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Assembly and Cofactor Attachment to a Mitochondrial Flavoprotein

by

Karen Marie Robinson 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Biochemistry

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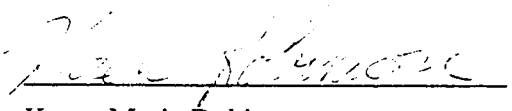

Karen Marie Robinson
4056 Aspen Drive East
Edmonton, Alberta, Canada

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Assembly and Cofactor Attachment to a Mitochondrial Flavoprotein* submitted by Karen Marie Robinson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. B. Lemire (supervisor)

Dr. J. Weiner

Dr. M. Ellison

Dr. F. Nargang

Dr. M. Yaffe (external examiner)

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ABSTRACT

Succinate dehydrogenase (SDH; EC 1.3.99.1) is a mitochondrial respiratory complex composed of four nuclear-encoded subunits: a flavoprotein that covalently binds FAD, an iron-sulfur protein, and two smaller hydrophobic membrane anchoring subunits. Using the *Saccharomyces cerevisiae* flavoprotein subunit as a model, the processes of import, cofactor attachment, and assembly were investigated.

A *S. cerevisiae* strain that was disrupted in *SDH1*, the gene encoding the flavoprotein, was constructed and used to clone and sequence the entire *SDH1* gene. The disruption mutant contains no detectable enzymatic activity, yet the other respiratory complexes assayed are not affected, suggesting that their assembly is independent of each other. Additionally, the iron-sulfur protein is not detectable in a *SDH1* disruption mutant, implying that the flavoprotein is required to stabilize the iron-sulfur protein. The *SDH1* sequence revealed that the yeast flavoprotein is highly homologous to other SDH flavoproteins and the related complexes, the fumarate reductases. Furthermore, these studies showed that the flavoprotein could be imported into isolated mitochondria and proteolytically processed *in vitro*.

A mutant flavoprotein that binds FAD non-covalently was constructed and characterized. Experiments with the mutant flavoprotein demonstrated that covalent FAD attachment is not required for either flavoprotein import into mitochondria, for proteolytic processing, or for its assembly into the holocomplex. Moreover, a holocomplex containing the mutant flavoprotein retains the ability to reduce fumarate to succinate, but is unable to oxidize succinate to fumarate. With the flavoprotein mutant as a control, an assay for covalent attachment of FAD was developed.

We explored the process of cofactor attachment both *in vitro* and *in vivo*. Our studies determined that cofactor attachment occurs after import, inside the mitochondria, and independently of the other SDH subunits. Moreover, these experiments suggested that FAD attachment is a post-translocational event that requires the flavoprotein to adopt a

partially folded conformation, that it is modulated by substrate molecules, and that it requires ATP. Additionally, our results demonstrate that proteolytic removal of the presequence is a prerequisite for cofactor attachment. Curiously, we found that the mitochondrial chaperonin binds the flavoprotein both before and after FAD is attached.

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ABBREVIATIONS AND NOMENCLATURE

ADP	adenosine diphosphate
AMP	adenosine monophosphate
Amp	ampicillin
apo-Fp	flavoprotein without FAD attached
Arg	arginine
ATP	adenosine triphosphate
bc1 complex	cytochrome bc1 complex, or complex III
BCS1	bc_1 synthesis
βME	2-mercaptoethanol
bp	nucleotide base pair
BSA	bovine serum albumin
CII-4	smallest membrane subunit of bovine SDH (complex II)
CoA	coenzyme A
COX	cytochrome oxidase
Cys	cysteine
Cyt. c	cytochrome c
DCPIP	dichlorophenol indophenol
DTT	dithiothreitol
ε	extinction coefficient
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
ETC	electron transport chain
F_1/F_0 ATP synthase	ATP phosphohydrolase (H^+-transporting, EC 3.6.1.34)
FAD	flavin adenosine dinucleotide
FADH•	FAD semiquinone radical
flavinylation	for a protein to have an FAD moeity covalently attached
FMN	flavin monoadenosine
Fp	flavoprotein
FRD	fumarate reducatase
GroE	GroEL and GroES
GrpE	glucose regulated protein
6-HDNO	6-hydroxy-D-nicotine oxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
His	histidine
holo-Fp	flavoprotein with FAD attached
H90S	the SDH flavoprotein mutant that has the histidine at position 90 mutated to a serine codon
Hsp10	heat shock protein 10
Hsp60	heat shock protein 60
Hsp70	heat shock protein 70
IMP	intermediate mitochondrial protease
Ip	iron-sulfur protein
ISP	import site protein
kb	kilobase
kDa	kilodalton
KLH	Keyhole limpet hemocyanin
MAS	mitochondrial assembly
MCD	magnetic circular dichroism

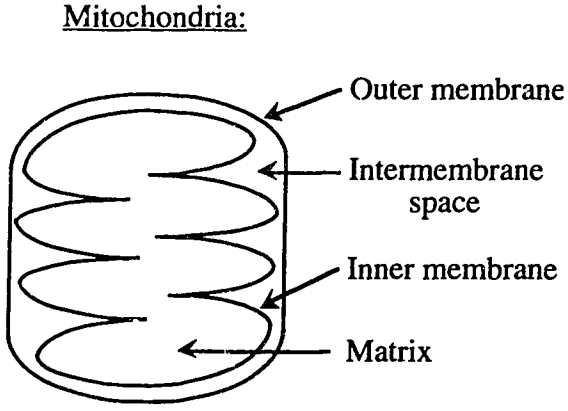
Mdj1p	mitochondrial DnaJ protein
Met	methionine
mFp	mature flavoprotein
mHsp70	mitochondrial heat shock protein 70
MIM	mitochondrial inner membrane
MIP	mitochondrial intermediate protease
MOM	mitochondrial outer membrane
MPP	matrix processing peptidase
MPP	matrix processing protease
MQ	menaquinone
MQH_2	menaquinol
mRNA	messenger RNA
NADH/NAD	nicotinamide adenine dinnucleotide
OXA1	oxidase assembly
PEP	processing enhancing protein
Pet	petitte mutant
P_i	inorganic phosphate
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid
pre-Fp	precursor flavoprotein
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
sulfo-LC-SPDP	sulfosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate
TCA	trichloroacetic acid
Tris-Cl	tris(hydroxylmethyl)amino methane hydrochloride
Tris-SO_4	tris(hydroxylmethyl)amino methane sulfate
ts	temperature sensitive mutant
Tyr	tyrosine

Chapter 1

Introduction

Organelle biogenesis is a very intricate and fascinating process that occurs in all eukaryotic cells. Of particular interest is the biogenesis of mitochondria, an organelle that provides most of the cell's energy and is also responsible for the biosynthesis of an array of different metabolites. The mitochondria have two distinct membrane layers, the outer and inner membranes, that enclose two separate soluble compartments: the intermembrane space and the mitochondrial matrix (Figure 1-1). Furthermore, mitochondria contain DNA that encodes several mitochondrial proteins. Thus, in addition to the coordination of two separate genomes, mitochondrial biogenesis involves protein targeting, import from the cytoplasm into the mitochondria, localization to various sub-compartments, cofactor insertion, and often subsequent assembly of these proteins into multisubunit membrane-bound complexes. Succinate dehydrogenase (SDH) is a mitochondrial protein that undergoes each of these processes and hence is an excellent model with which to study mitochondrial biogenesis. This thesis addresses SDH assembly, with particular emphasis on the assembly and cofactor attachment of the flavoprotein (Fp).

Figure 1-1.



1-1. IMPORT

Although mitochondria have their own genome, mitochondrial biogenesis requires the import of nuclear-encoded proteins since less than 10% of mitochondrial proteins are

encoded by the mitochondrial genome. Those proteins encoded by the mitochondrial DNA are transcribed and translated inside the matrix and then localized to the inner membrane. The remainding proteins are nuclear-encoded, translated in the cytosol, targeted to the mitochondria, translocated across one or two membranes, and localized to the appropriate sub-compartment. Targeting signals are most often in the form of a cleavable amino-terminal presequence, although some are internal signals. A protein with a cleavable presequence is referred to as a precursor; after removal of the presequence, the protein is called a mature protein. After targeting to the mitochondria, proteins are inserted and translocated across the outer membrane. If the protein is to be translocated across the inner membrane as well, import continues at sites where the outer and inner membranes are in close contact (Pfanner *et al.*, 1990b). Insertion across the inner membrane is followed by proteolytic processing of the protein, complete translocation, and folding and assembly. Thus, the import of proteins into mitochondria involves components in the cytoplasm, the mitochondrial outer membrane, the mitochondrial inner membrane, and the mitochondrial matrix (summarized in Table 1-1). The process of mitochondrial import has been extensively studied and many of the details are known (for reviews see Glick *et al.*, 1992; Schatz, 1992; Kiebler *et al.*, 1993), although not presented here. The following is a general overview of import into mitochondria.

1-1a. Targeting to the Mitochondria.

Most proteins are targeted to the mitochondria by a cleavable amino-terminal presequence that is both necessary and sufficient for targeting and sorting. Generally composed of both hydrophobic and positively charged amino acid residues, presequences are thought to form amphipathic alpha helices (von Heijne, 1986). Often, these presequences are also enriched in serine and threonine residues. The matrix processing protease (MPP) cleaves presequences from precursor proteins, producing the mature

proteins (Hawlitschek *et al.*, 1988; Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988). For some precursors, protease processing happens in two steps: the MPP cleaves off part of the presequence, leaving an intermediate form of the protein with eight amino acids of the presequence still remaining; the octapeptide is then removed by the mitochondrial intermediate protease (MIP) (Kalousek *et al.*, 1988; Isaya *et al.*, 1994).

All outer membrane proteins and some intermembrane space proteins either have amino-terminal presequences that are not cleaved or have internal signal sequences (Pfaller *et al.*, 1990; Mayer *et al.*, 1993). No obvious consensus sequence for internal signal sequences is yet apparent (Kiebler *et al.*, 1993a). Additionally, some proteins have a bipartite signal sequence consisting of a regular amino-terminal presequence followed directly by a sorting signal. These sorting sequences, which are stretches of hydrophobic amino acids, direct localization to the intermembrane space or to the membranes (Gasser *et al.*, 1982a; Gasser *et al.*, 1982b; Kaput *et al.*, 1982; Teintze *et al.*, 1982). The bipartite presequences are also often cleaved in two steps: MPP cleaves the amino-terminal segment followed by cleavage of the sorting signal by the intermediate mitochondrial protease (IMP) (van Loon *et al.*, 1986; Behrens *et al.*, 1991; Schneider *et al.*, 1991). Hence, various targeting signals localize proteins to the different subcompartments of the mitochondria.

1-1b. Import-Competent State.

Tightly folded proteins cannot be imported into mitochondria (Eilers and Schatz, 1986; Chen and Douglas, 1987). Co-translational import averts this problem, as it prevents the growing polypeptide chain from having time to fold tightly (Verner, 1993). By keeping the protein unfolded, the ribosomes themselves allow co-translational import. Although co-translational import is likely to be more frequent *in vivo*, post-translational import can occur both *in vivo* and *in vitro* (Reid and Schatz, 1982a; Reid and Schatz, 1982b). The precursors in post-translational translocation are kept in a loosely folded or

import competent state by the binding of Hsp70 to the precursor proteins (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Murakami *et al.*, 1988). Upon translocation across the outer membrane, Hsp70 releases the precursor protein in an ATP dependent manner (Pfanner *et al.*, 1990a; Glick *et al.*, 1992c). Thus the precursor proteins are kept in a loosely folded conformation allowing translocation to occur both post- or co-translationally.

1-1c. Translocation across the Outer Membrane.

After targeting to the mitochondria, precursor proteins are translocated across the outer membrane. Receptor proteins on the outer membrane first bind the precursor proteins. Examples of receptor proteins from *Neurospora crassa* are MOM19 (Söllner *et al.*, 1989) and MOM72 (Söllner *et al.*, 1990) (mitochondrial outer membrane) and the yeast analogs, Mas20p (Ramage *et al.*, 1993; Lithgow *et al.*, 1994) and Mas70p (Hines *et al.*, 1990) (mitochondrial assembly), respectively. Although the receptors have different affinities for various precursors, they can overlap in function. From the receptor, the precursor protein is transferred, with the aid of MOM22 in *N. crassa* (Kiebler *et al.*, 1993b), to a group of proteins that form a translocation channel. In *N. crassa* the proteins MOM38, MOM30, MOM8, and MOM7 constitute part of the translocation machinery (Kiebler *et al.*, 1990; Söllner *et al.*, 1992). In yeast, the MOM38 homolog, import site protein (ISP)42 (Vestweber *et al.*, 1989; Baker *et al.*, 1990) and ISP6 (Kassenbrock *et al.*, 1993) are part of the translocation machinery. The exact composition of the translocation channel varies depending on the isolation procedure used, which led to the prediction that the translocation channel is a dynamic structure that assembles and disassembles depending on what precursor is being imported and on the state of translocation (Pfanner *et al.*, 1991). After insertion into the translocation channel, the precursor is translocated across the membrane.

Table 1-1. Components of the Mitochondrial Import Machinery.

Location:	Name:	Known or possible function:
Cytoplasm	Hsp70[a]	helps keep proteins loosely folded
Mitochondrial Outer Membrane	MOM19[b]/Mas20p[c], MOM72[d]/Mas70p[e]	receptor proteins for precursors
	MOM22[f,g]	transfer protein from receptors to translocation channel
	MOM38[g]/ISP42[h]	forms part of translocation channel
	MOM30, MOM7, MOM8[i] and ISP6[j]	part of the translocation machinery
Mitochondrial Inner Membrane	MIM44[k, l]	binds precursors
	MIM17[k, m], MIM23[k, n]	form translocation channel
	IMP[o], Imp1p, Imp2p[p]	proteolytically removes sorting signals
Mitochondrial Matrix	mHsp70[q], Mdj1p[r], GrpE[s]	bind and pull in translocating
	MPP[t]/Mas2p[u], PEP[t]/Mas1p[v]	two subunits of matrix processing protease, cleave presequences
	MIP[w]	cleaves octapeptide sequence
	Hsp60[x] & Hsp10[y]	fold and assemble proteins

a. (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Murakami *et al.*, 1988), b. (Söllner *et al.*, 1989) c. (Ramage *et al.*, 1993; Lithgow *et al.*, 1994), d. (Söllner *et al.*, 1990), e. (Hines *et al.*, 1990), f. (Kiebler *et al.*, 1993b), g. (Kiebler *et al.*, 1990), h. (Vestweber *et al.*, 1989; Baker *et al.*, 1990), i. (Moczko *et al.*, 1992; Söllner *et al.*, 1992), j. (Kassenbrock *et al.*, 1993), k. (Maarse *et al.*, 1992), l. (Scherer *et al.*, 1992; Blom *et al.*, 1993; Horst *et al.*, 1993), m. (Maarse *et al.*, 1994), n. (Dekker *et al.*, 1993; Emtage and Jensen, 1993; Ryan and Jensen, 1993), o. (Behrens *et al.*, 1991; Schneider *et al.*, 1991; Isaya *et al.*, 1994), p. (Nunnari, 1993), q. (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Scherer *et al.*, 1990), r. (Rowley *et al.*, 1994), s. (Bolliger *et al.*, 1994), t. (Böhni *et al.*, 1983; Hawlitschek *et al.*, 1988), u. (Jensen and Yaffe, 1988), v. (Witte *et al.*, 1988), w. (Kalousek *et al.*, 1988; Isaya *et al.*, 1994), x. (Cheng *et al.*, 1989), y. (Rospert *et al.*, 1993; Hohfeld and Hartl, 1994)

How are proteins pulled across the outer membrane? Outer membrane proteins and some intermembrane space proteins require only the translocation machinery of the

outer membrane for import (Mayer *et al.*, 1993), at which stage their import is complete and is followed either by assembly into the outer membrane or the intermembrane space. Other precursors subsequently require interactions with the translocation machinery of the inner membrane. The translocation machinery of both the outer and inner membrane is non-selective enough to allow the passage of both hydrophobic and highly charged stretches of amino acids. By Brownian motion, the translocating proteins may be able to move back and forth through the translocation channel (Simon *et al.*, 1992). The translocating channels of both the mitochondrial outer and inner membrane are thought to act as passive pores that associate with the translocating proteins very weakly, thereby allowing passive diffusion (Unger *et al.*, 1994). Thus, there must be some interaction or driving force that causes the net movement of the proteins across membranes. Hence the driving force of translocation across the outer membrane may be folding, assembly into an oligomeric complex, or interaction with the inner membrane translocation machinery.

During import, precursor proteins often span both membranes simultaneously, forming translocational contact site intermediates (Schleyer and Neupert, 1985; Pfanner *et al.*, 1990b; Rassow *et al.*, 1990). However, these translocating proteins are exposed to the intermembrane space (Rassow and Pfanner, 1991), suggesting that the outer and inner membrane translocation machineries are not likely to be joined by a sealed channel. Interestingly, intermembrane space proteins are released into the intermembrane space compartment, but matrix proteins are never soluble in the intermembrane space during import. Recent studies indicate that the translocation machineries of both membranes act independently (Segui-Real *et al.*, 1992) though *in vivo* they are usually coupled (Glick and Schatz, 1991; Pfanner *et al.*, 1992; Pfanner *et al.*, 1994).

1-1d. Translocation across the Inner Membrane.

After the presequence has been translocated across the outer membrane, it is inserted into the mitochondrial inner membrane. Insertion into the inner membrane

requires a membrane potential. The potential may have an electrophoretic effect on the positively charged presequence, or it may be required for the function of one of the proteins involved in translocation. In yeast, the inner membrane translocation machinery is composed in part by MIM17, MIM23, and MIM44 (for mitochondrial inner membrane) (Pfanner *et al.*, 1994). MIM44 may function in the initial binding of the precursor (Blom *et al.*, 1993), while MIM17 and MIM23 likely form part of the translocation channel (Emtage and Jensen, 1993; Ryan and Jensen, 1993; Kübrich *et al.*, 1994). Mitochondrial Hsp70 (mHsp70) interacts with MIM44 and is also a part of the inner membrane translocation machinery (Schneider *et al.*, 1994). In an ATP-dependent manner, mHsp70 binds the precursor causing its translocation across the membrane (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Schneider *et al.*, 1994; Stuart *et al.*, 1994b). Like the translocation machinery of the mitochondrial outer membrane, the composition of the translocation machinery of the inner membrane is predicted to be a dynamic structure which assembles and disassembles (Pfanner *et al.*, 1994). Once the mHsp70 has pulled enough of the precursor into the matrix, the presequence is cleaved. Following translocation and protease processing, mHsp70 transfers matrix targeted proteins to a mitochondrial chaperonin, Hsp60 (Manning-Krieg *et al.*, 1991) (see next section). Thus, in contrast to translocation across the outer membrane, the energy for import across the inner membrane is provided by both ATP and a membrane potential.

How some proteins with a bipartite signal sequence are sorted has been a source of considerable debate. The "conservative sorting" hypothesis predicts that these precursor proteins are translocated entirely across the inner membrane into the matrix (Hartl *et al.*, 1987; Koll *et al.*, 1992; Schwarz *et al.*, 1993). The hydrophobic sorting signal exposed after removal of the first domain of the presequence then directs re-export across the inner membrane into the intermembrane space where it is cleaved by the IMP. In contrast, the "stop transfer" hypothesis predicts that only the amino terminus of the precursor is translocated across the inner membrane where the presequence is cleaved;

the sorting signal prevents translocation of the remainder of the protein (Gasser *et al.*, 1982b; Glick *et al.*, 1992a; Glick *et al.*, 1992b; Rospert *et al.*, 1994). The IMP then cleaves the sorting signal and the protein assembles either into the inner membrane or the intermembrane space. The location of the IMP does not rule out either possibility, as it is found on the inner membrane, facing the intermembrane space (Schneider *et al.*, 1991; Nunnari, 1993). A significant difference between the two hypotheses is that conservative sorting predicts the interaction of precursor proteins with Hsp60, which is soluble in the matrix, while the stop-transfer model suggests that most portions of the precursor protein are never exposed to the matrix. Notably, *in vivo* studies indicate that loss of Hsp60, or Hsp60 function, does not inhibit the import of two of these proteins with a bipartite signal(Hallberg *et al.*, 1993). Futhermore, the binding of these proteins to Hsp60 *in vitro* has recently been demonstrated to be artifactual (Rospert *et al.*, 1994), casting doubt on the "conservative sorting" theory.

Thus, nuclear-encoded proteins are localized to mitochondria by a process that involves targeting, interactions with one or two independent translocation machineries, and proteolytic processing. Energy for these events are partly provided by ATP and a membrane potential. After import, mitochondrial proteins are then folded and assembled into functional complexes.

1-2. FOLDING AND ASSEMBLY.

Assembly of complexes composed of peripheral and integral membrane domains that can contain 10 to 20 subunits and various cofactors may be an extremely complicated process and require the activities of different protein (de Vries and Marres, 1987). Upon import, the subunits initially interact with two different types of molecular chaperones, mHsp70 and Hsp60 (see above; Stuart *et al.*, 1994). These chaperones are members of highly conserved families of proteins that participate in protein translocation and folding (for reviews see Georgopoulos and Welch, 1993; Becker and Craig, 1994; Hartl *et al.*,

1994)). For the construction of the multi-subunit complexes, the individual subunits must also be brought together and the different cofactors inserted. In contrast to targeting and import, assembly of mitochondrial enzymes, such as the electron transport chain (ETC) complexes, is not well understood.

1-2a. The Role of Hsp70 in Folding.

The mitochondrial Hsp70 (mHsp70) is part of a family of Hsp70s that binds unfolded proteins and prevent them from aggregating (Georgopoulos and Welch, 1993; Becker and Craig, 1994; Hartl *et al.*, 1994). The prokaryotic equivalent is DnaK, so named because it was first identified as being required for bacteriophage DNA replication (Georgopoulos and Herskowitz, 1971; Georgopolous, 1977; Yochem *et al.*, 1978); subsequently it was also determined to be essential for host growth at elevated temperatures (Georgopolous, 1977). This family of chaperones binds polypeptides that are being translated or translocated across membranes (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Beckmann *et al.*, 1990; Nelson *et al.*, 1992). When bound by Hsp70s, the polypeptide is in an extended conformation (Landry *et al.*, 1992); hence, it is speculated that these chaperones bind the polypeptide backbone (Georgopoulos and Welch, 1993; Becker and Craig, 1994). Release of polypeptides requires the hydrolysis of ATP by Hsp70 (Flynn *et al.*, 1989; Liberek *et al.*, 1991a; Liberek *et al.*, 1991b). Hsp70s may also mediate partial folding of polypeptides as upon their release the polypeptides contain some secondary structure (Manning-Krieg *et al.*, 1991; Langer *et al.*, 1992a). Each Hsp70 protein has one protein binding site (Gething and Sambrook, 1992), therefore multiple Hsp70 molecules may simultaneously bind one polypeptide chain (Hartl *et al.*, 1994; Stuart *et al.*, 1994a). By binding the emerging polypeptide, Hsp70s prevent premature folding and thus misfolding and aggregation of nascent chains (Beckmann *et al.*, 1990; Nelson *et al.*, 1992).

Two families of proteins modulate the activity of Hsp70s. In the prokaryotic

system, DnaK interacts with DnaJ and glucose regulated protein E (GrpE). GrpE was so named because it was initially identified as a protein that is induced in cells starved of glucose (Pouysségur *et al.*, 1977). DnaJ and its homologs stimulate Hsp70 ATPase activity and the GrpE class of proteins acts as nucleotide exchange factors (Liberek *et al.*, 1991a). In yeast mitochondria, the DnaJ and GrpE equivalents are called mitochondrial DnaJ (Rowley *et al.*, 1994) or Mdj1p, and GrpE (Bolliger *et al.*, 1994), respectively. Interestingly, in yeast only GrpE and not Mdj1p, is essential for viability.

Mitochondrial Hsp70 has several functions. With the initial binding of the translocating polypeptide, mHsp70 prevents it from slipping backwards, and hence causes import to be uni-directional (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991). Without mHsp70 binding, precursors with fewer than 30 to 40 residues in the matrix are able to slip out of the mitochondria (Unger *et al.*, 1994). Additionally, the energy of binding by mHsp70 may help to unfold portions of the polypeptide remaining on the cytoplasmic side of the mitochondria (Cyr *et al.*, 1993; Stuart *et al.*, 1994b). In accord with the "Brownian ratchet" hypothesis (Simon *et al.*, 1992), mHsp70 may drive the translocation of the precursor by binding it, and thus trapping it as more portions of the protein emerge from the *trans* side of the membrane. Alternatively, the binding of mHsp70 to the precursor may also cause conformational changes to the precursor and affect its interactions with the membrane translocation machinery (Schneider *et al.*, 1994), driving its translocation across the mitochondrial membranes (Pfanner *et al.*, 1994). Like other Hsp70s, mHsp70 also prevents the misfolding and aggregation of the translocating chain. Finally, mHsp70 transfers the polypeptide to Hsp60 in a partially folded state (Manning-Krieg *et al.*, 1991).

1-2b. Hsp60/Hsp10 Function in Folding and Assembly.

A great deal of information concerning the structure and folding activity of Hsp60 is derived from studies on the *Escherichia coli* homologue, GroEL. The high resolution

crystal structure of GroEL has been solved (Braig *et al.*, 1994), and shows that the protein exists as a complex composed of two stacked rings each containing seven 60 kDa monomers. Each of the rings has a large central cavity where the polypeptides bind (Langer *et al.*, 1992b; Ishii *et al.*, 1994). Proteins bound by GroEL possess some secondary structure and therefore are predicted to be present as compact folding intermediates or in a "molten globule" states (Martin *et al.*, 1991; Landry *et al.*, 1992; Hayer-Hartl *et al.*, 1994). The cavity in each ring is predicted to be large enough to hold the folding intermediate of a 35 kDa protein (Hartl, 1994). By holding proteins in a cavity, GroEL may shield them from unproductive interactions with themselves and other proteins that might lead to misfolding or aggregation (Becker and Craig, 1994; Hartl *et al.*, 1994).

GroEL promotes the folding of proteins in conjunction with another protein, GroES; the mitochondrial equivalent is Hsp10 (Lubben *et al.*, 1990; Rospert *et al.*, 1993; Hohfeld and Hartl, 1994). Protein folding by GroEL is thought to occur by multiple rounds of binding and release from GroEL, which has multiple protein binding sites (Becker and Craig, 1994; Hartl *et al.*, 1994). For proteins to be released by GroEL, hydrolysis of ATP is required (Goloubinoff *et al.*, 1989; Buchner *et al.*, 1991; Martin *et al.*, 1991). The ATPase activity of GroEL is coupled to its protein folding activity through its interaction with GroES (Martin *et al.*, 1991; Mendoza *et al.*, 1991). GroES has a ring structure of seven 10 kDa monomers that caps the central cavity of GroEL (Chandrasekhar *et al.*, 1986); The association of GroES to the ring of GroEL causes conformation changes within GroEL that induce drastic movements of the GroEL domains, producing an even larger enclosed central space which could accomodate a folding protein (Chen *et al.*, 1994) Moreover, GroES provides additional protection to folding intermediates within the cavity from interactions with outside proteins (Becker and Craig, 1994). Once the protein has folded completely, it no longer possesses hydrophobic surfaces for GroEL to bind to; hence the folded protein is released (Becker

and Craig, 1994; Hartl, 1994; Hartl *et al.*, 1994; Hayer-Hartl *et al.*, 1994). GroEL has been shown to mediate the folding and assembly of a large number of proteins *in vitro* (Goloubinoff *et al.*, 1989; Buchner *et al.*, 1991; Höll-Neugebauer *et al.*, 1991; Martin *et al.*, 1991; Mendoza *et al.*, 1991; Viitanen *et al.*, 1991), including itself (Cheng *et al.*, 1990).

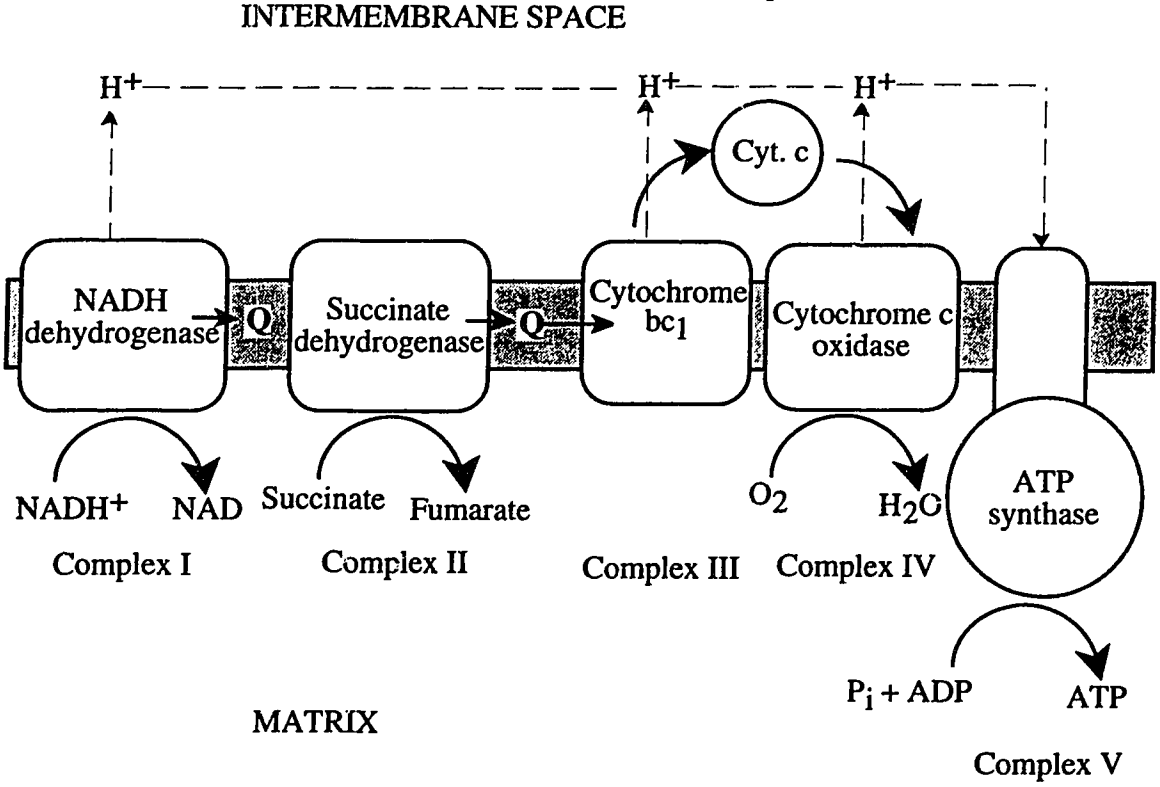
In mitochondria, in an analogous fashion to GroEL and GroES, Hsp60 and Hsp10 binds to the newly imported proteins and release them once folded (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). How or if Hsp60 participates in either establishing interactions between different subunits of the various membrane bound complexes, or in attachment of cofactors is not known.

1-2c. Assembly of Multisubunit Membrane-bound Complexes.

The assembly of multisubunit complexes, such as those of the electron transport chain (ETC, see Figure 1-2) appears to occur in a specific stepwise manner as opposed to a random order. The process may start with intramitochondrial pools of different subunits, as has been shown for the F_1/F_0 ATP synthase (Lewin and Norman, 1983). Complex assembly has been examined by investigating the function of individual subunits. These studies have shown that different subunits of a complex may be required for assembly but not catalytic activity, and *vice versa.* For example, studies with cytochrome c oxidase have demonstrated that the Cox VIa subunit in *Saccharomyces cerevisiae,* is not required for complex assembly, but does affect activity (Taanman and Capaldi, 1993). Alternatively, Cox VIb is essential for assembly of cytochrome c oxidase, but purified complex that does not contain this subunit retains full enzyme activity (LaMarche *et al.*, 1992). These experiments have also demonstrated that the cytochrome c oxidase, (Weilburski *et al* 1982) F_1/F_0 ATP synthase, (Lewin and Norman, 1983) and cytochrome bc_1 (Crivellone *et al.*, 1988; Fu and Beattie, 1991; Schmitt and Trumpower, 1991) complexes are assembled by ordered pathways via

distinct assembly intermediates. Likewise, biosynthesis of NADH:ubiquinone oxidoreductase occurs through assembly intermediates: the peripheral and integral membrane portions of this complex are constructed independently and then are joined together (Friedrich *et al.*, 1989; Tuschen *et al.*, 1990; Nehls *et al.*, 1992). Thus mitochondrial complexes are assembled in an ordered and sequential fashion.

Figure 1-2. Schematic of the Mitochondrial Electron Transport Chain.



Accessory proteins that are not part of the final complexes are required for assembly of many multi-subunit proteins. Numerous of these proteins have been identified in yeast (see Table 1-2). Possible functions for these non-structural proteins include membrane insertion of the subunits, establishing association between subunits, protein modification, subunit folding, and cofactor biosynthesis or insertion. Clarifying the roles of these non-structural proteins requires further investigation.

Proteolytic processing can also be involved in complex assembly and cofactor attachment. For example, the Rieske iron-sulfur protein of the bc1 complex has a

presequence that is cleaved in two steps. However, the second cleavage does not occur until after the protein has been assembled into the complex (Graham *et al.*, 1993). Protease function can be interconnected with cofactor attachment as well. Cytochome c_1 is cleaved in two steps; the second cleavage step requires prior attachment of heme (Nicholson *et al.*, 1989). Thus, the presequence may be either essential for assembly or cofactor attachment, or may prevent these processes from occuring at different steps in the complex formation.

Table 1-2. Non-structural Proteins required for Assembly of Different Complexes.

Required for Assembly of:	Protein:	Proposed function:	Ref.:
Cytochrome bc_1 complex (Complex III)	BCS1	required for insertion of Fe/S cluster	a
Cytochrome c oxidase (Complex IV)	COX10 & COX11	Heme A biosynthetic enzymes	b
	SCO1	?	c
	Pet117 & Pet 191	?	d
	OXA1	?	e
F_1/F_0 ATP synthase (Complex V)	ATP10	involved in the assembly of the F_0 portion	f
	ATP11 & ATP12	needed at a late stage of assembly of the F_1 portion	g
	ATP13	required for the transcription or translation of subunit 9	h

a. (Nobrega *et al.*, 1992), b. (Nobrega *et al.*, 1990; Tzagoloff *et al.*, 1990; Tzagoloff *et al.*, 1993), c. (Schulze and Rödel, 1989; Krummeck and Rodel, 1990), d. (McEwen *et al.*, 1993), e. (Bonnefoy *et al.*, 1994), f. (Ackerman and Tzagoloff, 1990a), g. (Ackerman and Tzagoloff, 1990b; Bowman *et al.*, 1991; Ackerman *et al.*, 1992), h. (Ackerman *et al.*, 1991)

1-2d. Cofactor Attachment.

In addition to the assembly of individual subunits, complex formation requires the insertion or attachment of cofactors, a process that may require additional proteins. The covalent attachment of various cofactors is enzymatically catalyzed. For example, heme

lyases catalyze the covalent attachment of heme to c-type cytochromes (Nargang *et al.*, 1988; Nicholson *et al.*, 1989; Dumont *et al.*, 1991). NADH is required to reduce the heme prior to attachment. Furthermore, enzymes are known to catalyze the covalent attachment of biotin and lipoic acid (Schmidt *et al.*, 1969; Gross and Wood, 1984). Both modifications also require ATP hydrolysis. The timing of cofactor attachment varies: it may occur after import, as is the case for pyrodoxal phosphate to mitochondrial aspartate aminotransferase (Sharma and Gehring, 1986), heme to cytochrome c (Dumont *et al.*, 1991), and biotin to pyruvate carboxylase (Ahmad and Ahmad, 1991); or it may happen either before or after import as is the case for biotin to the alpha subunit of propionyl-CoA carboxylase (Taroni and Rosenberg, 1991). Whether the insertion or the attachment of most cofactors requires additional proteins is not known.

Obviously, the assembly of the ETC presents a very complicated problem. Many of the mechanisms and details of this process remain to be elucidated. To study assembly, we used SDH as a model complex. The structure and function of SDH is now discussed, followed by what is known about its assembly.

1-3. STRUCTURE AND FUNCTION OF SDH AND FRDS.

The SDH and fumarate reductase (FRD) complexes are remarkably similar in activity, localization, and function (for recent reviews see: Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). Both enzymes catalyze the oxidation of succinate to fumarate, and the reduction of fumarate to succinate. The kinetic properties of these complexes have been extensively studied (Vinogradov, 1986). In prokaryotes, SDH and FRD are located in the cytoplasmic membrane; in eukaryotic cells, SDH is located on the inner membrane of the mitochondria, facing the matrix. In prokaryotes, such as *E. coli*, the expression of SDH and FRD depend on oxygen levels (Cole *et al.*, 1985). SDH is expressed in aerobically growing cells, although its expression is repressed by high concentrations of glucose due to catabolite repression. As part of the Krebs cycle, SDH

oxidizes succinate to fumarate, donating the reducing equivalents directly to the quinone (Q) pool; the terminal electron acceptor is ultimately oxygen. Thus, SDH is a component of the aerobic electron transport chain. Correspondingly, FRD is also part of a different electron transport chain. In anaerobically grown prokaryotes, FRD is expressed in the presence of fumarate when no electron acceptor with a higher redox potential is present. Using menaquinol (MQH_2) as an electron donor, FRD catalyzes the reduction of fumarate to succinate, thereby regenerating the menaquinone (MQ) pool. Hence, in the absence of oxygen, FRD allows the cells to use fumarate as the terminal electron acceptor. The similarity of these two complexes is further demonstrated by the ability of *E. coli* FRD, expressed aerobically, to complement an *E. coli* SDH mutant (Guest, 1981). Thus, SDH and FRD from prokaryotes and eukaryotes form a family of succinate:quinone oxidoreductases.

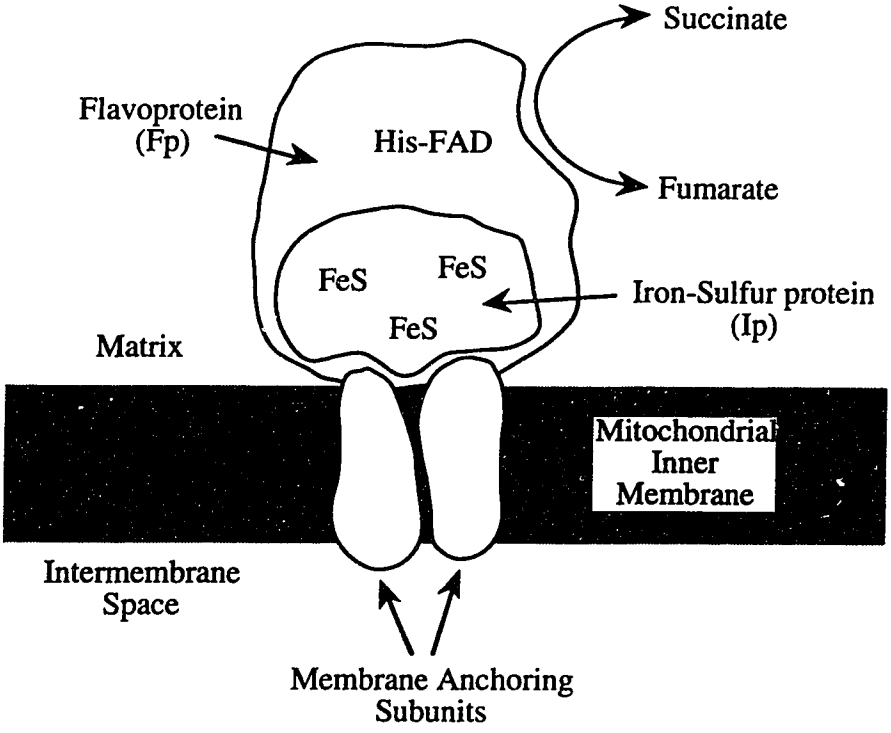
In spite of their similarities, at least one notable difference between SDH and FRD has been found. As detected by voltammetry studies (Sucheta *et al.*, 1992; Sucheta *et al.*, 1993) and spectrophotometric kinetics assays (Ackrell *et al.*, 1993), SDH has a low-potential activity switch-off that is not found in FRD; for example, the more reducing the conditions, the more difficult it is for SDH to reduce fumarate. Therefore, although the complexes are very analogous they have some mechanistic differences.

1-3a. Structure of SDH and FRD.

SDH and FRD are usually composed of three to four non-identical subunits: a flavoprotein (Fp) and an iron-sulfur protein (Ip), that are attached to the membrane by one or two smaller hydrophobic anchor-subunits (see Figure 1-3 and Table 1-3). These hydrophobic subunits are integral membrane proteins that contain the two quinone binding sites and may also ligand one or two b-type hemes. The active site is located in the Fp, which also contains covalently bound FAD. The other cofactors, three different iron-sulfur clusters, are found in the Ip subunit. The Fp and the Ip can form a soluble

catalytic dimer that is able to reduce fumarate or oxidize succinate using artificial electron donors or acceptors. Only when the dimer is associated with the membrane-anchoring subunits, thereby forming the holoenzyme, is the succinate:quinone oxidoreductase activity present.

