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University of Alberta

Structural and Functional Characterization of Chicken Apolipoprotein A-I

by Robert Scott Kiss 

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

Department of Biochemistry

Edmonton, Alberta

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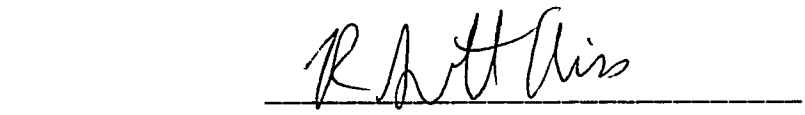

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Structural and Functional Characterization of Chicken Apolipoprotein A-I submitted by Robert Scott Kiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Understanding the relationship between structure and function of apolipoprotein (apo) A-I is necessary to elucidate the beneficial aspects of apoA-I in preventing atherosclerosis. Towards this goal, chicken apoA-I was chosen for study since, unlike human apoA-I, it was found to be monomeric in the lipid-free state, a characteristic that facilitates structural and functional study. Data collected with near- and far-UV circular dichroism experiments support a model in which lipid-free chicken apoA-I is organized as an amphipathic α-helix bundle. Chicken and human apoA-I have similar lipoprotein binding properties. In mouse peritoneal macrophages, human apoA-I showed a greater ability to promote cholesterol efflux than chicken apoA-I, while chicken and human apoA-I were shown to have comparable abilities to promote cholesterol mobilization in human skin fibroblasts. Similarities between chicken apoA-I and mammalian apoE, as well as the identification of a region of chicken apoA-I which resembles the receptor binding region of apoE, prompted the assessment of potential receptor binding properties of chicken apoA-I. Two independent assays provided evidence that chicken apoA-I can interact with the human low density lipoprotein receptor. To investigate the requirement of the presence of particular residues for high affinity receptor binding, we cloned and bacterially synthesized chicken apoA-I, producing a recombinant protein physically and functionally indistinguishable from plasma-derived chicken apoA-I. Mutations in the apoE-like region of chicken apoA-I (Glu157Arg/Glu158Lys) were created and showed that the double mutant of chicken apoA-I had enhanced receptor

binding properties as compared to the wild-type protein. A chimeric protein was constructed and synthesized where the 21 terminal residues of the fifth helix of *M. sexta* apolipophorin-III were substituted with residues 131-151 of human apoE. This chimeric construct exhibited physicochemical and functional characteristics of apoLp-III and receptor binding characteristics of apoE. Therefore, residues 131-151 were capable of receptor binding in the context of a functional helix bundle exchangeable apolipoprotein. In summary, chicken apoA-I can serve as a model of apoA-I in further structure/function studies for understanding the role of apoA-I in the prevention of atherosclerosis, and assist in the understanding of apolipoprotein:lipoprotein receptor interactions.

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On a personal note, I am very grateful to my family. Their quiet yet unwavering support has motivated me to finish a very long race. My family

means a lot to me, although I didn't tell them that nearly often enough over the past six years.

And to Iris, my wife and love of my life, I owe everything. Ich habe dich ganz doll lieb.

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Abbreviations

ACAT	acylCoA:cholesterol acyltransferase
ANS	8-anilino-1-naphthalene-sulfonic acid
apo	apolipoprotein
BSA	bovine serum albumin
CD	circular dichroism
CE	cholesteryl ester
DMEM	Dulbecco's modified Eagle's medium
DMPC	dimyristoylphosphatidylcholine
DPPC	dipalmitoylphosphatidylcholine
DSA	DOXYL stearic acid
FAFA	fatty acid-free albumin
FBS	fetal bovine serum
HDL	high density lipoprotein
HPLC	high performance liquid chromotography
IPTG	isopropyl β-D-thiogalactopyranoside
LCAT	lecithin:cholesterol acyltransferase
LDLp	low density lipophorin
LDL	low density lipoprotein
LPC	lysophophatidylcholine
LPDS	lipoprotein deficient serum
PLC	phospholipase C
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SFM	serum free medium
SR-BI	scavenger receptor BI
TAG	triacylglycerol
TLC	thin layer chromotography
TFE	trifluoroethanol
VLDL	very low density lipoprotein

Chapter 1

Introduction to apolipoproteins and their role in atherosclerosis

1.1 Atherosclerosis

Atherosclerosis is the number one cause of death in industrialized countries and accounts for 50 % of all mortality in the USA, Europe and Japan (1). This disease is the result of an excessive inflammatory-fibroproliferative response to the injury of arterial wall fibroblasts and smooth muscle cells. Although there are many risk factors for developing atherosclerosis, such as genetic predisposition, smoking, diabetes, obesity or high fat/cholesterol diet, atherosclerosis is also a disease of lipid metabolism. High levels of circulating fat and cholesterol contribute to the development of atherosclerosis. Therefore, the maintenance of proper lipid levels in the blood and the homeostasis of lipid levels in tissues is essential for prevention of atherosclerosis.

1.2 Lipoproteins

Plasma lipoproteins are organized complexes of lipids and protein, responsible for carrying hydrophobic dietary and endogenous lipids through the aqueous environment of the blood. Lipoprotein particles have a common structure of a hydrophobic neutral lipid core (triacylglycerol (TAG) and cholesteryl ester) surrounded by a surface monolayer of amphipathic lipids (phospholipid and unesterified cholesterol) and proteins, termed apolipoproteins. Lipoproteins are classified according to their density, based on their lipid content, and are usually separated from one another by isopycnic gradient ultracentrifugation. Chylomicrons (density ~ 0.95 g/mL), the largest and richest in TAG, are secreted by the intestine after a meal and are responsible for transport of dietary lipids. Very low density lipoproteins (VLDL, density < 1.006 g/mL) are

secreted by the liver for transport of TAG to peripheral tissues for storage or energy utilization. Low density lipoproteins (LDL, density range 1.019 - 1.063 g/mL), remnants of VLDL that have been acted upon by plasma lipases depleting the TAG-rich core and leaving a cholesteryl ester-rich particle, deliver cholesterol to the peripheral tissues. High density lipoproteins (HDL) are small, dense particles, containing phospholipid, cholesterol and cholesteryl ester, which can vary in their size and structure. In humans, there are HDL_1 (density < 1.063 g/mL), HDL_2 (density range 1.063 - 1.12 g/mL), HDL_3 (density range 1.12 - 1.21 g/mL) and pre-β HDL (density > 1.21 g/mL) the latter so defined by its pre-β mobility by agarose gel electrophoresis. HDL is believed to play a role in transport of cholesterol from the peripheral tissues back to the liver.

1.2.1 "Bad" versus "good cholesterol"

LDL has been termed "bad cholesterol" because the cholesterol associated with LDL tends to be proatherogenic. On the other hand, HDL has been termed "good cholesterol" because the cholesterol associated with HDL tends to be destined for return to the liver and thus be anti-atherogenic.

LDL, a cholesteryl ester-rich lipoprotein, contains as a structural component apo B-100 which is also a ligand for lipoprotein receptors such as the LDL receptor and the LDL receptor-related protein (LRP). LDL's role is to deliver cholesterol to tissues that require cholesterol. Brown and Goldstein elucidated the receptor-mediated pathway of LDL endocytosis (reviewed in (2)). LDL, via apoB-100, interacts with LDL receptors on the surface of cells. The LDL receptors are clustered into specialized structures called "coated pits" or invaginations on the cell surface. The LDL is internalized into an

endosome and the interior of the endosome becomes acidified, disrupting the LDL:LDL receptor charged interactions, allowing for the LDL receptors to be recycled to the cell surface. Further decreases in pH in the endosome and fusion with a lysosome result in the degradation of apoB-100 to amino acids and the hydrolysis of cholesteryl ester to free cholesterol. The cholesterol can then be utilized by the cell or resterified by the intracellular enzyme acyl CoA:cholesterol acyltransferase (ACAT) and stored intracellularly as a cholesteryl ester droplet. Influx of cholesterol can result in reduction of the activity of endogenous cholesterol biosynthetic enzymes and a decrease in synthesis of new LDL receptors. The cell can thereby regulate intracellular levels of cholesterol. In conditions where peripheral tissues have high levels of cholesterol, plasma LDL will no longer be taken up by cells, and the LDL will be subject to oxidation and accumulation at sites of arterial wall injury, leading to atherosclerosis (1).

No pathway exists for the breakdown of cholesterol in peripheral tissues, but cholesterol can be metabolized in other tissues. A pathway can exist whereby cholesterol can be extracted from peripheral tissues and delivered, either directly or indirectly, to the liver or other non-placental steroidogenic tissues for metabolism. Cholesterol in the liver can be either directly secreted into bile or converted to bile acids and then secreted into the intestine for excretion from the body. In steroidogenic tissues, cholesterol can be converted to steroid hormones and utilized by the body. The reverse cholesterol transport pathway is believed to be responsible for the main anti-atherogenic effects of HDL (3). In addition, HDL may be playing a role by preventing oxidation of LDL or reducing the potential atherogenic effects of

oxidized LDL (4-7). HDL may also be inhibiting inflammatory responses to arterial wall injury, preventing atherogenesis (8, 9).

1.3 Apolipoproteins

Apolipoproteins (apo), found as both integral and exchangeable components of lipoproteins, are responsible for many roles in fat transport and metabolism. Apolipoproteins can serve a structural role in lipoprotein structure, a role in modulating enzyme activity, or as a ligand for receptor-mediated uptake of lipoproteins. The apolipoprotein composition of a lipoprotein can determine its metabolic fate. Furthermore, the apolipoprotein content of a lipoprotein can change as the lipid content is altered by lipid transfer proteins and plasma lipases. Thus, lipoprotein metabolism is dependent on the dynamics of lipid transfer and metabolism as well as apolipoprotein composition.

The apolipoproteins and their functions are described in **Table 1-1**. Most exchangeable apolipoproteins are composed of repeats of 11 amino acid residues. Predominantly, these repeats are predicted to assume class A amphipathic α-helical conformations (10-12). The amphipathic nature of the α-helices that compose exchangeable apolipoproteins, that is helices having a hydrophobic and a hydrophilic face, are proposed to be the functional element necessary for lipid binding. On the other hand, exchangeable apolipoproteins, when free in solution, must sequester the hydrophobic faces of the amphipathic α-helices. The three-dimensional structures of three exchangeable apolipoproteins, apolipophorin III (apoLp-III; *Manduca sexta* apoLp-III, *Locusta migratoria* apoLp-III) and the N-terminus of human apoE, have been determined (13-16). All three proteins exhibit helix bundle structures such that

the hydrophobic faces are sequestered in the interior of the bundle and hydrophilic faces can interact with the aqueous environment. Upon introduction of a lipid surface, the helix bundle has been postulated to undergo an opening about hinge regions, exposing the hydrophobic interior for binding to lipid. The dual nature of these proteins, to be soluble in the absence of lipid and stably bind to lipid, is a unique aspect of exchangeable apolipoproteins.

1.3.1 Apolipoprotein E

ApoE is a glycosylated exchangeable apolipoprotein of 34 kDa (299 amino acids). It is composed of two independently folded domains joined by a short linking region (17). The C-terminal domain (residues 216-299) has been shown to be responsible for high affinity lipid binding. The N-terminal domain (residues 1-191) adopts a water-soluble, globular conformation and forms a 4-helix bundle of amphipathic α-helices, as determined by the crystal structure (16). ApoE is capable of interacting with lipoprotein receptors such as the LDL receptor or LRP. Helix 4 of the N-terminal domain has been implicated in containing the residues responsible for receptor binding (18). The overall amphipathic nature as well as the positioning of positively charged residues is necessary for effective apoE receptor binding. Eight basic amino acids between residues 136 and 150 are thought to interact with the cysteine-rich repeat regions of the LDL receptor, which are the functional receptor binding units. The cysteine residues in each repeat form 3 disulfide bonds which are essential for ligand binding. As well, a calcium ion is coordinated by four conserved acidic residues and two nearby carbonyl oxygens to maintain the overall structure of the repeat (19). The crystal structure of LDL receptor

repeat 5 shows that most of the conserved acidic residues are involved in coordinating the calcium ion and are not free to interact with the positive charges on the receptor binding region of apoE (19). It was suggested that other conserved acidic residues could be involved in ligand binding, that apoE could be interacting at the interface between repeats, or that the conformation of the repeat could change upon ligand binding. Elucidation of the structure of the complex of a ligand bound to a binding repeat would resolve this issue.

The importance of apoE can be interpreted from the rapid progression of atherosclerosis in apoE knockout mice (20, 21). The ability of apoE to redistribute among lipoproteins in response to fat metabolism and bind to multiple lipoprotein receptors is crucial for its role in lipoprotein metabolism.

1.3.2 Apolipoprotein A-I

ApoA-I, a 28 kDa exchangeable apolipoprotein, is a key structural component of high density lipoprotein (HDL), and is an essential activator of the plasma enzyme, lecithin:cholesterol acyltransferase (LCAT). As well, apoA-I plays a role in mediating cholesterol efflux in the reverse cholesterol transport pathway (3, 22). Recently, it was shown that apoA-I is a ligand for a class B scavenger receptor (SR-BI) and, as such, apoA-I can mediate cholesterol delivery to the liver and non-placental steroidogenic tissues (23-26). In fact, HDL apolipoprotein turnover may be regulated by obesity or leptin signaling (27). Importantly, the plasma concentration of apoA-I is inversely correlated with the incidence of atherosclerosis (3). The discovery of a naturally-occurring mutation in human apoA-I (apoA-I_{Milano}) which provides increased protection from atherosclerosis (28), highlights the need to understand how

apoA-I interacts with lipid surfaces, cell surfaces and receptors and may help gain insight into the role of apoA-I in cholesterol homeostasis.

Human HDL has apoA-I as its main structural component, with lesser amounts of other exchangeable apolipoproteins, such as apoA-II, apoE, apoC-I, II, and III. ApoA-I performs the main functions of HDL, facilitating formation of HDL, stabilizing the lipid complex, and mediating uptake and delivery of cholesterol (reviewed in (3)). **Figure 1-1** shows a generalized scheme of the functions of apoA-I. ApoA-I can interact with the cell surface, extracting cholesterol from the cell membrane via a diffusion mechanism or a mediated mechanism. ApoA-I can form a complex with phospholipid and the extracted cholesterol to form a discoidal pre-β-HDL. Through the action of LCAT, the cholesterol is esterified to produce cholesteryl ester, which will sequester itself in the growing core of the pre-β HDL particle due to its hydrophobicity. With the continued efflux of cholesterol from peripheral tissues and the conversion to cholesteryl ester by LCAT, the HDL will eventually become spherical with a core of cholesteryl ester. The HDL can then either exchange the cholesteryl ester with the TAG of VLDL or LDL particles through the action of a cholesteryl ester transfer protein or deliver the cholesterol directly to the liver or steroidogenic tissues via SR-BI. Cholesterol delivered to the liver can be secreted directly into bile or converted to bile acids and excreted from the body, or delivered to steroidogenic tissues to be metabolized to steroid hormones and utilized. The result is a net reduction of cholesterol in the body. The return of cholesterol to the liver for further metabolism (the reverse cholesterol transport pathway) is likely responsible for the beneficial effects of apoA-I and HDL in preventing heart disease.

The main role of apoA-I is believed to be in reverse cholesterol transport. However, Jolley *et al.*, using apoA-I knockout mice, suggested that reverse cholesterol transport still occurred normally despite the absence of apoA-I (29). In contrast, Stein *et al.* showed that there was impaired reverse cholesterol transport in apoA-I knockout mice (30, 31). It is clear that apoA-I is required for cholesteryl ester accumulation in steroidogenic cells and for normal steroid production (31). In addition, in apoE-deficient mice, overexpression of apoA-I was capable of suppressing atherosclerosis progression (32). ApoA-I levels are correlated with an anti-atherogenic effect and further study of apoA-I is necessary to completely elucidate its role in preventing atherosclerosis.

1.3.3 Structure of apolipoprotein A-I

As HDL is formed, apoA-I must have the ability to change conformation upon going from a lipid-free state to a pre-β HDL-bound protein, and subsequently to a spherical HDL-bound protein. It has been suggested that apoA-I forms an amphipathic α-helix bundle in the lipid-free state, such that the hydrophobic faces are sequestered in the core of the bundle and the hydrophilic faces are exposed to the aqueous environment (33-36). The structures of three other exchangeable apolipoproteins, apolipophorin III (apoLp-III; *Manduca sexta* apoLp-III, *Locusta migratoria* apoLp-III) and the N-terminus of human apoE, have been determined and all exhibit helix bundle structures (13-16). Similar to human apoE, apoA-I has been proposed to have two domains (34): an N-terminal domain, which is folded and globular, and a C-terminal domain which has an increased affinity for lipid surfaces. It is postulated that upon lipid

interaction, the helix bundle opens up about hinge regions, exposing the hydrophobic core to the lipid surface. The arrangement of helical segments of apoA-I on pre-β HDL-disc structures and spherical HDL are different , but have not been fully elucidated. Researchers had originally speculated that apoA-I may bind to disc complexes with the helical segments parallel to the acyl chains of the phospholipids ("picket-fence model") (37, 38). Fourier transform infrared spectroscopy experiments performed on human apoA-I:phospholipid discs supported the picket fence model (39). Recently, the three dimensional structure of N-terminal truncated human (Δ1-43)apoA-I has been elucidated at 4 Å resolution (40). The N-terminal truncated apoA-I, although in a lipid-free form, simulates a lipid-bound apoA-I (36, 41, 42). From the structure, the authors postulated that apoA-I formed an antiparallel dimer extended "belt" around the periphery of bilayer disc complexes or spherical lipoproteins (40). This model fails to address the possibility of the reversible interaction of apoA-I with lipid surfaces and the possibility of an odd number of apoA-I proteins binding to the lipid surface. Important questions remain about the structure of full-length apoA-I in the absence and presence of lipid, and the conformational changes that occur upon lipid binding.

1.4 Cholesterol efflux

Apolipoprotein- or HDL-mediated cholesterol efflux is a mechanism whereby peripheral cells can rid themselves of excess cholesterol. Cholesterol efflux is believed to be the rate-limiting step of the reverse cholesterol transport pathway. Studies have suggested that HDL or apoA-I can promote cholesterol efflux either by a passive or an active process. Passive aqueous diffusion of

membrane free cholesterol ond phospholipid to the acceptor particle can occur following a concentration gradient (43). Microsolubilization involves association of lipid-free (poor) apoA-I with the cell membrane and facilitates membrane free cholesterol efflux by solubilizing cholesterol and phospholipid (44-46). On the other hand, studies have shown that efflux of cholesterol is an active process by the cell since the addition of golgi apparatus disruptors and protein kinase C inhibitors can reduce the rate of efflux (47, 48). Some cells are resistant to cholesterol efflux (49), and mild protease treatment of the surface of the cells inhibits efflux (50), suggesting that cell surface proteins are necessary for efflux (reviewed in (51)). HDL and apoA-I are both potent stimulators of mediated cholesterol efflux, but other apolipoproteins (52), as well as synthetic amphipathic α-helical peptides, are capable of promoting cholesterol efflux (53). The ability to promote efflux may be based on the number and hydrophobicity of amphipathic α-helices, and in this respect, apoA-I is a potent mediator of cholesterol efflux.

It was recognized in 1985 that some tissues displayed the ability to selectively uptake cholesteryl ester from HDL (54). More recently, some receptors have been reported to play a role in HDL metabolism (55). In particular, HDL binds to SR-BI saturably, with high affinity, and SR-BI is responsible for the selective uptake of CE with no uptake of apolipoprotein (23). As well, it has been shown that apoA-I is likely the physiological ligand for SR-BI binding (23, 24). SR-BI has also been implicated in cholesterol efflux (56). SR-BI may merely be a docking site on cells and the cholesterol state of the cell may determine the direction of net cholesterol transfer. However, knockout studies of SR-BI in mice showed that steroidogenic tissues

were depleted of cholesterol, indicating the importance of SR-BI in delivery of cholesterol in steroidogenic tissues (25). Therefore, apoA-I also plays a role in cholesterol delivery, by serving as a ligand for the SR-BI-mediated selective uptake of cholesteryl ester.

1.5 Comparison of chicken and human apoA-I

In the lipid-free state, human apoA-I is known to have concentration-dependent self-association properties (57-61). In 1978, Swaney demonstrated, by crosslinking studies, that chicken apoA-I is monomeric in the lipid-free state (62). This unique feature could be advantageous in the study of the structural and functional properties of apoA-I. Human apoA-I is composed of 243 amino acids while chicken apoA-I has 240 amino acids. Chicken apoA-I shows 48% sequence identity and 66% sequence similarity with human apoA-I (**Figure 1-2**). From sequence alignment, chicken and human apoA-I are postulated to have similar structure based upon the organization of the primary sequence into 22 residue repeats, putatively forming amphipathic α-helical segments (10, 11). These helical segments are also usually punctuated by proline residues (12). It is likely that based on the sequence similarity chicken apoA-I would possess similar properties to human apoA-I and that its monomeric nature would facilitate further study.

1.6 Lipoprotein metabolism in chickens

Chickens have a similar lipoprotein system for cholesterol homeostasis as humans. In laying hens, the most abundant lipoprotein is VLDL which serves as direct precursor for egg production. In the rooster, the most abundant

lipoprotein is HDL, which is thought to serve a similar role as HDL in mammals. Chickens have lipoprotein receptors specific for oocytes, involved in the uptake of major yolk precursors, and specific for somatic cells, which are involved in the regulation of cellular cholesterol homeostasis. These receptors resemble VLDL receptors, LDL receptors and LRP (63, 64). The presence of a non-functional VLDL receptor, the result of a naturally-occurring mutation in chickens, results in severe hyperlipidemia and premature atherosclerosis in chickens (64), similar to LDL receptor deficiencies in humans (such as familial hypercholesterolemia). Chickens possess the lipoprotein vitellogenin (reviewed in (65)), as do most egg producing species, that serves as a substrate lipoprotein for oocyte synthesis by binding to the oocytic LDL and LRP receptors (66). This lipoprotein contains 75% phospholipids (of which 74% is phosphatidylcholine and 16% is phosphatidylethanolamine) and 25% other lipids (of which 63% is triacylglycerol and 16% is cholesterol and cholesteryl ester). Of particular interest is the fact that mammalian apoE can bind to these same oocytic chicken receptors (67). Both apoE and lipovitellin-1, the receptor binding protein component of vitellogenin, require lipid association for functional receptor binding (67, 68), which suggested that a similar receptor:ligand binding interaction had evolved in the chicken and mammalian species. An interesting feature is that chickens lack apolipoprotein E (apoE), a ligand for the mammalian LDL receptor and LRP. ApoE plays a key role in cholesterol metabolism, and it is not clear how chickens cope without this apolipoprotein, as vitellogenin does not play a major role in cholesterol homeostasis (only 4% of associated lipid is cholesterol).

1.7 Comparison of chicken apoA-I and mammalian apoE

Previous studies have shown that chicken apoA-I, unlike human apoA-I which is expressed only in liver and intestine, is expressed in many tissues, including skin, liver, intestine, kidney, lung, muscle, nervous and steroidogenic tissues. Chicken apoA-I exhibits a tissue expression pattern similar to mammalian apoE (69, 70). In the case of nerve injury, both chicken apoA-I in chicken optic nerve, and rat apoE in rat optic nerve, become highly expressed in response to injury (71). It was believed that apoE in mammals and chicken apoA-I may play a role in nerve regeneration. Since chickens lack apoE, it was postulated that chicken apoA-I could be assuming some of the functions of an apoE (72). Chicken apoA-I and human apoE show very little overall sequence similarity (< 10%). However, it was demonstrated that a specific region of chicken apoA-I shows a high degree of similarity to the receptor binding region of apoE (72). Upon alignment, the "apoE-like" region of chicken apoA-I (residues 146-165) and the receptor binding region of human apoE (residues 131-150) show 50% sequence identity and 65% sequence similarity. In addition, the apoE-like region of chicken apoA-I is predicted to maintain positive charge and overall amphipathic α-helical character, similar to the receptor binding region of apoE. This striking resemblance, taken together with the similarities between chicken apoA-I and mammalian apoE, has brought about the hypothesis that chicken apoA-I may be performing the physiological role of apoE in the regulation of cholesterol homeostasis in the chicken.

1.8 Thesis objectives

1. Characterize physicochemical properties of chicken apoA-I by a variety of biophysical and spectroscopic techniques.

2. Characterize key functional properties of chicken apoA-I, namely lipid binding and cholesterol efflux ability.

3. Develop a system to bacterially synthesize chicken apoA-I for production of chicken apoA-I mutants by site-directed mutagenesis to carry out structure/function analysis of chicken apoA-I.

4. Characterize functional receptor binding properties of chicken apoA-I.

5. Characterize the receptor binding properties of residues 131-151 of human apoE in the context of another helix bundle exchangeable apolipoprotein.

The properties of chicken apoA-I will be investigated, not only by defining its physical and functional properties in comparison to human apoA-I, but also by investigating the receptor binding properties of chicken apoA-I in comparison to human apoE. As well, the receptor binding properties of residues 131-151 of human apoE will be investigated. Chicken apoA-I is a unique apolipoprotein, but it is hoped that its application to understand the physiological role of apolipoproteins as well as the basic structure/function

properties of exchangeable apolipoproteins will aid in the understanding of the role of apolipoproteins in preventing atherosclerosis.

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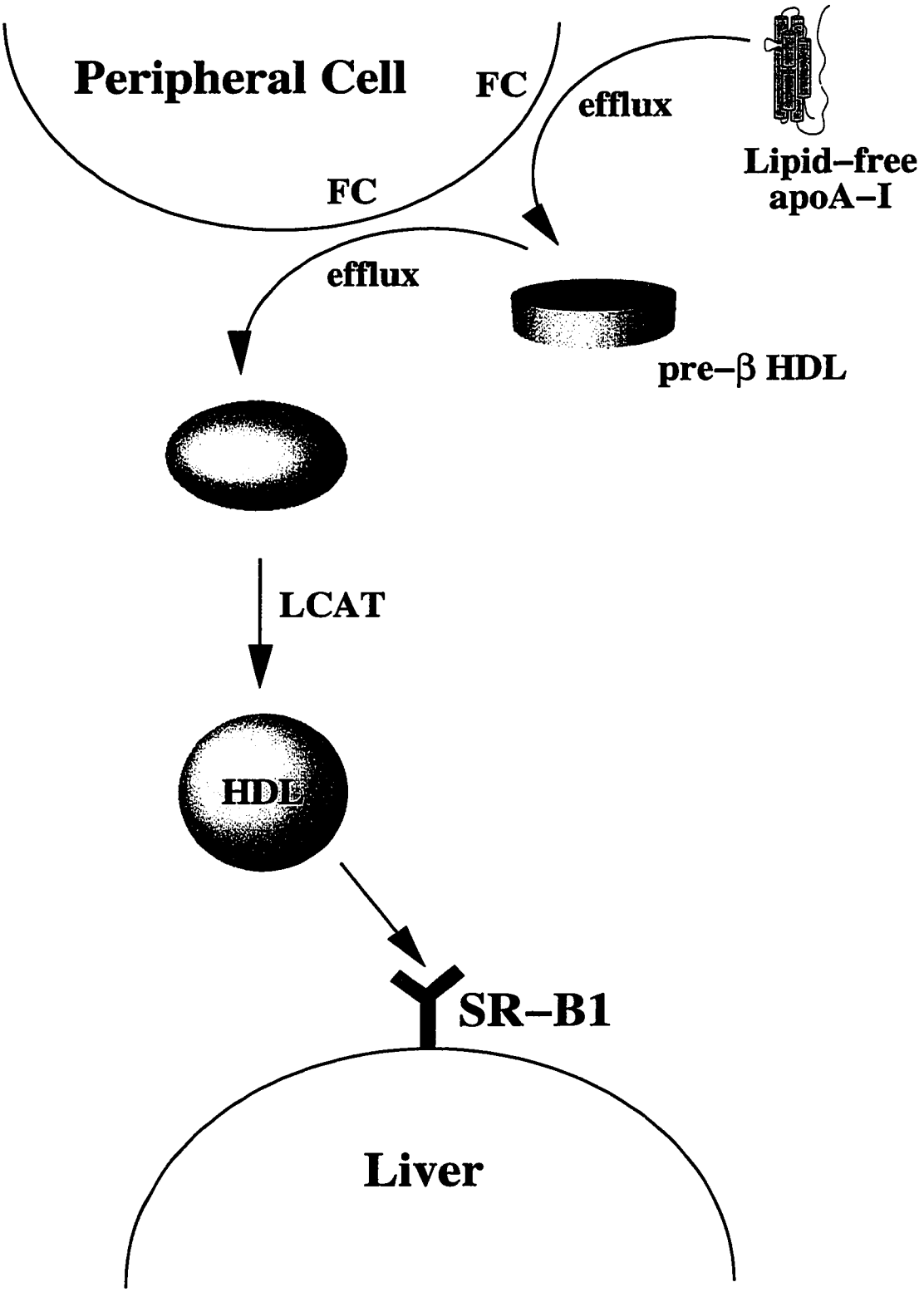
Apolipoprotein	Lipoprotein distribution	Function(s)
ApoA-I	All HDL classes	Cholesterol efflux; LCAT activation
ApoA-II	HDL_1, HDL_2, HDL_3	Inhibits apoA-I activity
ApoB-48	Chylomicrons	Chylomicron secretion
ApoB-100	VLDL, LDL	VLDL secretion; LDL receptor ligand
ApoC-I	VLDL, HDLs	Unknown
ApoC-II	VLDL, HDLs	Activates lipoprotein lipase
ApoC-III	VLDL, HDLs	Inhibits apoC-II activity
ApoE	VLDL, HDL_1	Cholesterol efflux; LDL receptor ligand

Table 1-1: Human plasma apolipoproteins. The major human apolipoproteins, their lipoprotein distribution and their respective functions are outlined. There are other lipoprotein-associated proteins which have not been included in this list because their function is uncertain or not associated with their lipoprotein-bound forms.

