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NAME OF AUTHOR/NOM DE L'AUTEUR Charles Timmis Grubmeyer

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NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. Mary Spencer

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THE ENERGY COUPLING ATPase COMPLEX OF PEA COTYLEDON MITOCHONDRIA

by

© CHARLES TIMMIS GRUBMEYER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

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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 "The Energy Coupling ATPase of Pea Cotyledon Mitochondria" submitted by Charles Timmis Grubmeyer in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Mary Spencer
Supervisor

Joel H. Weiner

W.M. Bragg

Armand Hordo

Date September 11, 1978

Harvey S. Penefsky
External Examiner

ABSTRACT

The energy coupling system (ATPase - ATP synthetase) of pea cotyledon mitochondria has been studied in both soluble and membrane-bound forms.

With a partially purified soluble ATPase, I was able to show many similarities to the F_1-ATPase of mammalian mitochondria. The enzyme was found to be extremely cold labile. Inhibition patterns with NaF and NaN_3 and stimulation by 2,4-dinitrophenol were also typical of F_1-ATPases from mammalian sources. The enzyme hydrolysed GTP, ITP, and ATP, but not CTP, UTP, ADP, or IDP. ATPase and ITPase activities were strongly inhibited by ADP, and to a lesser extent by IDP. Distinctive properties of the pea mitochondrial ATPase were its high rate of Ca-ATPase, and the stimulation of ATPase activity caused by NaCl.

The stimulation by NaCl was investigated further. The effect of chloride salts was independent of the type of cation in the salt. With sodium salts I found that the degree of stimulation depended on the nature of the anion present. Stimulatory anions included oxyanions such as bicarbonate, but anions such as chloride and bromide were also effective, as were anions of organic acids. My experiments clearly demonstrated that salt stimulations were caused or regulated by anions. The enzyme was not stimulated by trypsin treatments, which are known to destroy the specific polypeptide inhibitor of ATPase in submitochondrial particles. These results showed that the inhibitor was not present in the preparation and therefore the stimulations of the enzyme by the anions were not caused by dissociation or destruction of the inhibitor. I found that the anion-stimulated activity was ATP-specific. It was less sensitive to inhibition by azide than was activity without added anions. The relatively high GTPase acti-

vity of the enzyme was not anion-stimulated and was relatively insensitive to azide. The anion stimulations were very similar to those reported with other F_1-ATPases from yeast and mammalian tissues, except that oxyanion specificity was not observed with the pea enzyme.

Submitochondrial particles freshly prepared by sonication from pea cotyledon mitochondria showed low oligomycin-sensitive ATPase activity which increased 10-fold on exposure to trypsin, a treatment known to destroy the specific ATPase inhibitor polypeptide. Pea submitochondrial particle ATPase was activated to a similar extent by "aging" in vitro. At pH 7.0 1 mM ATP prevented the "aging" process. Freshly prepared submitochondrial particles showed a substrate specificity similar to that of the soluble pea mitochondrial ATPase, with GTPase>ATPase. "Aged" or trypsin-treated submitochondrial particles showed equal activity with the two substrates. NaCl and $NaHCO_3$, which stimulated the ATPase activity of the soluble pea enzyme, were also stimulatory to the GTPase and ATPase activity of freshly prepared submitochondrial particles, and only to the ATPase activity of trypsin-treated or "aged" submitochondrial particles. The ATPase activity of rat liver submitochondrial particles was stimulated by HCO_3^-, but inhibited by Cl^-. I conclude that Cl^- stimulation is an intrinsic property of the pea mitochondrial ATPase.

Submitochondrial particles were also shown to catalyse ATP synthesis coupled to substrate oxidation. ATP synthesis was sensitive to the electron transport inhibitor KCN, the uncoupler CCCP, and the coupling factor inhibitor oligomycin. The kinetics of ATP synthesis indicated a high affinity for phosphate (Km = 0.18 mM). ADP kinetics showed negative cooperativity, with Km values of 0.01 and 0.1 mM. A curve of pH effects on oxygen uptake, ATP synthesis and ATPase did not indicate a clear

relationship between ATPase and ATP synthesis. The effects of chloride and bicarbonate anions on [redacted] activities also failed to indicate any clear relation[redacted]drial particles also catalysed an ATP-Pi exchange reaction.

A more highly purified preparation of the soluble ATPase was obtained from submitochondrial particles. The purification method, employing DEAE cellulose chromatography, is described. The enzyme obtained was over 90% pure on gel electrophoresis. On SDS gels the enzyme showed subunit composition typical of F_1-ATPases except that α and β subunits were not resolved. Electrophoresis in 8 M urea resolved the two major subunits, however. Kinetic analyses indicated Vmax for the ATPase reaction was about 18 units/mg protein, with a Km of 0.29 mM. Negative cooperativity was observed in Lineweaver-Burke plots. Kinetic parameters are also given for the high rates of GTPase and anion-stimulated ATPase activity. Submitochondrial particles also showed negative cooperativity in Lineweaver-Burke plots. The aging-activation of submitochondrial particle ATPase was shown to be similar kinetically to removal of a noncompetitive inhibition, such as that caused by the ATPase inhibitor.

I was also able to show, in a brief Appendix, that corn submitochondrial particles also possess an ATPase activity that is sensitive to oligomycin. This result contradicts recent reports that monocotyledonous plants possess an unique oligomycin-insensitive energy-coupling ATPase.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Mary Spencer, for her continual support throughout the course of my research. I began this work as a very physiological problem: a study of the effects of plant growth regulators on a soluble ATPase. It was only with Dr. Spencer's help and willingness to explore that the pr[redacted] and shifted emphasis to the biochemistry of energy couplin[redacted]

Not all the work I describe was performed by me. I would like to thank Ian Duncan for his help with doing assays for every chapter of this thesis, for suggesting new experiments, and for discussing the interpretation of results. The results described in Chapter VI represent a joint effort by Dr. Dara Melanson, Ian Duncan, and myself. I would like to thank both of my coworkers for all their help.

For excellent discussions during the course of my work I have to thank many people: Dr. Gerald Bomhoff, who always asked for more experiments, better data, and less blind reliance on "the literature"; Dr. Arnost Horak who was always willing to discuss my results and suggest new experiments; and lastly Pawan Bassi, Ian Duncan, Ken Eastwell, Terry James, Dave Kristie, Dara Melanson, Donna McIvor, and Mark Whisson for listening to as much as four years worth of ATPase talk. Communication is essential to good research and I feel I gained from all the comments I received.

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INTRODUCTION

The mitochondrial synthesis of ATP by phosphorylation of ADP has been the subject of intense study for the last twenty to thirty years. Many advances have been made in this field, including the crucial discovery by Mitchell (Boyer et al., 1977) that the ATP synthesis reaction is linked to substrate oxidation by way of energy-rich proton gradients. The actual mechanism by which this energy is used to phosphorylate ADP remains largely unknown, however.

A step in the elucidation of this mechanism was made in 1960 when the group of Racker was able to solubilize and purify a protein factor (F_1) from beef heart mitochondria which, when added to F_1-depleted submitochondrial particles,was able to restore their ability to phosphorylate ADP (Pullman et al., 1960, Penefsky et al., 1960). In its soluble form F_1 was also found to be a highly active ATPase, although it could not catalyse any other partial reactions of oxidative phosphorylation unless bound to its native site on the inner mitochondrial membrane. Since this time many researchers have followed the assumption that studying the ATPase reaction catalysed by F_1 provides a valid approach toward understanding the mechanism of ATP synthesis. Intensive research has thus gone into discovering the catalytic, ligand-binding, and physical properties of F_1-ATPase, all of which appear to indicate that the F_1-ATPase is a very complex enzyme; perhaps, in its membrane form, "the most complex enzyme system known to man" (Pedersen, 1975).

Following the lead of Racker and his co-workers, other groups have isolated F_1-type ATPases from membranes of bacteria, yeast mitochondria, and higher plant chloroplasts (see Nelson, 1976; Pedersen,

1975; Penefsky, 1974), indicating that the enzyme is basic to the energy metabolism of most organisms. Very little research has been done on the ATPase of plant mitochondria, however, and aside from a few brief reports (Malhotra and Spencer, 1974a, b; Nagaraja and Patwardhan, 1974; Peterson and Heisler, 1963; Yoshida and Takeuchi, 1970) no one has attempted to solubilize, purify and characterize the F_1-ATPase of mitochondria from a higher plant. Not only would such a study be useful for understanding the bioenergetics of plant mitochondria, but it might also advance our understanding of the basic mechanism of F_1-type ATPases.

CHAPTER I

LITERATURE REVIEW

The energy-coupling ATPase systems of mitochondria, chloroplasts, and bacteria have been the subject of an immense literature in recent years. Fortunately, many excellent reviews exist that form a coherent picture from the variety of results in the literature. For F_1-ATPases from mitochondria, the reader is referred to the reviews by Pedersen (1975) and Penefsky (1974). The review by Senior (1973) is also of value. Chloroplast ATPase is well-discussed by Nelson (1976); a less current review on bacterial F_1-ATPase is that of Abrams and Smith (1974). Reviews on energy-coupling are also available; of particular interest is a recent multi-section review by leaders in the field of oxidative phosphorylation (Boyer et al., 1977). A review on photophosphorylation appeared recently (Jagendorf, 1977). Also of interest is a review by Kozlov and Schulachev (1977) that attempts to devise a mechanism for F_1-ATPases, and one by Harris (1978) that attempts to resolve the research on roles for nucleotides bound to coupling factors.

With this complete review literature readily available there appears to be little purpose in attempting a broad review of the field here. I have, rather, attempted in the discussion section of each chapter to compare my results with those in the literature, and to draw these comparisons together in a final discussion chapter. There are, however, two areas which seem worthy of discussion here because they are not well-reviewed, and are only touched on during my discussion sections. These topics are the control of F_1-ATPase activity, and the current state of plant mitochondrial ATPase research.

A. Control of F_1-ATPase Activity

A major function of the mitochondrion, and a central focus of the cell's bioenergetics network, is the synthesis of ATP by phosphorylation of ADP, using the energy of substrate oxidation. Thus the ability of the coupling factor F_1 to hydrolyse ATP at a very high rate comes as a shock. As discussed by Racker (1976), however, most workers resolve this conflict by considering the ATPase activity as an aberration (as opposed to a simple reversal) of a normal ATP synthesizing system. Commonly held theories, for example that ATPase involves a separate catalytic site from ATP synthesis (Pedersen, 1975, 1976; Penefsky, 1974b) or that ATPase is expressed only after a damaging conformational change has occurred (Warshaw et al., 1967), express a general feeling that ATPase activity is more an artifact of isolation than a natural activity. A more interesting approach, which appears to lead to further experimentation, is that F_1-ATPase is simply a well-controlled enzyme in vivo, that it is quite capable of hydrolysing ATP in its native state, but is kept from doing so at high rates by a battery of control systems. This seems a worthwhile hypothesis because it leads to predictions that can be tested, and may help to close the conceptual gap between a large body of work on ATPase, and an even larger body of work on oxidative phosphorylation.

What are the possible mechanisms for control? It seems possible to identify at least four: control by product inhibition, control by ATP export, control by the inhibitor polypeptide, and control through the kinetic properties of the enzyme.

1. Control by Product Inhibition

If one accepts the hypothesis of Mitchell that ATP synthesis and electron transport are linked by an energy-rich proton gradient the reaction of ATP hydrolysis catalysed by intact mitochondria or submitochondrial particles is:

$$ATP \rightleftharpoons ADP + Pi + H^+ \text{ gradient}$$

ADP has been found to be a relatively effective inhibitor of membrane-bound F_1-ATPase with a K_i of 80 μM in beef heart submitochondrial particles (Hammes and Hilborn, 1971), although very wide discrepancies in K_i values do exist in the literature (Pedersen, 1975). Internal ADP levels in intact mitochondria would normally be under control of the adenine nucleotide transporter, which under conditions of membrane energization would increase the internal ADP/ATP ratio, intensifying the inhibition by added ADP.

Free Pi is apparently a rather weak inhibitor of the ATPase reaction. For example Pullman et al. (1960) reported that 40 mM Pi was without effect on ATPase activity of beef heart F_1-ATPase in the presence of 6 mM ATP.

The most interesting of the three possible product inhibitors is the proton gradient shown to be produced by the reaction (Moyle and Mitchell, 1973). The potency of this inhibition has not been directly measured, and it is not clear what the critical experiment would be. In the presence of an ATP-regenerating system (Pullman et al., 1960) to remove ADP, and using the H^+ release assay (see Van de Stadt, 1973) or the NADH-linked assay of Pullman et al. (1960), the rate of ATPase activity should slow as an H^+ gradient is built up. This should hold true when well-coupled submitochondrial particles are used; uncoupler should release this inhibition. This experiment has been done by Van de Stadt

et al. (1973, Fig. 4) who found that particles with or without uncoupler showed a similar slow rate of the ATPase reaction. The authors interpreted this to indicate that the ATPase was not active enough to develop a proton gradient sufficient to inhibit the reaction. The ATPase of those particles, however, was shown to be under control of the inhibitor polypeptide (discussed below). During ATP hydrolysis, as the inhibitor dissociated from the ATPase because of the increasing proton gradient, uncoupler became effective in producing an enormous activation of ATPase activity. The authors concluded that ATPase activity could be directly regulated by the rate of dissipation of a high energy state (H^+ gradient) but that this regulatory mechanism would be important only when the ATPase was not being controlled by the inhibitor polypeptide. These experiments could be extended, but this would require a system in which inhibitor-free ATPase was used, and H^+ gradients could be directly measured or known.

It should be pointed out that the potency of product inhibition (K_i values) is not related to the position of the final equilibrium of the reaction. The position of this equilibrium is, of course, also of interest, since products of the ATPase reaction are also the substrates of the ATP synthesis reaction. Considering only ADP and P_i as products (as they are in a well-buffered assay using F_1-ATPase or a membrane-bound system with uncoupler) the equilibrium constant for ATP hydrolysis is about 2×10^5 M (assuming a standard free energy of ATP hydrolysis of -7.3 kcal (Lehninger, 1970)). In other words, ATP is not expected to be measurable at equilibrium. When the H^+ gradient is present however, this equilibrium can be shifted completely toward ATP synthesis, so that no net ATPase reaction is observed when ATP is added to respiring mitochondria (e.g. [redacted]uchi, 1975) and isolated respiring mitochondria can

maintain a high ATP level in state IV. This does not mean that respiring well-coupled mitochondria are not capable of ATPase activity, only that the net equilibrium will favor ATP synthesis. The potency of product inhibition will be reflected in how quickly the γ-phosphate of ATP becomes labelled when unlabelled ATP is added to respiring submitochondrial particles with ^{32}Pi. This ATP-Pi exchange (with respiration) appears worthy of more investigation for determining the effect of the proton gradient on ATPase kinetics, and on Ki values for product inhibition.

2. Control by ATP Export

The inner mitochondrial membrane is not permeable to charged substances, and special transporter systems exist for the charged substrates of oxidation and phosphorylation to move across the inner membrane (see Wiskich, 1977). With ATP, the transport system is of special interest for two reasons: it will only exchange internal adenine nucleotides with external adenine nucleotides (no net nucleotide transport is catalysed) and it is normally electrogenic, importing an ADP^{3-} and exporting an ATP^{4-}, thus requiring that one H^+ be exported by electron transport or ATPase (Vignais, 1976). Thus, mitochondria have a nucleotide pool of fairly fixed size whose composition will be determined by the relative concentration of the nucleotides present (ADP and ATP), but also by the energetic state of the membrane. In other words, in conditions favoring net oxidative phosphorylation, ADP will be taken up preferentially over ATP, while ATP will be preferentially exported. In this way mitochondria respiring in a mixture of ADP and ATP without Pi will concentrate ADP inside the mitochondria and ATP outside. Similarly, mitochondria

hydrolysing ATP in the absence of oxidizable substrate should export ATP and concentrate ADP, inhibiting the hydrolysis reaction. The degree to which this occurs with whole mitochondria could be determined to investigate how effectively the adenine nucleotide translocator does inhibit ATPase. Under normal ATPase experimental conditions, where ADP is kept very low, the translocator may not be important as a controlling influence.

3. Control by Inhibitor Polypeptide

Probably the most important mechanism by which the ATPase reaction is controlled is by the specific ATPase inhibitor polypeptide, discovered in 1963 by Pullman and Monroy (1963). These workers found that they could isolate, from whole mitochondria, a low molecular weight protein (mol wt 15,000) which could inhibit the ATPase activity of either F_1 or submitochondrial particles. The inhibitor conferred cold-stability on the F_1-ATPase. The inhibitor has since been purified from rat-liver (Chan and Barbour, 1976) and also occurs in chloroplast F_1-ATPase (see Nelson, 1976). Some debate centers on whether the inhibitor is identical with subunit ε of the F_1-ATPase, or is a sixth subunit (Pedersen, 1975). The most detailed work, that of Knowles and Penefsky (1972a, b), definitely supports the former possibility, since they were able to show that subunit ε and the Pullman and Monroy inhibitor co-electrophoresed on SDS gels. They showed that amounts of subunit ε were proportional to inhibitor content with their F_1-ATPase preparation (which was low in inhibitor) and the preparation of Pullman et al. (1960) (which contained a large amount of inhibitor).

In discussing the regulation of ATPase activity, however, the

important point is what factors control the interaction between the inhibitor and the F_1-ATPase. Pullman and Monroy (1963) found that high pH (over 7.0) prevented the inhibitor from inhibiting the F_1-ATPase. Later work (Horstman and Racker, 1970) showed that low levels of ATP and Mg^{2+} (20-100 μM) were required for the inhibitor to inhibit the ATPase activity of inhibitor-depleted submitochondrial particles. ADP could not replace ATP. From these data (Pullman and Monroy, 1963; Horstman and Racker, 1970) possible regulatory properties could be foreseen: the high internal pH and the low ATP/ADP concentration ratio during oxidative phosphorylation by mitochondria would favor non-association of the inhibitor with the ATPase, while the high ATP/ADP ratio of state IV would favor association of the inhibitor.

More experiments on the regulatory properties of the inhibitor polypeptide were reported by Asami et al. (1971) who found the inhibitor was effective against all ATP-driven energy linked reactions of submitochondrial particles, including the NADH-$NADP^+$ transhydrogenase, reverse electron transport, and membrane energization by ATP (measured as an enhancement of ANS fluorescence). None of these reactions was inhibited by the inhibitor when driven by succinate instead of ATP. When the inhibitor was present ATP synthesis by the submitochondrial particles was stimulated in a manner that mimicked the effect of an ATP trapping system. The authors suggested that the inhibitor polypeptide is a "directional regulator" of the coupling system.

Following the lead of Asami et al. (1971), Van de Stadt et al. (1973) investigated the regulatory function of the inhibitor in beef heart submitochondrial particles. These workers determined that the inhibitor was noncompetitive versus ATP in the ATPase assay and did not

affect the Ki of ADP. More importantly, they showed that the extent of inhibition caused by a set amount of inhibitor was proportional to the ATP/ADP ratio in the incubation medium, not merely the concentration of ATP. In addition the oxidation of NADH also reduced the extent of the inhibition. The presence of phosphate had no effect. When inhibited particles were allowed to hydrolyse ATP the rate was low and not stimulated by the simultaneous addition of an uncoupler. If hydrolysis was allowed to proceed several minutes before uncoupler was added, however, ATPase was greatly stimulated by the uncoupler. The same uncoupler stimulation of ATPase also occurred when particles were allowed to oxidize NADH for a short period before uncoupler addition. The authors concluded that membrane energization by either substrate oxidation or ATP hydrolysis weakens the interaction between the inhibitor and the ATPase. They hypothesize a regulatory scheme in which the ATPase is inhibited by the inhibitor whenever the ATP/ADP ratio increases, or the energization of the membrane ceases. This is clearly an ideal situation for regulating ATPase activity. Van de Stadt et al. (1973) also proposed the existence of a bound, noninhibitory state for the inhibitor. This was based on experiments in which partially inhibited particles were incubated with Mg-ATP causing a further inhibition. The physiological significance of the bound-noninhibited versus the fully dissociated state is not known.

In a later paper Van de Stadt and Van Dam (1974) showed that interaction between the inhibitor and the ATPase was weakened by high ionic strength, causing dissociation of the inhibitor, with some anion specificity being shown for this effect. They also showed that submitochondrial particles low in inhibitor showed little change in the Km for ATP or

the Ki for ADP in the ATPase reaction, but when these particles were assayed with uncoupler present, the Km ATP dropped from 193 μM to 50 μM, and the Ki for ADP dropped from 132 μM to 70 μM. Using this data and the finding that the presence of inhibitor caused a change in the binding and fluorescence properties of the ligand aurovertin (Van de Stadt et al., 1974; Van de Stadt and Van Dam, 1974) the authors proposed that inhibitor binding causes a conformational change in the membrane-bound F_1-ATPase.

A subject of some debate is the role of the inhibitor during the forward reaction of ATP synthesis. No effect on ATP synthesis was noted in the experiments of Pullman and Monroy (1963) or Asami et al. (1971), suggesting that ATPase may occur through a separate, inhibitor-regulated site. It is clear from the work of Van de Stadt et al. (1973) however, that the conditions of oxidative phosphorylation (membrane energization with ADP) are those that cause inhibitor dissociation, so that no effect on ATP synthesis should be expected in normal assays. A unique approach to this problem has been taken by Harris and Crofts (1978) who used millisecond light flashes to activate photophosphorylation in chloroplasts. They were able to show that the rate at which ATP synthesis ability appeared coincided with the rate at which inhibitor was displaced from the F_1-complex, as judged by the activation of ATPase. Although the chloroplast results may not be directly related to the situation in mitochondria, it does appear likely that inhibitor must be removed for either ATPase or ATP synthesizing activity.

4. Kinetic Control of the ATPase

A final mechanism through which some control over ATPase activity

is exerted is by the kinetic properties of the enzyme itself. These control properties are more evident with the soluble F_1-ATPase than with submitochondrial particles, raising some question as to their importance in the mitochondrion.

Early experiments by Lardy's group (Lambeth and Lardy, 1971) showed that the rat liver enzyme was stimulated by certain anions (bicarbonate, chromate, sulfite and maleate) but not by others (chloride, sulfate). Further work (Ebel and Lardy, 1975) examined the kinetics of the anion stimulations. It was found that the soluble rat liver enzyme displayed pronounced negative cooperativity when no stimulatory anions were present in the assay mixture. Addition of a stimulatory anion could completely remove the negative cooperativity and also increase the Vmax of the enzyme. These workers measured "fold-activation" and Ka for a variety of anions and found values of 1-9.4 for fold-activation and 0.06-33 mM for Ka values. There was no apparent correlation between these two measurements when anions were ranked in order of effectiveness. In addition Ebel and Lardy showed that azide and thiocyanate anions, which strongly inhibited Vmax, caused increased negative cooperativity in Lineweaver-Burke plots when ATP was the substrate. Bicarbonate and other stimulatory anions competed with inhibitory anions to exert their effects, leading Ebel and Lardy (1975) to hypothesize a regulatory anion binding site that could bind either stimulatory or inhibitory anions. The ATPase activity of rat liver submitochondrial particles also showed cooperativity, although much less so than the soluble enzyme, and anions could stimulate ATPase activity of this preparation only 2-fold. Interestingly, the very high GTPase and ITPase activities of the soluble enzyme showed linear kinetics, and anions were without substantial effect. This led Ebel and Lardy

(1975) to propose that there were either two types of catalytic sites, or that the enzyme possessed a nucleotide-binding regulatory site; in either case the regulation of cooperativity appeared to involve both anions and nucleotides. Pedersen (1976), using rat liver submitochondrial particles and F_1-ATPase, obtained results similar to those of Ebel and Lardy (1975) with respect to effects of anions, nucleotides, and membrane binding. He also showed that the ATP-driven transhydrogenase reaction and the ATP-Pi exchange reaction were not at all similar in their kinetics, substrate specificity, or anion sensitivity to the ATPase reaction. These results on ATP-driven reactions were interpreted to indicate multiple catalytic sites on the ATPase, each responsible for separate activities. Close examination of the data indicates this interpretation was not warranted, however.

Further research by Lardy's group (Schuster et al., 1975; Schuster et al., 1976) explored the regulation of ATPase kinetics further, using the nonhydrolyzable nucleotide analogs AMP-PNP, GMP-PNP, and IMP-PNP. The results far from clearing up the nucleotide specificity and function of the various sites, resulted in the rejection of any simple system of nucleotide and anion regulation (Schuster et al., 1976).

The group of Hess, working on soluble yeast F_1-ATPase have also explored the effects of anions. They were able to show (Takeshige et al., 1976) that different anions had clearly different effects on the negative cooperativity of the enzyme. In the presence of sulfate, for example, the enzyme showed no cooperativity, and a Km ATP (0.2 mM) similar to the low affinity site when no anion was present. With sulfite, no cooperativity was noted and the Km ATP was 0.04 mM, identical to that of the high affinity site for ATP when no anion was present. Neither anion had

a substantial stimulatory effect on Vmax. Bicarbonate, which did stimulate Vmax, had no effect on cooperativity or Km values. From further research on the effects of sulfite and sulfate (Recktenwald and Hess, 1977), Hess proposed that yeast F_1-ATPase has three $\alpha\beta$ pairs of subunits. Each pair possesses a catalytic site which binds MgATP and an allosteric regulatory site which binds MgATP or anions. Binding at this site would result in an increased Km (when ATP or sulfate is bound) or no effect on Km (sulfite). Sulfite would effectively compete with ATP at the regulatory site so that no ATP-induced curvature of kinetic plots would be noted when sulfite was present. This model requires no interaction between catalytic sites (no true cooperativity) but is perhaps overly convenient in ignoring Vmax effects of bicarbonate and azide, and the interesting lack of effects of anions on GTPase.

A recent report (Lopez-Moratalla et al., 1977) that anion effects can be completely absent when ATPase is isolated from the liver of fasted rats is interesting and may eventually provide a clue as to the mechanism and significance of anion effects. This report was on whole mitochondrial ATPase, however, so that data may reflect the effects of other regulatory systems.

Another report that has not been adequately followed up is that of Moyle and Mitchell (1975) who showed that the ATPase could exist in inactive or active states. Magnesium ions were able to induce the inactive state in a time-linked manner, while some anions could reverse the Mg^{2+} effect, again in a time-linked manner. No kinetic plots were shown, but the authors felt that the kinetic effects of anions (stimulation of the apparent Vmax) were best considered as an increase in the proportion of active catalytic sites. The significance of this report remains to be

evaluated.

Finally, one must ask what the physiological significance of the anion stimulations and cooperativity is. With anions, bicarbonate is the most interesting, since it is a product of the Krebs cycle. It could thus serve to activate the ATPase when respiration is active. This is of no use at all, of course, unless bicarbonate also stimulates the Vmax for oxidative phosphorylation, an effect which has not been carefully searched for in mammalian systems. The physiological significance of cooperativity toward ATP remains a mystery.

B. Plant Mitochondrial ATPase

1. ATPase in Intact Plant Mitochondria

In plants, the ATPase activity of intact mitochondria has frequently been studied. Forti (1957) found that pea mitochondria showed an ATPase that was stimulated by 2,4-dinitrophenol or aging of the preparation for several hours. Forti (1957) accepted the concept of Lardy and Wellman (1953) that ATPase activity does not represent a specialized ATP-hydrolysing enzyme, but is a reversal of oxidative phosphorylation. Another early report on plant mitochondrial ATPase is that of Aloni and Poljakoff-Mayber (1962) who studied lettuce mitochondria. On the basis of pH profiles these authors hypothesized the existence of 2 or 3 separate enzymes hydrolyzing ATP.

A major source of controversy in much of the more recent research on plant mitochondrial ATPase concerns the effect of 2,4-dinitrophenol. Following the early report of Forti (1957) that 2,4-dinitrophenol stimulated the ATPase activity of pea mitochondra by 100%, work by Reid et al. (1964) showed that cauliflower mitochondrial ATPase was not stimulated

by 2,4-dinitrophenol. Blackmon and Moreland (1971), working with mung bean mitochondria reported results similar to those of Forti (1957). Blackmon and Moreland (1971) found that 2,4-dinitrophenol at 0.08 mM was sufficient to uncouple mitochondria and to activate ATPase 4-fold. Oligomycin inhibited this stimulation. Interestingly, substrate oxidation also inhibited the 2,4-dinitrophenol-stimulated ATPase activity although the inhibition varied from 36-74%, depending on the respiratory substrate. Destruction of mitochondrial structure by sonication activated the ATPase by about 4-fold, and sonicated mitochondria were not stimulated by 2,4-dinitrophenol. Blackmon and Moreland (1971) also found that the 2,4-dinitrophenol stimulation of whole mitochondria ATPase was eliminated by 0.4 M sucrose, which was routinely present in the assays of Reid et al. (1964). This was not the cause of the failure of 2,4-dinitrophenol to stimulate cauliflower mitochondrial ATPase, however, as Reid et al. (1964) showed that at lower (0.2 M) sucrose, 2,4-dinitrophenol was actually inhibitory. Passam and Palmer (1973), working with Jerusalem artichoke mitochondria, found that ATPase activity in 0.3 M sucrose was only stimulated 33% by addition of 2,4-dinitrophenol, CCCP, or FCCP in concentrations sufficient to uncouple respiration. Changing the sucrose concentration had no effect. These authors found, however, that succinate oxidation in the presence of CCCP released a high rate of ATPase, a finding similar to that of Van de Stadt et al. (1973) with beef heart submitochondrial particles. An anomalous finding in the work of Passam and Palmer (1973) was that the respiration-released ATPase activity was not inhibited by oligomycin. This may be related to the author's earlier finding (Passam and Palmer, 1971) that the coupling activity of Jerusalem artichoke mitochondria is very easily solubilized

in the absence of Mg^{2+}. Other findings on 2,4-dinitrophenol effects are that the ATPase activity of mitochondria from potato tubers (Jung and Laties, 1976), from bean cotyledons (Olson and Spencer, 1968) and from Cicer arietinum seeds (Nagaraja and Patardhan, 1974) is only slightly stimulated by 2,4-dinitrophenol, and that the ATPase of mitochondria from sweet potato (Carmeli and Biale, 1970) and corn (Bottrill and Hanson, 1969) is substantially stimulated.

This problem has been attacked by Jung and Hanson (1973, 1975), who showed that a short period of respiration before the addition of 2,4-dinitrophenol caused the release of a high rate of ATPase activity in either corn mitochondria, which are normally 2,4-dinitrophenol stimulated, or cauliflower mitochondria, which are not (Jung and Hanson, 1973). Evidence was given that this "respiratory priming" involved the generation of a membrane potential, which in turn might have activated the ATP transporter to provide substrate for the ATPase reaction. Thus "the lack of 2,4-dinitrophenol-stimulated ATPase activity appears to be a problem of ATP transport and not a characteristic of the F_1-ATPase per se" (Jung and Hanson, 1973). A later paper showed that respiratory priming or cauliflower mitochondria could be replaced by incubation with ATP (Jung and Hanson, 1975). Analysis of Mg^{2+} and nucleotide content indicated that unprimed corn mitochondria had higher levels of these components than did unprimed cauliflower mitochondria. After priming, the cauliflower mitochondria had accumulated Mg^{2+} to the level shown by corn mitochondria, and were also able to accumulate adenine nucleotides through an atractyloside-insensitive site. These accumulations were believed to be responsible for the priming effects. Another possibility, which appears likely, is that priming represents a buildup of membrane

potential that results in dissociation of the ATPase inhibitor polypeptide, as proposed by Van de Stadt et al. (1973).