Figure 1-3. Schematic of SDH.



1-3b. Genes Encoding SDH & FRD.

The analogous subunits from different sources of SDH and FRD are conserved as demonstrated by their amino acid sequences, by their functions, or by both. The genes for SDH and FRD from various prokaryotic sources have been isolated and sequenced. In prokaryotic cells, the FRD and SDH genes are encoded in operons, which are transcribed as polycistronic mRNAs. The Fp, Ip, and anchoring subunits of SDH and FRD in prokaryotic cells are coded by the *SDHA*, *SDHB*, *SDHC*, and *SDHD* genes or *FRDA*, *FRDB*, *FRDC*, and *FRDD* genes respectively. The FRD subunits are conserved

enough amongst different species that the Fp subunit from *P. vulgaris* interacts and forms a functional holoenzyme with the other subunits from *E. coli* (Honore and Cole, 1985).

All of the genes for the SDH subunits in eukaryotic cells are predicted to be nuclear-encoded, although only a few of them have been isolated. In yeast, the genes *SDH1*, *SDH2*, *SDH3* and *SDH4* encode the Fp (Chapman *et al.*, 1992; Robinson and Lemire, 1992; Schülke *et al.*, 1992), Ip (Lombardo *et al.*, 1990), and anchoring subunits (Bullis and Lemire, 1994; Daignan-Fornier *et al.*, 1994) respectively and are now sequenced. To date, the other complete eukaryotic SDH nucleotide sequences that have been published includes the genes enoding: the Fp from humans (Morris *et al.*, 1994), and bovine heart (Birch-Machin *et al.*, 1992); the Ip from humans (Kita *et al.*, 1990),and fruitfly (Au and Scheffler, 1994); and the CII-3 membrane subunit from bovine heart (Cochran *et al.*, 1994). Although the membrane subunits across different species have very little if any sequence identity, the general hydrophobicity plot of these subunits is conserved (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). Furthermore, the sequences of the Fp and Ip reveal that they have a very high degree of sequence identity from a wide range of both prokaryotic and eukaryotic sources. As the structures and functions of SDH and FRD are so conserved, knowledge about one complex can be used to predict what may happen with another.

Table 1-3. Structure of Succinate Dehydrogenases and Fumarate Reductases.

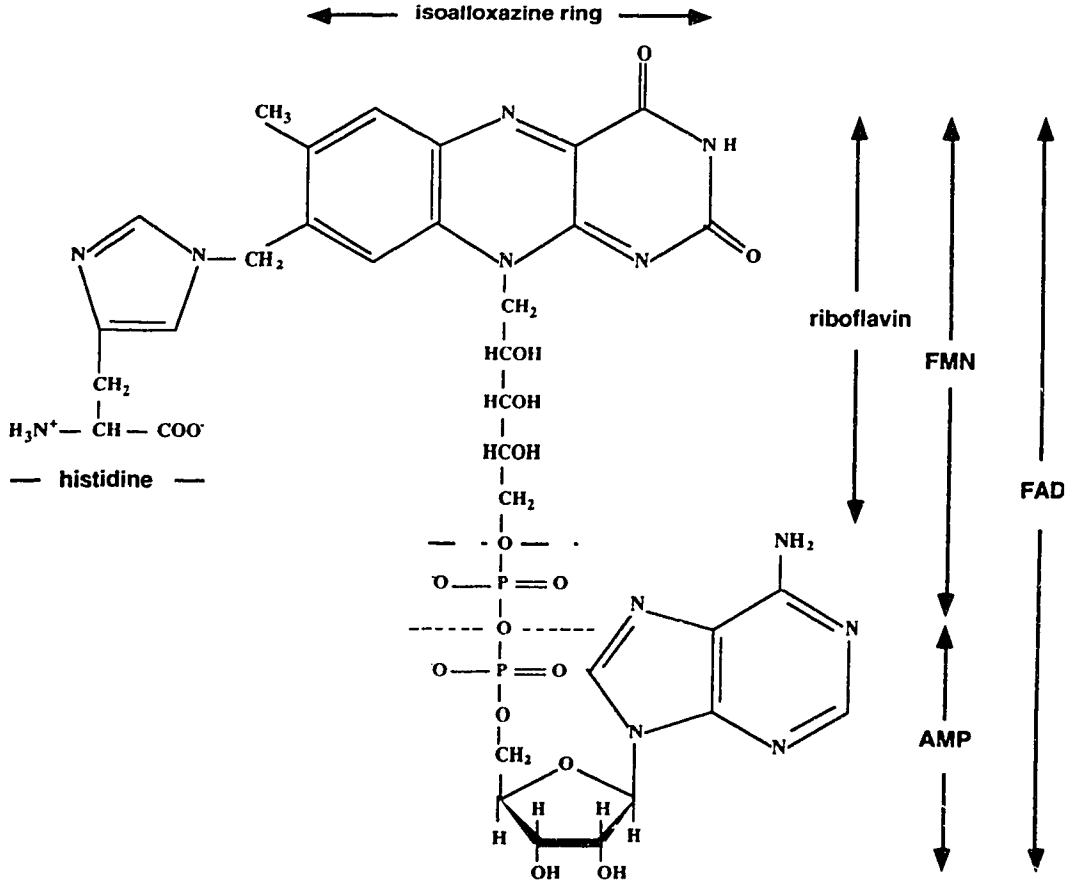
Subunit:	Location	Encoded by:	Cofactor:	Binding sites of:
Flavoprotein (Fp)	peripheral membrane	*SDH1*, *SdhA* , or *FrdA*	covalently bound FAD	substrates: fumarate and succinate
Iron sulfur protein (Ip)	peripheral membrane	*SDH2*, *SdhB*, or *FrdB*	three Fe-S clusters	-
Anchoring subunit (s)	integral membrane	*SDH3* and*SDH4*, *SdhC* and *SdhD*, or *FrdC* and *FrdD*	zero, one or two hemes	two quinone binding sites

1-3c. The Flavoprotein.

Located in a highly conserved region of the Fp, the active site, or substrate binding site differs in conformation depending on the enzyme's redox state and has a role in controlling enzyme activity. Various dicarboxylates bind at the active site, including succinate, fumarate, L-chlorosuccinate, and malate, all of which are substrates for the enzyme. The substrate binding site has different affinities for succinate or fumarate depending on whether the FAD is reduced or oxidized (Kotlyar and Vinogradov, 1984; Ackrell *et al.*, 1989). The K_d for succinate is lower when the enzyme is oxidized, while the K_d for fumarate is lower when the enzyme is reduced. Malonate and oxaloacetate also bind at the active site and thus are competitive inhibitors. Malonate is thought to mimic the transition state of the reaction, as it binds with approximately the same affinity regardless of the oxidation state of the enzyme. Oxaloacetate, binds with much higher affinity to the oxidized enzyme. When malate, a metabolite of the Krebs cycle, is oxidized by the enzyme the product is enol-oxaloacetate which gradually de-activates the enzyme (Ackrell *et al.*, 1974; Belikova *et al.*, 1988; Panchenko and Vinogradov, 1991). *In vivo*, oxaloacetate keto-enol-tautomerase converts the enol-oxaloacetate to keto-oxaloacetate which can then be consumed by enzymes such as L-glutamate-oxaloacetate transamidase or citrate synthase. Thus, oxaloacetate keto-enol-tautomerase, by allowing the removal of enol-oxaloacetate and thereby preventing the deactivation of SDH, is suggested to be involved in the regulation of SDH *in vivo* (Belikova *et al.*, 1988). Not surprisingly, SDH is often isolated with pre-bound oxaloacetate and must be re-activated by being reduced (Kearney, 1957; Kearney *et al.*, 1972). Since the oxidation state of the FAD affects the affinities of most substrates, the FAD is likely in close proximity to the substrate binding site. Whether the substrate binding site remains intact in mutants of SDH that lack FAD is unknown. The binding of different dicarboxylates, which are also Krebs cycle intermediates, at the active site may be a method of controlling enzyme activity or assembly *in vivo* (as discussed in the next section).

FAD is th.. first electron acceptor of the SDH complex, or the terminal electron donor of the FRD complex. As FAD has a semiquinone radical state (FADH•) it is able to accept two electrons from succinate and then donate them one at a time to the iron-sulfur clusters. The FAD therefore functions to bridge one electron and two electron carriers.

Figure 1-4. 8-α-[N (3)-histdyl]-FAD linkage.



1-3d. Attachment of FAD.

The Fp binds FAD by covalent and non-covalent interactions. The FAD is covalently attached to the Fp by an 8-α-[N (3)-histidyl]FAD linkage at a conserved histidine residue (see Figure 1-4). Additionally two other conserved regions are predicted to form an AMP binding fold: a Rossman nucleotide binding fold located upstream of the

site of covalent attachment, and a section near the middle of the Fp that is thought to form the top of the AMP binding fold (Schulz *et al.*, 1982; Wierenga *et al.*, 1983; Cole *et al.*, 1985). Hence, aside from the covalent attachment, the Fp most likely surrounds the FAD. Accordingly, antiserum against riboflavin fails to inhibit enzyme activity (Barber *et al.*, 1987), demonstrating that the isoalloxazine ring is probably buried in the protein. The importance of these non-covalent contacts is emphasized in two classes of mutants. In the first class, mutations downstream of the His residue that is the point of attachment, prevent both covalent and non-covalent binding of FAD (Hederstedt, 1983; Maguire *et al.*, 1986). The second class of mutants is at the conserved His residue that is the site of attachment (Blaut *et al.*, 1989). When this histidine is altered to another residue in the *E. coli* FRD, the mutated Fp can still tightly bind FAD non-covalently.

1-3e. Role of Covalent FAD-Protein Bond.

If the FAD can be tightly bound non-covalently, why is it covalently attached? A possible explanation is that the covalent attachment of FAD is required to raise the midpoint potential of FAD, thus allowing the oxidation of succinate in addition to the reduction of fumarate. Accordingly, the covalently attached FAD of *E. coli* FRD does have a substantially higher potential than free FAD (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). Mutational studies in which the histidine residue that is the site of covalent attachment was changed to Tyr, Ser, Cys or Arg codons in *E. coli* FRD also support this theory (Blaut *et al.*, 1989). All of these mutant enzymes lost SDH activity, although they still bound FAD non-covalently and all of them with the exception of the His to Arg mutant, retained considerable fumarate reductase activity. Additionally, *Desolfovibrio multispirans* FRD (He *et al.*, 1986) and a soluble monomeric FRD from yeast (Muratsubaki and Katsume, 1985), bind FAD only non-covalently and have only uni-directional activity; these FRDs cannot oxidize succinate. In contrast, SDH from *Mycobacterium phlei*, which may bind FAD only non-covalently, still has succinate

dehydrogenase activity (Reddy and Weber, 1986). Therefore, although covalent attachment of FAD does increase the redox potential enabling the oxidation of succinate, other non-covalent interactions may also be able to raise the potential of the FAD.

1-3f. The Iron-Sulfur Protein.

The Ip contains three different iron-sulfur clusters: center 1, a [2Fe-2S] cluster, center 2, a [4Fe-4S] cluster, and a center 3, a [3Fe-4S] cluster. The structures of these centres were determined by a variety of techniques including electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) (Ackrell *et al.,* 1992). Only centers 1 and 3 are reducible by succinate, prompting speculation that center 2 has no role in electron transfer but is instead a vestige of an earlier iron-sulfur protein (Manodori *et al.*, 1992). All of the clusters are thought to be ligated by cysteines located in the Ip, with the exception that a ligand for the [2Fe-2S] cluster of *E. coli* SDH may be an aspartate residue (Werth *et al.*, 1992). While the data thus far supports the hypothesis that all of the centres are ligated solely by the Ip, it is still possible that center 2 bridges the Ip and Fp.

1-3g. The Anchoring Subunit (s).

The membrane topologies, though not the amino acid sequences, of the anchoring subunits from different species are conserved (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). However, a membrane subunit for the yeast SDH does contain some sequence identity to an anchoring subunit of the bovine SDH (Daignan-Fornier *et al.*, 1994). In complexes that have two membrane subunits, such as the *E. coli* SDH and FRD, each anchor subunit has three membrane spanning domains. Where only one anchor subunit is present, as in the case for *Bacillus subtilis* SDH and *Wolinella succinogenes* FRD, the single subunit is proposed to cross the membrane five times.

The heme groups, that are likely co-ordinated by histidine residues in the anchoring subunit(s), may participate in the electron transfer mechanism. Some SDH and FRDs have two heme groups such as *B. subtilis* and *W. succinogenes*; one is a high redox potential heme and the other is a low redox potential heme (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). The redox midpoint potential of some heme groups is too low to be reducible by succinate. For example, the bovine cardiac SDH contains a heme group which is not reduced when the enzyme oxidizes succinate (Yu *et al.*, 1987). Additionally, SDH from yeast and FRD from *E. coli* most likely do not have any heme groups (Cecchini *et al.*, 1985; Cole *et al.*, 1985). Therefore, the function or role of the heme group (s) is unclear.

In the yeast *S. cerevisiae*, SDH is a four subunit enzyme, containing at least four types of cofactors. Furthermore, all of the subunits in yeast are encoded in the nucleus and are therefore accessible to genetic and molecular biological techniques. Thus yeast SDH provides a sophisticated yet alterable model for examining assembly of mitochondrial complexes.

1-4. ASSEMBLY OF SDH AND FRD.

SDH and FRD assembly has been examined with *in vitro* and *in vivo* systems. Experiments in which the holoenzyme was reconstituted from purified or partially purified subunits have been used to study the assembly of both bacterial and cardiac enzymes. These experiments demonstrated that the anchoring subunits, which were initially thought to be contaminants of SDH and FRD preparations, are components of the complexes, since re-association of the isolated soluble heterodimer with the anchoring subunits *in vitro* regenerates the quinone/quinol reducing/oxidizing activities (Cole *et al.*, 1985; Ackrell *et al.*, 1992). In contrast, the *in vivo* experiments have been done almost exclusively in bacterial systems. Hence, additional complications may arise in the eukaryotic system because of subunit import into the mitochondria. In general, the

present body of work suggests that the assembly of SDH and FRD begins with attachment of the prosthetic groups to the appropriate subunits, followed by the subunits associating with each other to form the holoenzymes.

1-4a. Assembly of the Anchor Subunits.

Several lines of experimentation have demonstrated that SDH and FRD assembly begin with the insertion of the anchoring subunit(s) into the membrane. Pulse-chase experiments were performed in *E. coli* in which the cells were subsequently lysed and fractionated and the location of the FRD subunits in either the soluble or membrane fraction was determined. These experiments demonstrated that the *FrdC* and the *FrdD* subunits are rapidly targeted to the membrane (Latour and Weiner, 1988). Furthermore, the anchoring subunit of *B. subtilis sdh* mutants lacking either the Fp or Ip subunit, is still correctly targeted, suggesting that localization of the anchoring subunit to the membrane is the initial step in SDH assembly (Hederstedt and Rutberg, 1980; Hederstedt *et al.*, 1982). This conclusion was substantiated by mixing membranes containing the correctly targeted anchoring subunits with lysates from cells containing soluble Fp and Ip subunits; the soluble subunits were able to bind the anchoring subunit. Moreover, when heme auxotrophs of *B. subtilis* are starved for heme, the anchoring subunit is present in the membrane, without heme (Friden and Hederstedt, 1990) The localization of this apo-subunit to the membrane demonstrates that membrane insertion of the anchoring subunit is one of the first steps of assembly, occurring prior to heme attachment (see below).

1-4b. Binding of Peripheral Subunits.

After the heme is inserted, the Fp and Ip subunits bind to the anchoring subunit(s). This order of assembly is indicated by pulse-chase experiments with a *B. subtilis* heme auxotroph mutant. Upon heme starvation, the Fp and Ip subunits are unable to associate with the anchoring subunit; instead the soluble subunits accumulate in the cytoplasm

(Hederstedt and Rutberg, 1980). With the addition of heme, the soluble proteins are chased into the membrane, even in the absence of protein synthesis, indicating that the anchor subunit was present but unable to bind with the other subunits. Reconstitution studies also show a better binding of the Fp and Ip subunits in membrane preparations with higher heme contents (Ackrell *et al.*, 1992). However, titration experiments demonstrate that the amount of binding observed exceeds the level of heme suggesting (Ackrell *et al.*, 1980; Ackrell *et al.*, 1992) that some active conformation is retained by the anchoring subunit even in the absence of heme. These results argue that heme attachment may be a prerequisite for the assembly of multisubunit proteins *de novo.*

In FRD and SDH enzymes that have two anchoring subunits, both are necessary for interactions with the Fp and Ip subunits. Reconstitution studies with the cardiac SDH show that just one of the anchoring subunits (CII-4), is not sufficient for attachment of the soluble subunits when the other anchoring subunit has been removed by protease digestion (Ackrell *et al.*, 1992). The requirement for both anchoring subunits is demonstrated more definitively by experiments with the *E. coli* FRD. In these experiments, the FRD subunits were expressed in different combinations. No significant membrane attachment of the soluble subunits is observed if only one of the anchoring subunits is expressed along with the Fp and Ip subunits (Cole *et al.*, 1985; Cecchini *et al.*, 1986). Additionally, when either of the anchoring subunits expressed has a carboxyl-terminal truncation, the binding of the soluble subunits to the membrane is impaired (Westenberg *et al.*, 1990). Thus, membrane attachment of the soluble subunits requires both full length anchoring subunits.

1-4c. Assembly of the Iron Sulfur Protein.

Little is known about the mechanism by which the iron-sulfur clusters are assembled. The iron-sulfur clusters can form independently of each other, as demonstrated by the assembly of center 1 in the *E. coli* FRD Ip with a carboxyl-terminal truncation (Johnson

et al., 1988). Iron-sulfur clusters of some proteins such as ferredoxins, can be assembled *in vitro* by incubating apo-protein with sulfide, a thiol, and ferrous (Fe^{2+}) iron under reducing conditions (Hederstedt and Ohnishi, 1992). Alternatively, center 3 of the mammalian enzyme can be restored with either Na_2S, Fe_2SO_4 and a reducing agent or Fe^{2+} and rhodanese, a thiosulfate thioreductase (Ackrell *et al.*, 1992). More work remains to be done to determine if iron-sulfur cluster formation is an autocatalytic event or if additional enzymes are required.

Two of the iron-sulfur clusters of the Ip most likely assemble before the Ip associates with the Fp. When the *E. coli* FRD Ip subunit is expressed in the absence of the Fp, centers 1 and 3 are still detected, demonstrating that the Fp is not required for their assembly (Johnson *et al.*, 1988). The lack of center 2 formation is possibly because center 2 may bridge the Fp and Ip. The presence of full length wild-type Fp however, slows proteolytic degradation of the Ip (Hederstedt *et al.*, 1985), suggesting that the Fp and Ip may form an assembly intermediate. Therefore, although the Fp is not needed for all of the iron-sulfur clusters assembly, it may help to stabilize the Ip.

Prior assembly of the iron-sulfur clusters may or may not be mandatory for the Ip and the Fp to associate. Fe limiting conditions may prevent iron-sulfur cluster formations. However, under such conditions the Fp and Ip subunits themselves are not detectable; hence its impossible to assay the state of their assembly. The absence of the subunits might be due to regulation at the transcriptional or translational level, or caused by rapid proteolysis (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992).

Iron-sulfur cluster formation plays a role in the attachment of the Ip subunit to the membrane. A carboxyl-terminal truncation of the *E. coli* FrdB subunit which assembles only the center 1 cannot associate with the anchoring subunits (Johnson *et al.*, 1988). Further experiments using site directed mutagenesis of this Ip demonstrated that assembly of center 3 and possibly of center 2 are essential for membrane attachment (Manodori *et al.*, 1992) These results are in accord with the observation that reconstitution of the

mammalian holoenzyme requires the center 3 cluster to be intact (Ackrell *et al.*, 1992). Thus, formation of at least one of the iron-sulfur clusters is a prerequisite for membrane attachment.

1-4d. Order of Assembly.

The order of subunit assembly is unclear. Some results suggest that the Ip and Fp assemble as a heterodimer before attaching to the membrane. In *E. coli* the FRD Fp and Ip form a soluble, active, heterodimer when they are overexpressed in the absence of the anchoring subunits (Lemire *et al.*, 1982) A preassembled heterodimer of *E. coli* FRD or the mammalian SDH subunits can also be reconstituted *in vitro* with their respective anchoring subunits (Cecchini *et al.*, 1986; Ackrell *et al.*, 1992). In contrast, pulse chase experiments suggest that the Ip attaches to the membrane before association with the Fp (Latour and Weiner, 1988). Additionally, in heme auxotrophs of *B. subtilis* starved for heme, the Fp and Ip are both expressed and soluble, but not associated with each other (Hederstedt and Rutberg, 1980). A similar result is seen with some *B. subtilis* mutants of the anchoring subunit; the Fp and Ip are found in the cytoplasm, but not associated with each other (Hederstedt and Rutberg, 1980; Hederstedt *et al.*, 1982). In some cases, the Ip is able to form a soluble active dimer with the Fp which can then attach to the anchoring subunits, but this is not necessarily the order of assembly for every SDH or FRD complex *in vivo*.

1-4e. Flavoprotein Assembly.

In contrast to iron-sulfur cluster formation, the binding of FAD by the Fp is not essential for assembly with either the Ip or the anchoring subunits. To examine the role of covalent binding of FAD in the *E. coli* FRD, Blaut *et al.* (Blaut *et al.*, 1989) changed by site-directed mutagenesis, the His-44 codon, which is the point of FAD attachment, to a Ser, Tyr, Cys, or Arg codon. The resulting mutants were able to bind FAD only non-covalently, but were still assembled into a holoenzyme; the holocomplexes from the His

to Ser, Tyr or Cys mutated Fp also retained significant levels of FRD activity. Moreover, different point mutations in the *B. subtilis* SDH Fp gene prevent covalent attachment, or abolish FAD binding completely; yet these mutant Fp still assemble with the Ip and the anchoring subunit (Hederstedt, 1983; Maguire *et al.*, 1986).

Although the binding of FAD is not needed for the formation of the holoenzyme, it most likely occurs before the Fp is assembled with the other subunits. In *B. subtilis* mutants when the assembly of SDH has been prevented by either the lack of heme or of a functional anchoring subunit, the Fp is found in the cytoplasm, with covalently attached FAD (Hederstedt and Rutberg, 1980; Hederstedt *et al.*, 1982). Other experiments have examined flavinylation by expressing the *E. coli* FRD Fp in the absence of the other subunits. The Fp has covalently bound FAD (Cole *et al.*, 1985), but has no activity (Cecchini *et al.*, 1986). The lack of activity is in contrast to experiments with *W. succinogenes* where the FRD Fp expressed by itself has activity (without Ip) (Ungen and Kroger, 1981).

The assembly of SDH begins with the localization of the anchoring subunits to the membrane. The insertion of some of the various cofactors into the appropriate subunits is then mandatory for assembly to progress. Next, either the Fp and Ip form a dimer that then assembles with the anchoring subunits or the Ip and Fp bind the anchoring subunits in a stepwise manner.

1-5. COVALENT ATTACHMENT OF FAD.

Covalently bound FAD is not unique to SDH and FRD enzymes; several proteins covalently bind FAD by a variety of linkages (Decker, 1993). Most of these flavoproteins are dehydrogenases or oxidases involved in catabolism. Aside from the N3 of histidine, FAD is found attached to some flavoproteins at N1 of histidine, and at cysteine and tyrosine residues. An assortment of different flavoproteins, with different

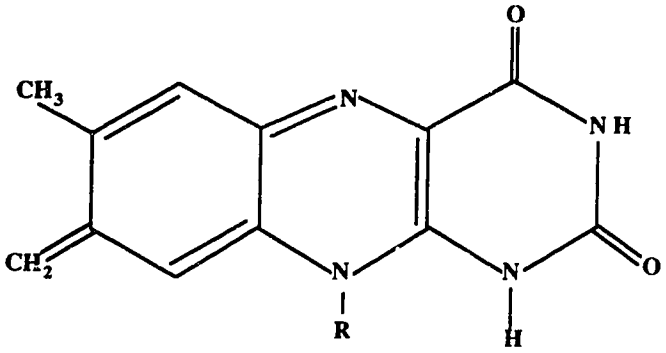
linkages can be found in the same organism. While higher eukaryotes have at least four flavoproteins which covalently bind FAD, SDH may be the only one in yeast (Singer *et al.*, 1965). Almost all of the flavoproteins in eukaryotes are localized to the mitochondria. Details concerning how FAD is attached to flavoproteins (flavinylation) are limited.

1-5a. Chemistry of Bond Formation.

Two theories explaining the mechanism of formation of the covalent flavin-protein bond are: the ATP method and the quinone methide intermediate (Decker, 1993). Significantly, formation of a covalent bond requires expenditure of free energy. In the ATP method, ATP provides the energy needed for bond formation by activating the flavin or the protein. The flavin could be activated by hydroxylation and then phosphorylation at the 8-α carbon or the accepting amino acid residue could be phosphorylated. Either of these phosphorylated species would be highly reactive, thereby permitting the creation of the flavin-protein bond. However, hydroxylation of the flavin would most likely require oxygen, which has not been shown to be a requirement for flavinylation. Alternatively, the quinone methide intermediate theory suggests that the flavin binds the protein as a quinone methide species (Figure 1-5), which would result in a covalently bound, reduced flavin. Re-oxidation of the flavin provides the required energy. However, flavinylation can proceed under anaerobic conditions, although either excess free FAD in the reaction mix or the protein itself may accept the electrons from the reduced flavin (Brandsch and Bichler, 1991). In addition, the FAD derivative 5-deaza-FAD, which is unable to form a quinone methide species cannot be covalently attached to a flavoprotein (Brandsch and Bichler, 1991). This latter mechanism requires a base to extract a proton from the FAD and a precise protein conformation to hold the FAD at the necessary place and distance from the amino acid. While mechanisms

employing ATP would most likely require another protein, a reaction involving the quinone methide species could conceivably occur without additional proteins.

Figure 1-5. Quinone Methide Form of FAD.



1-5b. Protein modification.

Flavinylation is a protein modification that could occur either co- or post-translationally. In the primary sequence of the different flavoproteins, no flavinylation signal is apparent. No sequence homology is seen among flavoproteins of different functions aside from the His residues at which FAD binds (Lang *et al.*, 1991). However, most of these proteins have a putative Rossman nucleotide binding fold upstream of the site of attachment. Possibly, conformation is more important for identifying proteins for flavinylation than the amino acid sequence.

1-5c. FAD Attachment to 6-HDNO.

Much of the information about FAD attachment is derived from studies with the flavoprotein 6-hydroxy-D-nicotine oxidase (HDNO) from *Arthrobacter oxidans*. HDNO binds FAD at its His-71 residue by an 8α-(N3)-histidyl flavin linkage, the same linkage as seen in SDH and FRD. Involved in catabolism of D-nicotine and D-nornicotine, HDNO oxidizes 6-hydroxy-D-nicotine to 6-hydroxy-methylmyosmine and the subsequent hydrolysis of the latter to [6-hydroxypydridyl(3)]-(N-methylaminopropyl)-

ketone (Decker and Brandsch, 1991). The enzyme is composed of a single protein with a molecular mass of approximately 48 kDa. Considerable work has been done with HDNO because the apoprotein can be isolated and then flavinylated *in vitro* (Brandsch and Bichler, 1991).

1-5d. Is FAD Attachment Autocatalytic?

Studies with apo-HDNO show that FAD attachment does not require any other proteins. When HDNO is expressed in *E. coli*, approximately 60% of the protein has covalently bound FAD, demonstrating that flavinylation does not require any host specific factors (Brandsch and Bichler, 1985). The amount of apo-protein can be increased to over 95% by adding diphenylene iodinium, a compound which inhibits FAD attachment possibly by interfering with protein conformation (Brandsch and Bichler, 1987b; Brandsch and Bichler, 1991). Under appropriate conditions, the purest preparations of apo-HDNO are able to covalently bind FAD (see below; Brandsch and Bichler, 1991). Thus, the covalent attachment of FAD is autocatalytic, at least for HDNO.

1-5e. Allosteric effectors.

Flavinylation does require certain factors which may modulate holoenzyme formation. Early experiments suggested that an ATP regenerating system is needed (Brandsch and Bichler, 1987b). However, subsequent studies demonstrated that what is actually required are phosphorylated three-carbon compounds (Nagursky *et al.*, 1988; Brandsch and Bichler, 1989). The three-carbon compounds, such as phospho*enol*pyruvate and glycerol-3-phosphate, are proposed to act as allosteric effectors, allowing the apo-protein to obtain the conformation needed for flavinylation. In fact, FAD attachment can occur in lysates that have been treated with apyrase, an enzyme which hydrolyses ATP and ADP to AMP (Brandsch and Bichler, 1992).

Interestingly, high concentrations of glycerol alone can stabilize the apoprotein, allowing covalent attachment of FAD although not to the same extent as observed in the presence of glycerol-3-phosphate (Brandsch and Bichler, 1991). Apparently, when the protein is expressed in *E. coli*, the limiting factor *in vivo* for flavinylation is the concentration of the allosteric effectors (Brandsch and Bichler, 1992). Curiously, in *A. oxidans*, the metabolites which induce expression of 6-HDNO are not the same molecules required for FAD attachment (Brandsch and Bichler, 1989). While flavinylation is promoted by glycolytic intermediates, 6-HDNO expression is induced by D- and L- nicotine in stationary phase cells. This requirement for certain allosteric effectors may be a method of controling holoenzyme biogenesis *in vivo* (Brandsch and Bichler, 1989).

1-5f. Does FAD Attachment Occur Co- or Post-translationally?

Inconsistency in the literature exists as to whether FAD attachment occurs co- or post-translationally. Studies using *E. coli* lysates irradiated with ultraviolet light suggest that FAD attachment occurs co-translationally (Brandsch and Bichler, 1986). In accord with this finding are experiments demonstrating that carboxyl-terminal truncations of HDNO are still flavinylated, but the evidence for FAD attachment in these studies is not convincing (Brandsch and Bichler, 1987a). However, recent mutagenesis experiments show that the amino acid residues located 11 and 12 residues from the carboxyl terminus are essential for flavinylation (Brandsch *et al.*, 1993). Thus, this latest finding strongly argues that FAD attachment is a post-translational modification, although these discrepancies as to when flavinylation occurs have not been reconciled.

1-5g. Chaperone Involvement.

The involvement of the GroE proteins in the folding and cofactor attachment of HDNO has been examined. HDNO could not be co-immunoprecipitated by GroEL anti-serum from crude cell lysates (Brandsch and Bichler, 1992). Since these

immunoprecipitations were done in the presence of 1% Triton X-100, a condition that is known to disrupt chaperone-protein association, this result cannot exclude the possibility that GroEL interacts with HDNO *in vivo*. Furthermore, anti-GroEL serum is able to immunoprecipitate a mutated version of HDNO, demonstrating that the chaperonin does interact with HDNO (Brandsch *et al.*, 1993). (Notably, these immunoprecipitations were done in the absence of detergent). Addition of the GroE proteins *in vitro* does catalytically increase the rate of FAD attachment to native apo-HDNO (Brandsch and Bichler, 1992). However, this increase in flavinylation is due to nonspecific effects from the increase in overall protein concentration; the increase seen is not ATP dependent. The GroE proteins do, however, catalyze re-folding and FAD attachment of denatured holoenzyme and denatured apoprotein respectively (Brandsch and Bichler, 1992). Interestingly, the rates of re-folding and holoenzyme formation are parallel, lending support to the arguement that GroE participates in FAD attachment by correctly folding the protein. These studies also revealed that the apoprotein can adopt a conformation that renders it incompetent to covalently bind FAD. Another set of experiments demonstrated that mutation of certain cysteine residues to serine affected the extent of both flavinylation and the interactions with the GroE proteins (Brandsch *et al.*, 1993). Some of the mutant proteins examined could no longer covalently bind FAD. The lack of attachment is attributed to an inability to obtain the correct protein conformation rather than the requirement of the cysteine residue for the chemical mechanism of bond formation. Many of these studies examining flavinylation strongly argue that correct protein conformation is critical for cofactor attachment. Therefore, the chaperones may catalyze the folding of the apoprotein to a conformation required for FAD attachment but do not catalyze the formation of the covalent flavin-protein bond itself.

1-5h. Flavinylation of SDH and FRD.

Our understanding of cofactor attachment to the SDH and FRD flavoproteins is at a preliminary stage. Unlike HDNO, when the Fp of *B. subtilis* SDH is expressed in *E. coli*, it is not flavinylated (Hederstedt *et al.*, 1987). This lack of FAD attachment implies that host specific factors are needed, or that FAD attachment in SDH is not autocatalytic, or both. Analogous to HDNO, the prokaryotic SDH and FRD Fp require allosteric effectors for FAD attachment. These effectors are citric acid cycle intermediates such as succinate, fumarate, or citrate; not the same molecules required for flavinylation of HDNO (Brandsch and Bichler, 1989). Thus, while the need for allosteric effectors may be a common feature to flavinylation, the molecules that act as effectors vary for the different flavoproteins. The need for citric acid cycle intermediates for flavinylation of SDH and FRD may be a mechanism of controling their biogenesis *in vivo*. Whether FAD attachment in the SDH and FRD Fp occurs co- or post-translationally is not known, although work by Cecchini indicates that FAD attachment in the Fp of *E. coli* FRD requires the entire flavoprotein and occurs post-translationally (Cole *et al.*, 1985). Whether other proteins are required for folding or catalysis of the flavin-protein bond in SDH and FRD is also not known. Some insights into the flavinylation of SDH and FRD are gained by examining the process in HDNO, but additional complications may arise with the SDH and FRD Fp because they are parts of multimeric complexes. Furthermore, SDH is found in the mitochondria of eukaryotes, hence, the targeting and import of the Fp adds another level of complexity to FAD attachment.

1-6. THESIS PROBLEM.

To gain a better understanding of import and assembly of mitochondrial complexes, we examined these processes for the yeast SDH. Having only four subunits, which are all nuclear-encoded, SDH is a simpler respiratory complex. Additionally, in *S. cerevisiae*, genes can be easily manipulated by molecular biological and yeast genetic

techniques, providing a powerful tool for investigating events *in vivo*. Moreover, this system is also sophisticated as yeast SDH contains four or five different cofactors. Therefore, using the yeast SDH, the relationships between import, cofactor attachment, and assembly could be explored both *in vivo* and *in vitro*.

We were intrigued by a number of basic questions about SDH and its Fp. How is SDH assembled? Why is FAD covalently attached rather than non-covalently bound by the Fp? Is the FAD attached by an autocatalytic mechanism or by a specific enzyme? Does FAD attachment occur after either import or assembly, or is it a prerequisite for either event?

To thoroughly take advantage of the yeast system, some of our preliminary goals were to isolate, clone, and sequence the yeast SDH genes. At the start of my studies, only the Ip subunit had been cloned and sequenced. We had previously tried to isolate the SDH genes by screening for SDH mutants; a genetic screen had the advantage that it could identify not only the structural genes, but also other proteins that were required for assembly. Unfortunately, our mutant screens up to that point had failed to identify any of the SDH genes. However, we reasoned that the sequence homologies seen between the SDH and FRD Fps might enable us to amplify a portion of the Fp gene by using degenerate oligonucleotide primers in a polymerase chain reaction. With the fragment of the gene that was amplified, I was able to create a disruption mutant for the Fp and isolated the entire *SDH1* gene from a genomic library. This was the first complete eukaryotic SDH Fp gene sequence.

Several insights into mitochondrial biogenesis and SDH function and assembly, both expected and otherwise, were gained from the disruption mutant and the *SDH1* sequence themselves. An *SDH1* disruption mutant is unable to grow on minimal media using non-fermentable carbon. However, slow growth is seen with non-fermentable carbon sources on rich media; the same growth phenotype is observed although to varying degrees with disruption mutants in other the SDH genes. Hence, *sdh* mutants do

have the classical petite phenotypes expected from the loss of a Kreb's cycle and respiratory chain enzyme but our mutant screens were unlikely to succeed as we had been screening for a complete absence of growth. An *sdh1* mutant lacks detectable SDH activity, although the other respiratory complexes assayed are not effected, suggesting that their assembly is independent of each other. Additionally, Ip was not detectable in the *SDH1* disruption mutant, implying that the Fp and Ip may form an assembly intermediate and be unstable in the absence of the other. The *SDH1* sequence revealed that the yeast Fp is highly homologous to other SDH and FRD, and accurately predicted cleavage site of the presequence and the histidine residue that ligates FAD. Contrary to prokaryotic sequences, the point of FAD attachment in yeast Fp is not the first histidine residue to appear in the sequence, discrediting the notion that FAD is attached to flavoproteins co-translationally at the first histidine to emerge from the ribosome. With the cloned *SDH1* gene, I did *in vitro* import experiments during which the Fp was imported into and proteolytically processed by isolated mitochondria. With the disruption mutant and sequence, we were able to further examine SDH Fp assembly.

One of the most striking characteristics of the SDH Fp is its covalently attached cofactor. Aside from the relevance to mitochondrial biogenesis, I found the process of FAD attachment an interesting puzzle conceptually; how does the cofactor, that is encompassed by this protein, become covalently linked to the protein, and why would the this linkage be necessary? To examine FAD attachment, we first had to have a means to distinguish between the apo- and the holo-Fp.

During the search to find a system that could detect FAD attachment, it became obvious that a mutant Fp which could not covalently bind FAD was an essential control. Even though it cannot covalently attach the cofactor, the Fp mutant that I constructed still binds FAD non-covalently. We also obtained some information about SDH function and assembly from the mutant itself. Only SDH activity is dependent on covalent FAD

attachment; it is dispensable for import, for assembly of the SDH holocomplex, and for FRD activity.

I then expended a considerable amount of time and effort trying to identify a technique that could quickly and reliably differentiate between the mutant or apo-Fp, and holo-Fp. After trying a wide variety of techniques (which I list in Appendix A only as a warning for future investigations into such an assay), I managed to raise an antiserum directed against the cofactor. This antiserum recognizes only the cofactor, and not the protein component of the Fp, hence it will only bind to the holo-Fp, but not the mutant or apo-Fp. With the anti-serum, modification of the Fp could be examined *in vivo* and *in vitro*.

Using the antiserum and with the mutant Fp as a control, I examined cofactor attachment first by studying the process *in vivo*. Pulse-chase experiments indicated the time-frame of flavinylation, and that the modification occurs after the Fp has been proteolytically processed. This was the first time that the rate of flavinylation of a protein was determined *in vivo*. These experiments also demonstrated that the carbon source which the cells were grown on drastically affects the rate and extent of FAD attachment. I was enticed by this observation since one possible explanation for the effect of carbon source is the differential expression of another protein which catalyzes the modification of the Fp. By this time, the genes for all of the other SDH subunits were available, and disruption mutants in each of the SDH genes had been constructed. Therefore, I investigated the possible roles of the other SDH subunits in cofactor attachment to the Fp and demonstrated that none of them were essential for this modification. The results from the *in vivo* experiments suggested to us that FAD is attached to the Fp inside the mitochondria, before assembly of the subunit into the holoenzyme.

To further unravel the mechanism of cofactor attachment, I developed *in vitro* flavinylation systems. To begin with, I examined FAD attachment to the Fp that had been imported into isolated mitochondria. The import experiments indicated that the Fp

is modified post-translocationally, that the flavin is attached as FAD, and that some degree of folding was necessary. Although this system was fundamental for understanding the process of FAD attachment, it had the limitation that the modification could only be examined under conditions that would allow import into the mitochondria. To continue investigating FAD attachment, I developed a flavinylation system with mitochondrial lysates. Overall, the studies with mitochondrial lysates were not as definitive or as satisfying as the prior experiments on flavinylation, although this system does provide a useful starting point for future experiments. Our results with this system did demonstrate that modification is inhibited by the presequence, and that this process needs a matrix protein component and ATP. Interestingly, with native immunoprecipitations, we showed that the mitochondrial chaperonin Hsp60 binds the Fp both before and after FAD has been attached. Both of the *in vitro* systems showed that substrate molecules dramatically stimulate cofactor attachment. Along with some details about flavinylation, the *in vitro* experiments demonstrated that FAD attachment occurs inside the mitochondria, after import, presequence cleavage, and partial folding of the Fp. From my studies we can propose a model for the assembly of the Fp which is discussed in chapter 7.