Figure 1-1: Schematic diagram representing the generalized functions of apoA-I. Lipid-free apoA-I, represented as a helix bundle, interacts with the surface of a peripheral cell causing efflux of phospholipid and cholesterol (FC) forming a discoidal pre-β HDL particle. The pre-β HDL particle can promote further efflux of cholesterol. The plasma enzyme lecithin:cholesterol acyltransferase (LCAT) converts cholesterol into cholesteryl ester which will sequester itself in the core of the HDL particle. Further efflux and LCAT activity will result in a spherical HDL particle. HDL can then travel through the blood to either the liver or steroidogenic tissues for SR-BI-mediated selective uptake of cholesteryl ester. These steps form the reverse cholesterol transport pathway.


Peripheral Cell
FC
FC
efflux
Lipid–free
apoA–I
efflux
pre–β HDL
LCAT
HDL
SR–B1
Liver

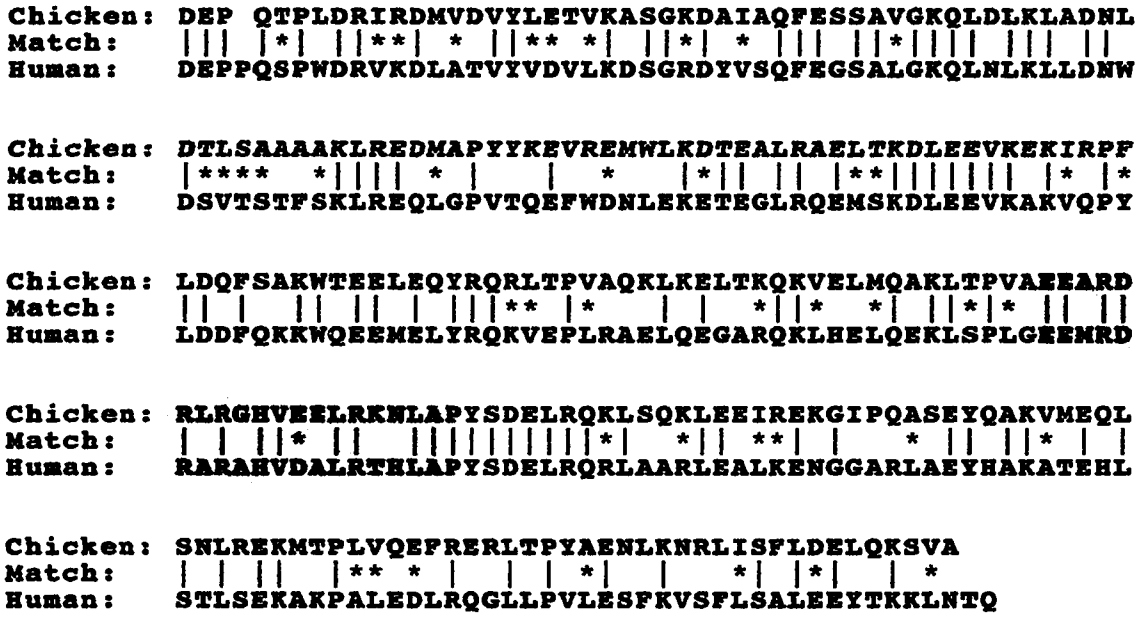


Figure 1-2: Sequence alignment of human and chicken apolipoprotein A-I. The aligned sequence of human apoA-I (243 amino acids) and chicken apoA-I (240 amino acids) showed overall identity of 48 % (115/240) and overall sequence similarity of 66 % (159/240). One letter amino acid codes were used to identify the amino acids. Conservative substitutions were accepted as S-T; M-I-L-V-A; D-E; K-R; F-Y-W. The yellow box highlights residues 146 to 165 of chicken apoA-I which form the apoE-like region.

Chapter 2

Physical Characterization of chicken apolipoprotein A-I: amphipathic α-helix bundle organization of lipid-free chicken apolipoprotein A-I

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2.1 Introduction

Apolipoprotein (apo) A-I is a 28 kDa exchangeable apolipoprotein that plays an important role in plasma cholesterol homeostasis (1). ApoA-I activates the enzyme lecithin:cholesterol acyl transferase and serves as the major structural component of high density lipoprotein (HDL). As a lipid-poor protein, apoA-I mediates removal of cholesterol from peripheral tissues, facilitating its return to the liver via the reverse cholesterol transport pathway (2, 3). This pathway is believed to be responsible for the inverse correlation between HDL levels and risk of cardiovascular disease (4).

Recently, the three dimensional structure of N-terminal truncated human (Δ1-43)apoA-I has been elucidated at 4 Å resolution (5). The structure clearly depicts the preponderance of amphipathic α-helices in the protein and suggests a possible molecular organization of lipid-bound apoA-I. It is conceivable that antiparallel dimers of apoA-I form an extended "belt" around the periphery of spherical lipoproteins or bilayer disc complexes with hydrophobic regions of the protein in direct contact with the lipid surface. At the same time, important questions remain about the conformation of full length apoA-I in the absence of lipid and the nature of lipid binding-induced structural adaptations.

Several approaches have been used to study the structure-function relationship of human apoA-I, including spectroscopic studies (6, 7), deletion mutants (8), N- and C-terminal truncation (9-12), and helix-swapping mutagenesis (13). Other approaches include study of HDL recombinants containing only apoA-I (14-16). In spite of these efforts, the molecular basis for dual existence of apoA-I in lipid-poor and lipid-associated states remains

unclear. The characteristic property of human apoA-I to oligomerize in the absence of lipid over a broad concentration range (17-19) suggests hydrophobic lipid binding sites in this protein may be protected from solvent exposure through formation of intermolecular helix-helix contacts.

ApoA-I from chicken plasma shares 48% sequence identity and 66% sequence similarity with human apoA-I (as determined by SEQSEE sequence homology program; 20). Unlike human apoA-I, chicken apoA-I is monomeric in the lipid-free state over a broad concentration range and comprises > 95% of the protein component of chicken HDL (21, 22). Circular dichroism (CD) studies reveal lipid-free chicken apoA-I possesses an exceptionally high α-helix content (22, 23) while sequence analysis indicates helical segments are amphipathic (24). These features suggest that chicken apoA-I is a good candidate for structure-function studies, including study of lipid binding-induced conformational changes. In this regard, chicken apoA-I contains two tryptophan residues, at positions 74 and 107. Both tryptophans are centrally located in the protein sequence and are predicted to reside on the hydrophobic face of distinct amphipathic α-helices.

In the present study, spectroscopic techniques have been used to examine different conformational states of chicken apoA-I. Near-UV CD studies provided evidence for a conformational change upon lipid association while far-UV CD data indicate the protein retains its high content of α-helix secondary structure in either state. Water-soluble and lipid-based quenching agents have been employed to monitor the microenvironment of apoA-I tryptophan residues in different conformational states. The results are consistent with organization of lipid-free apoA-I as a helix bundle.

Furthermore, the data indicate that chicken apoA-I adopts a relatively superficial association with the HDL phospholipid monolayer. The ramifications of these results in terms of the physiological role of apoA-I in lipoprotein metabolism are discussed.

2.2 Experimental Procedures

Materials: 5-, 7-, 10- and 12- DOXYL stearic acid (DSA) were obtained from Aldrich (St. Louis, MO). 8-anilino-1-naphthalene sulfonic acid (ANS), dimyristoyl-phosphatidylcholine (DMPC), and trifluoroethanol (TFE) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Lipoproteins and apolipoproteins: Chicken HDL was isolated from chicken blood by sequential ultracentrifugation between the density limits 1.12 and 1.21 g/mL. In order to isolate chicken apoA-I, chicken HDL was lyophilized, extracted repeatedly with chloroform:methanol (3:1, vol/vol), and dried. The resultant protein pellet was assessed for purity by polyacrylamide gel electrophoresis, high pressure liquid chromotography and electrospray ionization mass spectrometry. Human apoA-I was a kind gift of Dr. Gordon Francis and was prepared according to Yokoyama *et al.* (25). ApoA-I/DMPC discs were prepared by the cholate dialysis method (26) with a lipid to protein weight ratio of 2.5 to 1 (w/w). The disc preparation was adjusted to a density of 1.21 g/mL and subjected to ultracentrifugation in a VTi 65.2 rotor at 65,000 rpm for 1.1 h at 4 °C to separate unbound apolipoprotein.

Circular dichroism experiments: A Jasco J-720 spectrapolarimeter (Jasco Inc., Easton, MD) connected to a Epson Equity 386/25 computer was used to obtain circular dichroism (CD) measurements. Samples were kept at constant temperature (25 °C) by a thermostatted cell holder connected to a circulating water bath (Lauda, Westbury, NY). The instrument was calibrated regularly with ammonium *d*-(+)-10-camphorsulfonate at 290.5 and 192 nm and with *d*-(-)-pantoyllactone at 219 nm. The average of ten scans was used to increase signal to noise ratio and smoothing was employed to remove the high frequency noise. Distortion of CD spectra was minimized by keeping the voltage of the photomultiplier below 500 V. The cell used for wavelengths below 250 nm was 0.02 cm (calibrated for cell length). Far-UV spectra were obtained using protein concentrations between 0.5 and 1.0 mg/mL. Near-UV spectra were obtained using a protein concentration of 1.0 mg/mL with a 1.0 cm microcell. Modified Provencher-Glöckner analysis using the Contin program (Version 1.0) gave predictions of secondary structure from the CD data (27), and, as such, may give slightly different values than those previously published (22).

Spectroscopy: Fluorescence spectroscopy was performed on a LS 50 luminescence spectrometer (Perkin Elmer, Beaconsfield, England). All measurements were conducted at 24 °C with the excitation and emission slit widths set at 5 nm. Increasing concentrations of quenchers were added to the protein sample and tryptophan emission spectra were obtained (295 nm excitation). KI solutions contained 1 mM sodium thiosulfate to suppress free

iodide formation (28). A correction for the inner filter effect was taken into account when using acrylamide (29). Concentrations of fatty acid spin labels were determined by electron spin resonance. Fluorescence intensity values were corrected for dilution effects. The effective Stern-Volmer quenching constants (K_{sv}) for aqueous quenchers in lipid-free and lipid-bound states were calculated (29). K_{sv} values were calculated from the equation $Fo/F = 1 + K_{sv}[Q]$, where Fo and F are corrected fluorescence measurements at the emission maximum wavelength in the absence and presence of quencher, respectively, and [Q] is the concentration of quencher. A plot of Fo/F vs. [Q] yields a straight line, whose slope is K_{sv}. (30). Values are average of three independent measurements ± SD. In the case of spin-labelled fatty acids, the Stern-Volmer equation does not apply since the quencher is not uniformly distributed throughout the unit volume (30-32). We have, therefore, computed apparent Stern-Volmer quenching constants (K_{app}) for the purposes of comparison.

8-anilino-1-napthalene-sulfonic acid (ANS) fluorescence emission spectra were obtained in the presence of human and chicken apoA-I (50 μg/mL, 1.8 μM). 250 μM ANS in 10 mM sodium phosphate buffer (pH 7.5) was excited at a wavelength of 395 nm with slitwidths of 5 nm. Since ANS fluorescence in buffer is negligible (33), spectra were recorded in the presence of a minimum 100 fold excess of ANS with respect to protein (mol/mol). Carbonic anhydrase (50 μg/mL, 1.8 μM), a globular, water soluble protein, served as a negative control. Bovine serum albumin (50 μg/mL, 0.7 μM), known to have hydrophobic binding pockets, was employed as a positive control.

Analytical ultracentrifugation: Sedimentation velocity runs were carried out at 20 °C and 50,000 rpm using a Beckman XLI Analytical Ultracentrifuge and Absorbance optics following the procedures outlined in the instruction manual published by the Spinco Business Center of Beckman Instruments, Inc., Palo Alto, CA (1997). Runs were performed for 4 hours during which a minimum of 20 scans were taken. The sedimentation velocity data were analyzed using the Transport Method contained in the Beckman Analysis Program (Optima XL-A/XL-I Data Analysis Software, Version 4.0 Copyright (c) 1997). The program Sednterp (Sedimentation Interpretation Program, Version 1.01) was used to calculate the intrinsic sedimentation constant, $S^{\circ}_{20,w}$, the frictional ratio, f/fo, and the axial ratio, a/b. Sednterp calculates the partial specific volume and degree of hydration from the amino acid composition of the protein using the methods of Cohn and Edsall (34) and Kuntz (35), and also calculates the solvent density and viscosity using known values from physical tables. The density and viscosity of TFE were taken into account for the calculation of $S^{\circ}_{20,w}$ in that solvent, and the partial specific volume and degree of hydration were assumed not to change upon addition of TFE, a finding noted by MacPhee *et al.* (36) in studies of the effect of TFE on the solution behavior of amphipathic peptides.

2.3 Results

2.3.1 Circular dichroism spectroscopy

To evaluate conformational adaptations of lipid-free chicken apoA-I postulated to occur upon lipid binding, near-UV CD spectra were collected under different conditions (**Figure 2-1**). The spectrum of apoA-I in buffer was dominated by contribution from the 7 tyrosines in the protein, giving rise to an extremum at 278 nm. The shoulder at 295 nm can be attributed to the two tryptophans (W74 and W107). Little effect of the 5 phenylalanines in the protein was observed. Spectra of apoA-I bound to HDL or to DMPC disc particles differed significantly from the spectrum of lipid-free apoA-I. In addition to subtle differences in fine structure, the major spectral change corresponded to a large decrease in ellipticity in the region between 260 nm and 290 nm, indicative of a conformational change upon lipid binding (37, 38). As well, extrema for DMPC-bound apoA-I at 263, 270 and 293 nm were similar but slightly blue-shifted compared to HDL-bound apoA-I. This can be attributed to small differences in the environment of some tyrosines and tryptophans, indicating that apoA-I conformations in DMPC- and HDL-bound states are similar, but nonetheless unique.

Far-UV CD spectra of chicken apoA-I in buffer alone or complexed to lipid particles (spherical HDL or DMPC discs) are generally similar, with major troughs at 208 and 222 nm (22). Modified Provencher-Glöckner analysis of spectral data indicates α-helix is the predominant secondary structure component present in the different states, with minor amounts of other conformers (**Table 2-1**). Differences in α-helix content between apoA-I bound to HDL versus DMPC disc particles suggests subtle differences in

apoA-I conformation when associated with these morphologically and compositionally distinct lipid particles. The observation that α-helix remains the predominant secondary structure element in both lipid-free and lipid-associated states is consistent with the hypothesis that amphipathic α-helical segments in lipid-free apoA-I reposition upon lipid interaction, exposing hydrophobic sites which become available for contact with the lipid surface.

2.3.2 Tryptophan fluorescence emission of chicken apoA-I

Fluorescence spectra of apoA-I in buffer display an emission maximum at 331 nm, similar to that observed for DMPC disc complexes or HDL particles (Table 2-1). Compared to the emission wavelength maximum of free tryptophan in aqueous solution (347 nm), apoA-I tryptophan fluorescence emission is blue-shifted in all three states. Thus, in lipid-free and lipid-bound states, W74 and W107 reside in a hydrophobic environment, shielded from the aqueous milieu. Previous fluorescence studies of apoA-I from other species revealed that chicken apoA-I is distinctive in terms of the magnitude of the blue shift in its tryptophan fluorescence emission wavelength maximum (21). The observed blue shift in the absence of lipid indicates that monomeric apoA-I adopts a tertiary structure that maintains a hydrophobic environment in the vicinity of W74 and W107. Given the preponderence of amphipathic α-helix in the protein, and precedent established from structural studies of other apolipoproteins (39-42), it is reasonable to consider that helix-helix interactions may serve to sequester the tryptophan residues. By extension, it is conceivable that lipid binding induces a conformational adaptation in apoA-I wherein helix-helix contacts are replaced by helix-lipid interactions, with

presentation of helices containing W74 and W107 to the hydrophobic lipid milieu.

2.3.3 Fluorescence quenching studies

Three commonly used quenching agents, KI, CsCl and acrylamide, were employed as aqueous quenchers of the intrinsic fluorescence of the two tryptophan residues in apoA-I. **Figure 2-2** shows acrylamide quenching curves obtained for lipid-free, DMPC-bound and HDL-bound apoA-I. Quenching curves obtained with KI and CsCl revealed small differences between the different states of apoA-I, noting that Cs^+ was a particularly ineffective quenching agent. Comparison of Stern-Volmer quenching constants for the different quenchers indicates similar accessibility in the lipid-free and lipid-bound states (**Table 2-2**). In the case of the neutral quenching agent, acrylamide, the similar K_{sv} values obtained in the presence and absence of lipid suggests that lipid-free apoA-I adopts a fold that sequesters W74 and W107 from the aqueous environment. The observed increased ability of KI to quench apoA-I fluorescence in DMPC discs indicates that the environment of the tryptophans is more accessible in the disc-bound state than in lipid-free or HDL-bound states. This result is consistent with interaction of the protein around the periphery of the bilayer disc as opposed to penetration between polar head groups of the phospholipid, as proposed for spherical HDL particles (43).

2.3.4 Dye binding experiments

ANS is a dye whose intrinsic fluorescence increases upon binding to a hydrophobic surface or cavity (33). In the absence of protein, ANS has a very low quantum yield with an emission wavelength maximum of 515 nm (excitation 395 nm). Addition of either human or chicken apoA-I induced a significant enhancement in ANS fluorescence quantum yield together with a 35 nm blue shift in its wavelength of maximum emission (**Figure 2-3**). The comparable induction of fluorescence elicited by human and chicken apoA-I suggests the relative amount of ANS-accessible hydrophobic surface in these proteins is similar. Bovine serum albumin induced a large enhancement in ANS fluorescence spectra, consistent with the known hydrophobicity of this protein. On the other hand, carbonic anhydrase had little effect on ANS fluorescence. Data obtained with the latter two control proteins indicate the sensitivity of ANS fluorescence to exposed hydrophobic surface on proteins, and its suitability for study of lipid-free apoA-I. Since human apoA-I exists predominantly as a monomer at the concentration employed in this experiment (17, 19), the similar level of ANS fluorescence enhancement observed suggests human and chicken apoA-I adopt similar global folds to achieve sequestration of hydrophobic lipid binding regions, most likely through intramolecular helix-helix interactions.

2.3.5 Effect of trifluoroethanol on apoA-I structure

Far-UV CD spectra in the absence and presence of the lipid mimetic cosolvent, trifluoroethanol (TFE), reveal significant differences in the ratio of ellipticity at

222 nm and 208 nm (22). In other predominantly α-helical peptides, a 222 nm/208nm ellipticity ratio of ~ 1.0 indicates interhelical contacts, such as those present in coiled coil or helix bundle structures (44-46). Alternately, values in the range of 0.90 indicate an elongated helix with little or no interhelical contacts (45-47). The ratio of 1.02 observed for lipid-free apoA-I in buffer is consistent with an α-helix bundle organization while the observed decrease in ratio of ellipticity at 222nm/208nm to 0.90 in the presence of 50 % TFE (v/v) suggests realignment of apoA-I helices, perhaps by replacement of helix-helix contacts by helix-TFE contacts. In this case, individual helices would no longer be constrained to maintain a bundle configuration. In the present context we hypothesized that TFE-induced disruption of helix-helix contacts in apoA-I would be reflected in the hydrodynamic properties of the protein. Calculations based on sedimentation velocity experiments reported here reveal a $S^{\circ}_{20,w}$ of 2.31 S and an axial ratio (a/b) of 3.8 in buffer, in good agreement with earlier studies (22). In the presence of TFE, the calculated $S^{\circ}_{20,w}$ is 2.22 S and the corresponding axial ratio increased to 4.5, suggesting the protein has adopted a more asymmetric average conformation, which may be caused by partial helix bundle opening due to TFE-induced disruption of hydrophobic helix-helix contacts. This interpretation is consistent with the known effects of TFE on *Manduca sexta* apolipophorin III tyrosine fluorescence, which are similar to changes which occur upon binding to lipid (48, 49).

2.3.6 Lipid-based quenchers

Spin-labelled fatty acids were employed as lipid soluble quenchers to determine the average depth of penetration of W74 and W107 into the surface

monolayer of spherical HDL particles. Using a series of DOXYL fatty acids in which the DOXYL moiety is located at different positions along the acyl chain, the depth of binding of apoA-I tryptophan residues was monitored (50). An advantage of the present system is the ability to employ natural chicken HDL directly, since a uniform population of particles containing apoA-I as practically the sole apolipoprotein component can be obtained from chicken plasma. Addition of spin-labelled fatty acids to HDL results in partitioning of the quencher into the surface monolayer of the particle. In each case, increasing the quencher concentration resulted in increased quenching of apoA-I tryptophan fluorescence (**Figure 2-4**). Comparing K_{app} values, the data show that 5-DSA is the most effective quencher followed by 7-, 10- and 12-DSA (**Table 2-3**). These data are consistent with the hypothesis that HDL-associated apoA-I presents the hydrophobic face of helices containing W74 and W107 to the lipoprotein surface. Furthermore, W74 and W107 do not appear to penetrate deeply into the HDL surface monolayer, a property which may be related to the ability of apoA-I to interact reversibly with lipoprotein surfaces.

2.4 Discussion

A unique feature of amphipathic exchangeable apolipoproteins is an ability to exist stably in lipid-free and lipid-associated states. This property is related to their ability to transfer among lipoprotein particles and, in the case of apoA-I, to serve as progenitor of nascent lipoprotein particle assembly. ApoA-I is particularly interesting in this regard because of the physiological significance of its ability to promote cellular cholesterol efflux as a part of the reverse

cholesterol transport pathway (4). At present, however, the molecular basis for the existence of alternate lipid-free and lipid-bound states of apoA-I is not clear. In the present study we have addressed this question through study of apoA-I from chicken plasma. A unique characteristic of this protein is its existence in buffer as a monomer (21, 22) unlike the homologous apoA-I from human plasma (17, 19). The underlying hypothesis of the present study is that lipid-free chicken apoA-I adopts a helix bundle molecular architecture. This hypothesis was evaluated by comparing spectroscopic properties of apoA-I in the absence of lipid as well as in association with phospholipid bilayer discs and spheroidal HDL.

Near-UV CD spectroscopy experiments, which report on the environment of aromatic residues in the protein, revealed dramatic spectral differences between lipid-free and lipid associated apoA-I. These data can be compared to data reported for human apoA-I (37, 38) and provide evidence for helix repositioning upon interaction with lipid. A difference noted between human and chicken apoA-I in terms of their near-UV CD spectra is that, whereas chicken apoA-I displays a strong negative ellipticity in buffer alone, human apoA-I gives rise to positive ellipticity values between 270 and 288 nm (37). Also, whereas chicken apoA-I ellipticity values decrease dramatically upon lipid interaction, human apoA-I lipid interaction induced an increase in ellipticity values. Thus, although both proteins display major spectral differences between their respective lipid-free and lipid-bound states, there is no apparent correlation between the changes observed. Support for the conclusion that the changes in both proteins are indicative of a conformational change, however, can be seen from studies of an unrelated α-helix bundle

exchangeable apolipoprotein, *Locusta migratoria* apolipophorin III, whose X-ray structure has been determined (39). This protein, which possesses two tryptophans and lacks both tyrosine and cysteine, displays unusually well defined near-UV CD spectra (51). Lipid association of this protein, which occurs via conformational opening of the helix bundle (39), gives rise to characteristic changes in near-UV CD spectra, including a complete reversal in sign of extrema and a 7 nm red shift. When taken together with far-UV CD data indicating chicken apoA-I retains a high content of α-helix secondary structure in the absence and presence of lipid (Table 2-1), it is plausible to consider that lipid-binding of chicken apoA-I is accompanied by repositioning of α-helical segments in the protein.

The two tryptophan residues in chicken apoA-I are located in the N-terminal half of the linear protein sequence (positions 74 and 107) and are predicted to reside on the hydrophobic face of distinct amphipathic α-helices. We have used these intrinsic fluorophores as reporters of local environmental changes which arise from lipid association of the protein. Fluorescence spectra of apoA-I in lipid-free and lipid-bound states were similar, exhibiting a significant blue shift in their corresponding wavelength of maximum emission (excitation 295 nm) compared to free tryptophan. These data indicate that, even in the absence of a lipid surface, W74 and W107 are sequestered from the aqueous environment. Assuming that the blue shift in the lipid bound states results from interaction of the tryptophan residues with the hydrophobic lipid surface, it is reasonable to conclude that helix-helix interactions in the lipid-free state creates a hydrophobic environment in the vicinity of the tryptophan reporter groups.

Tryptophan fluorescence quenching experiments were performed on apoA-I in lipid-free and lipid-bound states. With acrylamide as quencher, K_{sv} values observed in the different states were comparable, ranging from 1.1 to 1.6 M^{-1} (Table 2-2). These data indicate that, in the absence of lipid, apoA-I assumes a conformation which shields W74 and W107 from aqueous quenchers to an extent that is similar to lipid-bound apoA-I wherein tryptophan residues are shielded by contact with the lipid milieu. Cs^+ was generally a poor quenching agent, yielding K_{sv} values of 0.1 M^{-1} in each of the three states. These data indicate that the average accessibility to this positively charged quencher does not change in lipid-free versus lipid associated apoA-I. In terms of KI quenching of apoA-I tryptophan fluorescence, similar K_{sv} values were observed for lipid-free and HDL-bound protein. The observed higher K_{sv} value for DMPC-associated apoA-I provides evidence that the protein adopts a unique conformation in bilayer disc particles versus that in spherical lipoprotein particles.