Further work on activation of plant mitochondrial ATPase has been done by Jung and Laties (1976), who were able to show that trypsin treatments could activate the ATPase of sonicated potato mitochondria. This implicates the inhibitor polypeptide of Pullman and Monroy (1963) in the regulation of plant mitochondrial ATPase. Work on castor bean mitochondrial ATPase (Takeuchi, 1975) was also interpreted to show the presence of the inhibitor polypeptide. Mitochondria of castor bean showed low ATPase and little stimulation by the uncoupler FCCP. When the mitochondria were oxidizing succinate, no ATPase activity was detectable, but addition of FCCP with succinate released a high rate of ATPase activity. Submitochondrial particles showed a 10-fold stimulated rate of ATPase activity, which was not inhibited by succinate and only slightly stimulated by succinate plus FCCP. Trypsin or pH 9.2 treatments to remove the inhibitor from submitochondrial particles stimulated ATPase activity 10-fold further. The author concluded that ATPase is normally regulated by the inhibitor polypeptide. The situation with respect to how association between the inhibitor and the ATPase is controlled appears complex however, since respiratory priming to activate ATPase can be not required (corn), required (cauliflower, castor bean) or not effective (potato). Further research may resolve these differences.

An additional minor point concerns the effect of oligomycin, a specific inhibitor of the membrane-bound F_1-ATPase. Two groups have shown that the ATPase activity of sonicated corn mitochondria is relatively insensitive to oligomycin (Jung and Hanson, 1973; Sperk and Tuppy, 1977). This was interpreted by the latter authors to indicate

the presence of a unique oligomycin-insensitive ATPase. These studies are discussed further in the Appendix.

Work in this laboratory (Phillips, 1971) showed that whole pea mitochondria possessed low rates of ATPase activity which was not stimulated by 2,4-dinitrophenol in 0.3 M sucrose, and was slightly stimulated in 0.3 M mannitol. The activity was inhibited by oligomycin and NaN_3, and was found to be stimulated by Na^+ and K^+ ions. Activity was also increased by the growth regulator ethylene.

2. Soluble Preparations of Plant F_1-ATPases

The soluble form of the F_1-ATPase has not been extensively studied by any group. In a short communication, Peterson and Heisler (1963) described the isolation of a soluble ATPase from acetone extracts of cauliflower mitochondria. The enzyme was not cold labile, but was stimulated by 2,4-dinitrophenol. No other properties were given. Similarly, in a more complete report, Yoshida and Takeuchi (1970) purified and partially characterized a soluble ATPase from castor bean mitochondria. The enzyme was cold labile, showed ATPase activity that was stimulated by 2,4-dinitrophenol, and a high rate of ITPase activity that was not stimulated by 2,4-dinitrophenol. Another report on a soluble plant mitochondrial ATPase is that of Nagaraja and Patwardhan (1974) who isolated the enzyme from Cicer arietinum seeds. The enzyme was cold labile, but no other properties were described.

Work in this laboratory resulted previously in the isolation of a soluble ATPase from pea cotyledon mitochondria (Malhotra and Spencer, 1974a, b). The method used was that of Horstman and Racker (1970), with minor changes. The preparation was used to study the earlier finding with in-

tact pea mitochondria (Phillips, 1971) that Na^+ and K^+ ions, added as their Cl^- salts, stimulated ATPase. This stimulation was also observed with the soluble enzyme (Malhotra and Spencer, 1974a). In addition, the enzyme was stimulated by mixtures of ethylene and carbon dioxide (Malhotra and Spencer, 1974b). The enzyme required Mg^{2+} for activation (Malhotra and Spencer, 1974a).

A recent report on a soluble ATPase from plant mitochondria is that of Sperk and Tuppy (1977), who used sonication to solubilize the ATPase from mitochondria of a variety of monocotyledonous and dicotyledonous plants. The ATPase activity remaining in the supernatant after the membrane fraction had been removed by centrifugation was cold labile in the case of dicotyledons and cold-stable in the case of monocotyledons. When the ATPase activity was chromatographed on Sephadex the monocotyledon mitochondrial ATPase ran with an apparent molecular weight of 50,000 daltons. The dicot ATPases ran as molecules larger than 200,000 daltons, in keeping with the molecular weight of 347,000 proposed by Knowles and Penefsky (1972a, b) for beef heart F_1-ATPase. Using the data on molecular weight and cold lability, and the oligomycin-insensitivity of monocot mitochondrial sonicates noted earlier, the authors concluded that monocotyledonous plants possess an unique energy-transducing ATPase system. This interpretation seems unwarranted, however, in view of the almost universal occurence of the F_1-type structure (Penefsky, 1974). Further research may be warranted to determine whether monocots possess an F_1-ATPase that is capable of a rapid association-dissociation in the cold or on Sephadex.

CHAPTER II

METHODS AND MATERIALS

A. Tissue Used

Pea seeds (Pisum sativum L. cv. Homesteader) were soaked in water for 6 hr then planted in trays of vermiculite. Seeds were germinated for four days in the dark at 27°C and cotyledons were harvested. Although Phillips (1971) found that ATPase activity of intact pea cotyledon mitochondria was highest after five days of germination, I found five-day-old peas were more likely to be diseased and their more extensive roots made them harder to work with.

For the work in the Appendix, corn seeds (Zea mays L. cv. Earli-King) were washed in 3% sodium hypochlorite solution for 15 min, then rinsed extensively and soaked in water for 6 hr. Seeds were planted in trays of vermiculite and grown in the dark at 27°C for three days. Shoots were then harvested.

B. Isolation of Mitochondria

Mitochondria were isolated following a previously published method (Solomos et al., 1972) with the following minor variations. Grin[illegible] was done for 5 min and the homogenate strained with a single layer of "miracloth" (Calbiochem). The homogenate was then centrifug[illegible] at 1[illegible] x g for 7 min. The supernatant layer was then centrifuged for [illegible] at 20,000 x g. The pellet layer was dark brown with a rim of light-colored material. The light material was removed by suction with a pasteur pipette, as was a floating layer of lipid-like material. The

pellets were resuspended in 40 ml of 0.25 M sucrose and centrifuged at 20,000 x g for 7 min. The light layer was again removed by suction. Mitochondria prepared by this method showed good respiratory control, as discussed in Chapter VI. 500 ml of dry pea seeds gave about 1200 ml of cotyledons and 250 mg of mitochondrial protein.

Mitochondria from corn epicotyls were isolated following exactly the same method, except that the dark brown pellet was surrounded by a green layer, which was removed.

Mitochondria from rat liver were isolated using exactly the same method as for peas, except that the low-speed pellet was resuspended and recentrifuged three times and the supernatants pooled for high speed centrifugation. Grinding was done in a Potter-Elvehejm homogenizer for 1 min.

C. Isolation of Enzyme

The ATPase preparation used in Chapters III and IV was prepared by the method of Horstman and Racker (1970) modified as described by Malhotra and Spencer (1974a). This method is reprinted here with minor changes. All steps were carried out at room temperature unless otherwise stated.

To about 250 mg of mitochondrial protein, 150 ml of 0.15 M sucrose, 2 ml of 0.2 M solution of tris-ATP (pH 7.4), and 2 ml of 0.2M EDTA solution (pH 7.4) were added, and the mitochondria were sonically disrupted with a Branson sonifier (model J-32). The temperature was allowed to rise to 45°C during the first 10 min and to 52-55°C during the second 10 min. The preparation was allowed to cool to room temperature and centrifuged at 100,000 x g for 90 min.

The pH of the supernatant layer was adjusted to 5.4 with 1 N acetic

acid (isoelectric precipitation). The white suspension obtained was centrifuged for 5 min at 16,000 x g. The supernatant layer was decanted from the precipitate and adjusted to a pH of 6.7 with 2 M tris. The volume of the clear supernatant layer was made to 160 ml by the addition of 0.15 M sucrose, and 4 ml of 0.5% protamine sulfate solution was then added to it. The precipitate thus formed was discarded after centrifugation at 20,000 x g for 5 min and to each 100 ml of the supernatant layer, 40 ml of 0.5% protamine sulfate solution was added. After 5 min, the suspension was centrifuged at 15,000 x g for 10 min. After removal of the supernatant layer, the precipitate was dissolved in 10 ml of the following buffer: 0.4 M ammonium sulfate, 0.25 M sucrose, 10 mM tris, and 2 mM EDTA, made to pH 7.5 at 25°C with sulfuric acid. To this solution, an equal volume of saturated ammonium sulfate (pH 7.2) was added slowly with stirring at room temperature. The suspension was placed on ice for 10 min and was then centrifuged at 15,000 x g for 10 min at 4°C. The precipitate obtained was warmed to room temperature and was dissolved in 10 ml of the following buffer: 0.25 M sucrose, 10 mM tris, 2 mM EDTA, and 4 mM ATP (pH 7.5 with sulfuric acid). An equal volume of saturated ammonium sulfate solution (pH 7.2) was added dropwise with stirring and the preparation was stored overnight at 4°C.

The entire preparation was centrifuged at 15,000 x g for 5 min at 4°C. The precipitate was warmed to room temperature and dissolved in the buffer (0.25 M sucrose, 10 mM tris, 2 mM EDTA, and 30 mM ATP, made to pH 7.4 at 25°C with sulfuric acid) to give a final concentration of 4 mg/ml. The solution was transferred to a test tube that was placed in a 72°C bath to allow the temperature to rise to 64°C while continuously stirring with the thermometer. The solution was then held at 64°C for

4 min by quickly transferring it to 64°C bath. The suspension was allowed to cool to 30°C and then centrifuged at 20,000 x g for 10 min to discard the residue. An equal volume of saturated ammonium sulfate solution (pH 7.2) was added to the supernatant layer and the mixture was kept in ice for 10 min. The suspension was centrifuged at 20,000 x g for 5 min at 4°C. The precipitate thus obtained was warmed to room temperature and dissolved in an appropriate amount of the following buffer: 0.25 M sucrose, 10 mM tris, and 2 mM EDTA, pH 8.0 at 25°C. Aliquots of this solution containing 100 µg of protein were transferred to small test tubes and mixed with an equal volume of saturated ammonium sulfate solution (pH 8.0). The suspensions were then stored at -30°C. These preparations were stable for 2-3 months.

Before an assay, a tube containing the enzyme was thawed at 30°C and centrifuged for 10 min at 20,000 x g. The pellet was dissolved in 1 ml of the following buffer: 0.25 M sucrose, 10 mM tris and 2 mM EDTA adjusted to pH 7.2 with HCl at 20°C. Solution containing ATPase was kept at room temperature.

D. Preparation of Submitochondrial Particles

For preparation of submitochondrial particles the mitochondria were suspended in 10 ml of 0.25 M sucrose and either frozen at -20°C or used immediately (all experiments in Chapter VI were done with mitochondria that were used immediately). The fresh or thawed mitochondrial suspension was diluted with 50 ml of "submitochondrial particle buffer" (0.25 M sucrose and 50 mM TES, brought to pH 7.0 with tris at 20°C). The mitochondria were sonicated in a 100 ml beaker at 0°C for two 1 min bursts at 90% full power on the Artek Sonic Dismembrator (Model 300), using

the full-size tip. The sonicate was centrifuged at 20,000 x g for 10 min to remove unbroken mitochondria, and the supernatant centrifuged at 100,000 x g for 60 min at 4°C. The pellet (submitochondrial particles) was suspended on 0.25 M sucrose, diluted to a protein concentration of 6 mg/ml, divided into 0.5 ml portions in glass tubes, and frozen at -40°C. The submitochondrial particles contained about 25-40% of the total protein of the sonicated mitochondria.

To assay ATPase, a tube of submitochondrial particles was thawed, diluted 1/5 in submitochondrial particle buffer, and assayed immediately. Freezing and storage had no effect on ATPase activity, and frozen submitochondrial particles are referred to as "freshly prepared".

Trypsin-treated submitochondrial particles were prepared by diluting 9 mg of submitochondrial particles in 10 ml of submitochondrial particle buffer, adding 0.5 mg pancreatic trypsin and reacting 15 min at 30°C. 2.5 mg lima bean trypsin inhibitor was added, and reacted 5 min. The preparation was stable to freezing at -40°C.

Aged particles were prepared by diluting frozen submitochondrial particles 1/5 in submitochondrial particle buffer and allowing them to stand in test tubes in a water bath. For the most convenient and repeatable aging procedure, I allowed submitochondrial particles to stand 3-5 hr at 30°C.

E. ATPase Assay Without ATP Regeneration

The ATPase assay used in Chapters III-VI was that of Malhotra and Spencer (1974a) which sensitively measures phosphate release in the absence of ATP regeneration. Assay medium, in a final volume of 2.00 ml, was 0.3 M sucrose, 25 mM TES, 3 mM $MgCl_2$ and 3 mM ATP, brought to

pH 8.0 with tris at 20°C. The assay, at 30°C, was started by the addition of enzyme or submitochondrial particles.

The assay was usually run for 10 min and was terminated by the addition of 1 ml of an ice-cold mixture of 0.12 M glycine, 1.8 M $NaClO_4$, and 0.3 N HCl. Tubes were then placed in ice for 6 min. When fresh submitochondrial particles were used the precipitated protein was removed by centrifugation. This was not necessary with soluble enzyme or more active submitochondrial particle preparations.

To assay phosphate released, 2 ml of the quenched reaction mixture was added to 2 ml of the Mozersky et al. (1966) molybdate reagent (2.1 M H_2SO_4, 0.6 M $NaClO_4$, and 12.5 mM ammonium molybdate). To this was added 4 ml of isobutanol:benzene (1:1) and tubes were capped and shaken 15 sec. After a 2 min centrifugation to separate aqueous and non-aqueous layers, the absorbance of the non-aqueous layer was measured at 313 nm.

In Chapter VI ATPase was measured using the phosphate release procedure, but assay medium was 0.3 M sucrose, 4 mM $MgCl_2$, 50 mM TES, 20 mM glucose, and 3 mM ATP, brought to pH 7.2 with tris at 20°C. The reaction was run for 10 min at 25°C.

F. ATPase Assay With ATP Regeneration

For the kinetic assays described in Chapter VII an ATP regenerating system was added as suggested elsewhere (Pullman et al., 1960). Assay medium was 0.3 M sucrose, 25 mM TES, 1 mM $MgSO_4$, 2 mM phosphoenol pyruvate, and 50 µg pyruvate kinase, brought to pH 8.0 with KOH at 20°C. Mg-ATP was added to the desired concentration from stock solutions equimolar in $MgSO_4$ and ATP. (Mg-ATP concentration was determined by absorbance at 259 nm at pH 7.0 assuming a millimolar extinction coefficient of 15.4

(Ebel and Lardy, 1975)). The tubes were placed in a water bath at 30°C and allowed to incubate 5 min. Submitochondrial particles or ATPase protein was added to start the reaction which was allowed to run for 15 min. The reaction was quenched as described earlier and protein was removed by centrifugation. Phosphate was measured as described earlier.

G. Assay for ATP Synthesis

The assay for ATP synthesis in Chapter VI measured esterification of ^{32}P-orthophosphate. The medium for the assay, in a final volume of 1 ml was 0.3 M sucrose, 4 mM $MgCl_2$, 20 mM glucose, 4 mM K_2HPO_4 (containing approximately 200,000 cpm $^{32}PO_4$), 50 mM TES, 5 units of hexokinase, 2 mM ADP, and either 0.88 mM NADH or 8 mM succinate, brought to pH 7.2 with tris. Minor changes in this assay medium were as noted in the figures and tables. The reaction was started by the addition of submitochondrial particles (100-200 µg protein), and was allowed to proceed for 6-15 min at 25°C. To stop the reaction, 1 ml of quench solution (0.3 M HCl, 0.12 M glycine, and 1.8 M $NaClO_4$) was added and the tubes placed on ice for 5 min. After a 6 min centrifugation to remove precipitated protein, the supernatants were transferred to clean test tubes, 2.0 ml of the Mozersky et al. (1966) molybdate reagent (2.1 N H_2SO_4, 0.6 M $NaClO_4$, and 12.5 mM ammonium molybdate) was added, and the tubes allowed to sit at room temperature for 2-6 min. The phospho-molybdate complex formed was removed by three extractions with 4 ml portions of isobutanol:benzene (1:1). The esterified $^{32}PO_4$ was then measured by adding a 1 ml portion of the aqueous residue to 10 ml of Aquasol, and counting by liquid scintillation. Blank tubes contained all assay components plus 1 µg of oligomycin. One unit of activity is defined as

the amount required to esterify 1 μmole of PO_4/min under the above assay conditions.

H. Assay of Oxygen Uptake

Assay of O_2 uptake used the same medium as ATP synthesis, except that no $^{32}PO_4$ or hexokinase were present, and the final volume was 3 ml. The oxygen electrode was used to follow O_2 consumption. Three ml of assay medium at 25°C contained 675 n mole of O_2.

I. Protein Assays

Protein was assayed using the method of Lowry et al. (1951) for all experiments in Chapters III and IV. For experiments in Chapters V-VII the coomassie blue G-250 binding method of Sedmak and Grossberg (1977) was used. The method is sensitive, very repeatable and requires about 5 min to complete. When BSA was used as the protein standard, the method of Lowry et al. (1951) or Sedmak and Grossberg (1977) gave identical estimates of the protein of soluble ATPase or submitochondrial particles. A nearly identical method (Bradford, 1976), available commercially from Calbiochem, was not as accurate.

J. Definition of Units

Units of ATPase or ATP synthesis were defined as the amount of enzyme or submitochondrial particle protein required to catalyse the conversion of 1 μmole of substrate to product in 1 min, under the specified conditions.

K. Electrophoresis

Electrophoresis of the soluble enzyme in Chapter III was carried out according to Davis (1964) on 5% acrylamide gels. For the work described in Chapter VII, it was found that optimal purity was observed when ATPase assay medium was used for the sample solution, gel buffer, and electrode buffer. The gels (5% acrylamide) were run at 4 mamp/tube for 2 hr.

Gels were stained with three different stains to visualize protein bands. Amido black (0.5%) in 20% ethanol and 7% acetic acid, was not very sensitive but was easily destained using electrophoresis. Coomassie blue G-250 was used in 3.4% $HClO_4$. The stain was taken up over a 24 hr period and destaining was not required. Coomassie blue R-250 (0.25%) was dissolved in 50% methanol and 7% acetic acid. Staining was carried out for 5-10 hr at 40°C. Destaining was carried in 20% methanol, 7% acetic acid by diffusion at 20-40°C.

ATPase activity was localized on polyacrylamide gels using the method of Horak (1972). Gels were immersed in 10 ml of ATPase stain medium (0.3 M sucrose, 25 mM TES, 5 mM ATP and 50 mM $CaCl_2$ brought to pH 8.0 with tris) at 30°C and gently shaken for 30-60 min. A white band of $(Ca)_3(PO_4)_2$ precipitate indicated ATP hydrolysis activity.

SDS gel electrophoresis was carried out exactly according to Weber and Osborn (1969). Protein standards were BSA (68,000), catalase (60,000), pyruvate kinase (57,000), enolase (41,000), lactic dehydrogenase (36,000) and cytochrome c (11,700). Samples of protein standards or ATPase were dissolved in 10 mM phosphate buffer, to which was added 1% SDS and 1% mercaptoethanol. Solutions were heated at 98-100°C for 5 min. Glycerol and bromophenol blue were added and the samples layered onto 10% acrylamide gels. Electrophoresis was done at 10 mamp/tube for 3-6

hr, and the gels stained with amido black or coomassie blue R-250.

5% acrylamide gels containing 8 M urea were run exactly according to Knowles and Penefsky (1972a). ATPase protein was dissolved in electrode buffer containing 8 M urea. Immediately before use a drop of bromophenol blue and a few extra crystals of urea were added and the samples were layered onto gels. Electrode buffer above the sample also contained 8 M urea. Electrophoresis was carried out for 2 hr at 5 mamp/tube. Gels were stained with coomassie blue R-250.

L. Materials

Protamine sulfate used for ATPase purification was from Eli Lilly Co. Bovine pancreatic trypsin was from Calbiochem Inc. All other biochemicals were from Sigma Chemical Co., Inc. Baker's yeast hexokinase was Sigma product H-4502 (200-300 units/mg protein). Rabbit muscle pyruvate kinase was Sigma product P-1506 (2 times recrystallized precipitate in $(NH_4)_2 SO_4$ 465 units/mg protein). It was centrifuged for 5 min at 20,000 x g and the pellet was dissolved in 20 mM tris buffer (brought to pH 8.0 with H_2SO_4 at 20°C) to a protein concentration of 2.5 mg/ml. Aquasol and ^{32}P-orthophosphate in 20 mM HCl were from New England Nuclear Co. The ^{32}P-orthophosphate was used without further purification; however, no pyrophosphate contamination was noted, and the oligomycin blanks described in Chapter VI insured that pyrophosphate contamination was not a problem.

M. Variation in Results

For the results in this thesis, no standard errors or measure of variation were formally calculated. All the experiments reported, how-

ever, were done at least two separate times, often by two different experimenters. Each experiment contained duplicates or, in most cases, triplicates of every level of treatment. The result actually reported is in most cases the median value of a representative assay. When an effect varied between two assays a third or fourth assay was done. Care was taken to include proper controls for each treatment and to arrange the assay so that any change in the activity of the enzyme during an assay was not crucial (for example age-activation of submitochondrial particles). The method of handling variation in two special cases is given in more detail below.

For submitochondrial particles there were several sources of variation. Between batches, the ATPase activity of "fresh" particles varied considerably, as noted in Chapter V. All experiments on effects of inhibitor or stimulators were thus done many times on separate batches. If the tube containing fresh submitochondrial particles for assay was kept in the hand or in the water bath at 30°C a gradual increase in activity resulted in replicate tubes at the end of an assay having higher activity than those at the beginning. In contrast, if the tube containing fresh or aged submitochondrial particles was kept in ice and the syringe used was not carefully wiped after injecting particles into the assay medium, the stock solution of particles quickly lost up to 30% of its activity, resulting in replicate assay tubes with lower activity at the end of an assay. The best solution to these problems was to keep the submitochondrial particles in ice but to carefully clean the syringe after each injection. Assays were arranged so that the entire series of treatments was done three times in sequence so that age-activation or cold-inactivation could be easily detected.

For the kinetic assays reported in Chapter VII all points on Lineweaver-Burke plots were medians of triplicates. The lines on Lineweaver-Burke plots were plotted by eye. Least-squares methods for plotting lines would be more accurate with most enzymes, and methods exist for weighting points using the reciprocal of the variance at each substrate concentration. These methods do not seem suited to an analysis of data showing negative cooperativity, however, and so they were not used.

CHAPTER III

PARTIAL CHARACTERIZATION OF THE SOLUBLE ATPase

My initial work on the partially purified enzyme attempted to characterize the ATPase enzyme isolated by the Malhotra and Spencer (1974a) method. Comparisons made between the pea ATPase and F_1-ATPases from other tissues showed they were similar in many respects.

A. Enzyme Assay System and Specific Activity

To establish optimal conditions for the ATPase assay, I tested the effects of enzyme concentration and reaction time. Between 0.5 and 10 µg ATPase protein the phosphate released was directly proportional to the enzyme concentration. With 2 µg enzyme the reaction time course was linear to at least 30 min, the maximum time tested. In most experiments reported here the standard assay was run with 2 µg protein for 10 min.

The enzyme I obtained had a specific activity of 3.8 µmole phosphate liberated/min/mg protein when assayed as described. This is lower than current literature values for mammalian F_1-ATPases (Pedersen, 1975; Penefsky, 1974). Treatments with trypsin or 50 mM dithiothreitol (Nelson et al., 1972) did not increase the specific activity of the pea enzyme (not shown).

The presence of protein impurities was partially responsible for the low observed activity. Gel electrophoresis (Fig. 1) showed that the enzyme contained approximately 40% protein impurities. Specific staining showed, however, that all ATPase and GTPase activity was associated with the major protein band.

Figure 1. Polyacrylamide gel electrophoresis of pea mitochondrial ATPase. The system of Davis (1964) was used. The gel concentration was 5% and gels were run 1 h at 1.5-2.0 mA/tube. A: gel stained for protein with coomassie blue R-250. B: stained for ATPase using 5 mM ATP and 50 mM $CaCl_2$ in normal assay medium, 30°C for 20 min. C: stained for GTPase using conditions as in B, but with 5 mM GTP replacing ATP. 50 µg protein was applied.


C

B. Cold Lability and Ion Stimulations

The pea mitochondrial ATPase showed pronounced cold lability when dissolved in the tris-sucrose-EDTA medium (Table 1). After 20 min at 0° most of the ATPase activity was lost. Inclusion of 4 mM tris-ATP in the incubation medium resulted in only a slight protective effect. When 10% methanol was added to the incubation medium cold lability was considerably slowed. The enzyme was stable for up to 8 hr at 0° when kept as a precipitate in the tris-sucrose-EDTA medium containing 50% saturated $(NH_4)_2SO_4$. Solutions at -40° were stable for several months.

Table 1 also shows, in agreement with previous results (Malhotra and Spencer, 1974a), that the activity of the untreated enzyme was stimulated to about 245% of the normal rate by inclusion of 100 mM NaCl in the assay medium. Inclusion of 20 mM $NaHCO_3$ gave activity of 320% of the basal rate. NaCl-stimulated activity was also cold labile, suggesting that it was not catalysed by a separate enzyme.

C. Nucleotide Specificity

The pea mitochondrial ATPase was able to hydrolyse several triphosphonucleotides, although only ATP, GTP, and ITP gave high rates of activity (Table 2). With CTP and UTP as substrates the activity was barely detectable under standard assay conditions. Table 2 also shows that ADP and IDP were not hydrolysed by the enzyme preparation, ruling out a nonspecific phosphatase activity. The addition of NaCl stimulated the ATPase, but not GTPase or ITPase, activity of the enzyme.

D. ADP Inhibition

Table 1. Cold lability of purified ATPase from pea cotyledon mitochondria.

20 min pre-assay treatment	ATPase Activity (μmole Pi/min/mg protein)	
	No added NaCl	With 100 mM NaCl
25°	3.7	9.1
0°	0.4	2.3
0° + 4 mM tris-ATP	0.7	3.0
0° + 10% methanol	2.6	7.7
0° + 50% saturated $(NH_4)_2SO$ (8 hr)	3.8	9.1

Note: The standard assay mixture (in 2 ml final volume) was 0.3 M sucrose, 3 mM $MgCl_2$, 3 mM tris-ATP and 25 mM TES brought to pH 8.0 with tris at 25°C. The reaction was carried out for 10 min at 30°C and contained 2 μg enzyme protein. Before assay the ATPase solution (0.20 mg protein in 1 ml of 0.25 M sucrose, 2 mM EDTA and 10 mM tris, pH 7.4 at 25°C) was preincubated at 25°C or 0°C (in ice). Where noted the preincubation mixture also contained 4 mM tris-ATP, 10% methanol, or 50% saturated $(NH_4)_2SO_4$. The slight carry over of Pi and ATP from ATP in the preincubation mixture was compensated for by suitable blanks.

Table 2. Nucleotide specificity of pea mitochondrial ATPase.

Nucleotide	Enzymatic activity (μmole Pi/min/mg protein) No added NaCl	+ 100 mM NaCl
ATP	3.8	9.3
ITP	9.9	8.5
GTP	18.9	17.0
UTP	0.8	0.8
CTP	0.3	0.9
ADP	0	0
IDP	0	0

Note: Assay conditions as in Table 1, except that 3 mM ATP was replaced by the indicated nucleotides at a concentration of 3 mM. GTP, UTP, ITP, and IDP were used as sodium salts; ATP, ADP, and CTP were used as tris salts. Where indicated the assay mixture also contained 100 mM NaCl.

ADP was found to inhibit the pea mitochondrial ATPase (Table 3). At an ATP concentration of 3 mM, 50% inhibition occurred at 0.4 mM ADP. ITPase activity was more strongly inhibited by 0.5 mM ADP than was ATPase activity. Both ITPase and ATPase activities were inhibited by IDP, which was a less effective inhibitor than ADP.

E. Divalent Cation Requirements

Pea mitochondrial ATPase was previously shown to require a divalent cation for full activity (Malhotra and Spencer, 1974a). Fig. 2 shows that maximal activation by $MgCl_2$ occurred when it was equimolar with ATP, in agreement with previous results (Malhotra and Spencer, 1974a). At $MgCl_2$ concentrations up to three times greater than the ATP concentration ATPase activity remained high.

When 3 mM $MgCl_2$ was replaced by 3 mM $CoCl_2$ ATPase activity fell to 75% of the $MgCl_2$-activated rate (Fig. 2). At concentrations of $CoCl_2$ of 6 mM or 9 mM the activity decreased. With 3 mM $CoCl_2$, inclusion of 100 mM NaCl gave a 123% stimulation of activity. $CaCl_2$ at 6 mM or 9 mM gave 264% of the rate with 3 mM $MgCl_2$. With 3 mM $CaCl_2$, inclusion of 100 mM NaCl resulted in a 45% inhibition of activity.

In order to test whether the accompanying anion had any effect on Mg^{2+} activation, $MgSO_4$ and $Mg(CH_3COO)_2$ salts were also tested. Fig. 3 shows that both salts gave 75% of the $MgCl_2$ activity at 3 mM. At higher concentrations $MgSO_4$ and $Mg(CH_3COO)_2$ were inhibitory.

F. Inhibitors

NaN_3 was found to be a potent inhibitor of pea mitochondrial ATPase (Fig. 4). A concentration of 3.5×10^{-6}M NaN_3 was required for 50%

Table 3. ADP and IDP inhibition of pea mitochondrial ATPase.

Substrate (3 mM)	Inhibitor	Enzymatic activity (μmole Pi/min/mg protein)	% Inhibition
ATP	-	3.8	0
ATP	ADP (0.25 mM)	2.4	37%
ATP	ADP (0.50 mM)	1.5	61%
ATP	ADP (1.00 mM)	0.8	79%
ATP	-	3.9	0
ATP	ADP (0.50 mM)	2.2	44%
ATP	IDP (0.50 mM)	3.3	15%
ITP	-	11.8	0
ITP	ADP (0.50 mM)	2.6	78%
ITP	IDP (0.50 mM)	7.9	33%

Note: Reaction conditions as in Table 1, except that 3 mM ATP was replaced by 3 mM ITP where noted. ADP or IDP were added as noted.

Figure 2. Divalent [illegible]tion requirements of pea mitochondrial ATPase. Assay [illegible]able 1, except that in this experiment 3 mM Mg[illegible] replaced by the indicated concentrations of $MgCl_2$(0), $CoCl_2$( ), or $CaCl_2$(Δ).