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Chapter 2

Isolation and Characterization of a *S. cerevisiae* Mutant Disrupted for the SDH Flavoprotein Subunit

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2-1. INTRODUCTION

Succinate dehydrogenase (SDH) participates in both the membrane-bound electron transfer chain on the mitochondrial inner membrane and the tricarboxylic acid cycle located in the matrix. Together, the Fp and Ip subunits form a catalytically active dimer capable of oxidizing succinate or reducing fumarate using artificial electron acceptors or donors. Two hydrophobic subunits function in anchoring the catalytic dimer to the inner membrane, and are required for transfering electrons to the natural acceptor, ubiquinone (Hatefi and Galante, 1980). In *Saccharomyces cerevisiae,* each of the four subunits is believed to be nuclear-encoded, cytosolically synthesized, and transported into mitochondria for assembly into the SDH complex.

Since *S. cerevisiae* is a facultative anaerobe, a yeast mutant deficient in respiration is still capable of growth on glucose and other fermentable carbon sources. Accordingly, the iron-sulfur subunit was recently cloned and its disruption analyzed (Gould *et al.*, 1989; Lombardo and Scheffler, 1989; Lombardo *et al.*, 1990). Hence, we expected a yeast *sdh1* mutant to be viable and that its phenotype could be investigated.

A number of sequences or partial sequences of SDH flavoproteins have been determined. By exploiting the sequence similarities of this family of enzymes in a polymerase chain reaction, we were able to isolate a partial genomic clone of the flavoprotein subunit (*SDH1*).

Using the partial *SDH1* clone for targeted gene disruption, we have constructed and characterized a yeast *sdh1* mutant. The flavoprotein mutant is unable to utilize glycerol as a carbon source for growth, has greatly reduced levels of SDH-dependent activities, and contains significantly lower levels of covalently-attached FAD. Disruption of the *SDH1* gene also results in the simultaneous loss of the iron-sulfur subunit from mitochondrial membrane fractions.

2-2. MATERIALS AND METHODS

Strains and Vectors. The *Saccharomyces cerevisiae* strain MH125 (MATa, trp1, ura3-52, leu2-3, leu2-112, his3, his4, rme, ρ^+) was used as the source of chromosomal DNA for amplification reactions and to construct the strain sdh1L6 (MATa, trp1, ura3-52, his3, his4, rme, SDH1::LEU2, ρ^+) by targeted gene disruption. MH124 (MATα, trp1, ura3-52, leu2-3, leu2-112, his3, his4, rme, ρ^+) was mated to the disruption mutant to create a diploid for analysis of marker segregation. The *E. coli* strain UT580 (Δ(lac-pro), rK-, mK+, Tn10, supD, lacIq, F', traD, pro+, 'LacZΔM15) was used for all DNA manipulations. The amplification product was first cloned into pBluescript II (Stratagene, La Jolla, CA 93037, U.S.A.).

Media. The yeast media used were SD, a synthetic glucose medium containing 0.67% yeast nitrogen base and 2% glucose; YPG, a complex medium containing 1% yeast extract, 2% peptone, and 3% glycerol; YPD, a complex medium containing 1% yeast extract, 2% peptone, and 2% glucose; YPGal, a complex medium containing 1% yeast extract, 2% peptone, and 2% galactose; YPD-0.6%, 1% yeast extract, 1% peptone, 0.6% glucose, 0.1% KH_2PO_4, 0.12% ammonium sulfate, pH 6.2 (Singer *et al.*, 1965); sporulation medium containing 0.1% yeast extract, 0.05% glucose, and 1% potassium acetate. *E. coli* strains were grown in Luria broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 100μg/ml ampicillin for plasmid selection.

Polymerase Chain Reaction and Cloning. Oligonucleotides were used to amplify an internal portion of the *SDH1* gene from genomic DNA (Figure 2-1). 10μl of 10x polymerase buffer (500mM KCl, 100mM Tris-HCl, pH 8.4, 25mM $MgCl_2$, 200μg/ml gelatin), 10μl of a dNTP solution (8mM each dATP, dGTP, dCTP, dTTP), 10μl of a 10μM solution of each primer, 1μg of genomic DNA and H_2O to 100μl were mixed and overlayed with mineral oil. 2.5 units of *Taq* polymerase (BRL, Gaithersburg, MD. 20877 U.S.A.) was added and cycling was initiated. Denaturation was for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and polymerization for 2 minutes at 72°C. The cycle

was repeated 25 times. $MgCl_2$ concentrations between 2.5 and 4.0 mM were found to be optimal; lower annealing temperatures resulted in a larger number of undesired amplified products. The amplified products were ethanol precipitated, digested with the restriction enzyme, Xho1, and ligated into the likewise digested pBluescript II.

Mutant Construction. The amplified product was cloned into YIplac128 which carries the selectable marker, LEU2 (Gietz and Sugino, 1988). Approximately 15 µg of the resulting plasmid, Y1-*SDH1*, was digested at the unique Bgl II site and used to transform MH125 . Leucine prototrophs were selected on SD medium supplemented with 20 µg/ml each of histidine, uracil, tryptophan, adenine sulfate, and lysine. At least six stably transformed leucine prototrophs had lost the ability to grow on YPG and were further examined.

Southern Analysis. Genomic DNA from MH125 and sdh1L6 was isolated and subjected to Southern analysis using a digoxigenin-dUTP labeled probe prepared in a polymerase chain reaction (Southern, 1975). Probe preparation, hybridization, and detection using the chemi-luminescent compound, Lumi-Phos, were as described by the manufacturer (Boehringer Mannheim Biochemicals, Laval, Quebec H7V 3Z9).

Enzyme Assays. Succinate-DCPIP reductase activity is measured as the malonate-sensitive reduction of DCPIP (dichlorophenol indophenol) at 23 ^{o}C in 50 mM Tris-SO_4, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM NaCN, pH 7.4, in a Pye Unicam SP1750 split beam spectrophotometer. DCPIP is added to 70 µM, phenazine methosulfate to 120 µg/ml, succinate and malonate to 20 mM each. The extinction coefficient for DCPIP is 21 $cm^{-1}mM^{-1}$ (Baginsky and Hatefi, 1969). Succinate-cytochrome c reductase activity is measured as the malonate-sensitive reduction of cytochrome c in 100 mM Tris-SO_4, pH 7.4, 1.6 mg/ml horse heart cytochrome c (Sigma Chemical CO., St. Louis, Missouri 63178), 1 mM NaCN. Succinate and malonate are added to 20 mM each. For antimycin A-sensitive NADH- or glycerol-1-phosphate-cytochrome c reductase activities, freshly prepared NADH is added to 0.1 mM or glycerol-1-phosphate to 10 mM. A deflection of

0.1 absorbance units corresponds to 0.52 x 10^{-8} moles of cytochrome c reduced. Succinate-ubiquinone-1 activities were determined as described (Kita *et al.*, 1989). Ubiquinone-1 was a generous gift of R. B. Gennis. Succinate and NADH oxidase activities were measured with a Clark type electrode (Rank Brothers, Cambridge, England).

Western Blot Analysis. The approximately 0.4 kb Xho1/Hind111 fragment of the amplified product was cloned into the vector, pATH10, to create a trpE-Fp fusion protein that could be isolated in an insoluble form upon indole-acrylic acid induction (Koerner *et al.*, 1991). Rabbit polyclonal antiserum was raised using electroeluted fusion protein as antigen (Dunbar and Schwoebel, 1990). The anti-iron-sulfur protein serum prepared by Bernard Lemire was raised using a trpE-Ip fusion protein as well. Proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell Inc., Keene, NH 03431) as described by the manufacturer (Tyler Research Instruments, Edmonton, Canada, T6E 4Y5). Detection of antigens was with a goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Bio/Can Scientific, Mississauga, Ont. L5L 1C7) and the ECL western blot detection system (Amersham Canada Limited, Oakville, Ont. L6L 5T7).

Measurement of Covalent Flavin. Covalently-attached flavin was measured on a Perkin Elmer Luminescence Spectrometer Model LS50 using riboflavin as a standard as described previously (Singer *et al.*, 1971; De Kok *et al.*, 1975). Values are averages of three independent determinations. Background levels have been subtracted.

Miscellaneous Methods. Published procedures were used for transforming *E. coli* (Mandel and Higa, 1970), for recombinant DNA methods (Maniatis, 1982), and for plasmid isolation from *E. coli* (Birnboim and Doly, 1979). Yeast transformation was performed by electroporation in a BRL Cell Porator (Bethesda Research Laboratories, Gaithersburg, MD 20877) as described by the manufacturer. Microdissection of asci was performed with a de Fonbrune type micromanipulator (Technical Products International

Inc., St. Louis, Missouri 63045) after digestion with B-glucoronidase (Sigma Chemical CO., St. Louis, Missouri 63178). Affinity purification of antibodies has been described (Sambrook, 1989). SDS-polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970).

2-3. RESULTS

Synthesis of a Partial SDH1 Clone. The complete nucleotide sequences of the *E. coli* and the *B. subtilis* SDH flavoprotein subunits have been published (Wood *et al.*, 1984; Phillips *et al.*, 1987). The proteins demonstrate considerable sequence similarity in certain regions of their primary structures such as their sites of FAD attachment. In addition, the sequence of the tryptic, flavin-containing peptide of the bovine SDH flavoprotein is available (Singer *et al.*, 1973). Annette von Kieckebusch-Gück used these sequences to design degenerate oligonucleotides that could serve to amplify an internal fragment of the yeast *SDH1* gene in a polymerase chain reaction. In Figure 2-1, the amino acid and the derived primer sequences are presented. Primer A is designed to peptide regions immediately following the histidine residues to which are attached the FAD cofactors (His44 in the *E. coli* protein; His40 in the *B. subtilis* protein; the location of the histidyl-flavin within the bovine sequence has not been determined). Primer B is designed to regions of the polypeptides thought to be involved in the formation of the top of the AMP binding domain (Cole *et al.*, 1985; Phillips *et al.*, 1987). In both cases, the degeneracy of the oligonucleotide primer is a balance between the need to accomodate differences between the known sequences and *S. cerevisiae* codon usage (Bennetzen and Hall, 1982; Sharp *et al.*, 1988).

With these primers, I amplified a fragment of the yeast *SDH1* gene from genomic DNA with a polymerase chain reaction. The amplification reaction generated a major product migrating at 0.95 kb, as well as minor products migrating at 0.8 and 0.4 kb upon agarose gel electrophoresis (data not shown). The expected product size based on the

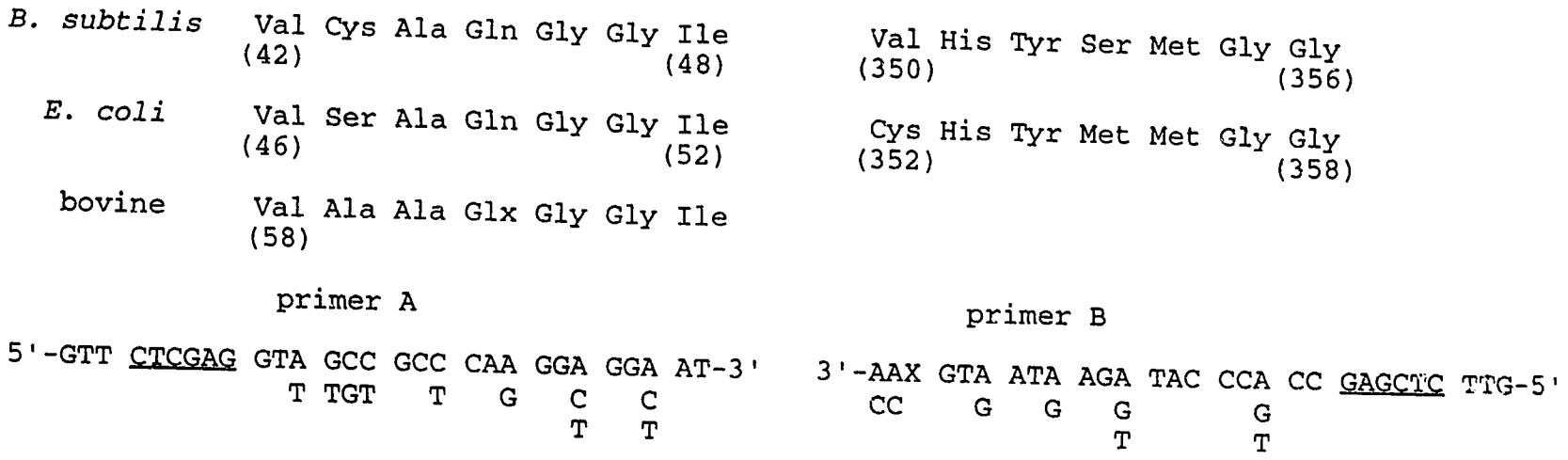


FIGURE 2-1. Design of oligonucleotide primers. Primer A spans the regions of the *B. subtilis*, *E. coli*, and bovine flavoprotein subunits immediately following the sites of covalent flavin attachment. The location of the flavin peptide sequence within the bovine flavoprotein subunit sequence was determined subsequently (Birch-Machin *et al.*, 1992). Primer B spans regions of the *B. subtilis* and *E. coli* subunits in the central portions of the flavoprotein subunits believed to participate in cofactor binding. Each amino acid sequence was backtranslated to all possible DNA sequences. Infrequently used codons were omitted to reduce oligonucleotide degeneracy. The three 5' most nucleotides are present to facilitate digestion of the amplified product at the Xho1 restriction site (underlined). X = any nucleotide.

choice of primers within the *E. coli* and the *B. subtilis* flavoprotein subunits is about 0.96 kb. The major product was cloned and partial nucleotide sequence was determined by the dideoxy chain termination method using double stranded plasmid DNA as templates. The 0.95 kb fragment displayed considerable similarity with the *E. coli* SDH flavoprotein gene in both its nucleotide and predicted amino acid sequences (see chapter 3). When amplification reactions were performed at 52 °C, a major product of 0.93 kb was obtained; it was identified as a portion of the yeast 16S rRNA.

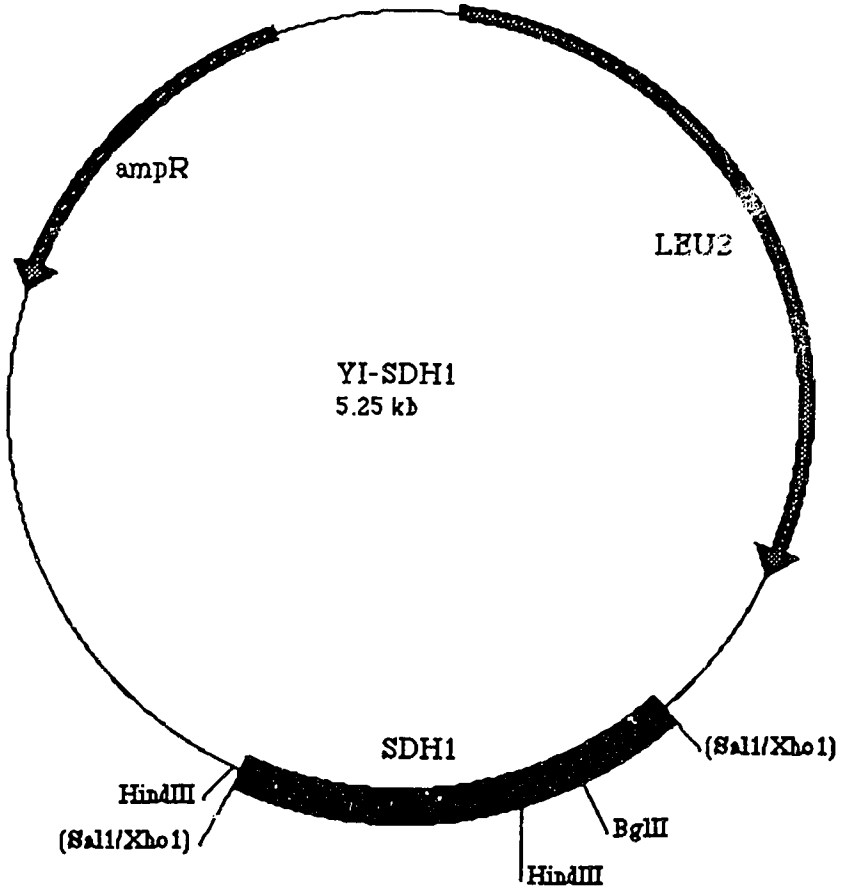
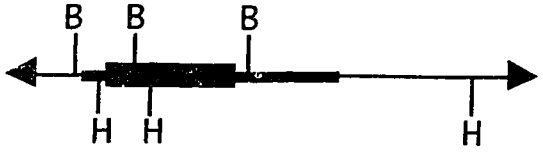


Figure 2-2. *Restriction Map of Y1-SDH1.* The 0.95 kb polymerase chain reaction amplification product was cloned into the Sal1 digested vector, YIplac128, to create the plasmid Y1-SDH1. The vector carries the B-lactamase (ampR) and the LEU2 genes for selection in *E. coli* and yeast, respectively.

Mutant Construction. A targeted gene disruption mutant, sdh1L6, was constructed by first cloning the partial SDH flavoprotein gene into the integration vector, YIplac128, which carries the LEU2 gene as a selectable marker. The resulting plasmid, Y1-SDH1 flavoprotein, was linearized at the unique Bgl II site and transformed into MH125 (Figure 2-2). Integration by homologous recombination into the chromosomal copy of the *SDH1* gene should result in both N- and C-terminally truncated copies of the *SDH1* gene separated by the vector and selectable marker sequences (Figure 2-3B and 2-3C). This arrangement was confirmed by Southern analysis (Figure 2-4). Chromosomal DNA from MH125 and from 6 independent isolates of sdh1L6 was digested with Bgl II; with the 0.95 kb amplification product as probe, MH125 displays two hybridizing bands that migrate at 1.0 and 0.42 kb. As expected, the sdh1L6 isolates display the same two bands as well as an additional band of 5.3 kb that results from the insertion of the integration plasmid.

2-3A. Map of a Wild-type Chromosome



2-3B. Map of the*SDH1* Disruption Chromosome

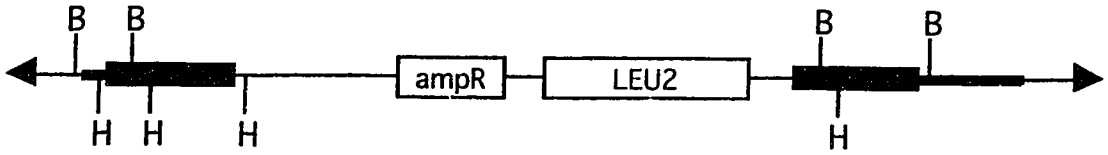
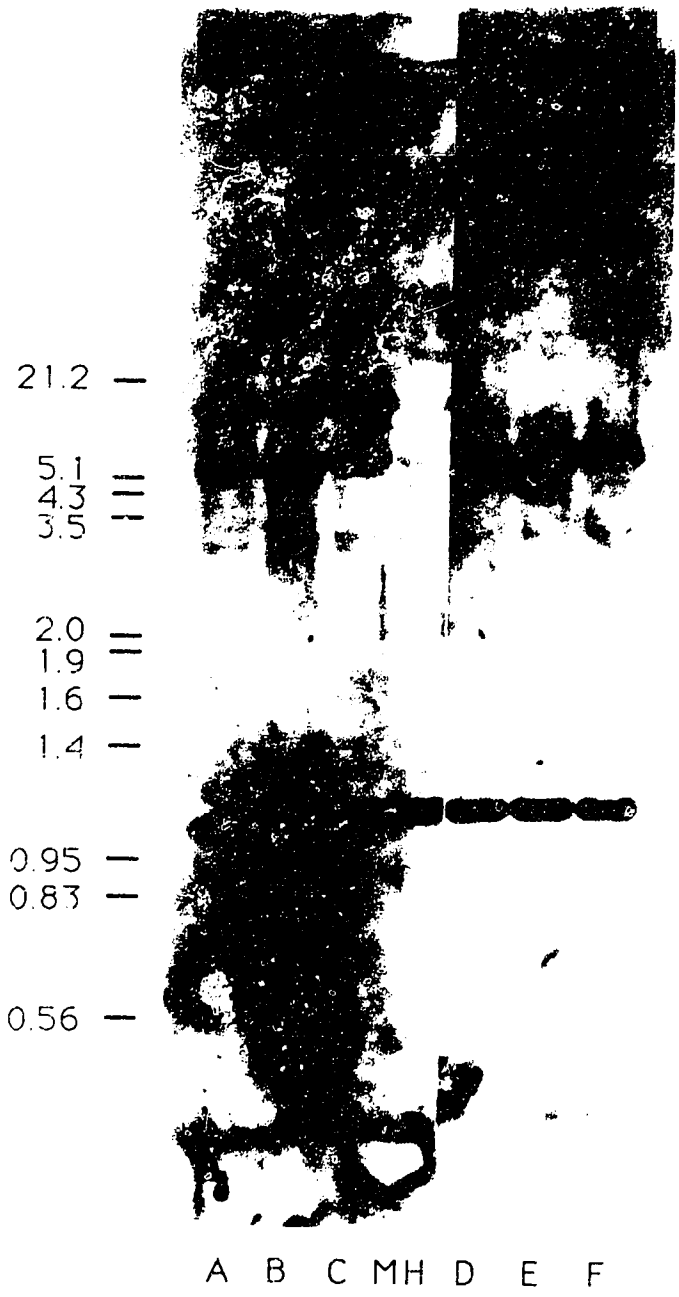


Figure 2-3. Restriction map of the chromosomes. *A.* restriction map in the wild type strain, MH125, is presented. The thick line corresponds to the cloned amplification product. The intermediate size line corresponds to the *SDH1* gene not represented in pY1-SDH1 flavoprotein; its length is based on an estimated protein product size of 67 kDa. B=Bgl11; H=Hind111. *B.* Restriction map of a *SDH1* disruption mutant is presented. Digestion of pY1-SDH1 flavoprotein with Bgl11 and transformation into MH125 led to the targeted integration of the linearized plasmid at the *SDH1* locus present in sdh1L6. This recombination event produces both 5' and 3' truncations of the *SDH1* gene separated by plasmid sequences.

Figure 2-4 Targeted Disruption of the SDH1 gene. Southern blot analysis of MH125 (MH) and 6 isolates (A-F) of sdh1L6 genomic DNA reveals the presence of an additional hybridizing species at 5.3kb in sdh1L6. Each lane contains 2µg of genomic DNA that was digested with Bgl11, electrophoresed on a 1% agarose gel, transferred to Hybond-N as described by the supplier (Amersham Canada Limited, Oakville, Ont. L6L 5T7), and subjected to Southern analysis. The positions of size markers in kb are indicated on the left.


21.2
5.1
4.3
3.5
2.0
1.9
1.6
1.4
0.95
0.83
0.56
A B C MH D E F

sdh1L6 demonstrates very slow growth on YPG and no growth on minimal glycerol media. A diploid between MH124 and sdh1L6 is proficient for growth on glycerol indicating the disruption mutation is recessive. Sporulation of the diploid and microdissection of asci results in a 2:2 segregation for growth on glycerol, as expected. Furthermore, the petite phenotype cosegregates (30 of 30) with the LEU2 marker indicating that the two traits are tightly linked.

Table 2-1. Enzymatic activities in submitochondrial particles.

Assay activity	MH125	sdh1L6	
succinate oxidase[a]	173	ND	(<5%)
succinate-cytochrome c reductase[b]	14.7	1.7	(12%)
succinate-ubiquinone reductase[c]	39	ND	(<5%)
succinate-DCPIP reductase[d]	290	ND	(<3%)
TCA precipitable flavin[e]	55	ND	(<10%)
NADH oxidase[a]	320	330	(103%)
G-1-P-cytochrome c reductase[b]	16.5	11.3	(69%)
NADH-cytochrome c reductase[b]	41.7	41.5	(100%)
cytochrome c oxidase[f]	790	1450	(184%)

Strains were grown to stationary phase in YPD-0.6%, harvested, resuspended in 20mM HEPES-KOH, pH7.4 containing 1mM phenylmethylsulphonyl fluoride, lysed by 3 passages through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 20,000psi and 4°C. Approximately 75% cell breakage was attained. Unlysed cells and envelopes were removed by centrifuging at 11,000 x g for 10 minutes, and the supernatant was spun at 16(x g for 60 minutes. The pellet of submitochondrial particles was resuspended in 20mM HEPES-KOH, pH 7.4 and assayed as described under experimental procedures. [a] Activities are expressed as natoms of oxygen $min^{-1}mg^{-1}$; [b] nmoles of cytochrome c reduced $min^{-1}mg^{-1}$; [c] nmoles of ubiquinone-1 reduced $min^{-1}mg^{-1}$; [d] nmoles of DCPIP reduced $min^{-1}mg^{-1}$; [e] pmoles of flavin mg^{-1}; [f] nmoles of cytochrome c oxidized $min^{-1}mg^{-1}$; ND, not de le.

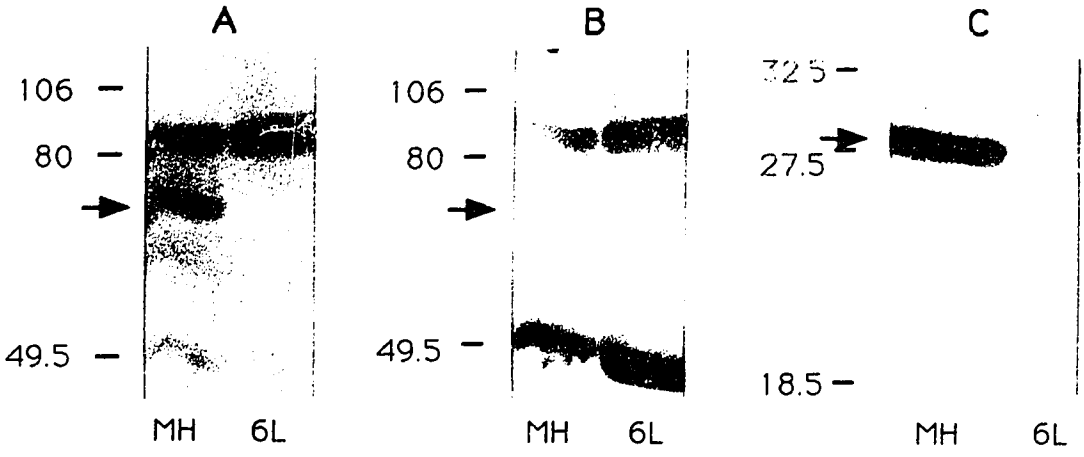
Characterization of the Mutant. Submitochondrial particles of mutant and wild-type strains were prepared an lyzed for the presence of various enzymatic

activities (Table 2-1). sdh1L6 shows greatly decreased levels of malonate-sensitive succinate oxidase, succinate-cytochrome c reductase, succinate-ubiquinone-1 reductase, and succinate-DCPIP reductase activities as compared to the parent levels. Thus this disruption negates succinate:oxidoreductase activity with both the natural and artificial electron acceptors. In contrast, the levels of NADH oxidase, glycerol-1-phosphate-cytochrome c reductase, antimycin A-sensitive NADH-cytochrome c reductase, and cytochrome c oxidase activities are comparable to those in MH125. These findings clearly indicate that the disruption has specifically affected the SDH complex.

Quantitation of Covalently-bound Flavin. A characteristic feature of the succinate-fumarate oxidoreductases is the unusual covalent attachment of an FAD molecule to a histidine residue near their N-termini. SDH is the only known enzyme in *S. cerevisiae* to possess such a cofactor (Singer *et al.*, 1965). The amount of flavin-derived fluorescence in a trichloroacetic acid insoluble form is therefore a direct measure of the levels of succinate dehydrogenase. The mutant sdh1L6's submitochondrial membrane preparations contain no detectable flavin-derived fluorescence above background levels (Table 2-1).

Western Blot Analysis. We raised antibodies to the SDH flavoprotein and Ip subunits by constructing and purifying fusion proteins that served as immunogens (Koerner *et al.*, 1991). Affinity-purified SDH flavoprotein antibodies detected an antigenic protein of 67kDa in wild-type, but not mutant mitochondrial membranes. This protein was not detected with pre-immune serum (Figure 2-4A and 4B). We also used antibodies directed against the iron-sulfur subunit (Bernard Lemire, unpublished data). A 28kDa protein is identified in MH125 membranes, but not in mutant membranes (Figure 2-4C). No signal was present when the Ip pre-immune serum was used (data not shown).

Figure 2-5 Western blot analysis of mutant and wild-type mitochondria. Mitochondria from MH125 and sdh1L6 grown on YPGal were isolated as described (Daum *et al.*, 1982). Fifty µg mitochondrial protein per lane was separated by SDS-gel electrophoresis and transferred to nitrocellulose membranes for western blot analysis as described in the methods section. The antibodies used are: A, affinity purified Fp antibodies; B, Fp pre-immune serum; C, Ip subunit antiserum. The arrows refer to the Fp subunit in A and B, and to the Ip subunit in C. The positions of size markers in kDa are indicated on the left.


A
106
80
49.5
MH
6L
B
106
80
49.5
MH
6L
C
32.5
27.5
18.5
MH
6L

2-4. DISCUSSION

This report describes the disruption of the gene for the flavoprotein subunit of the yeast mitochondrial enzyme, succinate dehydrogenase, by targeted recombination with a partial clone. The yeast gene is sufficiently closely related to its *E. coli* and *B. subtilis* counterparts to be amplified in a polymerase chain amplification reaction using degenerate oligonucleotides as primers. The regions of similarity used for primer design are involved in formation of the flavin cofactor binding site (Ackrell *et al.*, 1992; Hederstedt and Ohnishi, 1992). Further sequence analysis of the partial and full length clones examines whether these similarities extend to other regions of the polypeptides (see chapter 3).

The disruption mutant, sdh1L6, is unable to grow on glycerol as a carbon source. Southern analysis of the genomic DNA and genetic analysis of the linkage between the petite phenotype and the LEU2 marker indicate that recombination has occurred at the *SDH1* locus. The absence of covalently-attached flavin in mutant membrane preparations strongly supports this conclusion. Additionally, antibodies to the Fp do not detect a 67kDa protein in the disruption mutant Disruption of the flavoprotein subunit also results in the loss of the iron-sulfur subunit from submitochondrial membranes suggesting that assembly is required for its stability or membrane attachment.

The disruption mutant remains respiration-competent; it retains a normal level of NADH oxidase activity. Being blocked in the tricarboxylic acid cycle, it is however, incapable of oxidative phosphorylation. Disruption of the *SDH1* gene and loss of respiration-derived energy apparently does not significantly affect the cell's ability to synthesize and assemble other enzymes of the inner membrane electron transport chain.

The availability of the *SDH1* gene greatly facilitated the study of flavoprotein subunit synthesis, transport, and assembly with cofactors and other subunits into a functional enzyme. In particular, the temporal and spatial relationships of flavin attachment to membrane translocation of the newly synthesized flavoprotein subunit could be investigated.

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Chapter 3

Isolation and Nucleotide Sequence of a *S. cerevisiae* Gene for the SDH Flavoprotein Subunit

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3-1. INTRODUCTION

To investigate the mechanisms and order of import and covalent FAD attachment to the SDH Fp, the complete gene encoding this subunit (*SDH1*) was isolated. With the entire sequence available, numerous approaches for studying import and assembly could be utilized. For example, site specific mutations and truncations of the *SDH1* gene could be constructed and characterized. Additionally, *in vitro* import experiments into isolated mitochondria could be performed with *in vitro* translated Fp.

Previously, a partial fragment of the *SDH1* gene had been isolated by amplifying an internal portion of the gene with a polymerase chain reaction. Since yeast is a facultative anaerobe, none of the *SDH* genes are essential; an *sdh* mutant is still viable for fermentative growth (Lombardo and Scheffler, 1989, Lombardo *et al.*, 1990; Robinson *et al.*, 1991). Hence, with the partial clone of the *SDH1* gene, an *SDH1* disruption mutant was constructed and characterized (see chapter 2; Robinson *et al.*, 1991). Thus, I was able to screen a yeast genomic plasmid library for the *SDH1* gene by complementation of the mutant for growth on non-fermentable carbon sources, and by colony hybridization.

In this chapter, the isolation, cloning and sequencing of a complete genomic clone for the flavoprotein subunit gene, *SDH1*, is described This is the first complete nucleotide sequence of a eucaryotic *SDH1* gene to be determined; it predicts a hydrophilic protein of 70,185 Da (640 amino acids) bearing strong resemblance to the flavoprotein subunits of other succinate dehydrogenases and fumarate reductases. The *in vitro* expressed protein is synthesized as a precursor that can be imported into isolated, energized mitochondria. Gene mapping indicates that at least two of the succinate dehydrogenase subunits are located on different chromosomes.

3-2. MATERIALS AND METHODS

Strains, Vectors, and Media. The *Saccharomyces cerevisiae* and *E. coli* strains, as well as media, have previously been described in chapter 2, except D273-10B

(MATα; ATCC 25657). The vectors pRS416 and pBluescript11 were obtained from Stratagene (La Jolla, CA).

Cloning and Sequencing. The 0.95-kb polymerase chain reaction product discussed in chapter 2, was labeled with digoxigenin-dUTP as described by the manufacturer (Boehringer Mannheim), and used as a probe to screen 4,000 colonies of a yeast genomic library in the vector YCp50 (Rose *et al.*, 1987). DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using both single and double stranded template DNA (Mierendorf and Pfeffer, 1987). The sequencing reactions and the sequencing gel eletrophoresis were performed by the Department of Biochemistry DNA Core Laboratory (University of Alberta, Edmonton, Canada). Exonuclease digestions for the production of nested deletions were performed according to the supplier (Stratagene, La Jolla, CA). All sequence was determined using information obtained from both strands of the DNA.

Mitochondrial Protein Import. Mitochondria were isolated and import experiments performed as described (Gasser *et al.*, 1982). Coupled *in vitro* transcription and translation in rabbit reticulocyte lysate was performed as described by the supplier (Promega Corporation, Madison, WI) using Tran^{35}S-Label (ICN Biomedicals, St-Laurent, Quebec). Published procedures were used to assay inaccessibility to externally added proteinase K (Hurt *et al.*, 1985) and for polyacrylamide gel electrophoresis and fluorography (van Loon *et al.*, 1986)

Other Methods. Enzyme assays, Southern and western blot analyses, the determination of covalently bound flavin, transformations, and recombinant DNA methods are described in chapter 2.

3-3. RESULTS AND DISCUSSION

Isolation and Subcloning of the SDH1 Gene. A yeast genomic library was screened by colony hybridization with a previously isolated partial gene fragment that had

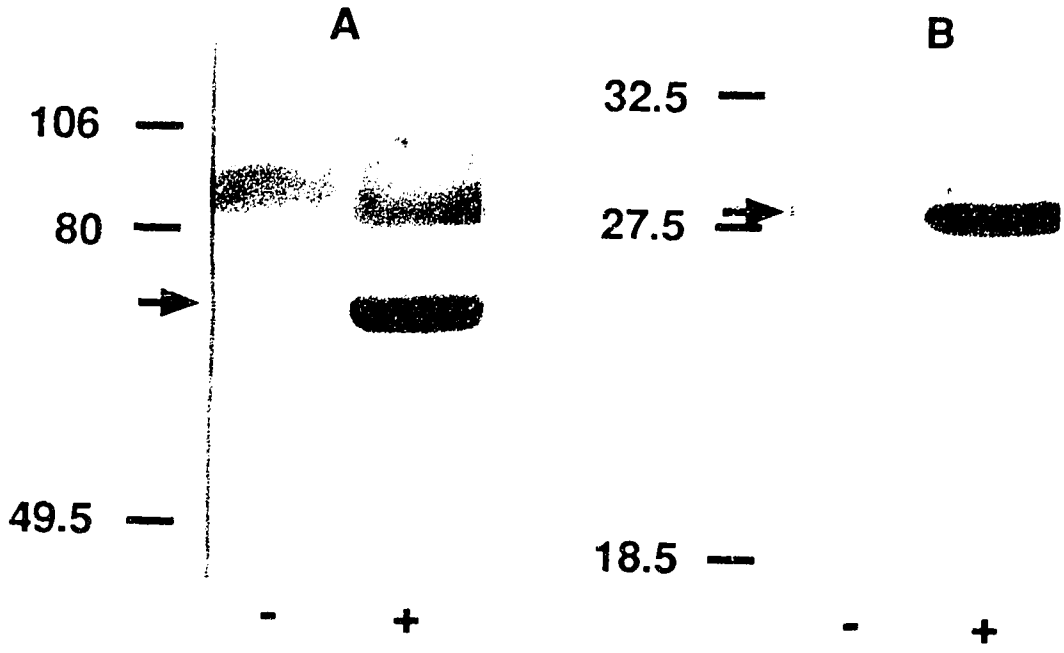
been labelled with digoxigenin-dUTP in a polymerase chain reaction (see chapter 2; Robinson *et al.*, 1991). Of approximately 4,000 colonies, three demonstrated strong hybridization. Plasmid DNA from each of the positives was isolated and further examined by restriction mapping; the three plasmids were identical. The *SDH1* gene was localized to a 3.5-kb *BamHI* fragment by Southern analysis. This fragment was cloned into the vector, pRS416, an autonomously replicating, single-copy yeast-*E. coli* shuttle vector carrying the yeast selectable marker, *URA3,* to produce the plasmid, pSDH1. In the following experiments, pRS416 and pSDH1 were found to be retained by at least 70% of the cells in each culture.

Complementation of an SDH1 Mutant. To determine whether pSDH1 encoded a functional gene, sdh1L6, a mutant constructed by targeted gene disruption of the *SDH1* gene, was transformed with the plasmid. sdh1L6 grows very slowly on glycerol; however, sdh1L6 transformed with pSDH1 displayed growth similar to MH125, the parental wild-type strain (data not shown).

Western Blot Analysis. Mitochondria from sdh1L6 transformed with either pSDH1 or the pRS416 vector were isolated and analyzed by western blot analysis. The subunit was not detectable in the mutant transformed with pRS416, but was clearly e as a 67 kDa protein in the cells bearing pSDH1 (Figure 3-1*A*). Expression of the flavoprotein subunit from pSDH1 was also accompanied by the re-appearance of the iron-sulfur subunit (Figure 3-1*B*).

pSDH1 Restores Succinate Dehydrogenase Activity to sdh1L6. Membranes were isolated from the parental wild-type strain, MH125, and from sdh1L6 transformed with either pRS416 or pSDH1. Succinate-dichlorophenol indophenol (DCPIP) reductase activity is not detectable in membranes from the mutant plus vector, but is comparable in wild-type membranes and membranes from the mutant plus pSDH1 (Table 3-1).

Figure 3-1. Western blot analysis. Mitochondria from sdh1L6 transformed with the vectors pRS416 (-) or with pSDH1 (+) were isolated as described (Daum *et al.*, 1982). Mitochondrial protein (50 µg per lane) was separated by SDS-gel electrophoresis, transferred to nitrocellulose membranes and analyzed by western blot analysis. The antibodies used are: *A*, affinity-purified Fp antibodies; *B*, iron-sulfur subunit antiserum. The arrows refer to the flavoprotein subunit in *A* and to the iron-sulfur subunit in *B*. The positions of size markers in kDa are indicated on the left.


A
B
106
80
49.5
32.5
27.5
18.5
-
+
-
+

pSDH1 Restores Trichloroacetic Acid-Precipitable Flavin Levels in sdh1L6. The only yeast membrane protein with a covalently attached flavin in yeast is the succinate dehydrogenase flavoprotein subunit (Singer *et al.*, 1965). Therefore, the amount of trichloroacetic acid-precipitable flavin is a direct measure of succinate dehydrogenase content. While membranes from the mutant plus vector have no trichloroacetic acid-precipitable flavin above background levels, membranes from the mutant plus pSDH1 contain levels comparable to those of wild-type membranes (Table 3-1).

Table 3-1. Succinate dehydrogenase levels in submitochondrial particles.

	MH125	sdh1L6 +pRS416	sdh1L6 +pSDH1
Succinate-DCPIP reductase[a]	267	ND (<3%)	232 (87%)
Trichloroacetic acid-precipitable flavin[b]	66	ND (<10%)	61 (92%)

Strains were grown to stationary phase and submitochondrial particles were isolated and assayed for succinate dehydrogenase activity or for the presence of covalently attached FAD as described (see chapter 2; Robinson *et al.*, 1991). ND, not detectable. [a] Activities are expressed in nanomoles of DCPIP reduced $min^{-1}mg^{-1}$; [b] Picomoles of flavin mg^{-1}

The genomic DNA insert in pSDH1 contains a functional copy of the succinate dehydrogenase flavoprotein subunit gene; the plasmid hybridizes to a partial clone we had previously isolated (Robinson *et al.*, 1991), is able to complement an *sdh1-* mutant, and restores succinate dehydrogenase activity, polypeptides recognized by antisera to the Fp and Ip subunits, and the levels of covalent FAD detected in submitochondrial membranes.

Nucleotide Sequence Determination. The nucleotide sequence of the pSDH1 insert was determined by sequencing cloned restriction fragments and nested deletions constructed by exonuclease digestion. Sequence determination was performed with M13 universal primers and on one occasion, with a specifically-designed oligonucleotide primer.