In other studies, the effect of human apoA-I and chicken apoA-I on ANS fluorescence quantum yield were compared under conditions wherein both proteins are predominantly monomeric in solution. Interestingly, no differences in ANS binding were detected. Stryer (33) showed that ANS binds to hydrophobic regions on proteins, causing a dramatic increase in ANS quantum yield and a significant blue shift in its wavelength of maximum fluorescence intensity. The similar induction of ANS fluorescence observed for human and chicken apoA-I suggest both proteins possess similar amounts of dye-accessible hydrophobic surface. By extension, it is reasonable to consider that human and chicken apoA-I (48 % sequence identity) adopt similar global

folds in solution and that structural data obtained on one protein will have implications for the other.

In an effort to examine this aspect in further detail we conducted studies in the presence of the lipid mimetic co-solvent TFE. TFE has previously been shown to induce additional α-helix in lipid-free apoA-I, as seen by a significant increase in negative ellipticity, especially at 208 nm (22). Others (44-47) have shown that the ratio of ellipticity at 222 nm to 208 nm can be used to evaluate the existence of interhelical contacts in coiled coil peptides. Since exchangeable apolipoproteins contain predominantly α-helical secondary structure, they are suitable candidates for similar analyses. Initially, we compared the ratio of ellipticity at 222 and 208 nm for three monomeric helix bundle exchangeable apolipoproteins whose three dimensional structures are known (*Locusta migratoria* apoLp-III (39, 51), *Manduca sexta* apoLp-III (40 and unpublished data) and human apoE N-terminal domain (42, 52)). In these proteins 208nm/222nm ellipticity ratio values approaching 1.0 or above were observed in buffer, in keeping with the fact that they are known to be stabilized by intramolecular helix-helix interactions. In the presence of TFE these proteins display a decrease in the corresponding ratio, consistent with the concept that TFE effectively disrupts hydrophobic contacts between neighboring amphipathic α-helices. The observation that chicken apoA-I follows the same pattern provides support for the presence of helix-helix interactions, most likely as an amphipathic α-helix bundle, in the lipid free state. To verify that disruption of helix-helix contacts in chicken apoA-I occurs upon introduction of TFE, sedimentation velocity experiments were performed. Calculations based on these data revealed an increase in protein axial ratio

(a/b) from 3.8 to 4.5 in the presence of 50 % TFE (v/v). The formation of a more asymmetric structure in the presence of TFE is conceivably due to partial opening of the helix bundle structure, as a result of replacement of helix-helix contacts in the bundle by helix-TFE contacts, which are not constrained to retain the bundle conformation.

To address questions related to the interaction of apoA-I with the surface of HDL particles, the effect of different lipid-based quenching agents was evaluated. Spin-labelled DOXYL moieties covalently bound to the 5, 7, 10 or 12 position of stearic acid were employed in studies designed to assess the depth of penetration of apoA-I into the surface of HDL. The data indicated that 5-DSA was the best quencher, suggesting that W74 and W107 reside in close proximity to the surface of the lipoprotein. We concluded from this data that chicken apoA-I has a relatively superficial association with the surface of chicken HDL, in general agreement with the ability of apoA-I to interact reversibly with lipoproteins. A recent study showed that another exchangeable apolipoprotein, *M. sexta* apoLp-III, also adopts a superficial location on lipoprotein surfaces (53), although displacement studies show that apoA-I has a higher lipid binding affinity than apoLp-III (54). While it is tempting to speculate that the binding affinity of exchangeable apolipoproteins is not correlated to depth of penetration, it is recognized that the tryptophan reporter groups on apoA-I are localized to specific regions of the protein. It is conceivable that the C-terminus of apoA-I could be responsible for enhanced binding, independent of the N-terminal half which contains the two tryptophan probes (9, 10).

Recently, Borhani *et al.* (5) reported the crystal structure of human (Δ1-43) apoA-I at 4 Å resolution. The structure revealed a continuously curved, horseshoe shaped series of 10 amphipathic α-helices, with four truncated apoA-I molecules arranged as pairs of antiparallel dimers. The interaction of molecules A and B to form a stable dimer is due to electrostatic and hydrophobic contacts. The molecular organization of the A/B dimer produces a structure which presents two faces, one predominantly hydrophilic and the other, strongly hydrophobic. Interactions between the hydrophobic surfaces of two dimers stabilizes the dimer structure and gives rise to the observed tetrameric organization.

On the basis of comparative biophysical studies of full length apoA-I and (Δ1-43)apoA-I, it has been suggested that the structure reported by Borhani *et al.* (5) is representative of the conformation of a lipid-bound apoA-I (11). Indeed, it is envisioned that apoA-I dimers, aligned as depicted in the X-ray structure, could form a "belt" around phospholipid bilayer discs or the circumference of spheroidal HDL. It is conceivable that tetramer formation by (Δ1-43)apoA-I in the absence of lipid provides an alternative mechanism to protect the hydrophobic surface of the antiparallel dimers from solvent exposure. By extension, it is plausible that, in the presence of lipid, helix-lipid interactions serve to stabilize the dimer. It is important to recognize that the tetrameric organization observed is not an intermediate state in the lipid binding interactions of apoA-I. Indeed, full length apoA-I does not adopt a similar lipid-free organization, presumably prevented by virtue of its N-terminal 43 amino acids (11). Whereas the N-terminal deletion induces spontaneous formation of the continuously curved, horseshoe conformation, which is

stabilized by intermolecular contacts, full length apoA-I is induced to undergo a conformational change only as a result of lipid contact.

The data presented in the present study extend this model by providing evidence that apoA-I can adopt a globular amphipathic α-helix bundle conformation that is stabilized by intra-molecular helix-helix interactions. Furthermore it is clear that lipid contact induces a significant conformational change wherein the molecule replaces helix-helix interactions for helix-lipid interactions. Previous studies of lipid-free human apoA-I (12, 55-57) are also consistent with a helix bundle organization of apoA-I and the concept that lipid binding triggers formation of an altered conformational state that retains its overall α-helix content yet presents a significant hydrophobic surface to the lipid milieu. Future studies of this intriguing system will be directed toward confirmation that the continuously curved, horseshoe structure reported by Borhani *et al.* (5) in fact resembles the conformation adopted by full length apoA-I in the presence of lipid. In addition, further high resolution structural information is needed to more accurately define the apparent helix bundle organization of apoA-I in the absence of lipid and to distinguish the unique conformations assumed in disc particles and spherical lipoproteins. Success in this endeavor will provide an understanding of the molecular basis for the pivotal role apoA-I plays in the reverse cholesterol transport pathway.

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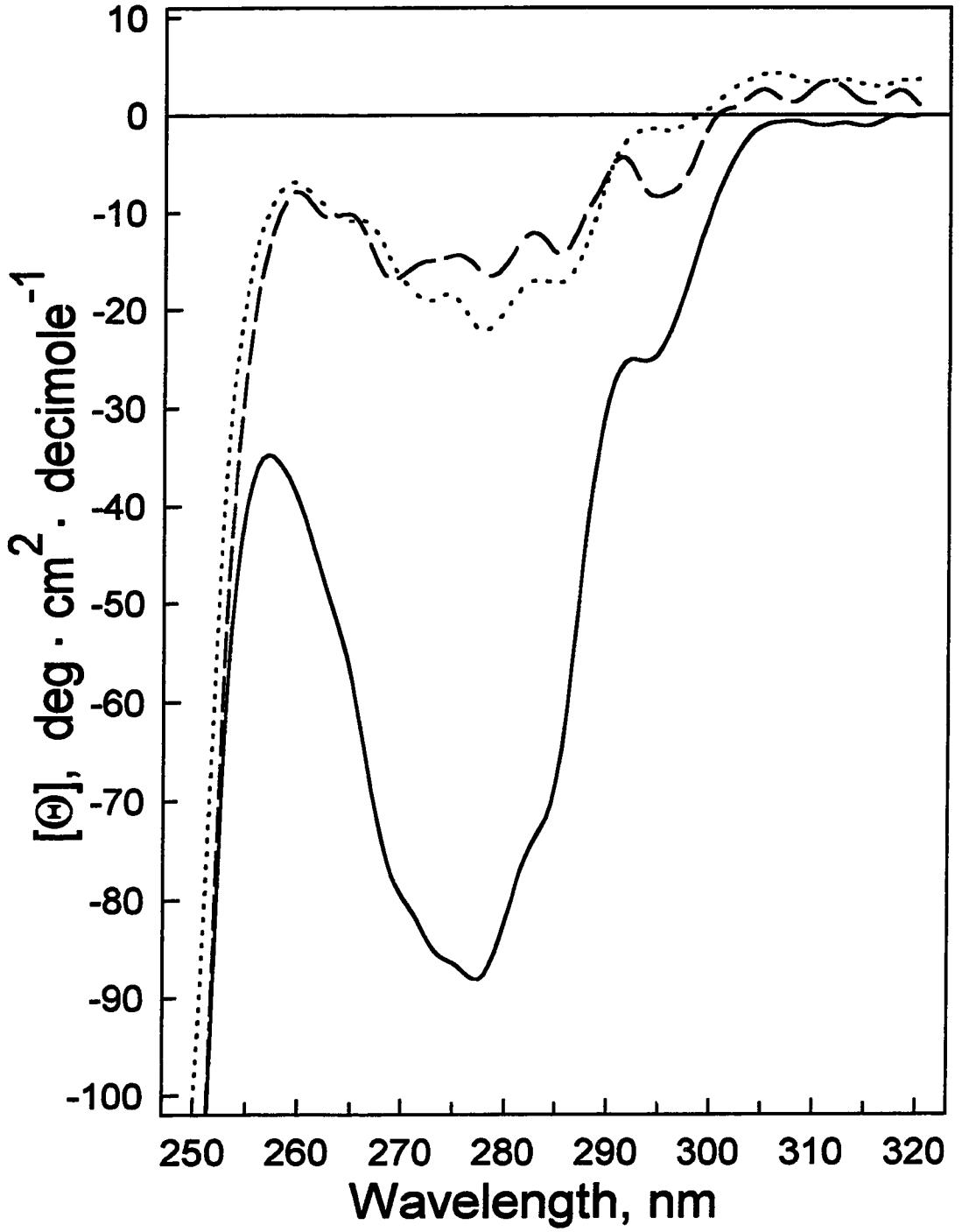
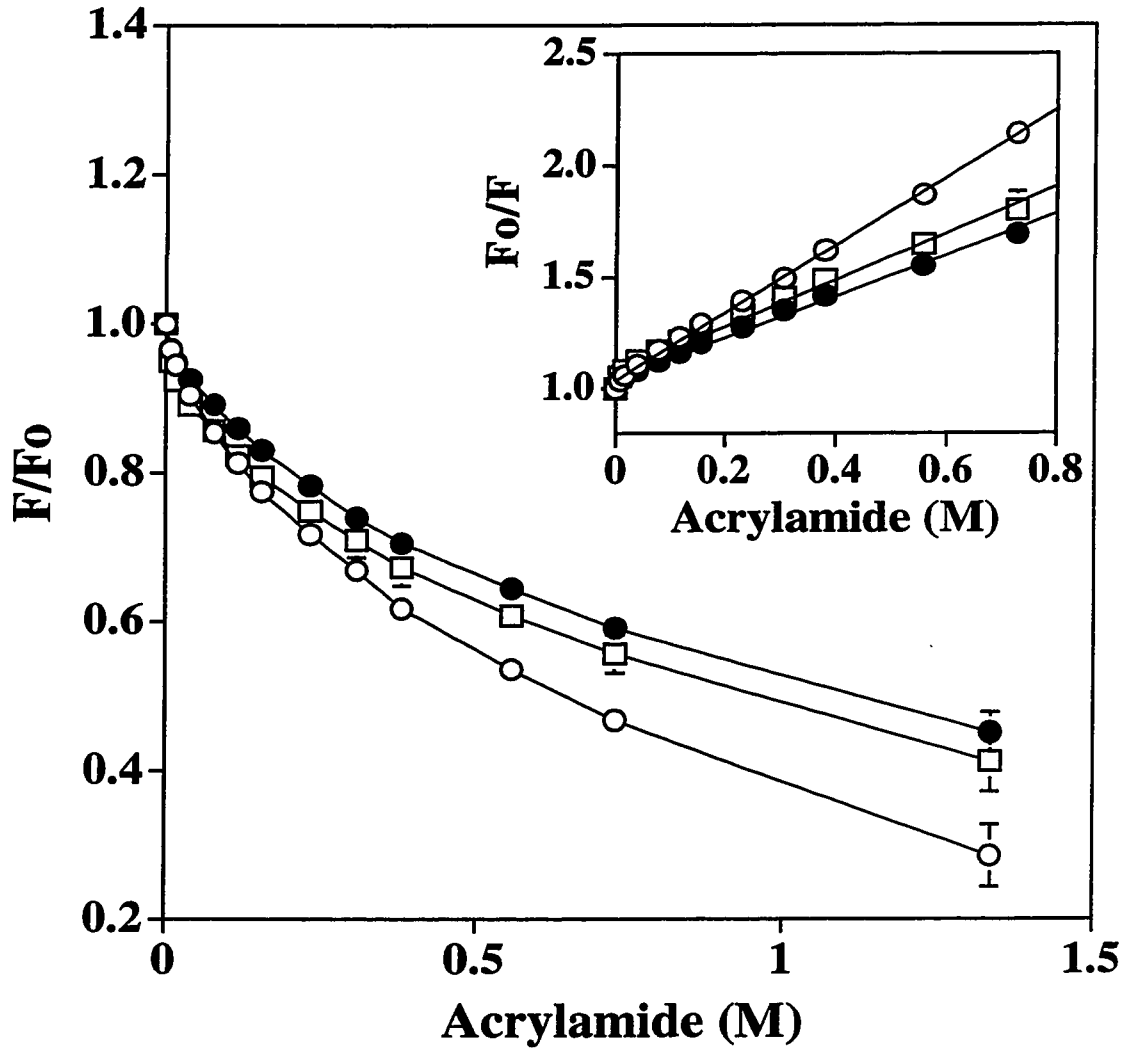
Figure 2-1: Near-UV CD spectra of lipid-free apoA-I (*straight line*), DMPC-bound apoA-I (*dashed line*) and HDL-bound apoA-I (*dotted line*).


10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
[Θ], deg · cm2 · decimole-1
250
260
270
280
290
300
310
320
Wavelength, nm

apoA-I	Secondary structure conformers α-helix %	β-sheet %	β-turn %	remainder %	scale factor	fluorescence emission λmax (nm)
Lipid-free	72±2	8±2	12±1	9±1	1.001	331
DMPC-bound	68±2	7±3	16±1	9±1	0.990	331
HDL-bound	86±1	0±0	8±1	3±1	0.997	329

Table 2-1: Properties of chicken apoA-I. Percentage of secondary structure conformers were derived from modified Provencher-Glöckner analysis (27) of far-UV CD spectra as described in the text. The percentage error for the values was derived from the relative goodness of fit of the spectra in comparison to a standard set of 16 reference proteins plus poly L-glutamate (employed as a 100 % helical reference). The scale factor represents the overall goodness of fit and should theoretically be 1.0. The wavelength of maximal fluorescence emission was determined by using the excitation wavelength of 295 nm.

Figure 2-2: Fluorescence quenching of tryptophans by acrylamide. Increasing concentrations of acrylamide were added to a 50 μg/mL solution of chicken apoA-I in the lipid-free (*open circles*), DMPC-bound (*filled circles*), and HDL-bound (*open squares*) states. Emission spectra were obtained from 300 - 450 nm with excitation at 295 nm (excitation and emission slit widths set at 5 nm). Relative fluorescence (F/Fo) is plotted as a function of quencher concentration. *Inset*, Stern-Volmer plot of acrylamide quenching of apoA-I tryptophan fluorescence. Values are average of three independent measurements ± SD.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
F/Fo
0
0.5
1
1.5
Acrylamide (M)
2.5
2.0
1.5
1.0
Fo/F
0
0.2
0.4
0.6
0.8
Acrylamide (M)

	K_{sv} (M^{-1})		
	lipid-free apoA-I	DMPC-bound apoA-I	HDL-bound apoA-I
CsCl	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
KI	0.6 ± 0.1	1.3 ± 0.1	0.6 ± 0.0
Acrylamide	1.6 ± 0.1	1.1 ± 0.1	1.2 ± 0.1

Table 2-2: Effective Stern-Volmer constants (K_{sv}) for aqueous quenchers. Stern-Volmer quenching constants (K_{sv}) for aqueous quenchers in lipid-free and lipid-bound states were calculated as described under experimental procedures. Values reported represent the mean ± SD of three separate determinations.

Figure 2-3: ANS fluorescence spectra were obtained in the presence of 50 μg/mL bovine serum albumin (A), human apoA-I (B), chicken apoA-I (C), and carbonic anhydrase (D). Protein samples were excited at 395 nm and the emission spectra from 400 to 560 nm monitored (excitation and emission slit widths set at 5 nm). Spectra were recorded in 10 mM sodium phosphate (pH 7.5).

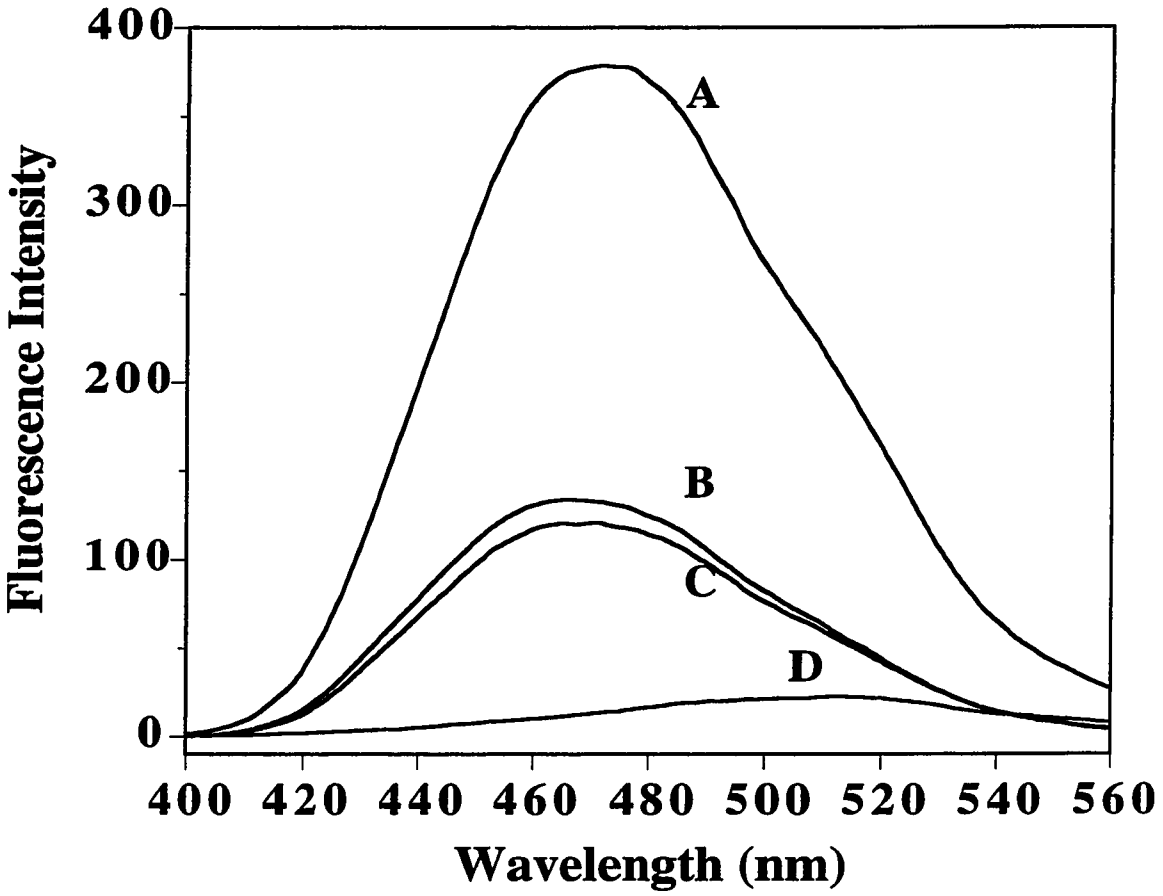
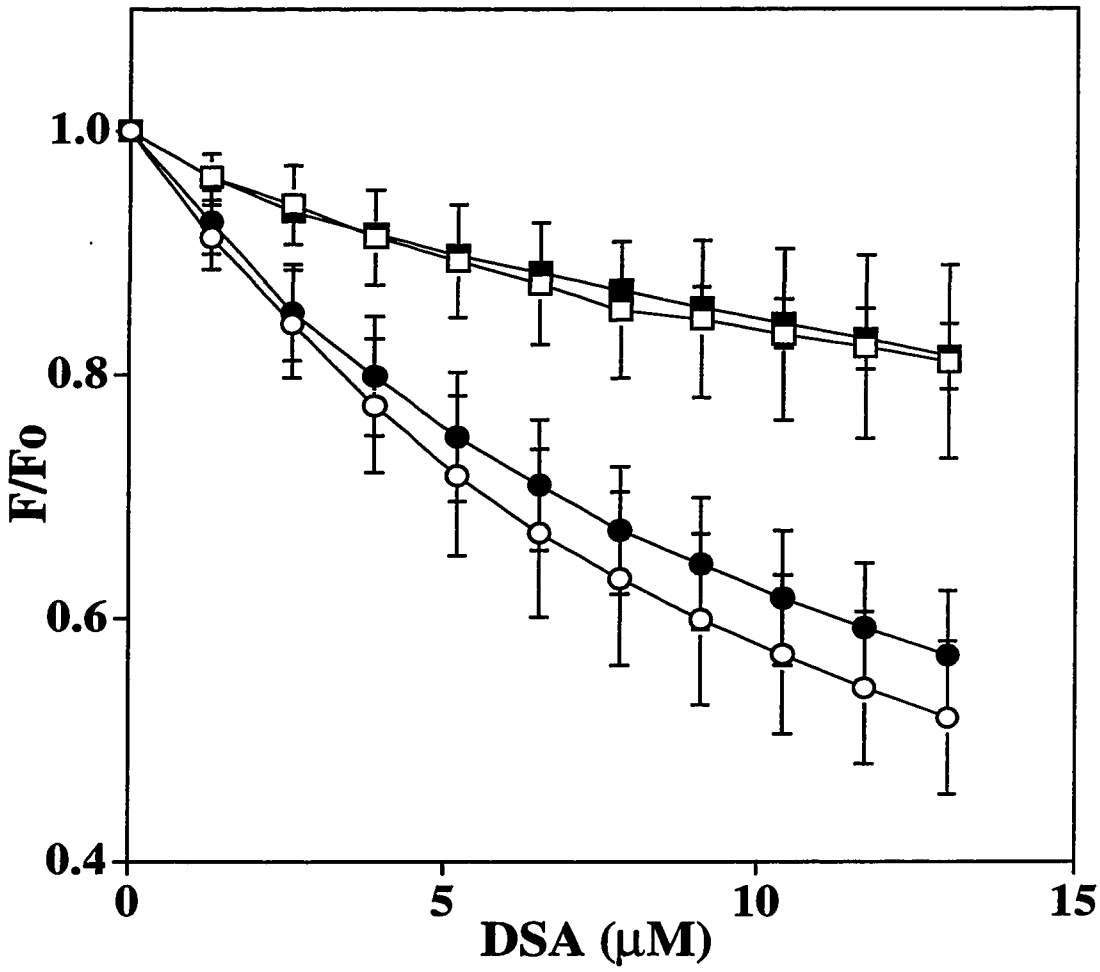

400
300
200
100
0
Fluorescence Intensity
A
B
C
D
400 420 440 460 480 500 520 540 560
Wavelength (nm)

Figure 2-4: Fluorescence quenching of tryptophans by lipid-soluble quenchers. Increasing concentrations of spin labelled fatty acids: 5-DSA (*open circles*), 7-DSA (*filled circles*), 10-DSA (*open squares*) and 12-DSA (*filled squares*) were added to 50 μg/mL of HDL-bound chicken apoA-I. Fluorescence measurements were conducted at an excitation wavelength of 295 nm (excitation and emission slit widths set at 5 nm), scanning emission from 300 - 450 nm. Relative fluorescence (F/Fo) is plotted as a function of quencher concentration.


1.0
0.8
0.6
0.4
F/Fo
0
5
10
15
DSA (μM)

Quencher	K_{app} (x 10^{-4} M^{-1})
5- DSA	7.5 ± 0.9
7- DSA	6.0 ± 0.8
10- DSA	2.3 ± 0.5
12- DSA	1.7 ± 0.1

Table 2-3: Apparent Stern-Volmer constants (K_{app}) for lipid-soluble quenchers. The apparent Stern-Volmer quenching constants (K_{app}) for DSA lipid-soluble quenchers of HDL-bound apolipoprotein A-I tryptophan fluorescence were calculated as described in experimental procedures. Values reported represent the mean ± SD of three independent measurements.

Chapter 3

Functional characterization of chicken apolipoprotein A-I: similarity between chicken and human apolipoprotein A-I

3.1 Introduction

Apolipoprotein (apo) A-I, as the main structural component of high density lipoprotein (HDL), plays an important role in plasma cholesterol homeostasis. Clinical studies have demonstrated an inverse correlation between cardiovascular disease and plasma HDL levels, suggesting that HDL and apoA-I serve a protective role against atherogenesis (1, 2). As a surface component of HDL, apoA-I serves to stabilize exposed hydrophobic surfaces and facilitates the solubilization of hydrophobic lipids in the aqueous environment of the blood (3). As well, apoA-I mediates cholesterol efflux from peripheral tissues and activates the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), which catalyzes the conversion of HDL cholesterol to cholesteryl ester (4). Recently, apoA-I has been found to serve as a ligand for the class B scavenger lipoprotein receptor SR-BI, mediating specific uptake of cholesteryl ester to the liver and non-placental steroidogenic tissues (5, 6). These processes facilitate removal of cholesterol from peripheral tissues and transport the cholesterol to the liver as part of the reverse cholesterol transport pathway (7) accounting for apoA-I's anti-atherogenic role.

Evaluation of the structure/function relationship of apoA-I and the regions of apoA-I responsible has aided in understanding the beneficial nature of apoA-I. Mutagenesis and truncation experiments have identified regions of apoA-I which interact with lipid surfaces (8-12). It appears that there is no specificity for any particular sequence, but that the C-terminus (residues 190-243) and possibly residues 44-65 at the N-terminus are involved in lipid binding. It appears that the C-terminus is also responsible for self-association properties (9). Likewise, the regions of apoA-I that activate LCAT have been

determined (8, 13 ,14). The central helices (between residues 123 and 186) are likely involved, but the C-teminus may also be implicated in LCAT activation (9, 15). Stimulation of cholesterol efflux by apoA-I has been investigated (reviewed in (16)). Use of monoclonal antibodies indicated that residues 140-147 and 149-150 were likely involved (17), however, apoA-I truncated to residue 135 had significant efflux ability (18). In fact, other apolipoproteins and even peptides were capable of promoting cholesterol efflux (12, 19-21), indicating that the only structural requirement is the presence of amphipathic α-helices. Physiologically, however, apoA-I, in its lipid-free or lipid-bound states, performs these functions and is responsible for normal cholesterol homeostasis.

Further studies of apoA-I showed that chicken apoA-I was monomeric in the lipid-free state (22), unlike human apoA-I, which oligomerizes in the absence of lipid (23, 24). This feature was exploited to facilitate study of apoA-I in the absence of lipid. Chicken and human apoA-I share 48 % sequence identity and 66 % sequence similarity and share similarities upon physical characterization (22; Chapter 2). Lipid-free chicken apoA-I has an α-helical content of 72 % as compared to lipid-free human apoA-I which has 55 % (25). It is believed that the high α-helical content of chicken apoA-I may allow it to maintain a stable monomeric helix bundle structure (Chapter 2).