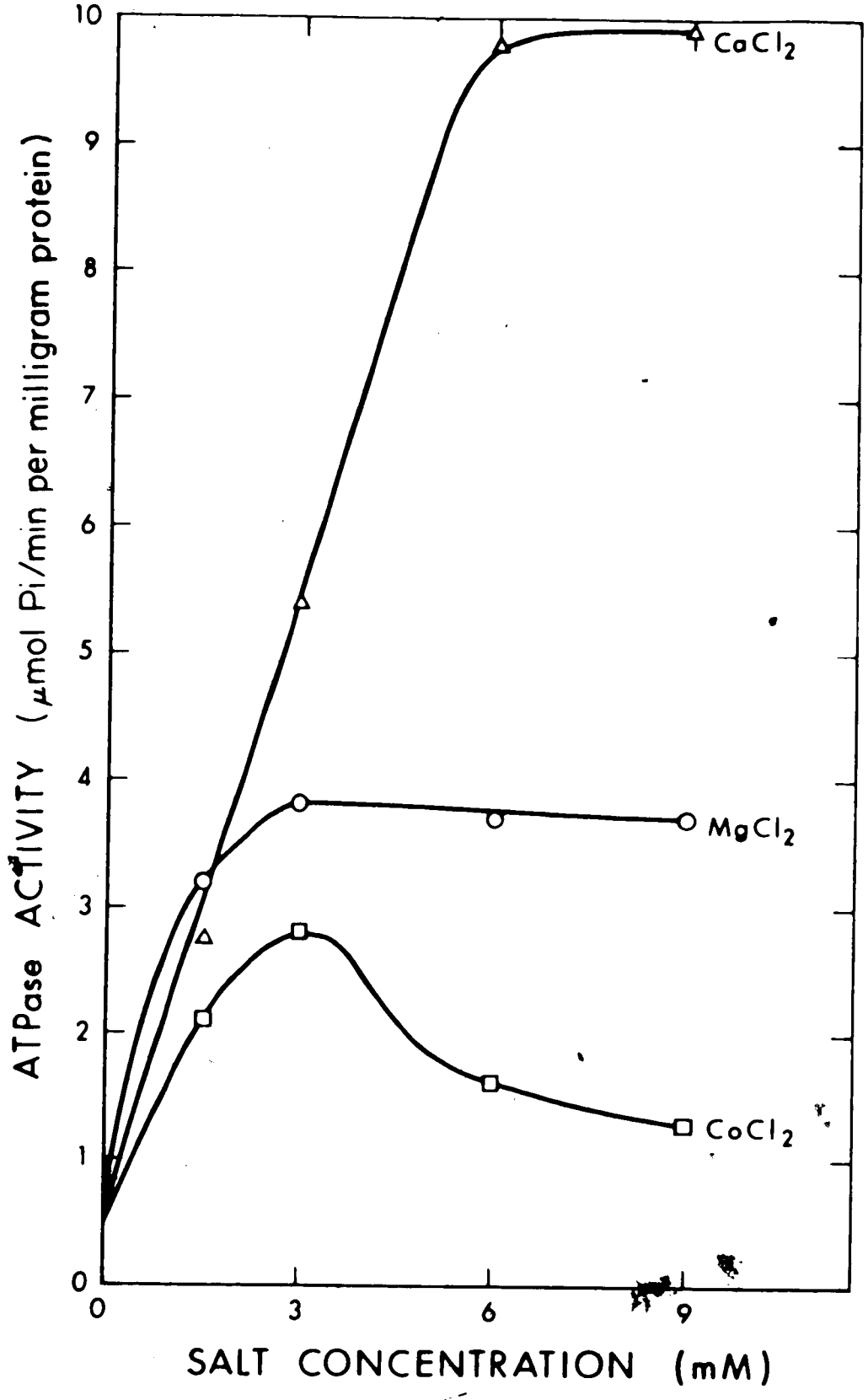
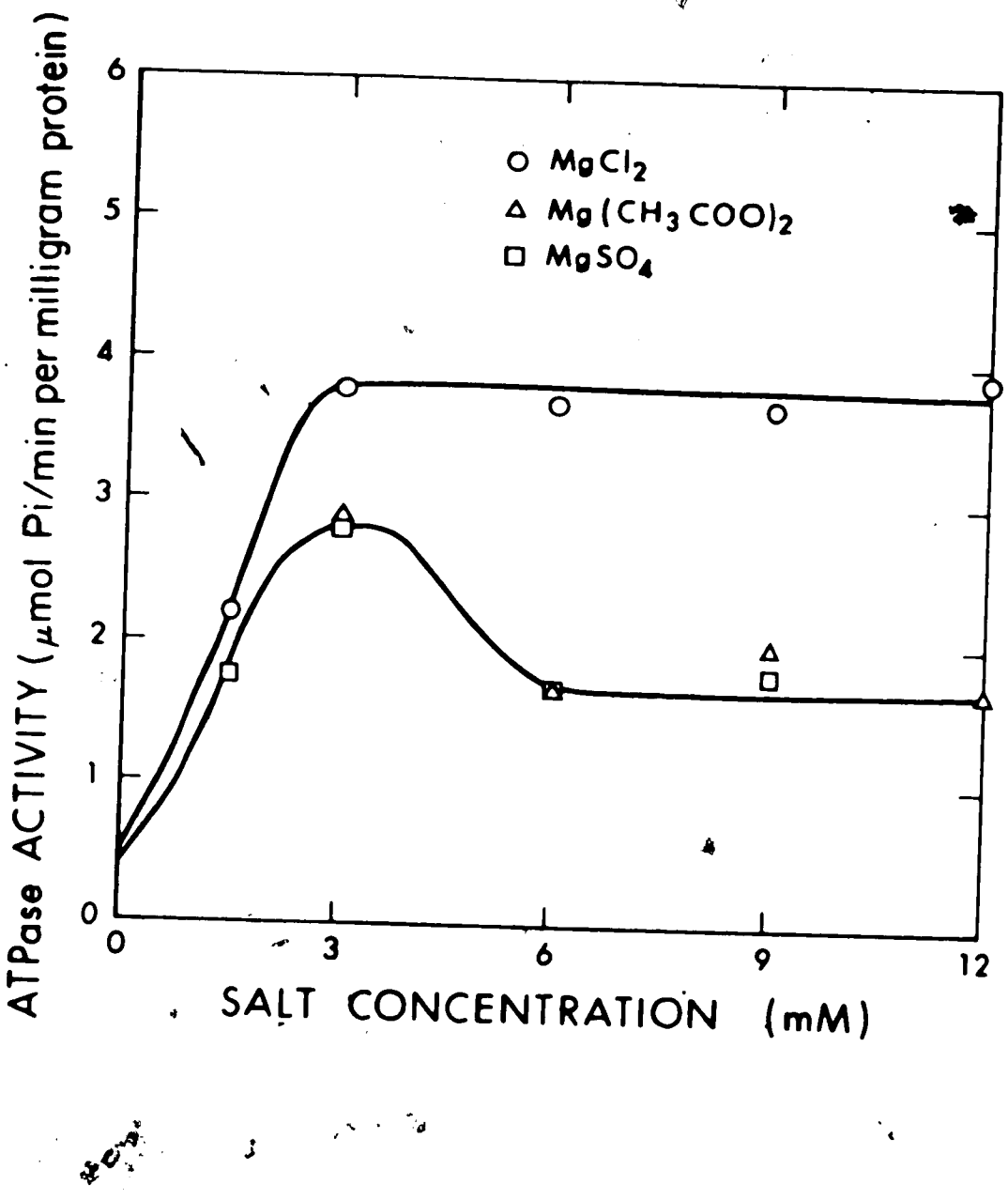

ATPase ACTIVITY (μmol Pi/min per milligram protein)
SALT CONCENTRATION (mM)
CaCl2
MgCl2
CoCl2
0
1
2
3
4
5
6
7
8
9
10
0
3
6
9

Figure 3. Effects of anion on Mg^{2+} activation of pea mitochondrial ATPase. Assay conditions as in Fig. 2, except that 3 mM $MgCl_2$ was replaced by the indicated concentrations of $MgCl_2$(○), $MgSO_4$( ), or Mg $(CH_3COO)_2$(△).


ATPase ACTIVITY (μmol Pi/min per milligram protein)
SALT CONCENTRATION (mM)
MgCl2
Mg(CH3COO)2
MgSO4
0
1
2
3
4
5
6
0
3
6
9
12

inhibition of ATPase activity in the absence of NaCl. When 100 mM NaCl was present ATPase activity was less sensitive to azide, 50% inhibition occurring at 1.6×10^{-5}M NaN_3.

KCN at 0.5 or 5 mM had no effect on the ATPase from pea mitochondria.

Sodium fluoride produced a partial inhibition of activity. At 20 mM NaF the inhibition was approximately 29%.

p-Chloromercuribenzene sulfonic acid, which binds to free sulfhydryl groups, inhibited ATPase activity. A concentration of 5×10^{-8}M PCMBS gave a 31% inhibition.

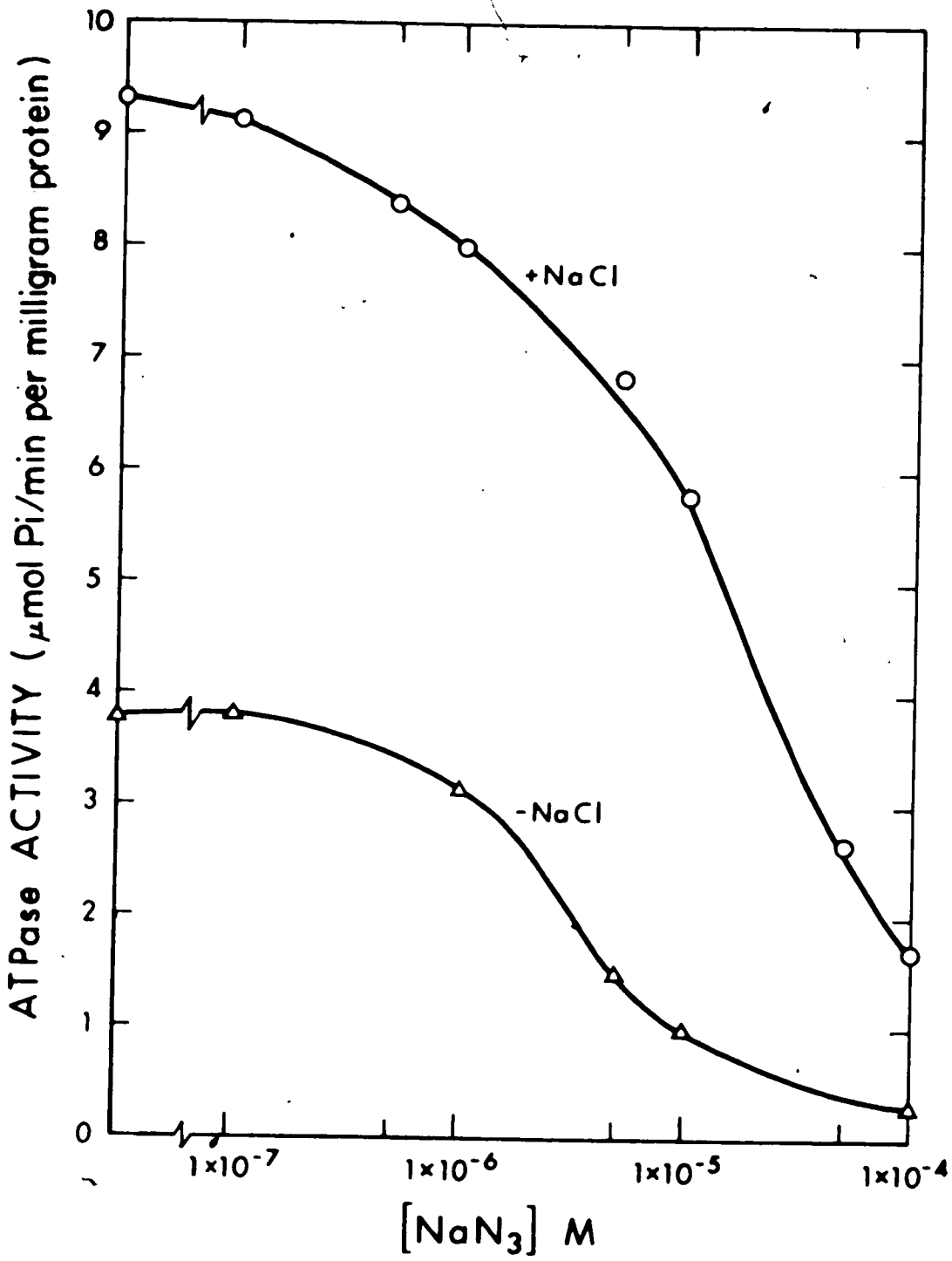
2,4-Dinitrophenol, which stimulates the ATPase activity of mitochondria and some preparations of purified F_1-ATPase (Pullman et al., 1960; Yoshida and Takeuchi, 1970), was also found to stimulate the activity of pea mitochondrial ATPase. A concentration of 5×10^{-4}M DNP stimulated activity by 128%. The activity of the NaCl-stimulated enzyme was not further increased by DNP.

A specific inhibitor of F_1-ATPase in submitochondrial particles, oligomycin (1-10 µg), added in 10-100 µl of ethanol, did not inhibit ATPase activity beyond an inhibition found to be caused by the ethanol alone.

G. Discussion

The assay method used in these experiments gave repeatable results, with linearity toward both time and enzyme concentration. Other F_1-ATPase assay methods require auxiliary enzyme systems to rephosphorylate ADP in order to obtain a linear time course (Pullman et al., 1960). The sensitivity of the phosphate test employed here means that only a small fraction of the available substrate need be used (0.05 - 0.2

Figure 4. Azide inhibition of pea mitochondrial ATPase. Assay conditions as in Table 1, with the addition of the indicated concentrations of NaN_3. NaCl (100 mM) was present where noted. Scale for azide concentration is logarithmic.


ATPase ACTIVITY (μmol Pi/min per milligram protein)
10
9
8
7
6
5
4
3
2
1
0
+NaCl
-NaCl
1×10⁻⁷
1×10⁻⁶
1×10⁻⁵
1×10⁻⁴
[NaN3] M

μmole of the 6 μmole ATP available) and a correspondingly small concentration of the inhibitory product ADP is formed, lessening the need for auxiliary enzyme systems.

The specific activity values reported for the pea enzyme are about twice as high as those previously reported for the same enzyme preparation (Malhotra and Spencer, 1974a). This likely arises from differences in the assay technique. In the present work enzyme, instead of ATP, was added to start the reaction, and the dissolved enzyme was not exposed to cold temperatures.

The specific activity of the pea enzyme was lower than current literature values for mammalian F_1-ATPases, which range from 60–100 μmoles phosphate/min/mg protein (Pedersen, 1975; Penefsky, 1974). This discrepancy could indicate that the pea enzyme was isolated in a latent state. However, when treatments that release the latency of chloroplast or mammalian F_1-ATPases were tried on the pea enzyme, including trypsin treatments, and preincubation with 50 mM dithiothreitol (Nelson et al., 1972) they were ineffective. A heat-activation step, already included in the method, caused only slight activation (Malhotra and Spencer, 1974a). Another possible reason for the low activity of the pea enzyme is that the assay medium does not include chloride or bicarbonate anions, used in some other assay methods. These anions were found to stimulate the pea enzyme 3-fold, as discussed later.

Cold lability is an unusual enzyme property that has always been found with purified mammalian F_1-ATPases since its original observation by Pullman et al. (1960; Penefsky, 1974). Yoshida and Takeuchi (1970) have shown that a preparation of castor bean mitochondrial ATPase was also inactivated by cold treatments, as is the chloroplast F_1-ATPase

involved in photophosphorylation (Nelson et al., 1972). The property has been studied with beef heart mitochondrial F_1-ATPase by Penefsky and Warner (1965), who found that a variety of alcohols afforded cold protection. Their report that the enzyme at 0° loses 50% of its activity in 6 min when kept at a protein concentration of 0.1 mg/ml is in keeping with the results reported here.

Pullman et al. (1960) noted that ATP, which stabilized their beef heart mitochondrial ATPase against a slow inactivation at room temperature, was not effective as a cold protective agent. A similar lack of ATP effect was noted here. This is in contrast to results with the yeast mitochondrial ATPase, which is cold-protected by 2 mM ATP or ADP (Ryrie, 1975).

In the experiments reported by Penefsky and Warner (1965) as well as the work of other groups (Pullman et al., 1960; Ebel and Lardy, 1975) no stimulation of activity was found to occur when NaCl or KCl was included in the assay medium. The salt stimulation found (Table 1) with the pea mitochondrial enzyme will be described in detail in Chapter IV.

The results reported in Table 2 indicated that the pea enzyme has a broader nucleotide specificity than was earlier suspected (Malhotra and Spencer, 1974a). Beef heart F_1-ATPase results reported by Pullman et al. (1960), indicated that ATPase, GTPase, and ITPase activities were approximately equal. UTP gave 60% of the ATPase rate, while CTP was not hydrolysed by their preparation. However, the specifity of soluble rat liver F_1-ATPase appears very different, since Vmax values for the GTPase of that enzyme are 2 and 4-fold greater than the Vmax values for ATPase activity (Pedersen, 1976). The only report on the substrate specificity

of a soluble plant mitochondrial ATPase is that of Yoshida and Takeuchi (1970) who showed ITPase activities 100% higher than ATPase values, in complete agreement with the results reported here.

Further support for the similarity between the pea enzyme and the F_1-ATPase of rat liver is that only ATPase activity, and not GTPase or ITPase activities, was affected by the addition of NaCl. This closely resembles the kinetic effects of bicarbonate anion on the rat liver enzyme (Pedersen, 1976).

The high rates of GTPase activity reported here may seem to merit renaming the enzyme nucleoside triphosphatase rather than ATPase. It should be pointed out, however, that in intact mitochondria the adenine-specific nucleotide transporter enzyme imposes an ATP specificity on the ATPase reaction. For example Phillips (1971), using intact pea mitochondria isolated and assayed by the methods used here, found that GTPase activity was considerably less than ATPase activity. However, in experiments reported in Chapter V, I found that submitochondrial particles freshly prepared from pea mitochondria showed substrate specificity similar to that of the isolated enzyme, with GTPase>ATPase. These particles, in which ATPase activity could be completely inhibited by oligomycin, demonstrate that GTPase activity is likely not caused by denaturation during the enzyme purification procedure.

In addition, it should be noted that the anionic composition of the assay medium has a dramatic effect on the relative rates of GTPase and ATPase activity. In the presence of 20 mM $NaHCO_3$, when ATPase was stimulated to 12.0 μmole P*i*/min/mg protein, and GTPase activity was unaffected at 18.9 μmole P*i*/min/mg protein, the ratio of activities was reduced from 5:1 to 3:2. A similar effect occurs in 100 mM NaCl

(Table 2), where the GTPase:ITPase:ATPase ratio is reduced from 5:3:1 to 2:1:1.

ADP was found to be a strong inhibitor of the pea cotyledon mitochondrial ATPase. Early work with beef heart mitochondria F_1-ATPase (Pullman et al., 1960) indicated that an ADP:ATP ratio of 1:2 gave 50% inhibition of ATPase activity. Other workers have reported a wide range of values for the inhibitory power of ADP. Pedersen (1975) has reviewed the discrepancies in the literature and finds that variations in anionic composition of the assay medium or purity of the nucleotide solutions used may account for the disagreement.

The finding that IDP is inhibitory to the pea mitochondrial ATPase is in contrast to the lack of inhibition observed with beef heart mitochondrial F_1-ATPase (Pullman et al., 1960). The same workers found that ADP was a more powerful inhibitor of ITPase activity than ATPase activity. This finding agrees with the results presented here.

The divalent cation requirements of the pea enzyme were somewhat surprising in that free Mg^{2+} did not seem to inhibit the enzyme (Fig. 2). In contrast, excess Mg^{2+} has been found to inhibit many ATPases, such as the Na^+ - K^+ ATPase from crab nerve (Skou, 1960), a cell wall ATPase of barley (Hall and Butt, 1969) and the F_1-ATPase from chloroplasts (Nelson et al., 1972). However, Pullman et al. (1960) found that a 3-fold excess of Mg^{2+} caused only very minor inhibition of their preparation of beef heart mitochondrial F_1-ATPase.

The high $CaCl_2$-activated rate of ATPase activity is unusual for mitochondrial ATPases. Selwyn (1967), detailing the specific cation requirements of purified F_1-ATPase from beef heart mitochondria, found highest activation with $MgSO_4$ as the activator; $CoCl_2$ and $CaCl_2$ gave

96% and 7% of the $MgSO_4$ rate respectively. Tzagoloff et al. (1968) found with their preparations of F_1-complexes from beef heart mitochondria that the $CaCl_2$-activated rate varied between 3% and 83% of the $MgCl_2$-activated rate, depending on the stage of purification. With the spinach chloroplast F_1-ATPase the $MgCl_2$-activated rate is only 3% of the $CaCl_2$-activated rate under normal assay conditions (Nelson et al., 1972).

The cation activation curves with $MgSO_4$ and $Mg(CH_3COO)_2$ were puzzling, in that with these salts a clear optimum was seen at 3 mM Mg^{2+}, in contrast to the curve with $MgCl_2$. A possible explanation for this finding is that high Mg^{2+} concentrations do inhibit the enzyme, but a stimulatory effect of chloride is capable of masking this behavior. Alternatively, sulfate and acetate could be inhibitors. This hypothesis is tested in the next chapter.

The pea cotyledon enzyme was shown to be typical of F_1-ATPases in its inhibitor sensitivities.

NaN_3 was a very potent inhibitor, causing a 50% inhibition at 3.5×10^{-6}M. Pullman et al. (1960) showed that NaN_3 is a potent inhibitor of beef heart mitochondrial F_1-ATPase. A concentration of 4×10^{-5}M resulted in 80% inhibition. Other workers, using rat liver F_1-ATPase, have found that NaN_3 is a less effective inhibitor when the ATPase activity is stimulated by bicarbonate anion (Ebel and Lardy, 1975). With the pea enzyme NaN_3 lost effectiveness when NaCl was present.

The pea ATPase was insensitive to KCN, which is normally inhibitory to the same metallo-enzymes inhibited by NaN_3. A similar lack of cyanide inhibition has been shown with purified rat liver F_1-ATPase, although the ATPase activity of intact mitochondria or submitochondrial particles was inhibited by cyanide (Weiner and Lardy, 1974).

NaF was a relatively weak inhibitor of pea mitochondrial ATPase. This result compares with the 20% inhibition by 20 mM NaF in experiments with F_1-ATPase reported by Pullman et al. (1960).

The PCMBS inhibition was found to be quite potent. This result differs from those reported from many F_1-ATPases, which were not inhibited by mercurials (Pedersen, 1975; Senior, 1973). However, Pullman et al. (1960) found that 5×10^{-4}M PCMB completely inhibited the DNP stimulation of purified beef heart mitochondrial F_1-ATPase. Iodoacetamide at 5×10^{-4}M failed to inhibit the activity of the pea mitochondrial ATPase.

The lack of an oligomycin inhibition is in keeping with the finding that the protein required for oligomycin sensitivity of F_1-ATPase is destroyed by the heat treatments used to purify the enzyme (see Pedersen,(1975).

The many similarities between pea mitochondrial ATPase and solubilized mammalian F_1-ATPase, including cold lability, substrate specifity for purine nucleotides, stimulation by $NaHCO_3$, inhibition patterns by ADP, IDP, NaF, KCN, NaN_3, and divalent cation requirements, all point to these two enzymes being closely related. The novel features of the pea enzyme, especially high relative GTPase activity, may be partially caused by the assay buffer employed,which did not contain chloride, bicarbonate or other stimulatory anions.

CHAPTER IV

ANIONIC STIMULATIONS OF THE SOLUBLE ATPase

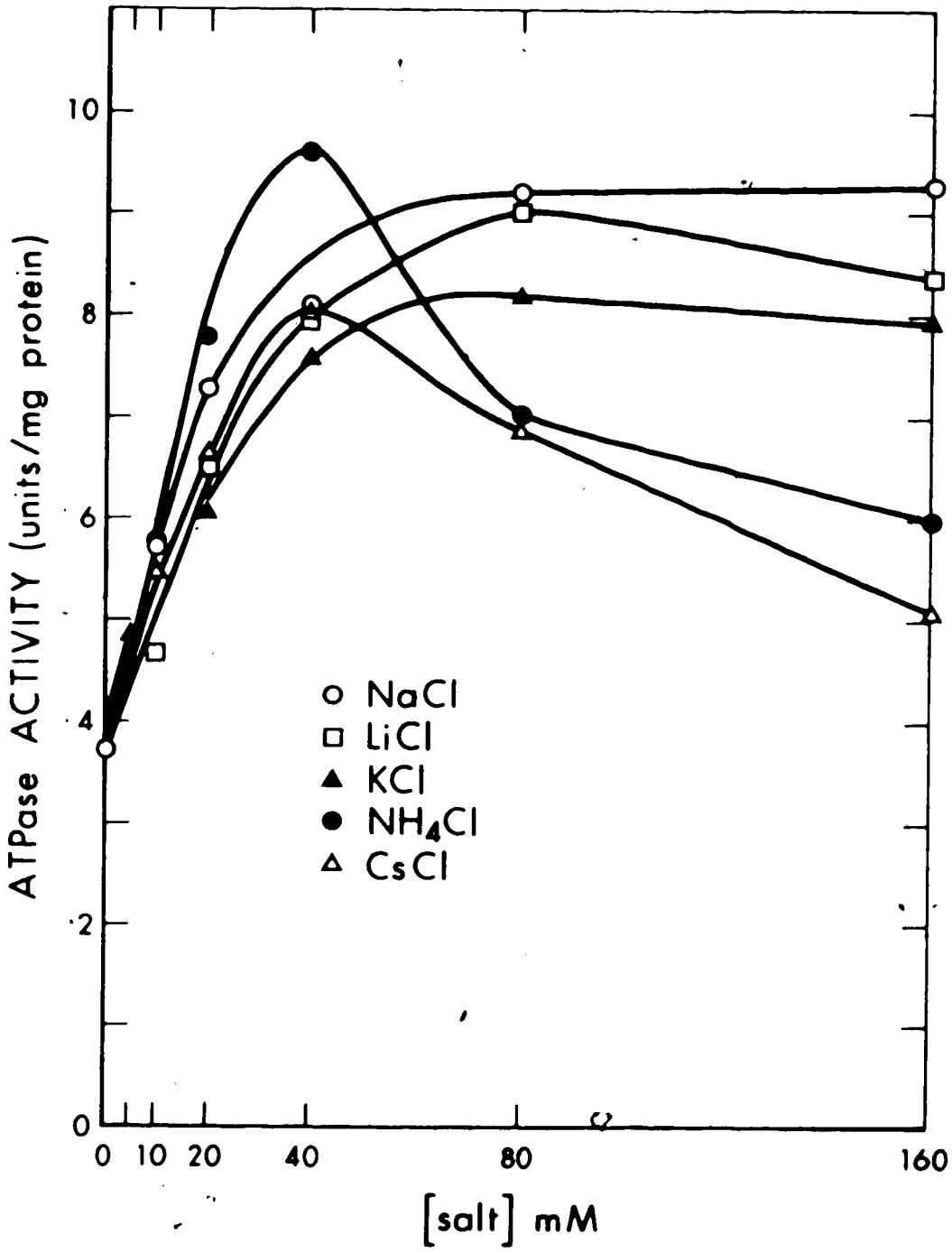
A. Nature of the Salt Stimulations

Previous work in this laboratory showed that isolated pea mitochondrial ATPase was stimulated by NaCl and KCl (Malhotra and Spencer, 1974a, b). To further characterize this stimulation I initially did experiments to determine the roles of the cation and anion. When I tested Cl^- salts of a variety of cations, all gave patterns of stimulation similar to that produced by NaCl (Fig. 5). The curves showed similar effects of salt concentrations of 5 to 40 mM. Above 40 mM, rates of ATPase activity with NH_4Cl and CsCl dropped, while activities in the presence of NaCl, LiCl, and KCl remained high. High concentrations of CsCl (80-160 mM) resulted in the formation of a precipitate during the assay. The cause of the NH_4Cl inhibition was unknown. In the cases of all cations tested the lowest concentration at which I found maximal activity was 40-80 mM. The magnitude of the maximal activity varied between 221% and 259% of the basal rate[1], depending upon the salt added. The similarity of results obtained with the variety of salts used appeared to rule out a selective cation stimulation.

When a variety of anions were added as their Na^+ salts (Fig. 6), I found that the stimulations of ATPase activity varied widely, even

[1] Basal rate refers to the activity measured in the absence of added (non-buffer) salts. Stimulated rate refers to the full activity measured in the presence of the added salt.

Figure 5. Effects of cation-Cl salts on activity of solubilized ATPase from pea mitochondria. The assay was done as in Table 1, except that salts were present in the indicated concentrations.


ATPase ACTIVITY (units/mg protein)
10
8
6
4
2
0
0 10 20 40 80 160
[salt] mM
NaCl
LiCl
KCl
NH4Cl
CsCl

at concentrations as low as 5-10 mM, suggesting a specific anionic effect. The most stimulatory anion was I^-, followed by Cl^- and Br^-; a less active anion was SO_4^{2-}, while NO_3^- was inhibitory. When I tested Na^+ salts of three organic acids, the effect again varied with the anion (Fig. 7). Acetate and formate were both stimulatory, although formate only mildly so, while citrate was inhibitory.

Since some of the salts tested in Figs. 5-7 were inhibitory at higher concentrations, I tested the effects of NaCl concentrations above 160 mM, to determine if NaCl could inhibit also. Fig. 8 shows that NaCl concentrations of 0.2 M and higher were inhibitory.

B. Properties of NaCl-Stimulated Activity

Having established the anionic nature of the stimulations I carried out experiments to determine whether the properties of the NaCl-stimulated activity were similar to those of the basal activity.

When I examined the divalent cation specifity of the NaCl stimulations (Table 4), I found that this stimulation was most pronounced with Mg^{2+} as the divalent cation; in the presence of Co^{2+}, less NaCl stimulation was observed (Part A). In part B, with Ca^{2+} as divalent cation, the basal rate was high and NaCl inhibited activity. Mg^{2+} added with SO_4^{2-} as counterion supported a high rate of activity in the presence of NaCl (Part C).

Fig. 9 shows that in the presence of 0.1 M NaCl the Mg^{2+} optimum of the enzyme was sharpened slightly. Maximal activity occurred at 1.5-3.0 mM Mg^{2+}.

I tested the effects of several inhibitors on both the basal and NaCl-stimulated rates of activity. Table 5 shows the PCMBS inhibition

Figure 6. Effects of Na-anion salts on activity of pea mitochondrial ATPase. Assay conditions as in Table 1. Salts were present in the indicated concentrations.

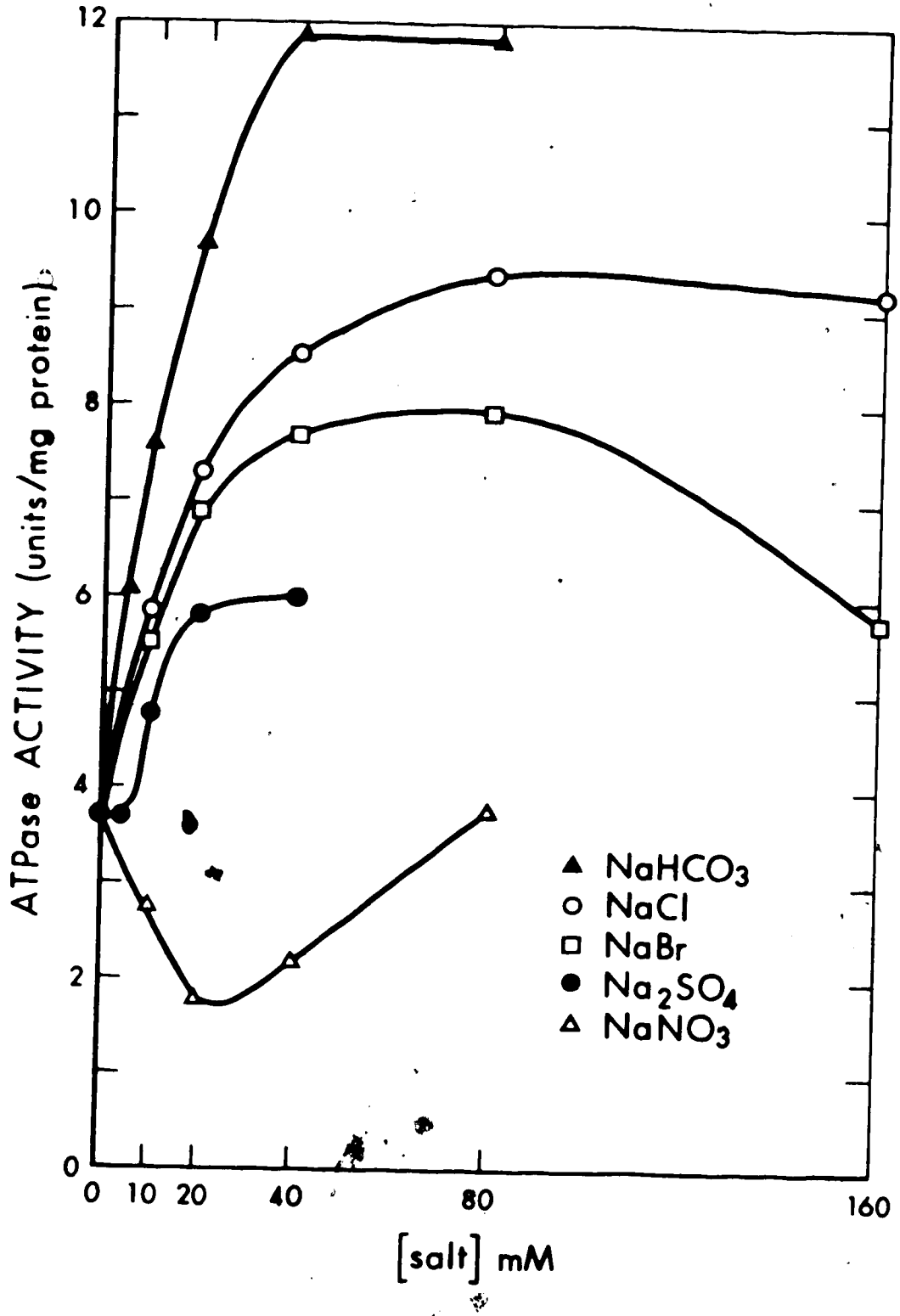

12
10
8
6
4
2
0
ATPase ACTIVITY (units/mg protein)
0 10 20 40 80 160
[salt] mM
▲ NaHCO3
○ NaCl
□ NaBr
● Na2SO4
△ NaNO3

Figure 7. Effects of Na^+ salts of organic acids on activity of pea mitochondrial ATPase. Assay conditions as in Table 1. Salts were present as noted.