The sequence was determined from both strands for the entire open reading frame and flanking regions (Figure 3-2). The sequence begins at a *BamHI* site 272 nucleotides upstream of the initiator methionine codon, proceeds through an open reading frame of 1920 nucleotides and ends nearly 500 nucleotides downstream. Two potential TATAA boxes in the promoter region (positions -137 to -141 and -155 to -161) are shown in bold type (Figure 3-3). A possible polyadenylation signal, TTTTTAT (2143 to 2149), in a region of sequence with high A+T content is underlined (Figure 3-3; Irniger *et al.*, 1991).

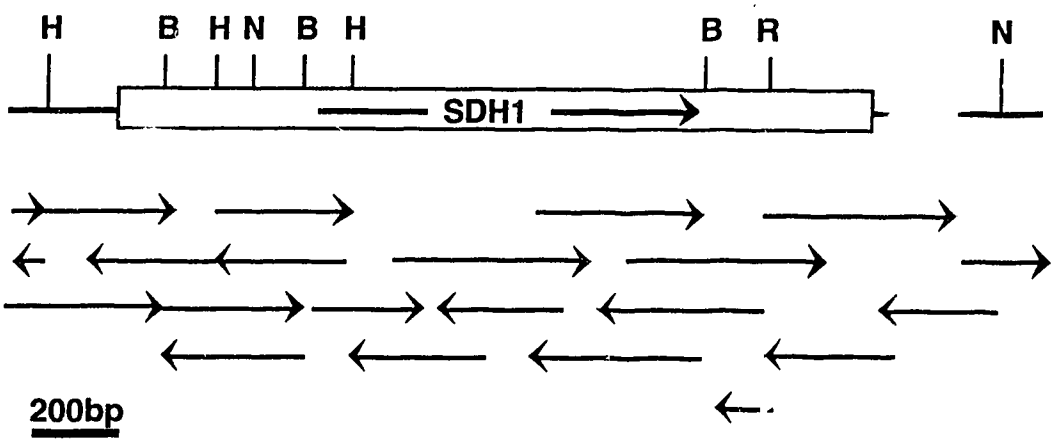


Figure 3-2. Sequencing strategy for SDH1. The top line represents part of the 3.5-kb *BamHI* fragment of pSDH1. The *SDH1* open reading frame is represented by the box. The arrows beneath indicate directions and lengths of sequences obtained from various subcloned fragments. The restriction sites indicated are: B, *BglII*; H, *HinDIII*; N, *NdeI*; R, *EcoRI*.

The predicted size of the Fp precursor is 70,185 Da and as expected, it displays considerable sequence similarity with other flavoprotein subunits of this family (Table 3-2). The yeast Fp subunit was most closely related to its *E. coli* homologue. Surprisingly, it is least similar to the only other mitochondrially derived flavoprotein subunit sequenced at the time, a partial sequence from a human placental cDNA library (Malcovati *et al.*, 1991). This is in marked contrast to the case for the iron-sulfur subunits of yeast and human where extensive sequence identity occurs (68.7%; Gould *et al.*, 1989). However, the human

Figure 3-3. Nucleotide sequence of SDH1 and flanking regions. The sequence extends over 2.64 kb of the genomic insert in pSDH1. The reading frame identified as *SDH1* starts with a methionine initiation codon at nucleotide +1 and terminates with an ochre codon at nucleotide +1921. Only the sequence of the sense strand is shown. The amino acid sequence of the succinate dehydrogenase flavoprotein subunit is shown below the nucleotide sequence. Shown in bold type are two potential TATAA boxes and underlined is a possible polyadenylation signal.

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                                                  GGATCCGGGCCTCTTCTATTGGTTGTTTGTTTGCTCAAACCCGTTATATATTCAGGCATCCTCGACTCTAACCTT   -198
 TTGCCACGTCGAGGCGGCTTGAAGCTTAAATAGCACTAATAAACTAGTAGACACATTATAACTAACCAGTAGTATACTTTGCACTTTCGATATTCTTT   -100
TCACTAATCTCCTCCCCAACCCCTTATTGAAGATAAAAAGAAAGAAAGAAAGAAAAAAATCCAATTTCATAGTACGAAGAAGAACGAGAATAAAG    -1

ATG CTA TCG CTA AAA AAA TCA GCG CTC TCC AAG TTG ACT TTG CTC AGA AAC ACA AGA ACA TTT ACA TCG TCA GCT     75
MET Leu Ser Leu Lys Lys Ser Ala Leu Ser Lys Leu Thr Leu Leu Arg Asn Thr Arg Thr Phe Thr Ser Ser Ala     25

TTG GTG CGC CAA ACG CAG GGC TCT GTA AAC GGT TCC GCG TCC AGA TCT GCA GAC GGG AAG TAC CAC ATA ATA GAT     150
Leu Val Arg Gln Thr Gln Gly Ser Val Asn Gly Ser Ala Ser Arg Ser Ala Asp Gly Lys Tyr His Ile Ile Asp     50

CAC GAG TAT GAC TGT GTG GTA ATC GGT GCC GGT GGT GCC GGC CTT AGA GCG GCC TTT GGT CTT GCC GAG GCG GGC     225
His Glu Tyr Asp Cys Val Val Ile Gly Ala Gly Gly Ala Gly Leu Arg Ala Ala Phe Gly Leu Ala Glu Ala Gly     75

TAC AAG ACT GCT TGT ATA TCC AAG CTT TTC CCC ACC AGA TCC CAC ACT GTT GCT GCT CAG GGT GGT ATC AAT GCC     300
Tyr Lys Thr Ala Cys Ile Ser Lys Leu Phe Pro Thr Arg Ser His Thr Val Ala Ala Gln Gly Gly Ile Asn Ala     100

GCT CTG GGA AAT ATG CAC AAG GAT AAC TGG AAA TGG CAT ATG TAC GAT ACT GTG AAA GGA TCT GAT TGG CTA GGT     375
Ala Leu Gly Asn Met His Lys Asp Asn Trp Lys Trp His Met Tyr Asp Thr Val Lys Gly Ser Asp Trp Leu Gly     125

GAC CAG GAC TCC ATC CAT TAC ATG ACC AGG GAA GCG CCC AAA TCG ATC ATT GAA CTG GAA CAC TAT GGT CTT CCT     450
Asp Gln Asp Ser Ile His Tyr Met Thr Arg Glu Ala Pro Lys Ser Ile Ile Glu Leu Glu His Tyr Gly Val Pro     150

TTT TCA AGA ACT GAA AAC GGT AAG ATC TAC CAA AGA GCC TTT GGT GGT CAG ACC AAG GAG TAC GGT AAG GGT CCT     525
Phe Ser Arg Thr Glu Asn Gly Lys Ile Tyr Gln Arg Ala Phe Gly Gly Gln Thr Lys Glu Tyr Gly Lys Gly Ala     175

CAG GCC TAT AGA ACA TGC GCT GTC GCA GAC AGG ACA GGA CAT GCT CTT TTA CAC ACG CTT TAT GGC CAA GCT TTA     600
Gln Ala Tyr Arg Thr Cys Ala Val Ala Asp Arg Thr Gly His Ala Leu Leu His Thr Leu Tyr Gly Gln Ala Leu     200

AGA CAT GAC ACA CAT TTC TTT ATT GAG TAC TTT GCC CTC GAT CTG TTG ACC CAT AAT GGC GAG GTC GTT GGT GTC     675
Arg His Asp Thr His Phe Phe Ile Glu Tyr Phe Ala Leu Asp Leu Leu Thr His Asn Gly Glu Val Val Gly Val     225

ATC GCT TAT AAT CAG GAA GAC GGT ACC ATC CAC AGA TTC AGA GCA CAC AAG ACC ATT ATT GCC ACT GGT GGC TAT     750
Ile Ala Tyr Asn Gln Glu Asp Gly Thr Ile His Arg Phe Arg Ala His Lys Thr Ile Ile Ala Thr Gly Gly Tyr     250

GGT AGA GCA TAC TTC TCT TGT ACC TCT GCT CAC ACA TGT ACG GGT GAC GGT AAT GCC ATG GTT TCG CGT GCT GGT     825
Gly Arg Ala Tyr Phe Ser Cys Thr Ser Ala His Thr Cys Thr Gly Asp Gly Asn Ala Met Val Ser Arg Ala Gly     275

TTC CCC TTG CAA GAT TTA GAG TTT GTT CAA TTC CAT CCT TCA GGT ATA TAT GGG TCT GGT TGC TTA ATC ACT GAA     900
Phe Pro Leu Gln Asp Leu Glu Phe Val Gln Phe His Pro Ser Gly Ile Tyr Gly Ser Gly Cys Leu Ile Thr Glu     300

GGT GCT CGT GGT GAA GGT GGT TTT TTG GTT AAT TCT GAA GGT GAA AGA TTC ATG GAA CGT TAC GCT CCT ACG GCC     975
Gly Ala Arg Gly Glu Gly Gly Phe Leu Val Asn Ser Glu Gly Glu Arg Phe Met Glu Arg Tyr Ala Pro Thr Ala     325

AAG GAT CTA GCT TGT AGA GAT GTC GTT TCC AGA GCA ATC ACC ATG GAG ATC AGA GAA GGC AGA GGT GTT GGT AAG     1050
Lys Asp Leu Ala Cys Arg Asp Val Val Ser Arg Ala Ile Thr Met Glu Ile Arg Glu Gly Arg Gly Val Gly Lys     350

AAA AAG GAC CAC ATG TAC TTA CAA TTG AGC CAT CTA CCT CCG GAA GTT CTA AAG GAA AGA TTG CCA GGT ATC TCT     1125
Lys Lys Asp His Met Tyr Leu Gln Leu Ser His Leu Pro Pro Glu Val Leu Lys Glu Arg Leu Pro Gly Ile Ser     375

GAA ACA GCA GCC ATT TTT GCT GGT GTA GAC GTC ACC AAG GAA CCT ATT CCC ATT ATT CCT ACC GTC CAC TAT AAC     1200
Glu Thr Ala Ala Ile Phe Ala Gly Val Asp Val Thr Lys Glu Pro Ile Pro Ile Ile Pro Thr Val His Tyr Asn     400

ATG GGT GGT ATT CCC ACG AAG TGG AAT GGT GAG GCA TTA ACC ATT GAT GAA GAA ACT GGC GAA GAC AAG GTT ATT     1275
Met Gly Gly Ile Pro Thr Lys Trp Asn Gly Glu Ala Leu Thr Ile Asp Glu Glu Thr Gly Glu Asp Lys Val Ile     425

CCC GGT TTA ATG GCT TGT GGT GAA GCC GCT TGT GTT TCT GTC CAT GGT GCC AAT AGA TTA GGT GCC AAT TCC TTG     1350
Pro Gly Leu Met Ala Cys Gly Glu Ala Ala Cys Val Ser Val His Gly Ala Asn Arg Leu Gly Ala Asn Ser Leu     450

TTG GAT CTT GTT GTC TTT GGT CGT GCT GTT GCC CAT ACG GTT GCT GAC ACT TTA CAG CCT GGG TTG CCA CAC AAA     1425
Leu Asp Leu Val Val Phe Gly Arg Ala Val Ala His Thr Val Ala Asp Thr Leu Gln Pro Gly Leu Pro His Lys     475

CCA CTA CCT TCT GAT TTG GGT AAA GAA TCC ATC GCA AAC TTG GAT AAA CTA AGA AAT GCT AAT GGT TCA AGA TCT     1500
Pro Leu Pro Ser Asp Leu Gly Lys Glu Ser Ile Ala Asn Leu Asp Lys Leu Arg Asn Ala Asn Gly Ser Arg Ser     500

ACG GCA GAA ATT AGA ATG AAT ATG AAA CAA ACT ATG CAA AAG GAT GTT TCC GTC TTT AGA ACA CAA TCA TCT TTA     1575
Thr Ala Glu Ile Arg Met Asn Met Lys Gln Thr Met Gln Lys Asp Val Ser Val Phe Arg Thr Gln Ser Ser Leu     525

GAT GAA GGT GTT CGG AAC ATT ACT GCA GTA GAG AAG ACC TTT GAT GAT GTG AAG ACG ACC GAT AGA TCA ATG ATC     1650
Asp Glu Gly Val Arg Asn Ile Thr Ala Val Glu Lys Thr Phe Asp Asp Val Lys Thr Thr Asp Arg Ser Met Ile     550

TGG AAT TCT GAC TTG GTT GAA ACT CTG GAG CTA CAG AAC TTA TTA ACC TGT GCC TCC CAA ACA GCT GTT TCC GCT     1725
Trp Asn Ser Asp Leu Val Glu Thr Leu Glu Leu Gln Asn Leu Leu Thr Cys Ala Ser Gln Thr Ala Val Ser Ala     575

GCT AAT AGA AAG GAA TCC CGT GGT GCT CAT GCA AGA GAG GAT TAT CCA AAT AGA GAT GAC GAA CAT TGG ATG AAG     1800
Ala Asn Arg Lys Glu Ser Arg Gly Ala His Ala Arg Glu Asp Tyr Pro Asn Arg Asp Asp Glu His Trp Met Lys     600

CAT ACA TTA TCC TGG CAA AAG GAC GTC GCT GCC CCA GTG ACT TTG AAA TAC AGA AGG GTT ATC GAT CAC ACT TTG     1875
His Thr Leu Ser Trp Gln Lys Asp Val Ala Ala Pro Val Thr Leu Lys Tyr Arg Arg Val Ile Asp His Thr Leu     625

GAC GAA AAG GAA TGT CCT TCC GTA CCT CCA ACT GTA AGA GCC TAC TAA                                         1923
Asp Glu Lys Glu Cys Pro Ser Val Pro Pro Thr Val Arg Ala Tyr  .                                          640