Previously, to determine whether the difference in self-association and stability properties between chicken and human apoA-I are manifest in their respective lipid binding affinities, displacement studies were conducted using insect low density lipophorin (LDLp) as a model lipid surface. LDLp contains two nonexchangeable apolipoproteins, apoLp-I and apoLp-II, and an

exchangeable apolipoprotein, apoLp-III. In the previous experiment ((22) and in accordance with Liu *et al.* (26)) it was found that when lipid-free radiolabeled chicken apoA-I (both chicken and human) was incubated with insect LDLp, radioactivity was recovered at a density corresponding to that of LDLp (particle density = 1.03 g/mL; (27)). Therefore, similar to human apoA-I, chicken apoA-I can quantitatively displace the resident apolipoprotein, apoLp-III, from the LDLp particle (22, 26), indicating that chicken apoA-I has a higher lipid binding affinity than apoLp-III, in a manner analogous to human apoA-I. Similar experiments were performed using chicken HDL (density range 1.125-1.21 g/mL) or human HDL_3 (density range 1.125-1.21 g/mL) as model lipid surfaces. Incubation of lipid-free ^3H-labeled chicken apoA-I with chicken HDL or human HDL_3, or lipid-free ^3H-labeled human apoA-I with chicken HDL or human HDL_3, and subsequent density gradient ultracentrifugation, resulted in all cases in association of radioactivity with the HDL particles (unpublished observations). Both human and chicken apoA-I could effectively compete for HDL binding. Lipoprotein binding competition experiments were also performed by incubating ^3H-labeled and unlabeled apoA-I's with LDLp. On the basis of their relative abilities to decrease the specific radioactivity associated with LDLp, it was concluded that chicken and human apoA-I have approximately similar affinities for the surface of LDLp, with that of chicken apoA-I being slightly higher (22). As is the case between human apoA-I and apoA-II (28), insect apoLp-III and human apoA-I (26), and human apoE and apoC-III (29), if one apolipoprotein possessed significantly higher affinity than the other, it would be expected that nearly complete displacement of the weaker lipid binding protein would result. In this case, both chicken and

human apoA-I were equal in their ability to compete for binding to a lipoprotein surface.

In the present study, the functional similarity between human and chicken apoA-I has been further addressed. The relative lipid binding affinities of human and chicken apoA-I for phospholipase C-treated low density lipoprotein, the ability to transform phospholipid vesicles into discoidal complexes, the ability to mediate cholesterol efflux, and the ability to promote cholesterol mobilization were all evaluated. Chicken apoA-I was found to be functionally similar to human apoA-I in all aspects except cholesterol efflux from mouse peritoneal macrophages.

3.2 Experimental Procedures

Materials: [^{14}C]-oleate and [1, 2-^{3}H]cholesteryl oleate (45.4 Ci/mmol) were obtained from Amersham (Toronto, Canada). Phospholipase C (PLC), from *Bacillus cereus*, penicillin-streptomycin solution, dithionitrobenzoic acid, bovine serum albumin (BSA), dimyristoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Bio-Whittaker (Walkersville, MD), and fetal bovine serum (FBS) and lipoprotein deficient serum (LPDS) were obtained from Hyclone (Logan, UT). RPMI 1640 medium was obtained from Flow Laboratories. Whatman PE SIL-G thin layer chromotography (TLC) plates were obtained from Fisher Scientific (Edmonton, Canada).

Lipoproteins and apolipoproteins: Chicken HDL and apoA-I were prepared as described in Chapter 2. Human low density lipoprotein (LDL) and HDL_3 was isolated from the blood of healthy male volunteers by sequential ultracentrifugation between the density ranges of 1.019-1.063 for LDL and 1.125-1.21 for HDL_3. ApoB- and apoE-containing particles were removed from the HDL_3 fraction by heparin-agarose affinity chromotography. Human apoA-I was isolated by delipidation of HDL_3 and subsequent DEAE-cellulose chromotography (30).

Binding of chicken apoA-I to PLC-treated LDL: Human LDL (100 μg protein) in 10 mM Tris-HCl, pH 7.5, was incubated with 160 milliunits of *Bacillus cereus* phospholipase C (PLC), in the absence or the presence of human or chicken apoA-I. In the time course assay, 30 μg of apoA-I protein was used and sample turbidity was measured at 340 nm on a Spectromax 250 microtiter plate reader (Molecular Devices, Sunnyvale, CA) at indicated timepoints. For the concentration dependent assay, varying amounts of apoA-I protein were added for an incubation time of 1 h, and then sample turbidity was read as before. For the control proteins, human LDL (50 μg) was incubated with 160 mU of PLC for 120 minutes at 37 °C in the absence and presence of bovine serum albumin and carbonic anhydrase (25 μg each). At the indicated time points, sample absorbance at 340 nm was measured. The control protein experiment was performed by Dr. Paul Weers.

Transformation of phospholipid vesicles: The ability of apoA-I to transform bilayer vesicles of DMPC and DPPC into disc complexes was studied. Discs were made following a modification of the sonication protocol of Wientzek *et al.* (31). In brief, phospholipid was dissolved in a small volume of chloroform:methanol (3:1, v:v) and dried under a $N_{2\ (g)}$ stream. The thin film was dispersed into 10 mM sodium phosphate (pH 7.5) by vortexing and then sonicated until the solution was clear. Chicken apoA-I was added at a lipid:protein ratio of 5:1 (w/w) and incubated for 16 h at 24 °C for DMPC and 42 °C for DPPC. The density of the solution was adjusted to 1.21 g/mL with KBr to a final volume of 2.5 mL and put into a 5 mL Quick-Seal centrifuge tube. The solution was overlaid with saline, the tube sealed, and spun at 65,000 rpm for 75 minutes at 4 °C in a VTi65.2 rotor. After centrifugation, fractions containing discs were pooled and dialyzed against 10 mM sodium phosphate (pH 7.5) before use. Discs were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE) (32). Samples for electron microscopy were adsorbed to carbon-coated grids, rinsed three times with buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$) and negatively stained with sodium phosphotungstate, pH 7.0, for 10 s. Grids were photographed in a Philips EM420 operated at 100kV. Crosslinking studies were performed on DMPC and DPPC discs using the crosslinking agent dimethylsuberimidate (Pierce, Rockford, IL). Increasing concentrations of dimethylsuberimidate (20 mg/mL in 1M triethanolamine/HCl, pH 9.7) were incubated with disc complexes (50 μg) for 90 min at room temperature (33). Samples were then applied to a 4 - 20% sodium dodecyl sulfate (SDS)-polyacryalamide gel for analysis.

Preparation of [^{3}H]cholesteryl oleate-labeled acetylated LDL: [1, 2-^{3}H]cholesteryl oleate was incorporated into LDL according to the method of Nishikawa *et al.* (34). Egg phosphatidylcholine was sonicated with radiolabeled cholesteryl oleate to produce vesicles. The vesicles were incubated at 37 °C with plasma of density > 1.006 with dithionitrobenzoic acid. The LDL was then bound to a dextran sulfate-cellulose column and eluted with an increasing salt gradient and further purified by ultracentrifugation. The radiolabeled LDL was then acetylated by treatment with acetic anhydride (35). The labeled acetylated LDL had a specific radioactivity between 2000 and 4000 cpm/μg of protein.

Culturing of mouse peritoneal macrophages: Mouse peritoneal macrophages (MPM) were obtained and cultured according to Hara and Yokoyama (36). Briefly, mouse peritoneal macrophages were obtained from male or female ICR mice (20-30 g) by peritoneal lavage with PBS. The cells were pelleted by centrifugation, washed once with RPMI 1640 medium, and then resuspended in RPMI 1640 medium containing 20% FBS. Aliquots of the cells (2 x 10^6 cells in 1 mL per dish) were put into 3.5 cm plastic culture dishes. After 2 h, the cells were washed twice with RPMI 1640 medium alone, and then used for subsequent assays.

Loading macrophages with cholesteryl ester: Following the method of Brown *et al.* (37), macrophages were loaded with cholesteryl ester by incubating with 1 mL of RPMI 1640 medium, containing 2 mg/mL BSA, with 50 μg/mL of [1, 2-^{3}H]cholesteryl oleate-labeled acetylated LDL protein for 24

h. Afterwards the macrophages were washed twice with RPMI 1640 medium containing 10% FBS and once with RPMI 1640 medium alone. The macrophages were then incubated in RPMI 1640 medium containing 2 mg/mL BSA for 24 h, and then washed once again with RPMI 1640 medium alone.

Incubation of the cells with apolipoproteins and extraction of lipids: Following loading with radiolabels, the macrophages were incubated with RPMI 1640 containing 2 mg/mL BSA in the presence of varying concentrations of lipid-free human or chicken apoA-I at 37 °C for 24 h. The culture medium was removed and centrifuged for 2 min at 10,000 rpm in a Beckman microfuge to eliminate contamination of cellular components. The lipids were then extracted from the medium following the method of Bligh and Dyer (38). The macrophages were washed once with PBS, and then extracted *in situ* with hexane/isopropyl alcohol (3:2) (37). After delipidation, 0.1 N NaOH was added to the macrophages, and after 30 min, an aliquot was removed for protein determination by the Lowry method (39). The mean amount of cell protein per dish was 90 μg/dish. The specific activity of cholesterol in the medium and cholesterol in the cell (both free and esterified cholesterol) was determined by gas-liquid chromatography according to Hara and Yokoyama (36) and was approximately 3000 cpm/μg cholesterol.

Separation of lipid species by TLC: Aliquots of the lipid extracts (both from the cell and medium) were separated by silica gel TLC. Hexane/diethyl ether/acetic acid (85:30:1) was used to separate free and esterified cholesterol, triglyceride, and free fatty acid, while chloroform/methanol/acetic acid/water

(75:45:12:6) was used to separate phospholipids. Lipid spots, as visualized by I_2 staining, on the TLC plate corresponding to each lipid fraction were scraped from the plate for radioactivity determination (36).

Human skin fibroblast cholesterol mobilization: Normal human skin fibroblasts (EB91-290) were cultured in DMEM containing 10% FBS (40). Fibroblasts were plated at a density of 15, 000 cells per 16 mm well and grown at 37 °C with 5% CO_2 until confluent (approximately 7 days). The protocol of Francis *et al.* (41) was followed to load the cells with cholesterol. In brief, confluent cultures were washed twice with phosphate buffered saline containing 2 mg/mL fatty acid free albumin (PBS-FAFA) and then incubated in DMEM containing 2 mg/mL FAFA and 30 mg/mL cholesterol for 48 h. Cells were rinsed twice with PBS-FAFA and incubated for another 24 h in DMEM containing 1 mg/mL FAFA to equilibrate cholesterol pools. Cholesterol-loaded cells were then incubated for 16 h in DMEM with 1 mg/mL FAFA and different concentrations of HDL or apoA-I. The cells were then washed once with PBS, and incubated for one hour at 37 °C with DMEM containing 9 mM [^{14}C]-oleate bound to 3 mM BSA (42). Cells were chilled on ice and the medium was removed and discarded. The cells were rinsed twice with ice-cold PBS containing 2 mg/mL BSA, twice with PBS, and stored at -20 °C for later extraction. Cells were extracted with hexane:isopropanol (3:2, v/v) (43). TLC on PE SIL G plastic backed plates, developed in hexane/diethyl ether/acetic acid (130:40:1.5, v/v/v), was used to separate cholesterol species. TLC plates were stained with I_2 vapour and cholesteryl ester spots were identified by co-migration with standards. The I_2 stain was allowed to fully evaporate, and the

cholesteryl ester spots were cut from the TLC plate for determination of radioactivity by liquid scintillation counting. Cell protein was determined by the Lowry method (39).

3.3 Results

3.3.1 Binding of chicken apoA-I to PLC-treated LDL

ApoA-I interaction with spherical lipoprotein particles was examined by utilizing a functional lipoprotein binding assay. In this assay, PLC served to cleave the phosphocholine headgroups from the phosphatidylcholine surface component of LDL. The diacylglycerol (DAG) which was generated by PLC, causes perturbation of the monolayer surface of LDL leading to particle destabilization and ultimately to aggregation (44, 45). It was shown that addition of exchangeable apolipoproteins to PLC-lipolyzing LDL prevents aggregation presumably by binding to the hydrophobic DAG on the surface (46). The extent of LDL aggregation can be observed spectrophotometrically by measuring absorbance at 340 nm. Over a time course, LDL in the absence of PLC showed no turbidity development, while addition of PLC caused a rapid increase in turbidity (**Figure 3-1a**). Both human and chicken apoA-I were equally competent in preventing turbidity development and, thus, PLC-induced aggregation of LDL. A concentration-dependent assay was performed to determine the relative ability of apolipoproteins to prevent PLC-treated LDL aggregation. Addition of increasing concentrations of apoA-I revealed that both chicken and human apoA-I could reduce PLC-dependent aggregation in a concentration dependent manner. (**Figure 3-1b**). In conclusion, both chicken and human apoA-I had similar abilities to bind to the

lipolyzed LDL and prevent aggregation. A trivial explanation for the observed results obtained is that addition of any protein to this assay could prevent PLC-induced turbidity development. This possibility can be eliminated, however, on the basis of control experiments (**Figure 3-1c**) which revealed that neither bovine serum albumin nor carbonic anhydrase are able to prevent PLC-induced LDL aggregation (P. M.M. Weers and R. O. Ryan, unpublished observations)

3.3.2 Phospholipid disc complex formation Exchangeable apolipoproteins have the ability to transform phospholipid vesicles into bilayer disc complexes. Using the model phospholipids DMPC and DPPC, chicken apoA-I was examined for its ability to produce disc complexes using the sonication method. These complexes were analyzed by nondenaturing PAGE and electron microscopy. Nondenaturing PAGE (**Figure 3-2**) revealed that the DMPC:chicken apoA-I discs migrated as a major species of approximately 300 kDa (Lane 1). Sedimentation equilibrium results identified a single species of 319, 140 Da, indicating that the discs were homogeneous. The DPPC:chicken apoA-I discs were homogeneous and approximately 750 kDa as estimated by nondenaturing PAGE (Lane 2). The disc complexes were examined by electron microscopy (**Figure 3-3**). The differences in molecular mass as determined from the non-denaturing PAGE corresponded to the differences observed in the electron micrographs. Crosslinking studies were performed to allow an estimation of the number of apolipoproteins bound per disc complex. Our studies revealed that there were 2 apoA-I molecules bound to DMPC disc complexes, whereas 4 apoA-I molecules could bind to DPPC disc complexes.

When the lipid:protein ratio (w/w) was varied from 2:1 to 5:1, there was an increase in the molecular mass from approximately 230 kDa to 300 kDa for DMPC:chicken apoA-I discs and from 250 kDa to 750 kDa for DPPC:chicken apoA-I discs.

3.3.3 Cholesterol efflux in mouse peritoneal macrophages

To test the relative ability of human and chicken apoA-I to promote cholesterol efflux, mouse peritoneal macrophages were employed according to the method of Hara and Yokoyama (36). Macrophages were loaded with [1, 2-^{3}H]cholesteryl oleate and then incubated with lipid-free human and chicken apoA-I. Efflux of cholesterol could be measured as the amount of cholesterol (μg/mg cell protein), or percent of originally loaded cellular cholesterol, that was found in the medium after incubation with human or chicken apoA-I. Up to 15 % of the total cellular cholesterol can be effluxed by exchangeable apolipoproteins. Using this system, efflux of cholesterol to human apoA-I has been studied extensively (36), showing that even small amounts of human apoA-I can effectively promote cholesterol efflux (**Figure 3-4**). In comparison, chicken apoA-I accepted cell-derived cholesterol to a lesser extent. In fact, three fold as much chicken apoA-I was necessary to promote a similar amount of cholesterol efflux as human apoA-I (at half maximal efflux). The results showed that chicken apoA-I was capable of promoting cholesterol efflux from mouse peritoneal macrophages, but was not as efficient as human apoA-I.

3.3.4 ApoA-I-induced cholesterol mobilization in human skin fibroblasts

Cholesterol-loaded human skin fibroblasts were incubated with lipid-free human and chicken apoA-I to assess the ability of these exchangeable apolipoproteins to promote cholesterol mobilization. Human and chicken apoA-I were incubated with the cells for 24 h, before [^{14}C]-oleate was added. Because of the esterification of free cholesterol in the cell, oleate will become incorporated into newly synthesized cholesterol ester and act as a gauge of the intracellular cholesterol levels (37). It would be expected that if an apolipoprotein could promote cholesterol efflux, the net pool of cholesterol accessible for esterification by the intracellular enzyme acylCoA:cholesterol acyltransferase (ACAT) would be depleted in the cell, thus resulting in decreased incorporation of [^{14}C]-oleate into cholesteryl ester. Cholesterol-loaded cells incubated in the absence of any apolipoprotein show a very high incorporation of labeled oleate into cholesterol ester. In the presence of increasing amounts of apoA-I, the amount of [^{14}C]-oleate incorporated into cholesteryl ester decreased indicating a decrease in the cholesteryl ester pool. Comparing lipid-free human and chicken apoA-I, the decrease of [^{14}C]-oleate incorporated into cholesteryl ester plotted as a function of apoA-I protein concentration is similar (**Figure 3-5**). This experiment suggested that the relative abilities of human and chicken apoA-I to promote cholesterol mobilization is similar. A parallel experiment using human HDL_3 and chicken HDL was performed. Human HDL_3 contains predominantly apoA-I, with lesser amounts of apoA-II and other apolipoproteins, while chicken HDL contains only apoA-I. The relative abilities of human HDL_3 and chicken HDL to

promote cholesterol mobilization were quite similar, with chicken HDL being a slightly better substrate (**Figure 3-6**).

3.4 Discussion

ApoA-I is an important apolipoprotein due to its involvement in cholesterol transport and homeostasis. As the main structural component of HDL, an activator of LCAT and a mediator of cholesterol efflux in the reverse cholesterol transport pathway, apoA-I serves a protective role in preventing atherosclerosis. An understanding of the structure and function of apoA-I will lead to the ability to harness the beneficial effects of apoA-I in treatment of heart disease.

Chicken apoA-I, which shares 48 % sequence identity with human apoA-I, is unique amongst apoA-I's in that it is monomeric in the lipid-free state. Physical and structural analyses of proteins can be greatly facilitated by working with a monomeric protein, thus chicken apoA-I was an attractive candidate for study. Due to the high degree of sequence similarity (66 %), structure/function studies performed on chicken apoA-I would have relevance to apoA-I, in general. In this chapter, the functional properties of chicken apoA-I were examined and compared with the known functional properties of human apoA-I.

The relative lipid binding affinity of human and chicken apoA-I were analyzed by executing competition assays. Initially, competition of chicken apoA-I with human apoA-I for their native HDL particles gave results indicating that chicken and human apoA-I had similar lipid binding affinities (unpublished observations). The limitation of this assay was that there was no

net addition of apolipoproteins and only a limited exchange of apoA-I proteins. Additionally, HDL_3 was not the best lipoprotein substrate since there were other apolipoproteins on the HDL_3 that could interfere with the competition. Subsequently, competition assays were performed with the lipoprotein, LDLp. The resident exchangeable apolipoprotein on LDLp, apoLp-III, has a lower lipid binding affinity than both human and chicken apoA-I, such that both apoA-I's can displace apoLp-III (22, 26, Figure 5-2, Figure 5-3). By selectively radiolabeling human or chicken apoA-I and coincubating them with LDLp, a measure of the relative lipid binding affinity could be obtained. The results demonstrated that human apoA-I and chicken apoA-I had similar lipid binding affinities such that neither was capable of significant displacement of the other (22).

The PLC assay measures the ability of exchangeable apolipoproteins to prevent aggregation of lipolyzed LDL particles by binding to the LDL surface. This assay served as another means to measure the relative abilities of chicken and human apoA-I to bind to a lipoprotein substrate. Both chicken and human apoA-I had similar abilities to bind PLC-treated LDL and prevent aggregation. This effect was not only time dependent but also concentration dependent. The similarity observed between chicken and human apoA-I in lipoprotein binding (both LDLp and PLC-treated LDL binding) indicated that the difference in self-association properties in the lipid-free state were not apparent in the lipid binding affinities.

Pre-β HDL particles are formed when apoA-I forms a complex with phospholipid and cholesterol at the surface of peripheral cells. It is believed that pre-β HDL serves as an active recruiter of cholesterol at the surface of

peripheral cells and mediates cholesterol efflux (47). These nascent disc-like complexes are then converted to spherical HDL through the action of LCAT (3). Therefore, the ability of apoA-I to form pre-β HDL is an essential part of the reverse cholesterol transport pathway.

Using model phospholipids, DMPC and DPPC, the ability of chicken and human apoA-I to transform phospholipid vesicles into bilayer disc complexes (which resemble pre-β HDL particles) was compared. At a lipid:protein ratio of 5:1 (w/w), DMPC:chicken apoA-I discs and DPPC:chicken apoA-I discs, using the sonication method, produced discs of 319 kDa and 750 kDa, respectively. In comparison, DMPC:human apoA-I discs and DPPC:human apoA-I discs, using the cholate dialysis method at the same lipid:protein ratio, were about 300 kDa (48) and 750 kDa (49). Different lipid:protein ratios could vary the size of DMPC:chicken apoA-I discs or DPPC:chicken apoA-I discs, similar to the effect seen with DMPC:human apoA-I discs (48) or DPPC:human apoA-I discs (49). DMPC:human apoA-I discs and DPPC:human apoA-I discs of similar sizes to the chicken apoA-I discs were shown to contain 2 and 4 apoA-I proteins, respectively (48, 49), the same number of apoA-I proteins on DMPC:chicken apoA-I and DPPC:chicken apoA-I discs. Taken together, chicken and human apoA-I created similarly-sized discs at the same lipid:protein ratios. The sizes of these disc complexes could be varied at different lipid:protein ratios. Both chicken and human apoA-I discs contained the same number of apoA-I proteins per disc particle for similarly-sized discs. Therefore, it was concluded that chicken and human apoA-I behave similarly in producing phospholipid disc complexes. It should be noted, though, that the relative propensity to form discs may be different. A

time course experiment examining the ability of chicken and human apoA-I to solubilize DMPC vesicles would address this issue.

When lipid to protein ratios vary, it is possible that chicken and human apoA-I can undergo conformational changes to accomodate the varying amounts of disc surface area (50). The conformation of apoA-I on the disc complexes is controversial. Originally, the model of human apoA-I structure on discoidal complexes suggested that the helices were aligned parallel to the acyl chains of the phospholipids in the bilayer (51), and this was further supported by Fourier transform infrared spectroscopy data (52). Recently, the elucidation of the crystal structure of human apoA-I (residues 44-243) (53) provided strong evidence that the helices may be perpendicular to the acyl chains. This model was supported by other apolipoprotein disc complex models (31, 54). The actual orientation of helical segments of apoA-I on disc complexes may, in fact, be both parallel and perpendicular to the acyl chains. As pre-β HDL particles are converted to spherical HDL particles, apoA-I must undergo some realignment of helical segments to accomodate the addition of new lipid. The pre-β HDL must occassionally accomodate an odd number of apolipoproteins, arguing against models that have proteins in symmetrical alignments. As well, the process must be reversible, as apoA-I must be able to associate and dissociate from the HDL particles when required. It is not yet possible to elucidate the model of apoA-I association.

As a cellular model, mouse peritoneal macrophages can be cultured and loaded with cholesterol to resemble the major histological and biochemical features of foam cells. Foam cells are monocytes/macrophages that have intracellular accumulation of lipids and are the primary pathological hallmark of

the progression of atherosclerosis (55, 56). Treatment of mouse peritoneal macrophages with apolipoproteins mimics cellular cholesterol efflux as a step in the reverse cholesterol transport pathway (37, 57, 58). The relative ability of chicken and human apoA-I to promote efflux of cholesterol was assayed in the cholesterol-loaded mouse peritoneal macrophage model. The results indicated that human apoA-I was better at promoting efflux of cholesterol. For 50 % of maximal efflux, only 5 μg of human apoA-I, while 15 μg of chicken apoA-I was required.

To address the effect of apolipoproteins on the promotion of cellular cholesterol mobilization, cultured cells were utilized. Human skin fibroblasts have been extensively used as a peripheral tissue model in receptor-mediated lipoprotein uptake and apolipoprotein-mediated cholesterol efflux. Human skin fibroblasts were loaded with cholesterol and then treated with HDL or apoA-I, both having the ability to promote cholesterol efflux. Since cholesterol is constantly being esterified to cholesteryl ester, hydrolyzed, and reesterified, labeling of cholesterol can be accomplished by addition of [^{14}C]-oleate as a fatty acid substrate for the esterification reaction by ACAT (37). Therefore, intracellular addition or removal of cholesterol will alter the pool of cholesteryl ester and change the amount of [^{14}C]-oleate incorporation. This indirect measure of cholesterol levels in cells has been shown to be correlated to the extent of cholesterol efflux (21, 41, 59, 60). The results showed that chicken and human apoA-I behaved similarly in their respective abilities to promote cholesterol mobilization. Similar to other published data (60), only low concentrations of apoA-I (5 μg) were necessary to promote maximal effect. Chicken HDL and human HDL_3 also showed similar abilities to promote

cholesterol mobilization. Similar to the results of Wang *et al.* (60), HDL was slightly less efficient than free apoA-I at promoting cholesterol efflux. As well, it appears that chicken HDL was a slightly better substrate for cholesterol mobilization than human HDL_3. Although the difference is minor, the relative resistance of apoA-II containing HDL particles to activate LCAT and mediate cholesterol efflux may account for the difference (61-63). In summary, chicken and human apoA-I, and chicken HDL and human HDL_3 show similar abilities to promote cholesterol mobilization.

Further understanding of the relative functions of chicken and human apoA-I could be gained from the comparison of the ability of human and chicken apoA-I to activate lecithin:cholesterol acyltransferase (LCAT). LCAT facilitates the conversion of HDL cholesterol to cholesteryl ester and thereby plays a role in generating spherical HDL particles in the reverse cholesterol transport pathway. It is not obvious whether measuring LCAT activity of the apoA-I's with their respective plasma LCAT's or comparing both using human LCAT would be the most physiological or informative. The specificity of chicken and human LCAT for their substrates is variable (64) and the structure and expression of chicken LCAT is different than mammalian LCATs (65). On the other hand, it has been shown that some exchangeable apolipoproteins and amphipathic α-helical peptides are capable of activating human LCAT (66, 67), suggesting a lack of specificity excepting amphipathic α-helical secondary structure. It would be informative to investigate the LCAT activation properties of chicken apoA-I in comparison to human apoA-I.

In 1980, a group in Milano, Italy, identified a natural mutation in human apoA-I (68, 69). The carriers of apoA-I_{Milano} showed decreased

incidences of heart attacks or progression of atherosclerosis despite having risk factors for atherosclerosis. Researchers have shown that an Arg to Cys mutation at position 174 of human apoA-I is responsible for this beneficial effect (69). The structural changes in apoA-I_{Milano} manifest in the Arg174Cys mutation result in altered functional properties that, in this case, were extremely beneficial. Homodimers of apoA-I_{Milano} produced phospholipid disc complexes that were poorer substrates for LCAT activation (70, 71). Other researchers demonstrated that apoA-I_{Milano} could activate LCAT normally, but was not as efficient in promoting cholesterol efflux (72). It is not clear how the poorer ability of apoA-I_{Milano} in reverse cholesterol transport functions can be reconciled with the beneficial aspects observed in carriers of apoA-I_{Milano}. Therefore, further examination of the structure/function relationship of apoA-I is critical for elucidating the beneficial mechanism of apoA-I action.