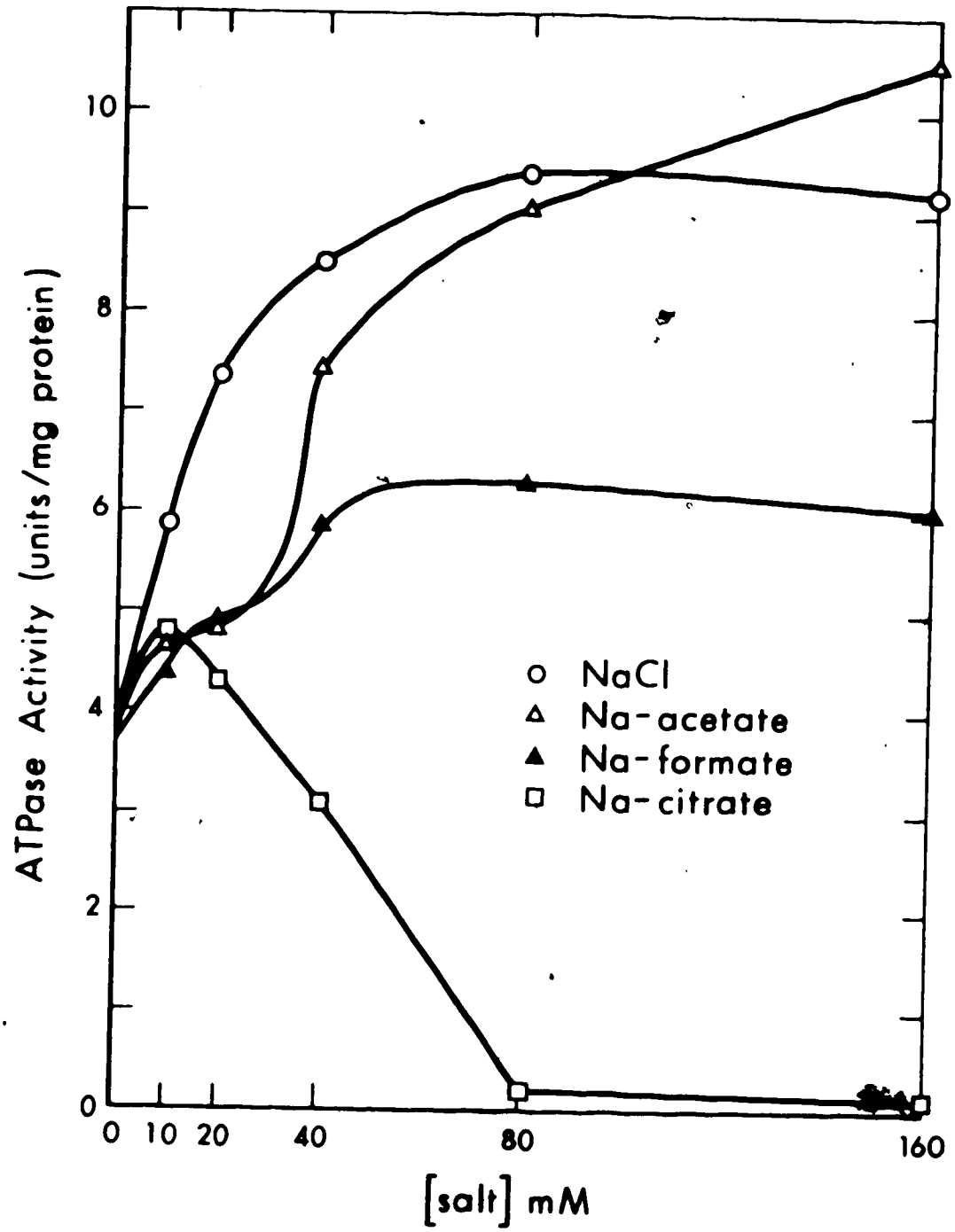

ATPase Activity (units/mg protein)
10
8
6
4
2
0
0 10 20 40 80 160
[salt] mM
NaCl
Na-acetate
Na-formate
Na-citrate

Figure 8. Effect of high NaCl concentrations on activity of pea mitochondrial ATPase. Assay conditions as in Table 1. NaCl was present as noted.

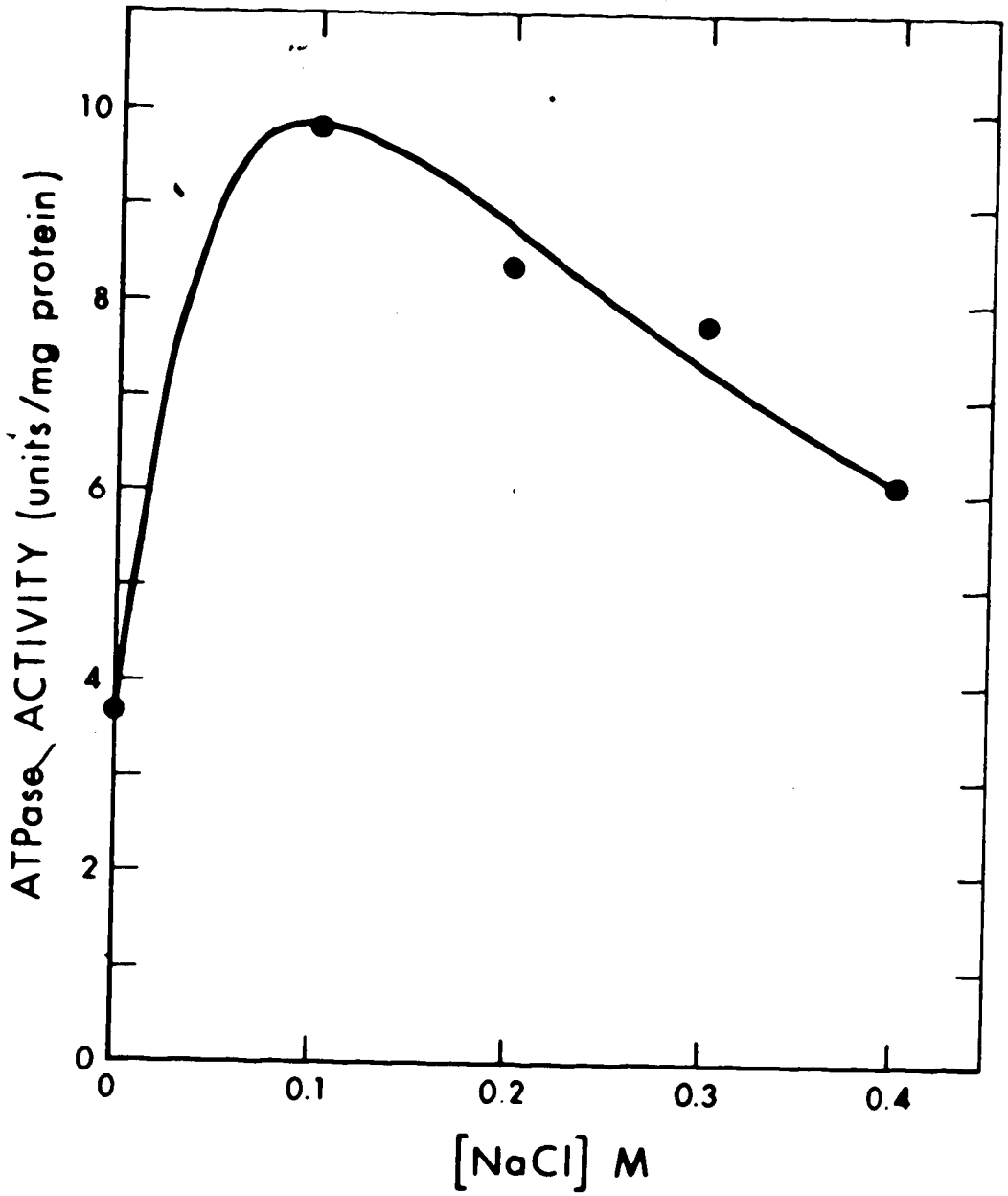
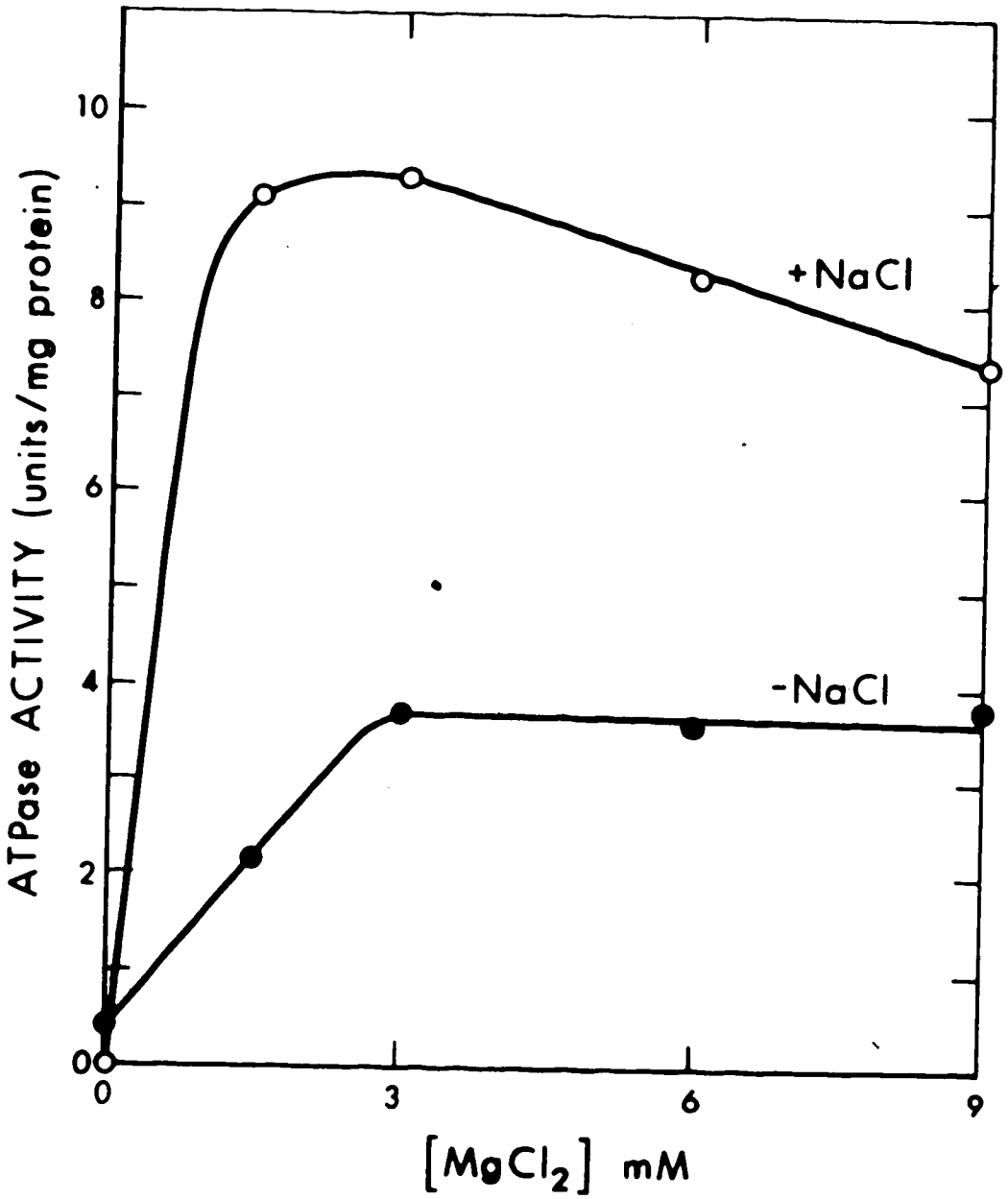

ATPase ACTIVITY (units/mg protein)
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
[NaCl] M

Table 4. Effect of 100 mM NaCl on ATPase activity.

	Cation	ATPase activity: No added NaCl	+ 100 mM NaCl	% Stimulation by NaCl
A.	$MgCl_2$ 3 mM	3.8	9.3	145
	$CoCl_2$ 3 mM	1.7	3.8	123
B.	$MgCl_2$ 3 mM	3.8	9.4	147
	$CaCl_2$ 3 mM	7.7	4.3	-44
	$MgCl_2$ 6 mM	3.6	7.4	105
	$CaCl_2$ 6 mM	11.8	7.7	-35
C.	$MgSO_4$ 3 mM	2.8	10.6	278

Note: Reaction conditions as in Fig. 1, except that 3 mM $MgCl_2$ was replaced by 3 mM or 6 mM concentrations of the indicated salts. Where noted 100 mM NaCl was present in the assay medium. Activity is in units/mg protein.

Figure 9. Effect of NaCl on the Mg^{2+} requirements of pea mitochondrial ATPase. Assay conditions as in Table 1, except that $MgCl_2$ concentration was varied and NaCl (100 mM) was present as noted.


ATPase ACTIVITY (units/mg protein)
10
8
6
4
2
0
+NaCl
-NaCl
0
3
6
9
[MgCl2] mM

of the basal rate of activity noted in the last chapter; the PCMBS effect was enhanced by NaCl at all tested concentrations of PCMBS. Iodoacetamide, which had little effect on the basal rate, was slightly inhibitory in the presence of NaCl. The inhibition by 0.5 mM ADP was not substantially changed by the addition of 100 mM NaCl. I also found that 5% ethanol in the assay medium reduced basal activity and severely inhibited the NaCl-stimulated rate.

To test whether the stimulation by NaCl might actually be the result of release of the enzyme from an inhibited state, such as that caused by the specific ATPase inhibitor polypeptide (Horstman and Racker, 1970; Pullman and Monroy, 1963; Van de Stadt and Van Dam, 1974), I preincubated the enzyme with 0.1 M NaCl. Table 6 shows that this preincubation treatment had no effect. A variety of trypsin treatments, known to destroy the specific polypeptide inhibitor of mitochondrial ATPase (Horstman and Racker, 1970; Jung and Laties, 1976; Takeuchi, 1975), failed to stimulate the activity of the pea mitochondrial ATPase (Table 6).

One of the characteristics of the direct anion stimulations reported for rat liver F_1-ATPase and SMP by Ebel and Lardy (1975), and Pedersen (1976) is that anions stimulate ATPase activity more than GTPase or ITPase activities. These workers also found that N_3^- preferentially inhibited ATPase over other substrates, and that the N_3^- inhibition was reduced by the presence of stimulatory anions. I thus investigated substrate specificity and azide inhibition of the Cl^- stimulated activity. Table 7 shows that the NaCl stimulation of enzyme activity was specific for ATP among the nucleotides tested. GTPase and ITPase activities, which were high in the absence of NaCl, were not increased

Table 5. Effects of inhibitors on NaCl-stimulated activity of ATPase from pea cotyledon mitochondria.

Treatment	No added NaCl		100 mM NaCl	
	Activity	% Inhibition	Activity	% Inhibition
Control	3.7	0%	9.3	0%
5×10^{-5}M PCMBS	0.9	77%	0.6	94%
5×10^{-6}M PCMBS	0.8	78%	1.3	87%
5×10^{-7}M PCMBS	1.8	52%	2.1	77%
5×10^{-8}M PCMBS	3.0	20%	5.3	43%
5×10^{-4}M Iodoacetamide	4.0	-5%	7.6	18%
5×10^{-6}M Iodoacetamide	4.0	-5%	7.5	19%
5×10^{-4}M ADP	1.5	60%	4.5	52%
Control	3.8	0%	9.3	0%
100 µl Ethanol	2.7	29%	3.4	64%

Note: Assay was performed as in Fig. 1, with inhibitors added as noted. Activity is in units/mg protein.

Table 6. Effects of treatments designed to release the polypeptide inhibitor from the ATPase from pea cotyledon mitochondria.

Treatment	ATPase Activity Units/mg Protein	% Control
Control (preincubated without NaCl)	3.8	100%
Preincubated with 100 mM NaCl	3.7	97%
Control	3.8	100%
0.2 µg trypsin	4.2	111%
2.0 µg trypsin	4.1	108%
25 µg trypsin	3.0	80%

Note: NaCl preincubation was for 25 min in a medium of 0.25 M sucrose, 2 mM EDTA, and 10 mM Tris, at pH 8.0, with or without 100 mM NaCl. For trypsin treatments, the indicated amounts of trypsin were incorporated into assay medium without ATP. 2 µg enzyme was added and allowed to react 6 min at 30°C. The reaction was stopped by the addition of a 10-fold excess (by weight) of lima bean trypsin inhibitor, and after 5 min Tris-ATP was added to start the reaction. In blank tubes trypsin and trypsin inhibitor were both added before ATPase.

by addition of the salt. In addition, Table 7 shows that the Cl^- anion was antagonistic to the inhibition of ATPase activity caused by N_3^-. GTPase activity, which was not Cl^- stimulated, was also not severely inhibited by N_3^-. Inclusion of Cl^- appeared to reduce the N_3^- inhibition in this case also.

C. Discussion

Stimulations of enzyme activity by univalent cations have been reported for many enzymes and are well documented for mammalian Na^+-K^+ ATPases (Skou, 1965). In addition, several plant ATPases have been reported to be cation-stimulated (Dodds and Ellis, 1969; Hall and Butt, 1969; Karlsson and Kylin, 1974; Lin et al., 1977). Based on evidence similar to that presented in Fig. 5 of this chapter, Adolfsen and Moudrianakis (1973) concluded that a non-specific cation activation occurred in their preparations of beef heart and bacterial coupling factors. A univalent cation stimulation of the 2,4-dinitrophenol-induced ATPase activity of intact rat liver mitochondria has also been noted (Amons et al., 1968). Early work on the isolated pea cotyledon mitochondrial ATPase (Malhotra and Spencer, 1974a) indicated that the enzyme was stimulated by Na^+ and K^+ ions (added as their Cl^- salts). Somewhat similar results were found with the ATPase activity of intact mitochondria from pea cotyledons (Phillips, 1971). The results of Fig. 6 and 7 of this paper, however, demonstrate that if any cation stimulation of pea mitochondrial ATPase occurs, it is relatively non-selective. The variation among the Na^+ salts that stimulated activity (-100% - +313%) is much more pronounced than that between the tested Cl^- salts (221-259%). This suggests that the stimulations are primarily

Table 7. Substrate specificity of anion effects on activity of ATPase from pea cotyledon mitochondria.

Substrate	NaN_3	Nucleoside triphosphatase activity Without NaCl Activity	% Inhibition	100 mM NaCl added Activity	% Inhibition
ATP	-	3.7	-	9.3	-
ATP	5×10^{-6} M	1.6	57%	6.7	28%
GTP	-	18.1	-	16.2	-
GTP	5×10^{-6} M	13.9	23%	14.8	9%
ITP	-	9.9	-	8.5	-

Note: Assay was as in Fig. 1, except that 3 mM ATP, GTP, or ITP was present as noted. Activity is given in units/mg protein.

anionic.

The Mg^{2+} optimum of the NaCl-stimulated activity was sharper than was the Mg^{2+} optimum of the basal activity. The probable cause of this change is that the Cl^- normally present in the basal assay mixture as the counterion of Mg^{2+} stimulated the enzyme at high Mg^{2+} concentrations, masking the slight inhibition by free Mg^{2+}. This conclusion is supported by the finding in Chapter III that $MgSO_4$ used as the activator shows a curve with a clear optimum at 3 mM Mg^{2+}.

The anions that are stimulatory do not show any trends of size, valency or other properties that might suggest a mode of action. However, the anions tested may not all work by the same mechanism; citrate for example, has been found to inhibit bacterial and beef heart coupling factors, probably by chelating an enzyme-bound Mg^{2+} ion (Adolfsen and Moudrianakis, 1973). At high concentrations many of the salts showed an inhibitory effect that may not share the same mechanism as the stimulations. Other workers have found that high salt concentrations cause subunit dissociation of soluble F_1-ATPase (Penefsky and Warner, 1965), and this may explain the inhibition by high NaCl concentration shown in Fig. 8.

Three types of mechanisms could be considered for the anion stimulations: In the first of these a contaminating or denatured ATPase could be present, which could be Cl^--activated. The enzyme used in these experiments was about 60% pure. However, I showed in the preceding chapter by specific staining of electrophoresis gels that only one band of nucleoside triphosphatase activity was present in the preparation when ATP or GTP were used as substrates in the presence of 100 mM Cl^-. I was also able to show (Appendix I) that the oligomycin-

sensitive ATPase activity of corn submitochondrial particles was also stimulated by NaCl, indicating that denaturation during the purification of the soluble enzyme was not a likely cause for the NaCl stimulations.

Two other possible explanations of anion stimulation that were considered in detail were dissociation of an inhibitor polypeptide, or alternatively, a direct effect of anions on the enzyme. In the experiment shown in Table 6 I attempted to find out whether the anion stimulations resulted from dissociation of the naturally occurring inhibitor polypeptide (Pullman and Monroy, 1963). The polypeptide can be caused to dissociate from the beef heart F_1-ATPase by ions (Horstman and Racker, [illegible] Van de Stadt and Van Dam, 1974) and some anion specificity for this effect is shown, with effectiveness ranked acetate>Cl^->NO_3 (Van de Stadt and Van Dam, 1974). However, pre-assay incubation of the pea enzyme with NaCl, which might have been expected to release the inhibition, did not affect activity (Table 6). The inhibitor from beef heart is extremely sensitive to trypsin (Horstman and Racker, 1970; Pullman and Monroy, 1963). Trypsin treatments have been found to stimulate the ATPase activity of castor bean submitochondrial particles about 10-fold, indicating that plant mitochondria do possess a trypsin-sensitive ATPase inhibitor (Takeuchi, 1975). I have also found that corn SMP are trypsin stimulated (Appendix I). However, none of the trypsin treatments used here with the soluble enzyme (Table 6) was effective in stimulating activity or in changing the effect of added NaCl. I conclude that the anion stimulations of the soluble enzyme reported here are not caused by dissociation of an inhibitor polypeptide.

A direct effect of anions on the enzyme, similar to that found with mammalian and yeast ATPases (Ebel and Lardy, 1975; Recktenwald and

Hess, 1977) seems a more likely hypothesis, mainly because of the ATP specificity and NaN_3-anion interaction (Table 7).

The NaCl stimulation of activity was found to be specific for Mg-ATP as a substrate (Tables 4 and 7). Among the nucleotides tested, the high GTP and ITP hydrolysing activities were not further increased by NaCl, while among the divalent cations only Mg^{2+} and Co^{2+} supported a NaCl-stimulated rate. In the l[illegible] chapter I showed that the soluble pea ATPase had a high Ca-ATPase activity, which is rare among mitochondrial ATPases. The decrease in Ca-ATPase caused by NaCl (Table 4) is thus intriguing. It is possible that free Ca^{2+} has a direct stimulatory effect on the enzyme, and that this effect is inhibited by NaCl. This possibility is supported by the finding that maximal activity with Ca^{2+} was found at concentrations (6-9 mM) beyond that required to complex the 3 mM ATP present. Sone et al. (1969) have reported that the soluble mitochondrial ATPase from Endomyces yeast shows substantial Ca-ATPase activity in normal (Tris-acetate) assay medium. Mg-ATPase activity of the Endomyces enzyme was stimulated 4- to 5-fold by 2,4-dinitrophenolate or maleate anions, but Ca-ATPase activity was only slightly stimulated.

The ATP specifity of the anion stimulations reported here paralleled the ATP specificity for the effects of HCO_3^-, and other oxyanions on the V_{max} and Km of soluble rat liver F_1-ATPase (Ebel and Lardy, 1975; Pedersen, 1976). For example, the rat liver F_1-ATPase preparation of Pedersen (1976) showed a substrate specificity similar to that of the pea enzyme (GTPase>ITPase>ATPase) under normal assay conditions; the addition of 20 mM HCO_3^- stimulated the V_{max} of ATPase activity 5-fold, but had no effect on GTPase or ITPase activities. The rat liver F_1-ATPase preparation of Ebel and Lardy (1975) showed substrate specifi-

city of ITPase>GTPase=ATPase; in the presence of HCO_3^-, ATPase was stimulated 3-fold, while GTPase and ITPase were stimulated only 1.6-fold. Similarly, the soluble castor bean mitochondrial ATPase showed ITPase> ATPase activity. Addition of 2,4-dinitrophenol stimulated ATPase by 88%, but only stimulated ITPase slightly (Yoshida and Takeuchi, 1970).

The finding that the N_3^- inhibition [illegible] by NaCl is directly comparable to the antagonism between N[illegible]O found with soluble mammalian F_1-ATPases (Ebel and Lardy, [illegible]beth and Lardy, 1971). Ebel and Lardy (197[illegible] that addition of 10 mM HCO_3^- causes a 5-fold decrease in the in[illegible]of ATPase activity by NaN_3. ITPase activity, which was relatively [illegible]nsitive to HCO_3^- stimulation, was also less affected by NaN_3. Addition of HCO_3^- decreased the N_3^- inhibition of ITPase activity. These results (Ebel and Lardy, 1975) were analogous to those reported by other groups (Moyle and Mitchell, 1975; Sone et al., 1969), as well as to those reported for the pea enzyme in Table 7

The PCMBS inhibition of the basal rate is rare for soluble F_1-ATPases, although the Endomyces mitochondrial ATPases (Sone et al., 1969) was also inhibited by mercurials, and was not inhibited by iodoacetate. The increased sensitivity to -SH reagents in the presence of NaCl may indicate that NaCl helps to expose a sulfhydryl group. Other workers have also reported a PCMB sensitivity of anion-stimulated ATPase activity (Pullman et al., 1960).

In contrast to NaN_3, whose effectiveness as an inhibitor was decreased by NaCl, ethanol was a very effective inhibitor of the NaCl-stimulated activity and a poor inhibitor of the basal rate. Ethanol has been found to stimulate beef heart F_1-ATPase (Penefsky and Warner, 1965).

ADP was an equally effective inhibitor of basal and anion-stimulated activities.

The stimulations of soluble pea mitochondrial ATPase reported here are somewhat similar to the oxyanion stimulations reported with other F_1-ATPases. Similarities occur in substrate specificity, anion-N_3^- antagonism, and in the case of HCO_3^-, in the concentration of anion required. An important difference, however, is in the anion specificity: Cl^- anion has been tested and found to have no stimulatory effects on beef heart and rat liver F_1-ATPase preparations (Lambeth and Lardy, 1971; Penefsky, 1965). On the basis of the oxyanion specificity observed with mammalian F_1-ATPases, a specific oxyanion binding site on the enzyme has been proposed (Ebel and Lardy, 1975; Recktenwald and Hess, 1977). Such a site, if present on the pea enzyme, does not show an absolute oxyanion specifity.

CHAPTER V

ATPase ACTIVITY OF SUBMITOCHONDRIAL PARTICLES

The low activity of the soluble enzyme, and the fact that it was impure, indicated that a new purification method should be devised, starting with submitochondrial particles rather than whole mitochondria. The ATPase of the particles, however, was interesting enough to merit investigation.

A. ATPase Activity of Freshly Prepared Submitochondrial Particles

Using pea cotyledon submitochondrial particles prepared as described in Chapter II, I found that between batches ATPase activity varied from 0.07 to 0.29 units/mg protein (Table 8 , controls), with most batches in the range of 0.07-0.10 units/mg protein. The activity required a divalent cation (Table 8 , expt. 1). With Mg^{2+} the optimal concentration was 3 mM; at this concentration Ca^{2+} was only 28% as effective. I found that oligomycin, a specific inhibitor of membrane-bound mitochondrial ATPases, inhibited the ATPase activity of submitochondrial particles by more than 90% in most batches (expt. 2). Submitochondrial particles that were not 90% or more sensitive to oligomycin were not used in these studies. To insure that only oligomycin-sensitive ATPase was being measured, each assay included blanks that contained every assay component plus 1 µg of oligomycin.

To test for possible contamination by adenylate kinase and non-specific phosphatase activity I tested the hydrolysis of ADP and β-glycero phosphate. Neither of these substrates was hydrolysed at a detectable rate.

I found that NaN_3, a potent inhibitor of soluble mammalian F_1-ATPases (Ebel and Lardy, 1975; Pullman et al., 1960) and of the soluble pea mitochondrial ATPase (Chapter III), was also effective against the ATPase activity of submitochondrial particles (Table 8, expt. 3).

The respiratory and phosphorylative properties of pea submitochondrial particles are reported in Chapter VI. However, to insure that the H^+ gradient produced by the ATPase reaction in submitochondrial particles (Moyle and Mitchell, 1973) was not inhibiting the rate of ATP hydrolysis, I tested the effect of addition of an uncoupler and an electron transport substrate on the ATPase reaction. Table 8, (expt. 4) shows that addition of 16 μM CCCP had no effect on the ATPase activity of submitochondrial particles, with or without added succinate. This concentration of CCCP completely uncoupled respiration from phosphorylation in both mitochondria and submitochondrial particles of peas (see Chapter VI). It thus appears unlikely that a proton gradient sufficient to inhibit ATPase was formed under these assay conditions. The stimulation by succinate is intriguing and may be caused by inhibitor dissociation, as noted with beef heart submitochondrial particles (Van de Stadt et al., 1973).

Addition of an ATP regenerating system (Pullman et al., 1960) had no stimulatory effect on ATPase activity, showing the reaction was not being inhibited by buildup of ADP (not shown). Table 8, (expt. 5) shows that ADP was a weak inhibitor of the ATPase reaction of submitochondrial particles, compared to its effect on the soluble enzyme where 0.5 mM ADP caused 61% inhibition under the same conditions (Chapter III, Table 3.

Atractyloside (20 μM), a specific inhibitor of adenine nucleotide transport (Vignais, 1976; Wiskich, 1977), was only slightly inhibitory (Table 8, expt. 6), indicating that the submitochondrial particles were nearly completely in the "inside-out" conformation.

I found that submitochondrial particles could be stored in 0.25 M sucrose at -40 C for up to several months with no change in rates of ATPase activity.

B. Presence of the Inhibitor Polypeptide

Since the ATPase activity of fresh submitochondrial particles was relatively low (0.1 unit/mg protein, compared to 2-10 unit/mg protein for mammalian preparations) (Bruni and Bigon, 1974; Racker and Horstman, 1967), it appeared likely that the preparations contained the naturally occurring ATPase inhibitor polypeptide. Since the inhibitor is known to be easily destroyed by trypsin (Jung and Laties, 1976; Pullman and Monroy, 1963; Racker and Horstman, 1967), I tried treating fresh submitochondrial particles with trypsin. Table 9 shows trypsin treatments did induce an approximately 20-fold increase in ATPase activity of submitochondrial particles with low initial activity. The ATPase activity of trypsin-treated submitochondrial particles remained sensitive to oligomycin, and all activity was found in the pellet after a 1 hour centrifugation at 100,000 x g, indicating that the enzyme was still membrane-bound.