TTTGAACCTCATTGTATTTTACGGAAAAGAATATCATACTCTTCTTTAATTGCACTTTTTTTGTGCGTTTGCATTTTTTACCACTGACTCACTAATTTGT     2023
ATATATACCTATTAATATACATTTACATAAAGTTTCTTCTTATACATACTCTATTTATTTAGTTATTTATTAACTTACTATTTATTTATTTATTTATTTA     2123
TTTATTTATTACTTTCAATTTTTTATCGAGGCATTTCCTTAGTTCTCCAATTTTTTTTCTCATTAGCCAGATGTGTGTTTTTCTGGCCCTCACAAAAAAT     2223
GAGCAGGAGAAAGTCATATGGCGAACGTAAATATGTAACTAAAAATTAAGATGGGCAGACATTTATCATTTTGCTTATGACTAAACCGCGAATTGCTGTA     2323
CAAGGGTGCTGTCATGGTCAGCTAAACCAAATTTATAAAGAA                                                             2365
```

Table 3-2. Comparison of amino acid sequence identities between flavoprotein subunits.

	scSDH1	ecSDHA	pvFRDA	ecFRDA	wsFRDA	bsSDHA	huSDH1
scSDH1	100	53	41	40	35	31	31
ecSDHA		100	42	42	34	31	32
pvFRDA			100	84	36	34	33
ecFRDA				100	36	33	33
wsFRDA					100	31	29
bsSDHA						100	77
huSDH1							100

The primary structure of the *S. cerevisiae* Fp subunit (scSDH1; this chapter) was compared with other flavoprotein subunits: the *E. coli* SDHA subunit (ecSDHA; Wood *et al.*, 1984), the *Proteus vulgaris* FRDA subunit (pvFRDA; Cole, 1987), the *E. coli* FRDA subunit (ecFRDA; Cole, 1982), the *Wollinella succinogenes* FRDA subunit (wsFRDA; Lauterbach *et al.*, 1990), the *Bacillus subtilis* SDHA subunit (bsSDHA; Phillips *et al.*, 1987), and the human placental Fp subunit (huSDH1; Malcovati *et al.*, 1991). Comparisons were performed by Bernard Lemire with the FASTDB program (Brutlag *et al.*, 1990). The values represent % identities (matches / matches + mismatches + gaps).

sequence does not contain the amino terminus of the polypeptide with the highly conserved regions involved in flavin attachment and binding. Additionally, subsequent to these studies the sequence for the bovine heart and the human heart Fp were determined (Birch-Machin *et al.*, 1992; Morris *et al.*, 1994). These mammalian sequences also share little homology to the partial sequence of the human placental Fp (Table 3-3). Comparsions of the eukaryotic sequences reveal that the yeast SDH Fp is more closely related to the bovine and human heart Fps (respectively, see Table 3-3), than to the bacterial Fps. Furthermore, the bovine and human heart Fps and the yeast Fp have significant homologies starting from the N-termini of the mature Fp to the carboxyl termini or their sequences (Morris *et al.*, 1994). Even without comparisons to the more recent eukaryotic sequences, it is clear that pSDH1 encodes the *S. cerevisiae SDH1* gene.

Table 3-3. Comparison of amino acid sequence identities between eukaryotic SDH Fps.

Mature \ Entire Fp	Yeast	Bovine Heart	Human Heart
Yeast	100	67.1	63.5
Bovine Heart	69.7	100	93.2
Human Heart	65.0	95.2	100
Human Placental	31	16	27.9

The primary structure of the *S. cerevisiae* Fp subunit was compared with other eukaryotic SDH Fp: the bovine heart Fp (Birch-Machin *et al.*, 1992), the human heart Fp (Morris *et al.*, 1994) and the human placental Fp (Malcovati *et al.*, 1991). Values were calculated and obtained from: (Birch-Machin *et al.*, 1992; Robinson and Lemire, 1992; Morris *et al.*, 1994). The values represent % identities (matches / matches + mismatches + gaps).

The alignment of the yeast Fp polypeptide sequence with related flavoprotein subunits is displayed in Figure 3-4 The FAD cofactor of the bovine succinate dehydrogenase is covalently bound via the 8α-methyl group of the isoalloxazine ring to the N(3) of a histidine (Singer and Edmondson, 1974). The sequence of a 23 residue flavopeptide from this enzyme was determined (Singer *et al.*, 1973) and comparison with the yeast sequence reveals that 17 of 23 positions are identical; this identifies His-90 as the probable site of flavin attachment in the yeast protein. In contrast to the other flavoprotein subunits listed in Figure 3-4, this histidyl residue is not the first in the yeast sequence, but is preceded by His-47 and His-51. The histidines that are the site of FAD attachment to the bovine and human Fps are also not the first histidine residue to appear in these sequences (Birch-Machin *et al.*, 1992; Morris *et al.*, 1994). This observation is inconsistent with the notion that FAD is attached cotranslationally to the first histidine residue encountered, but rather suggests that some minimal tertiary structure is necessary to accomodate the cofactor (Hamm and Decker, 1978) or that the process is post-translational (Cecchini *et al.*, 1985). The role of the presequence and the mechanism of flavin attachment to a mitochondrially localized protein was further investigated (discussed in chapters 5 and 6).

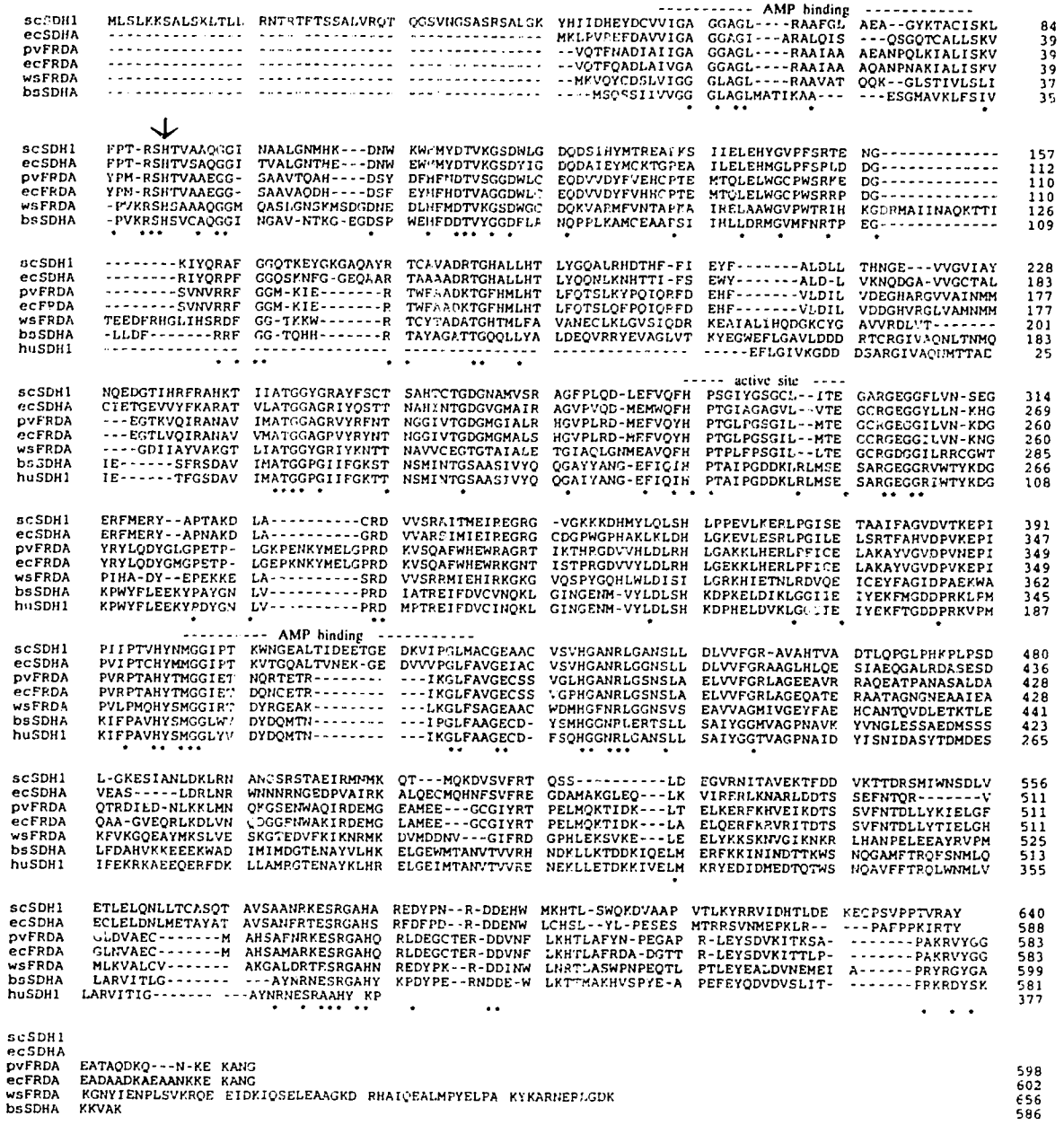


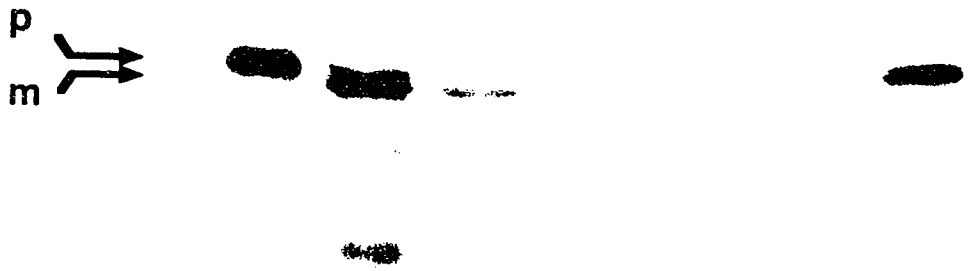
Figure 3-4. Homologies between fumarate-succinate oxidoreductase flavoprotein subunits. The *S. cerevisiae* Fp subunit is aligned with other flavoprotein subunits; see Table 3-2 for a description of the sequences used. Gaps have been introduced to optimize homology. Residue numbering on the right begins with the initiating methionine or valine codons for all sequences except the human Fp subunit where the first residue does not represent the amino terminus. Residues conserved in all of the sequences are indicated with an asterisk below the alignment. Segments constituting the AMP binding regions and the active site are indicated above the alignment. The His residue to which the covalent FAD cofactor is believed to be attached is indicated with an arrow.

From comparisons with glutathione reductase, a non-covalent flavoprotein for which 3-dimensional structural data exists, the succinate dehydrogenase and fumarate reductase flavoprotein subunits are believed to interact non-covalently with the AMP moiety of the FAD in 2 regions (Schulz *et al.*, 1982; Wierenga *et al.*, 1983; Cole *et al.*, 1985). One such region contains a Rossman nucleotide binding fold which contacts the bottom of the AMP and is located near the amino terminus. The second region appears in the center of the polypeptide and forms the top of the AMP-binding domain (Figure 3-4). The residues in these regions, along with those near the site of FAD attachment, are among the most conserved.

It is believed that a histidine residue plays an important role in the function of the fumarate-succinate oxidoreductases. This residue is conserved in the sequence His-Pro-Thr present in all flavoprotein subunits except the yeast subunit where this sequence is His-Pro-Ser (residues 287-289). Near this sequence are the putative active site cysteine residues of the *E. coli* SDHA (Cys-257) and *E. coli* FRDA (cys-248) subunits (Wood *et al.*, 1984). The analogous cysteine is conserved in the *P. vulgaris* FRDA (Cys-248;Cole, 1987) and the *W. succinogenes* FRDA (Cys-272; Lauterbach *et al.*, 1990), but not in the *B. subtilis* or the human Fps, where alanines are located; it is for this reason that the *B. subtilis* enzyme is believed to be insensitive to thiol-reactive reagents (Phillips *et al.*, 1987). Although, after these studies this region in the human placental Fp was resequenced, revealing a sequencing error; the analogous cysteine is actually conserved in this Fp as well, explaining why the human placental SDH is sensitive to thiol-reactive reagents (Morris *et al.*, 1994). In the yeast sequence, the analogous residue is Ala-302; however, there is a cysteine residue nearby (Cys-296) that may account for the enzyme's sensitivity to *p*-chloromercuribenzoate (Singer *et al.*, 1957).

Figure 3-5 Import of the Fp into isolated mitochondria. Mitochondria from D273-10B were isolated as described (Daum *et al.*, 1982). Plasmid pSDH1 was linearized, transcribed *in vitro* with T7 RNA polymerase, and the mRNA translated in a cell free reticulocyte lysate. Import reactions were performed with 150 µg of mitochondria in the presence of 10 mM succinate and 2 mM NADH for 30 min at 30 °C. The mitochondria were re-isolated and analyzed by SDS-gel electrophoresis and fluorography. *Lane 1,* 10% of the amount of lysate added to each import reaction; *lane 2*, mitochondria that have imported the Fp precursor; *lane 3,* same as *lane 2* but treated with 250 µg proteinase K/ml; *lane 4,* mitochondria incubated with the Fp precursor, re-isolated, disrupted by the addition of 1% Triton X-100, and treated with proteinase; *lane 5,* mitochondria incubated with the Fp precursor in the presence of 10 µM valinomycin; *lane 6,* same as *lane 5* but treated with proteinase; *lane 7,* a mock import reaction in the absence of mitochondria; *lane 8,* same as *lane 7* but treated with proteinase. p, precursor form; m, mature form.

	1	2	3	4	5	6	7	8
Mitochondria		+	+	+	+	+	-	-
Valinomycin		-	-	-	+	+	-	-
Proteinase K		-	+	+	-	+	-	+
Triton X-100	10%	-	-	+	-	-	-	-

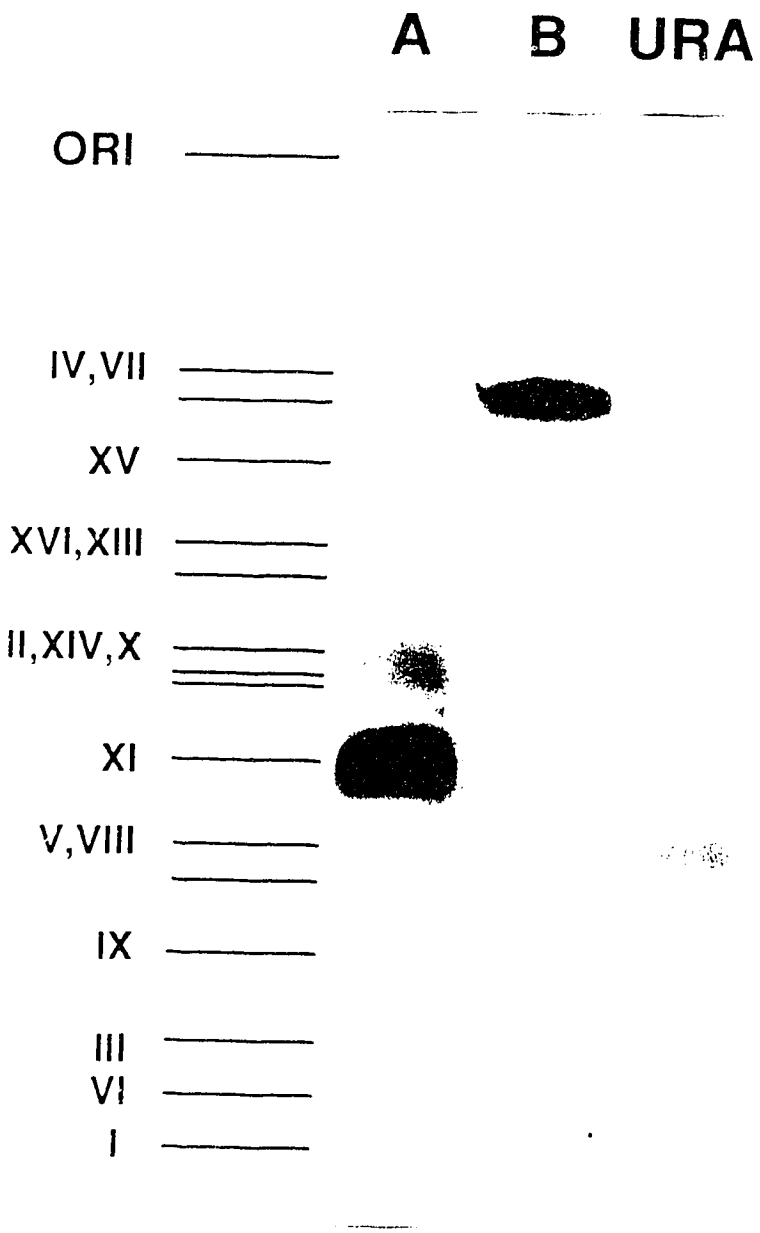


Import into Isolated Mitochondria. The *SDH1* gene was transcribed *in vitro* with T7 RNA polymerase and the mRNA translated in a cell free reticulocyte lysate in the presence of ^{35}S-labelled methionine. As expected, the Fp polypeptide is able to bind to mitochondria (Figure 3-5, *lanes 2* and *5*). Import into mitochondria is membrane potential dependent (*lanes 5* and *6*); only in the presence of energized mitochondria is a faster migrating species seen (*lanes 2* and *3*). This species is resistant to externally added proteinase (*lane 3*) except when mitochondrial integrity is disrupted with detergent (*lane 4*). As is commonly observed with *in vitro* synthesized precursor proteins, a fraction is present in an insoluble form (*lane 7*). This fraction is however, totally sensitive to proteinase (*lane 8*).

The amino terminus of the Fp precursor contains the consensus sequence for cleavage upon import by two separate proteases: Rx(F)xx(S) where R = arginine; x=any amino acid; (F) = phenylalanine or other hydrophobic residues; and (S)=serine, threonine or glycine (Hendrick *et al.*, 1989). In the yeast Fp subunit, this motif is composed of Arg-19, Phe-21, and Ser-24 and would predict that the yeast Fp precursor is first cleaved between Thr-20 and Phe-21 by the matrix processing protease, and that a subsequent cleavage to a mature species occurs between Arg-28 and Gln-29 by the mitochondrial intermediate protease. The purpose of such a dual cleavage event has not been elucidated. The former cleavage can be inhibited *in vitro* by the addition of *o*-phenanthroline, a metal chelating agent (data not shown). We have not detected an intermediate in our *in vitro* import reactions, although amino-terminus of the mature protein was determined (Schülke *et al.*, 1992; Bullis and Lemire, 1994), and confirms this prediction.

Chromosomal Mapping. The chromosomal location of the *SDH1* gene was mapped by probing a nylon membrane to which had been transferred *S. cerevisiae* chromosomal DNA separated by CHEF electrophoresis. As controls, two known genes, the succinate dehydrogenase iron-sulfur subunit (*SDH2)* gene on chromosome VII and the *URA3* gene on chromosome V were also mapped (Lombardo *et al.*, 1990).

Figure 3-6 Mapping of the SDH1 gene onto chromosome XI. Chromosomal DNA from *S. cervisiae* that had been separated by CHEF and transferred to a nylon membrane (Chromo-blot from Clontech Laboratories, Palo Alto CA) was probed. The roman numerals on the left indicate the positions of the individual chromosomes. *A,* probed with a fragment of the *SDH1* gene; *B,* probed with the iron-sulfur subunit gene; *URA*, probed with the *URA3* gene; *Ori*, origin.


A
B
URA
ORI
IV,VII
XV
XVI,XIII
II,XIV,X
XI
V,VIII
IX
III
VI
I

For the *SDH1* gene, a strong signal corresponding to chromosome number XI was observed (Figure 3-6). The mapping of *SDH1* to chromosome number XI was subsequently confirmed upon the sequencing of the entire XI chromosome (Dujon *et al.*, 1994). A weaker signal corresponding to chromosome XIV may indicate the presence of a related gene in the genome.

The *SDH1* and *SDH2* genes are located on different chromosomes in the *S. cerevisiae* genome. In all procaryotic organisms in which fumarate reductases or succinate dehydrogenases have been examined, the enzymes are expressed from operons encoding all of the subunits. Therefore, mitochondrially-localized, multisubunit enzymes likely have evolved from the translation of polycistronic messages to the expression of individual subunits from different transcriptional units within the nucleus. This evolution requires that each subunit gene must not only acquire transcriptional signals but also an in-frame mitochondrial targeting sequence. It is improbable that each of the succinate dehydrogenase subunit genes would simultaneously undergo these changes. Gene transfer as an evolutionary process is a basic tenet of the endosymbiotic theory which proposes that mitochondria and chloroplasts were once free-living organisms (Gray and Doolittle, 1982). Regardless of whether the nuclear, precursor-encoding succinate dehydrogenase genes originated from the mitochondrial progenitor or not, it is probable that at some time during evolution, the enzyme was composed of both nuclear and mitochondrially encoded subunits in a situation similar to those of complexes I, III, IV, and V in most modern eucaryotes (Attardi, 1988).

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Chapter 4

Covalent Attachment of FAD to the Flavoprotein of *S. cerevisiae* SDH is not Necessary for Import and Assembly into Mitochondria

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4-1. INTRODUCTION

SDH and FRD Fps and other proteins such as 6-HDNO have FAD covalently attached via an 8α-[N(3)-histidyl]FAD linkage. The function of this covalent attachment is unclear since it is not required for all catalytic activities. For example, when the histidine residue that is the site of covalent attachment for 6-HDNO is changed to cysteine, serine, or tyrosine residues, the resulting proteins cannot covalently bind FAD, but mutants with the cysteine or serine substitution mutants maintain enzymatic activity. Mutants in *Escherichia coli* FRD that are unable to bind FAD covalently have also been constructed by changing the conserved histidine that is the site of FAD attachment, to serine, cysteine, arginine, or tyrosine (Blaut *et al.*, 1989). Similarly, all of these FRD mutants were able to assemble, bind FAD non-covalently, and with the exception of the arginine substitution, retained FRD activity. However, without the covalent attachment of FAD, the FRD enzymes have no significant SDH activity.

It was of interest to determine whether an eucaryotic SDH mutant would differ from the analogous *E. coli* FRD mutant. A notable distinction between these enzymes, as demonstrated by voltammetry studies, is that SDH has a low-potential activity switch-off not observed in FRD (Sucheta *et al.*, 1992; Sucheta *et al.*, 1993). Therefore, although SDH and FRD are highly homologous and catalyze the same reactions, they have some mechanistic differences. Additionally, with an eucaryotic SDH Fp mutant the role of covalent cofactor attachment in translocation into the mitochondria and subsequent proteolytic processing could be investigated. Furthermore, a mutant Fp that was unable to covalently bind FAD but behaved like the wild-type protein in most respects would be a valuable control for examining modification of the wild-type Fp.

Hence, to study FAD attachment and assembly of the SDH Fp in *Saccharomyces cerevisiae*, we created a mutant that is unable to covalently bind FAD. We predicted that His-90 is the site of FAD attachment in the Fp subunit (see chapter 3; Robinson and Lemire, 1992) and by analogy with the *E. coli* FRD changed this histidine to a serine. The

yeast SDH mutant Fp is still imported into mitochondria where it is processed and assembled with other subunits into a holoenzyme that contains non-covalently bound FAD, retains FRD activity, but has lost SDH activity.

4-2. MATERIALS AND METHODS

Strains, Media, and Vectors. The *S. cerevisiae* strains MH125 (MATa, ura3-52, leu2-3, leu2-112, his3, his4, rme, ρ+) and sdh1L6 (MATa, ura3-52, leu2-3, leu2-112, his3, his4, rme, sdh1::LEU2, ρ+) are the sources of either mitochondria or submitochondrial particles for enzyme assays, for covalently bound flavin determination, and for Western blot analysis (these methods are described in chapter 2; Robinson *et al.*, 1991). By targeted gene disruption, the strain sdh1W3 (MATa, ura3-52, leu2-3, leu2-112, his3, his4, rme, sdh1::TRP1, ρ+) was constructed from MH125. The strain RbS1W8C (rib, leu2, his3, his4, ura3-52, trp1, sdh1::TRP1, ρ+) has its riboflavin deficiency and *sdh1* disruption mutations originating from the strains HK859 (ATCC 22091) and sdh1W3, respectively. Mitochondria from the strain D273-10B (MATα; ATCC 25657) were used for *in vitro* import experiments and histochemical staining. The *E. coli* strains, the plasmids pRS416 and pSDH1, and the media used have been described (see chapter 2; Robinson *et al.*, 1991).

Oligonucleotide-Directed Mutagenesis. Mutagenesis was performed using an oligonucleotide-directed *in vitro* mutagenesis kit according to the manufacturer's instructions (Amersham Canada Limited, Oakville, Ontario). The template for mutagenesis was single stranded DNA derived from the plasmid pSDH1. The oligonucleotide was designed to change *SDH1* His-90 (a CAC codon) to a serine (TCC) codon. After the mutagenesis, the *Bgl II* fragment containing the mutated region was cloned into pBluescriptII SK- (Stratagene, La Jolla, CA) and its sequence was confirmed using an Applied Biosystems 373A DNA Sequencer (La Jolla, CA). The *SDH1* gene was

reconstructed to create the plasmid pS1H90S, which is identical to pSDH1 except for the two introduced base changes.

Isolation of Submitochondrial Particles and Mitochondria. Import competent mitochondria were isolated from D273-10B as described (Gasser *et al.*, 1982). A small scale procedure was used to isolate mitochondria from RbS1W8C or sdh1L6 (Ljungdahl *et al.*, 1989). Submitochondrial particles were prepared by cell lysis in a French press as described (see chapter 2; Robinson *et al.*, 1991). For radiolabeled mitochondria, the strain RbS1W8C transformed with either pSDH1, pS1H90S, or pRS416 was grown to late log phase on YPGal supplemented with 20 mg/ml D-[2-^{14}C]riboflavin (24 mCi/mmol; Amersham Canada Limited, Oakville, Ontario). Protein concentrations were estimated by absorbance at 280 nm.

Non-Denaturing Gel Electrophoresis. Blue native gel electrophoresis was performed as described by Schägger and von Jagow, (1991) with the following modifications. Isolated mitochondria were resuspended to a concentration of 9.4 µg/µl in 0.75 M 6-aminocaproic acid (Sigma), and solubilized by the addition of lauryl maltoside (Boehringer Mannheim) to a concentration of 1.5%. Solubilized mitochondria were centrifuged at 100 000 x *g* for 30 minutes and the supernatant mixed with a 20X loading buffer [5% Serva Blue G (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany), 0.5 M 6-aminocaproic acid, 25% glycerol]. The samples were electrophoresed on a 16 cm, 7 to 16.5% acrylamide gradient gel at 100 V for 2 hrs followed by 200 V for 20 hrs. When the dye front had migrated approximately one third of the way down the gel, the cathode buffer was replaced with cathode buffer without Serva Blue G.

Gel Slicing and Measurement of Radioactivity. Lanes of the blue native gel were sliced at a spacing of 2 mm. The gel slices were digested with 70% hyamine hydroxide (ICN Biomedicals, St. Laurent, Quebec) in liquid scintillation vials overnight at 37°C. 10 ml of Ecolite liquid scintillation fluid (ICN) was added and the vials were kept in the dark overnight. Samples were counted twice for 5 minutes.

Histochemical Stain for Succinate Dehydrogenase Activity. After blue native gel electrophoresis, the lane containing the solubilized D273-10B mitochondria was incubated overnight at 30°C in 50 mM Tris-HCl, 120 mM succinate, 0.1 mg/ml tetranitro blue tetrazolium (2,2',5,5'-tetra-*p*-nitrophenyl-3,3'[3,3'-dimethoxy-4,4'diphenylene] ditetrazolium chloride; Sigma), 1 mM phenyl methyl sulfonyl fluoride, pH 7.2 (Owen *et al.*, 1982). The lane was fixed and destained with 30% methanol, 10% acetic acid until the protein-bound Serva Blue G had been removed.

Electron Paramagnetic Resonance Spectroscopy. EPR spectra of washed submitochondrial particles were recorded using a Bruker ESP300 EPR spectrometer (Bruker Spectrospin, Milton, Ontario) equipped with an Oxford Instruments ESR-9 flowing helium cryostat. Instrument conditions and temperatures are described in the figure legend. Spectra were corrected for protein concentration and tube calibrations.

Other Methods. Reduced benzyl viologen-fumarate oxidoreductase activity was measured as the fumarate-dependent oxidation of dithionite reduced benzyl viologen (Dickie and Weiner, 1979). Fp antiserum was obtained against a peptide consisting of residues 167-177 conjugated to keyhole limpet hemocyanin (Alberta Peptide Institute, Edmonton, Alberta). Coupled *in vitro* transcription, translation in rabbit reticulocyte lysate in the presence of Trans ^{35}S-Label (ICN Biomedicals, St. Laurent, Quebec), and import into isolated mitochondria have been described (see chapter 3; Robinson and Lemire, 1992). Other methods including phenazine methosulfate-mediated succinate-dichlorophenol indophenol reductase activity, Western blot analysis, the determination of covalently bound flavin, and the antiserum against the Ip subunit have been described (see chapter 2; Robinson *et al.*, 1991).

4-3. RESULTS

Rationale and Mutant Construction. The sites of covalent flavin attachment are highly conserved amongst the members of the succinate-fumarate oxidoreductase family. In *E. coli* FRD, mutations affecting the conserved histidine to which FAD is attached result in a noncovalent association between the cofactor and enzyme (Blaut *et al.*, 1989). The serine substitution has the least effect on enzyme function. To investigate the assembly and flavinylation of a eucaryotic SDH, where import and processing are additional steps in the assembly pathway, we constructed the analogous histidine to serine mutation in the *S. cerevisiae* Fp at the predicted position of FAD attachment, His-90 (chapter 3; Robinson and Lemire, 1992). The serine substitution was chosen because the analogous mutation in the *E. coli* FRD Fp, has the least deleterious effect on FRD activity; if it was possible, we wanted a Fp mutant for future studies that would act like the wild-type protein except for the loss of covalent attachment to FAD.

Import of the Mutant Fp. The import and proteolytic processing of the H90S Fp (the mutant flavoprotein with the histidine at position 90 converted to a serine residue) into mitochondria was compared to the wild-type Fp. *In vitro* translated precursor proteins were incubated with isolated mitochondria which were re-isolated and analyzed by SDS-PAGE (Figure 4-1). Both the wild-type and the H90S Fp precursors bind to mitochondria (lanes 3 and 4, respectively). They are imported and proteolytically cleaved to a mature size (lanes 3 to 6) where they are resistant to externally added proteinase (lanes 5 and 6) unless the mitochondria have been solubilized with detergent (lanes 7 and 8). Import and processing of both precursors is inhibited by the uncoupler, valinomycin (lanes 9 to 12), indicating a requirement for a membrane potential. No difference in migration between the wild-type and mutant precursors or mature Fps could be distinguished with SDS-PAGE.

Figure 4-1. Import of Fps into isolated mitochondria. *In vitro* translated wild-type or H90S Fp precursors were added to 200 μg of D273-10B mitochondria in the presence of 10mM NADH for 30 min at 30 °C. Mitochondria were reisolated and analyzed by SDS-PAGE and fluorography. *Lanes 1 and 2,* 10% of the amounts of the wild-type and H90S Fp lysates added to each import reaction, respectively; *lanes 3 and 4,* mitochondria that have been incubated with the wild-type or H90S Fp precursors, respectively; *lane 5 and 6,* same as *lanes 3 and 4* but treated with 50 μg proteinase K for 15 min at 4 °C; *lane 7 and 8,* same as *lanes 3 and 4* but mitochondria were treated with 1% Triton X-100 before the addition of proteinase K; *lanes 9 and 10,* mitochondria incubated with wild-type or H90S Fp precursors in the presence of 6 μM valinomycin, respectively; *lanes 11 and 12,* same as *lanes 9 and 10* but treated with proteinase K.

Lane number	1	2	3	4	5	6	7	8	9	10	11	12
Valinomycin			-	-	-	-	-	-	+	+	+	+
Proteinase K			-	-	+	+	+	+	-	-	+	+
Triton X-100			-	-	-	-	+	+	-	-	-	-
flavoprotein	Wt 10%	Mut 10%	Wt	Mut	Wt	Mut	Wt	Mut	Wt	Mut	Wt	Mut

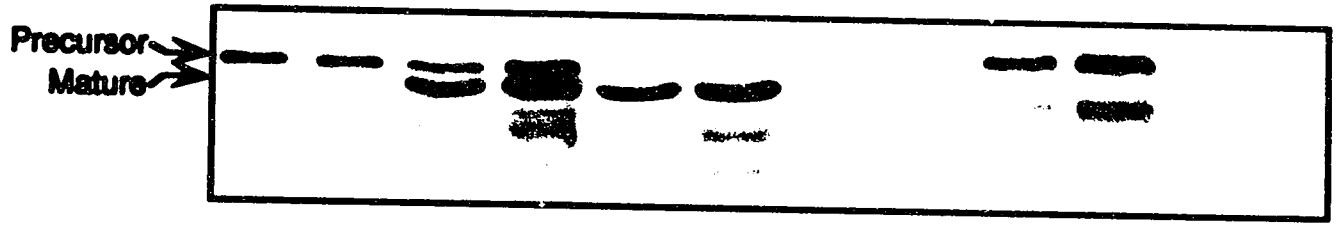
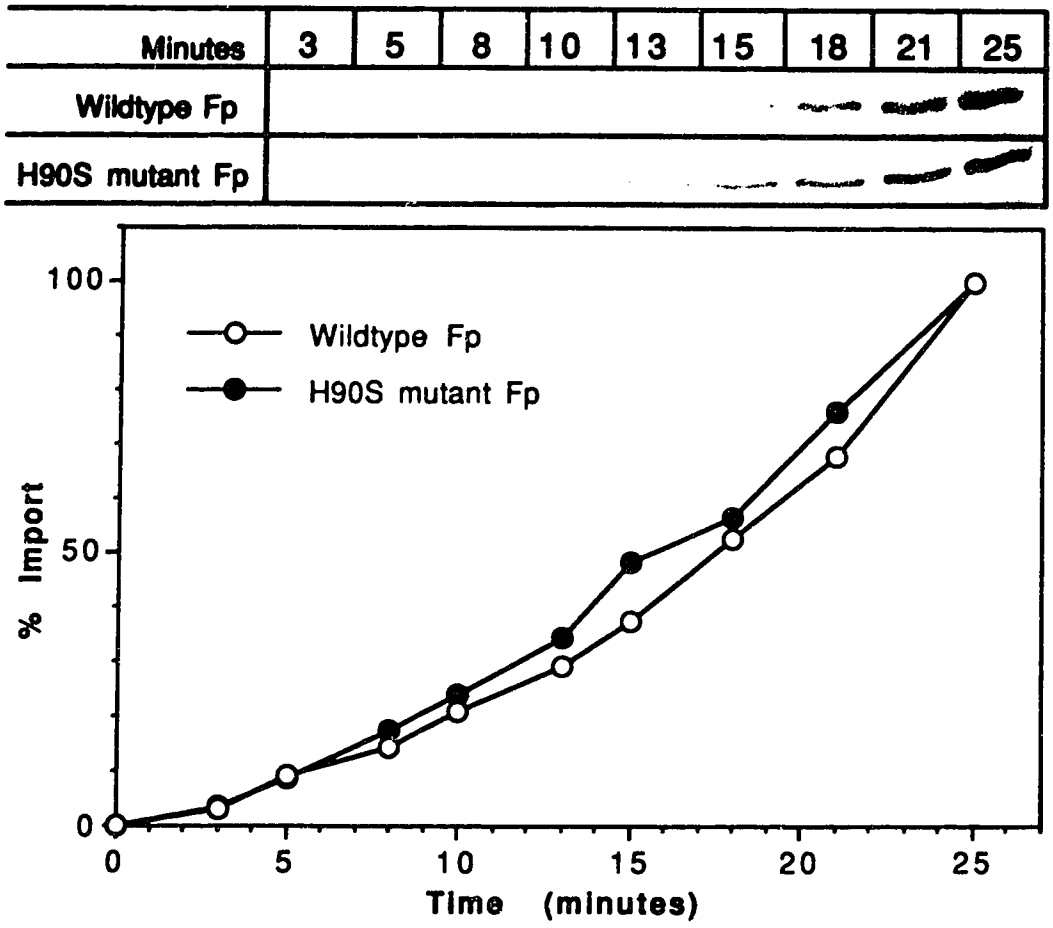


Figure 4-2. Time course for Fp import. Import reactions were performed as in Figure 4-1 except 50 μg of mitochondria and 19 °C were used. At the time points indicated, an aliquot was removed and import was stopped by the addition of 6 μM valinomycin. Proteinase *K* was added to degrade unimported protein. The upper panel shows the autoradiograms for the wild-type and mutant Fps. The bands were quantified by densitometry using a Chromoscan 3 (Joyce Loebl). Import after 25 minutes was arbitrarily set at 100%.


Minutes
3
5
8
10
13
15
18
21
25
Wildtype Fp
H90S mutant Fp
100
50
0
% Import
Wildtype Fp
H90S mutant Fp
0
5
10
15
20
25
Time (minutes)

Thus, the import and proteolytic processing of the wild-type and H90S Fp precursors are apparently the same.

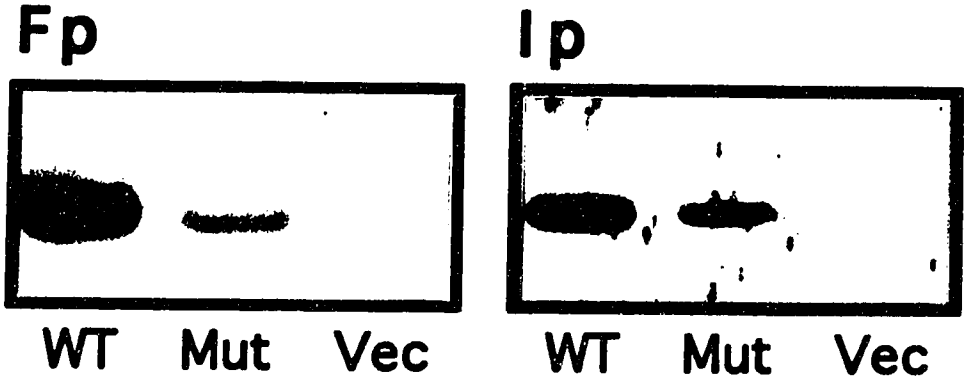
To determine if there is a difference in the rate of import between the wild-type and H90S Fp precursors, a time course experiment was performed (Figure 4-2). At the indicated times, import was stopped by the addition of valinomycin and cooling on ice; protein that had not been imported was degraded by externally added proteinase. No difference in the import rates of the wild-type and H90S Fp precursors was detected.

Complementation. To ascertain if the H90S Fp is capable of supporting SDH activity *in vivo*, we tested for complementation of the strain, sdh1L6, which is disrupted in the *SDH1* gene and therefore cannot grow with glycerol as the sole carbon source. Only pSDH1, which encodes the wild-type Fp, and not pS1H90S, which encodes the mutant Fp, or the parental vector pRS416 complemented for growth on glycerol (Table 4-1). We therefore concluded that the His90→Ser mutation eliminates SDH activity *in vivo*.

Assembly of the Fp and Ip Subunits. To examine the effects of the His90→Ser mutation on SDH assembly, mitochondria or submitochondrial particles were isolated from the disruption strain transformed with either pSDH1, pS1H90S, or pRS416. In submitochondrial particles from sdh1L6 carrying the plasmids pSDH1 or pS1H90S, wild-type and H90S Fp are detected by Western blot analysis (Figure 4-3). The Fp is not detected in the submitochondrial particles from the sdh1L6 containing the vector pRS416. The wild-type Fp is present at approximately three times the level of the H90S Fp as estimated by densitometry. The size of the mutant Fp corresponds to that of the mature protein, indicating that it is imported and proteolytically processed *in vivo*. Again, no size difference that might arise from differential flavinylation is detected between the wild-type and H90S proteins.

The presence of the Ip subunit was also examined by Western blot analysis (Figure 4-3). The Ip subunit is not detected in membrane fractions from sdh1L6 plus pRS416, but is present with the pSDH1 plasmid and in reduced amounts with the pS1H90S plasmid.

Figure 4-3. Western blot analysis. Submitochondrial particles (Fp panel) or mitochondria (Ip panel), were isolated from sdh1L6 transformed with the plasmids pSDH1 (Wt), pS1H90S (Mut), or pRS416 (Vec). Protein samples (50 μg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to Western blot analysis. The antibodies used are: Fp, affinity purified Fp subunit antibodies; Ip, Ip subunit antiserum.


Fp
Ip
WT
Mut
Vec
WT
Mut
Vec

Quantification of Covalently Bound FAD. In *S. cerevisiae*, the SDH Fp subunit is the only membrane bound protein with a covalently attached flavin; hence the amount of trichloroacetic acid-precipitable flavin directly reflects the levels of Fp (Singer *et al.*, 1965; Robinson *et al.*, 1991). Acid precipitable flavin measurements were performed to determine whether, like *E. coli* FRD, the His90→Ser Fp mutation prevented the covalent attachment of FAD (Table 4-1). Submitochondrial particles from MH125 and sdh1L6 transformed with pSDH1 both contain covalently bound FAD; the lower amount in sdh1L6 plus pSDH1 is attributable to plasmid loss in approximately 60% of the cells during growth in the absence of selection. The same membranes from sdh1L6 plus pS1H90S or pRS416 contain no acid-precipitable flavin above background levels, demonstrating that the His90→Ser mutation prevents the covalent attachment of FAD.

Iron-sulfur Centres of the Ip Subunit. The three iron-sulfur clusters in the Ip subunit are: a [2Fe-2S], a [3Fe-4S], and a [4Fe-4S] cluster called centres 1, 3, and 2, respectively. Richard Rothery examined the Ip centres 1 and 3 by EPR spectroscopy (Figure 4-4). In air or ferricyanide-oxidized samples of submitochondrial particles containing either the wild-type or the H90S Fp, centre 3, the [3Fe-4S] cluster, is clearly detectable (the spectrum of sdh1L6 containing pRS416 has been subtracted). Centre 1, the [2Fe-2S] cluster, is also detected in the dithionite-reduced submitochondrial particles containing either the wild-type or H90S Fp (Figure 4-4). The centre 1 and centre 3 signals in the samples containing H90S Fp are greatly reduced compared to the wild-type signals. This can be explained by the lower amounts of Ip present as judged by Western blot analysis. However, Ip centres 1 and 3 are clearly both assembled in the samples containing the H90S Fp. Due to low concentration of SDH in submitochondrial particles containing either the wild-type and H90S Fp it was not possible to determine the effect of the His90→Ser mutation on the redox potential of the FAD/FADH·/$FADH_2$ couples.

Figure 4-4. EPR detection of the iron-sulfur centres. The EPR spectra of oxidized (Ox) and reduced (Red) submitochondrial particles isolated from sdh1L6 transformed with pSDH1 (Wt) or pS1H90S (Mut) minus the oxidized spectrum (1X) or the reduced spectrum (0.75X) of the submitochondrial particles isolated from sdh1L6 transformed with pRS416, respectively. Spectra of ferricyanide-oxidized (200 μM potassium ferricyanide) samples were recorded at 10 K; spectra of dithionite-reduced (5 mM dithionite) samples were the sum of five scans accumulated at 20 K. Both the oxidized and reduced spectra were recorded under the following conditions: microwave power, 20 mW; microwave frequency, 9.48 GHz; modulation amplitude, 10 G at 100 kHz; gain, $1X10^5$. The x-axis corresponds to the field intensity in Gauss.

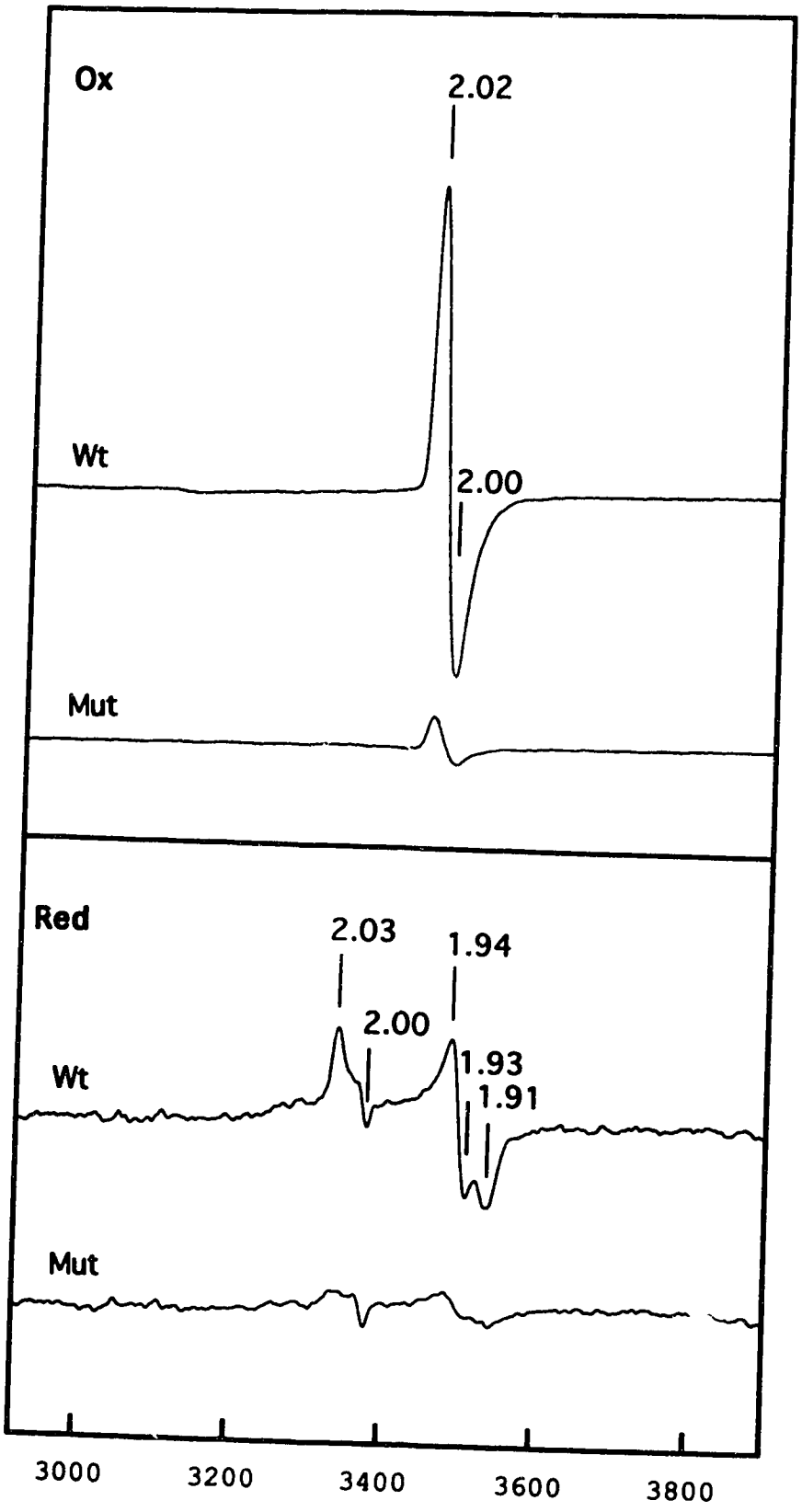

Ox
2.02
Wt
2.00
Mut
Red
2.03
1.94
2.00
Wt
1.93
1.91
Mut
3000
3200
3400
3600
3800

TABLE 4-1. Effects of the His90→Ser mutation on growth, acid-precipitable flavin, SDH and FRD activities.

Parameter	Strain			
	MH125	sdh1L6 + pSDH1	sdh1L6 + pS1H90S	sdh1L6 + pRS416
growth on glycerol	+	+	-	-
acid-precipitable flavin[a]	47	25	ND (<5)	ND
succinate-dichlorophenol indophenol reductase activity[b]	238	97	ND (<7)	ND
benzyl viologen-FRD activity[c]	90	54	15	ND (<2)

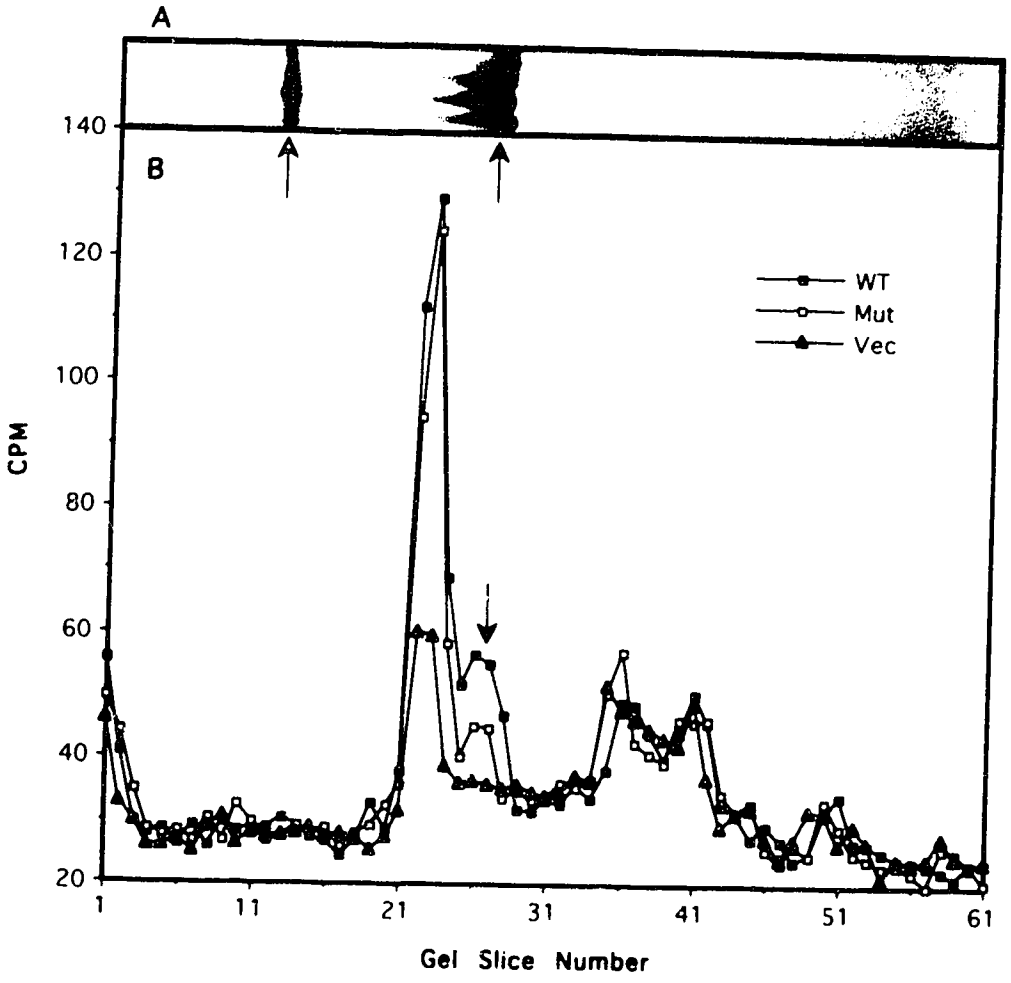
Complementation studies were performed on minimal medium containing glycerol as the sole carbon source and the required auxotrophic nutrients. Strains were grown to late stationary phase and submitochondrial particles were isolated and assayed for the presence of covalently attached FAD and SDH or FRD activities (Dickie and Weiner, 1979; Robinson *et al.*, 1991). Plasmids were retained in approximately 40% of the cells after growth without selection. Acid-precipitable flavin contents and activities are expressed in: [a] picomoles of flavin mg^{-1}; [b] nanomoles dichlorophenol indophenol reduced $min^{-1}mg^{-1}$; [c] nanomoles benzyl viologen oxidized $min^{-1}mg^{-1}$. ND, Not detectable.

Enzyme Activities. Submitochondrial particles from MH125 and sdh1L6 transformed with either pSDH1, pS1H90S, or pRS416 were isolated and assayed for SDH and FRD activities (Table 4-1). Succinate-dichlorophenol indophenol reductase activity is present in the samples with the wild-type Fp but not in samples containing the H90S Fp. In contrast, reduced-benzyl viologen fumarate oxidoreductase activity is observed in both wild-type samples and the samples containing the H90S Fp. However, unlike the wild-type submitochondrial particles, the samples that contained the H90S Fp did not show an increase in the rate of oxidation of benzyl viologen with formation of the oxidized dye (Ackrell *et al.*, 1993). Neither SDH or FRD activity is detectable in the samples from the disruption strain plus the vector, pRS416.

Noncovalently Bound FAD. The detection of reduced-benzyl viologen fumarate oxidoreductase activity suggested that the H90S Fp binds FAD noncovalently. We expected that noncovalently attached FAD should remain associated with this mutant Fp during non-denaturing gel electrophoresis. To show this, cells were grown with ^{14}C-labeled riboflavin which will serve as metabolic precursor for FAD. However, only riboflavin auxotrophs will accumulate exogenously-added riboflavin (Perl *et al.*, 1976). Furthermore, as the wild-type holoenzyme and the H90S Fp holoenzyme are expected to co-migrate, the presence of noncovalently associated FAD in the mutant holoenzyme must be determined in a strain that is also disrupted in the *SDH1* gene. Therefore, to examine the FAD content of the H90S Fp, I constructed by standard genetic techniques the strain RbS1W8C, which is a riboflavin auxotroph disrupted in the *SDH1* gene.

RbS1W8C transformed with pSDH1, pS1H90S, or pRS416 was grown in the presence of radiolabeled riboflavin. Mitochondria were isolated, and the proteins were resolved by a non-denaturing gel system, namely blue native gel electrophoresis. The radiolabeled lanes of the gel were sliced and radioactivity was measured (Figure 4-5B). All covalent and noncovalent flavoproteins of mitochondria will be labelled and detected by this approach. To identify the position of the SDH holoenzyme following electrophoresis, mitochondria isolated from D273-10B, a normal healthy yeast strain used in mitochondrial studies, were resolved on the same gel as the radiolabeled samples and the gel lane was subjected to histochemical staining for SDH activity. Two bands are visualized by this histochemical staining. The higher molecular weight band (Figure 4-5A, open arrow) may be an aggregate of the SDH holoenzyme. Several observations support the conclusion that the lower molecular weight band identified by activity staining (Figure 4-5A, closed arrow) is the SDH holoenzyme. First, staining is succinate dependent. Second, staining is absent in sdh1L6 mitochondria that do not contain a functional SDH. Thirdly, resolution of the SDH staining band into individual subunits by denaturing gel electrophoresis produces four bands of the sizes expected for the subunits of SDH. Finally, the largest of the four bands,

Figure 4-5. Content of covalently and noncovalently bound FAD in SDH. Mitochondria were isolated from D273-10B or RbS1W8C transformed with the plasmids pSDH1 (Wt), pS1H90S (Mut), or pRS416 (Vec) grown on YPGal supplemented with ^{14}C-riboflavin. Mitochondria were solubilized and resolved by blue native gel electrophoresis (Schägger and von Jagow, 1991). *A* The lane containing the D273-10B mitochondria after histochemical staining for SDH activity (Owen *et al.*, 1982). The closed arrow indicates the position of the SDH holoenzyme visualized with activity staining. The open arrow indicates an oligomer of the SDH holoenzyme. *B* The lanes containing the radiolabeled mitochondria were sliced, digested, and the radioactivity was measured. The counts per minute (CPM) for each gel slice were plotted against the slice number. The direction of migration is from left to right. The arrow indicates the peak of radiation due to bound flavin in the wild-type and H90S SDH holoenzymes.


A
B
140
120
100
80
60
40
20
CPM
WT
Mut
Vec
1
11
21
31
41
51
61
Gel Slice Number

the 67 kDa protein, was isolated and identified as the Fp subunit by amino terminal sequence determination (Bullis and Lemire, 1994). The identity of the large peak of radioactivity seen in gel slices 22-23 in both the wild-type and H90S Fp samples is unknown. It does not co-migrate with the major band of SDH activity. Also, the peak of radioactive material is present, although reduced, in the disruption mutant plus vector sample. For these reasons, we believe this peak is not relevant to the present study.

A smaller peak of radioactive material is observed to migrate with the succinate-dependent tetrazolium dye reducing band in mitochondria containing either the wild-type or H90S Fp (Figure 4-5B, closed arrow), but not in the disruption strain transformed with pRS416. The reduced amount of radiation migrating with SDH in the sample containing the H90S Fp as compared to the wild-type is most likely due to reduced Fp levels present as judged by Western blot analysis (Figure 4-3). We attribute this peak to radiolabeled FAD noncovalently associated with the H90S Fp SDH holoenzyme.

We did not detect a peak of radiation corresponding to the slower migrating form of SDH seen in Figure 4-5A. There are two likely explanations for this. Firstly, this form of SDH is most prominent under fully respiratory conditions such as in the mitochondria from lactate-grown D273-10B (Figure 4-5A). In contrast, the data in Figure 4-5B are from galactose-grown RbS1W8C; the levels of SDH in these mitochondria are much lower than the levels shown in Figure 4-5A. Secondly, the slower migrating form of SDH has only a fraction of the SDH activity of the major band. Therefore, it is not surprising that the amount of radiolabeled FAD associated with this form of SDH might be too small to detect.

4-4. DISCUSSION

In recent years, a great deal has been learned about the targeting and import of proteins into mitochondria. However, the mechanisms of cofactor attachment and assembly of complex enzymes after import remain poorly understood. To study these processes using *S. cerevisiae* SDH as a model, we have constructed a Fp mutant unable to

covalently bind FAD. The mutant, H90S Fp, has His-90, the predicted site of FAD attachment, converted to a serine residue.

Both *in vivo* and *in vitro* experiments demonstrate that the H90S Fp is imported and proteolytically processed. *In vitro*, the precursor H90S Fp is translocated into isolated mitochondria at the same rate as the wild-type Fp where it is proteolytically cleaved to the mature size. *In vivo*, only the mature size of the mutant Fp is detected by Western blot analysis demonstrating correct targeting and proteolytic processing. That import and processing of the H90S Fp are not affected as compared to the wild-type Fp suggests that covalent flavinylation may normally occur after import is completed.

Cofactor attachment can take place before or after import. The attachment of heme to cytochrome c (Dumont *et al.*, 1991), of pyridoxal phosphate to mitochondrial aspartate aminotransferase (Sharma and Gehring, 1986), and of biotin to pyruvate carboxylase (Ahmad and Ahmad, 1991) all occur after import. Interestingly, the biotinylation of the α-subunit of propionyl-CoA carboxylase can either precede or follow import and proteolytic processing (Taroni and Rosenberg, 1991). In contrast, the processing of the intermediate form of cytochrome c_1 to the mature size is dependent on the covalent attachment of heme (Nicholson *et al.*, 1989). Further studies, with the aid of the H90S Fp, determined the order of flavinylation and import (see chapter 5).

In both FRD and SDH, FAD is bound by a combination of covalent and noncovalent interactions. The presence of the flavin cofactor is required for the benzyl viologen-FRD activity (Blaut *et al.*, 1989). Therefore, the presence of benzyl viologen-FRD activity and the detection of radiolabelled FAD associated with the H90S Fp holoenzyme demonstrates that the mutant Fp is still able to bind FAD noncovalently, as is the analogous mutant in the *E. coli* FRD Fp (Blaut *et al.*, 1989). In contrast, several *B. subtilis* Fp mutants have completely lost the ability to interact with FAD (Hederstedt, 1983). One *B. subtilis* mutation, the conversion of a conserved glycine five amino acids downstream of the site of FAD attachment to an aspartic acid residue, exemplifies the role