In this chapter, the functional properties of chicken apoA-I were examined and compared to human apoA-I. Chicken apoA-I was found to have a similar lipid binding affinity based on competition assays with human apoA-I. Chicken and human apoA-I had similar abilities to produce disc complexes with model phospholipids. In human skin fibroblasts, lipid-free chicken and human apoA-I had similar abilities to promote cholesterol mobilization, as did chicken HDL and human HDL_3. In mouse peritoneal macrophages, lipid-free human apoA-I had a greater ability than lipid-free chicken apoA-I to promote cholesterol efflux. Therefore, with the exception of the ability to mediate cholesterol efflux in macrophages, chicken and human apoA-I were functionally similar. Chicken apoA-I will be characterized in terms of LCAT activation, conformational changes upon lipid binding and potential receptor

interactions to fully elucidate the functional aspects of chicken apoA-I. With the benefit of a monomeric apoA-I and its functional characterization, elucidation of the structure/function properties of apoA-I may be enhanced.

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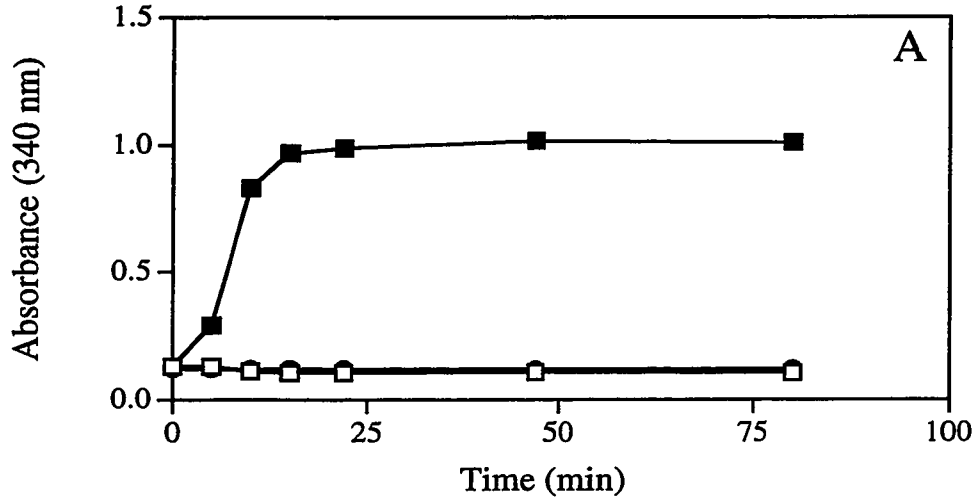
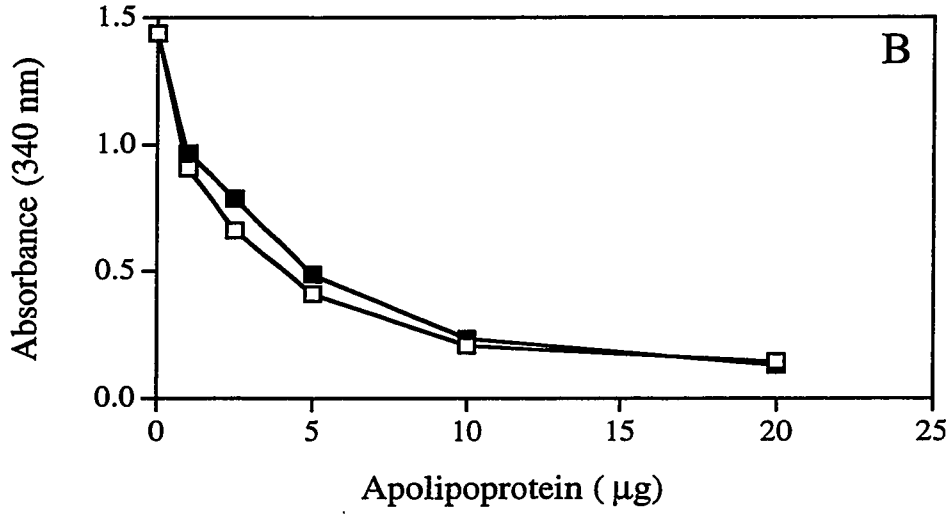
Figure 3-1a: Binding and stabilization of PLC-treated LDL by chicken and human apoA-I. Human LDL (100 μg) was incubated with 160 mU of PLC for 80 minutes at 37 °C in the absence and presence of chicken and human apoA-I (30 μg each). Control incubations containing no added apolipoproteins (closed circles) or no PLC (open circles), were included. At the indicated time points, sample absorbance at 340 nm was measured. Open squares, human apoA-I; closed squares, chicken apoA-I.

Figure 3-1b: Apolipoprotein concentration-dependent prevention of PLC-induced aggregation of LDL. Human LDL (150 μg) was incubated with 160 mU of phospholipase C (PLC) in the absence and presence of increasing amounts of chicken and human apoA-I. The absorbance at 340 nm was determined after 60 min. Open squares, human apoA-I; closed squares, chicken apoA-I.

Figure 3-1c: Effect of control proteins on PLC-induced aggregation of LDL. Human LDL (50 μg) was incubated with 160 mU of PLC for 120 minutes at 37 °C in the absence and presence of bovine serum albumin and carbonic anhydrase (30 μg each). Control incubations containing no added apolipoproteins (closed squares) or no PLC (open squares), were included. At the indicated time points, sample absorbance at 340 nm was measured. Open circles, bovine serum albumin; closed circles, carbonic anhydrase. (P. M. M. Weers and R. O. Ryan, unpublished data)


A
Absorbance (340 nm)
1.5
1.0
0.5
0.0
0
25
50
75
100
Time (min)


B
Absorbance (340 nm)
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Apolipoprotein (µg)

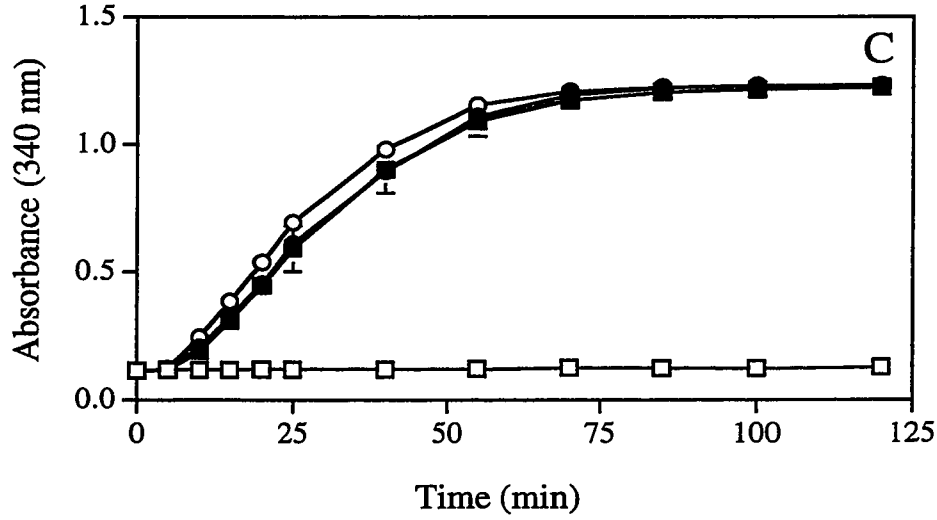

C
Absorbance (340 nm)
1.5
1.0
0.5
0.0
0
25
50
75
100
125
Time (min)

Figure 3-2: Chicken apoA-I disc complexes analyzed by non-denaturing PAGE. DPPC and DMPC chicken apoA-I discs were made according to the Experimental Procedures. DMPC:chicken apoA-I discs (Lane 1) and DPPC:chicken apoA-I discs (Lane 2) were analyzed on a 4 - 20% non-denaturing polyacrylamide gel and compared with non-denaturing gel standards. Corresponding particle sizes are 669 kDa, 17 nm; 440 kDa, 12.2 nm; 232 kDa, 10.4 nm; 140 kDa, 8.4 nm; 67 kDa, 7.3 nm.

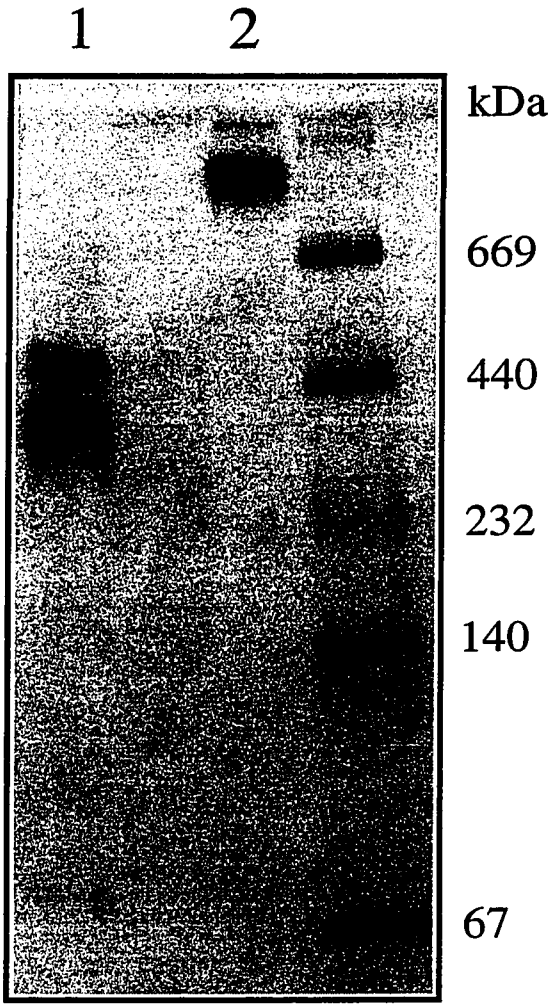
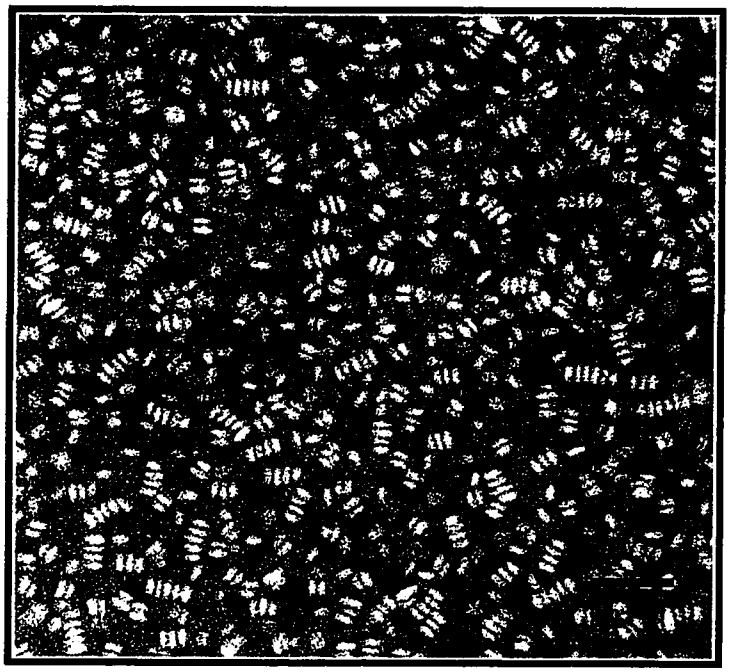
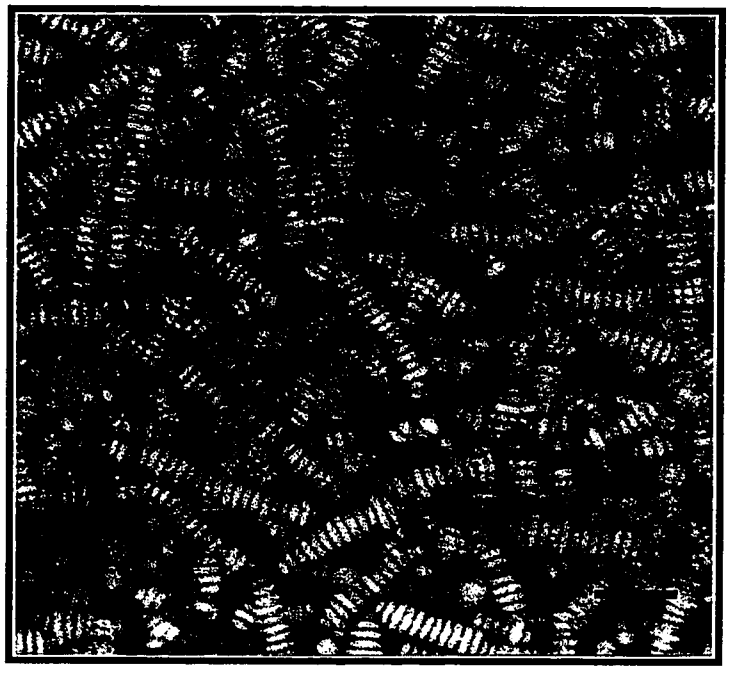

1
2
kDa
669
440
232
140
67

Figure 3-3: Electron micrographs of chicken apoA-I disc complexes. DMPC:chicken apoA-I disc complexes (A) and DPPC:chicken apoA-I disc complexes (B) were prepared for electron microscopy according to Experimental procedures. The bars represent 50 nm and both panels are of the same magnification.



A



B

Figure 3-4: Cholesterol efflux in mouse peritoneal macrophages by chicken and human apoA-I. Macrophages in monolayer were incubated with 50 μg/mL of acetylated LDL containing [^{3}H]cholesteryl oleate for 24 h at 37 °C, washed, and incubated for another 24 h in the absence of lipoproteins. Each dish of cells was given 1 mL of RPMI 1640 medium with 2 mg/mL bovine serum albumin containing increasing amounts of human apoA-I (open squares) or chicken apoA-I (closed squares) and incubated for 24 h. The cholesterol in the medium was measured as described in Experimental Procedures. Results are the average of three independent determinations ± SD. The values are expressed as the micrograms of cholesterol released into the medium/milligram cell protein. The average cell protein per dish was 90 μg.

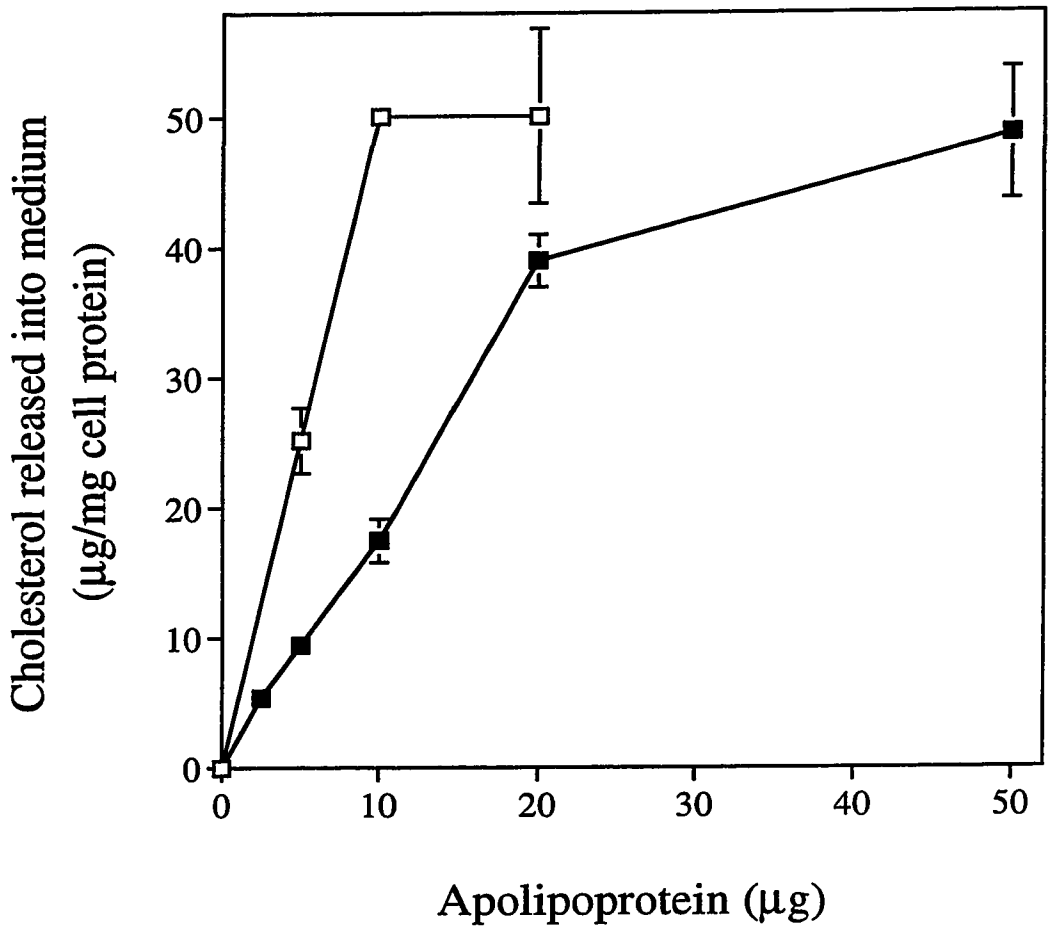

Cholesterol released into medium
(µg/mg cell protein)
0
10
20
30
40
50
0
10
20
30
40
50
Apolipoprotein (µg)

Figure 3-5: Promotion of cholesterol mobilization by human and chicken apoA-I in human skin fibroblasts. Confluent cultures of human fibroblasts loaded with cholesterol were incubated with the indicated concentrations of human apoA-I (open squares) or chicken apoA-I (closed squares) for 16 h. After the incubation, cells were washed, incubated with [^{14}C]-oleate for 1 h. Cellular lipids were extracted, separated by TLC and then the amount of cellular cholesteryl [^{14}C]-oleate was determined. Values are expressed as a percentage of 380.15 picomoles of [^{14}C]-oleate incorporated into cholesteryl oleate/mg of cellular protein, corresponding to serum free medium (SFM)-treated cells. The results are the mean of 3 determinations ± SD.

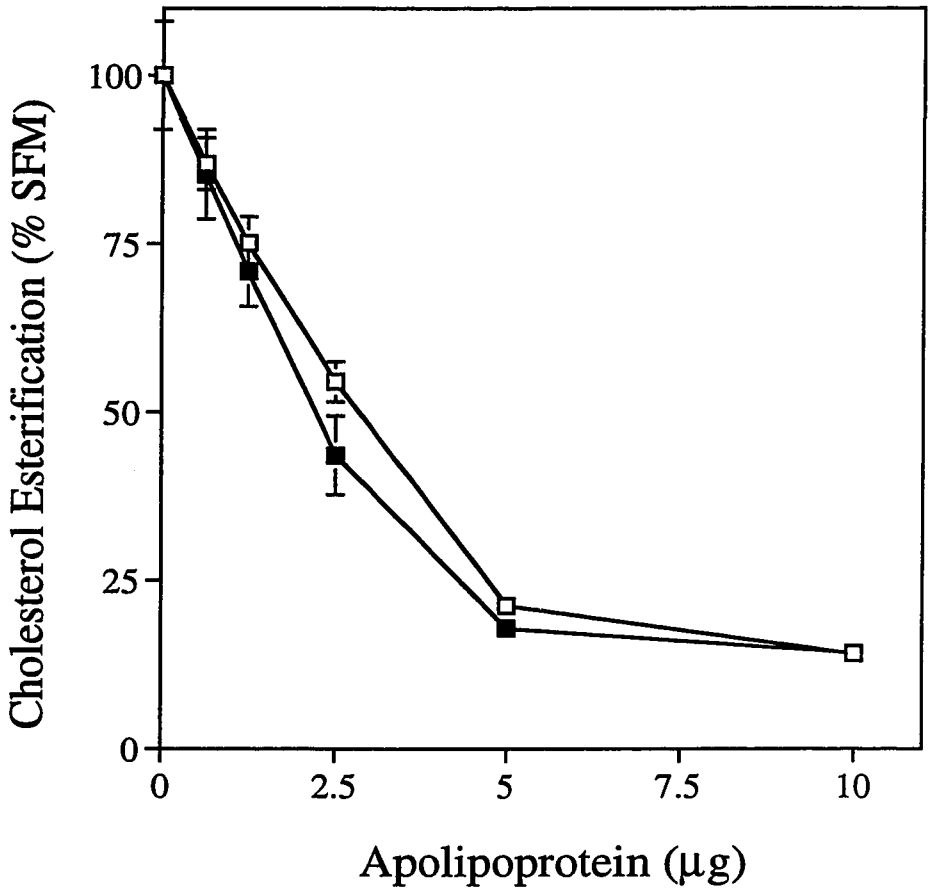
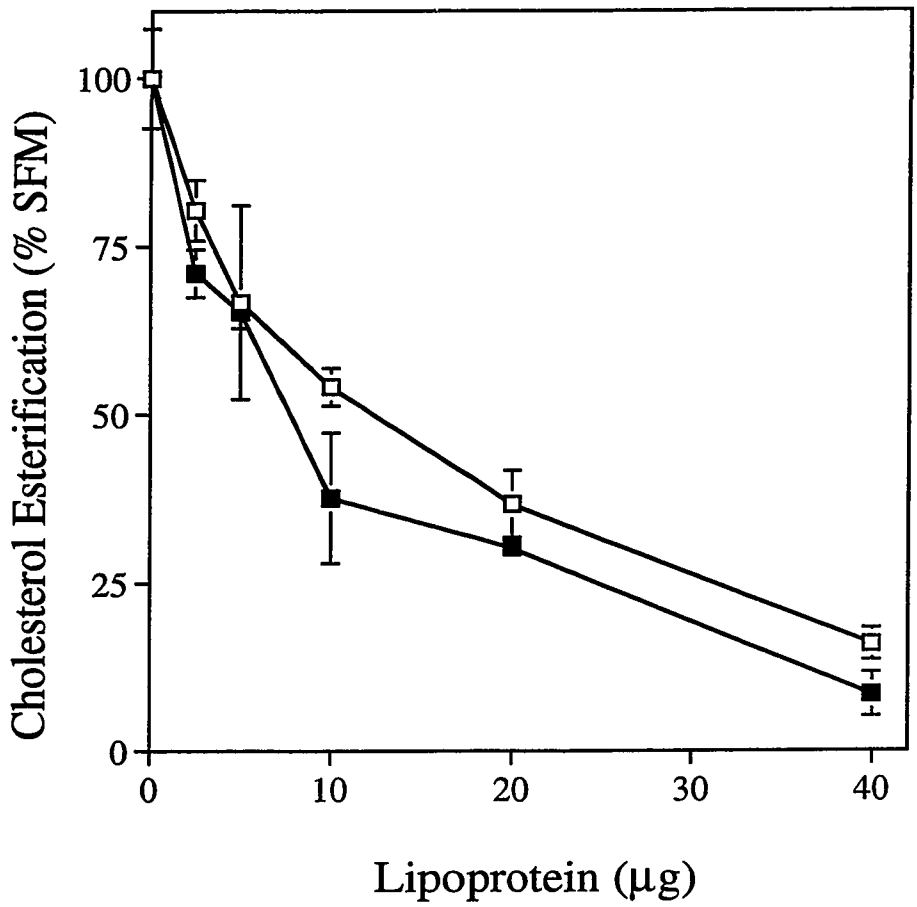

Cholesterol Esterification (% SFM)
100
75
50
25
0
0
2.5
5
7.5
10
Apolipoprotein (µg)

Figure 3-6: Promotion of cholesterol mobilization by human HDL_3 and chicken HDL in human skin fibroblasts. Cholesterol-loaded fibroblasts were incubated with increasing concentrations of human HDL_3 (open squares) and chicken HDL (closed squares) under the same conditions as in Figure 3-5. Values are the mean of three independent determinations ± SD and are expressed as a percentage of 380.15 picomoles of [^{14}C]-oleate in cholesteryl oleate/mg cell protein corresponding to serum free medium (SFM)-treated cells.


Cholesterol Esterification (% SFM)
100
75
50
25
0
0
10
20
30
40
Lipoprotein (µg)

Chapter 4

Bacterial synthesis and characterization of recombinant chicken apolipoprotein A-I

This work was previously published:

Kiss, Robert S., Kay, Cyril M., and Ryan, Robert O. (1998) *Protein Expression and Purification* **12**, 353-360.

4.1 Introduction

Apolipoprotein A-I (apoA-I) from humans is a 243 amino acid protein involved in cholesterol transport and homeostasis (1). ApoA-I is responsible for the activation of the plasma enzyme lecithin:cholesterol acyl transferase (LCAT; 2-4) and is the main protein component of high density lipoprotein (HDL). In plasma, apoA-I is believed to be present in both lipid-poor and lipid-rich states (5). In the lipid-poor state, apoA-I is an acceptor of cellular cholesterol and, as such, can induce cholesterol efflux from cells (6, 7). ApoA-I has been proposed to be involved in the return of cholesterol from peripheral tissues to the liver (the reverse cholesterol transport pathway) and this may account for its beneficial effects in preventing heart disease (5). Study of these processes is hindered because apoA-I from mammalian sources self associates in the absence of lipid (8) and this tendency to oligomerize is a major impediment to obtaining structural information on this protein.

Chicken apoA-I (240 amino acid residues) is a close relative of human apoA-I, sharing 48% sequence identity and 66% sequence similarity (as determined by SEQSEE sequence homology program; 9). Chicken apoA-I is practically the sole apolipoprotein component of chicken HDL (10, 11). Unlike human apoA-I, chicken apoA-I was found to be monomeric in the absence of lipid (12). Physical characterization revealed that, as with the 22 kDa N-terminal domain of human apolipoprotein E (apoE3(1-183); 13) and insect apolipophorin III (14, 15), chicken apoA-I may exist as an amphipathic α-helix bundle (Chapter 2). Likewise, it has been shown that chicken apoA-I is functionally analogous to human apoA-I in terms of LCAT activation (16), lipoprotein binding (12, Chapter 3), and the ability to induce cholesterol efflux

(Chapter 3). Thus, chicken apoA-I is a good candidate for further structural and physical characterization.

Other studies have shown a unique tissue distribution of chicken apoA-I mRNA compared to that of mammalian apoA-I. Chicken apoA-I mRNA is found in skin (17), muscle tissue (18), nerve tissue (19), kidney (20), as well as small intestine and liver (21), whereas human apo A-I is predominantly found in small intestine and liver (22). Interestingly, the tissue expression pattern of chicken apoA-I more closely resembles that of mammalian apoE than apoA-I (23). Indeed, nerve ablation studies indicate that chicken apoA-I resembles apoE in mammalian systems in terms of its synthesis at sites of nerve injury (24). Chicken apoA-I, like apoE, has been proposed to play a role in nerve regeneration. The fact that chickens do not possess apoE has led to the postulate that chicken apoA-I may fulfill functional roles in the chicken that are met by apoE in mammals (18, 23). In order to address hypotheses related to the structural and functional properties of chicken apoA-I, we have cloned chicken apoA-I for production of full length recombinant apoA-I in a bacterial system.

4.2 Experimental Procedures

Plasmid vector construction: A plasmid encoding the cDNA of chicken apoA-I (a kind gift of Dr. Alan Attie, University of Wisconsin) served as template for amplification of the coding sequence of chicken apoA-I. Oligonucleotides containing nonannealing Msc I and Hind III restriction sites served as primers for DNA amplification. The amplification product was cloned into the pET-22b(+) plasmid (Novagen, Cambridge, MA) directly downstream

of the plasmid-encoded pelB leader peptide (which functions to target the expressed protein to the periplasmic space of the cell). The final construct included a stop codon immediately following the nucleotides encoding the C-terminal amino acid of chicken apoA-I. Thus, no carboxyl tags or extensions were employed in the present strategy. The sequence of the chicken apoA-I/pET plasmid DNA insert was verified by the chain termination sequencing procedure (24, ABI Prism dye terminator cycle sequencing reaction ready kit, Perkin Elmer).