C. Activation of ATPase by Aging

Early in my work I found that when fresh submitochondrial particles were kept at room temperature or higher ATPase activity increased dra-

Table 8. Characterization of ATPase activity of fresh submitochondrial particles.

Assay as in Table 1. In treatments marked *, the blank contained all assay components except submitochondrial particles. In all other expts., blanks contained all assay components plus 1 µg oligomycin. For values marked "nil", detection limit was below 5% of control value.

Expt.	Treatment	ATPase Activity units/mg protein	% Control
1	no cation	nil	0
	3 mM $MgCl_2$	0.29	100
	3 mM $CaCl_2$	0.08	28
2*	control	0.26	100
	+ 1 µg oligomycin	[redacted]	4
3	control	0.16	100
	+ 5 µM NaN_3	0.12	75
	+ 50 µM NaN_3	0.06	38
	+ 100 µM NaN_3	0.04	25
4	control	0.09	100
	+ 1.5 µM CCCP	0.09	100
	+ 8 mM succinate	0.13	140
	+ CCCP + succinate	0.13	140
5	control	0.07	100
	+ 1 mM ADP	0.03	43
6	control	0.08	100
	+ 20 µM atractyloside	0.07	88

tically. This response, which I called "aging", was studied in more detail. A variety of analogous temperature activation phenomena have been reported, both with plant (Jung and Hanson, 1976) and animal mitochondrial systems (Pullman et al., 1960; Warshaw et al., 1968).

Table 9 shows that the rate of aging depended on the pH of the incubation medium. At pH 6.6 the rate was slowed compared to pH 7.0. At pH 7.4 and 8.0 aging was initially fast, but this was followed by lower rates. All further experiments on aging were done at pH 7.0.

The aging response was also directly related to the temperature of the incubation (Fig.10, A-C). At 0°C aging was barely detectable, (Fig. 10A), while at 50°C maximal activity was achieved in 10 min (Fig. 10C). At room temperature (20°C) maximal activity was found at 20-24 hr. I was unable to achieve significant activation at 60°C, with or without addition of 1 mM ATP, in contrast to other workers (Jung and Laties, 1976; Warshaw et al., 1968). At all temperatures tested, ATPase reached a maximal level, then decreased (Fig. 10C; decline not shown for 10°C, 20°C, 30°C). The rate of activity loss was greatest at high temperatures.

The sulfhydryl reducing agent dithiothreitol (DTT) (50 mM) increased the rate of aging at 30°C (10B), but did not protect the activated submitochondrial particles against loss of activity. Dithiothreitol also activates the ATPase of plastid membranes (Nelson, 1976).

I found that at 20°C aging was prevented by inclusion of 1 mM ATP (Fig. 10D). When 1 mM $MgCl_2$ was added in addition to ATP, the aging-activation was reduced compared to controls, but was not prevented, perhaps because of hydrolysis of the added ATP. Addition of 1 mM EDTA with 1 mM ATP completely reversed the ATP effect (not shown), suggesting

Table 9. Trypsin and aging activations of pea submitochondrial particle ATPase.

For trypsin activation, 3 µg submitochondrial particles in a[redacted]dium at 30°C was treated with 2.5 µg trypsin for 6 min, then 15 µ[redacted]sin inhibitor was added. After 5 min, ATP was added to start th[redacted]y. For aging activation, submitochondrial particles in sucrose[redacted] diluted with submitochondrial particle buffer at the indicated pH, [redacted]assayed immediately or allowed to age at 20°C for the indicated t[redacted] Assays were done as described in Table 1.

Pre-assay Treatment	ATPase activity (units/mg protein)
[redacted]	0.07
Trypsin, 6 min	1.40
pH 6.6, assayed immediately	0.09
pH 6.6, aged 3 hr	0.46
pH 6.6, aged 8 hr	0.90
pH 7.0, assayed immediately	0.09
pH 7.0, aged 3 hr	0.58
pH 7.0, aged 8 hr	1.10
pH 7.4, assayed immediately	0.09
pH 7.4, aged 3 hr	0.68
pH 7.4, aged 8 hr	0.96
pH 8.0, assayed immediately	0.32
pH 8.0, aged 3 hr	0.75
pH 8.0, aged 8 hr	0.92

that an endogenous cation [illegible] be required for the ATP effect. EDTA alone increased the rate of aging. At higher temperatures ATP delayed the onset of aging but once initiated did not inhibit its rate (Fig. 10B).

The ATPase of aged particles was still completely sensitive to oligomycin, and all activity was found in the pellet after 1 hr. of centrifugation at 100,000 x g at 4°C.

I found that trypsin-treated particles were not further activated by aging (not shown), suggesting that aging, like trypsin treatment, stimulates via release or destruction of the inhibitor.

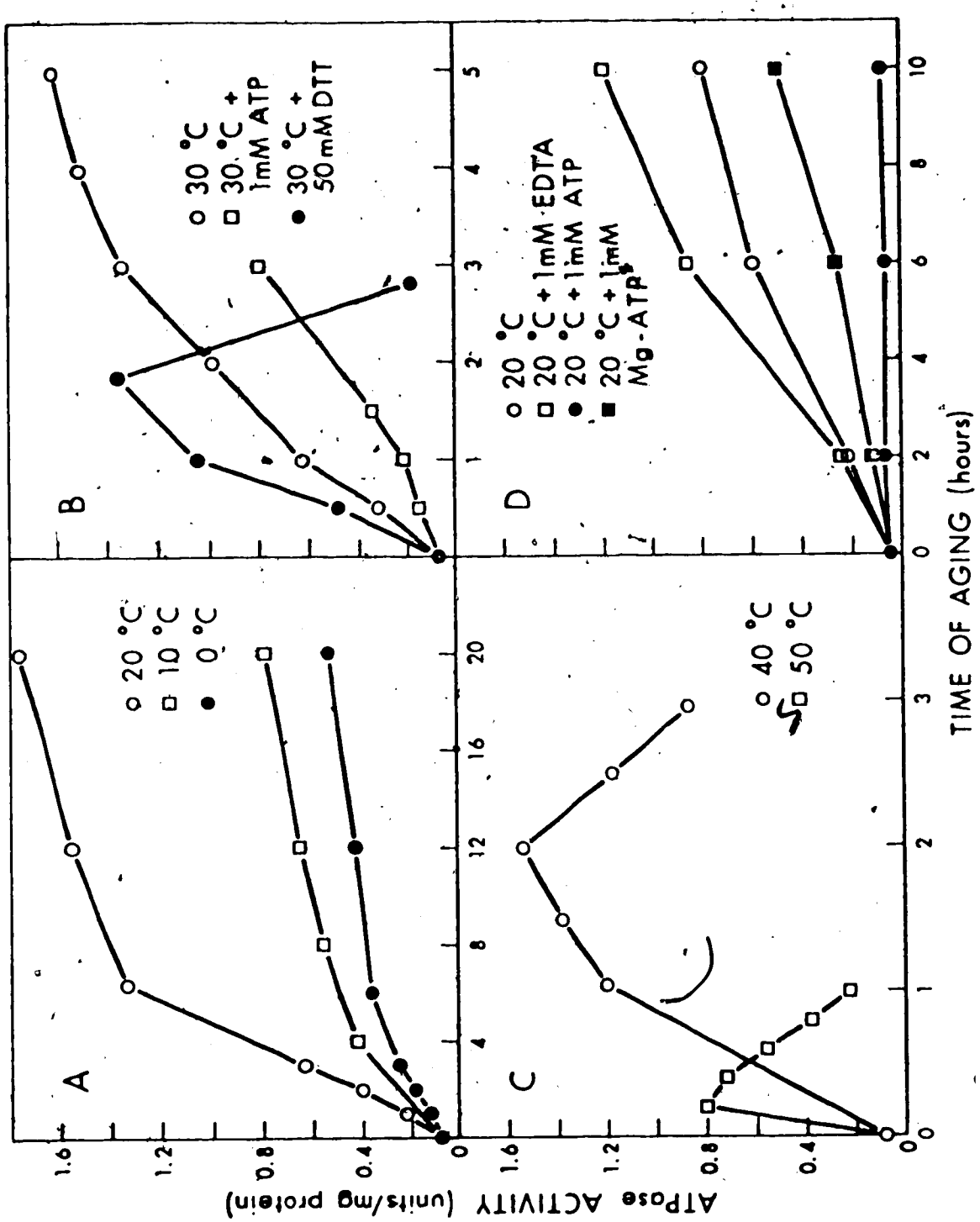
D. Other Activation Treatments

I tried two other methods of releasing the inhibitor, but neither was successful. I found that sonication at pH 9.2, used to release the inhibitor with beef heart (Knowles and Penefsky, 1972a) and castor bean submitochondrial particles (Takeuchi, 1975) produced only a slight activation compared to trypsin or aging (Table 10, expt. 1). Passing submitochondria through Sephadex G-50 Coarse in the presence of 0.25 M KCl, which yields low-inhibitor beef heart submitochondrial particles (Racker and Horstman, 1967), resulted in complete loss of ATPase activity with pea submitochondrial particles (Table 10, expt. 2).

E. Attempts to Reverse Aging

If aging is the result of inhibitor dissociation, then it should be possible under the proper conditions to re-inhibit aged submitochondrial particles. With mammalian systems it has been found that maximal association of the inhibitor with the enzyme occurs at pH 7.0 or below, at low

ure 10. Effect of temperature on rate of aging. All activation treatments were done at submitochondrial particle concentrations of 1.0 mg protein/ml. In Fig. 1B 50 mM dithiothreitol (DTT) was added where noted. In Fig. 1B and 1D, 1 mM ATP, 1 mM Mg^{2+}, 1 mM MgATP were added as noted. Assay was as described in Table 1 and blanks contained all assay components plus 1 µg oligomycin.


ATPase ACTIVITY (units/mg protein)
TIME OF AGING (hours)
A
B
C
D
20 °C
10 °C
0 °C
30 °C
30 °C + 1mM ATP
30 °C + 50 mM DTT
40 °C
50 °C
20 °C
20 °C + 1mM EDTA
20 °C + 1mM ATP
20 °C + 1mM Mg-ATP

ionic strength, and in the presence of low (100 μM) concentrations of Mg-ATP (Horstman and Racker, 1970; Pullman and Monroy, 1963). I performed several experiments in which aged pea cotyledon submitochondrial particles were exposed to these conditions, but at no time was aging reversed.

When the submitochondrial particles were kept at 0 C, however, I found that an ATP-dependent loss of activity occurred in both fresh and aged particles (Table 10, expt. 3-5). While the effect was small (10-30%) it was reproducibly detected in over 5 batches of fresh and aged submitochondrial particles. This effect may represent a partial reversal of aging, but it is important to note that when a preparation of particles was aged to give two different levels of activity, both lost approximately the same amount of activity on ATP-cold treatment (Table 10, expt. 4 & 5). A similar ATP-dependent cold inactivation has been noted with beef heart submitochondrial particles (Bruni et al., 1977), and was thought to represent a dissociation of the inhibitor-free enzyme when ATP was bound. However, when I tried to repeat the ATP-dependent cold inactivation with trypsin-treated submitochondrial particles, no effect was noted (Table 10, expt. 6), suggesting that the effect requires the presence of inhibitor.

F. Substrate Specificity and Anion Effects on Submitochondrial Particles

Fresh, aged and trypsin-treated submitochondrial particles provide a good comparison system for investigating the significance of the substrate specificity and anion stimulations observed earlier with the soluble enzyme.

Studies on the soluble enzyme in Chapter III showed that GTP was

Table 10. Activation and de-activation of ATPase activity of pea cotyledon submitochondrial particles.

In expt. 1, pea mitochondria were sonicated in submitochondrial particle buffer that had been brought to pH 9.3 with NH_4OH. The rest of the isolation proceeded normally.
In expt. 2, 100 mg of fresh submitochondrial particles was applied to a column of Sephadex G-50 Coarse (2.5 x 30 cm) that had been equilibrated with 2 mM EDTA, 250 mM KCl, 75 mM sucrose, and 30 mM tris-sulfate, pH 8.0. The particles were allowed to pass through the column in 1 hr.
In expt. 3-6, the pre-assay de-activation treatments were done in submitochondrial particle buffer. When added, Mg^{2+} or ATP were at 20 µM. Aging and trypsin treatments were as described in Methods. All assays were done as described in Table 1.

Expt.	Material	Pre-assay Treatment	ATPase Activity (units/mg protein)
1.	pH 9.3 sonicated submitochondrial particles	none	0.18
2.	Fresh submitochondrial particles	KCl/Sephadex	nil
3.	Fresh submitochondrial particles	0°C, 5 min	0.12
		0°C, 5 min + Mg^{2+}	0.12
		0°C, 5 min + ATP	0.10
		0°C, 5 min + Mg-ATP	0.10
4.	Submitochondrial particles aged 30°C, 1 hr	22°C, 1 hr + ATP	0.62
		0°C, 1 hr	0.61
		0°C, 1 hr + ATP	0.49
5.	Submitochondrial particles aged 30°C, 2 hr	0°C, 1 hr	1.12
		0°C, 1 hr + ATP	0.96
6.	Trypsin-treated submitochondrial particles	0°C, 1 hr	1.01
		0°C, 1 hr + ATP	0.96

by far the most active substrate, followed by ITP, with ATP the least active purine nucleotide. Using fresh submitochondrial particles I observed that GTP was hydrolysed at about twice the rate of ATP (Table 11). This specificity was not preserved after aging or trypsin-treatment, however, when ATP and GTP were hydrolysed at about equal rates.

Chapter IV showed that Cl^-, HCO_3^- and other anions stimulated ATP hydrolysing activity of the soluble pea ATPase, but had little effect on GTPase or ITPase activity. This effect was thought to result from a direct interaction of the anions with the enzyme, but could also have been the result of inhibitor dissociation or an artifact caused by solubilization of the enzyme. With submitochondrial particles, I found (Table 11) that HCO_3^- anion was stimulatory to the ATPase activity of fresh, aged, and trypsin-treated particles, indicating that HCO_3^- stimulations are not an artifact of enzyme solubilization, and are not caused only by the presence of the inhibitor.

With Cl^- as the anion, where 100 mM concentrations were required for maximal activation, the situation changed slightly. I found that with fresh submitochondrial particles, NaCl stimulated ATPase by about 1.7-fold, slightly greater than the stimulation caused by HCO_3^-. With aged or trypsin-treated particles the stimulation was 1.3 fold, less than that caused by HCO_3^-. This suggests that 100 mM NaCl can cause some inhibitor dissociation with fresh submitochondrial particles, and perhaps also cause some enzyme dissociation to lower activity with trypsin-treated particles.

Other investigators have found with mammalian F_1-ATPases that Cl^- anion has no effect on the kinetics of F_1-ATPase, while HCO_3^- is stimulatory (Ebel and Lardy, 1975; Lambeth and Lardy, 1971; Pedersen, 1976).

Brunl and Bigon (1974) who found a Cl^- activation of beef heart submitochondrial particles, attributed it to loss of inhibitor. To test whether this was the case with pea submitochondrial particles, I examined the effect of anions through the entire time course of trypsin activation. Fig. 11 indicates that NaCl and $NaHCO_3$ both retained their stimulatory effect, even when ATPase activity had peaked.

Effects of anions became more clear when they were examined with an alternative substrate. When I tested trypsin-treated submitochondrial particles I found that GTPase activity was not stimulated by 20 mM HCO_3^- (Table 11), and was slightly inhibited by 100 mM Cl^-, again suggesting a destructive effect of Cl^-. With fresh submitochondrial particles I found that GTPase was stimulated by Cl^- and by HCO_3^-, indicating that either can partially release the inhibition.

In Chapter IV, I showed that 10% ethanol severely inhibits the anion stimulations with the soluble pea enzyme but has less effect on the basal activity. I found this was true with trypsinised submitochondrial particles when ATP was used as the substrate and HCO_3^- was the activating anion (Table 11). I found that with ATPase the substrate trypsin-treated submitochondrial particles were inhibited by NaCl in the presence of 10% ethanol. Table 11 also shows that 0.1 mM N_3^- anion was an effective inhibitor of trypsin-treated submitochondrial particles.

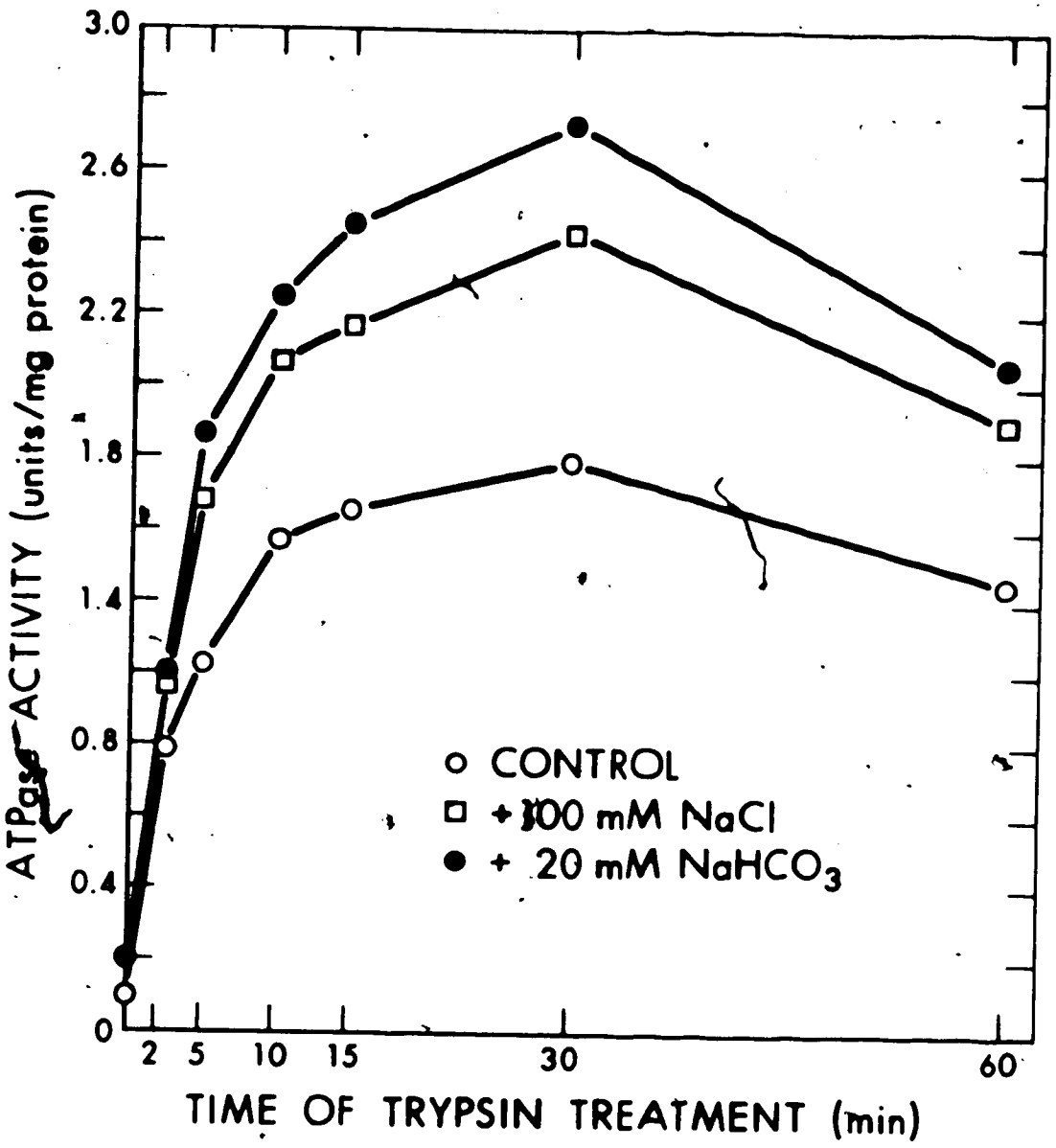
To insure that Cl^- stimulations were not an artifact of the assay system, I also tested the ATPase activity of rat-liver submitochondrial particles. Rat liver F_1-ATPase has been reported to be HCO_3^- stimulated and inhibited by Cl^- (Ebel and Lardy, 1975; Lambeth and Lardy, 1971). Table 11 shows this was also the case with rat liver submitochondrial

Table 11. Nucleoside triphosphatase activity of pea cotyledon submitochondrial particles: Substrate specificity and anion effects.

Methods are given in the text. Results with the soluble pea enzyme are from Chapters III and IV.

Material	ATPase (units/mg protein)			GTPase (units/mg protein)		
	no anion	100 mM NaCl	20 mM $NaHCO_3$	no anion	100 mM NaCl	20 mM $NaHCO_3$
Fresh submitochondrial particles	0.11	0.19	0.18	0.19	0.30	0.27
Submitochondrial particles aged 4.5 hr/30°C	1.73	2.19	2.53	1.88	1.82	2.04
Trypsin-treated submitochondrial particles	1.65	2.04	2.36	1.62	1.26	1.62
Trypsin-treated submitochondrial particles (assay + 10% ethanol)	1.29	1.05	1.31			
Trypsin-treated submitochondrial particles (assay + 0.1 M NaN_3)	0.32	0.70	0.51			
Rat liver submitochondrial particles	0.62	0.49	0.84			
Soluble pea mitochondrial ATPase	3.8	9.3	11.9	18.9	17.0	—

Figure 11. Time course of trypsin activation. Trypsin treatment was as described in Methods. Assay was done as described in Table 1.


3.0
2.6
2.2
1.8
1.4
0.8
0.4
0
ATPase ACTIVITY (units/mg protein)
2
5
10
15
30
60
TIME OF TRYPSIN TREATMENT (min)
CONTROL
+ 300 mM NaCl
+ 20 mM NaHCO3

particles in the present assay system.

G. Discussion

The experiments described in this chapter explore the properties of the pea cotyledon ATPase in its native, membrane-bound, oligomycin-sensitive state. Both inhibited (fresh) and activated (trypsin-treated or aged) preparations were investigated. It is important to note that in these experiments the submitochondrial particle preparation was relatively uncontaminated by ATPase activities that were not sensitive to oligomycin. The small amount of oligomycin - insensitive activity found remained in the supernatant layer after a second centrifugation at 100,000 x g and was not likely membrane-bound; it was probably F_1-ATPase that had been solubilized by sonication. The preparation was also substantially free of adenylate kinase and non-specific phosphatase.

The ATPase activity of freshly prepared pea submitochondrial particles showed several differences from the solubilized enzyme described in Chapter III. The soluble enzyme from peas catalysed a high rate of Ca-ATPase (300% of Mg-ATPase), while the submitochondrial particles showed low Ca-ATPase; typical of mammalian submitochondrial particles and F_1-ATPases (Penefsky, 1974). As with mammalian systems (Ebel and Lardy, 1975) pea submitochondrial particles showed less inhibition by NaN_3 (Table 9, expt. 5; Table 11) than did the soluble pea enzyme, which required 3.5 μM NaN_3 for 50% inhibition (Chapter III, Fig. 4). It may be that conformational changes required for NaN_3 action are restricted when the enzyme is bound to the membrane.

The ATPase of freshly-isolated submitochondrial particles appeared to be associated with the ATPase inhibitor polypeptide, since trypsin

treatments caused a 10-fold or greater increase in ATPase activity. The inhibitor has been found with other F_1-ATPase and submitochondrial particle preparations from mitochondria of plants (Jung and Laties, 1976), mammals (Horstman and Racker, 1970; Pullman and Monroy, 1963), yeast (Satre et al., 1975) and from chloroplasts (see Nelson, 1976). Using castor bean submitochondrial particles, Tukeuchi (1975) has shown a 10-fold increase in ATPase on trypsin treatment, and Jung and Laties (1976) have found that the ATPase activity of sonicated potato mitochondria is also trypsin-activated, although it was unclear in the latter work what proportion of the activity was membrane bound.

The discovery of a more gentle method than trypsin for removing the inhibitor (aging), allowed me to investigate some of the conditions that might control association of the inhibitor with the ATPase. These conditions are similar to those noted with mammalian submitochondrial particles and F_1-ATPases: high pH, elevated temperature, absence of ATP, and absence of divalent cations favor loss of inhibitor.

Pullman and Monroy (1963) and other workers (Horstman and Racker, 1970) have shown that pH values of 7.0 or lower give rise to maximal association of the inhibitor with the F_1-ATPase of beef heart. Similarly, the initial rate of aging of pea submitochondrial particles was lowest at pH 6.6, and rose steadily with higher pH values.

The loss of inhibition with heat treatment is also well known in mammalian ATPases and is the basis for a heat activation step in some of the purification procedures (Horstman and Racker, 1970; Pullman et al., 1960). It should be noted that the heat activation reported here is different than that reported by Warshaw et al. (1968), in which oligomycin sensitivity of submitochondrial particles was lost and the

F_1-ATPase apparently solubilised. I was not able to use the 60°C activation treatments of Jung and Laties (1976), since loss of activity generally occurred, with or without added ATP.

For association of the polypeptide inhibitor with beef-heart F_1-ATPase, low levels of ATP and Mg^{2+} have been shown to be required (Horstman and Racker, 1970; Pullman and Monroy, 1963). In a similar fashion, ATP slowed the aging of pea submitochondrial particles. The divalent cation requirement appeared complex: EDTA increased the rate of aging, and negated the effect of ATP, but with MgATP, aging proceeded at an appreciable rate. This may result from hydrolysis of the added ATP when Mg^{2+} is present.

It appears that pea submitochondrial particles from which the inhibitor has been removed are somewhat unstable under the conditions that cause inhibitor release. Aging for prolonged periods, the presence of DTT, or elevated pH values all resulted in eventual loss of activity. The instability of the activated ATPase may explain why more drastic treatments such as 60°C heat, Sephadex chromatography in 0.25 M KCl, or sonication at pH 9.2 all failed to activate the pea cotyledon submitochondrial particles.

The work on the pea ATPase inhibitor has been hampered by an inability to reverse aging (that is, to re-associate the inhibitor with the ATPase), although I tried acidic pH, ATP, Mg, and lower ionic strength in a variety of combinations. Initially I thought that the ATP-dependent cold inactivation of fresh and aged submitochondrial particles shown in Table 10, was a possible "aging reversal" technique. However, after considerable effort, I was unable to obtain more than the 10-20% inhibition shown in Table 10. I believe that the cold-inactiva-

tion represents inhibition by a portion of the inhibitor which is in a "bound non-inhibitory" state, as proposed by Van de Stadt et al. (1973). That the inactivation is not an effect of ATP binding to inhibitor-free ATPase molecules and causing them to dissociate, as proposed elsewhere (Bruni et al., 1977), is shown by the fact that increasing the proportion of inhibitor-free ATPase molecules (aging) does not increase the amount of cold inactivation, and by the fact that trypsin-treated particles, in which the inhibitor is destroyed, are not susceptible to cold-inactivation (Table 10, expt. 6).

The substrate specificity of the pea submitochondrial particles was intriguing, since it was altered from that of the soluble enzyme, which shows approximately 5-fold higher activity with GTP than ATP (Chapter III, Table 2). With activated (aged or trypsin-treated) submitochondrial particles, activity was nearly equal with the two substrates. Lower relative GTP hydrolysing activity with membrane bound ATPase was found by Pedersen (1976) when he compared soluble rat liver F_1-ATPase to rat liver submitochondrial particle ATPase. It is difficult to account for the difference in substrate specificity between activated and fresh pea submitochondrial particles, however. It may be that the high relative GTPase activity is caused by a slight inhibitor dissociation. It appears clear from the results with activated submitochondrial particles that the high GTPase seen with the soluble enzyme is not an intrinsic property of the membrane-bound enzyme.

One of the purposes of my research with pea submitochondrial particles was to show that the Cl^- and HCO_3^- stimulations noted with the soluble enzyme in Chapter IV were not artifacts of isolation or a result of inhibitor dissociation. The data in Table 11 and Fig. 11

show that with trypsin-treated or aged submitochondrial particles, where the inhibitor has been removed, the anion effects remain, and are specific for ATPase activity, as they are with the soluble enzyme (Table 11). Interpretation of the results with fresh submitochondrial particles is more difficult, and requires that NaCl and $NaHCO_3$ act to release the polypeptide inhibitor, thereby stimulating both ATPase and GTPase activities, as well as specifically stimulating the ATPase activity via a direct interaction with the enzyme.

The effect of anions on the ATPase of submitochondrial particles is much reduced over that noted with the soluble enzyme in Chapter IV. This has also been observed with rat liver submitochondrial particles and F_1-ATPase (Ebel and Lardy, 1975; Pedersen, 1976). However, from this work it appeared quite definite that anion stimulations are an intrinsic property of the pea enzyme, and that Cl^-, in addition to HCO_3^-, is stimulatory. This may be important in determining what shape (Ebel and Lardy, 1975) or chemical (Lardy et al., 1975) properties determine if an anion is stimulatory.

It should be pointed out that the experiments described in this chapter did not directly prove the existence of the polypeptide inhibitor in the particle preparation, and it is possible that the activations noted here are not the result of inhibitor dissociation. However, the many similarities between the aging and trypsin activations reported here, and those in the literature make a strong case for the presence of the inhibitor.

CHAPTER VI

OXIDATIVE PHOSPHORYLATION BY SUBMITOCHONDRIAL PARTICLES

In order to characterize the pea submitochondrial particles more fully, and also in the hope of exploring the relationship of ATPase to ATP synthesis, I also carried out a series of experiments to measure oxidative phosphorylation by pea submitochondrial particles.

A. Oxygen Uptake by Mitochondria and Submitochondrial Particles

I found that mitochondria and submitochondrial particles prepared as described in Chapter II showed typical behavior in the assay for O_2 uptake (Table 12). Mitochondria showed RCR values of 2-3 with succinate and 3-4 with the NADH-linked substrate malate plus TPP (not shown). State IV respiration of the mitochondria was not inhibited by oligomycin, a specific inhibitor of the ATPase-ATP synthetase complex, but this inhibitor prevented the increase of respiration observed when ADP was added. In the presence of an uncoupler (16 μM CCCP) the rate of O_2 uptake was only slightly greater than the rate in state III. Atractyloside (20 μM), which competitively inhibits ADP transport into the mitochondrion (Vignais, 1976) produced a one-third inhibition of the state III rate when 1 mM ADP was present. Mersalyl (10 μM), a sulfhydryl blocking reagent that inhibits phosphate transport in mitochondria (Wiskich, 1977), blocked the state III increase in respiration.

When submitochondrial particles were oxidizing NADH or succinate, ADP produced a slight to moderate stimulation of O_2 uptake (Table 12) (between batches ADP response with NADH as substrate varied from 10-40%). No state III to state IV transition was observed (not shown).

Upon addition of uncoupler, respiration was increased substantially beyond state III rates. Oligomycin inhibited the initial state IV rate slightly, and prevented any increase in respiration on addition of ADP. Oligomycin had no effect on the rate of uncoupled respiration. With NADH as substrate I found that rates were stimulated by addition of 10 µg of oxidized cytochrome c to the submitochondrial particles. Mersalyl (10 µM) completely blocked the increase in respiration observed on addition of ADP plus Pi. Mersalyl had no effect on uncoupled rates of respiration. Atractyloside (20 µM) had no effect on respiration of submitochondrial particles (not shown).

B. Oxidative Phosphorylation by Mitochondria and Submitochondrial Particles

I found that both mitochondria and submitochondrial particles were capable of good rates of oxidative phosphorylation, as measured by ^{32}Pi esterification (Table 13). The assay was linear with respect to time and concentration of mitochondria or submitochondrial particles up to the point of O_2 depletion (result not shown). The assay was usually run to use 40-60% of the available O_2. With submitochondrial particles the assay required hexokinase for maximal activity (Table 13), since pea submitochondrial particles catalyse an active ATPase reaction which was stimulated by the presence of oxidizable substrate (Chapter V, Table 8).