of noncovalent interactions with the cofactor (Maguire *et al.*, 1986). Several regions in the Fp sequence have been suggested to interact noncovalently with the cofactor (Hederstedt and Ohnishi, 1992).

Numerous observations suggest that the covalent attachment of FAD to the Fp subunit is not necessary for its assembly with the other subunits of SDH. We and others (Lombardo *et al.*, 1990; Robinson *et al.*, 1991) have shown that neither the Fp nor the Ip subunits are stable when expressed alone. Schmidt *et al.*, (1992) have suggested that the Ip and Fp subunits are quickly degraded unless they are assembled into a stabilizing complex. Therefore, in the samples containing the H90S Fp, the detection of the Ip subunit strongly suggests that it has been stabilized by assembling with the mutant Fp. The assembly of the Ip subunit is further demonstrated by the detection of two of its iron-sulfur centres by EPR spectroscopy. Additionally, the covalent attachment of FAD is not required for assembly of the SDH holoenzyme; this assembly of the holoenzyme with the mutant Fp was indicated by the co-migration of the wild-type and H90S containing enzymes on the blue native gel system and by the detection of FRD activity in the samples with H90S Fp. Since this FRD activity detected in the samples containing the H90S Fp is membrane associated, the H90S Fp and Ip subunits are likely assembled into a holoenzyme with the membrane anchoring subunits. Similarly, both *B. subtilis* SDH and *E. coli* FRD Fp mutants that bind FAD noncovalently or not at all, still assemble into a membrane bound holoenzyme (Hederstedt, 1983; Blaut *et al.*, 1989).

SDH activity, but not FRD activity, in either FRD or SDH requires FAD to be covalently attached. Fp mutants in *E. coli* FRD that are able to bind FAD only noncovalently have no significant succinate phenazine methosulfate activity but almost wild-type levels of FRD activity (Blaut *et al.*, 1989). Similarly, this H90S Fp has no SDH activity as demonstrated by the inability of a plasmid encoding the mutant Fp to complement an *SDH1* disruption mutant and by the lack of detectable phenazine methosulfate mediated-succinate-dichlorophenol indophenol reductase activity. The

membranes containing the H90S Fp possess FRD activity, though at a lower level than membranes from the disruption strain transformed with pSDH1. However, membranes from the mutant have correspondingly lower levels of the Fp subunit as determined by Western blot analysis. This demonstrates that the covalent attachment of FAD in an SDH is not required for FRD activity.

Blaut *et al.*, (1989) suggest that the covalent attachment of FAD in *E. coli* FRD raises its midpoint potential and allows the oxidation of succinate. In agreement with this hypothesis, the *Desulfovibrio multispirans* FRD has noncovalently bound FAD and is unable to oxidize succinate (He *et al.*, 1986). Although the *E. coli* FRD is an excellent SDH (Cecchini *et al.*, 1986), yeast SDH is a very poor FRD. Yeast membranes have 50 to 100 times less FRD activity than the cytoplasmic membrane of anaerobically grown *E. coli* (Lemire *et al.*, 1982). Therefore, due to the low activity levels and the low enzyme abundance in the membrane preparations, we are unable to test this hypothesis by measuring the FAD midpoint potential of the H90S Fp by either kinetic or EPR analyses.

H90S Fp holoenzyme levels are consistently lower than those of the wild-type as judged by Western blot analysis, EPR spectroscopy, noncovalently associated radiolabeled FAD, and FRD activities. The decrease is not due to decreased import or processing of the H90S Fp subunit, as both seem unaffected *in vitro*. Brandsch *et al.*, (1989) have observed that the covalent attachment of FAD to 6-hydroxy-D-nicotine oxidase makes the enzyme much less susceptible to proteolysis than the apoenzyme. Therefore the absence of covalent FAD attachment in the H90S Fp may either make the Fp inherently less stable before assembly or cause the resulting mutant holoenzyme to be less stable to the membrane isolation procedures employed. Alternatively, without covalent attachment of FAD, a decreased rate of assembly of the Fp into the holoenzyme may allow more of it to be degraded.

The H90S Fp is targeted, translocated, and assembled like the wild-type protein but does not covalently bind its FAD cofactor. The H90S Fp holoenzyme has no detectable

SDH activity, but retains FRD activity. These findings indicate that covalent attachment of the FAD is not necessary for the import and assembly of an eucaryotic SDH Fp. This mutant Fp proved a valuable tool for dissecting the mechanism of flavinylation and the assembly pathway of SDH.

4-5. BIBLIOGRAPHY

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Chapter 5

Covalent Attachment of FAD to the Yeast SDH Flavoprotein Requires Import into Mitochondria, Presesquence Removal, and Folding

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5-1. INTRODUCTION

The relationships between the attachment of covalent cofactors to mitochondrial proteins and their import has been examined for several proteins. In some cases, such as the addition of pyridoxal phosphate to mitochondrial aspartate aminotransferase or the addition of biotin to pyruvate carboxylase, import and processing of the precursor proteins proceed without the coenzyme (Sharma and Gehring, 1986; Ahmad and Ahmad, 1991). In contrast, the biotinylation of propionyl-CoA carboxylase is independent of its import into mitochondria and can occur either before or after translocation (Taroni and Rosenberg, 1991). Interestingly, heme attachment to cytochrome *c* is essential for its irreversible localization to the intermembrane space (Dumont *et al.*, 1991). Covalent bond formation between the cofactor and the apoprotein is usually a catalyzed process (Schmidt *et al.*, 1969; Gross and Wood, 1984; Nargang *et al.*, 1988; Nicholson *et al.*, 1989). However, investigations with the 6-hydroxy-D-nicotine oxidase (6-HDNO) of *Arthrobacter oxidans* suggest that covalent FAD attachment to the purified apoprotein is autocatalytic (Brandsch and Bichler, 1991).

We report here the results of investigations carried out both *in vivo* and *in vitro* on the flavinylation of the yeast SDH Fp. In previous work, we constructed a mutant *SDH1* gene that encodes an apoprotein unable to undergo cofactor addition because the normally modified histidine had been converted to a serine (chapter 4; Robinson *et al.*, 1994). The resulting His90Ser Fp is imported into mitochondria, binds FAD non-covalently, and is assembled into an SDH holoenzyme. Covalent FAD attachment is thus, dispensable for both import and assembly and so the mutant Fp serves as a useful control for examining the flavinylation of the wild-type Fp. Flavinylation is assayed by immunoprecipitation with an anti-FAD serum that recognizes the holo-Fp, but not the apo-Fp or the mutant Fp (appendix A; Robinson and Lemire, 1995). We show here that FAD attachment *in vivo* occurs after import and proteolytic processing of the apo-Fp and that the rate of FAD attachment varies markedly with the carbon source that the cells are grown on. Interestingly, the Ip subunit

is stimulatory for FAD attachment. *In vitro,* carboxyl-terminal truncations of the apo-Fp completely eliminate modification by FAD while Krebs cycle intermediates function as effector molecules. Our results are consistent with a model where flavinylation is a post-translocational process that may be autocatalytic and occur during the folding of the mature Fp.

5-2. MATERIALS AND METHODS

Strains, Media, and Plasmids. Yeast strains used are described in Table 5-1. The *SDH1*, *SDH2*, *SDH3* and *SDH4* disruption mutants were made by myself, Bernard Lemire, Stephen Oyedotun, and Bonnie Bullis respectively. Standard yeast growth media have been described (see chapter 2; Robinson *et al.*, 1991).

TABLE 5-1. *Saccharomyces cerevisiae* strains.

Strain	Genotype	Parent/Source
D273-10B	Matα rho+	ATCC 25657
DAUL2	Mata ade2 ura3-del lys2 rho+	lab collection
Sdh1Ad1	Mata ura3-del lys2 sdh1::ADE rho+	DAUL2
MH125	Mata trp1 ura3-52 leu2-3,112 his3 his4 rme rho+	lab collection
sdh1L6	Mata trp1 ura3-52 his3 his4 rme sdh1::LEU2 rho+	MH125
sdh2L1	Mata trp1 ura3-52 his3 his4 rme sdh2::LEU2 rho+	MH125
sdh3W22	Mata ura3-52 leu2-3,112 his3 his4 rme sdh3::TRP1 rho+	MH125
sdh4W2	Mata ura3-52 leu2-3,112 his3 his4 rme sdh4::TRP1 rho+	MH125

For cloning experiments, the *E. coli* strains, UT580 and DH5α, were used. The plasmids, pSDH1 and pS1H90S, have been described (see chapters 3 and 4; Robinson and Lemire, 1992; Robinson *et al.*, 1994). The plasmid, pSfRHAC, which encodes the entire *SDH1* gene without any 5'-untranslated sequence, was created in a two step process. pSDH1 was cut with *Sfa*N1, the ends filled in with the Klenow fragment of DNA polymerase, and

digested with *Eco*R1 (see Figure 5-1). The *Sfa*N1/*Eco*R1 fragment encoding the Fp amino-terminus was cloned into *Eco*RV and *Eco*R1 digested pBluescriptII SK- (Stratagene, La Jolla, CA) placing the *SDH1* coding sequence under the expression of the T7 promoter and creating pSfR1. To reconstruct the entire *SDH1* coding sequence, pSDH1 was digested with *Nde*1, the ends filled in, cut with *Eco*R1, and the *Nde*1/*Eco*R1 fragment encoding the Fp carboxyl-terminus was cloned into pSfR1 digested with *Eco*R1 and *Sma*1. The *SDH1* gene obtained from pSfRHAC, was inserted into the multicopy vector, YEplac195 (Gietz and Sugino, 1988), and placed under the control of the inducible yeast copper metallothionein promoter (*CUP1*) obtained from the vector, Yep96 (Ellison and Hochstrasser, 1991), to create the plasmid, pCuSDH1. To place *SDH2* under control of the *CUP1* promoter, the *SDH2* open reading frame was amplified by a polymerase chain reaction and inserted into the vector, pTCu, which is YEplac112 (Gietz and Sugino, 1988) into which the *CUP1* promoter had been inserted, to create pCuB. The integrity of the *SDH2* gene was established by its ability to complement an *SDH2* disruption mutant. To select for the *SDH2* gene under the *CUP1* promoter in the yeast strain Sdh1Ad1, the *LYS2* gene (Fleig *et al.*, 1986) was inserted into pCuB, making the plasmid pKCu2.

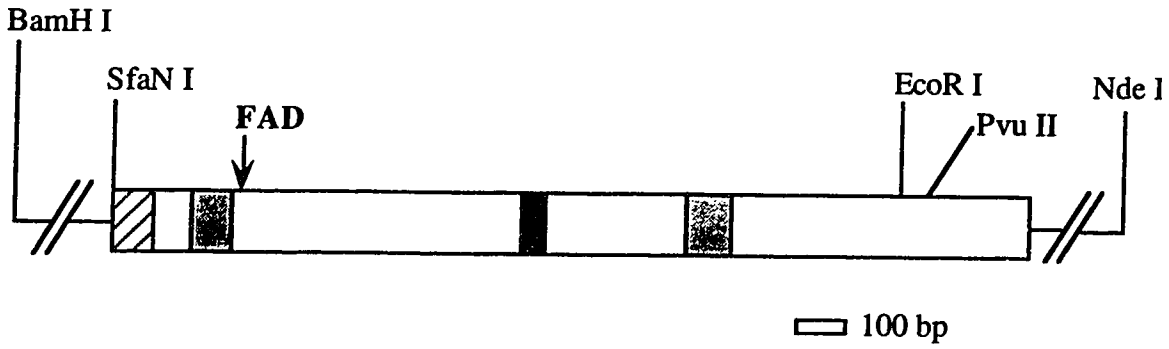


Figure 5-1. Restriction map of the yeast *SDH1* gene. The *SDH1* open reading frame is depicted as a box while flanking regions are depicted as lines. The hatched box represent sequences encoding the mitochondrial targeting sequence, the stippled boxes, the AMP binding domains, and the filled box, the active site. The arrow indicates the His-90 codon which encodes the histidine to which the FAD is covalently attached.

***In Vivo* Labeling.** Labeling was performed based on a previously published procedure (Brandt, 1991). Cells were grown overnight on semi-synthetic media containing 0.15 % yeast extract as well as the necessary auxotrophic markers, and a carbon source of either 2% glucose, galactose, or lactate. MH125 and related strains were supplemented with 0.1% glucose when grown on galactose. When the OD_{600} of the culture was between 0.8 and 1.0, it was quickly harvested and resuspended in pre-warmed labeling buffer containing auxotrophic markers and 2% carbon source. Radiolabeled methionine was added (Tran^{35}S-Label, 81 µCi/ml; 1037 Ci/mmol; ICN Biomedicals, Mississauga, Ont.) and the cells were labeled for 3 min at 30 °C with vigorous shaking. Cycloheximide and cold methionine were added to 100 µg/ml and 2 mM, respectively, and the chase continued at 30 °C with vigorous shaking. At the indicated times, 0.5 ml aliquots were taken, lysed with NaOH and 2-mercaptoethanol, and precipitated with trichloroacetic acid (TCA) (Yaffe and Schatz, 1984). Lysates were pelleted (14,000 *x g* for 10 min), resuspended in 1 ml of 2% sodium dodecyl sulfate (SDS) and 1% TCA. 0.1 ml 55% TCA was added to reprecipitate proteins and the sample incubated at room temperature for 10 min and then on ice for an additional 10 min. We found it necessary to wash the precipitates in SDS and to reprecipitate with TCA for the the anti-FAD serum to efficiently immunoprecipitate holo-Fp. Lysates were pelleted, washed with 1 ml ice-cold acetone, resuspended in 115 µl 2% SDS, 100 mM Tris-HCl, pH 7.5, and heated to between 65 and 90 °C for 10 min. Insoluble material was removed by centrifugation at 14,000 *x g* for 10 min and the supernatant used for immunoprecipitations. The efficiencies of the labeling and the chase were monitored as described (Brandt, 1991).

***In Vitro* Transcription, Translation, and Import.** Template mRNA was produced from the plasmids, pSDH1, pS1H90S, or pSfRHAC using T7 RNA polymerase and translated in rabbit reticulocyte lysate as described by the supplier (Promega Corp., Madison, WI). Carboxyl-terminal truncated Fps were produced from the plasmid, pSfRHAC, that had been digested with the restriction enzymes, *Eco*R1 or *Pvu*11, before

transcription and translation. Import competent mitochondria were isolated and *in vitro* import experiments containing 400 µg of mitochondria or mitoplasts in a volume of 320 µl were performed essentially as described using 10 µl of lysate per import reaction (chapter 4; Gasser *et al.*, 1982; Robinson *et al.*, 1994). Import reactions were supplemented with 20 mM succinate, 10 mM malate, and 50 µM FAD unless stated otherwise. Import was allowed to proceed for 1 hr at 30 °C. After proteinase K treatments (50 µg for 15 min at 0°C), re-isolated mitochondria or mitoplasts were resuspended in 1 ml of 2% SDS, 0.1 M Tris-HCl, pH 7.5, and prepared for immunoprecipitation as described above. 90% of the sample was used for immunoprecipitation and 4.5% was seperated by denaturing gel electrophoresis to determine protein import.

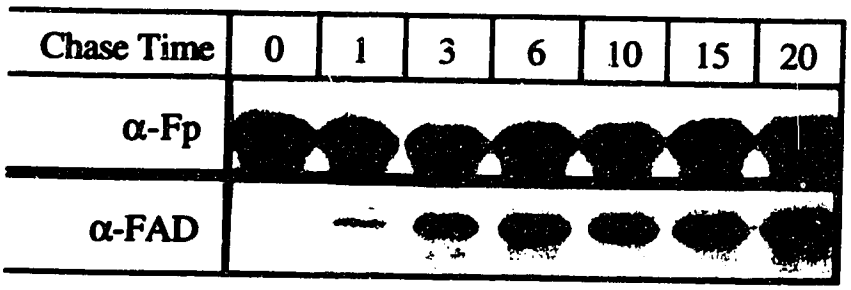
Immunoprecipitations. Immunoprecipitations were performed as described (Brandt, 1991) with the following modifications. 45 µL of *in vivo* cell lysate was added to 1 ml of BTNTE (2 mg/ml BSA, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 0.02% NaN_3) containing 5 µl preimmune serum and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated 1 hr at room temperature with rotation and centrifuged at 14,000 *x g* for 10 min. 8 µl protein A-sepharose beads (Sigma Chemical Co., St Louis, MO, USA, binding capacity of 20 mg human IgG/ml) were added to the supernatants incubated as above. The beads were removed by centrifugation, 10 µl of either anti-FAD serum or anti-Fp serum (Robinson *et al.*, 1991) added, and samples incubated overnight with rocking at 4 °C. Aggregated protein was removed by centrifugation at 14,000 *x g* for 10 min and 16 µl protein A-sepharose beads were incubated with the supernatants for 1 hr at room temperature. The beads were pelleted with a brief centrifugation, the supernatant removed, and the beads washed twice with NaTNTE (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% Triton X-100, 5 mM EDTA), twice with TNTE (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA), and finally twice with NTE (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA). The beads were resuspended in 20 µl of IPLB (0.1M Tris-Cl, pH 6.8, 5% SDS, 5 mM EDTA,

0.005% bromophenol blue, 25% glycerol, 5% 2-mercaptoethanol), heated for 10 min at 65 to 90 °C, spun briefly, and the supernatant resolved by SDS polyacrylamide gel electrophoresis. The gels were treated for fluorography, exposed to x-ray film, and when desired, the bands were quantified with a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.). For immunoprecipitations from *in vitro* translation experiments, 90 µl of lysate was added to 1 ml BTNTE containing 1 mM phenylmethylsulfonyl fluoride and 10 µl protein A-sepharose beads. Samples were treated as above except that 20 µl of anti-FAD serum and 30 µl of protein A-sepharose beads were used.

5-3. RESULTS

***In vivo* Flavinylation of the Yeast Fp.** We have previously shown that the anti-FAD serum only recognizes flavinylated protein and that this recognition can be competed for with free FAD or riboflavin, thus confirming its specificity (see Appendix A; Robinson and Lemire, 1995). The anti-Fp serum immunoprecipitates both apo- and holo-Fp forms of the protein. Therefore, the amount of FAD-modified Fp can be expressed as the amount of Fp immunoprecipitated by the anti-FAD serum divided by the total amount of Fp immunoprecipitated by the anti-Fp serum. The *in vivo* rate of FAD attachment to the Fp was examined by labeling cellular proteins in the wild-type strain, D273-10B, with ^{35}S-methionine and chasing with excess cold methionine and cycloheximide. Aliquots taken during the chase period were analyzed by immunoprecipitation with the anti-FAD and the anti-Fp sera. Only the mature size Fp is detected by both sera in this experiment. All the Fp molecules had reached the mitochondrial matrix and been proteolytically processed to the mature size. The precursor form of the Fp is not detected because import is too rapid under these conditions (Brandt, 1991). As seen in Figure 5-2, panel α-Fp, the total amount of labeled Fp is constant throughout the chase period indicating that the chase has been effective and that the mature Fp is stable over this time period. In contrast, the amount of flavinylated Fp immunoprecipitated with the anti-FAD serum is initially

Figure 5-2. The *in vivo* flavinylation of the Fp. D273-10B was grown in sulfate-free medium with lactate as the carbon source to an OD_{600} of approximately 1.0. Cells were harvested, resuspended in labeling buffer, labeled with ^{35}S-methionine for 3 min, and chased with cycloheximide and cold methionine. Aliquots were taken during the chase at the times indicated above individual lanes and immunoprecipitated with the anti-Fp serum (α-Fp) or the anti-FAD serum (α-FAD). Immunoprecipitates were performed and analyzed as described in 'Materials and Methods'.


Chase Time
0
1
3
6
10
15
20
α-Fp
α-FAD

undetectable and increases rapidly with time (Figure 5-2, panel α-FAD), demonstrating that the Fp is modified post-translationally after its presequence has been removed. Although it did not exceed 15%, flavinylation occured with a half-time of about 5 minutes. We believe the low yield is in part due to the lower efficiency of the anti-FAD serum in immunoprecipitations as compared to the anti-Fp serum (KMR, unpublished observations).

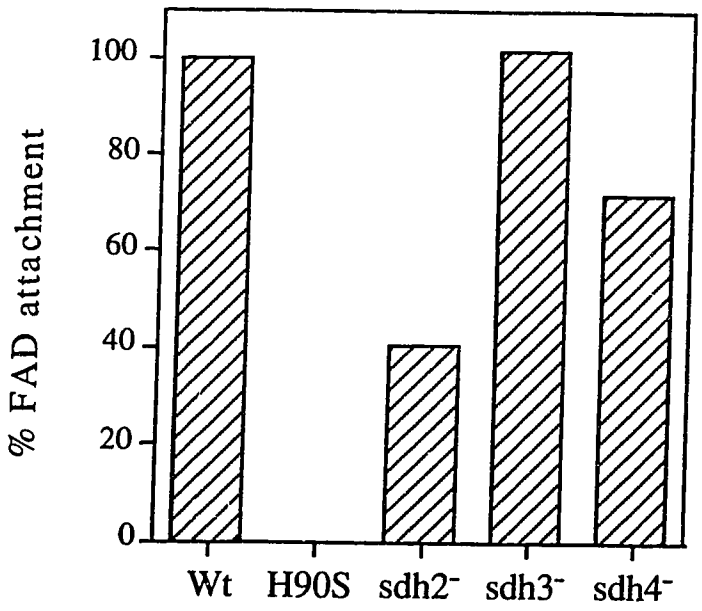


Figure 5-3. Roles of the SDH2, SDH3, and SDH4 subunits in Fp flavinylation. MH125, the wild-type strain (Wt), sdh1L6 carrying the plasmid, pS1H90S (H90S), sdh2L1 (sdh2-), sdh3W22 (sdh3-), and sdh4W2 (sdh4-) were grown in sulfate-free medium with galactose as the carbon source to an OD_{600} of approximately 1.0. Cells were harvested, resuspended in labeling buffer, labeled with ^{35}S-methionine for 3 minutes and the chased with cycloheximide and cold methionine. Aliquots were removed after chasing for 20 min and immunoprecipitated with the anti-Fp or the anti-FAD sera. The extent of Fp modification was determined by calculating the ratio of holo-Fp to total Fp present in each strain. The levels of flavinylated Fp in the mutant strains are compared to the amount detected in the wild-type, which is set to 100%. The values presented are from a single experiment but comparable data were obtained in two replicates.

Since flavinylation of the Fp occurs post-translationally and either co- or post-translocationally, we investigated whether the presence of the other SDH subunits could influence FAD attachment. A positive result would suggest that modification is a late step in the assembly of the SDH complex. The wild-type parent strain, MH125, and the

derived *SDH2, SDH3,* and *SDH4* disruption mutants (Table 5-1) were labeled, chased, and subjected to immunoprecipitations (Figure 5-3). As a control, sdh1L6, an *SDH1* disruption mutant transformed with the plasmid, pS1H90S, encoding the flavinylation-incompetent His90Ser Fp was also analyzed. We compared the amount of FAD-modified Fp in each mutant strain to the amount detected in the wild-type strain, which was set to 100%. As expected, the His90Ser Fp was not immunoprecipitated by the anti-FAD serum, demonstrating the specificity of the serum for the cofactor. Flavin attachment to the Fp was consistently reduced 2-3 fold in the *SDH2* mutant, but was not significantly affected in the *SDH3* or the *SDH4* mutants. Thus, FAD attachment to the Fp can proceed in the absence of any one of the other subunits, although the Ip subunit may enhance the process.

Since most covalent flavoproteins are localized to mitochondria (Hederstedt and Ohnishi, 1992), we investigated the flavinylation of the Fp under respiratory (lactate as the carbon source) and non-respiratory (glucose as the carbon source) conditions where mitochondrial function and development are quite different. We transformed the *SDH1* disruption strain, Sdh1Ad1, with the plasmid (pCuSDH1) containing the *SDH1* gene under the control of the *CUP1* promoter. Uninduced levels of expression from this promoter are not significantly affected by the carbon source (Hottiger *et al.*, 1994). When grown on lactate, the Fp will be expressed from the *CUP1* promoter while the other subunits will be expressed from chromosomally-encoded genes; when grown on glucose, only the Fp will be present as expression of the *SDH2, SDH3*, and *SDH4* genes will be repressed (Lombardo *et al.*, 1992; Daignan-Fornier *et al.*, 1994). Since the level of FAD attachment was reduced in the absence of Ip subunit (Figure 5-3), Sdh1Ad1 was also transformed with both pCuSDH1 and pCuB; the latter plasmid has the *SDH2* gene under the *CUP1* promoter. In this case, the Fp and Ip subunits will both be expressed when grown on glucose. We labeled the cellular proteins of cells grown on either glucose or lactate with radioactive methionine and removed aliquots for immunoprecipitation by the anti-Fp or the anti-FAD sera during the chase period. The data are presented as the percentage of counts

precipitated by the anti-FAD serum as compared to the counts precipitated by the anti-Fp serum (Figure 5-4). Both the rate and the extent of FAD attachment are severely reduced when cells are grown on glucose as compared to when they are grown on lactate. Furthermore, the presence of the Ip subunit does not change the kinetics of cofactor attachment when the cells are grown on glucose. The difference in FAD attachment with carbon source may arise from a requirement for an additional protein whose expression is repressed by glucose or from a need for a metabolite that is less abundant when the cells are grown on glucose.

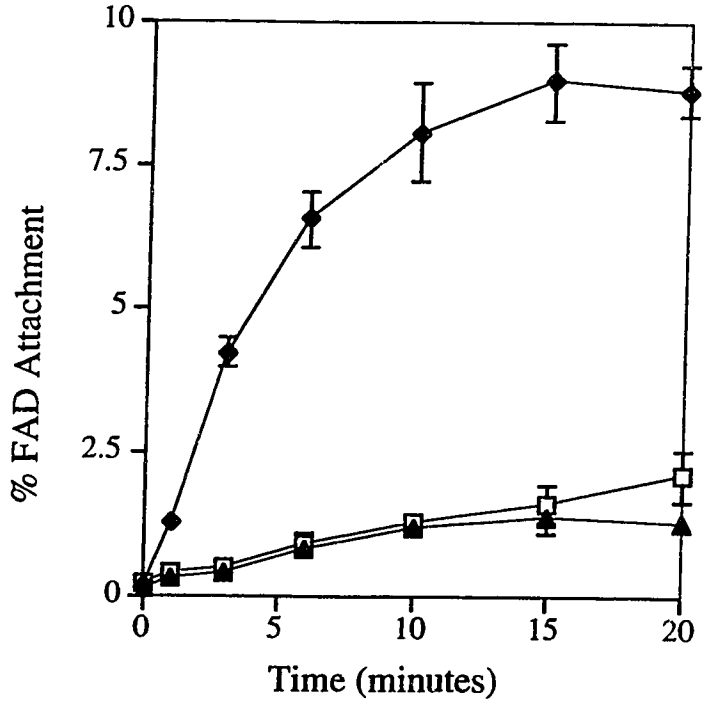


Figure 5-4. Effect of carbon source on FAD attachment. Sdh1Ad1 carrying the plasmid pCuSDH1, or both plasmids, pCuSDH1 and pCuB, was grown in sulfate-free medium with either lactate or glucose as the carbon source, labeled, chased, and analyzed as described in the legend to Figure 5-2. The expression of the *SDH1* gene is under control of the *CUP1* promoter in all cases. When present as pCuB, the expression of the *SDH2* gene is also under control of the *CUP1* promoter. Otherwise, the *SDH2, SDH3,* and *SDH4* genes are chromosomally encoded. The symbols represent Sdh1Ad1 carrying pCuSDH1 grown on lactate (diamonds), grown on glucose (triangles), and Sdh1Ad1 carrying pCuSDH1 and pCuB grown on glucose (squares).

***In vitro* Flavinylation of the the Yeast Fp.** To test whether modification by FAD could occur *in vitro* and thus provide a more convenient and manipulatable system for further investigations, we examined Fp flavinylation during import into both isolated whole mitochondria and mitoplasts (mitochondria that have had their outer membranes disrupted). *In vitro* translated wild-type or His90Ser precursor proteins were incubated with isolated mitochondria or mitoplasts, the organelles re-isolated, and cofactor attachment assayed by immunoprecipitation with the anti-FAD serum (Figure 5-4). If import is blocked by the ionophore valinomycin, modification of the surface-bound Fp is not detected (lanes 2 and 3, 'α-FAD' row), indicating that FAD is not attached prior to import. We found a significant proportion of the wild-type Fp precursor could be imported into mitoplasts, processed ('5% of imported Fp' row), and modified by FAD (lane 5, 'α-FAD' row), demonstrating that the FAD could be attached to the Fp *in vitro*. In these experiments, mitoplasts imported, and hence modified, larger quantities of the Fp than intact mitochondria (compare lanes 4 and 5; Hwang *et al.*, 1989). However, the fraction of imported Fp that was flavinylated in mitochondria and mitoplasts is approximately the same. FAD attachment might be occurring either during or after import. To distinguish between these possibilities, we performed import reactions and examined flavinylation both with and without proteinase treatment. Proteinase treatment selectively removes proteins that have not been fully imported. In these experiments, the extent of FAD attachment is not affected by proteinase treatment (compare lanes 5 and 7) suggesting that flavinylation requires complete import of the Fp into the mitochondria or mitoplasts. The His90Ser Fp (lane 8), although imported to the same extent as the wild-type, was not immunoprecipitated by the anti-FAD serum.

Since the conversion of FMN to FAD is performed by a cytosolic enzyme (Wu *et al.*, 1995), the implication is that mitochondria are able to transport FAD. The low levels of flavin attachment in our *in vitro* assays might be due to the loss of FAD during the isolation of mitochondria. Import reactions were performed in the absence of added flavin or in the

Figure 5-5. *In Vitro* import of the Fp and FAD attachment. Wild-type Fp (Wt) and His90Ser (H90S) Fp precursors were produced by *in vitro* transcription and translation in rabbit reticulocyte lysate. The precursors were imported into mitochondria (M) or mitoplasts (MP) and the organelles were re-isolated for analysis by SDS gel electrophoresis and fluorography (import) or by immunoprecipitation with anti-FAD serum (α-FAD) as described in 'Materials and Methods'.

Flavoprotein	Pre-Fp	WT	WT	WT	WT	WT	WT	H90S
Valinomycin	-	+	+	-	-	-	-	-
Proteinase K	-	-	-	-	-	+	+	+
Mitochondria	-	M	MP	M	MP	M	MP	MP
Lane Number	1	2	3	4	5	6	7	8

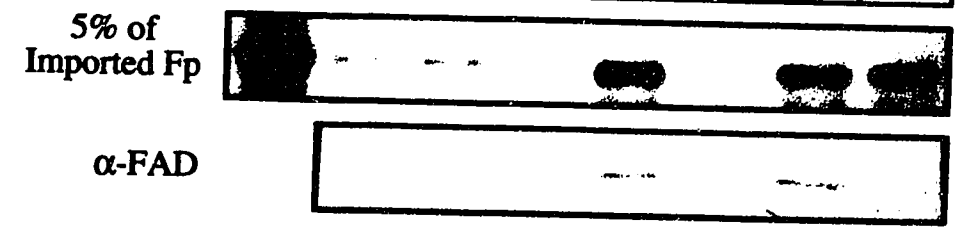


Figure 5-6. *In vitro* flavinylation requires added FAD. *A.* Wild-type Fp precursor, produced by *in vitro* transcription and translation, was imported into mitoplasts in the absence of additions (-), or in the presence of 50 μM riboflavin (Rb), (FMN), or FAD (FAD). After import, the mitoplasts were re-isolated for analysis by SDS gel electrophoresis and fluorography (lanes 1-5) or by immunoprecipitation with anti-FAD serum (lanes 6-9) as described in 'Materials and Methods'. The bands of Fp present from analysis by SDS-PAGE, and band of holo-Fp immunoprecipitated with the anti-FAD serum were quantitated and the ratio of holo-Fp to total Fp for each sample was calculated. For comparison, the ration of holo-Fp to total Fp for the import sample without addition of any flavin was set at 1.

	Pre-Fp	5% of Imported Fp				Immunoprecipitated with α-FAD			
Flavin Added		-	Rb	FMN	FAD	-	Rb	FMN	FAD
Lane Number	1	2	3	4	5	6	7	8	9
									
Fold Increase	-	-	-	-	-	1.0	0.7	0.8	3.2

presence of riboflavin, FMN, or FAD and cofactor attachment to proteinase-protected Fp assayed by immunoprecipitation with the anti-FAD serum (Figure 5-6). Import of the Fp into mitoplasts was not affected by the addition of riboflavin or FMN (lanes 2, 3, and 4), but was diminished by the addition of FAD to the import mix (lane 5). FAD attachment to the imported Fp in the absence of added flavin was minimal (lane 6) and the addition of either riboflavin or FMN did not increase protein modification (lanes 7 and 8, respectively). In contrast, cofactor attachment was substantially increased with the addition of FAD to the import mix (lane 9) suggesting that FAD is indeed transported across the mitochondrial inner membrane. The extents of flavinylation were quantified and the ratio of holo-Fp to total Fp present was calculated for each sample. For comparison between the samples, the ratio determined for modification without added flavin was set at 1 (Figure 5-6, row "Fold Increase"). These results suggest that FAD is the immediate substrate for covalent attachment and that riboflavin and FMN are incapable of supporting modification under these conditions.

An interesting observation pertaining to the flavinylation of the 6-HDNO, was the stimulation of modification by small molecules that could apparently act as allosteric effectors. We investigated whether a similar phenomenon occurs with the flavinylation of the Fp. Import reactions were performed with a variety of metabolites (Figure 5-7) and immunoprecipitations with the anti-FAD serum were used to determine the extent of cofactor attachment. Without the addition of any metabolites, FAD attachment was not detectable (Figure 5-7, blank). The 4-carbon Krebs cycle intermediates, succinate, fumarate, and malate resulted in the greatest stimulation of FAD attachment. Less effective, were malonate and oxaloacetate (not shown), competitive inhibitors of SDH and citrate. The addition of glycerol-3-phosphate, a compound which enhances the flavinylation of 6-HDNO, was not stimulatory (Brandsch and Bichler, 1989). Thus, FAD attachment to the Fp is stimulated by metabolites of the Krebs cycle or related molecules.

Figure 5-7. Flavinylation of the Fp requires effector molecules. *A.* Wild-type or His90Ser Fp precursors were imported into mitoplasts in the absence of any additions (blank) or in the presence of 20 mM succinate, fumarate, malonate, citrate, malate, or glycerol-3-phosphate. After import, the mitoplasts were re-isolated for analysis by SDS gel electrophoresis and fluorography (import) or by immunoprecipitation with anti-FAD serum (α-FAD) as described in 'Materials and Methods'. The amount of Fp present in both gels were quantitated and the ratio of holo-Fp to total Fp was calculated as described in figure legend 6. For comparison the ration of holo-Fp to total Fp of the import sample without any additions was set at 1.

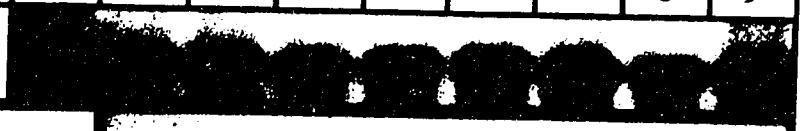
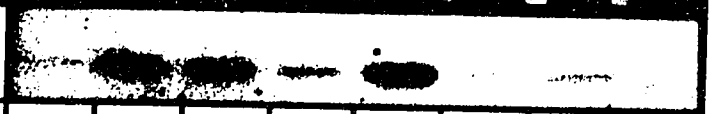
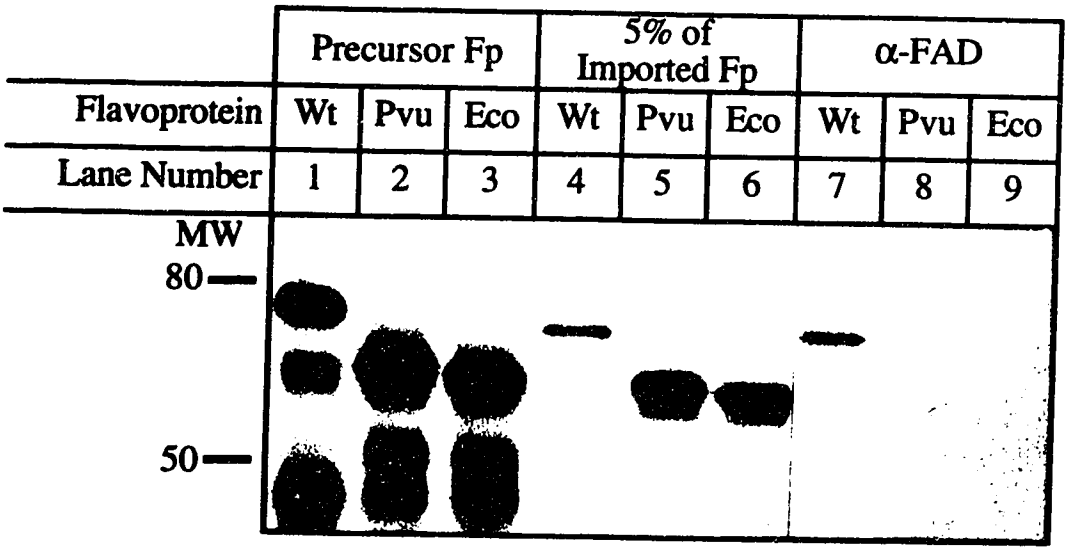
Additive	pFp	-	Suc	Fum	Mln	Mal	Oxl	Cit	G3P
Lane Number	1	2	3	4	5	6	7	8	9
5% of Imported Fp									
α-FAD									
Fold Increase	-	1.0	5.3	4.9	2.2	6.0	0.9	2.4	0.3

Figure 5-8. Carboxyl-terminal truncates of the Fp are not flavinylated. Full-length Fp (wt), or Fp with a carboxyl-terminal truncations at the *Pvu* II site, removing 70 amino acids (Pvu), or at the *Eco* RI site, removing 90 amino acids (Eco), were translated in rabbit reticulocyte lysate (lanes 1-3), imported into mitoplasts (lanes 4-6), and analyzed for FAD attachment by immunoprecipitation with the anti-FAD serum (lanes 7-9).


Precursor Fp
5% of Imported Fp
α-FAD
Flavoprotein
Wt
Pvu
Eco
Wt
Pvu
Eco
Wt
Pvu
Eco
Lane Number
1
2
3
4
5
6
7
8
9
MW
80
50

The stimulation of flavinylation by small effector molecules *in vitro* and by the presence of the Ip subunit *in vivo* suggests that the Fp must adopt some of its native structure before modification occurs. To test this possibility, we reasoned that if mitochondrial precursors are transported in an unfolded state (Eilers and Schatz, 1986; Deshaies *et al.*, 1988; Stuart *et al.*, 1993; Schwarz and Neupert, 1994) and modification precedes folding, then carboxyl-terminal truncations of the Fp should be without effect. We performed import reactions with Fp molecules that were truncated of 70 amino acids (Pvu) or 90 amino acids (Eco) at their carboxyl termini (Figure 5-8). Truncated precursors were efficiently synthesized (lanes 2 and 3) and imported to a protease protected location (lanes 5 and 6). However, cofactor attachment was undetectable with the truncated Fp molecules (lanes 8 and 9). Thus, almost the entire Fp is required for FAD attachment.

5-4. DISCUSSION

Many of the details of protein import into mitochondria have been elucidated in recent years, including some aspects of the folding process that follows translocation. However, the assembly of subunits into multisubunit complexes and the insertion of cofactors remains poorly understood. We have been utilizing the biogenesis of the yeast SDH as a model system with which to address these problems. One of the most distinguishing features of SDH enzymes is the presence of a covalently attached FAD coenzyme; it is with the addition of FAD that this work is concerned.

FAD attachment was monitored with the aid of an FAD-specific polyclonal antiserum that we have raised. The serum recognizes the holo-Fp subunit but not the apo-subunit. In addition, as a control, we have used a mutant Fp subunit that is capable of being transported into mitochondria, assembling into a membrane-bound enzyme, and binding FAD but only in a non-covalent manner.

FAD attachment occurs in mitochondria after the Fp is imported. Pulse-chase experiments (Figure 5-2) demonstrated that FAD is attached *in vivo* after the presequence has been cleaved, a process that occurs in the mitochondrial matrix. We have not detected

any flavinylated precursor protein even when import is blocked with uncoupler (KMR, unpublished data). Partly imported or surface-bound Fp, which can be distinguished from fully imported molecules by proteinase sensitivity, do not get modified. Finally, if FAD attachment is a co-translocational process, we would not anticipate any effects on the modification of carboxyl-terminal truncated Fp molecules since these would appear identical to the full-length Fp until their import is almost complete. FAD addition is not detected in truncated Fps, suggesting that attachment occurs post-translocationally when the entire Fp is available for folding and assembly. Modification of the carboxyl-terminal truncated Fps could also not be detected when these proteins were expressed *in vivo* under respiratory conditions (K.M.R. unpublished observations). Flavinylation studies on the 6-HDNO and on *E. coli* FRDA also indicate that cofactor attachment requires the entire length of these proteins and therefore must occur post-translationally (Cecchini *et al.*, 1985; Cole *et al.*, 1985; Brandsch *et al.*, 1993).

The immediate substrate for flavinylation is likely FAD since only FAD and not the precursors, riboflavin or FMN, increase the amount of cofactor attached *in vitro*. Recent work by Tzagoloff's group has demonstrated that the yeast flavin synthase, which adenylates FMN to FAD, is located in the cytosol (Wu *et al.*, 1995). Thus, even if riboflavin or FMN were transported into mitochondria, they would not be converted into FAD. Neither riboflavin or FMN are apparently attached to the Fp as they do not increase the amount of Fp immunoprecipitated by the anti-FAD serum (Figure 5-6) even though this serum recognizes these molecules (see appendix A; Robinson and Lemire, 1995). Studies with 6-HDNO have led to similar conclusions that the flavin moiety is attached to the protein as FAD rather than as riboflavin or FMN which is subsequently converted to FAD (Brandsch and Bichler, 1991). Our studies also support the suggestion that yeast mitochondria have an FAD transporter (Wu *et al.*, 1995).

Proteolytic processing can be a mandatory step for cofactor attachment. For example, cyctochrome *c1* is cleaved in two steps, first to an intermediate form and then to

the mature size. Heme attachment precedes cleavage of the intermediate to the mature form of the protein (Nicholson *et al.*, 1989). Our studies with the SDH Fp have shown that cofactor attachment occurs after presequence cleavage, although they do not address whether FAD addition could proceed if proteolytic processing were prevented.

Modification of the Fp with FAD appears to happen once the mature Fp molecule has adopted some structure. Three lines of evidence support this hypothesis; firstly, we demonstrated a decrease in the extent of flavin attachment to the Fp *in vivo* in an *SDH2* disruption mutant. The yeast SDH Fp and Ip subunits are postulated to form an assembly intermediate since in the absence of one, the other is proteolytically degraded (Lombardo *et al.*, 1990; Robinson *et al.*, 1991; Schmidt *et al.*, 1992). Thus, the Ip may stabilize the folding or assembly of the Fp and thereby assist in cofactor attachment. However, the Ip is not essential for the process and thus, Fp modification probably precedes assembly with the other SDH subunits. Similar conclusions have been reached in prokaryotic systems; FAD is attached to the SDH or FRD Fps independently of the other subunits (Hederstedt, 1980; Hederstedt *et al.*, 1982; Cole *et al.*, 1985).

Secondly, FAD attachment *in vitro* was greatly stimulated by the addition of Krebs cycle intermediates which may promote a conformation conducive to the process by acting as effectors molecules. Interestingly, the most efficient effectors, succinate, malate, fumarate and to a lesser extent oxaloacetate and malonate are all known to bind to the SDH active site located in the Fp subunit (Kotlyar and Vinogradov, 1984). It is tempting to suggest that these effector molecules are manifesting their effects on flavinylation by virtue of their binding to and perhaps stabilizing an appropriate conformation at the active site. Correspondingly, 6-HDNO also requires allosteric effectors, in this case, phosphorylated three carbon molecules, for its flavinylation while prokaryotic SDH and FRD Fps showed a dependence on Krebs cycle intermediates for FAD attachment (Brandsch and Bichler, 1989). The effectiveness of the intermediates differs between the prokaryotic proteins and the yeast Fp, but this may be due to differences in their individual uptake rates into

mitochondria or may reflect a more fundamental difference between prokaryotic and eukaryotic Fps. FAD attachment may be a mitochondrial event because of the requirement for substrate-like molecules that are present in the matrix. In addition, the need for effectors may explain why the rate of FAD attachment is reduced in cells grown on glucose; a condition that might result in lower concentrations of these molecules. Accordingly, the limiting factor for FAD attachment to 6-HDNO when expressed in *E. coli*, is proposed to be the concentration of the effector molecules (Brandsch and Bichler, 1992). This requirement for effectors may provide an additional level of control over the biogenesis of mitochondrial enzymes.

Thirdly, if flavinylation were a reaction that required a fully unfolded protein substrate, then one would not expect carboxyl-terminal truncations of the Fp to have any effect since the FAD-modified histidine (His-90) is at the amino terminus of the precursor protein. The truncations we tested, removed 70 or 90 residues, all of which are well beyond portions of the protein postulated to be involved in contacting the AMP moiety of the FAD (Figure 5-1).

The evidence that we have provided, although not conclusive, suggests that flavinylation of the Fp requires some structure be adopted and is consistent with an autocatalytic reaction mechanism in which the Fp catalyses flavinylation without the participation of other proteins. Such a mechanism has been proposed for the modification of 6- HDNO. One argument against autocatalysis is the existence of specific enzymes for heme addition to such proteins as cytochrome *c*, and cytochrome *c1* (Nargang *et al.*, 1988· Dumont *et al.*, 1991), for biotin addition to carboxylases (Gross and Wood, 1984), and for lipoic acid addition to α-ketoglutarate dehydrogenase (Schmidt *et al.*, 1969). A second argument is that the reduced rate of FAD attachment observed when the cells are grown on glucose could be accounted for by diminished levels of a flavinylation enzyme because of repressed expression on glucose. Alternatively, metabolites such as effectors molecules or even FAD itself may be limiting in glucose grown cells. Clearly, further work is required