Synthesis of recombinant apoA-I: E. coli BL-21 (DE3) cells transformed with the apoA-I/pET plasmid were used to synthesize recombinant protein. A single bacterial colony was used to seed a 3 mL culture of yeast tryptone medium with 200 μg/mL carbenicillin (Sigma, Oakville, ON) and grown at 37 °C. After the culture reached an optical density of 0.6 units (measured at 600 nm) the cells were pelleted at 10, 000 g. The cells were resuspended in 20 mL medium containing 500 μg/mL carbenicillin and cultured at 37 °C to an OD_{600} = 0.6. Following this, the cells were pelleted at 10, 000 g, resuspended in 250 mL medium, induced with isopropyl β-D thiogalacto-pyranoside (IPTG; 1mM final concentration) and cultured for 4 h. Cells were harvested by pelleting and resuspended in buffer (10 mM Tris-HCl, 1 mM glutathione, and 1 mM diisopropylflourophosphate). The suspension was sonicated with a tip sonicator until homogeneous. Cell debris was removed by centrifugation and the supernatant retained.

To assess if the protein was targeted to the periplasmic space, the protocol of Ames *et al.* was utilized (25). A 1.5 mL aliquot of induced culture was

centrifuged at 13, 000 g for 1 min. The supernatant was decanted and 15 μL of chloroform was added. The cells were vortexed and, after 15 min at 22 °C 75 μL of 10 mM Tris-HCl, pH 8, was added and the sample centrifuged at 13,000 g for 15 min. The supernatant (soluble periplasmic space fraction) was then analyzed.

To cleave the pelB leader peptide from the recombinant protein, a soluble, truncated form of leader peptidase (a kind gift of Dr. Ross Dalbey, Ohio State University; 26, 27) was incubated with the sonicated cell suspension (1:1000, 1:100, or 1:10, enzyme:substrate ratio) in the presence or absence of 0.1% Triton X-100 (BDH Chemicals, Toronto, ON) for 4 h at 37 °C. Leader peptidase-treated protein was isolated by high pressure liquid chromatography (HPLC) on a semi-preparative, reversed-phase RXC-8 Zorbax 300SB column (9.4 mm x 25 cm) on a Beckman high pressure liquid chromatograph. Fractions were monitored at 210 nm and eluted with a linear gradient of 0.5% B/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions containing purified protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analytical HPLC (RXC-8 Zorbax 300SB column, 2.1 mm x 15 cm) using a linear AB gradient of 2% B/min, pooled, lyophilized and stored at -20 °C until use.

Analytical procedures: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (28) and gels were stained with Amido Black 10B (Merck, Darmstadt, Germany). Immunoblot analysis was conducted by transferring SDS-PAGE gel contents

to a polyvinylidene difluoride membrane (Millipore Corp.) and probing with a chicken apoA-I rabbit polyclonal antibody at a dilution of 1:5000. An enzyme-linked secondary antibody (horseradish peroxidase-linked goat anti-rabbit IgG) and chemiluminescence reagents (Amersham) were used to detect antigen:antibody interactions. Electrospray ionization mass spectrometry was performed as previously described (29). A VG Quattro electrospray mass spectrometer (Fisons Instruments, Manchester, U. K.) was used to detemine molecular mass by taking the mean value of several multiply charged ions in a coherent system.

Spectroscopic studies: Circular dichroism (CD) spectroscopy was performed using a Jasco J-720 spectropolarimeter following protocols previously described (30). Fluorescence spectroscopy was conducted on a LS 50 Luminescence spectrometer (Perkin Elmer, Beaconsfield, England) at 24 °C. Tryptophan fluorescence emission spectra were recorded for plasma-derived and recombinant chicken apoA-I (10 μg/mL in 50 mM sodium phosphate buffer, pH 7.0) in the absence and presence of 0.4% lysophosphatidylcholine (LPC, Avanti Polar Lipids); emission spectra were obtained between 300 nm and 450 nm with excitation at 295 nm. Excitation and emission slit widths were set at 4 nm.

Lipid binding assays: Three different binding assays were used to assess the ability of recombinant apoA-I to interact with lipids. In the first assay (31), human low density lipoprotein (LDL, 150 μg protein) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, was incubated with 80 milliunits of *Bacillus*

cereus phospholipase C (PLC; Sigma Chemical Co., St. Louis, MO), in the absence or presence of plasma-derived or recombinant chicken apoA-I (50 μg). Sample turbidity was measured at 340 nm on a Spectromax 250 microtitre plate reader (Molecular Devices, Sunnyvale, CA) at indicated time points. In a second lipoprotein binding assay, plasma-derived and recombinant chicken apo A-I were assayed for their ability to prevent aggregation of human LDL induced by facilitated lipid transfer, as described by Singh *et al.* (32). Incubations were carried out in a volume of 200 μL containing 250 μg *Manduca sexta* high density lipophorin protein, 50 μg human LDL protein, 2 μg *M. sexta* lipid transfer particle and 50 μg chicken apoA-I (plasma-derived or recombinant). Sample turbidity was monitored at regular time intervals at 340 nm. Lastly, the ability of recombinant apoA-I to transform bilayer vesicles of dimyristoylphosphatidylcholine (DMPC) into disc complexes was studied as follows (33): DMPC (2.1 mg) was dissolved in chloroform:methanol (3:1, vol:vol) and dried to form a thin film in a test tube. Sodium cholate (2.7 mg) was added (cholate:phospholipid molar ratio 2:1), vortexed, and incubated at 37 °C for 30 minutes until the solution was clear. Plasma-derived or recombinant chicken apoA-I (0.9 mg) in buffer A (10 mM Tris, pH 7.4, 140 mM NaCl, 0.15 mM sodium azide, and 0.25 mM sodium EDTA) was added and the solution incubated for 60 min at 37 °C. The sample was then dialyzed exhaustively at 37 °C against buffer A and analyzed by pore limiting native gradient polyacrylamide gel electrophoresis (34).

4.3 Results and Discussion

4.3.1 Synthesis and purification of recombinant chicken apoA-I.

The nucleotide sequence encoding chicken apoA-I (residues 1-240) was ligated into the pET-22b(+) vector and introduced into *E. coli* BL21 (DE3). Following induction and culturing, the cells were harvested and examined for recombinant apoA-I production. SDS-PAGE analysis of the soluble fraction of cell pellets from cultures harboring the parent pET vector or the apoA-I/pET vector is shown in **Figure 4-1**. Comparison of the protein patterns revealed the presence of a prominent band at ~30,000 Da in induced cultures harboring the apoA-I/ pET plasmid grown at three different temperatures. The slower migration of the enriched protein compared to plasma-derived chicken apoA-I standard (lane 1) indicated it has a higher apparent molecular weight. Given that the plasmid construct employed encodes an 18 amino acid N-terminal leader sequence extension (the pelB sequence), we hypothesized that the apparent molecular weight difference was due to accumulation of an apoA-I-pelB fusion protein. This leader sequence is designed to target expressed proteins to the periplasmic space, where an endogenous leader peptidase should function to produce mature apoA-I. However, under the different culture temperature conditions examined, the molecular weight difference persisted, suggesting that either the recombinant protein was not a substrate for the leader peptidase or the protein was not targeted to the periplasmic space. Subsequently, the periplasmic space contents from an aliquot of cells expressing chicken apoA-I were isolated. SDS-PAGE analysis of these fractions indicated no accumulation of protein. It is likely, then, that the synthesized apoA-I protein was not targeted to the periplasmic space and

therefore could not be acted upon by the endogenous leader peptidase. It is probable that the nature of the synthesized protein itself hinders its transport. It is known from previous displacement studies (Chapter 3) that chicken apoA-I has a higher lipid binding affinity than another protein produced with this system (34) and this characteristic may prevent apoA-I from efficient targeting and cleavage.

4.3.2 Immunoblot analysis of recombinant chicken apoA-I

To confirm that the protein enriched in induced bacterial cultures harboring the apoA-I/pET vector was indeed chicken apoA-I, an immunoblot was performed. When the soluble fraction obtained from the bacterial cell pellet of induced cultures was probed with a polyclonal antibody directed against chicken apoA-I, the 30 kDa band was recognized along with a less intense reactive band, of mass corresponding to that of the chicken apoA-I standard (**Figure 4-2**). Control immunoblots probing bacterial cell pellets harboring the parent pET vector with the same antibody failed to reveal cross reactive proteins.

4.3.3 Signal peptidase cleavage of pelB leader sequence

To evaluate if the slower mobility of the major reactive band was due to failure of the endogenous leader peptidase to efficiently cleave the N-terminal pelB sequence, an isolated, soluble, recombinant leader peptidase was employed in *in vitro* cleavage experiments with recombinant apoA-I (**Figure 4-3**). At an enzyme:substrate ratio of 1:1000, specific cleavage of the 30 kDa band occurred, resulting in appearance of a protein product of approximately 28 kDa. Inclusion of Triton X-100 had no effect on the extent of cleavage at this

ratio while increasing the enzyme concentration to 1:100 (enzyme: apoA-I) resulted in nearly complete cleavage. Importantly, at a 1:10 ratio, additional lower molecular weight cleavage products appeared, suggesting that non-specific cleavage had occurred. On the basis of these data, subsequent cleavage experiments were performed at a ratio of 1:100 (peptidase: recombinant apoA-I) for 4 h at 37 °C in buffer lacking detergent.

4.3.4 Purification of recombinant chicken apoA-I

The ability of isolated leader peptidase to specifically cleave recombinant chicken apoA-I was incorporated into the purification scheme. Due to the relative hydrophobicity of recombinant apoA-I, reversed phase HPLC was used to remove contaminating proteins present in the soluble fraction of the bacterial cell pellet, resulting in a >95% pure protein preparation. Electrospray mass spectrometry of the isolated recombinant protein revealed a molecular weight of 27,961 ± 4 Da, in agreement with that predicted from the amino acid sequence of chicken apoA-I (35) as well as previous molecular weight determinations for apoA-I isolated from chicken plasma, which yielded values of 28,000 (10) and 28,170 (12). The results indicate that processing of chicken apo A-I by the leader peptidase was complete and specific. In **Figure 4-4**, an analytical HPLC trace of the soluble fraction of the cell pellet after treatment with leader peptidase is shown.

Unlike experience with synthesis of other exchangeable apolipoproteins using the pET vector (apoE3(1-183) (29); apoLp-III (34)), in the case of recombinant chicken apoA-I, the pelB leader peptide was not efficiently cleaved by the endogenous leader peptidase and the synthesized protein did

not specifically accumulate in the culture medium. These factors complicated the purification and limited the yield of recombinant protein. However, refinement of conditions have yielded expression levels of up to 50 mg apoA-I/L culture medium. Furthermore, addition of exogenous leader peptidase overcame the complication of the pelB leader peptide removal. As a result, adequate amounts of purified recombinant chicken apo A-I could be obtained using this system. The final yield was approximately 10 mg purified mature recombinant protein/L of bacterial cell culture.

4.3.5 Characterization of recombinant chicken apoA-I

The structural properties of recombinant apoA-I were evaluated spectroscopically. Far-UV CD analysis of recombinant apoA-I revealed the characteristic high content of α-helical secondary structure (**Figure 4-5**). Addition of 50% trifluoroethanol (TFE) resulted in a further induction of α-helix. The fact that this pattern was quite similar to that observed for plasma-derived chicken apoA-I (12) suggests that recombinant apoA-I adopts a folded conformation in solution similar to its natural counterpart.

Confirmation of proper folding of recombinant chicken apoA-I was obtained from tryptophan fluorescence emission spectra (**Figure 4-6**) recorded in the absence and presence of 0.4% LPC. LPC micelles act as a lipid surface for apolipoprotein binding and provide a potential hydrophobic environment for the two tryptophan residues in the protein (residues 74 and 107; 35). Emission spectra (excitation 295 nm) showed no shift in tryptophan fluorescence emission maximum (331 nm) upon addition of LPC, indicating no change in the relative hydrophobicity of the environment of the two

tryptophans. This trend is identical to the behavior of plasma-derived chicken apoA-I, which displayed a tryptophan fluorescence emission maximum of 331 nm. Helical wheel modeling of chicken apoA-I indicates that the two tryptophans reside on the hydrophobic face of amphipathic α-helices. In the lipid-free state, it is conceivable that these tryptophans are sequestered from the aqueous environment by helix-helix contacts while, in the lipid-bound state, the tryptophans interact with the lipid surface. Another exchangeable apolipoprotein, *L. migratoria* apoLp-III, contains one tryptophan in the middle of an amphipathic helix and one tryptophan in an exposed loop region (14). Upon binding to lipid, a 15 nm blue shift in fluorescence emission maximum occurred, indicating a transition to a more hydrophobic environment (36). Although high resolution structural data are not available for full-length apoA-I, the relative location of tryptophan residues in the protein and their fluorescence properties are supportive of the concept that chicken apoA-I exists as a α-helix bundle in the absence of lipid and, upon association with a lipid surface, substitutes helix-lipid interactions for helix-helix interactions which stabilize its lipid-free conformation (Chapter 2).

4.3.6 Functional analysis of recombinant chicken apoA-I

To examine apoA-I interaction with spherical lipoprotein particles a functional lipoprotein binding assay was employed. Human LDL aggregates following incubation with PLC due to hydrolysis of the phosphocholine head group from phosphatidylcholine molecules present in the surface monolayer of the particle, creating the membrane-destabilizing lipid, diacylglycerol. This results in LDL particle aggregation seen as an increase in sample turbidity which is

conveniently monitored spectrophotometrically. When functional amphipathic exchangeable apolipoproteins are included in the incubation with LDL and PLC, however, particle aggregation is prevented by apolipoprotein binding with the lipolyzed lipoprotein surface (31). As shown in **Figure 4-7**, recombinant apoA-I was effective in preventing aggregation of human LDL induced by treatment with PLC and this effect was comparable to plasma-derived chicken apoA-I.

A second lipoprotein binding assay was employed, in which the lipid content of human LDL was enriched by facilitated lipid transfer employing insect lipid transfer particle as the catalyst and insect high density lipophorin as lipid donor (32, 37). As with PLC treated LDL, lipid transfer-induced LDL particle aggregation was prevented by inclusion of recombinant apoA-I through formation of a stable binding interaction with the modified lipoprotein. Recombinant chicken apoA-I was functional and indistinguishable from plasma-derived chicken apoA-I in these lipid binding assays.

A hallmark property of exchangeable apolipoproteins is an ability to transform phospholipid bilayer vesicles into disc complexes. To examine the ability of recombinant chicken apoA-I to function in this manner, apoA-I was incubated with multilamellar bilayer vesicles of DMPC. Following incubation, the sample was analyzed by nondenaturing PAGE. The data revealed formation of a discrete population of particles in the range of 200 kDa, indicating disc particle formation.

4.4 Conclusions

In summary, the present results show that recombinant chicken apoA-I can be synthesized in bacteria and isolated to yield a protein product that is immunologically indistinguishable from plasma-derived chicken apoA-I. The isolated protein has a mass identical to that predicted for chicken apoA-I and contains similar amounts of secondary structure conformers as the plasma-derived protein. Furthermore, recombinant apoA-I is fully functional in lipid binding assays, indicating its suitability for studies designed to examine mechanistic details of its important biological roles. Studies employing site-directed mutagenesis to probe specific aspects of apoA-I function are described in Chapter 5.

Acknowledgement

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Figure 4-1: Bacterial synthesis of chicken apoA-I. Transformed bacterial cultures were grown at for 2.5 h, induced with IPTG and grown for another 4 h. Cell pellets were obtained by centrifugation, sonicated and an aliquot of the soluble fraction subjected to SDS-PAGE on a 12% acrylamide slab gel. Lanes 1-3, cells harboring chicken apoA-I/pET vector grown at 30, 27 and 20 °C, respectively; Lane 4, cells harboring the pET vector grown at 30 °C; Lane 5, plasma-derived chicken apoA-I.

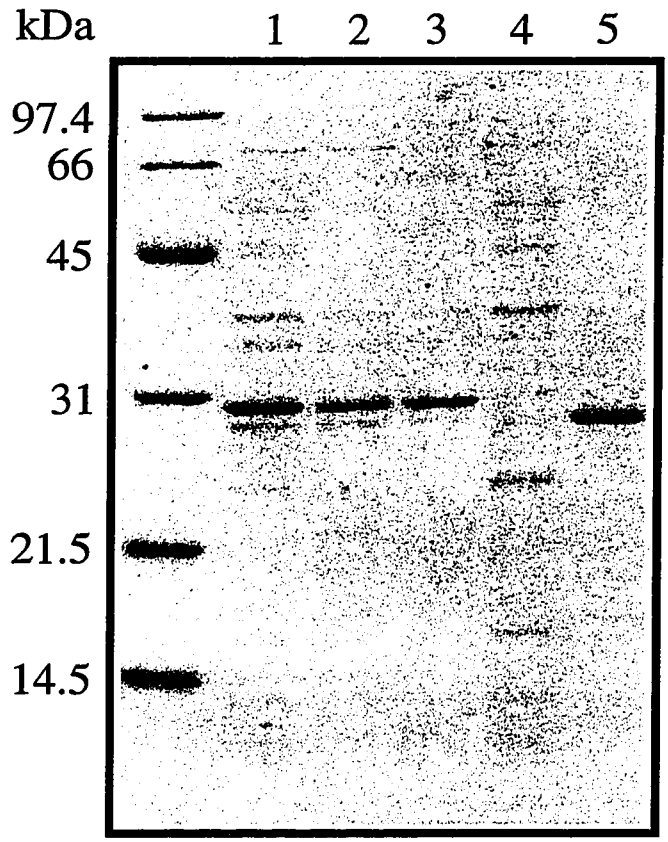

kDa
1
2
3
4
5
97.4
66
45
31
21.5
14.5

Figure 4-2: Immunoblot of recombinant chicken apoA-I. The immunoblot was probed with rabbit polyclonal anti-chicken apoA-I serum as described in Experimental Procedures. Lane 1, plasma-derived chicken apoA-I; Lane 2, soluble fraction from cells harboring pET vector; Lane 3, soluble fraction from cells harboring chicken apoA-I/pET vector.

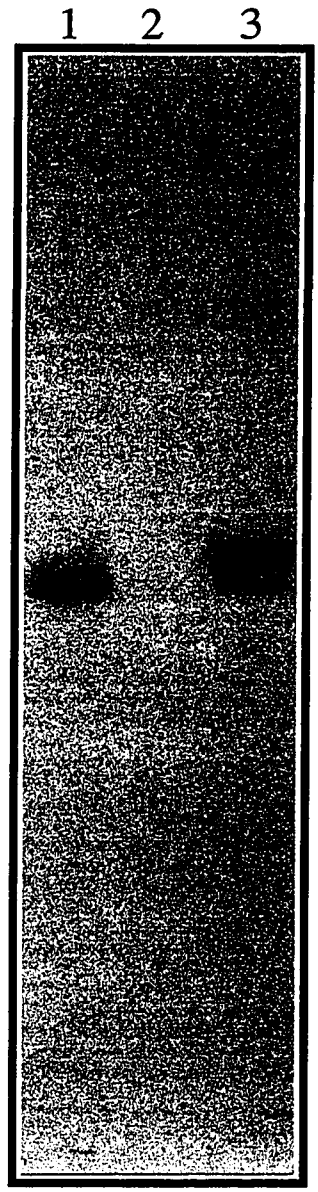

1
2
3

Figure 4-3: Cleavage of the pelB leader peptide from recombinant chicken apoA-I by exogenous leader peptidase. The soluble fraction of the cell pellet was treated with leader peptidase for 4 h at 37 °C. The extent of cleavage was evaluated by SDS-PAGE and immunoblotting. Lane 1, no exogenous leader peptidase; Lane 2, enzyme:substrate ratio of 1:1000; Lane 3, enzyme:substrate ratio of 1:1000 in the presence of 0.1% Triton X-100; Lane 4, 1:100 enzyme:substrate ratio in the absence of Triton X-100; Lane 5, ratio of 1:10 in the absence of Triton X-100.

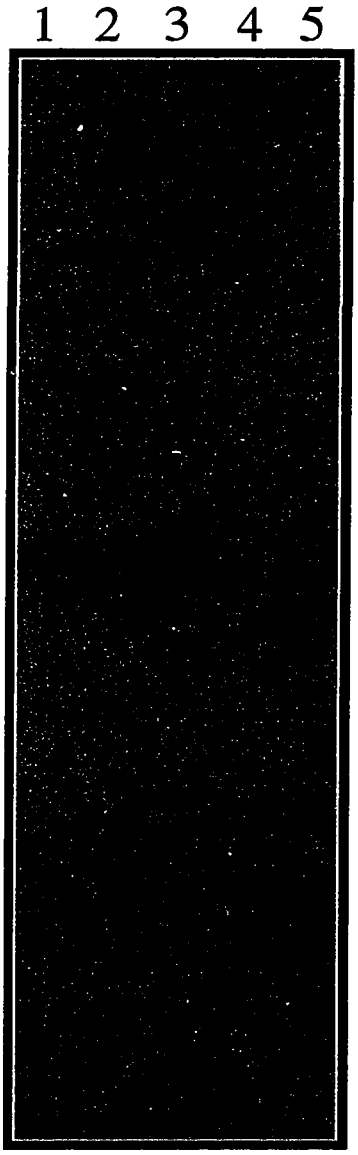

1 2 3 4 5

Figure 4-4: Reversed phase HPLC profile of recombinant chicken apo A-I. Soluble fractions from the sonicated cell pellet were chromatographed on a Zorbax C_8 reversed phase column as described.

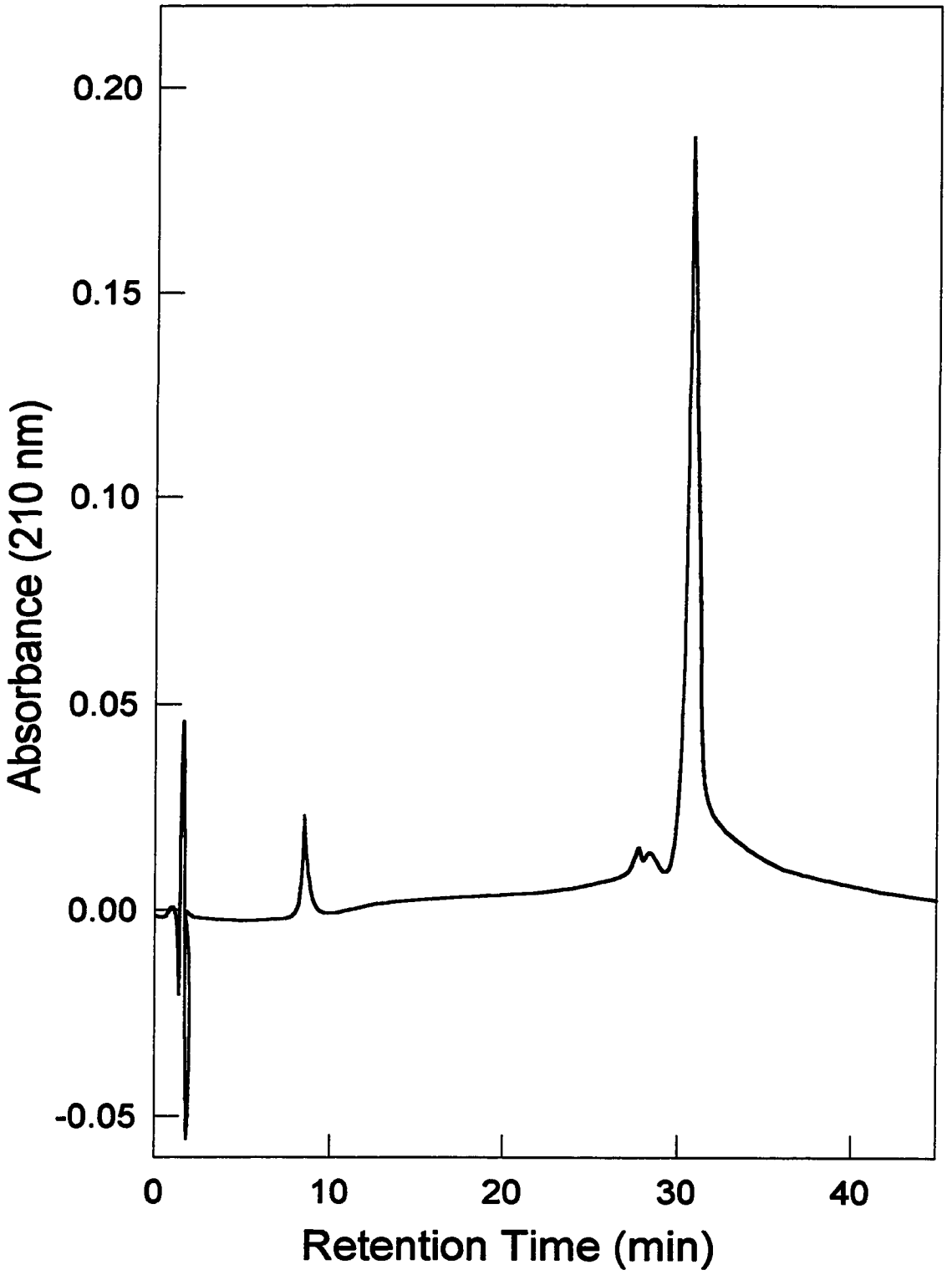

0.20
0.15
0.10
0.05
0.00
-0.05
Absorbance (210 nm)
0
10
20
30
40
Retention Time (min)

Figure 4-5: Far-UV circular dichroism spectra of recombinant chicken apoA-I. Spectra were obtained in the absence (——) or presence (- - -) of 50% TFE in 50 mM sodium phosphate, pH 7.

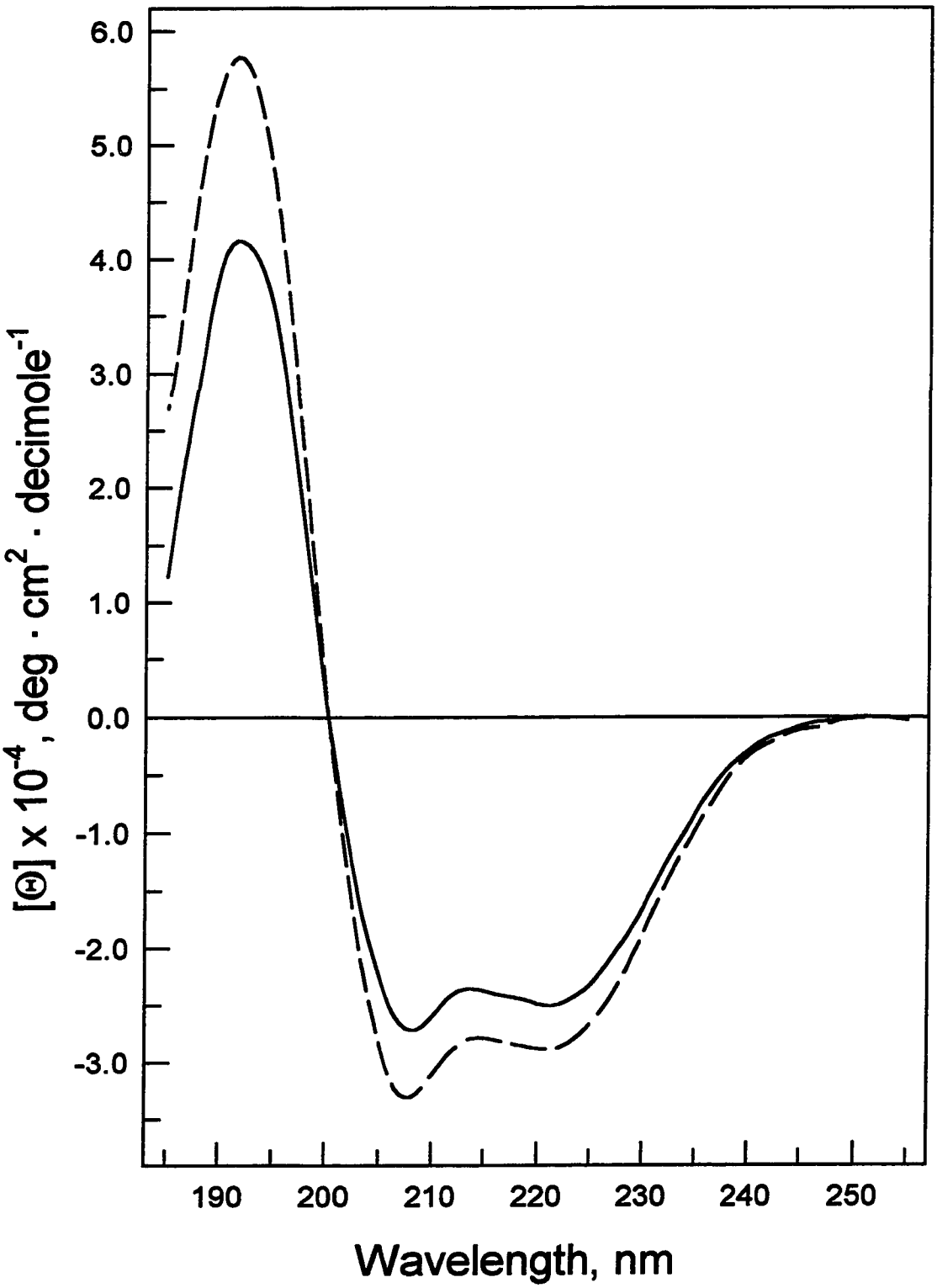

6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
[Θ] x 10^-4, deg · cm^2 · decimole^-1
190
200
210
220
230
240
250
Wavelength, nm

Figure 4-6: Fluorescence emission spectra of recombinant chicken apo A-I. Recombinant chicken apoA-I in 50 mM sodium phosphate, pH 7, was excited at 295 nm and emission recorded between 310 and 450 nm in the absence (—) or presence (- - -) of 0.4% LPC. Slit widths for excitation and emission were 7 nm.