Oxidative phosphorylation by mitochondria or submitochondrial particles was stopped completely by either uncoupler or oligomycin (Table 13). In all further experiments I used blanks containing all assay components plus 1 µg oligomycin, so that treatments that affected ^{32}Pi

Table 12. Oxygen uptake by mitochondria and submitochondrial particles of pea cotyledons.

Assay medium (in a final volume of 3 ml) was 0.3 M sucrose, 4 mM $MgCl_2$, 20 mM glucose, 4 mM K_2HPO_4, 50 mM TES, 2 mM ADP and oxidizable substrate (8 mM malate with mM thiamine pyrophosphate, 8 mM succinate, or 0.88 mM NADH) brought to pH 7.2 with tris at 25°C. Assay was done at 25°C in a Clark oxygen electrode.

			O_2 Uptake (n mol/min/mg protein)			
			Control		Treated	
Preparation	Substrate	Treatment	no ADP	+ ADP	no ADP	+ ADP
Mitochondria	Malate	none*	20.1	67.5	--	--
	Succinate	1 µg oligomycin*	69.2	153.7	61.3	61.3
		16 µM CCCP	75.2	147.6	--	192.4
		20 µM atractyloside	121.1	259.6	128.8	144.2
		10 µM mersalyl	110.3	246.2	--	87.6
Submitochondrial Particles	Succinate	16 µM CCCP**	45.0	45.0	--	83.0
	NADH	16 µM CCCP**	153.2	189.1	--	331.8
		1 µg oligomycin	131.4	160.5	111.9	111.9
		10 µg cytochrome c	87.8	117.2	159.3	199.8***
		10 µM mersalyl	60.6	87.5	65.6	56.2

* same batch of mitochondria
** from same batch of submitochondrial particles
*** rate with 16 µM CCCP was 313.2

carryover, pyrophosphate formation, or non-enzymatic phosphate ester hydrolysis would not give erroneous results. Atractyloside (20 μM) inhibited mitochondrial oxidative phosphorylation by 30%, but had no effect when used against submitochondrial particles, indicating that the adenine nucleotide transporter plays no role in oxidative phosphorylation in this preparation. When the phosphate transport inhibitor mersalyl was present (10 μM), phosphorylation by either mitochondria or submitochondrial particles was nearly completely stopped, at both low (0.1 mM) and high (4.0 mM) phosphate concentrations. Since a transport inhibitor should not inhibit inside-out particles, and respiration was neither inhibited nor uncoupled, I also tested the effect of 10 μM mersalyl on the ATPase reaction of submitochondrial particles. It was inhibited 100%, indicating that mersalyl is a direct inhibitor of the ATPase-ATP synthetase complex. KCN (0.2 mM) nearly completely stopped phosphate uptake. Addition of oxidized cytochrome c resulted in a 30% increase in oxidative phosphorylation when submitochondrial particles were oxidizing NADH.

For submitochondrial particles, P/O ratios with NADH as substrate were 0.5 to 1.4, and with succinate they ranged from 0.2 to 0.5. For most experiments I used NADH as substrate.

When submitochondrial particles were stored in sucrose for several weeks at -40°C, their ability to phosphorylate gradually decreased. All experiments were done with submitochondrial particles that were stored less than three weeks.

C. Kinetics of Oxidative Phosphorylation by Submitochondrial Particles

I found that the Pi kinetics of oxidative phosphorylation by sub-

Table 13. Oxidative phosphorylation by mitochondria and submitochondrial particles.

Assay medium (in a final volume of 1 ml) was 0.3 M sucrose, 4 mM $MgCl_2$, 20 mM glucose, 4 mM K_2HPO_4 (containing approximately 200,000 cpm ^{32}P-orthophosphate), 50 mM TES, 5 units of hexokinase, 2 mM ADP and oxidizable substrate (8 mM malate with 10 µM TPP, 8 mM succinate or 0.88 mM NADH) brought to pH 7.2 with tris at 25°C. Assay was run at 25°C for 6 min. Esterified PO_4 was determined as described in Methods.

Preparation	Substrate	Treatment	ATP Synthesis (units/mg protein) Control	Treated
Mitochondria	Malate	none	0.194	--
	Succinate	16 µM CCCP	0.364	0.001
		1 µg oligomycin	0.327	0.015
		20 µM atractyloside	0.128	0.083
		10 µM mersalyl	0.30?	0.034
Submitochondrial Particles	Succinate	none	0.081	--
	NADH	no hexokinase	0.256	0.212
		16 µM CCCP	0.358	0.005
		1 µg oligomycin	0.316	0.012
		20 µM atractyloside	0.172	0.161
		10 µM mersalyl	0.233	0.010
		0.2 mM KCN	0.174	0.002
		10 µg cytochrome c	0.196	0.281
		20 µM atractyloside*	0.022	0.024
		20 µM atractyloside**	0.059	0.050
		20 µM atractyloside***	0.119	0.114

* ADP at 2 µM, ** ADP at 20 µM, *** ADP at 200 µM

mitochondrial particles were different from those of several animal submitochondrial particle preparations. The Km for Pi was found to be 0.18 mM (Fig. 12), 20-50 fold lower than values for rat liver and beef heart submitochondrial particles (Bygrave and Lehninger, 1967; Schuster et al., 1977). The positive cooperativity toward Pi, noted by Schuster et al. (1977) with rat liver submitochondrial particles, was not detected in my experiments. Since the rat liver experiments were conducted at concentrations of ADP below 200 μM, I also tried repeating the Pi kinetics experiments at 50 μM ADP, and again found linear kinetics (not shown). To be certain that the low Km observed here was not caused by some component of the assay medium, I tried an experiment using rat liver submitochondrial particles. I found that freshly prepared rat liver submitochondrial particles showed a Km at least 10-fold higher than that found with pea submitochondrial particles.

The ADP kinetics of phosphorylation (Fig. 13) were not as simple as the Pi kinetics. In repeated attempts I consistently observed negative cooperativity, with Km values of 10 μM, and 100 μM. This could have been the result of two populations of particles, one inside-out and one right-side-in. The kinetics of right-side-in particles could be governed by an adenine nucleotide transporter with a high affinity for ADP, while the inside-out particles could give a higher Km. To test this, I tried inhibiting oxidative phosphorylation at low levels of ADP by adding 20 μM atractyloside. Table 13 shows that even at very low ADP levels, atractyloside had very little effect.

D. ATPase and Oxidative Phosphorylation

Since the ATPase reaction and substrate oxidation can occur at the

Figure 12. Phosphate kinetics of oxidative phosphorylation by submitochondrial particles. Assay was done as in Table 13, except that K_2HPO_4 concentrations were varied as noted.

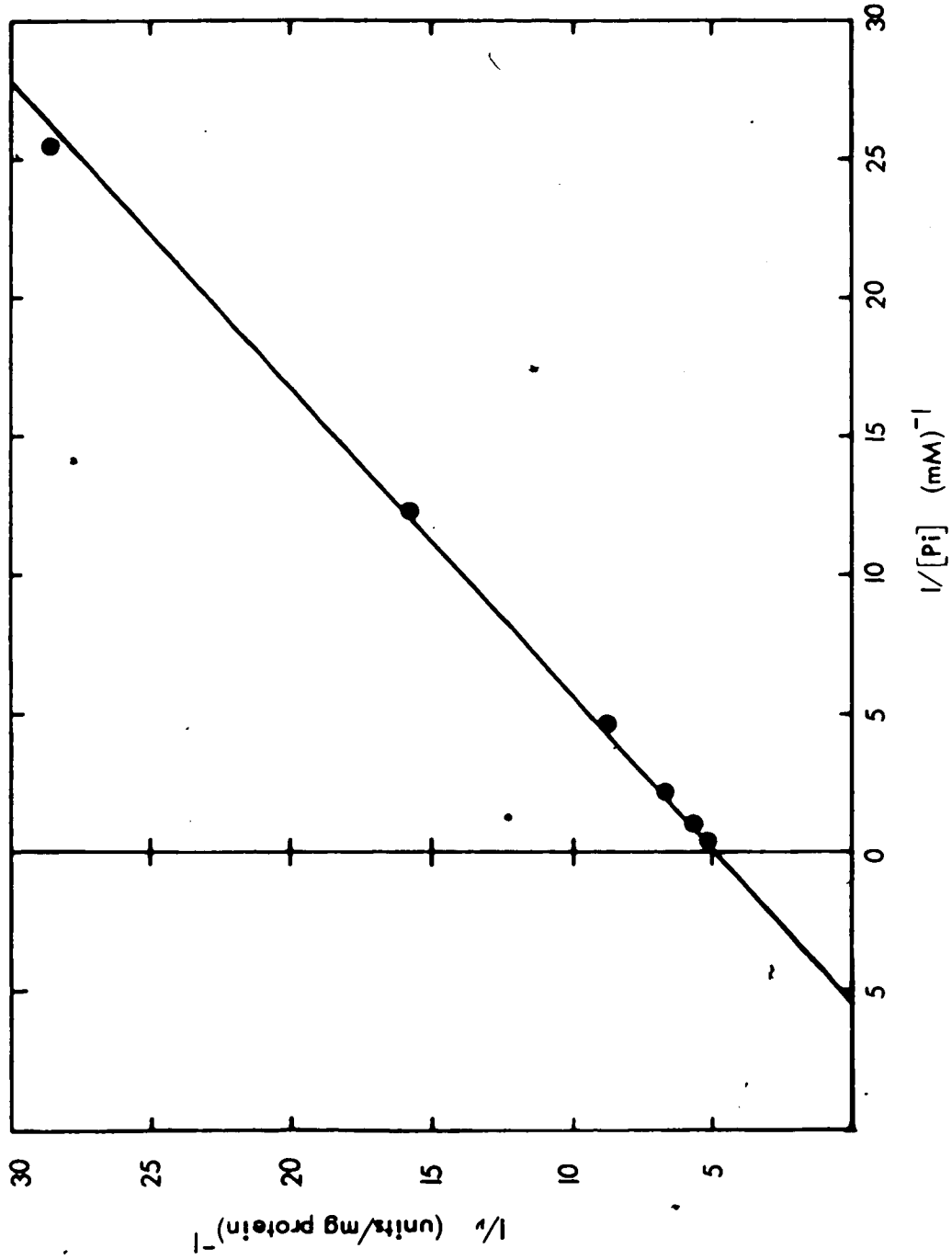
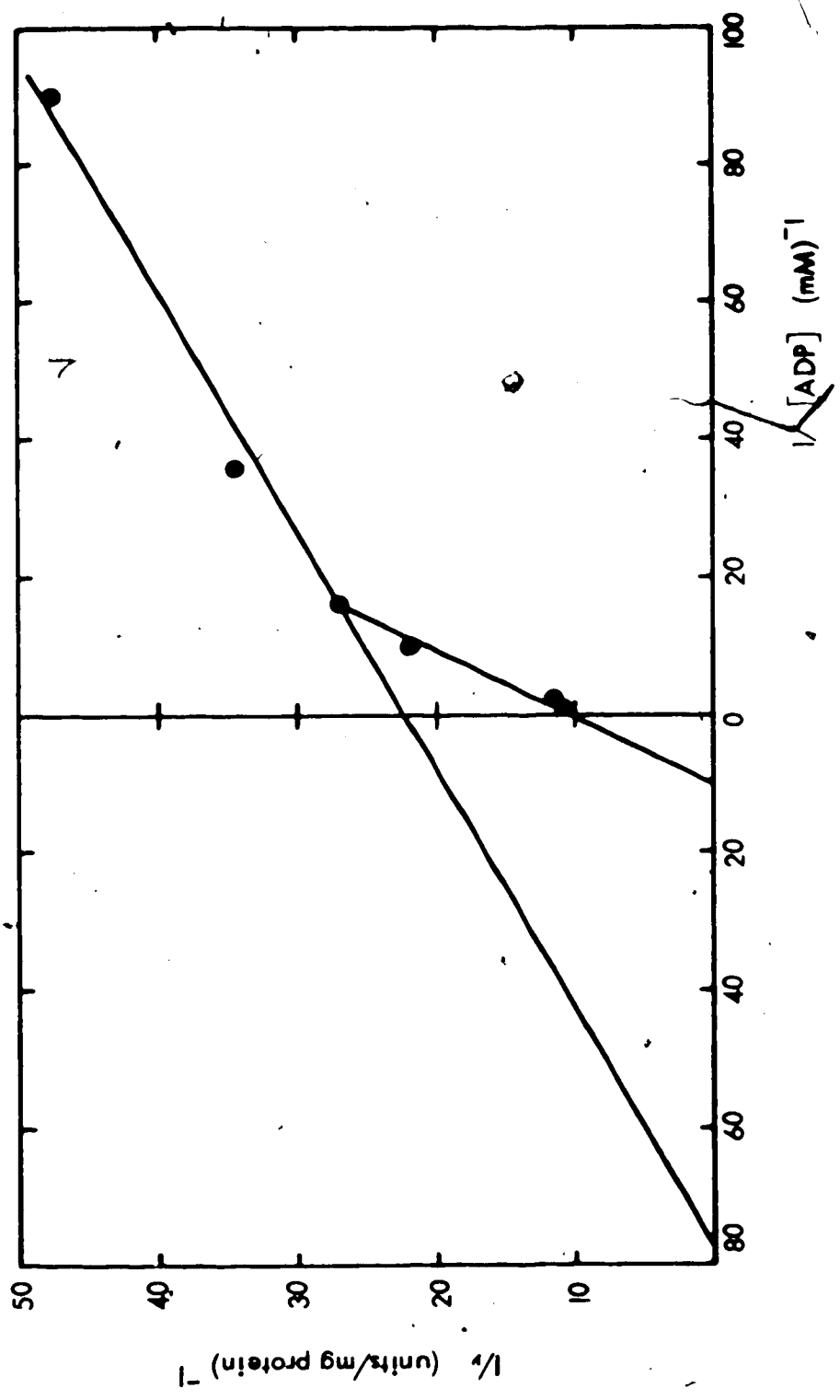

1/v (units/mg protein)$^{-1}$
30
25
20
15
10
5
0
5
10
15
20
25
30
1/[Pi] (mM)$^{-1}$

Figure 13. ADP kinetics of oxidative phosphorylation by submitochondrial particles. Assay was done as in Table 13, except that ADP concentrations were varied as noted.


1/v (units/mg protein) $^{-1}$
50
40
30
20
10
80
60
40
20
0
20
40
60
80
100
1/[ADP] (mM)$^{-1}$

same time (Chapter V), I undertook experiments to determine what, if any, relationship existed between rates of ATPase, ATP synthesis, and O_2 uptake.

When I performed a pH curve for the three reactions (Table 14), I found they each behaved differently. As expected from earlier work on the soluble pea mitochondrial ATPase (Malhotra and Spencer, 1974a), ATPase activity of submitochondrial particles increased steadily over the pH range 6.8-8.0. The rate of phosphorylation, however, showed a peak at pH 7.6. O_2 uptake in the presence of ADP and Pi, or with uncoupler, was maximal at pH 7.6. P/O ratios were highest at pH 7.2.

The stimulations of ATPase by Cl^- and HCO_3^- provided another test system for the relationship between ATPase and ATP synthesis. I found that in the pH 7.2 ATP synthesis buffer, the stimulation of oligomycin-sensitive ATPase by anions was reduced from that observed at pH 8.0 (Table 15). Phosphorylation, however, was not stimulated by anions but slightly reduced. To show that this reduction was not caused by the increased ATPase competing with hexokinase for the ATP I added extra hexokinase, which had no effect. Results with the O_2 electrode indicated that both $NaHCO_3$ and NaCl were inhibitors of O_2 uptake by submitochondrial particles.

Submitochondrial particles were also found to catalyse an ATP-Pi exchange reaction (Table 16). I found that exchange rates with 4 mM Pi and 1 mM ATP were increased by the addition of either 1 mM ADP or NADH.

E. Discussion

Submitochondrial particles with properties similar to those report-

Table 14. Effect of pH on oxidative phosphorylation and ATPase of pea cotyledon submitochondrial particles.

Assays for O_2 uptake and ATP synthesis as in Tables 12 and 13. Assay medium was 0.3 M sucrose, 20 mM glucose, 4 mM $MgCl_2$ and 50 mM TES, brought to the indicated pH with tris at 25°C. The assay was run for 10 min at 25°C.

pH	O_2 Uptake (n mol/min/mg protein) no ADP	+ ADP	+CCCP	ATP Synthesis (units/mg protein)	P/O	ATPase Activity (units/mg protein)
6.8	86.4	86.4	116.9	0.176	1.02	0.086
7.2	78.2	86.4	120.2	0.244	1.41	0.141
7.6	108.6	136.6	206.6	0.273	1.00	0.190
8.0	99.6	114.4	179.4	0.224	0.98	0.276

Table 15. Effect of bicarbonate and chloride on oxidative phosphorylation and ATPase of submitochondrial particles.

Assays for O_2 uptake, ATP synthesis, and ATPase were done as described in Tables 12, 13, and 14.

Treatment	O_2 Uptake (n mol/min/mg protein)	ATP Synthesis (units/mg protein)	P/O	ATPase (units/mg protein)
Control	83.0	0.130	0.78	0.196
20 mM $NaHCO_3$	68.2	0.124	0.91	0.238
100 mM NaCl	64.1	0.087	0.68	0.261
20 mM $NaHCO_3$ + 100 mM NaCl	43.2	0.085	0.99	--

Table 16. ATP-^{32}Pi exchange by pea cotyledon submitochondrial particles.

Assay was done as described for ATP synthesis (Table 13) except that assay medium contained 2 mM ATP, and ADP or NADH were only present where noted.

Conditions	ATP-^{32}Pi Exchange (units/mg protein)	
	no ADP	2 mM ADP
No substrate	0.011	0.021
1 mM NADH	0.040	0.075

ed here have been prepared from plant mitochondria by several workers. Wilson and Bonner (1970a) used osmotic shock to prepare mung bean submitochondrial particles that showed NADH and succinate oxidation rates similar to those reported here. The submitochondrial particles also catalysed energy-linked reactions (ATP-driven reverse electron transport and NADH-NADP transhydrogenase) (Wilson and Bonner, 1970b) but oxidative phosphorylation was not reported. Mung bean submitochondrial particles prepared by sonication (Beyer et al., 1968) were also shown to catalyse NADH and succinate oxidation. Passam and Palmer (1971) have found that submitochondrial particles from sonicated Jerusalem artichoke mitochondria were capable of phosphorylating ADP at rates of 0.1 unit/mg protein with NADH as substrate. A rate of 0.029 unit/mg protein was observed with succinate as substrate.

By using inhibitors I was able to show that the reactions we were studying were those of oxidative phosphorylation: an electron transport inhibitor (KCN), an uncoupler (CCCP) and an ATPase-ATP synthetase inhibitor (oligomycin), were each individually able to completely inhibit the uptake of ^{32}Pi into organic phosphate esters. Throughout the remainder of the experiments the use of blanks containing oligomycin insured that no other Pi esterifying system was interfering, while the addition of excess hexokinase kept ATPase interference to a minimum.

A series of experiments with atractyloside showed that the pea submitochondrial particles were indeed inside-out, and thus not kinetically governed by transporter systems. To insure the pea ADP transporter was not insensitive to atractyloside I also performed the experiments with whole mitochondria in Tables 12 and 13, which showed clear inhibitions of O_2 uptake and phosphorylation. The experiments with mersalyl

were thus somewhat surprising, as this compound is an inhibitor of Pi transport and direct effects on oxidative phosphorylation have not been observed (De Santis et al., 1975; Wiskich, 1977). However, the ATPase activity of pea submitochondrial particles, which does not require Pi transport, was also completely stopped by mersalyl, and Chapter III showed that the soluble ATPase of peas was inhibited by the sulfhydryl blocking compound PCMBS. It is known that the coupling factor CF_1 of chloroplasts is poisoned by sulfhydryl agents when the enzyme is in its energized state (see Schmid et al., 1977).

Storage of submitochondrial particles has been shown by several workers to result in a slow loss of oxidative phosphorylation (Passam and Palmer, 1971) and energy-linked functions (Wilson and Bonner, 1970b). In work with the ATPase activity of pea submitochondrial particles, I found that it is very stable toward prolonged storage time in 0.25 M sucrose at -40 C. Thus the loss of phosphorylating ability with time may be attributed to a parallel loss of membrane integrity leading to uncoupling.

The kinetics of oxidative phosphorylation were somewhat surprising, since the pea submitochondrial particles appear to have much stronger affinity for Pi than do any of the reported animal preparations. For example, Bygrave and Lehninger (1967), who investigated the Pi kinetics of intact and broken rat liver mitochondria, found that the concentration of Pi required for 1/2 maximal velocity increased from 0.25 mM for whole mitochondria, to 3.0 mM and 6.0 mM with digitonin-treated and sonicated mitochondria, respectively. Similarly, Schatz and Racker (1966) found Km values of 1-6 mM Pi, with Km inversely proportional to the rate of respiration. Schuster et al. (1977), although not report-

ing a Km value, show data indicating a Km for Pi of over 10 mM for beef heart submitochondrial particles. My own experiment indicated a Km of over 2 mM with rat liver submitochondrial particles. It thus appears that pea submitochondrial particles have an intrinsically higher affinity for Pi than do mammalian preparations. Whether this is a basic property of the pea enzyme system, or indicates that the phosphorylation system of mammalian submitochondrial particles is more easily damaged by sonication is not clear. In addition, the work of Schuster et al. (1977) indicated strong positive cooperativity towards Pi as a substrate with most pronounced cooperativity observed at 10-50 μM ADP. When I performed experiments at 50 μM ADP with pea submitochondrial particles, plots were still linear.

ADP kinetics of pea submitochondrial particles showed strong negative cooperativity, with Km values of 10 and 100 μM. With mammalian submitochondrial particles a wide range of values have been reported. With sonicated rat liver mitochondria, Bygrave and Lehninger (1967) found a Km for ADP of 300 μM, although Lineweaver-Burke plots were not shown, and the data of Schuster et al. (1977) suggest a Km of below 30 μM (at 10 mM Pi) for beef heart submitochondrial particles, with linear, non-cooperative kinetics. Catterall and Pedersen (1972) found a Km of 3.8 μM for phosphorylation of ADP by "inner membrane vesicles" prepared by digitonin treatment of rat liver mitochondria. No cooperativity was noted.

During my experiments I was interested in trying to discover what factors regulated the rate of oxidative phosphorylation. Several factors were definitely ruled out: Concentrations of ADP, Pi, and electron transport substrates were well above Km values, and since the submit-

chondrial particles were determined to be inside-out, substrate transport was also not limiting. Since O_2 uptake was stimulated considerably by uncoupler, I judged that under normal assay conditions (fresh submitochondrial particles, NADH as substrate) O_2 uptake must be limited by the rate at which the high-energy state can be dissipated. Since in conditions of phosphorylation (ADP present) the dissipation of the high-energy state is through ATP synthesis, I concluded that the ATP-synthetase reaction is normally rate-limiting in pea submitochondrial particles.

The pH curves for O_2 uptake, oxidative phosphorylation, and ATPase attempted to explore the relationship, if any, among these reactions. Interpretation of the results was not clear, however. The O_2 uptake showed a maximum at pH 7.6, which was also the maximum for ATP synthesis. The P/O ratios, however, indicated that oxidative phosphorylation was most efficient at pH 7.2. There appeared to be no relationship between ATP synthesis and ATPase rates in this case, since ATPase activity was highest at pH 8.0. However, lowered ATP synthesis at this pH could be a result of poor O_2 consumption, since P/O ratios stayed the same.

The experiments with NaCl and $NaHCO_3$ attempted to determine if control mechanisms of the ATPase also apply to the ATP synthesis reaction catalysed by the same enzyme. The experiment could be successful only if the ATP synthesis reaction, and not substrate oxidation, was rate-limiting. The stimulatory effect of uncoupler on NADH oxidation in the presence of ADP indicates that state III respiration is normally limited by ATP formation. The actual effect of the salts on ATP formation however, was inhibitory, not stimulatory as with ATPase. It is doubtful whether this represents a direct effect on the ATP synthesis system, or more likely, is the result of the inhibition of substrate oxidation.

High ionic strength ($I>0.1$) has been shown to inhibit pea cotyledon cytochrome oxidase (Bomhoff and Spencer, 1977), and similar effects of NaCl and $NaHCO_3$ on mitochondrial substrate oxidation have been noted (Bendall et al., 1960; Miller and Hsu, 1965). It is interesting, however, that with HCO_3^- the inhibition of ATP synthesis was slight compared to the effect on O_2 uptake, resulting in an increase in P/O ratios.

The ATP-^{32}Pi exchange reaction proceeded at a rate well below that of oxidative phosphorylation. At least three factors could be rate-limiting with this reaction: interference by ATPase, low membrane potential, and low levels of ADP. It is hard to judge how much, if any, interference is caused by the hydrolysis of the radioactive ATP formed by the reaction. Assuming that radioactive ATP is an equilibrium with the medium ATP, the very small amounts of radioactive ATP formed (1-6% of the amount of ATP added) would make interference by hydrolysis insignificant. That low membrane potential and lack of available ADP for the phosphorylative portion of the exchange might be limiting is suggested by the finding that addition of either oxidizable substrate (NADH) or ADP increased exchange rates considerably.

CHAPTER VII

PURIFICATION, SUBUNIT STRUCTURE AND KINETICS OF HIGHLY PURIFIED PEA F_1-ATPase

A. Preliminary Attempts at Purification

To devise a more rigorous purification method, I investigated a number of possible steps. I found that the optimum sonication time was 5 min. With shorter periods, little of the enzyme was solubilized, as judged by oligomycin sensitivity, while with longer sonication times there was a gradual loss of activity. Temperature during sonication was also important: below 45°C, I found that little of the enzyme was solubilized in a 5 min sonication, while at temperatures above 52°C activity was quickly lost. Activity of the soluble fraction was higher after sonication at pH 7.0 rather than 7.4 (the pH of the medium for DEAE chromatography). In order to obtain the most active soluble preparation I also tried age-activating the submitochondrial particles for 4 hr at 30°C before sonication. This resulted in lower ATPase activity in the soluble fraction however, and the activation step was abandoned.

After removal of the membrane fraction by centrifugation at 100,000 x g for 90 min, several different methods were tried for further purification. The pH 5.4 isoelectric precipitation step used previously was found to produce almost no precipitate when submitochondrial particles were the starting material, so this step was not used. I tried performing precipitation with $(NH_4)_2SO_4$ directly on the supernatant layer after the high speed centrifugation step. I found, however, that precipitation with 60% $(NH_4)_2SO_4$ followed by centrifugation at 20,000 x g for

10 min gave rise to a pellet which disintegrated easily and floated in the solution. It was not possible to solubilize the pellet and over 90% of the ATPase activity was lost. Following the finding that the enzyme would bind to DEAE cellulose, the following method was developed.

B. Method

A typical protocol for the purification method is given in Table 17.

Submitochondrial particles that had been stored at -20°C in 10 ml of 0.25 M sucrose were thawed in 50 ml of "DEAE buffer" (0.15 M sucrose, 20 mM tris, 2 mM ATP and 2 mM EDTA, brought to pH 7.4 at 20°C with H_2SO_4). The pH was then adjusted to 7.0 with 2 M H_2SO_4. The 100 ml beaker containing the preparation was placed in a small water bath at 40°C and allowed to equilibrate for several minutes. The full-size tip of the sonicator apparatus was also heated to 40°C in the water bath. Sonication was then carried out at full power on the Artek Sonic Dismembrator (Model 300) in the 40°C water bath, and the temperature of the sonicate was monitored. When the temperature reached 47°C (approximately 30 sec) timing was started and sonication continued for 5 min. The temperature was kept at 47-50°C by adding ice cautiously to the bath.

After sonication, the sonicate was immediately placed in 10 ml centrifuge tubes and centrifuged for 90 min at 100,000 x g, with the rotor temperature held at 22°C.

While the sonicate was being centrifuged a 1.5 x 20 cm column of DEAE cellulose was prepared. Twenty g of Whatman pre-swollen DE-52 microcrystalline cellulose was added to 100 ml of DEAE buffer. The suspension, in an Erlenmeyer flask, was deaerated for 2-3 min under

aspirator vacuum. The pH of the cellulose was then adjusted to 7.4 using 2 M H_2SO_4 and the cellulose was slowly poured into a glass column. The column was then washed with 50 ml of DEAE buffer at a flow rate of 1 ml/min using a peristaltic pump.

The yellow supernatant layer from the centrifugation step (the intensity of the yellow color varied considerably between batches, but this appeared to be unrelated to ATPase activity) was brought to pH 7.4 with 2 M tris. The supernatant layer gradually lost ATPase activity so it was quickly pumped onto the column at 2 ml/min, where it formed a pronounced yellow layer 1-2 cm thick. The column was washed using 50 ml of DEAE buffer, pumped at 1 ml/min. Elution was carried out using a 200 ml linear gradient of 0-0.2 M K_2SO_4 in DEAE buffer. A flowrate of 0.5-0.8 ml/min was used. Fractions of 2 ml were collected and protein content and ATPase activity were monitored (Fig. 14). Three major peaks emerged, with all ATPase activity in the middle peak (no ATPase activity was detectable in the effluents during sample application or column washing). Extending the gradient up to 0.3 M K_2SO_4 produced no additional protein peaks. If 1 M NaCl was then pumped through the column a sharp protein peak with no ATPase activity emerged. The yellow color remained on the column after the 1 M NaCl wash.

All fractions containing 30% or more of the ATPase activity of the most active fraction were pooled and solid $(NH_4)_2SO_4$ was added to 60% of saturation and mixed slowly until dissolved. After 10-15 min at room temperature the cloudy solution was centrifuged for 15 min at 30,000 x g. The pellet layer, which contained all ATPase activity, was dissolved in 1 ml of DEAE buffer and solid $(NH_4)_2SO_4$ was again added to 60% of saturation. The suspension was divided into 0.1 ml portions

in plastic centrifuge tubes and frozen at -20°C. Before an assay, a tube was thawed at 37°C, centrifuged 7 min at 20,000 x g and the pellet was dissolved in 1-3 ml of DEAE buffer. The enzyme activity of the frozen precipitate was stable for about a month, then activity began to decline.

The enzyme preparation obtained had a specific activity of 12 units/mg protein using the ATPase assay with no regenerating system. Activity was increased to 18-20 units/mg by inclusion of the ATP regenerating system described in Chapter II. The enzyme was cold-labile and oligomycin-insensitive (not shown).

C. Enzyme Purity

Using gel electrophoresis, I found that the enzyme preparation was over 90% pure (Fig. 15A). When I tested for ATPase activity, using the specific staining method of Horak (1972), all activity was found in the same location as the major protein band (not shown). The gels stained for protein appeared to contain two contaminating protein bands: a slowly moving component which barely entered the 5% acrylamide gels, and a faster moving very diffuse band. A 10 min cold treatment (0°C) before electrophoresis (at 0-5°C) intensified the fast-moving band (Fig. 15B), in agreement with the results of Knowles and Penefsky (1972a) with beef heart F_1-ATPase.

D. Subunit Composition

On SDS gels (10% acrylamide) the enzyme gave rise to one major protein band (Mol. wt=58,000) and three visible minor bands (Mol. wt= 65,000, 36,000 and 22,000) (Fig. 15A). Since some workers have found

Table 17. Purification of soluble pea mitochondrial ATPase.

Purification steps are described in the text. The non-regenerating ATPase assay described in Table 1 was used.

Material	Protein (mg)	ATPase Activity	
		Total units	Units/mg Protein
Submitochondrial particles	80	3.2	0.04
Whole sonicate	80	35.7	0.45
Pellet after centrifugation	64	4.5	0.07
Supernatant layer[1.]	16.5	21.5	1.30
DEAE effluent[2.]	1.5	15.0	10.0
First $(NH_4)_2SO_4$ precipitate	1.2	12.1	10.1
Second $(NH_4)_2SO_4$ precipitate[3.]	0.7	8.4	12.0

1. ATPase activity was not inhibited by oligomycin. The ATPase activity of the pellet layer was 60% inhibited by 1 µg of oligomycin.