to clarify the mechanism of flavinylation of the yeast Fp and its relationship to protein folding and subunit assembly.

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Chapter 6

Presequence Cleavage is required for Covalent Attachment of FAD to the Yeast SDH Flavoprotein Subunit

A version of this chapter has been submitted for publication: Robinson, K. M. and Lemire, B. D. (1995)

6-1. INTRODUCTION

The assembly of many mitochondrial proteins often involves proteolytic processing, cofactor insertion, and oligomerization, and can depend on the activities of special proteins such as the mitochondrial processing peptidase (MPP) (Arretz *et al.*, 1991; Glick *et al.*, 1992) and the chaperonin, heat-shock protein 60 (Hsp60) (Stuart *et al.*, 1994). The processing peptidase proteolytically removes cleavable presequences, a step that must occur before cofactor insertion or assembly into multisubunit complexes can proceed for some proteins (Nicholson *et al.*, 1989; Graham *et al.*, 1993). Proteins translocated into the matrix also interact with Hsp60 (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). Hsp60, a member of the chaperonin family, mediates the folding and assembly of some imported proteins in an ATP-dependent reaction (Georgopoulos and Welch, 1993; Becker and Craig, 1994). Both the processing peptidase and Hsp60 are indispensable for mitochondrial biogenesis, as they are both essential for viability in *Saccharomyces cerevisiae* (Baker and Schatz, 1991).

Covalent cofactor attachment to mitochondrial and non-mitochondrial proteins can also be mediated by special enzymes. For example, heme (Nargang *et al.*, 1988; Nicholson *et al.*, 1989; Dumont *et al.*, 1991), biotin (Gross and Wood, 1984), and lipoic acid (Schmidt *et al.*, 1969) are all attached to target proteins by specific enzymes. The mechanism of covalent FAD attachment for mitochondrial proteins such as the SDH Fp has not been elucidated, although the bacterial enzyme 6-HDNO which binds FAD via the same linkage attaches its cofactor to itself in an autocatalytic reaction (Brandsch and Bichler, 1991). Whether the formation of covalent protein-FAD bonds is autocatalytic for all flavoproteins remains to be documented.

Several observations indicate that proper protein folding is a prerequisite for covalent FAD attachment. Firstly, unrelated enzymes with 8α-N(3)-histidyl-FAD linkages show no sequence identity other than the histidine residue (Lang *et al.*, 1991). Since no consensus sequence is apparent, protein conformation itself may be the signal for FAD

attachment. Secondly, the 6-HDNO must also attain a correct protein conformation which can be stabilized by glycerol or sucrose, before it flavinylate itself (Brandsch and Bichler, 1992; Brandsch *et al.*, 1993). Thirdly, a number of observations indirectly suggest that FAD attachment to SDH Fp requires folding, or at least partial folding of the Fp as well, as described in chapter 5.

Previously, we showed that Fp modification can occur after import into isolated mitochondria. To further investigate the mechanism of Fp modification, I have developed an *in vitro* flavinylation system consisting of *in vitro* translated Fp and a mitochondrial lysate and assayed for flavin attachment by immunoprecipitation with an anti-FAD serum. Our results indicate that FAD attachment can occur without translocation of the Fp across the mitochondrial membranes. In contrast to results obtained *in vivo*, modification is not enhanced by the presence of the Ip subunit. It is stimulated by the presence of Krebs cycle intermediates such as succinate and malate and needs ATP. Interestingly, proteolytic processing of the presequence is mandatory for FAD attachment to the Fp. Finally, we show that the Fp interacts with Hsp60. The data are consistent with a model that requires the Fp to be proteolytically processed to the mature size and to acquire a partially folded conformation with the assistance of Hsp60, before modification with FAD.

6-2. MATERIALS AND METHODS

Media, Strains, and Plasmids. *Escherichia coli* and yeast strains and their growth media have been described (see chapter 2; Robinson *et al.*, 1991). The plasmids, pSDH1 and pS1H90S, encode the wild-type and a flavinylation-incompetent His90Ser Fp, respectively (chapters 3 and 4; Robinson and Lemire, 1992; Robinson *et al.*, 1994). The plasmid, pT7mFp, carries the *SDH1* gene that has been truncated at the amino-terminal end so as to encode an Fp subunit without a presequence in the vector, pBluescript II KS- (Stratagene, La Jolla, CA). By a polymerase chain reaction, a methionine codon was

introduced in frame immediately preceding the Gln-29 codon; this residue corresponds to the amino-terminal amino acid of the processed Fp precursor (Bullis and Lemire, 1994). The amino- and carboxyl-terminal *SDH1* coding sequences not contained in the internal 1.48-kilobase *Pst* I fragment were verified by sequencing. The internal *Pst* I fragment was replaced with the same fragment derived from a genomic source. The plasmid, pT7LSC, encodes a chimeric matrix-targeted precursor composed of the a mitochondrial targeting sequence fused to the large subunit of eucaryotic Rubisco expressed from the T7 promoter in pBluescript II SK- (Rospert *et al.*, 1994). The orignal plasmid encoding the LS chimeric protein was a generous gift of Sabine Rospert and Jeff Schatz. The entire *SDH2* open reading frame was amplified by a polymerase chain reaction and placed downstream of the T7 promoter in the vector, pBluescript II KS-, to produce the plasmid, pSDHB39.

Preparation of Matrix Lysates. Matrix lysates were prepared from mitoplasts (Jascur, 1991) suspended at 10 mg/ml protein in Flavinylation Buffer (20mM Hepes-KOH, pH 7.4, 5 mM ATP, 5 mM $MgCl_2$, 50 μM $MnCl_2$, 50 μM $ZnCl_2$, 50 μM FAD, 10 mM succinate, 10 mM fumarate, 10 μg/ml oligomycin, and the protease inhibitors phosphoramidon (1.25 μg/ml), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK; 2.5 μg/ml), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 5.0 μg/ml), 4-(2-aminoethyl)benzenesulfonyl fluoride (APMSF; 2.5 μg/ml), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64; 2.5 μg/ml), Pepstatin A (6.5 μg/ml), Aprotinin (52.5 μg/ml), benzamidine (5 mM), Leupeptin (51 μg/ml), α_2-macroglobulin (1 mg/ml), and phenylmethylsulfonyl fluoride (1mM)). Mitoplasts were disrupted by freezing in liquid nitrogen and thawing while sonicating in a Branson 1200 waterbath (Glick *et al.*, 1992). This freeze-thaw cycle was repeated four times, 5 mM β-mercaptoethanol was added, and the membranes were pelleted at 12,000 *x g* for 10 min. Solubilized mitochondria or mitochondrial membranes were prepared by resuspending in Flavinylation Buffer containing 1% Triton-X 100. 5 mM β-mercaptoethanol was added after solubilization.

FAD Attachment Reactions and Assay. For flavinylation reactions, 10 µl of rabbit reticulocyte lysate that had been programmed with mRNA encoding the Fp precursor was added to 40 µl of matrix lysate or detergent-solubilized mitochondria. After a 20 min incubation at 30 °C, the reaction was stopped by the addition of 1 ml of 4 % sodium dodecyl sulfate (SDS), and the proteins precipitated by the addition of trichloroacetic acid (TCA) to 11 % final concentration. Samples were incubated 10 min at room temperature, 10 min on ice, and centrifuged for 5 min at 12,000 *x g*. Samples were re-precipitated and washed with acetone as described in chapter 5. The final protein pellet was resuspended in 100 µl of 1% Triton X-100, 0.1 M Tris-HCl, pH 7.5. To determine the extent of proteolytic processing, 4.5 µl of each sample was analyzed by SDS gel electrophoresis and fluorography and the bands quantitated with a model BAS1000 phosphoimager (Fuji Photo Film Co., Ltd.). Covalent FAD attachment was monitored by immunoprecipitating modified Fp from 90 µl of each sample with anti-FAD serum as described in chapter 5.

Native Immunoprecipitatons with anti-Hsp60 serum. Flavinylation reactions incubated for 15 min at 30 °C were treated with 20 U/ml apyrase (Sigma, grade VIII) and further incubated for an additional 10 min. Native immunoprecipitations with anti-Hsp60 serum were performed as described (Rospert *et al.*, 1994) using 10 µl of serum for 12 µl of flavinylation reaction mix. Protein-A-sepharose bead-bound proteins were removed by heating at 65 °C in 15 µl IPLB (0.1 M Tris-HCl, 5% SDS, 5 mM EDTA, 0.005% bromophenol blue, 25% glycerol, 5% β-mercaptoethanol, pH 6.8) for 8 min. Beads were removed by centrifugation, re-extracted with IPLB as above, and the supernatants combined. The anti-native Hsp60 serum was a kind gift of Sabine Rospert and Jeff Schatz, as well as the native immunoprecipitation protocol.

Miscellaneous. *In vitro* translated Fp precursor was prepared in rabbit reticulocyte lysate as described by the supplier (Promega Corp., Madison, WI) in the presence of radiolabeled methionine (Trans[^{35}S]-Label; ICN Biomedicals). The anti-FAD and anti-Fp antisera have been described (see chapter 2 and Appendix A; Robinson *et al.*,

1991; Robinson and Lemire, 1995). All protease inhibitors and other reagents were from Sigma (St. Louis, MI).

6-3. RESULTS

Flavinylation is stimulated by mitochondrial matrix fractions. Previously, we have shown that FAD attachment to the Fp can happen *in vitro* during import into isolated mitochondria or mitoplasts (see chapter 5). Further investigation of the flavinylation reaction *in vitro* is however limited to conditions that are compatible with protein translocation. Furthermore, the mitochondrial inner membrane is a barrier that prevents full access to the matrix, the site of FAD attachment. In order to ascertain whether import and modification of the Fp are coupled and to remove the experimental limitations of maintaining import competent organelles, we developed an *in vitro* flavinylation system using mitochondrial lysates.

We first tested whether mitochondrial integrity and hence also import competence are necessary for flavinylation of the Fp to occur. Isolated mitochondria were solubilized with Triton-X 100 in Flavinylation Buffer, which contains ATP, $MgCl_2$, $ZnCl_2$, $MnCl_2$, FAD, succinate, fumarate, and several protease inhibitors (see 'Materials and Methods'). We included ATP and magnesium because FAD attachment might be an energy-dependent reaction, succinate and fumarate because these molecules greatly stimulate flavinylation of the Fp in mitoplasts (as seen in chapter 5), and the metals ions Zn^{+2} and Mn^{+2} because these are necessary for MPP function. Fp precursor that had been translated in rabbit reticulocyte lysate was incubated with the solubilized mitochondria; precursor stability and processing were monitored by SDS gel electrophoresis and fluorography while cofactor attachment was assayed by immunoprecipitation with the anti-FAD serum (Figure 6-1). Not only are detergent-solubilized mitochondria capable of proteolytically processing the Fp precursor (lane 1) to its mature size (lane 2), but they are also able to support flavinylation of a significant fraction of that mature Fp (lane 5).

Figure 6-1. FAD attachment activity is located in the mitochondrial matrix. Fp precursor was translated in rabbit reticulocyte lysate (lane 1) and 10 µl of lysate was incubated for 20 min at 30 °C with either mitochondria (Mit; lanes 2 and 5), mitochondrial membranes (Mem; lanes 3 and 6), or a mitochondrial matrix fraction (Sol; lanes 4 and 7) prepared as described in "Materials and Methods" from an initial mitochondrial concentration of 10 mg/ml . Aliquots (4.5% of total) were analyzed by SDS gel electrophoresis and fluorography to quantitate the Fp remaining and to determine the extent of proteolytic processing to the mature size (lanes 2 to 4). Immunoprecipitations with anti-FAD serum followed by SDS gel electrophoresis and fluorography were performed with 90% of the total sample to measure cofactor attachment to the Fp (lanes 5 to 7). Radioactivity in the Fp bands was quantitated with a model BAS1000 phosphoimager and the ratios of flavinylated Fp to total Fp calculated. The extent of flavinylation (% Holo-Fp) in the matrix fraction was set at 100% .

	Pre Fp	Protein			α-FAD		
Fraction		Mit	Mem	Sol	Mit	Mem	Sol
Lane Number	1	2	3	4	5	6	7
							
% Holo-Fp	-	-	-	-	87	26	100

To determine whether the flavinylating activity is located in the mitochondrial matrix or whether it is associated with a membrane, we fractionated mitoplasts. Mitoplasts suspended in Flavinylation Buffer were disrupted by freeze-thawing and sonication and the membrane fraction pelleted and solubilized with Triton-X 100 in Flavinylation Buffer. The soluble fraction is referred to as the matrix fraction. Flavinylation reactions containing *in vitro* translated Fp precursor were incubated with either the membrane (Figure 6-1, lanes 3 and 6) or the matrix (lanes 4 and 7) fractions and the extent of FAD attachment determined by immunoprecipitation with the anti-FAD serum. Both the membrane and the matrix fractions supported processing of the Fp precursor to the mature size (lanes 3 and 4, respectively). However, modification of the Fp with FAD was significantly more efficient in the matrix fraction (lane 7) compared to the membrane fraction (lane 6). Furthermore, the amount of [^{3}H]-Fp detected in the matrix fraction is marginally greater than that observed with solubilized mitochondria. The increase in the extent of flavinylation in reactions with solubilized mitochondria or with matrix fractions could stem from competition by wild-type Fp present in mitochondrial membranes for binding to the anti-FAD serum or from the presence of detergent. Flavinylation of the SDH Fp therefore requires matrix components.

We further examined the dependence of the flavinylation reaction on matrix components by preparing matrix lysates with different protein concentrations (Figure 6-2). Fp precursor (lane 1) was incubated in Flavinylation Buffer without matrix fraction (lane 2) or with matrix lysates prepared from mitochondria that were at 5, 10, or 20 mg/ml (lanes 3, 4, 5, respectively). Proteolytic processing (lanes 2 to 5) and FAD attachment (lanes 6 to 9) occur only in the presence of matrix lysate demonstrating that these activities are not attributable to the reticulocyte lysate or to the Flavinylation Buffer. Furthermore, the extent of modification was directly proportional to the concentration of the lysate used. In all further experiments, we used lysates prepared at 10 mg/ml.

Figure 6-2. Flavinylation is proportional to the concentration of the matrix lysate. *In vitro* translated Fp precursor (lane 1) was incubated 20 min at 30 °C in Flavinylation Buffer without matrix fraction (lanes 2 and 6) or with matrix lysates at concentrations of 5 (lanes 3 and 7), 10 (lanes 4 and 8), or 20 mg/ml (lanes 5 and 9). After incubation, the samples were analyzed by SDS gel electrophoresis and fluorography to monitor Fp levels and processing (lanes 2 to 5) or by immunoprecipitation with the anti-FAD serum to assay for cofactor attachment (lanes 6 to 9). The radioactivity in the Fp bands was quantitated and the ratios of flavinylated to total Fp calculated. Flavinylation in the 10 mg/ml sample was set to 1 and other samples were compared to it.

Sample	Pre Fp	Protein				α-FAD			
Matrix Lysate		-	5	10	20	-	5	10	20
Lane Number	1	2	3	4	5	6	7	8	9
									
Fold Increase	-	-	-	-	-	-	0.6	1	2.0

The flavinylation of imported Fp precursor during *in vitro* import could be greatly enhanced by the addition of certain citric acid cycle intermediates such as succinate, fumarate, or malate, but not others such as citrate or oxaloacetate or by glycerol-3-phosphate, a stimulator of FAD attachment to the flavoprotein, 6-HDNO (chapter 5). The inability of the latter three compounds to stimulate FAD attachment might be due to their slower uptake into mitoplasts. We re-assessed the efficacies of these molecules in flavinylation reactions performed with matrix lysates (Figure 6-3). FAD attachment was assayed by immunoprecipitation with the anti-FAD serum. In the absence of additives, very little holo-Fp could be immunoprecipitated (lane 2). Succinate, fumarate, and malate greatly increased the amount of holo-Fp detected (lanes 3, 4, and 6, respectively). Flavin attachment was only slightly or not at all stimulated by the competitive inhibitors, malonate and oxaloacetate, (lanes 5 and 7, respectively), or with citrate and glycerol-3-phosphate (lanes 8 and 9, respectively). The His90Ser Fp, which cannot be modified (chapter 4; Robinson *et al.*, 1994), was also incubated with a sample of the matrix lysate in the presence of succinate (lane 10). Only trace amounts of the His90Ser Fp are precipitated with the anti-FAD serum, demonstrating the serum's specificity for the cofactor. These results demonstrate that FAD attachment in matrix fractions is also greatly stimulated by the presence of the same Krebs cycle intermediates that stimulate the reaction in intact organelles.

We wished to further examine the specific role of ATP in FAD attachment (Figure 6-4). We increased the amount of ATP present in the lysate by either supplementing the flavinylation reaction with additional ATP after the reaction had proceeded for ten minutes (lanes 3 and 7) or by including an ATP regenerating system in the flavinylation reaction (lanes 4 and 8). Neither of these changes stimulated flavinylation compared to the untreated lysate (lanes 2 and 6). Another sample was pretreated with apyrase to hydrolyze both ATP and ADP to AMP (Glick, 1991). ATP depletion reduced processing of the precursor (lane 5) and cofactor attachment by about 6-fold (lane 9). The recovery of Fp

Figure 6-3. Cofactor attachment is stimulated by effector molecules. *In vitro* translated Fp precursor (lane 1) was incubated with matrix lysate without additions (lane 2) or with 10 mM succinate, fumarate, malonate, malate, oxaloacetate, citrate, or glycerol-3-phosphate (lanes 3 to 9, respectively). His90Ser Fp was also incubated with matrix lysate in the presence of succinate (lane 10). To determine the amount of Fp remaining and the extent of proteolytic processing, a portion of each sample was analyzed by SDS gel electrophoresis and fluorography ("Protein" row). Flavinylation was assayed by immunoprecipitation with the anti-FAD serum ("α-FAD" row). The radioactivity in the Fp bands was quantitated and ratios of flavinylated to total Fp calculated. The level of flavinylation in the extract without additives (lane 2) was set to 1 and other samples compared to it. Note, the band in lane 1 of the α-FAD row is a size marker for Fp precursor; it was not immunoprecipitated with the anti-FAD serum.

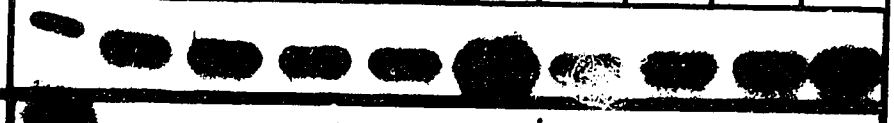
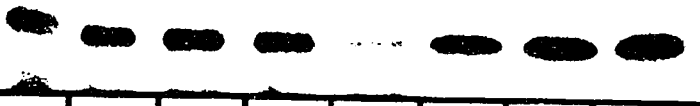
Additive	Pre-Fp	-	Suc	Fum	Mln	Mal	Oxl	Cit	G3P	Suc
Fp		Wt	Wt	Wt	Wt	Wt	Wt	Wt	Wt	H90S
Lane Number	1	2	3	4	5	6	7	8	9	10
Protein										
α-FAD										
Fold Increase	-	1.0	4.9	4.6	2.0	5.6	1.1	1.5	0.8	0.5

Figure 6-4. Flavinylation requires ATP. Untreated matrix lysates (lanes 2 and 6), lysates to which had been added an additional aliquot of 5 mM ATP after the first 10 min of incubation (lanes 3 and 7) or an ATP regenerating system (lanes 4 and 8), or lysates pretreated with apyrase (20 U/ml for 10 min at 30 °C; lanes 5 and 9), were incubated with *in vitro* translated Fp precursor (lane 1). A portion of each sample was analyzed by SDS gel electrophoresis and fluorography (lanes 2 to 5) or by immunoprecipitation with the anti-FAD serum (lanes 6 to 9) and the ratios of flavinylated to total Fp calculated. The level of flavinylation in the untreated lysate was set to 100%.

	pFp	Protein				α-FAD			
Lane Number	1	2	3	4	5	6	7	8	9
									
% Holo-Fp	-	-	-	-	-	100	82	83	16

from apyrase-treated matrix lysates was usually lower, possibly due to Fp instability or degradation (lane 5). Addition of either NADH or the ionophore, valinomycin, did not affect the amount of Fp modified (data not shown). Thus, ATP is required *in vitro* for FAD attachment.

One of the most interesting questions about the covalent attachment of FAD to flavoproteins is whether the reaction is catalyzed by an enzyme or whether an autocatalytic mechanism is utilized for some or all proteins. To determine whether a matrix protein is responsible for Fp modification activity, matrix lysates were pretreated in several ways that either inactivate proteins or remove small molecules and subsequently tested in flavinylation

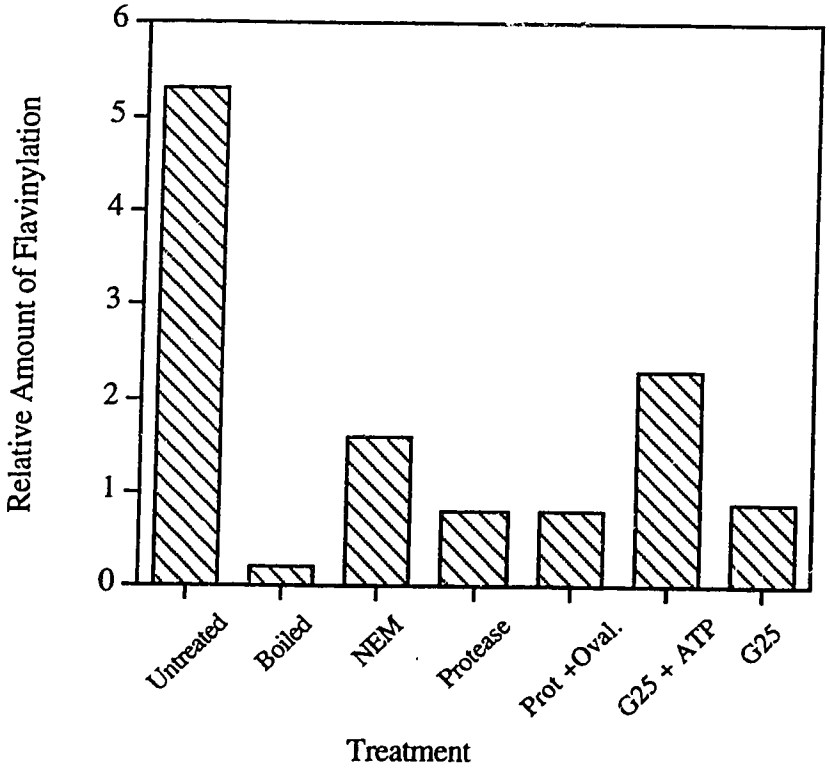


Figure 6-5. Flavinylation requires a matrix protein component. Fp precursor was incubated with matrix lysate that had been untreated, boiled for 10 min, incubated with 2.5 mM N-ethylmaleimide on ice for 30 min (NEM), treated with 2 µg/ml Proteinase K for 20 min at room temperature with (Prot + Oval) or without (Protease) the subsequent addition of 400 µg chicken ovalbumin, or applied to a Sephadex G-25 spin column with (G25 + ATP) or without (G25) the subsequent addition of 5 mM $MgCl_2$, 5 mM ATP, 50 µM FAD, 10 mM succinate, 10 mM fumarate, 50 µM $ZnCl_2$, and 50 µM $MnCl_2$ to the void volume fractions. The samples were analyzed as described in the legend to Figure 6-1. The level of flavinylation in the untreated sample was set to 100%.

reactions (Figure 6-5). Matrix lysate that had been boiled prior to addition of Fp precursor was essentially incompetent for flavinylation as compared to untreated lysate. Treatment with N-ethylmaleimide, a sulfhydryl-modifying reagent, or digestion with Proteinase K also inhibited FAD attachment, thus, implying that a protein component is involved in the flavinylation of the Fp. Re-addition of ovalbumin, an unrelated protein that might non-specifically stabilize the Fp, to protease-treated lysates did not restore FAD attachment activity, suggesting that at least one specific protein is required. Depletion of low molecular mass components from the matrix lysate could be accomplished with a Sephadex G-25 spin column; this results in an almost complete loss of flavinylation and reduced processing activity. Re-addition of ATP, Mg^{+2}, succinate, fumarate, Zn^{+2}, Mn^{+2}, and FAD could partially restore FAD attachment to the depleted lysate, demonstrating the efficiency of the spin column. Collectively, these data strongly argue that modification of the Fp requires both a matrix protein component and small molecules such as divalent cations and FAD.

Expression of the Ip subunit stimulates FAD attachment to the Fp subunit under respiratory conditions *in vivo*. To test whether the Ip subunit could also stimulate Fp modification *in vitro*, flavinylation reactions were supplemented with *in vitro* translated Ip (Figure 6-6). The extents of Fp processing or modification were not significantly changed by the presence of the Ip subunit (lanes 2 and 4, respectively) as compared to in its absence (lanes 1 and 3). This result is in contrast with *in vivo* observations see in chapter 5.

Proteolytic Processing is Required for FAD Attachment. Cofactor attachment to the Fp was only detected for the mature Fp and not the precursor form both *in vivo* and *in vitro* (see chapter 5), although the fraction of Fp present in the precursor form is usually quite small unless import or processing are inhibited. Hence, a possible candidate for the matrix lysate protein component required for Fp modification was the MPP, a mitochondrial matrix metalloprotease that requires Zn^{+2} or Mn^{+2} ions for its activity (Arretz *et al.*, 1991). We treated matrix extracts with EDTA to inactivate the MPP

Figure 6-6. The Ip subunit does not stimulate cofactor attachment to the Fp. 40µl of matrix lysate was incubated with 5 µl of reticulocyte lysate programmed with Fp mRNA plus either 5 µl of reticulocyte lysate programmed with Ip mRNA (lanes 2 and 4) or 5 µl of unprogrammed reticulocyte lysate (lanes 1 and 3). After incubation, samples were analyzed by SDS gel electrophoresis and fluorography (lanes 1 and 2) and by immunoprecipitation with the anti-FAD serum (lanes 3 and 4). The ratios of flavinylated to total Fp were calculated and the level of flavinylation in the sample without Ip was set to 1.

Sample	Protein		α-FAD	
Ip subunit	-	+	-	+
Lane Number	1	2	3	4
				
Fold Increase	-	-	1.0	0.9

Figure 6-7. Proteolytic processing is required for FAD attachment. *In vitro* translated Fp precursor (lane 1) was incubated with matrix lysates that had been prepared in ATP and divalent cation-free Flavinylation Buffer supplemented with 50 μM $MgCl_2$ and treated in the following ways: untreated (lanes 2 and 6); incubated with 0.5 mM EDTA for 10 min in the presence of 5 mM ATP followed by the addition of 0.1 mM $MnCl_2$ and 0.6 mM $ZnCl_2$ (lanes 3 and 7); incubated with 0.5 mM EDTA for 10 min in the absence of ATP followed by the addition of 0.1 mM $MnCl_2$ and 0.6 mM $ZnCl_2$ (lanes 4 and 8); or incubated with 0.5 mM EDTA for 10 min in the absence of ATP followed by the addition of 0.6 mM $MgCl_2$ (lanes 5 and 9). Samples were analyzed for protein content and processing (lanes 2 to 5) and the amount of FAD attachment (lanes 6 to 9).

Sample	Pre-Fp	Protein				α-FAD			
EDTA		-	+	+	+	-	+	+	+
Lane Number	1	2	3	4	5	6	7	8	9
									

in the presence or absence of ATP. We reasoned that the MPP might not be inactivated in the presence of ATP since Hsp60 might stabilize it. The peptidase's solubility and function have been shown to be intimately connected to the function of the mitochondrial Hsp60 (Glick *et al.*, 1992; Hallberg *et al.*, 1993). Matrix lysates were prepared in Flavinylation Buffer without ATP and divalent cations, treated with EDTA for 10 minutes to chelate endogenous Zn^{+2}, Mn^{+2}, and Mg^{+2} ions, and then re-supplied with one or more of the cations. Fp precursor (Figure 6-7, lane 1) was incubated with a matrix lysate that had been prepared in the normal way as a control. This resulted in substantial processing to the mature size (lane 2) and flavinylation of the mature Fp (lane 6). Lysates treated with EDTA in the presence of added ATP showed similar levels of Fp processing (lane 3) but somewhat reduced levels of flavinylation (lane 7). Similarly, lysates treated in the absence of ATP and re-supplied with all three divalent cations were able to process (lane 4) and flavinylate the Fp (lane 8). Only with lysates treated in the absence of ATP and re-supplied with Mg^{+2} was a significant increase in the levels of unprocessed Fp seen (lane 5). When the flavinylation products of this reaction were analyzed, only the mature-sized Fp was immunoprecipitated with the anti-FAD serum (lane 9). Even though substantial Fp precursor remains at the end of the flavinylation reaction (lane 5), none of it has been flavinylated (lane 9), suggesting that the precursor is in an incompetent state for FAD attachment.

Since the presequence appears to inhibit cofactor attachment, we engineered an amino-terminally truncated Fp that does not contain a presequence by placing a methionine codon immediately upstream of the Gln-29 codon which encodes the mature amino-terminal amino acid (chapter 3; Robinson and Lemire, 1992) and tested whether it could be modified in our assay system. The wild-type and the pseudomature Fps were translated (Figure 6-8, lanes 1 and 2, respectively), incubated with the matrix lysate (lanes 3 and 4) and subjected to immunoprecipitations with the anti-FAD serum to measure FAD attachment (lanes 5 and 6). Although the pseudomature Fp is efficiently translated and is

Figure 6-8. Pseudomature Fp is not flavinylated in matrix lysates. Wildtype (pFp or Wt) or pseudomature (mFp) Fp were translated in reticulocyte lysate (lanes 1 and 2, respectively), incubated with matrix lysate and analyzed by SDS gel electrophoresis and fluorography (lanes 3 and 4), and by immunoprecipitation with the anti-FAD serum (lanes 5 and 6).

Sample	-		Protein		α-FAD	
Flavoprotein	pFp	mFp	Wt	mFp	Wt	mFp
Lane Number	1	2	3	4	5	6
						

not degraded by the matrix lysate, no cofactor attachment was detectable. Therefore, FAD attachment requires the proteolytically processed wild-type Fp.

Hsp60 binds Fp. The requirement for proteolytic processing of the Fp prior to flavinylation could explain the need for a matrix protein component, but it does not account for an ATP requirement. Previous experiments shown in chapter 5 however, had suggested that FAD addition is to an Fp molecule that has adopted a folded conformation. For this reason, we decided to investigate whether the mitochondrial chaperonin, Hsp60, which is known to assist in the folding of proteins in an ATP dependent manner, has a role in Fp flavinylation (Becker and Craig, 1994). We first needed to determine whether the Fp interacts with Hsp60. Co-immunoprecipitation with specific anti-Hsp60 antibodies is the only reliable method of monitoring association with Hsp60 (Rospert ' 1994). The fusion protein, LS, which contains a matrix targeting signal fused to *Chlamydomonas* Rubisco, was used as a control for Hsp60 binding (Rospert *et al.*, 1994). *In vitro* translated LS or Fp precursor proteins were incubated with matrix lysate in the presence or absence of FAD. After a 15 minute incubation, apyrase was added to hydrolyse ATP and inhibit the release of bound proteins from Hsp60 and the samples incubated for an additional 10 minutes. Samples were divided and the proteins analyzed by denaturing gel electrophoresis and fluorography (Figure 6-9, lanes 3 to 7) or by immunoprecipitations with anti-native Hsp60 serum to determine Hsp60 association (lanes 8 to 12). As a control to show that the anti-Hsp60 serum does not recognize the Fp, a sample consisting of Fp in Flavinylation Buffer without matrix lysate was also subjected to the immunoprecipitation protocol (lane 12). Both the LS and Fp precursors were efficiently translated (lanes 1 and 2, respectively), proteolytically processed to mature forms (lanes 3 and 5, respectively), and co-immunoprecipitated with Hsp60 (lanes 8 and 10, respectively). Note that after immunoprecipitation with the anti-Hsp60 antibodies, LS migrates faster than usual on the gel because of co-migrating IgG heavy chains (Rospert *et al.*, 1994). In the absence of matrix lysate, the Fp was neither proteolytically processed (lane 7) nor immunoprecipitated

Figure 6-9. The Fp is co-immunoprecipitated with Hsp60. The LS fusion protein and the Fp precursor were translated (lanes 1 and 2, respectively) and incubated in Flavinylation Buffer with (lanes 3 to 6, 8 to 11) or without (lanes 7 and 12) matrix lysate. Each sample was incubated with 20 U/ml apyrase for 10 min at 30 °C. Where indicated, 50 μM FAD was added to the incubation mix. 20% of each sample was analyzed by SDS gel electrophoresis and fluorography (lanes 3 to 7). The remaining sample was analyzed by native immunoprecipitation with the anti-Hsp60 serum (lanes 8 to 12). Note that after immunoprecipitation with the anti-Hsp60 antibodies, LS migrates faster than usual on the gel because of co-migrating IgG heavy chains (Rospert *et al.*, 1994).

Sample	-		20% total protein					α-Native Hsp60				
Protein	LS	Fp	LS		Fp			LS		Fp		
Matrix Lysate	-	-	+	+	+	+	-	+	+	+	+	-
FAD	-	-	-	+	-	+	+	-	+	-	+	+
Lane Number	1	2	3	4	5	6	7	8	9	10	11	12

with the anti-native Hsp60 serum (lane 12). The presence of FAD did not have any effects on the processing of the LS and Fp precursors (lanes 4 and 6, respectively) or on their association with Hsp60 (lanes 9 and 11, respectively). The fractions of LS and Fp bound to Hsp60 under these conditions are 15% and 43%, respectively (lanes 8 and 9, and lanes 10 and 11 respectively). These results demonstrate that the Fp interacts with mitochondrial Hsp60 and that this interaction is independent of FAD.

Does the association of the Fp with Hsp60 precede or follow covalent cofactor attachment? We addressed this question by determining whether Fp found in association with Hsp60 is already flavinylated. Co-immunoprecipitations of the Fp with the anti-Hsp60 serum were used to separate Fp molecules into Hsp60 bound and unbound fractions (Figure 6-10). To semi-purify Fp in the unbound fraction in order to mimic the

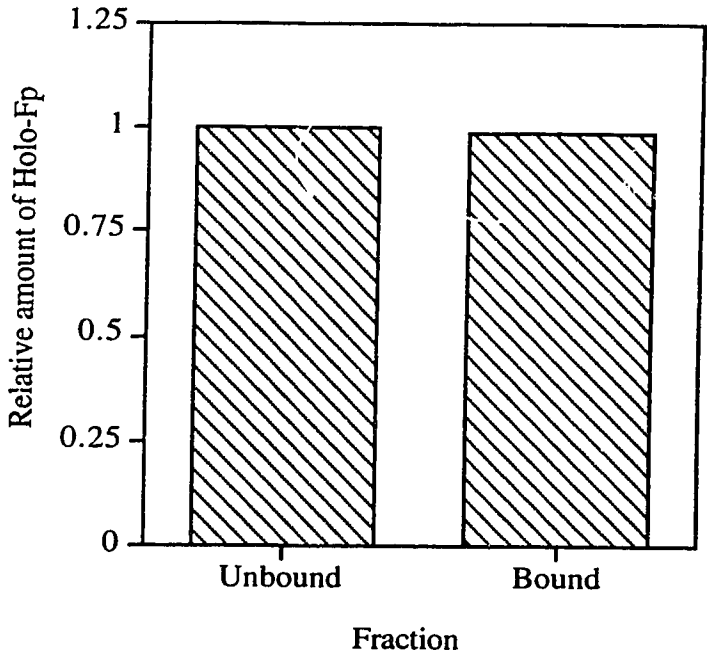


Figure 6-10. Both apo- and holo-Fp bind to Hsp60. Fp precursor was incubated with matrix lysate for 15 min at 30 °C, treated with apyrase (20 U/ml) for 10 min at 30 °C, and separated into Hsp60 bound and unbound fractions by immunoprecipitation with the anti-native Hsp60 serum. Fp in the unbound fraction was immunoprecipitated with anti-Fp serum. After a first immunoprecipitation with either the anti-Hsp60 or the anti-Fp sera, portions of the samples were analyzed by SDS gel electrophoresis and fluorography to determine the amount of Fp remaining and the remainder of the samples were immunoprecipitated with the anti-FAD serum to measure flavinylation. The fraction of Fp in the unbound fraction that was flavinylated was set to 1.

immunoprecipitation of the bound fraction with the anti-Hsp60 serum, Fp in the unbound fraction was immunoprecipitated with anti-Fp serum. Both Fp fractions were removed from the protein-A-Sepharose beads of the first immunoprecipitations and subjected to a second immunoprecipitation with the anti-FAD serum to determine the levels of FAD attachment in each fraction. The Hsp60 bound and unbound fractions (Figure 6-10) contain equal proportions of holo-Fp when compared to the amount of Fp recovered after the first immunoprecipitation.

6-4. DISCUSSION

During the biogenesis of SDH and FRD enzymes, a covalent FAD cofactor is added to the flavoprotein subunit. It is believed that covalent linkage of the cofactor is necessary to modify the midpoint potentials of the enzymes and to permit succinate oxidation. We have been studying the role of the covalent FAD and the mechanism by which this unusual cofactor is linked to the yeast SDH. In a recent series of experiments, we developed an assay procedure using an anti-FAD serum for immunoprecipitation of the modified protein that allows us to follow FAD addition both *in vivo* and *in vitro* during import of the Fp precursor into isolated mitochondria. Now, we have expanded our assay system to include flavinylation reactions in mitochondrial matrix fractions. The absence of the mitochondrial membranes removes a permeability barrier between the external space to which Fp precursor is added and the matrix where FAD attachment occurs. Membrane-impermeable reagents can be tested and experiments can be performed under conditions that need not allow translocation of proteins across the mitochondrial inner membrane.

Protein components in the matrix lysate fractions are necessary for the flavinylation of the Fp subunit to proceed. The extent of Fp modification is directly proportional to the protein concentration of the matrix lysate prepared (Figure 6-2). Furthermore, treatment of lysates by boiling or with the protein modifying reagent, N-ethylmaleimide, or digestion with proteinase K all inactivate the flavinylation activity of the lysates (Figure 6-5).

One protein component that is not required in the flavinylation reactions in matrix lysates is the Ip subunit. This observation is in contrast to Fp flavinylation *in vivo* (see chapter 5). The yeast SDH Fp and Ip subunits are believed to interact with each other to form an assembly intermediate (Lombardo *et al.*, 1990; Robinson *et al.*, 1991; Schmidt *et al.*, 1992). Similarly, the *E. coli* FRD subunits form an active heterodimer in the absence of the membrane subunits (Lemire *et al.*, 1982). The inability of the Ip to stimulate Fp flavinylation may be because its iron-sulfur clusters are not assembled under the conditions used here and thus, the Ip is in an inappropriate conformation. Alternatively, the Ip may interact with the membrane subunits, which are missing in these experiments, prior to its association with the Fp. Accordingly, the *E. coli* FRD Ip assembles with the membrane subunits first and subsequently with the Fp (Latour and Weiner, 1988). Similarly, the *B. subtilis* SDH Fp and Ip subunits will not form a heterodimer in the absence of the membrane subunit (Hederstedt and Rutberg, 1980; Hederstedt *et al.*, 1982).

Strangely, Hsp60 is not required for FAD attachment. Immunodepletion experiments I performed after this thesis was submitted demonstrated that Fp modification is not diminished by the removal of the chaperonin (results not shown). Additionally, experiments showed that there is enough FAD present in the reticlysate itself to support Fp modification and hence the native immunoprecipitations shown in this chapter did not test whether Hsp60 can bind the Fp in the absence of FAD. Notably the medium chain acyl-CoA dehydrogenase binds to hsp60 only when its flavin cofactor is present (Saijo and Tanaka, 1995).

One of the matrix proteins required is the MPP which removes the amino-terminal presequence from the Fp precursor and allows cofactor addition to proceed. Similarly, cytochrome c_1 must be proteolytically processed to its intermediate form by the MPP before the heme cofactor can be attached (Nicholson *et al.*, 1989). In our experiments, no modified precursor Fp is detectable even when the activity of the peptidase, a metalloenzyme, is inhibited by the chelating agent, EDTA, and a significant fraction of the

added Fp remains as precursor (Figure 6-7). It is unlikely that the lack of detection is because the anti-FAD serum does not recognize modified precursor. The proteins are denatured with TCA prior to the immunoprecipitation, and this is likely to expose the FAD to the antibodies. Furthermore, the antiserum detects covalently attached FAD in completely unrelated flavoproteins, even when the flavin is present in different linkages (Robinson and Lemire, 1995). The simplest explanation is that the presequence prevents flavinylation by inhibiting Fp folding, which we have suggested is essential for FAD attachment (see chapter 5). The presequence may prevent folding of the Fp by interacting with proteins such as the presequence binding factor (Murakami *et al.*, 1988; Murakami and Mori, 1990; Murakami *et al.*, 1992) or the mitochondrial import stimulation factor (Hachiya *et al.*, 1993) or by interacting with the remainder of the Fp molecule. By preventing folding, the presequence may delay flavinylation of the Fp until after its import into mitochondria.

That presequence cleavage is related to protein modification is also demonstrated by the lack of modification of a pseudomature Fp (Figure 6-8). The only differences between the true mature and the pseudomature Fps are the presence of an amino-terminal methionine residue in the pseudomature Fp and that the amino-terminal residue of the true mature Fp is predicted to be a Gln by the DNA sequence but was found to be a Glu by protein sequencing (Bullis and Lemire, 1994). The pseudomature Fp presumably has a Gln following the methionine. It is possible that the introduced initiating methionine interferes with protein folding; however, this is unlikely since the amino-terminus of the Fp is not conserved across species and is unlikely to be critical for proper folding (Morris *et al.*, 1994). Curiously, the amino acid sequence predicts that the yeast SDH Fp is cleaved in two-steps; first by the MPP to an intermediate species, and then the remaining octa-peptide is removed by the mitochondrial intermediate protease (MIP) producing the mature sized Fp. Correspondingly a yeast MIP mutant has no SDH activity, suggesting that this protease is required to process at least one of the SDH subunits. Thus, perhaps

modification can only occur to the intermediate sized Fp, and not the mFp or pseudomature Fp. However, we have never detected an intermediate Fp species, hence we could neither verify its existence nor examine its role in modification. Alternatively, the pseudomature Fp may not be modified because without the presequence to impede folding, it may misfold in the reticulocyte lysate and adopt a conformation incompatible with FAD attachment. Such a flavinylation incompetent state has been observed with the 6-HDNO (Brandsch and Bichler, 1992; Brandsch *et al.*, 1993).