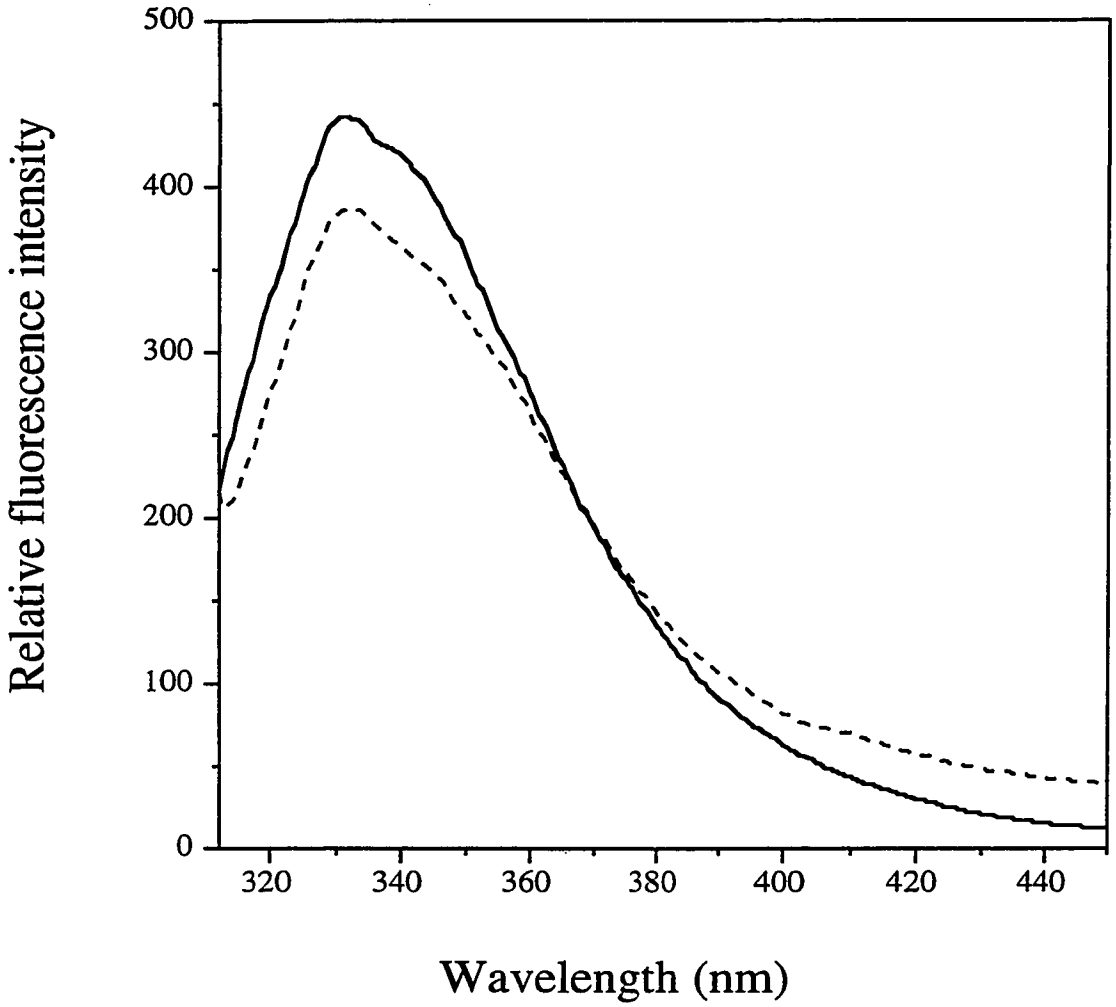
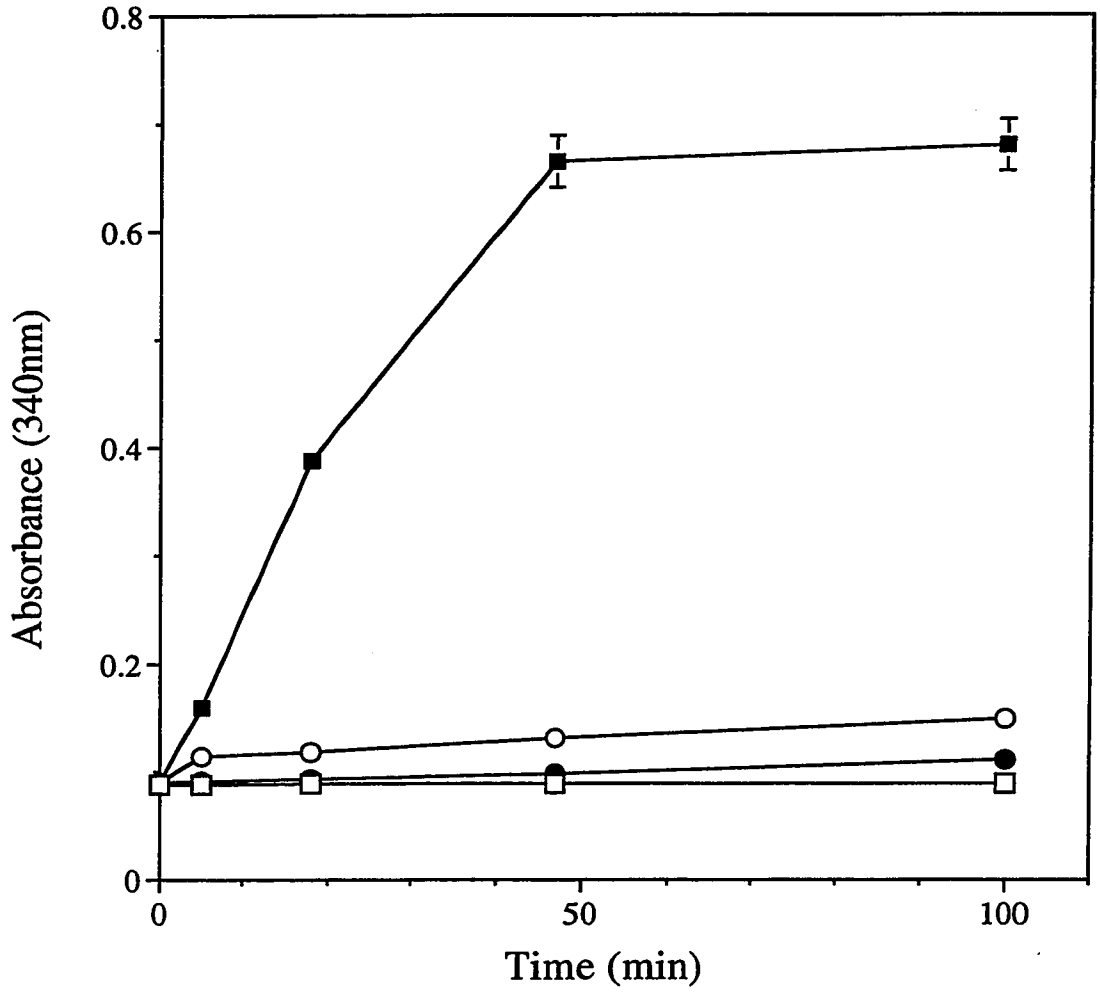

Relative fluorescence intensity
500
400
300
200
100
0
320
340
360
380
400
420
440
Wavelength (nm)

Figure 4-7: The effect of recombinant chicken apoA-I on the stability of phospholipase C-treated human LDL. Human LDL (150 μg protein) was incubated with 80 mU of phospholipase C for 2 h at 37 °C in the absence or presence of plasma-derived or recombinant chicken apoA-I (50 μg). The absorbance at 340 nm was determined at the indicated time intervals. Open squares; LDL alone; Closed squares, LDL plus PL-C; Open circles, LDL, PL-C plus recombinant apoA-I; Closed circles, LDL, PL-C plus plasma derived chicken apoA-I.


0.8
0.6
0.4
0.2
0
Absorbance (340nm)
0
50
100
Time (min)

Chapter 5

Chicken Apolipoprotein A-I can mediate receptor binding of hybrid lipoprotein particles

A part of this work was previously published:

Fisher, C. A., Kiss, R. S., Francis, G. A., Gao, P., and Ryan, R. O. (1999) *Comparative Biochemistry and Physiology* **In Press**

5.1 Introduction

Apolipoproteins play a major role in the regulation of lipid metabolism. Apolipoprotein (apo) A-I is the main structural component of high density lipoprotein (HDL), activates the plasma enzyme lecithin:cholesterol acyltransferase, and mediates cholesterol efflux from peripheral cells as part of the reverse cholesterol transport pathway (1). Recently, the discovery of scavenger receptor BI (SR-B1) and others from a family of HDL receptors has widened the role of apoA-I in the body, notably in steroidogenic tissues, by demonstrating that apoA-I can serve as a ligand for cell-surface receptors (2).

Human apoA-I is expressed in the liver and intestine, like chicken apoA-I, but chicken apoA-I is also expressed in skin, kidney, spleen, muscle, heart, adrenals, gonads and brain (3, 4). This distribution suggested that perhaps chicken apoA-I serves a larger role in the chicken. The tissue distibution of chicken apoA-I mRNA resembles mammalian apoE mRNA tissue distribution. In rat nerves, researchers found that apoE was highly expressed after nerve injury (5, 6). In chickens, ablation of the optic nerve resulted in high expression of chicken apoA-I (5). Therefore, in response to nerve injury, both mammalian apoE and chicken apoA-I were highly expressed at the site of injury. In light of the fact that chickens lack apoE (7), chicken apoA-I has been postulated to perform not only apoA-I associated functions, but also the physiological roles of apoE in the chicken (8).

ApoE is a 299 amino acid exchangeable apolipoprotein which has long been recognized to bind the low density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP) through its receptor binding region (residues 130-150) (9). The distribution of positive charges on the hydrophilic

face of the amphipathic helix #4 of the N-terminal domain of apoE has been shown to be responsible for receptor binding (10). Remarkably, chicken apoA-I contains a sequence that shows reasonable similarity to the receptor binding region of apoE. This "apoE-like region" has 50 % sequence identity and 65 % sequence similarity. The aim of this study was two-fold: firstly, the ability of chicken apoA-I to bind to an apoE receptor was evaluated; secondly, from a protein engineering perspective, by altering chicken apoA-I at only two positions (Glu157Arg/Glu158Lys; double mutant chicken apoA-I) where the apoE-like region more closely resembled the receptor binding region of human apoE, the receptor binding ability would be enhanced compared to the wild-type chicken apoA-I.

Standard lipoprotein receptor binding assay systems were utilized (11). The first assay involves binding apolipoproteins to a lipoprotein to compete with ^{125}I-labeled LDL for binding to LDL receptors. A limitation of this assay involves the necessity of complexing the apolipoprotein of choice to a suitable lipoprotein substrate for LDL receptor competition. ApoE, for example, must be bound to lipid in order to bind a lipoprotein receptor (12). The second assay involves labeling of the intracellular cholesteryl ester pool , catalyzed by the enzyme acylCoA:cholesterol acyltransferase (ACAT), as an indirect measure of receptor-mediated uptake of cholesterol-containing lipoproteins. A suitable lipoprotein substrate which contains cholesterol, with the apolipoprotein of choice bound, is required for receptor uptake. These two assays, the ^{125}I-LDL receptor competition assay and the cholesterol mobilization assay, were chosen to evaluate receptor binding.

The first assay involves a model lipoprotein called low-density lipophorin (LDLp) from the insect, *Manduca sexta*. LDLp contains two nonexchangeable apolipoproteins, apoLp-I and apoLp-II, and an exchangeable apolipoprotein, apoLp-III. ApoLp-III can be displaced from LDLp by other exchangeable apolipoproteins which possess a higher lipid binding affinity (13, 14). In this way, a hybrid lipoprotein containing the apolipoprotein of choice can be obtained for ^{125}I-LDL receptor competition assays.

Normally, LDL is a good substrate for the LDL receptor. Treatment of LDL with phospholipase C (PLC) results in a depletion of LDL surface phospholipids, exposure of hydrophobic surface and aggregation of LDL. However, exchangeable apolipoproteins can bind to the hydrophobic surface created by PLC and prevent aggregation (15). These apolipoprotein-bound, PLC-treated LDL particles can then be used in the cholesterol mobilization assay to evaluate the potentially enhanced effect of the bound apolipoproteins. Acetylation of lysine residues on apoB-100 on LDL (AcLDL) prevent receptor binding of LDL (16). AcLDL can then be treated with PLC and the apolipoprotein of choice can be bound to the AcLDL. These apolipoprotein-bound, PLC-treated AcLDL particles can then be used in the cholesterol mobilization assay to evaluate the uptake of lipoproteins solely by the effect of the added apolipoproteins as ligands. This assay allows for evaluation of cholesterol delivery by the AcLDL as a means of determining receptor binding and uptake of cholesterol.

Evaluation of the receptor binding properties of chicken apoA-I by two independent assays has demonstrated that chicken apoA-I does indeed

have receptor binding activity. Mutation (Glu157Arg/Glu158Lys) of chicken apoA-I resulted in enhanced receptor binding.

5.2 Experimental Procedures

Materials: [^{14}C]-oleic acid was obtained from Amersham (Toronto, Canada). *Taq* polymerase was obtained from Gibco BRL (Burlington, Canada) and *Pfu* polymerase was obtained from Stratagene (through PDI Bioscience, Aurora, Canada). *Bacillus cereus* phospholipase C (PLC) was purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagles medium (DMEM) was obtained from Bio-Whittaker (Walkersville, MD), and fetal bovine serum (FBS) and lipoprotein deficient serum (LPDS) were obtained from Hyclone (Logan, UT). Whatman PE SIL G flexible thin layer chromotography (TLC) plates were obtained through Fisher Scientific (Edmonton, Canada).

Lipoproteins and apolipoproteins: Chicken high density lipoprotein (HDL) and chicken apoA-I were purified according to Chapter 2. Recombinant human apoE3(1-183) was prepared according to Fisher *et al.* (17). Human apoA-I was a kind gift from Dr. Gordon Francis (University of Alberta). Insect apoLp-III was prepared according to Ryan *et al.* (18). Insect LDLp was isolated from the hemolymph of adult *M. sexta* following administration of 20 pmol of adipokinetic hormone following the protocol of Ryan *et al.* (18). Human low density lipoprotein (LDL) was purified by sequential ultracentrifugation between the density ranges 1.009 and 1.063 g/mL. Isolated human LDL was radioiodinated by the iodine monochloride method (19). The specific activity of the ^{125}I-LDL was 100 - 200 cpm/ng LDL protein.

Double mutant synthesis and characterization: Using a four-primer method, polymerase chain reaction (PCR)-based mutagenesis of the apoA-I/pET vector (20) was used to create the Glu157Arg/Glu158Lys double mutant. Two primers (overlapping the codons for residues 157 and 158; identical to the coding and non-coding strands, respectively, except for three nucleotide mutations) were used independently with primers flanking the full length apoA-I insert to generate, by *Pfu* polymerase PCR reactions, two mutant PCR fragments corresponding to the 5'end and the 3'end of apoA-I (each containing the mutation sites). These mutant fragments were then used as DNA templates to prime the synthesis, by a *Taq* polymerase PCR reaction, of the entire apoA-I sequence with the mutations intact. The full-length mutant sequence was subcloned into the TA vector (Invitrogen) before replacing the wild-type sequence in the apoA-I/pET vector by means of unique restriction sites. The DNA sequence was verified by a chain termination sequencing procedure (ABI Prism dye termination cycle sequencing reaction ready kit, Perkin Elmer). The double mutant chicken apoA-I protein was synthesized and characterized the same as wild-type recombinant protein (20, Chapter 4), and was found to behave the same as plasma-derived chicken apoA-I and wild-type recombinant chicken apoA-I.

Preparation of apolipoprotein-LDLp hybrid: Two mg of LDLp were incubated with 1 mg of apolipoprotein for 1 h at 37 °C. Following incubation, the sample was adjusted to a density of 1.125 and subjected to ultracentrifugation to re-isolate LDLp. The LDLp was dialyzed into PBS and

analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

^{125}I-LDL receptor competition assay: The ^{125}I-LDL competition assay was performed as described in Fisher *et al.* (17). In brief, human skin fibroblasts (EB91-290) were grown to 60% confluence in DMEM containing 10% FBS, then switched to media containing DMEM with 10% LPDS and grown to confluence. Upon confluence, cells were cooled on ice for 30 min, washed twice with PBS/FAFA and incubated for 2 h at 4 °C in DMEM containing 25 mM HEPES, 1 mg/ml FAFA, 2 μg/ml ^{125}I-LDL, and a specified amount of receptor binding competitor. After the 2 h incubation, the media was removed and the cells were washed five times with PBS/FAFA, and two times with PBS. To release the cell-associated radioactivity, the cells were treated with 0.1 N NaOH for 1 h at 24°C, and an aliquot was used to determine radioactivity by liquid scintillation counting. The bicinchoninic acid assay was used to determine cell protein using bovine serum albumin as a standard.

Preparation of PLC-treated Acetylated LDL: To 5 mg of ice cold LDL (in 0.9% NaCl, 1mM EDTA) an equal volume of a solution of saturated sodium acetate was added with continuous stirring. Acetic anhydride, in multiple 1.5 - 2.0 μL aliquots, was added over a 1 hour period until the total mass of acetic anhydride exceeded that of LDL by 1.5 times. The solution was incubated for one further hour and then dialyzed exhaustively against saline/EDTA (0.9% NaCl, 1 mM EDTA). Extent of acetylation was monitored by agarose gel electrophoresis (Beckman Paragon Lipogel) and stained with Sudan Black.

LDL (3 mg, either native or acetylated) in 50 mM Tris (pH 7.5), 150mM NaCl, 2 mM CaCl, was incubated with PLC, 100 munits/mg LDL protein, in the presence of 3 mg of exchangeable apolipoprotein. PLC-treated LDL was then spun at a density of 1.125 at 65,000 rpm in a VTi 65.2 rotor for 75 minutes at 4 °C. The buoyant fraction containing LDL was collected, dialyzed against saline/EDTA and used in the cell experiments.

Cholesterol mobilization assay: Human skin fibroblasts (EB91-290) were grown to 60% confluence in DMEM at 37 °C with 5% CO_2 in the presence of 10% FBS. The cells were then grown to confluence in DMEM with 10% LPDS. Cells were then washed with DMEM containing 1 mg/mL fatty acid free albumin (FAFA) for two hours at 37 °C. Afterwards, the cells were incubated for 24 h with varying lipoprotein or apolipoprotein concentrations in DMEM containing 1 mg/mL FAFA. Cells were washed briefly with warm phosphate buffered saline (PBS), and then incubated in DMEM containing 9 mM [^{14}C]-oleic acid bound to 3 mM albumin, at a 100:1 ratio (v/v) of DMEM:oleic acid for 1 h at 37 °C. After the incubation, the cells were washed twice with iced PBS containing 1 mg/mL albumin and twice with iced PBS. The cells were then frozen at -20 °C until further use. Cell lipids were extracted with hexane/isopropanol (3:1, v/v) and dried down. The lipids were resuspended in a small volume of $CHCl_3$ and applied to a TLC plate. Samples were resolved in a hexane/diethyl ether/acetic acid (130:40:1.5) solvent and stained with I_2 to identify bands. The bands corresponding to cholesteryl ester (as determined from standards) were removed from the TLC plate and radioactivity was determined with a scintillation counter.

5.3 Results

5.3.1 Similarity of human apoE receptor binding region and chicken apoA-I "apoE-like" region

Sequence comparison of residues 111-166 of human apoE3 and residues 126-181 of chicken apoA-I (**Figure 5-1a**) reveal that there is a striking similarity between the receptor binding region of apoE (residues 131-150) and the corresponding apoE-like region (residues 146-165) of chicken apoA-I. Within this 20-amino acid region, the proteins share 50 % sequence identity and 65 % sequence similarity, while the overall sequence similarity of these two proteins is approximately 10 %. Similar comparison of the receptor binding region of apoE with the comparable region of human apoA-I shows 40 % sequence identity and 60 % sequence similarity. A sequence search was performed using Bioccelerator (Version 1.9) to evaluate the similarity of the receptor binding region of human apoE (residues 131-150) to 77977 protein sequences in the Swissprot database. Chicken apoA-I (Escore=2.00) was more similar to the human apoE receptor binding region than human apoA-I (Escore=49.55), and human apoA-I was no more similar than unrelated non-lipoprotein binding proteins. From a protein engineering perspective, it was hypothesized that creation of a Glu157Arg/Glu158Lys double mutant chicken apoA-I would show enhanced receptor binding. This double mutant has an ever greater similarity to the receptor binding region of apoE (60 % sequence identity, 75 % sequence similarity; **Figure 5-1b**).

5.3.2 Displacement of apoLp-III from LDLp by exchangeable apolipoproteins

An independent assay was used to determine of chicken apoA-I's receptor binding ability. It had been shown (13, 14) that exchangeable apolipoproteins having higher lipid binding affinity can displace the resident exchangeable apolipoprotein, apoLp-III, on insect LDLp and form a stable lipoprotein complex.

Incubation of LDLp (**Figure 5-2**, Lane 1) with recombinant apoE3(1-183) *in vitro* resulted in a concentration dependent, saturable displacement of apoLp-III from the particle surface (Lane 2). At higher concentrations of apoE3(1-183) nearly all apoLp-III was replaced by apoE3(1-183). These data suggest that apoE3(1-183) has a higher lipid binding affinity for the surface of LDLp than its natural component, apoLp-III. In a previous study, it was demonstrated that human apoA-I has a higher lipoprotein binding affinity than apoLp-III ((15), Lane 3). To assess the relative lipid binding affinity of human apoA-I versus the N-terminal domain of apoE, apoE3(1-183)-modified LDLp was incubated with exogenous human apoA-I, reisolated and the apolipoprotein content assessed. Following incubation and re-isolation of the hybrid LDLp, apoA-I was the major exchangeable apolipoprotein on the particle surface, suggesting it has a higher lipid binding affinity than apoE3(1-183). When identical experiments were conducted with chicken apoA-I (**Figure 5-3**, Lane 2) and the double mutant chicken apoA-I (Figure 5-3, Lane 3), both chicken proteins were capable of displacing apoLp-III and binding to the hybrid LDLp. These data indicated that the relative lipid binding affinities of the apolipoproteins investigated in this study are chicken and human apoA-I > apoE3(1-183) > apoLp-III.

5.3.3 Competition of hybrid LDLp with ^{125}I-LDL for LDL receptor binding

The ability of various hybrid LDLp's to compete for binding of ^{125}I-LDL to LDL receptors on cultured human skin fibroblasts was determined (**Figure 5-4**). In the absence of competitor lipoprotein, efficient binding of ^{125}I-LDL was seen. When incubated with an excess of unlabeled (cold) LDL, a dramatic decrease in cell-associated radioactivity was observed. Natural LDLp was a poor competitor for ^{125}I-LDL binding, serving as a negative control. ApoE3(1-183)-LDLp was an effective competitor in this assay system, serving as a positive control. Chicken apoA-I-LDLp was capable of competing with some ^{125}I-LDL, indicating some receptor binding. Importantly, the double mutant chicken apoA-I-LDLp was an even more effective competitor. These results indicate that chicken apoA-I on the surface of LDLp was a competitor with ^{125}I-LDL for LDL receptor binding and the double mutant chicken apoA-I showed enhanced receptor binding.

5.3.4 Measurement of intracellular cholesterol levels

Human skin fibroblasts were grown to confluence in lipoprotein deficient serum to upregulate LDL receptors and endogenous cholesterol synthesis. Uptake of LDL was then monitored indirectly by incorporation of [^{14}C]-oleate into cholesteryl ester as a gauge of cholesterol levels in the cell. If there is no uptake of LDL, and therefore no influx of cholesterol into the cells, there will be little incorporation of [^{14}C]-oleate into cholesteryl oleate. On the other hand, uptake of LDL, and therefore an influx of cholesterol into the cells, will result in increased incorporation of [^{14}C]-oleate into cholesteryl ester.

Therefore, increased values of [^{14}C]-oleate incorporation into cholesteryl ester indicates receptor mediated uptake of LDL. Four different exchangeable apolipoproteins were utilized in these assays. Human apoE3(1-183) composes the N-terminal domain of apoE containing the receptor binding region and this protein serves as a positive control for receptor binding when bound to lipid. Insect apoLp-III is a 5-helix bundle exchangeable apolipoprotein which contains no receptor binding region and, thus, serves as a negative control. Human apoA-I, having less similarity in the apoE-like region, also serves as a negative control in some experiments. Chicken apoA-I will be evaluated in these experiments for receptor binding ability.

5.3.5 Effect of free apolipoproteins on LDL uptake

Initially, free apolipoproteins were added to LDL without any other treatment to assess the effect of free apolipoproteins on the cholesterol mobilization assay. It was a concern that the changes in the measured levels of cholesterol and cholesterol esterification by the influx of LDL would be confounded by cholesterol efflux mediated by free apolipoproteins. Addition of only serum free medium (SFM) resulted in low incorporation of the radiolabel into cholesterol ester, as expected (**Figure 5-5**). Addition of LDL resulted in an influx of cholesterol and an enhanced incorporation of the oleate label into cholesteryl ester. Addition of free apolipoproteins to LDL without any other treatments resulted in decreased incorporation of radiolabel. Indeed, unbound human apoE3(1-183), chicken apoA-I, and insect apoLp-III all decreased the apparent influx of cholesterol from the uptake of LDL (Figure 5-5). Free apolipoproteins reduced the measured level of radiolabel incorporation, and as

a result, all subsequent samples of LDL were reisolated by ultracentrifugation to ensure no free apolipoproteins were present.