2. Fractions containing 30% or more of the activity of the peak fraction were pooled for this determination.

3. Vmax in the ATP-regenerating assay of Fig. 17 was 18.0 units/mg protein.

Figure 14. DEAE chromatography elution profile. Chromatography was performed as described in this chapter. Protein was measured as described by Sedmak and Grossberg (1977, see Methods). ATPase was assayed as described in Table 1.

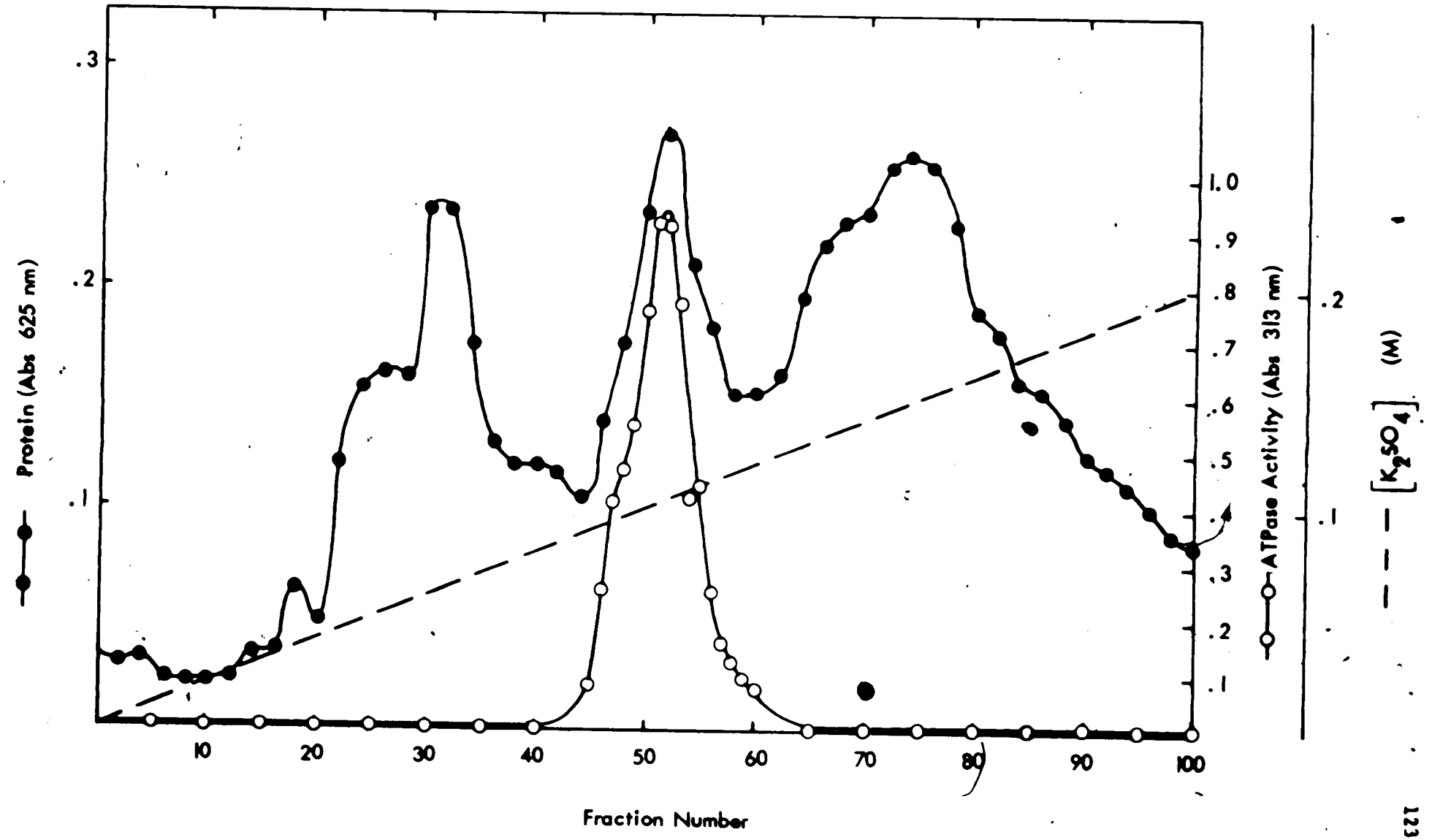
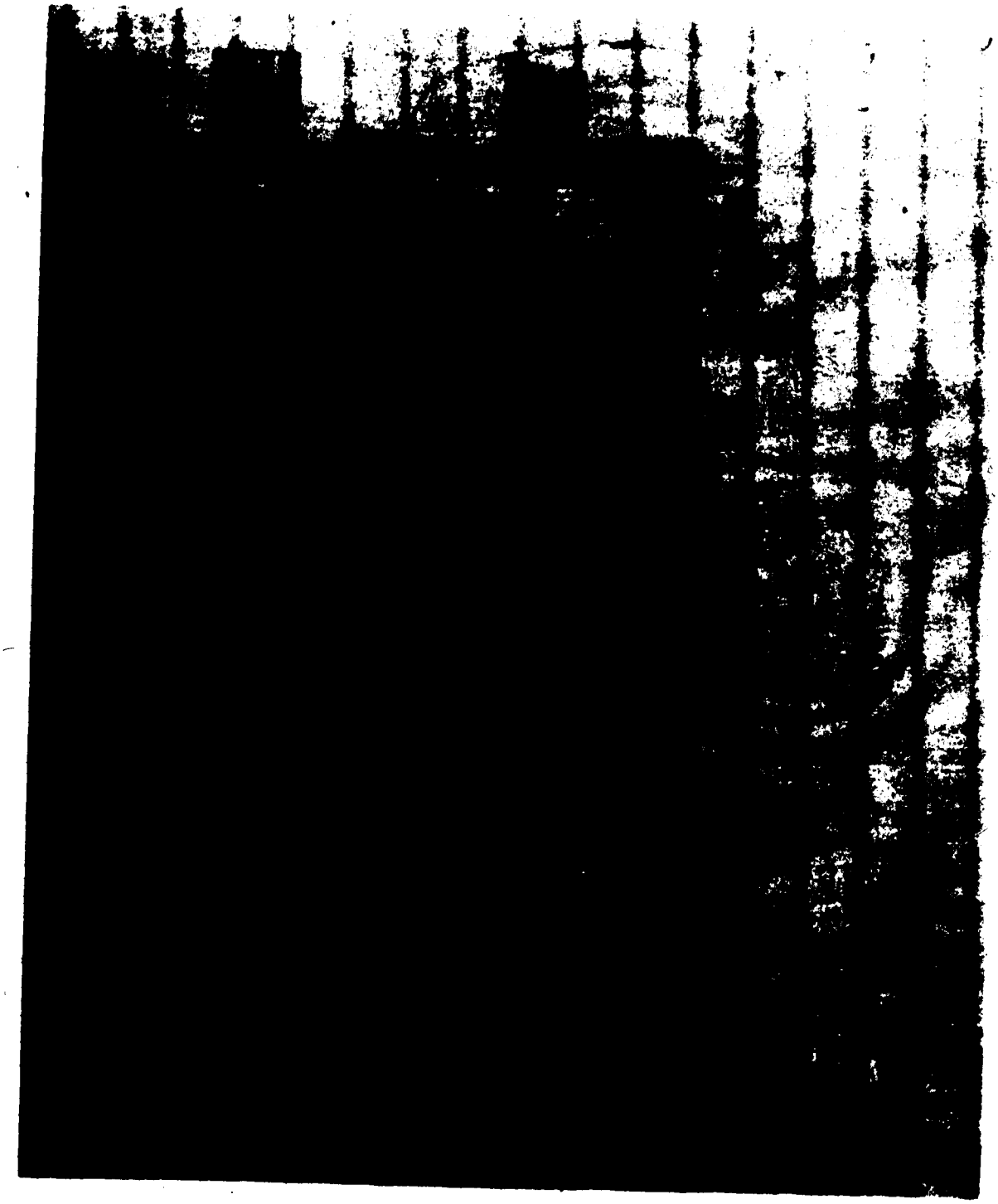

Protein (Abs 625 nm)
.3
.2
.1
10
20
30
40
50
60
70
80
90
100
Fraction Number
ATPase Activity (Abs 313 nm)
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.2
.1
$[K_2SO_4]$ (M)

Figure 15. Gel electrophoresis of soluble pea F_1-ATPase. 5% acrylamide gels contained ATPase assay medium. Assay medium was also used for sample and electrode buffer. Gels were run for 90 min at 4 mA/tube. In A, 50 µg of ATPase protein was applied, and gels were run at 25°C. In B, 50 µg of ATPase protein was applied after a 10 min incubation at 0°C. Gels were run at 0-5°C for 90 min at 4 mA/tube. Staining was done with Coomassie blue R-250 as described in Methods.



that SDS gels fail to resolve the α and β subunits well (Knowles and Penefsky, 1972a; Yoshida et al., 1977), I also tried the 8 M urea system employed by Knowles and Penefsky (1972a), which they found gave rise to a slow band composed of α and γ subunits, and a faster band of β subunits. Fig. 16B shows that urea gels of the pea enzyme contained two major protein bands in the approximate positions noted by Knowles and Penefsky (1972a) and by Yoshida et al. (1977). The proportion of stain was greater in the faster band. In addition, urea gels contained a third, more slowly migrating band. At high protein concentrations the protein precipated on the gel surface.

SDS gels of submitochondrial particles (Fig. 16C) gave rise to numerous bands, one of which appeared to coincide with the major band noted with the pure enzyme. Urea gels of submitochondrial particles failed, as all protein precipitated on the gel surface.

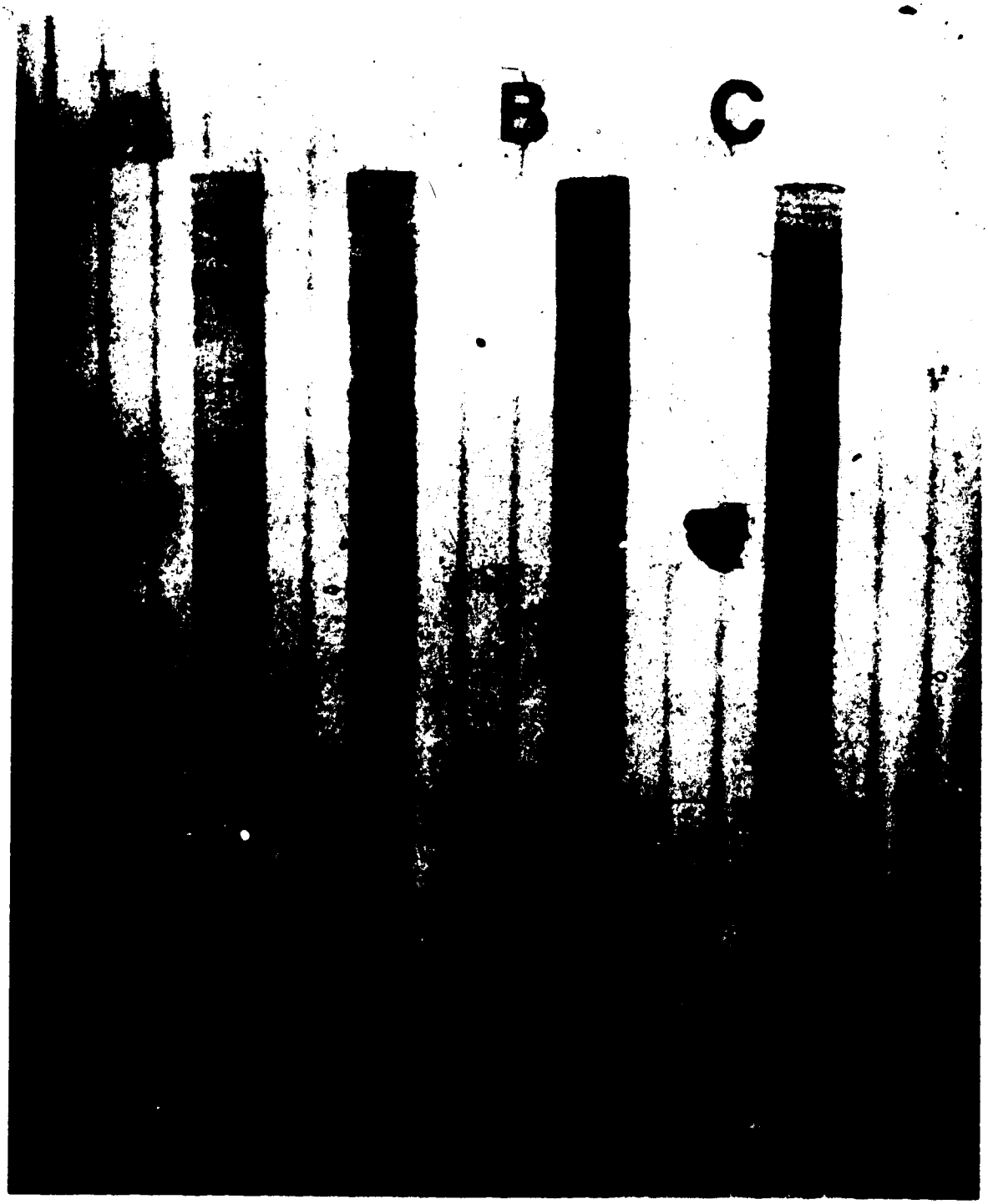
I tried several times to test for homogeneity and measure the molecular weight of the enzyme by chromatography on a column of 10% agarose gel (Bio-gel A-0.5 m) using the DEAE medium. This technique failed, however, as no enzyme activity was recovered in the column effluent.

E. Kinetics of the ATPase Reaction

The kinetics of the purified ATPase were examined using the ATP regenerating system described in Chapter II. Activities of the pure enzyme measured in this system were consistently about 50% higher than in the non-regenerating assay buffer, even though assays in both systems were linear with respect to time course.

Fig. 17 shows typical Lineweaver-Burke plots obtained with the pure

Figure 16. Gel electrophoresis of soluble pea F_1-ATPase in dissociating systems. In A and C, 10% acrylamide gels contained 1% SDS. Protein samples were treated with SDS and mercaptoethanol and gels were run as described by Weber and Osborn (1969, see Methods). In B, 5% acrylamide gels containing 8 M urea were run as described by Knowles and Penefsky (1972a, see Methods). In A, 10 and 50 µg of ATPase protein was applied. In B, 20 µg ATPase protein was applied. In C, 100 µg of submitochondrial particles was applied. All gels were stained with coomassie blue R-250.


B
C

enzyme. Kinetic parameters are summarized in Table 18. It can be seen that the enzyme displays pronounced negative cooperativity. However, data points above 0.2 mM Mg-ATP fell on a straight line with a Km of 0.17 mM and a Vmax of 17.8 units/mg protein. Fig. 17 also shows that either 100 mM NaCl or 20 mM $NaHCO_3$ increased Vmax dramatically, but had little effect on the curvature of the Lineweaver-Burke plots, in contrast to the results reported by other workers (Ebel and Lardy, 1975; Pedersen, 1976). In the presence of 100 mM NaCl the Km for Mg-ATP rose to 0.31 mM, while with 20 mM $NaHCO_3$, the Km was 0.20 mM.

When Mg-GTP was used as substrate Vmax was 118 units/mg protein. The Km for Mg-GTP was 1.3 mM, and negative cooperativity was observed.

I also examined the kinetics of Mg-ATP hydrolysis by submitochondrial particles. Fig. 18 (and Table 18) shows that the Km for the ATPase reaction was 0.07 mM. Curvature of the Lineweaver-Burke plots was much less than that noted with the soluble enzyme. Fig. 18 also shows that aging the submitochondrial particles for 5 hr at 30°C resulted in a 5-fold enhancement of Vmax in this case, but there was no change in the Km toward Mg-ATP. Table 18 also shows that 100 mM NaCl or 30 mM $NaHCO_3$ both raised the Km of the enzyme slightly, while increasing Vmax. With Mg-GTP the Km was 0.29 mM.

F. Discussion

The purification method described in this chapter was a considerable improvement over that used previously, as judged by the purity and specific activity of the final preparation.

The identity of the two contaminating protein bands could not be established; however several findings provide evidence that they are

Figure 17. Kinetics of the soluble F_1-ATPase. Assay medium was 0.3 M sucrose, 25 mM TES, 1 mM $MgSO_4$, 2 mM phosphoenol pyruvate, and 50 µg pyruvate kinase, brought to pH 8.0 with KOH at 20°C. Mg-ATP was added as indicated and the mixture equilibrated at 30°C for 5 min. Assay also contained 100 mM $NaHCO_3$ or 20 mM $NaHCO_3$ as noted. Assays were started by addition of enzyme and ran for 15 min.

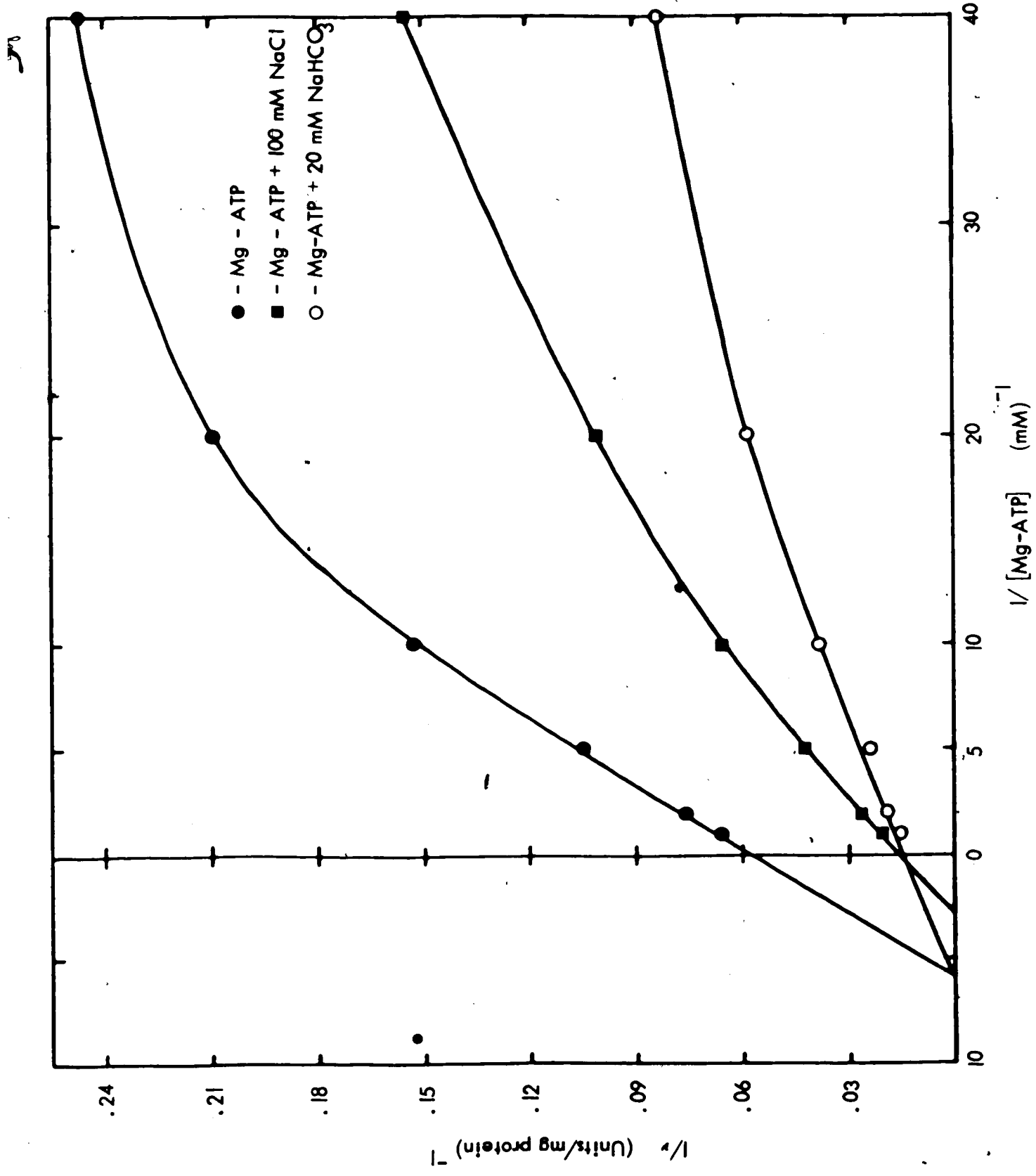

● - Mg - ATP
■ - Mg - ATP + 100 mM NaCl
○ - Mg-ATP + 20 mM NaHCO3
1/v (Units/mg protein)⁻¹
1/ [Mg-ATP] (mM)⁻¹
.24
.21
.18
.15
.12
.09
.06
.03
10
0
5
10
20
30
40

Figure 18. Kinetics of the ATPase reaction catalysed by submitochondrial particles. Assay was run as described in Fig. 17. Fresh particles or particles aged 5 hr at 30°C were added to start the reaction.

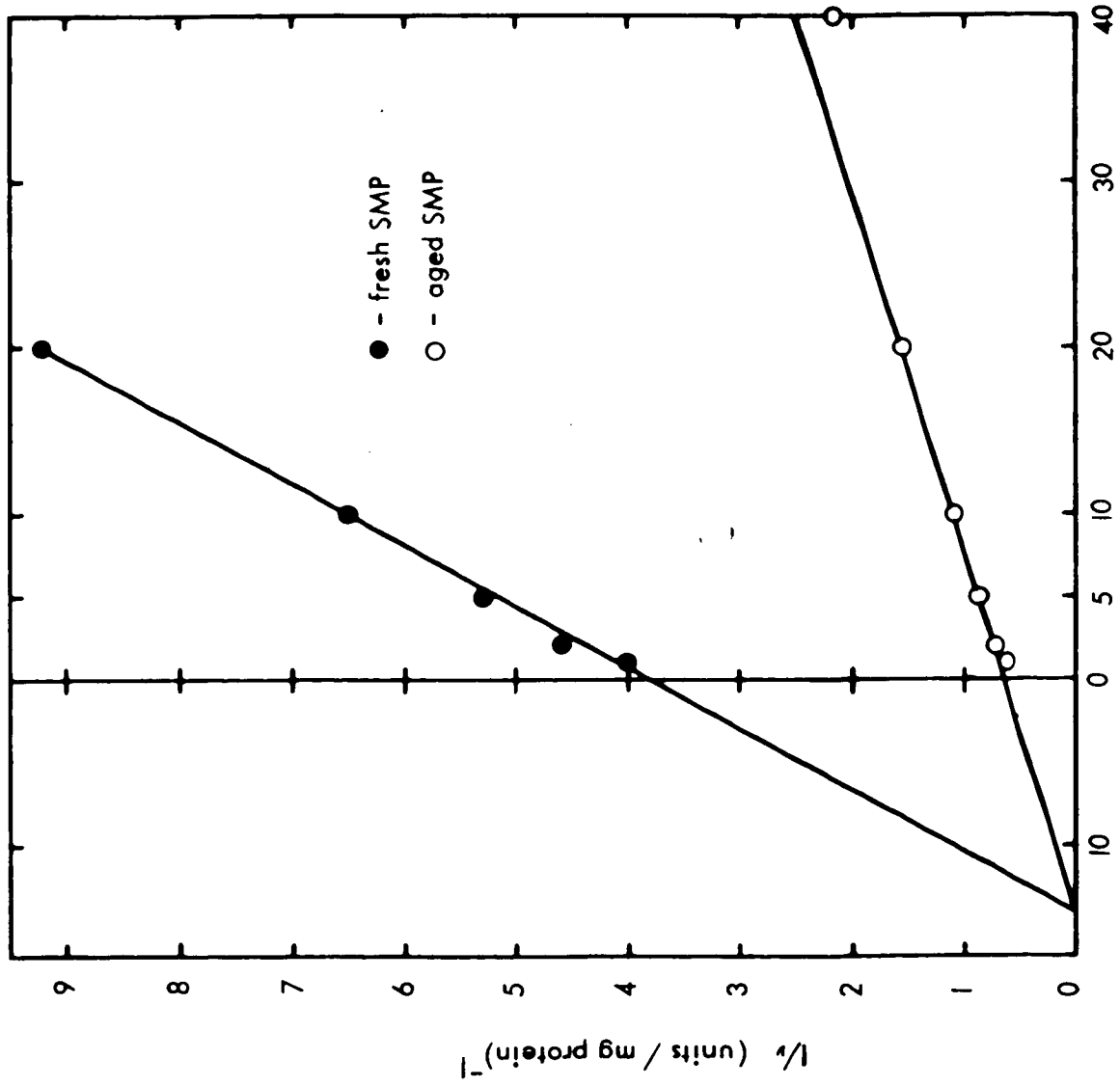

● - fresh SMP
○ - aged SMP
1/v (units / mg protein)⁻¹
1/[Mg - ATP] (mM)⁻¹
0
1
2
3
4
5
6
7
8
9
10
0
5
10
20
30
40

Table 18. Kinetics of soluble and submitochondrial particle ATPase.

Assay conditions as in Fig. 17. 100 mM NaCl and 20 mM $NaHCO_3$ were present as noted.

Preparation	Substrate	Anion	Km (mM)	Vmax (units/mg protein)
Soluble enzyme	ATP	none	0.13	17.5
		NaCl	0.33	66.7
		$NaHCO_3$	0.13	67.3
	GTP	none	1.2	111
Submitochondrial particles (fresh)	ATP	none	0.07	0.26
	GTP	none	0.12	0.46
Submitochondrial particles (aged)*	ATP	none	0.07	1.54

* Particles were aged for 5 hr at 30°C.

related to the main peak. When I tried precipitating all fractions along the ATPase peak after DEAE chromatography, I found that the proportion of protein in each of the bands remained the same, indicating that all three proteins co-chromatograph on DEAE cellulose. Cold treatment of the enzyme before electrophoresis appeared to increase the amount of protein in the faster moving band, although this was difficult to judge. This may indicate the the fast band was a dissociated subunit. The diffuse nature of the fast band may also indicate that it was dissociating from the main band during the electrophoresis run. When the pea enzyme was electrophoresed with the pH 9.5 tris-glycine electrode buffer of Davis (1964) both the fast and slow bands were present in larger quantities. While it is not possible to conclude that the enzyme is homogenous, it does seem likely that the impurities are a dimer or multimer of the main band, and a dissociated subunit.

Many other workers have reported that electrophoresis of pure F_1-ATPases gives rise to more than a single band. Knowles and Penefsky (1972a) noted that pure F_1-ATPase of beef heart gave one pronounced protein band, but a faster moving band was often present and increased in intensity when the ATPase was electrophoresed at 5°C. Warshaw et al. (1968) found their preparations of pure "factor A" (a latent F_1-ATPase) gave rise to several bands when electrophoresed at pH 9.5, but a single band when run at a lower pH. Azocar and Munoz (1977) found that the solubilized F_1-ATPase of E. coli k/2 was unstable in a variety of electrophoresis systems, and purity could only be demonstrated in one buffer system.

F_1-ATPases from many sources have been shown to have five types of subunits (here referred to as α, β, γ, δ, and ε). This has been found

with bacteria (e.g. Yoshida et al., 1977), yeast mitochondria (Tzagoloff and Meager, 1971), beef heart mitochondria (Knowles and Penefsky, 1972a) and chloroplasts (Nelson, 1976). In mammalian F_1-ATPases the subunit stoichiometry is probably $\alpha_3\beta_3\gamma\delta\varepsilon$, judged by molecular weight and stain intensity in SDS gels (Catterall and Pedersen, 1971). The two largest subunits, α and β (Mol. wt 54,000 and 49,000 from beef heart mitochondria (Knowles and Penefsky, 1972a, b) and 62,500 and 57,000 from rat liver (Catterall and Pedersen, 1971)), are often not clearly resolved on SDS gels (Knowles and Penefsky, 1972a; Senior and Brooks , 1971; Yoshida et al., 1977). They are, however, well resolved on 5% acrylamide gels containing 8 M urea (Knowles and Penefsky, 1972a; Yoshida et al., 1977). The minor subunits (γ, mol. wt=33,000; δ, mol. wt=16,000; ε, mol. wt=5,850 (Knowles and Penefsky, 1972a, b)) are well resolved on SDS gels. Subunit γ co-electrophoresed with α on 8 M urea gels (Knowles and Penefsky, 1972a, b).

The results with the pea enzyme indicated a similar subunit structure, with one major band (mol. wt=58,000) which probably contains both α and β subunits. That there are two distinct polypeptides in this major band was shown by electrophoresis in 8 M urea, where two major bands were visible. SDS gels also showed two less intensely staining protein bands (mol. wt=36,000, mol. wt=22,000) that roughly corresponded to weights reported for γ and δ subunits (Knowles and Penefsky, 1972a, b). The other faint band visible on SDS gels corresponded to molecular weight of 65,000. The significance of this band is not known. The band appears to be present as a major component on SDS gels of submitochondrial particles, and is thus possibly a contaminant of the soluble enzyme. On very heavily loaded gels (200 μg protein) a diffuse band was noted at

a mobility faster than that of cytochrome c (mol. wt=11,700). This may have been subunit ε; however the gels were too heavily stained to be reliable.

The kinetics of Mg-ATP hydrolysis by the pure enzyme were similar to those of rat-liver and beef heart F_1-ATPases. Negative cooperativity has been reported for these enzymes by Ebel and Lardy (1975), Schuster et al. (1976) and by Pedersen (1976). Those authors found, however, that negative cooperativity was relieved by those anions which also increased Vmax of the ATPase activity. This is in contrast to the results with the pea enzyme, which showed that Cl^- and HCO_3^- did not remove the curvature of Lineweaver-Burke plots. This raises the interesting possibility that anion effects on Vmax and cooperativity noted with mammalian F_1-ATPases may be separate phenomena, although Ebel and Lardy (1975), in a rather complete survey of anions as effectors of the F_1-ATPase of rat liver, did not report any anions that increased Vmax but had no effect on cooperativity.

Results of the kinetic studies on ATPase activity of the pure enzyme failed to disclose any major difference between HCO_3^- and Cl^- as stimulatory anions. Both appear to exert their major effect on Vmax, while slightly increasing Km. It is likely therefore, that they have their stimulatory effect on catalysis rather than on substrate binding. This is discussed further in the next chapter.

Using the ATP-regenerating assay I was also able to investigate the kinetics of the ATPase activity of submitochondrial particles. In agreement with literature on mammalian F_1-ATPases (Hammes and Hilborn, 1971; Ebel and Lardy, 1975; Pedersen, 1976), the membrane-bound enzyme had a significantly lower Km for Mg-ATP than did the pure enzyme (0.07

mM, compared to 0.17 mM for the soluble enzyme) and less curvature in the Lineweaver-Burke plots. In common with the soluble enzyme, the major effect of anions was on Vmax; no major change in Km values was noted. I was also able to show (Fig. 18) that the aging-activation discussed in Chapter V appeared kinetically to be relief of a non-competitive inhibition (Vmax increased, while Km remained constant). Van de Stadt et al. (1973) have shown that the ATPase inhibitor polypeptide is a non-competitive inhibitor versus Mg-ATP. This helps to confirm the hypothesis of Chapter V that aging-activation represents release of inhibitor.