Non-protein components are also required for the flavinylation of the Fp by matrix extracts. ATP is required possibly for cofactor activation as for biotin or lipoic acid (Schmidt *et al.*, 1969; Gross and Wood, 1984), but this is unlikely because 6-HDNO flavinylation is independent of ATP (Brandsch and Bichler, 1991). Alternatively, the ATP may be required for Fp release from either cytosolic (Pfanner *et al.*, 1990; Glick *et al.*, 1992) or mitochondrial heat shock proteins (Cheng *et al.*, 1989; Ostermann *et al.*, 1989) that are necessary for protein folding. The role of MPP which requires divalent cations is discussed below. Certain Krebs cycle intermediates, especially succinate, fumarate, and malate, which are substrates for SDH, stimutale FAD attachment. Strangely, both oxaloacetate and malonate which bind strongly to the enzyme active site located in the Fp, do not enhance FAD attachment. Perhaps the nascent active site has a different geometry from that in the fully assembled enzyme and does not recognize oxaloacetate or malonate. Alternatively, FAD attachment may require a molecule that can also be oxidized or reduced. Interestingly, citrate and succinate were the most efficient stimulators of *E. coli* SDH Fp modification (Brandsch and Bichler, 1989). Understanding the mechanism of effector molecule stimulation of FAD addition may require a more purified system since matrix lysates likely metabolize added molecules into other species that may be more or less effective in the flavinylation reaction. Additionally, in a more purified system which protein or activity that requires ATP could be identified.

Our results suggest a model in which FAD attachment is one of the earliest steps of the assembly pathway of the SDH Fp. After presequence cleavage the Fp attains some degree of folding, with the aid substrate-like molecules and possibly ATP dependent chaperones such as mitochondrial hsp70. Aside from the site of covalent attachment, the Fp has at least two other regions involved in non-covalent interactions with FAD; the Fp can tightly bind FAD non-covalently. Therefore, FAD itself may act as a nucleation site for folding of the Fp, as has been shown for the medium chain acyl-CoA dehydrogenase, a non-covalent flavoprotein (Saijo and Tanaka, 1995). The Fp is probably folded to state similar to the molten globule state, but with some tertiarary structure present to co-ordinate the FAD molecule and position it in the geometry necessary for covalent attachment to the His residue. After cofactor attachment, the Fp is recognized by the chaperonin hsp60. That hsp60 interacts with the Fp following cofactor attachment is suggested because it is not required Fp modification. Since almost half of the SDH Fp present is bound by hsp60, this interaction is probably real and thus hsp60 most likely plays a role in assembling the Fp into the SDH holo-complex.

In summary, we have developed an *in vitro* flavinylation assay using mitochondrial matrix fractions. Translocation across a membrane is not a prerequisite for FAD attachment to the Fp. At least one matrix protein appears to participate in Fp flavinylation, the MPP by removing the presequence from the Fp precursor. Curiously, although folding seems to be crucial for Fp modification, the chaperonin hsp60 is not required for cofactor attachment. The participation of MPP in cofactor addition does not eliminate the possibility that bond formation is an autocatalytic process; rather, it may, as we believe, signify the need for an appropriate conformation before modification can proceed. Further insights into the roles of MPP into the mechanism of FAD addition may await the development of an assay with purified components.

6-5. BIBLIOGRAPHY

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Chapter 7

Conclusions and Recommendations

The results I present in this thesis address the import, assembly and cofactor attachment of the SDH Fp, along with some aspects of the structure and function of the holoenzyme. In this chapter I will present a model for these processes, discuss some remaining unanswered questions, and express my thoughts on possible answers to those questions and on the means of attaining them.

In sum, my results suggest a model for Fp assembly that begins with its targeting to the mitochondria and its translocation across both mitochondrial membranes. In addition to localizing the Fp to the mitochondria, the presequence may also prevent modification of the Fp in the cytosol either by interacting with the mature portion of the Fp or by promoting an association with one or more cytoplasmic factors that maintain the Fp in a flavinylation incompetent state. After proteolytic processing of the presequence in the mitochondrial matrix, the Fp finds and folds around a FAD molecule and attains the correct conformation for covalent attachment with the aid of substrates. The requirement for a substrate-like molecule provides a means of control over holocomplex biogenesis at the assembly level. In the correct conformation, the His90 residue is precisely positioned with respect to the FAD molecule for the formation of a covalent bond between the protein and the cofactor. Bond formation may be catalyzed possibly by the Fp itself as it assembles with the other SDH subunits. This assembly of the Fp into the SDH holocomplex is probably assisted by the mitochondrial chaperonin Hsp60.

Despite the progress I have made, I do not have definitive answers to two basic questions about flavinylation. Why is the covalent bond needed? Is FAD attachment autocatalytic? I believe that the answers to these questions are attainable and that the yeast system, but more importantly the anti-FAD serum as a detection method, will prove very useful. Although cumbersome to work with the anti-FAD serum works, and I think would work with other *in vivo* and *in vitro* systems. Additionally, this serum has the tremendous advantage that it can be used to examine both snap shots, and steady state levels of modification with FAD *in vivo*.

With regards to the chemical mechanism by which a covalent bond is formed, I think that the 6-HNDO system is better suited for such studies. 6-HDNO is a monomeric bacterial protein that can attach FAD to itself. Also this protein is not proteolytically processed after translation. Hence, the 6-HDNO system has fewer variables and provides a simpler system for examining the chemical mechanism of the histidine-FAD bond formation. Although, I think the anti-FAD serum should be usable to assay for cofactor attachment. The anti-FAD serum may allow the attachment of different derivatives of FAD to be examined more readily as this detection system does not require the flavin to be radio-labeled. With insights gained from the 6-HDNO system, experiments could test more easily if the same chemical mechanism for the FAD-protein covalent bond formation in SDH and FRD Fps.

Why is FAD Covalently Attached?

The H90S Fp mutant, in accord with the equivalent *E. coli* FRD Fp mutant, demonstrated that covalently attached FAD is essential for SDH activity, as shown in chapter 4. In contrast, a SDH from *M. phlei* has SDH activity but with non-covalently bound FAD (Reddy and Weber, 1986; Ackrell *et al.*, 1992). This observation suggests that the midpoint potential of FAD can be raised by means other than covalent attachment. Perhaps covalent attachment is simply one of several ways to acquire SDH activity.

Another line of evidence supports the hypothesis that a function of covalent attachment is to give SDH a diode-like characteristic. An additional difference between the H90S Fp and the wild-type enzyme, is that the H90S mutant did not demonstrate the low-potential activity switch-off seen with wild-type SDH. The FRD activity of the H90S mutant is not inhibited by the initially reducing conditions of the benzyl-viologen assay. Since yeast SDH is very difficult to purify, the corresponding *E. coli* SDH Fp could be made and the resulting complex purified. Voltammetric studies examining both the *E. coli*

SDH Fp mutant and the *M. phlei* SDH which does not covalently bind FAD could be very informative.

Is FAD Attachment Autocatalytic?

A method for unequivocally demonstrating that the protein-flavin bond is formed by the Fp itself would be to show that purified apo-Fp can attach FAD. As seen in chapter 4, Fp expressed from the uninduced *CUP1* promoter has a low rate and extent of flavinylation when the cells are grown on glucose. If the *CUP1* promoter is induced under these conditions, massive amounts of Fp are made and imported into the mitochondria where aggregates without FAD attached are formed. After isolating the mitochondria, these aggregate bodies which contain both proteolytically processed and unprocessed Fp, can then be purified in the same manner that inclusion bodies are purified from *E. coli*. In this manner, large quantities of the apo-Fp can be isolated. The apo-Fp could be denatured and then slowly renatured by dialysis out of the denaturant in the presence of FAD, Mg^{+2}, and succinate to investigate if it is able to covalently bind FAD to itself. Although I think this method can definitively show that the formation of the FAD-protein bond is catalyzed by the protein itself, it would not disprove a role for other proteins *in vivo*. Furthermore, absence of detectable flavinylation by this approach does not refute the autocatalysis model.

Most flavoproteins may not be able to modify themselves. Brandsch's group published one paper on FAD attachment to the eukaryotic enzyme dimethylglycine dehydrogenase four years ago (Lang *et al.*, 1991). Although they have the cDNA to overexpress it in *E. coli*, are accomplished in purification, re-folding and flavinylation studies, and state in this paper their intentions to do flavinylation experiments with purified dimethylglycine dehydrogenase, they have not published anything on the attachment of FAD to this protein *in vitro*. This could be attributable to technical difficulties or that their 6-HDNO project has been very fruitful, consuming most of their time. Alternatively, they may not have demonstrated that this eukaryotic flavoprotein is autocatalytic because this

ability is unique to 6-HDNO. Significantly, most flavoproteins, like dimethylglycine dehydrogenase and the SDH and FRD Fps contain dinucleotide binding domains, but no such domain is apparent in 6-HDNO. Thus, 6-HDNO's folding may be more independent of FAD; it may exist for relatively long periods of time without misfolding until FAD is present for it to bind. In the flavoproteins with the dinucleotide domains, FAD may be too essential to their proper folding for them to maintain a flavinylation competent conformation for any length of time in its absence. Nevertheless, for *in vitro* experiments that attempt to show modification of the purified apo-Fp, negative results do not disprove that the bond is made by the protein.

Flavinylation Reactions.

The extent of modification that I observed with the *in vitro* flavinylation reactions, although measurable, was extremely limited. (I estimated roughly between 2 and 10% for the import experiments into isolated mitochondria and between 0.2 and 1.0% for the matrix lysate experiments, depending on what fraction of the modified protein is immunoprecipitated by the anti-FAD serum). Discovering the reason for the low efficiency may be the key to any success with in an *in vitro* system using less crude extracts or purified apo-proteins. Also for simple technical reasons, further experiments would be far easier to do if the extent of flavinylation were larger. Hence I think it is worthwhile discussing some possibilities for why the amount of FAD attachment *in vitro* is so limited.

Notably, the total amount of holo-Fp formation I detected during *in vitro* import into isolated mitochondria was about equal to what I saw with matrix lysates. This is surprising as the matrix lysates had approximately 5 to 10X fold more labeled protein available to modify since the import system rarely exceeded 20% import. Furthermore, unlike the import reactions, the lysate system does not contain endogenous Fp to compete for binding to the anti-FAD antibodies. One possibility is that a portion of the Fp aggregates; Fp that is aggregated can not be imported into the mitochondria and modified.

Although perhaps technically difficult, I think it would be interesting and informative to translate the Fp in the presence of the matrix lysate so that it has little time to misfold before being flavinylation competent.

Secondly, the amount of modification might increase if the translated Fp were incubated with denaturants to unfold aggregated or misfolded protein before adding them to the lysate. I performed some experiments with denatured Fp, although without success. I think that the concentration of denaturant remaining after dilution into the matrix lysate was excessive, as judged by the reduction in the extent of proteolytic processing of the Fp.

Thirdly, the low level of FAD attachment may be the result of the presence of an inhibitor in the reticulocyte lysate. Once the Fp is imported into mitochondria it may become protected from the inhibitor, whereas in the matrix lysate reactions the Fp remains exposed to the inhibitor. Problems with inhibitors may be averted by denaturing the proteins of the reticulocyte lysate or by diluting out the inhibitor with a much larger volume of matrix lysate. Alternatively, labeled apo-Fp with and without the presequence attached could be isolated from over-expression *in vivo* as described above, and used in the flavinylation reactions instead of *in vitro* translated Fp.

Even though a membrane potential is not essential for FAD attachment, its absence in the matrix lysate reactions could be a fourth reason for the small amount of Fp modification observed. This theory could be tested by adding an uncoupler to import reactions after a short incubation since only insertion, and not translocation of the remaining portion of the protein, is dependent on the membrane potential. Thus, the extent of Fp modification inside the mitochondria without a membrane potential present could be examined.

Finally, modification of the Fp may be far from maximal because the mixture is still missing a key ingredient; this ingredient could be a salt, a small molecule metabolite, a lipid component or a protein required for FAD attachment which is unstable especially after the mitochondria have been lysed. Unfortunately I know of no way to identify the missing

ingredient other than empirical trials. Proteolytic degradation of a key protein despite the variety and amounts of protease inhibitors present in the Flavinylation Buffer is a possibility. For example, the endogenous SDH Fp present in the membrane pellet removed in matrix lysates preparations is completely proteolytically digested to 40 to 50 kDa fragments. The mitochondria that I used in these experiments were a crude preparation and undoubtedly contained endoplasmic reticulum and vacuolar contaminants. Vacuolar membranes are likely to be rich in proteases. Hence, *in vitro* flavinylation experiments could be tried using: sucrose gradient density purified mitochondria, mitochondria from a protease deficient strain, even more protease inhibitors, or a combination of two or more of these modifications. The low levels of Fp modification may be an important clue to understanding the mechanism of Fp flavinylation, but a tremendous amount of time could obviously be spent examining the variety of possible reasons outlined above and yield nothing in the end.

Strangely, in some experiments I performed after submitting this thesis showed that the presence of protein-A-sepherose beads prebound with antiserum (any sera) as outlined in the native immunoprecipitation with anti-hsp60 serum drastically increased Fp modification. However, this increase was not seen when the reaction was done in the presence of untreated protein-A-sepherose beads of native immunoprecipitation buffer. Hence either the presence of bovine serum albumin (BSA,by non-specifically blocking hydrophobic surfaces?) or something in rabbit sera increases FAD attachment or the effectiveness of the immunoprecipitaiton with the anti-FAD sera. Determining which of these it is and why should be relatively straight forward and tremendously helpful.

Effector Molecules

Perhaps the substrate molecules stimulate FAD attachment by binding to and stabilizing the necessary conformation at the active site. Curiously, the extent of stimulation in FAD attachment by the effector molecules varies for the yeast and the *E. coli*

SDH Fp (see chapters 5 and 6; Brandsch and Bichler, 1989). While these discrepancies could be caused by general disparities between the two Fps amino acid sequences, they could also be due to specific differences in the affinities of the substrate binding site for the various molecules. The genes for assorted prokaryotic Fps and the cDNAs for the bovine and human SDH Fps are now available. It would be intriguing to test whether other Fps could be modified by the yeast matrix lysate. If the bovine Fp can not be modified in a heterologous system, making a similar lysate from bovine mitochondria would be relatively simple. Moreover, trying flavinylation reactions with Fps from other sources could be used to further explore the reasons for the disparities in the extent of stimulation by certain substrate-like molecules. A correlation between the enzymes affinity for the molecule, and the molecules stimulatory effect on Fp modification would be indicative of the binding at the active site.

In the *E. coli* FRD Fp that do not bind oxaloacetate as tightly as the wild-type protein, mutants have been isolated; these mutations are thought to be in the substrate binding site (Schroder *et al.*, 1991). A comparison of the flavinylation properties of mutant and wild-type Fps could further implicate binding of effector molecules to the active site in the modification reaction.

Finally, another means of examining the role of allosteric effector molecules in flavinylation would be to construct mutants in the substrate binding site for the yeast SDH Fp. I think such experiments are relevant since binding to the emerging active site as the protein folds is an assumption that has not been tested.

Effect of the Ip Subunit.

The presence of the Ip subunit increased the amount of FAD attachment to the Fp *in vivo* when the cells were grown on a non-fermentable carbon source, but had no effect on Fp modification when the cells were grown on glucose or in *in vitro* flavinylation experiments. As stated in chapter 6, this discrepancy is possibly due to an additional

requirement for either the membrane subunits or for the formation of iron-sulfur clusters. Determining whether the membrane subunits are needed should be relatively easy. Modification of the Fp could be examined in *sdh2-*, *sdh3-* and *sdh4-* yeast mutants, with or without a plasmid containing the *SDH2* gene. An increase (or lack there of), in flavinylation upon expression of the Ip subunit in this mutant would indicate that the membrane subunits are not (or are) required for the Ip to assist with Fp modification. Mutants in *E. coli* FRD Ip that do not assemble iron-sulfur clusters exist. Comparing the amounts of Fp modification between these mutants and the wild-type could determine whether iron-sulfur cluster assembly is necessary for the Ip to aid in Fp modification.

The Roles of Other proteins in Fp Modification

Although these results demonstrate that the modification enabling activity is in the mitochondrial matrix, I do not think a purification project for this activity should be undertaken until some likely candidates are first examined, namely MPP, and possibly MIP and hsp70. My guess is that these enzymes are essential and sufficient for FAD attachment.

The inability of the matrix lysate to modify the Fp precursor may be an artifact caused by proteins from the reticulocyte lysate binding to it. Both *in vivo* and in *in vitro* import reactions, the Fp is no longer in contact with cytoplasmic proteins when it is being folded and modified. Import experiments that disable MPP activity and allow precursors to be present in the matrix could test this hypothesis. MPP could be inactivated in import experiments by the membrane permeable chelator, ortho-phenanthroline, or by using heat treated mitochondria from temperature sensitive mutants of the MPP that are still import competent. In another approach, the cleavage site of the Fp could be mutated so that it is no longer recognized by the MPP, and then *in vivo* and *in vitro* experiments could be used to examine whether this mutant Fp is flavinylated. However, I think that the import

experiments that inactivate MPP alone could demonstrate if the presequence must be removed before the Fp is modified.

The amino acid sequence of the pre-Fp and N-terminal sequencing of the mFp predict that this protein is proteolytically processed in two steps. However, throughout my studies, I have never detected an intermediate sized species of the Fp. Possibly, this is because the presumed Fp intermediate species migrates with the mFp species on SDS-PAGE, although intermediate species were also not detected with any of the carboxyl-terminal truncations. Whether this presequence is cleaved in a two step process could be determined by fusing it to a small passenger protein like dihydrofolate reductase. More intriguingly, the yeast MIP gene has recently been identified (Isaya *et al.*, 1994), and the *mip1* mutant is deficient in SDH activity, suggesting that one or more of the SDH subunits is a substrate of this protease. With this mutant, an Fp intermediate could be trapped and identified. Moreover, it would be interesting to determine whether the Fp intermediate can be modifies.

Hsp70 may be require to simply prevent aggregation of the Fp before its translocation into the matrix is completed, and possibly to aid the Fp to aquire some of the necessary secondary structure. Experiments examining Hsp70's role in FAD attachment will be complicated by the presence of cytoplasmic Hsp70 in the reticulocyte lysate. However, this question could be approached possibly by importing urea denatured Fp into mitochondria isolated from $hsp70^{ts}$ mutants.

Rather unexpectedly, Hsp60's is not required for FAD attachment. This fact was determined by flavinylation reations in which Hsp60 was removed from the matrix lysate by immuno-depletion with anti-native Hsp60 serum. Since Hsp60 binds a large percentage of the Fp, this interaction is probably real and would suggest that Hsp60 plays a role in the assembly of the subunit into the holocomplex. Additionally, it would be interesting to determine if FAD is required for the chaperonin to bind the Fp. The cofactor may be so

fundamental for the proper folding of the Fp that the protein cannot acheive a molten-globule state that is recognizable to Hsp60 in its absence.

My work predicts a model for the modification and assembly of the SDH Fp. The model includes possible functions for additional proteins such as MPP. Using the methodology developed in this thesis, future studies could elucidate more clearly the mechanism of cofactor attachment and the roles of these other proteins in modification, folding, and assembly. Thus, additional insights into the assembly processes of respiratory complexes, and in turn mitochondrial biogenesis, will emerge.

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Appendix A

Anti-FAD Serum; Detecting Cofactor Attachment

Portions of this chapter are in press: Robinson, K. M. and Lemire, B. D. (1995) *Methods Enzymol.*

A-1. INTRODUCTION

To study FAD attachment to the Fp required an assay that could efficiently distinguish between the holo-Fp and the apo-Fp or the mutant H90S Fp. Ideally, the technique should be able to examine the progress of Fp flavinylation with pulse-chase experiments as well as the steady-state levels of modified Fp *in vivo* . Additionally, the detection method should be applicable to *in vitro* studies which would provide a more manipulatable system. In this appendix, I briefly list some of the techniques I tested but found unusable. Next, I describe the rationale for producing an anti-FAD serum, and the method I used to make the conjugate that elicited the anti-FAD response. Finally, the characterization of this antiserum is presented to demonstrate the serum specificity at recognizing the FAD moiety of the Fp and not the protein.

Methods tested.

By using radio-isotope that would label the FAD moiety itself, the holo-Fp but not the mutant or apo-Fp, should be specifically visualized. However, radiolabeled FAD is not commercially available nor is FAD likely to be transported into yeast cells. FAD is synthesized by the phosphorylation by ATP of riboflavin to FMN, followed by the adenylation by ATP of FMN to FAD. Therefore, I labeled the FAD using metabolic precursors of FAD, namely ^{14}C-riboflavin and ^{32}P-orthophosphate. The incorporation of label into the holo-Fp with either isotope was insufficient to easily visualize the protein; the exposure times required were weeks to months, instead of days . For example, the amount of radioactivity associated with the wild-type SDH from 200 µg of mitochondria is on the order of 100 cpm (see chapter 4, figure 4-5). Additionally, incubating the cells with ^{32}P-orthophosphate results in the labeling of hundreds of proteins, presumably due to their phosphorylation. Most of the phosphoproteins were resistant to both acid and alkaline treatments, and to incubation with phosphatases. Thus, as well as poor incorporation,

labeling with ^{32}P-orthophosphate was not specific, which made the identification of the Fp difficult.

Numerous techniques were attempted that exploited the physical or chemical characteristics of FAD. FAD has a molecular mass of 830 daltons, is fluorescent, and contains two negative charges at its phosphate groups. The 830 daltons size change was not significant enough to cause a shift in the migration for a 67 kDa protein. However, the difference between the apo- and holo-Fp could be accentuated if a peptide containing the His90 residue, the point of FAD attachment, was produced. Thus, the wild-type and H90S Fps were digested with Glu-C endopeptidase which resulted in peptide fragments of 7 and 8 kDa that contained the His90 residue. However, in a number of different gel systems I could not detect a difference in migration between the FAD-peptide fragment from the holo-Fp, and the corresponding peptide of the apo- or H90S Fp. Differentiating between the modified and non-modified Fp by digestions with other peptidases were similarly unsuccessful. Detecting the FAD attached to the Fp by its fluorescence proved to be insensitive, in addition to the technical difficulties associated with this method when *in vivo* experiments were performed. The holo- and apo-Fp should be separatable by iso-electric focusing gel electrophoresis because of the two negative charges present on the FAD, but again, I failed to detect a different isoelectric point between the holo- and apo-Fp.

Antisera raised against a portion of the FAD may have been able to detect the holo-Fp, but not the apo- or mutant Fp. However, commercially available antiserum against adenosine did not visualize any of the Fps by western blotting. Using the method for synthezing the conjugates that elicited the anti-adenosine serum (Erlanger and Beiser, 1964), I conjugated the isoalloxazine ring of riboflavin to BSA and injected this antisera into rabbits. The resulting serum was unable to recognize the Fp.

Rationale for the anti-FAD serum.

The availability of antibodies directed against FAD could be utilized for *in vivo* and *in vitro* studies on Fp modification by either immunoprecipitations or western blotting. Anti-flavin antibodies have previously been elicited by coupling the hapten *N*-6-(6-aminohexyl)-FAD to bovine serum albumin (Barber, *et al.*, 1987). This hapten is not commercially available but can be synthesized by a complex procedure (Morris and Buckler, 1983). However, we reasoned that peptide-bound FAD could also serve as a suitable hapten once coupled to a carrier via the peptide amino groups. One source of 8α-*N*(3)-histidyl-FAD available in large quantities is the *E. coli* FRD overexpressed with a multicopy plasmid (Lemire and Weiner, 1986). Thus, I subjected FRD Fp from *E. coli* membranes enriched in FRD to extensive proteolytic digestion and isolated the resulting FAD-peptides. This hapten was cross-linked to keyhole limpet hemocyanin, and the conjugate was used to raise antiserum directed against FAD. The antiserum recognizes only the holo-Fp, and neither the apo-Fp or the H90S Fp mutant. Additionally, excess FAD or FMN inhibits the recognition of the holo-Fp. Furthermore, the anti-FAD serum visualizes other non-related flavoproteins by western blotting.

Materials and Methods.

E. coli HB101 carrying the plasmid pFRD63, which encodes the entire *frd* operon, overproduces FRD 30- to 40-fold during anaerobic growth on glycerol-fumarate medium (Lemire, *et al.*, 1986). The membranes from this strain enriched with FRD were a generous gift from Joel Weiner. The yeast strain used in this work is, Sdh1Ad1 (*Mata, ura3, lys2, sdh1::ADE2*), also described in chapter 5. The plasmids pSDH1 which carries the wild-type *SDH1* gene and pS1H90S which is identical to pSDH1 except that the sequence encoding the flavin-binding histidine (His-90) is altered to encode a serine are described in chapters 3 and 4 respectively. Both plasmids are constructed in the vector, pRS416 (Stratagene). The procedure used to isolate mitochondria from yeast grown on

YPGal, and the production and the specificity of the anti-Fp subunit antibodies have been described in chapter 4. Pronase was obtained from Boehringer Mannheim. Keyhole limpet hemocyanin (KLH), the flavoproteins, *Pseudomonas* sarcosine dehydrogenase, *Alcaligenes* choline oxidase, *Schizophyllum commune* cholesterol oxidase, and bovine plasma monoamine oxidase were purchased from Sigma.

Isolation of an FAD-peptide hapten for antiserum production.

Solutions

A. 0.05% trifluoroacetic acid in H_2O

B. 0.05% trifluoroacetic acid in acetonitrile

C. 50 mM sodium borate, 300 mM NaCl, 0.5% SDS, pH 9.0

D. 0.1 M sodium acetate, 100 mM NaCl, 0.5% SDS, pH 4.5

E. 20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 0.02% sodium azide, pH 7.2

Crude *E. coli* membranes (140 mg protein, Table A-1) were precipitated with trichloroacetic acid (10% final). The pellet was washed with acetone and resuspended in 100 µl 20% sodium dodecyl sulfate (SDS) and 1 ml 8 M urea. Some material remained insoluble. From this step onward, samples were protected from light. The solution was diluted to 0.3% SDS, and adjusted to 2 M urea, 20 mM potassium phosphate, 2 mM $CaCl_2$, 1 mM EDTA, pH 7.4. Pronase (10 mg) was added and the solution incubated at 37 °C for 12 hrs. An additional 10 mg of pronase was added and the incubation continued for a further 52 hrs. The extent of digestion was monitored by SDS-polyacrylamide gel electrophoresis on 16.5% acrylamide, 6% bisacrylamide tricine gels and visual inspection of fluorescent flavopeptide species detected at pH 3.5 (Schägger and von Jagow, 1987). The sample was centrifuged at 100, 000 *g* for 30 min in a Beckman TL100.3 rotor to remove insoluble material.

FAD has a characteristic absorption spectrum with absorption maxima[1] at 264 nm ($\varepsilon = 10^{4.58}$), 375 nm ($\varepsilon = 10^{3.93}$), and 450 nm ($\varepsilon = 10^{4.05}$) the flavopeptides were monitored at 375 nm throughout the purification. The digested material was separated in multiple runs on a reverse phase C-8 HPLC column (Zorbax, 15 cm C-8, 300 Å pore size, 5 μm particle size, Rockland Technologies, Wilmington, DE) using a linear gradient of solutions A and B with solution B increasing at 2%/min and a flow rate of 1 ml/min. All HPLC runs were performed by Lorne Burke. Three flavopeptide peaks eluting between 10 and 12 min were pooled and dried.

Table A-1. Preparation of Carrier-Coupled Flavopeptides

Fraction	nanomols FAD	Percentage
Crude Membranes	560	100
Reverse phase HPLC	190	34
Mono Q FPLC	130	23
Derivatized flavopeptide	60	11
KLH-conjugate	36	6

Flavopeptides were resuspended in 15 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.0, and applied in multiple runs to an analytical Mono Q FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). Unexpectedly, the flavopeptides were not retained, however, a significant amount of contaminating material was. Flavopeptide fractions were pooled and dried.

Coupling the hapten to carrier protein and immunization.

To prevent flavopeptide molecules from dimerizing and to provide a long spacer arm between the hapten and the carrier, we derivatized both with the bifunctional crosslinker, sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate) essentially as described by the manufacturer (Pierce, Rockford,

IL). The flavopeptides were resuspended in 1 ml 0.1 M sodium borate, pH 9.0 and 1.3 mg sulfo-LC-SPDP was added. After incubation at room temperature with gentle agitation for 2 hrs, the sample was applied to a reverse phase HPLC C-8 column and eluted using a linear A to B gradient with solution B increasing at 1%/min. Unconjugated flavopeptides eluted between 11.5 and 13.5 min while conjugated material eluted between 22 and 23.5 min. Underivatized flavopeptides were re-incubated with sulfo-LC-SPDP as above but with an overnight incubation. HPLC profiles subsequently revealed that all flavopeptides had been derivatized; fractions were pooled and dried.

KLH (7 mg) was added to 0.5 ml buffer C and incubated at 42 °C for 48 hrs to dissolve the protein. After centrifugation at 15,000 *g* for 10 min, the supernatant was added to 1.2 mg sulfo-LC-SPDP and incubated overnight at room temperature with gentle agitation. Unconjugated crosslinker was removed by chromatography on a 1 x 7 cm Sephadex G-25 (Pharmacia LKB Biotechnology) column equilibrated in buffer D. Protein containing fractions were identified by monitoring absorbance at 280 nm. The extent of derivatization (3 to 4 crosslinker groups per KLH) was estimated by measuring the absorbance of the pyridine-2-thione at 343 nm ($\varepsilon = 10^{3.91}$). The KLH was concentrated to less than 1 ml with a 10-kDa molecular weight cutoff Ultra-MC filter unit (Millipore Ltd., Bedford, MA), reduced by the addition of dithiothreitol to 52 mM, and applied to a 1 x 12 cm Sephadex G-25 column equilibrated in buffer E. Derivatized protein was collected, immediately added to the dried derivatized flavopeptides, and incubated overnight at room temperature. Unbound flavopeptide was separated from the KLH coupled material on a 1 x 25 cm Sephadex G-25 column. The modified KLH (2 mg) was estimated to have 1 FAD per protein molecule.

Polyclonal antibodies were raised essentially as described except that the rabbit was given an initial injection of 1 mg of the KLH-conjugate and boosted at two week intervals with 0.5 mg of conjugate (Dunbar and Schwoebel, 1990).

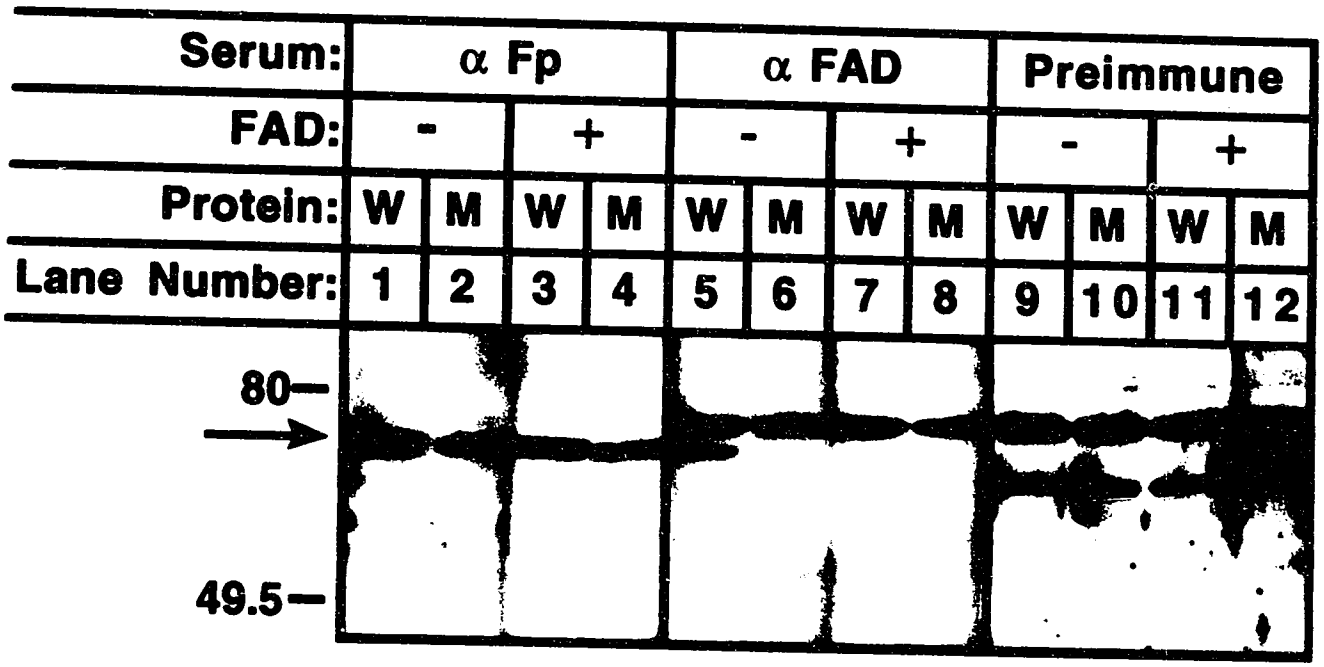
Comments.

To minimize the size of the peptide attached to the FAD, we performed an extensive proteolysis with a general proteinase. We did not characterize the structure of the flavin peptide(s). From competition studies detailed below, we concluded that the peptide backbone did not contribute significantly as an immunogen.

Specificity of the anti-FAD serum.

I prepared mitochondria from Sdh1Ad1 transformed with either pSDH1 or pS1H90S and subjected these to Western blot analysis with the anti-FAD serum to determine whether the yeast Fp subunit could be detected. With pS1H90S, SDH is assembled but with non-covalent FAD. As shown in Figure A-1, affinity purified anti-Fp antibodies recognize a single 67-kDa band in both wild-type (W; lane 1) and mutant (M; lane 2) mitochondria. This recognition is unaffected by the addition of free FAD during the incubation with the antibodies, lanes 3 and 4, respectively. The affinity-purified anti-FAD antibodies also recognize wild-type Fp (lane 5) but fail to detect the H90S-Fp subunit (lane 6), as expected with a non-covalent cofactor. The Fp subunit is not detected if free FAD is added while the blot is exposed to the antibodies (lane 7). Both the anti-FAD and the pre-immune sera detect an approximately 78 kDa protein. Since this protein is seen in both wild-type and mutant mitochondria (lanes 5 to 12) and the recognition is unaffected by free FAD (lanes 7, 8, 11, and 12), it is unrelated to the Fp subunit. Thus, we conclude that the anti-FAD serum recognizes the FAD cofactor attached to the *S. cerevisiae* Fp subunit.

Figure A-1. Western blot analysis of mutant and wild-type mitochondria. Mitochondrial protein (50 μg/lane) from Sdh1Ad1 carrying the plasmids pSDH1 (wild-type, W) or pS1H90S (mutant, M) was solubilized, separated by SDS polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose for Western blot analysis as described in chapter 2. Blots were incubated with affinity-purified anti-SDH1p antibodies (α-Fp, lanes 1-4), affinity-purified anti-FAD antibodies (lanes 5-8), or pre-immune serum diluted 1:3,000 (lanes 9-12) in the presence (+) or absence (-) of 1.5 mM FAD. The position of the SDH1p subunit is indicated by the arrow and the positions of the size markers are indicated on the left.


Serum:
α Fp
α FAD
Preimmune
FAD:
-
+
-
+
-
+
Protein:
W
M
W
M
W
M
W
M
W
M
W
M
Lane Number:
1
2
3
4
5
6
7
8
9
10
11
12
80
49.5

To further define the anti-FAD serum specificity, we tested other potential competitors. When incubated in the absence of competitor, the anti-FAD antiserum detects a 68 kDa band corresponding to the FRD flavoprotein subunit (Figure A-2, lane 1). FAD or FMN effectively compete for the recognition of covalent FAD by the serum (lanes 3 and 4). Riboflavin can partially compete (lane 5), but histidine and ATP can not (lanes 2 and 6, respectively). These results indicate the major epitope recognized by the anti-FAD serum is the phosphate group of the FMN moiety since riboflavin which lacks the phosphate, is an ineffective competitor. Furthermore, the peptide backbone is not recognized because all binding is competed for by FMN or FAD.

Several types of covalent linkages can be formed between FAD and a protein (Decker, 1982). Histidyl residues can be attached through the *N*(1) or the *N*(3) nitrogens to the 8α-methyl group of the isoalloxazine ring. Cysteine residues may form thioether linkages with either the 8α-methyl group or with carbon-6 of the ring. Finally, a tyrosyl(*O*)-8α-flavin linkage can occur. In addition to the *E. coli* FRD and the *S. cerevisiae* SDH with 8α-*N*(3)-histidyl linkages, we also tested an *Alcaligenes* choline oxidase and a *Pseudomonas* sarcosine dehydrogenase with the same linkage type, *Schizophyllum commune* cholesterol oxidase with an 8α-*N*(1)-histidyl linkage, and bovine monoamine oxidase with a cysteinyl(*S*)-8α-FAD for recognition by the anti-FAD serum. All flavoproteins with the *N*(3) or the *N*(1) linkages were efficiently recognized (Figure A-3, lanes 1 to 4) and the recognition could be competed by free FAD (not shown). Surprisingly, monoamine oxidase was not (lane 5), suggesting that the linkage chemistry is important in recognition. This contrasts with the previously described anti-FAD serum that recognized not only the histidyl and the cysteinyl linkages but also a tyrosyl linkage which we did not test (Barber, *et al.*, 1987).

Figure A-2. Specificity of the anti-FAD serum. *E. coli* FRD (4 μg per lane) was electrophoresed and transferred to nitrocellulose for Western blot analysis. Individual lanes were incubated with anti-FAD serum diluted 1:3,000 in the absence of competitor (lane 1) or the presence of 1.5 mM histidine (His, lane 2), 1.5 mM FAD (lane 3), 1.5 mM FMN (lane 4), 1.25 mM riboflavin (Rb, lane 5), or 1.5 mM ATP (lane 6).

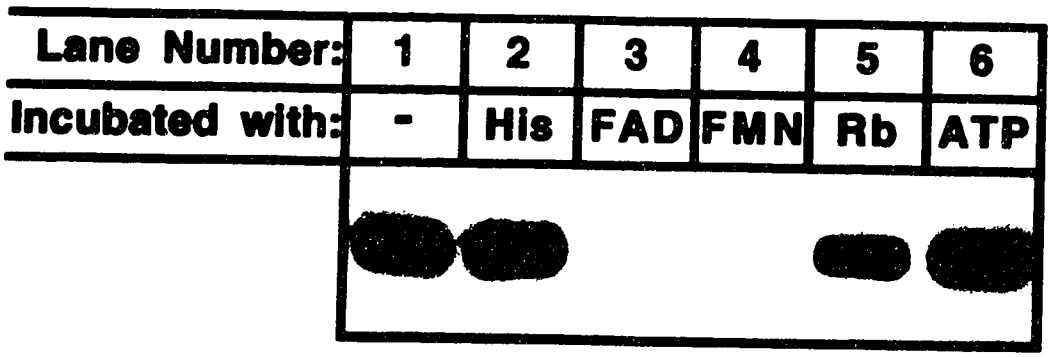
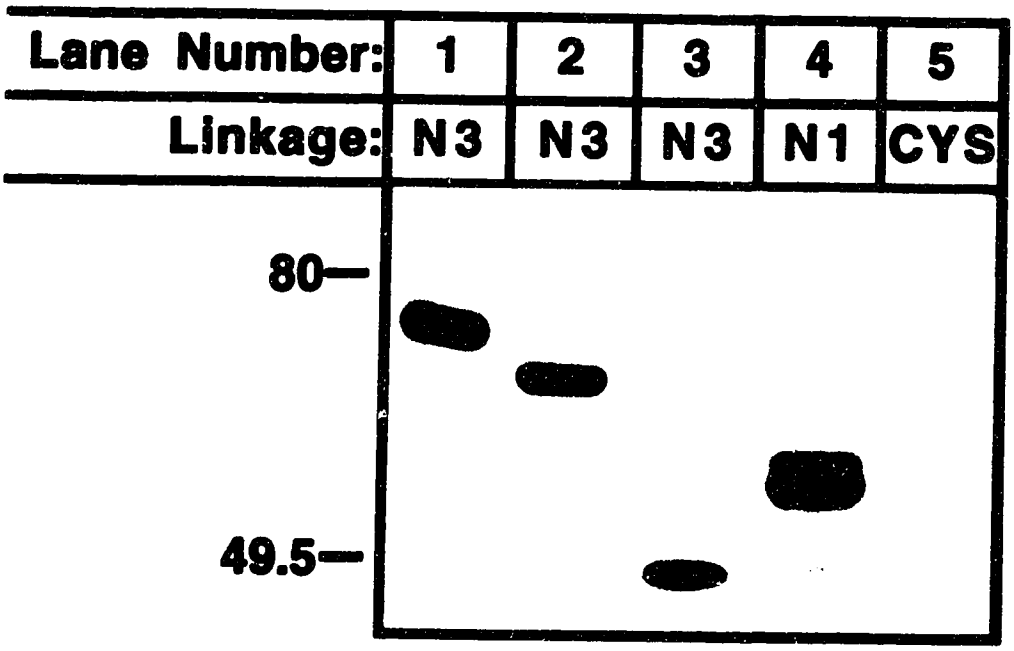

Lane Number:
1
2
3
4
5
6
Incubated with:
-
His
FAD
FMN
Rb
ATP

Figure A-3. Linkage specificity of the anti-FAD serum. *E. coli* FRD (lane 1), *Alcaligenes* choline oxidase (lane 2), *Pseudomonas* sarcosine dehydrogenase (lane 3), *S. commune* cholesterol oxidase (lane 4), and bovine monoamine oxidase (lane 5; 4 μg each) were subjected to Western blot analysis with the anti-FAD serum. Details of the FAD linkages are described in the text. The positions of the size markers are indicated on the left.


Lane Number:
1
2
3
4
5
Linkage:
N3
N3
N3
N1
CYS
80
49.5

A-2. FOOTNOTES

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