CHAPTER VIII

CONCLUSIONS AND A PERSPECTIVE

A. Similarities to Other F_1-ATPases

This study represents the first detailed characterization of the F_1-ATPase complex from mitochondria of a higher plant. The picture of the ATPase complex that emerges is one that is similar in many respects to that of mammalian mitochondrial F_1-ATPases. Similarities were first shown to occur in cold lability, a rare property among enzymes, but found with all soluble mitochondrial F_1-ATPases (Penefsky, 1974). I was also able to show similarities in the broad purine substrate specificity of the soluble enzyme, in the anion stimulations and their ATP specificity (Ebel and Lardy, 1975; Pedersen, 1976) and in sensitivity to inhibitors such as NaN_3, ADP, and NaF (Pullman et al., 1960). With submitochondrial particles I was able to demonstrate that both ATPase and ATP synthesis are sensitive to oligomycin, a feature of all mitochondrial F_1-ATPases bound to their native site on the cristal membrane. Freshly prepared submitochondrial particles also appeared to possess an ATPase inhibitor polypeptide, similar to that of beef heart F_1-ATPase (Horstman and Racker, 1970; Pullman and Monroy, 1963). The particles were able to catalyse oxidative phosphorylation that was typical in its sensitivity to inhibitors of electron transport, energy coupling, and ADP phosphorylation. A more highly purified preparation of the soluble enzyme showed subunit structure similar to that of F_1-ATPases on SDS gels, except that there was no resolution of α and β subunits. These appeared to be resolved, however, on the urea gel system of Knowles and

Penefsky (1972a), indicating a subunit structure typical of mitochondrial F_1-ATPases (Pedersen, 1975). Similarly, the kinetics of the soluble enzyme indicated negative cooperativity similar to that observed for rat liver F_1-ATPase by Ebel and Lardy (1975) and Pedersen (1976).

It is not unexpected that there should be so many similarities between pea and mammalian F_1-ATPases, since the enzyme is basic to energy metabolism and is already known to be similar among bacteria (Abrams and Smith, 1974; Yoshida et al., 1977), yeast and mammalian mitochondria (Penefsky, 1974), and chloroplasts of higher plants (Nelson, 1976). Some of the unique features of the pea F_1-ATPase (high Ca-ATPase, sensitivity to PCMBS, and stimulation by Cl^- ion) will be discussed later as they relate to future research.

B. Soluble and Membrane-bound Forms of the Enzyme

Each of the two different forms of the F_1-ATPase (soluble and membrane-bound) dealt with in my work has some validity in the study of the energy coupling system of pea mitochondria. The soluble form has as its main advantage the fact that it can be relatively easily purified away from all contaminating activities that could give misleading results. The membrane-bound system (submitochondrial particles), on the other hand, allows the study of the modification or control of catalytic activity that might occur through interaction with other membrane components or energization of the membrane. Each system also has a major drawback, however: the particulate system is impure and the soluble enzyme exhibits some properties that may be a product of solubilization.

With the pea enzyme, like those from mammals, the catalytic activities of the enzyme were definitely altered by solubilization. I found

that sensitivities to ADP, stimulatory anions, and NaN_3 were all reduced with the membrane-bound enzyme, as was the amount of Ca-ATPase activity, the relative GTPase activity, the Km values for nucleotides, and the curvature of Lineweaver-Burke plots. These results are similar to those reported with the rat liver F_1-ATPase complex (Ebel and Lardy, 1975; Pedersen, 1976). The cold lability of the soluble preparation also represents an alteration in properties from the membrane-bound enzyme.

The likeliest explanation for these results is that membrane binding in some way alters the conformation of the F_1 molecule or modifies conformational changes that may occur on catalysis or effector binding. This modification would most likely be a restriction of conformational changes in the membrane-bound enzyme form. This would explain, for example, how anions that are very stimulatory with the soluble form of the enzyme have less effect with the membrane-bound form. Similarly, cold lability which has been shown to proceed by way of an initial conformational change (Rosing et al., 1975), may be lessened when the conformation of the enzyme is restrained.

Future research on the pea enzyme will thus have to be based on the fact that neither of the systems currently available (the soluble enzyme and the submitochondrial particles) gives a completely reliable picture of the enzyme. Only by continuing to work with both preparations or attempting to purify the oligomycin-sensitive ATPase with all its associated membrane proteins (Soper and Pedersen, 1976; Serrano et al., 1976) can a complete knowledge of the enzyme be obtained.

C. Control of the ATPase

Of the control mechanisms discussed in Chapter I, two were directly

addressed in my work: control by the inhibitor polypeptide and kinetic control by anions. Clearly, control by the inhibitor polypeptide is the more important of the two with the membrane-bound enzyme. The freshly isolated submitochondrial particles had very low ATPase activity unless they were exposed to treatments that removed or destroyed the inhibitor. In isolated mitochondria under state IV conditions (no ADP present) it is likely the presence of the inhibitor which prevents ATP hydrolysis. The high internal ATP level would favor the maintenance of the inhibition, in the same way it inhibits the aging response with submitochondrial particles. In the case where unavailability of oxidizable substrate was limiting oxidative phosphorylation, and both ADP and ATP were present, the interaction between the inhibitor and the ATPase might gradually weaken (the ATPase would become activated), but inhibition of the ATPase reaction might then occur by the ADP inhibition and unavailability of ATP that would be imposed by the adenine nucleotide translocator. While further work on the control of ATPase in intact mitochondria would be of interest, the complexity of the control system makes design of rigorous experiments difficult. It is also difficult to design further experiments on the role of the inhibitor polypeptide, since I was unable to develop any system in which activated submitochondrial particles could be inhibited. It is possible, however, that the highly purified enzyme might be more sensitive or reactive toward inhibitor and could be used as an assay system. This would allow for purification of the inhibitor and further studies on its status in aging, and in oxidative phosphorylation.

A question that was approached, but not answered, in my work was the mechanism of the kinetic control by anions. Two mechanisms were definitely discarded: studies with fresh, aged, and trypsin-treated

submitochondrial particles indicated anion stimulations were not completely an artifact of solubilization, and were also not likely a result of inhibitor dissociation. After discarding these possibilities, several hypotheses remain that require a direct interaction of anions with the enzyme: anions could directly assist catalysis at the catalytic site, they could cause a conformational change that increases the number of catalytic sites, or they could cause a conformational change that in turn assists catalysis.

The first possibility, a direct role in assisting catalysis, does not seem to explain why the ATPase of submitochondrial particles is less stimulated by anions than the soluble enzyme, although if the shape of the catalytic site was altered during solubilization an anion requirement might be developed. In addition, however, it seems difficult to imagine a catalytic step in which such a variety of sizes, shapes, and charges of anions could participate. This is less of a problem with the rat liver F_1-ATPase preparation of Ebel and Lardy (1975) where a good deal of specificity was shown toward oxyanions of only certain shapes. The fact that with the pea enzyme only ATPase, and not GTPase, was stimulated by anions also argues against participation at the catalytic site, since the hydrolytic mechanism should be similar for the two substrates, and interaction of the anions with the purine base portion of the nucleotide would seem unlikely to change Vmax.

Either of the other two mechanisms would work if there was a regulatory anion binding site on the enzyme. Binding of a stimulatory anion could cause a conformational change that could in turn make available a previously blocked catalytic site, or increase Vmax at a catalytic

site that was previously available. Either of these two mechanisms would result in a stimulation of the observed Vmax. Km effects could not be predicted. It is not clear how either of these two hypotheses could be verified, however. An interesting first step would be to measure anion binding, and to determine whether the anion binding constants compare to the Ka values for activation of Vmax. An easy technique for studying ligand binding has been recently developed (Penefsky, 1977) and used to measure phosphate binding to the beef heart enzyme.

The idea of opening a blocked catalytic site agrees well with the abolition of negative cooperativity noted during anion stimulations by Ebel and Lardy (1975) and Pedersen (1976). It is relatively easy to imagine the F_1-ATPase as having three copies of a catalytic site. Substrate binding to one site might make the other two less available, and anion binding might release this cooperativity. In a closely related mechanism, an ATP-binding allosteric site might be inducing cooperativity which anion binding might release. If GTP could not bind at this regulatory site it would not induce coooperativity and thus anions would have no effect on GTPase. This hypothesis, in either of its two versions, does not completely fit my data, however, since the anion stimulations could not be explained as release of cooperativity and Vmax stimulations of 4.5-fold would require a large number of catalytic sites. A third version of the blocked-site hypothesis could be put forward in which each catalytic site has a separate regulatory site through which it can be activated by some anions and inactivated by other anions or compounds. In other words, anions would control the proportion of active to inactive sites. This idea of active and inactive states has been proposed by Moyle and Mitchell (1975), while the idea of each catalytic site having

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a separate anion-binding regulatory site was brought forward by Recktenwald and Hess (1977). If one accepts the latter authors' idea that the regulatory site also binds ATP then cooperativity in kinetics can be explained as allostery. It is not clear in this version, however, why a certain proportion of sites is not active without stimulatory anions being present, and why this proportion should be repeatable among enzyme batches.

An anion-induced conformational change that stimulates Vmax at a previously available catalytic site could be developed from the same assumptions as the last discussed hypothesis. If each catalytic site had a low activity with ATP as substrate and Vmax was increased by anion-binding at a regulatory site the most important effect of anions, increasing Vmax of ATPase, is explained. If this regulatory site also bound ATP then cooperativity in kinetics might be an allosteric stimulation of activity when ATP is bound, with anion binding being more active at producing the conformational change involved (giving rise to a higher Vmax). If both ATP and anion binding were required for the anion effect and the regulatory site was specific for ATP, then the lack of anion effects on GTPase activity is explainable.

All of these hypotheses, however, are too complex to receive any direct support from my data, and it is not clear what evidence could be obtained that would support or defeat any of them. The kinetic approach, used in conjunction with nucleotide analogs by Lardy's group (Schuster et al., 1975; Schuster et al., 1976) seems to have only confirmed that a final statement of nucleotide and anion-related regulatory properties will be complex. This is also supported by recent studies showing multiple nucleotide-binding sites (Penefsky et al., 1976).

D. Further Research on Pea F_1-ATPase

In comparison with other F_1-ATPases, work on the pea enzyme may be hampered by the relatively low amount of the enzyme available from a typical purification (1 mg) and also by the relative instability of the enzyme toward gel chromatography and electrophoresis. Assuming these problems to be perhaps limiting, but not a cause for hopelessness, the key question is which properties of the pea enzyme merit continued research. There are two ways of looking at this question. One could answer that plant mitochondrial ATPase merits study just because it is from a plant tissue. Thus research could continue to be comparative or descriptive. The other approach, however, would be to admit that pea F_1-ATPase is fairly similar to other F_1-ATPases in its essential properties and to search for special areas where the pea enzyme offers a chance to advance ATPase research. The second approach seems more interesting and productive.

One feature which may be offered by the soluble pea enzyme is that of its high relative Ca-ATPase activity. While this is different from other F_1-ATPases (Pedersen, 1975), the findings that make Ca-ATPase of special interest are that it is inhibited by Cl^- anion and is highest when the concentration of Ca^{2+} is above that of the ATP present. These may indicate a role for Ca^{2+} different from that of Mg^{2+}. It is possible that free Ca^{2+} can induce a change in enzyme properties similar to that caused by anions. Further work on the kinetics, substrate specificity and anion sensitivity of Ca-ATPase may provide some valuable information on anion effects and alternative roles for divalent cations in addition to substrate activation. A further point of interest concerning

the Ca-ATPase activity of the enzyme is that it was low (relative to Mg-ATPase) in submitochondrial particles. This resembles the situation with CF_1-ATPase, in which the soluble form has high Ca-ATPase in the absence of activating anions, while with light-activated plastid fragments Ca-ATPase is low compared to Mg-ATPase (Nelson et al., 1972).

The PCMBS inhibition of the soluble pea enzyme may also provide an opportunity for further research on the pea enzyme. It has not generally been found that compounds which react with sulfhydryl groups are inhibitors of F_1-ATPases (Pedersen, 1975), although Pullman et al. (1960) found that PCMB inhibited the stimulatory effect of 2,4-dinitrophenol on beef heart F_1-ATPase. In addition, the soluble ATPase of Endomyces yeast mitochondria was also inhibited by PCMBS. The finding that iodoacetamide did not inhibit the pea enzyme may indicate that the reactive sulfhydryl groups is not involved in catalysis. The bulky PCMBS group may block substrate binding or interfere with a conformational change needed in catalysis. It is intriguing that PCMBS was a somewhat more effective inhibitor of anion-stimulated activity than basal activity. A broad screening of sulfhydryl group reagents might disclose what structure is needed for maximal inhibition of ATPase in the presence and absence of anions. A kinetic study of enzyme previously reacted with an appropriate sulfhydryl reagent to produce a partial inhibition might show changes in substrate specificity or Km expected by blocking access to the catalytic site, or changes in anion effects or cooperativity if a conformational change is blocked. Using submitochondrial particles as a comparison system may provide additional information, although the fact that mammalian submitochondrial particles are inhibited by mercurials (Pedersen, 1975) may indicate that the

membrane portion may contain a catalytically essential sulfhydryl group. A final line of research on the pea enzyme and submitochondrial particles concerns the role of the inhibitor polypeptide. This area has received little attention since a few early studies (Pullman and Monroy, 1963; Horstman and Racker, 1970) in spite of some extremely interesting experiments in the labs of Ernster and Van Dam (Asami et al., 1973; Van de Stadt et al., 1973, 1974). The role of the inhibitor in the forward reaction of oxidative phosphorylation is extremely interesting. For the moment, however, this research is stalled, pending an ability to re-inhibit activated ATPase, and thus assay for the inhibitor.

These lines of research area, of course, only a few of the possible directions work on the pea enzyme could take.

REFERENCES

Abrams, A., & Smith, J.B. (1974) The Enzymes, 3rd Ed. 10, 395.

Adolfsen, R., & Moudrianakis, E.N. (1973) Biochemistry 12, 2926.

Amons, R., Van den Bergh, S.G., & Slater, E.C. (1968) Biochim. Biophys. Acta 162, 452.

Asami, K., Juntti, K., & Ernster, L. (1971) Biochim. Biophys. Acta 205, 307.

Azocar, O., & Munoz, E. (1977) Biochim. Biophys. Acta 482, 438.

Bendall, D.S., Ranson, S.L., & Walker, D.A. (1960) Biochem. J. 76, 220.

Beyer, R.E., Peters, G.A., & Ikuma, H. (1968) Plant Physiol. 43, 1395.

Blackmon, W.J., & Moreland, D.E. (1971) Plant Physiol. 47, 532.

Bradford, M. (1976) Anal. Biochem. 72, 248.

Bomhoff, G.H., & Spencer, M.E. (1977) Can. J. Biochem. 55, 1114.

Bottrill, D.E., & Hanson, J.B. (1969) Aust. J. Biol. Sci. 22, 847.

Boyer, P.D., Chance, C., Ernster, L., Mitchell, P., Racker, E., & Slater, E.C. (1977) Ann. Rev. Biochem. 46, 955.

Bruni, A., & Bigon, E. (1974) Biochim. Biophys. Acta 357, 333.

Bruni, A., Frigeri, A., & Bigon, E. (1977) Biochim. Biophys. Acta 462, 323.

Bygrave, F.L., & Lehninger, A.L. (1967) Proc. Natl. Acad. Sci. (USA) 57, 1409.

Carmeli, C., & Biale, J.B. (1970) Plant Cell Physiol. 11, 65.

Catterall, W.A., & Pedersen, P.L. (1971) J. Biol. Chem. 246, 4987.

Catterall, W.A., & Pedersen (1972) J. Biol. Chem. 247, 7969.

Chan, S., & Barbour, R. (1976) Biochim. Biophys. Acta 430, 426.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404.

De Santis, A., Borraccino, G., Arrigoni, O., & Palmieri, F. (1975) Plant Cell Physiol. 16, 911.

Dixon, M., & Webb, E.C. (1962) in Enzymes 2nd Ed., pp 337-340, Longmans, London.

Dodds, J.J.A., & Ellis, R.J. (1966) Biochem. J. 101, 31.

Ebel, R.E., & Lardy, H.A. (1975) J. Biol. Chem. 250, 191.

Forti, Giorgio. (1957) Physiol. Plant. 10, 898.

Hall, J.L., & Butt, V.S. (1969) J. Expt. Bot. 2, 751.

Hammes, G.G., & Hilborn, D.A. (1971) Biochim. Biophys. Acta 233, 580.

Harris, D.A. (1978) Biochim. Biophys. Acta 463, 245.

Harris, D.A., & Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87.

Horak, A. (1972) Ph.D. Thesis, University of Manitoba.

Horstman, L., & Racker, E. (1970) J. Biol. Chem. 245, 1336.

Jagendorf, A.T. (1977) in Encyclopedia of Plant Physiology (New Series) Vol. 5, p. 307. Springer-Verlag, New York.

Jung, D.W., & Hanson, J.B. (1973) Arch. Biochem. Biophys. 148, 139.

Jung, D.W., & Hanson, J.B. (1975) Arch. Biochem. Biophys. 168, 358.

Jung, D.W., & Laties, G.G. (1976) Plant Physiol. 57, 583.

Karlsson, J., & Kylin, A. (1974) Physiol. Plant. 32, 136.

Knowles, A., & Penefsky, H.S. (1972a) J. Biol. Chem. 247, 6617.

Knowles, A., & Penefsky, H.S. (1972b) J. Biol. Chem. 247, 6624.

Kozlov, I.A., & Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29.

Lambeth, D.O., & Lardy, H.A. (1971) Eur. J. Biochem. 22, 359.

Lardy, H.A., Schuster, S.M., & Ebel, R.E. (1975) J. Supramol. Struct. 3, 214.

Lardy, H.A., & Wellman, H. (1953) J. Biol. Chem. 201, 357.

Lehninger, A.L. (1970) Biochemistry Worth, New York.

Lin, W., Wagner, G.J., Siegelman, H.W., & Hind, G. (1977) Biochim. Biophys. Acta 465, 110.

Lopez-Moratalla, N., Franch, V., Paniagua, R., & Santiago, E. (1977) FEBS Lett. 79, 113.

Lowry, O.H., Rosebrough, N.J. Farr, A.L., & Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.

Malhotra, S.S., & Spencer, M. (1974a) Can. J. Biochem. 52, 491.

Malhotra, S.S., & Spencer, M. (1974b) Can. J. Biochem. 52, 1091.

Miller, G.W., & Hsu, W.H. (1965) Biochem. J. 97, 615.

Moyle, J., & Mitchell, P. (1975) FEBS Lett. 36, 55.

Mozersky, S.M., Pettinati, J.D., & Kolman, S.D. (1966) Analyt. Chem. 38, 1183.

Nagaraja, K.V., & Patwardhan, M.V. (1974) Plant Biochem. J. 1, 5.

Nelson, N., Nelson, H., & Racker, E. (1972) J. Biol. Chem. 247, 6506.

Nelson, N. (1976) Biochim. Biophys. Acta 456, 314.

Olson, A.O., & Spencer, M. (1968) Can. J. Biochem. 46, 283.

Passam, H.C., & Palmer, J.M. (1971) J. Expt. Bot. 22, 304.

Passam, H.C., & Palmer, J.M. (1973) Biochim. Biophys. Acta 305, 80.

Pedersen, P.L. (1975) Bioenergetics 6, 243.

Pedersen, P.L. (1976) J. Biol. Chem. 251, 934.

Penefsky, H.S., & Warner, R.C. (1965) J. Biol. Chem. 240, 4694.

Penefsky, H.S. (1974) The Enzymes 3rd Ed. 10, 375.

Penefsky, H.S. (1974b) J. Biol. Chem. 249, 3579.

Penefsky, H.S., Garrett, N.E., & Chang, to-min. (1976) in the Structural Basis of Membrane Function (Hatefi, Y., & Djavadi, L., Eds.) pp. 69-79, Academic Press Inc., New York, N.Y.

Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891.

Peterson, T.G., & Heisler, C.R. (1963) Biochem. Biophys. Res. Commun. 12, 492.

Phillips, D.R. (1971) Ph.D. Thesis, University of Alberta.

Pullman, M.E., & Monroy, G.C. (1963) J. Biol. Chem. 238, 3762.

Pullman, M.E., Penefsky, H.S., Datta, A., & Racker, E. (1960) J. Biol. Chem. 235, 3322.

Racker, E. (1976) in A New Look at Mechanisms in Bioenergetics Academic Press, New York.

Racker, E., & Horstman, L. (1967) J. Biol. Chem. 242, 2547.

Recktenwald, D., & Hess, B. (1977) FEBS Lett. 76, 25.

Reid, H.B. Jr., Gentile, A.C., & Klein, R.M. (1964) Plant Physiol. 39, 1020.

Rosing, H. Harris, D.A., Kemp, A. & Slater, E.C. (1975) Biochim. Biophys. 376, 13.

Ryrie, I.J. (1975) Arch. Biochem. Biophys. 168, 712.

Satre, M. DeJerphanion, M.B., Huet, J., & Vignais, P. (1975) Biochim. Biophys. Acta 387, 241.

Schatz, G., & Racker, E. (1966) J. Biol. Chem. 241, 1429.

Schmid, R., Jagendorf, A.T., & Hulkower, S. (1977) Biochim. Biophys. Acta 462, 177.

Schuster, S.M., Ebel, R.E., & Lardy, H.A. (1975) J. Biol. Chem. 250, 7848.

Schuster, S.M., Gertschen, R.J., & Lardy, H.A. (1976) J. Biol. Chem. 251, 6705.

Schuster, S.M., Reinhart, G.D., & Lardy, H.A. (1977) J. Biol. Chem. 252, 427.

Sedmak, J.J., & Grossberg, S.E. (1977) Analyt. Biochem. 79, 544.

Selwyn, M.J. (1967) Biochem. J. 105, 279.

Senior, A.E., & Brooks, J.C. (1971) FEBS Lett. 17, 327.

Senior, A.E. (1973) Biochim. Biophys. Acta 301, 249.

Serrano, R., Kanner, B.I., & Racker, E. (1976) J. Biol. Chem. 251, 2453.

Skou, J. (1960) Biochim. Biophys. Acta 42, 6.

Skou, J.C. (1965) Physiol. Rev. 45, 596.

Solomos, T., Malhotra, S.S., Prasad, S., Malhotra, S.K., & Spencer, M. (1972) Can. J. Biochem. 50, 725.

Sone, N., Furuya, E., & Hagihara, B. (1969) J. Biochem. 65, 935.

Soper, J.W., & Pedersen, P.L. (1976) Biochemistry 15, 2682.

Sperk, G., & Tuppy, H. (1977) Plant Physiol. 59, 155.

Takeshige, K., Benno, H., Bohm, M., [redacted]mermann-Telschow, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1605.

Takeuchi, Y. (1975) Biochim. Biophys. Acta 376, 505.

Tzagoloff, A., Byington, K.H., & MacLennan, D.H. (1968) J. Biol. Chem. 243, 2405.

Tzagoloff, A., & Meagher, P. (1971) J. Biol. Chem. 246, 7328.

Van de Stadt, R.J., De Boer, B.L., & Van Dam, K. (1973) Biochim. Biophys. Acta 292, 338.

Van de Stadt, R.J., Van Dam, K., & Slater, E.C. (1974) Biochim. Biophys. Acta 347, 224.

Van de Stadt, R.J., & Van Dam, K. (1974) Biochim. Biophys. Acta 347, 240.

Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1.

Warshaw, J.B., Lam, K.W., Nagy, B., & Sanadi, D.R. (1968) Arch. Biochim. Biophys. 123, 385.

Weber, K.,& Osborn, M. (1969) [redacted]. Biol. Chem. 244, 4406.

Weiner, M.W., & Lardy, H.A. (1974) Arch. Biochim. Biophys. 162, 568.

Wilson, S.B., & Bonner, W.D. Jr. (1970a) Plant Physiol. 46, 25.

Wilson, S.B., & Bonner, W.D. Jr. (1970b) Plant Physiol. 46, 31.

Wiskich, J. (1977) Ann. Rev. Plant Physiol. 28, 45.

Yoshida, K, & Takeuchi, Y. (1970) Plant Cell Physiol. 11, 403.

Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977) J. Biol. Chem. 252, 3480.

APPENDIX

APPENDIX

OLIGOMYCIN-SENSITIVE ATPase OF SUBMITOCHONDRIAL PARTICLES FROM CORN

A. Abstract

To test the hypothesis (Sperk and Tuppy, 1977) that mono-cotyledons contain a unique oligomycin-insensitive ATPase, I prepared submitochondrial particles and a soluble fraction from sonicated corn mitochondria (Zea mays L. cv. EarliKing). Although the ATPase activity of the whole sonicate was relatively insensitive to oligomycin, the corn submitochondrial particles possessed an ATPase activity that was nearly completely inhibited by oligomycin, and was activated by trypsin. This ATPase is similar to that from other sources (plants, animals, and microorganisms). The soluble fraction also contained an active ATPase, which was inhibited by azide and stimulated by sodium chloride and trypsin. The soluble fraction differed from other F_1-ATPases in that it was cold-stable.

B. Introduction

Research into the mechanisms of oxidative and photosynthetic phosphorylation has shown that the energy-transducing ATPases (ATP: phosphohydrolase EC 3.6.1.3) from a wide variety of organisms are extremely similar. Well-studied preparations from chloroplasts, from yeast and mammalian mitochondria (Penefsky, 1974), from bacteria (Abrams and Smith, 1974), and from pea mitochondria (Chapter III-VII) show similarities in many catalytic and structural properties. In particular, the mitochondrial ATPases in their membrane-bound state are noted for their

sensitivity to the antibiotic oligomycin (Pedersen, 1975; Penefsky, 1974). This sensitivity, conferred upon the F_1-ATPase by integral membrane components, is lost when the F_1-ATPase is solubilized (Pedersen, 1975). The soluble enzyme is noted for extreme cold lability (Penefsky and Warner, 1965) and a high molecular weight (380,000 daltons) (Pedersen, 1975).

Recently, workers from two laboratories have reported that the ATPase activity of sonicated corn mitochondria is not inhibited by oligomcyin (Jung and Hanson, 1973; Sperk and Tuppy, 1977). In addition, the solubilized form of the corn ATPase was shown to be stable in the cold, and of low molecular weight (40,000 to 60,000 daltons) as demonstrated by gel filtration (Sperk and Tuppy, 1977). The workers suggested that mitochondria of corn, and of the other monocotyledons tested, may possess a unique energy-transducing ATPase system (Sperk and Tuppy, 1977).

In this Appendix I report on the results of experiments designed to test this hypothesis. Submitochondrial particles, which are low in contaminating soluble enzymes, were prepared from sonicated corn mitochondria and were shown to be inhibited by oligomycin in the normal fashion; the remaining soluble fraction contained an ATPase that resembled F_1-ATPase.

C. Results and Discussion

The whole sonicate from corn mitochondria showed an ATPase activity that was only partially inhibited by oligomycin (Table 19, expt. 1). However, the particulate fraction (submitochondrial particles) prepared from the sonicate showed an ATPase activity that was more than 95% inhibited by low levels of oligomycin. Since the mitochondrial ATPases are genera-

lly associated with a trypsin-sensitive inhibitor polypeptide (Penefsky, 1974), I tried treating corn submitochondrial particles with trypsin. The treatment produced a 3-fold activation of the ATPase, which remained sensitive to oligomycin. My experiments with submitochondrial particles prepared from peas (Chapter V) showed that they were also very similar to typical submitochondrial particles from rat liver and yeast mitochondria, and to those from corn. They were inhibited by oligomycin when fully separated from soluble proteins and whole mitochondria, and they were activated by trypsin.

The soluble fraction remaining as a supernatant layer after the high-speed centrifugation was also found to contain an ATPase activity. This activity could be a nonspecific phosphatase, an F_1-ATPase that was solubilized during sonication, or the novel low molecular weight ATPase observed by Sperk and Tuppy (1977). With a 3 mM β-glycerophosphate as a substrate I was unable to detect any hydrolysis activity with the soluble fraction. This ruled out a typical nonspecific phosphatase as a component of the soluble fraction. I found that the ATPase activity of the soluble fraction was stimulated 2-fold by 0.1 M NaCl; this stimulation was similar to that for the soluble ATPase of pea mitochondria (Chapter III). To further determine whether the fraction was actually solubilized F_1-ATPase, I added 0.5 mM NaN_3 to the assay medium. Azide, which normally inhibits metallo-enzymes (Dixon and Webb, 1962), is also an inhibitor of F_1-ATPases (Pedersen, 1975) including that from peas (Chapter III). The soluble fraction was about 90% inhibited by 0.5 mM NaN_3 in the presence of 0.1 M NaCl; this inhibition is similar to that of the soluble pea enzyme under the same conditions (Chapter VI).

The soluble fraction was found to be stable to freezing at -40°C, and

to prolonged exposure to cold treatments at 0°C. This finding is in direct contrast to that observed with the purified ATPase from pea mitochondria and with other soluble F_1-ATPases (Penefsky, 1974), but it confirmed the experiments of Sperk and Tuppy (1977) with the partially purified corn enzyme. A variety of compounds have been found to stabilize the mammalian F_1-ATPases to low temperature, including the F_1-ATPase inhibitor polypeptide, the protein-phospholipid complex F_o, and mitochondrial phospholipids (Penefsky and Warner, 1965). The partial inhibition by oligomycin of the ATPase activity of the soluble fraction suggests that some of the F_1-ATPase might have complexed with an F_o-type protein-phospholipid.

If inhibitor polypeptide was stabilizing the enzyme, then trypsin treatment should result in increased activity and the appearance of cold-lability. My results show that trypsin did increase ATPase activity, but did not enhance cold lability (Table 19, Expt. 2). In this experiment I carried out sonication and centrifugation at 20°C to minimize any loss of F_1-ATPase caused by cold. The high activity of the whole sonicate and soluble fractions is in agreement with my findings on pea submitochondrial particles that ATPase activity of submitochondrial particles was stimulated by being kept at temperatures above 0°C (Chapter V).

The soluble fraction, although it possessed some catalytic properties that were similar to F_1-ATPases, can not be definitely classified. While it may be found to be a completely separate ATPase enzyme, it is likely either a complex of F_1-ATPase with some cold-protecting factor or a depolymerized form of the enzyme which is either capable of rapid reassociation under assay conditions, or which has catalytically active

Table 19. ATPase activity of submitochondrial particles and soluble fractions from corn mitochondria.

Material	Treatment	Rates of Pi release (Units/mg protein) Control	+ Oligomycin
Expt. 1			
Whole sonicate		0.14	0.09
Submitochondrial particles		0.22	n.d.
Trypsinized submitochondrial particles		0.75	n.d.
Soluble fraction		0.12	0.10
	0.1 M NaCl	0.27	0.18
	0.5 mM NaN_3 + 0.1 M NaCl	0.02	
	3 mM β-glycerophosphate as substrate	n.d.	
Expt. 2			
Whole sonicate		0.74	0.24
Submitochondrial particles		0.96	n.d.
Soluble fraction		0.41	
Trypsinized soluble fraction		0.73	
	1 hr at 25°C	0.53	
	1 hr at 0°C	0.45	

Assay conditions as in Table 1. In Expt. 2 sonication and centrifugation were done at 20°C to prevent any possible cold degradation of F_1-ATPase that had been solubilized.

A blank space indicates test was not made. The entry n.d. means activity was not detectable (less than 5% of control rates).

subunits.

Finally, I would like to comment on the widespread use of whole sonicate preparations to investigate plant mitochondrial ATPase (Blackmon and Moreland, 1971; Jung and Hanson, 1973; Jung and Laties, 1976; Sperk and Tuppy, 1977). These preparations can be misleading since they not only contain two distinct forms of the ATPase (soluble and membrane-bound), but also contain adenylate and nucleotide kinases, which confuse the results of substrate specificity studies. In addition they may contain other enzymes that react with nucleotides or assay components. Submitochondrial particles, which require little additional effort to prepare, are less subject to contamination and provide more informative results.

References

Abrams, A. and Smith, J.B. (1974) The Enzymes (3^d ed.) 10, 395.

Blackmon, W.J. and Moreland, D.E. (1971) Plant Physiol. 47, 532.

Dixon, M. and Webb, E.C. (1962) Enzymes (2^d ed.) Longmans, London pp. 337-340.

Jung, D.W. and Hanson, J.B. (1973) Arch. Biochem. Biophys. 158, 139.

Jung, D.W. and Laties, G.G. (1976) Plant Physiol. 57, 583.

Pedersen, P.L. (1975) Bioenergetics 6, 243.

Penefsky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694.

Sperk, G. and Tuppy, H. (1977) Plant Physiol. 59, 155.