5.3.6 Uptake of PLC-treated LDL

It had been shown previously that treatment of LDL with PLC resulted in LDL aggregation (Chapter 3), while inclusion of exchangeable apolipoproteins abrogated this aggregation. As a result, the function of apolipoproteins, measured as the ability of apolipoproteins to prevent PLC-induced LDL aggregation, could be measured conveniently in a microtitre assay. This assay was employed to selectively bind the apolipoprotein of choice to the LDL particle. Subsequent ultracentrifugation removed any excess PLC or apolipoproteins. Incubation of the cells with serum free medium (SFM) gave low [^{14}C]-cholesteryl ester counts (**Figure 5-6**). Addition of LDL resulted in an increased incorporation of oleate label. A control sample, LDL plus PLC, could not be used in this assay, as PLC treatment resulted in aggregation and precipitation of LDL. Upon binding of apoE3(1-183) to LDL by PLC treatment, a further increase of radiolabeled cholesteryl oleate was detected, as expected. When bound to lipid, apoE3(1-183) has a higher receptor binding affinity than apoB-100 on LDL (17) and also serves as a ligand for LDL uptake. Binding of chicken apoA-I resulted in a slight but insignificant increase in label incorporation, while apoLp-III had no effect. The assay, in this format, could not detect chicken apoA-I enhanced uptake of LDL.

5.3.7 Uptake of PLC-treated acetylated LDL By eliminating apoB-100 mediated uptake of LDL, the uptake due only to added apolipoproteins could

be measured. This was accomplished by acetylating LDL with acetic anhydride resulting in a shift in mobility on a Beckman lipogel (**Figure 5-7**). Agarose gel separation is based on charge and acetylation of LDL resulted in a increased migration of AcLDL (Lane 2), in comparison to LDL (Lane 1), to the positive pole (top of figure). AcLDL could then be treated with PLC to facilitate the binding of apolipoproteins. Incubation of cells with SFM gave low values for incorporation of the [^{14}C]-oleate label into cholesteryl oleate, while addition of LDL resulted in greatly increased label incorporation. Addition of AcLDL significantly reduced label incorporation, clearly indicating that acetylation of LDL inhibits uptake of LDL by a receptor-mediated process (**Figure 5-8**). Binding of apoE3(1-183) to the lipoprotein surface by PLC treatment resulted in an increase in label incorporation ($p < 0.01$), indicating that apoE3(1-183) enhanced AcLDL uptake and served as a good positive control. On the other hand, addition of apoLp-III or human apoA-I resulted in no increase in label incorporation, indicating that, as expected, apoLp-III and human apoA-I had no ability to enhance AcLDL uptake and served as good negative controls. Addition of chicken apoA-I to AcLDL resulted in a significant increase in label incorporation ($p < 0.05$), indicating that chicken apoA-I enhanced AcLDL uptake. Binding of the double mutant (Glu157Arg/Glu158Lys) chicken apoA-I to AcLDL resulted in a remarkable increase in label incorporation, demonstrating a greatly enhanced AcLDL uptake. It was concluded from this experiment that chicken apoA-I does indeed have some receptor binding activity. In addition, the double mutant chicken apoA-I showed greatly increased receptor binding.

5.4 Discussion

In 1987, Rajavashisth *et al.* (8) demonstrated that the mRNA tissue distribution of chicken apoA-I was similar to mammalian apoE and was different than mammalian apoA-I. Chicken apoA-I was shown to be expressed at the site of nerve injury, similar to mammalian apoE. As well, chickens lacked an apoE (7). The authors indicated that a helical region of chicken apoA-I showed significant similarity to the receptor binding region of human apoE. These factors led Rajavashisth *et al.* to postulate that, in the absence of a functional apoE, chicken apoA-I may perform functions analogous to that of mammalian apoE (8).

The similarity of the apoE receptor binding region and the apoE-like region of chicken apoA-I is striking (Figure 5-1). With the exception of residues 157 and 158 (both glutamates) of chicken apoA-I, the overall similarity is very good. The single histidine (residue 155) in chicken apoA-I aligns perfectly with histidine 140 of human apoE. As well, arginine 160 and lysine 161 of chicken apoA-I align correctly with arginine 145 and lysine 146 of human apoE which have been shown to be involved in receptor interaction. Other residues, such as arginine 136, alanine 144, and arginine 158 of human apoE, which are all involved in receptor binding, are identical or conserved in chicken apoA-I (21, 22). Both the net positive charge and the overall structure of the amphipathic helix are important determinants in receptor binding. For example, mutation of lysine 143 of human apoE to an alanine may indicate that loss of the positive charge or disruption of the amphipathic nature of the helix is responsible for impaired receptor binding. Overall topological distribution of

charged and hydrophobic residues of the apoE-like region of chicken apoA-I and the receptor binding region of human apoE, as visualized by helical wheel representations, are very similar, indicating that the amphipathic nature of these helices is maintained despite amino acid differences. Based on this degree of similarity, chicken apoA-I was examined for receptor binding.

It is noteworthy that apoE monoclonal antibodies specific for the receptor binding region (1D7 and 2E8, a kind gift from Drs. Ross Milne and Karl Weisgraber, respectively) showed no cross-reactivity with chicken apoA-I (personal observations). This result is not entirely unexpected as the epitopes for 1D7 and 2E8 are highly specific and any changes in the apoE sequence between residues 143 and 150 resulted in impaired antibody binding (21). It would be informative to show that the double mutant of chicken apoA-I could crossreact with receptor binding region-specific antibodies, and these experiments are planned.

Addition of exchangeable apolipoproteins to LDLp caused displacement of the resident apoLp-III, quantitative binding of apolipoproteins of higher lipid binding affinity and formation of hybrid LDLp particles. These hybrid LDLp particles could then be used in competition with ^{125}I-LDL to detect receptor binding (23). Chicken apoA-I-LDLp was able to compete with ^{125}I-LDL for binding to the LDL receptor, and the double mutant of chicken apoA-I showed greatly enhanced receptor binding ability.

An advantage of the LDLp system is the ability to add apolipoprotein units incrementally to the lipoprotein surface. This would allow for investigation of the effect of multiple copies of an apolipoprotein on a given particle to modulate receptor binding affinity. As such, Pitas *et al.* (24),

using apoE-phospholipid disc complexes, demonstrated that particles containing only one apoE protein had a lower binding affinity for the LDL receptor on fibroblasts than discs possessing two, three or four apoE proteins per particle. In these experiments, apolipoproteins were in excess when bound to the LDLp to assure maximal displacement of apoLp-III, and when bound to PLC-treated LDL. Kowal *et al.* (25, 26) found that enrichment of ß-VLDL with apoE was required to create an effective ligand for LRP binding. The intrinsically higher lipid binding affinity of apoE versus apoLp-III may be responsible for the ability of baculovirus-mediated synthesis of human apoE in *M. sexta* to generate apoE hybrid lipophorin particles which bind to the LDL receptor (27). Further studies of hybrid LDLp particles containing apoE3(1-183), chicken apoA-I and mutants should reveal additional details of apolipoprotein-receptor interactions.

Human skin fibroblasts were used as a model cell to test receptor binding activity. In this case, the fibroblasts were grown to confluence in lipoprotein deficient serum to upregulate the LDL receptor and endogenous cholesterol synthesis. Incubation of LDL particles with these cells at 37 °C results in rapid, receptor-mediated uptake of the LDL, and degradation by the lysosomes. Cholesteryl ester from the LDL is hydrolyzed to cholesterol by the cell and converted to cholesteryl ester by the enzyme ACAT. To evaluate the influx of cholesterol, a [^{14}C]-oleate label can be incorporated into newly formed cholesteryl ester (28, 29). Thus, a large incorporation of the [^{14}C]-oleate label into cholesteryl ester, indicates a large influx of cholesterol from lipoprotein uptake. A small incorporation of label indicates no lipoprotein uptake. The measurement of receptor binding by [^{14}C]-oleate label incorporation is indirect,

but has been shown to be a reliable assay for receptor-mediated lipoprotein uptake (11, 30-32).

The first experiment was designed to examine the effects of free apolipoproteins on the observed radiolabel incorporation into cholesteryl ester. Unbound apolipoproteins reduced the apparent influx of cholesterol, supposedly by promoting cholesterol efflux ((33); and also Chapter 3). This effect is supported by the data which shows that the apolipoproteins efficient in promoting cholesterol efflux have lower observed incorporation values. To eliminate this potential complication, all LDL and modified LDL samples were reisolated by ultracentrifugation.

In order to assess receptor binding ability, we took advantage of a functional lipoprotein binding assay that was developed in our laboratory. PLC-treated LDL was prevented from aggregating by the addition of exogenous exchangeable apolipoproteins (13). Depletion of the phosphocholine headgroups on the surface of LDL created a hydrophobic surface to which apolipoproteins could bind. Thus, binding of the exchangeable apolipoprotein of choice to PLC-treated LDL provided a suitable lipoprotein substrate for further experiments. Binding of apoE(1-183) to PLC-treated LDL resulted in a significant increase in LDL uptake. Binding of chicken apoA-I had no significant effect. It was not possible to evaluate chicken apoA-I mediated-receptor binding in addition to apoB-100-mediated receptor binding. Therefore, acetylation of LDL with subsequent binding of apolipoprotein (by PLC-treatment) allowed the effect of only the added apolipoprotein to be evaluated. As a result, chicken apoA-I was shown to

mediate receptor binding and the double mutant chicken apoA-I showed greatly enhanced receptor binding.

Treatment of LDL with acetic anhydride would result in acetylation of all accesible lysine residues on apoB-100, even those not in the receptor binding region. It is not known if acetylation of other lysines would affect apoB-100 stability, lipid binding or receptor binding of other apolipoproteins. Likewise, PLC may, if present, have an effect on cell growth or lipid metabolism. Lipoproteins treated with PLC were reisolated by ultracentrifugation to reduce the likelihood of this occurrence. As well, each experiment contained a positive control, apoE3(1-183), and a negative control, insect apoLp-III or human apoA-I, which were treated the exact same way as the experimental samples. Any effect of acetylation or PLC treatment would be replicated in the controls. Thus, we believe the results of the PLC-treated AcLDL experiments are accurate. In addition, the results were confirmed with an independent assay, the LDLp competition assay, requiring no treatment of the substrate lipoprotein.

Two independent assays were used to evaluate chicken apoA-I receptor binding. One assay directly examined chicken apoA-I's competition with ^{125}I-LDL for binding to the LDL receptor. The second assay examined the ability of chicken apoA-I and the double mutant of chicken apoA-I to enhance receptor-mediated uptake of AcLDL, and hence cholesterol influx, by evaluating the increase in [^{14}C]-oleate incorporation into cholesteryl ester. Together these assays give evidence that chicken apoA-I does have receptor binding activity, albeit lower than apoE receptor binding activity. Previously, Arg142 and Lys143 in human apoE were shown to be important for receptor

binding (21, 34). In chicken apoA-I, the comparable residues are Glu157 and Glu158. The presence of these negative charges could explain chicken apoA-I's lower receptor binding activity. Substitution of Arg for Glu157 and Lys for Glu158 resulted in enhanced receptor binding activity. This finding allows us to speculate that the apoE-like region of chicken apoA-I could be responsible for receptor binding. To confirm the role of the apoE-like region of chicken apoA-I, further mutations should be performed to disrupt the receptor binding activity of chicken apoA-I. While it is true that the results presented here do not give any information about the possible functions of chicken apoA-I in the chicken or the physiological role low affinity receptor binding would have, the results have provided a functional basis for further experimentation.

These findings are in dispute with data showing that chicken apoA-I is not a ligand for the chicken LDL receptor or LRP (35-37). However, these same authors concede that chicken apoA-I may be performing a ligand role in the chicken follicle to mediate delivery and uptake of lipids in egg maturation (38). The authors speculated that possibly an HDL receptor like SR-BI may be involved. It is conceivable that different assays or different chicken apoA-I substrates may give significant positive results in one assay and not the other. It has been recognized, though, that chicken apoA-I may be a ligand for receptor-mediated uptake, and elucidation of the true physiological role and receptor will be forthcoming. In the future, the role of chicken apoA-I in chickens will be further addressed by examining receptor binding in chicken fibroblasts.

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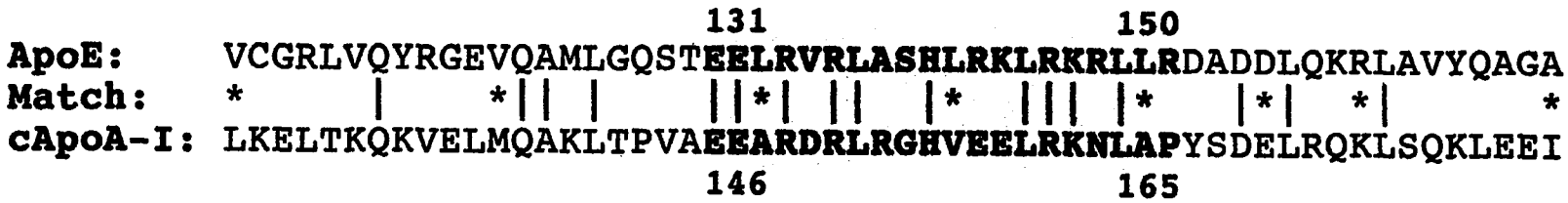
Figure 5-1a: Sequence comparison of human apoE and chicken apoA-I. The receptor binding region (residues 131-150) of human apoE, when aligned with residues 146-165 of chicken apoA-I, show a striking sequence similarity (indicated by yellow box). Identical residues are denoted by a line and conservative substitutions are denoted by a star. Conservative substitutions were accepted as S-T; M-I-L-V-A; D-E; R-K; F-Y-W. Sequence identity is 50% (10/20 residues) and sequence similarity is 65% (13/20 residues).

Figure 5-1b: Mutation of "apoE-like" region of chicken apoA-I. Using site-directed mutagenesis, a double mutation (Glu157Arg/Glu158Lys) created a chicken apoA-I with greater similarity in its apoE-like region to the receptor binding region of human apoE. Sequence identity was 60% (12/20 residues) and sequence similarity was 75% (15/20 residues).

A



sequence identity: 10/20 = 50%

sequence similarity: 13/20 = 65%

B

After mutation, apoE receptor binding region identity: 12/20 = 60%

sequence similarity: 15/20 = 75%

Figure 5-2: ApoLp-III displacement by exogenous apolipoproteins on LDLp. LDLp (2 mg protein) was incubated separately with apoE3(1-183) and human apoA-I (1 mg each) for 1 h at 37 °C. The hybrid LDLp was reisolated by density gradient ultracentrifugation and the apolipoprotein composition of the hybrid LDLp was analyzed by 4 - 20 % SDS-PAGE. Lane 1, control LDLp; lane 2, apoE3(1-183)-LDLp; lane 3, human apoA-I-LDLp; lane 4, LDLp plus apoE3(1-183) and human apoA-I; lane 5, apoA-I standard; lane 6, apoE3(1-183) standard; lane 7, apoLp-III standard. Gel was stained with Coomassie Brilliant Blue R-250.

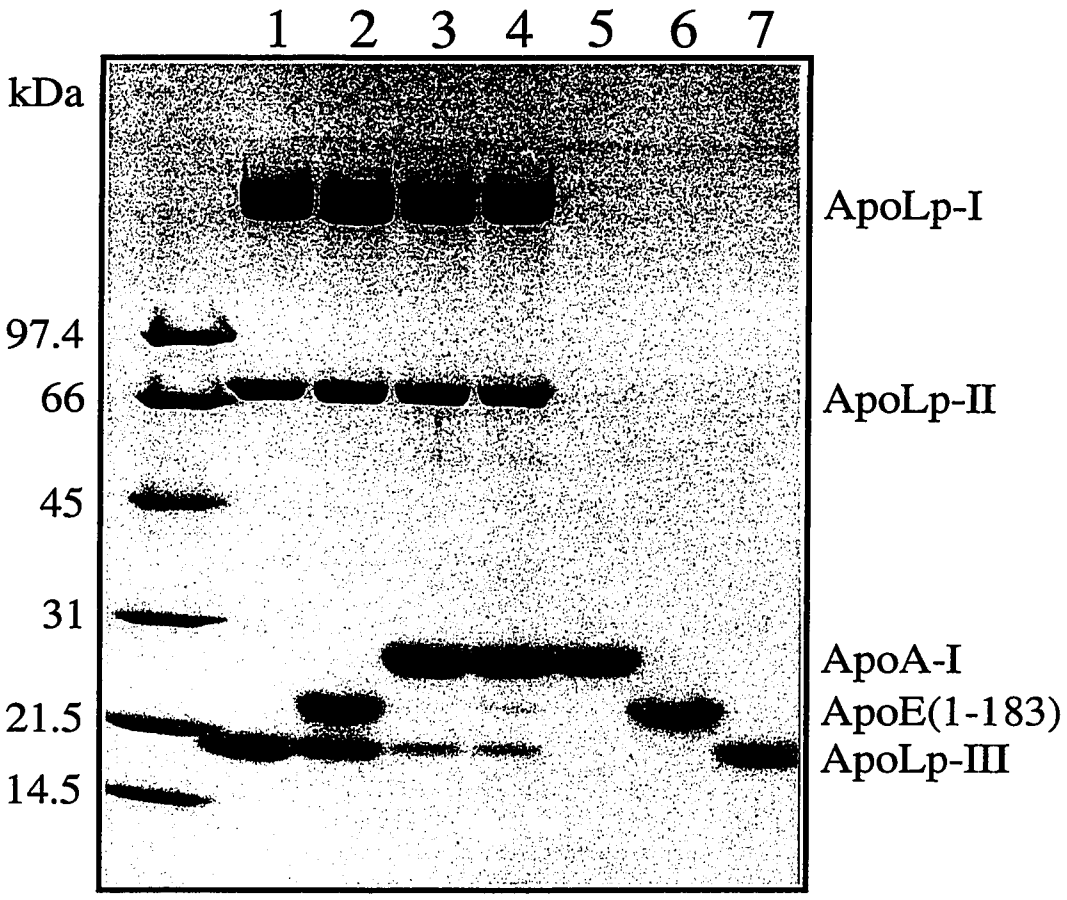

1 2 3 4 5 6 7
kDa
97.4
66
45
31
21.5
14.5
ApoLp-I
ApoLp-II
ApoA-I
ApoE(1-183)
ApoLp-III

Figure 5-3: Chicken apoA-I and double mutant chicken apoA-I displacement of apoLp-III from LDLp. LDLp (2 mg protein) was incubated separately with chicken apoA-I or double mutant chicken apoA-I (1 mg each) for 1 h at 37 °C. The hybrid LDLp was reisolated by density gradient ultracentrifugation and the apolipoprotein composition of the hybrid LDLp was analyzed by 4 - 20 % SDS-PAGE. Lane 1, control LDLp; lane 2, chicken apoA-I-LDLp; lane 3, double mutant chicken apoA-I-LDLp. Gel was stained with Coomassie Brilliant Blue R-250.

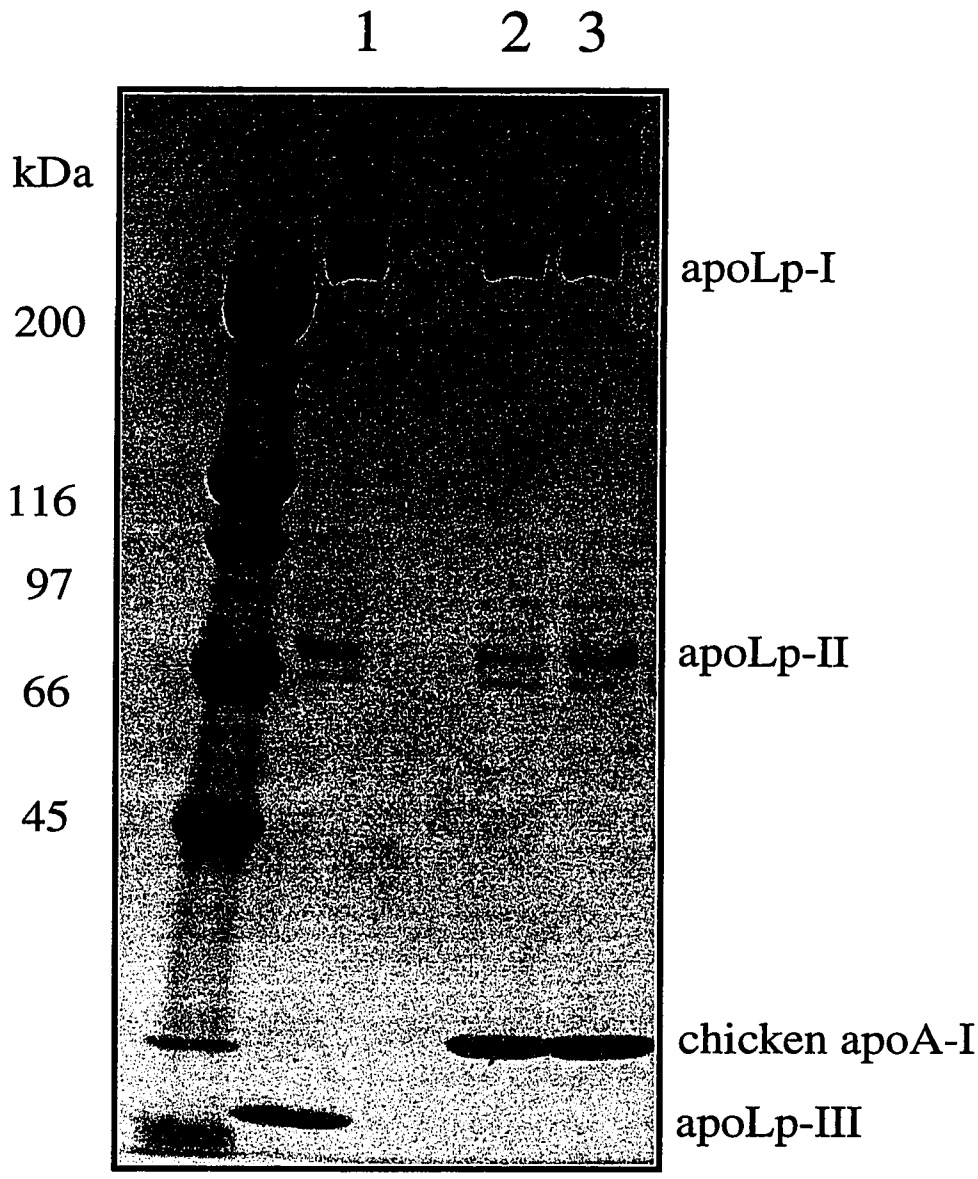

1
2
3
kDa
200
116
97
66
45
apoLp-I
apoLp-II
chicken apoA-I
apoLp-III

Figure 5-4: Enhanced ^{125}I-LDL competition with apoE3(1-183), chicken apoA-I, and the double mutant chicken apoA-I hybrid-LDLp. ^{125}I-LDL (2 μg/mL), alone or in the presence of competitor lipoprotein, was added to cell dishes for 2 h at 4 °C. Cell-associated radioactivity and protein was determined as described in Experimental Procedures. Unlabeled competitor LDL concentration was 100 μg/mL (positive control). LDLp and hybrid LDLp competitor lipoprotein concentrations were 25 μg/ml. 100 % binding corresponded to 70 763 cpm of ^{125}I-LDL/mg cell protein in serum free medium treated cells.

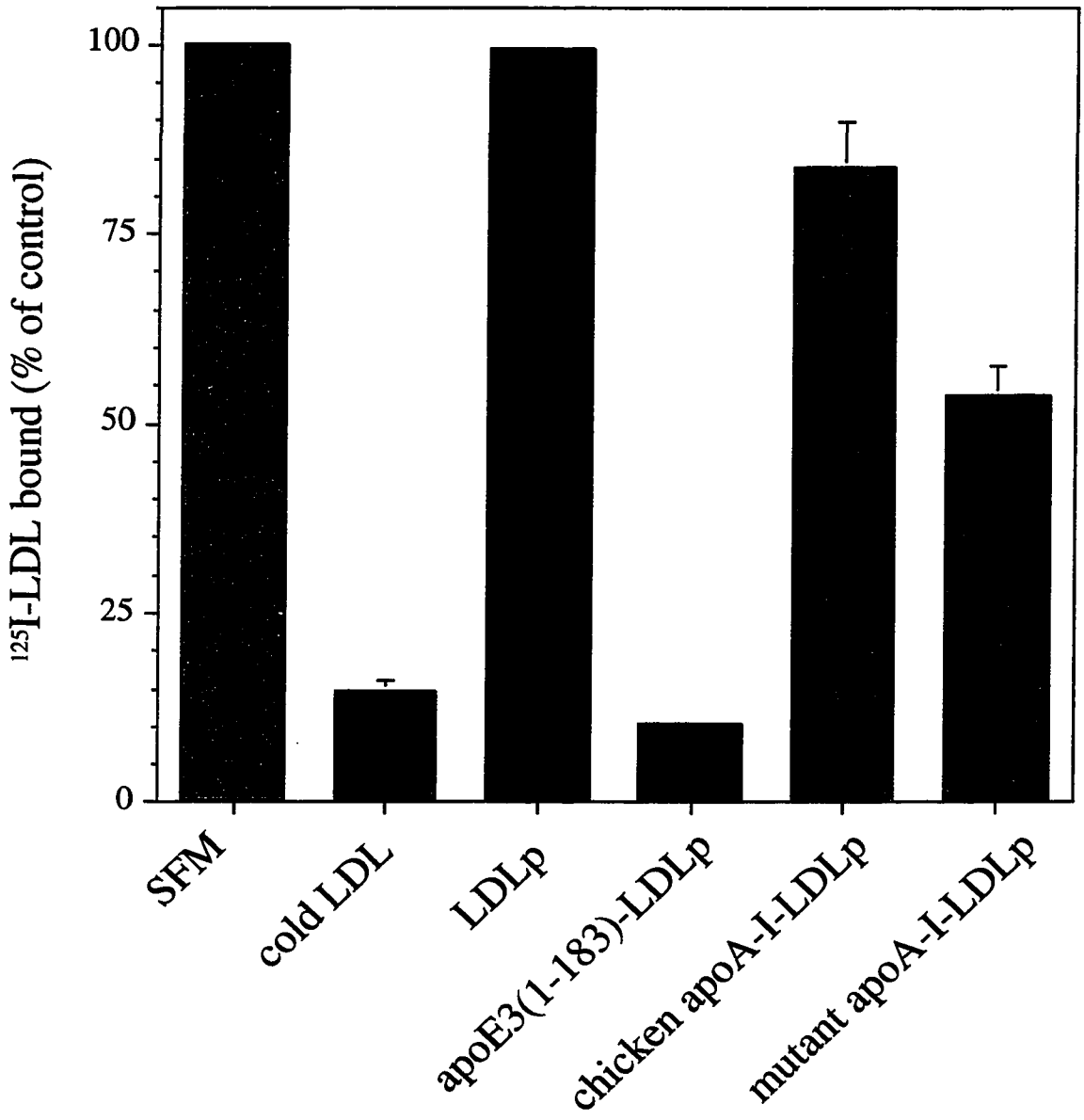

100
75
50
25
0
^{125}I-LDL bound (% of control)
SFM
cold LDL
LDLp
apoE3(1-183)-LDLp
chicken apoA-I-LDLp
mutant apoA-I-LDLp

Figure 5-5: Free apolipoproteins reduce intracellular cholesterol esterification. Human skin fibroblasts, grown to confluence in lipoprotein deficient serum, were incubated with DMEM containing 1 mg/mL bovine serum albumin (SFM) and 50 μg of LDL in the presence or absence of 50 μg of free apolipoproteins for 24 h. Cells were washed, incubated for 1 h with [^{14}C]-oleate and then cellular cholesteryl [^{14}C]oleate was measured according to Experimental Procedures. Results are the average of 3 determinations ± SD. Values are reported as pmols of [^{14}C]-oleate incorporated into cholesteryl ester/mg cell protein.

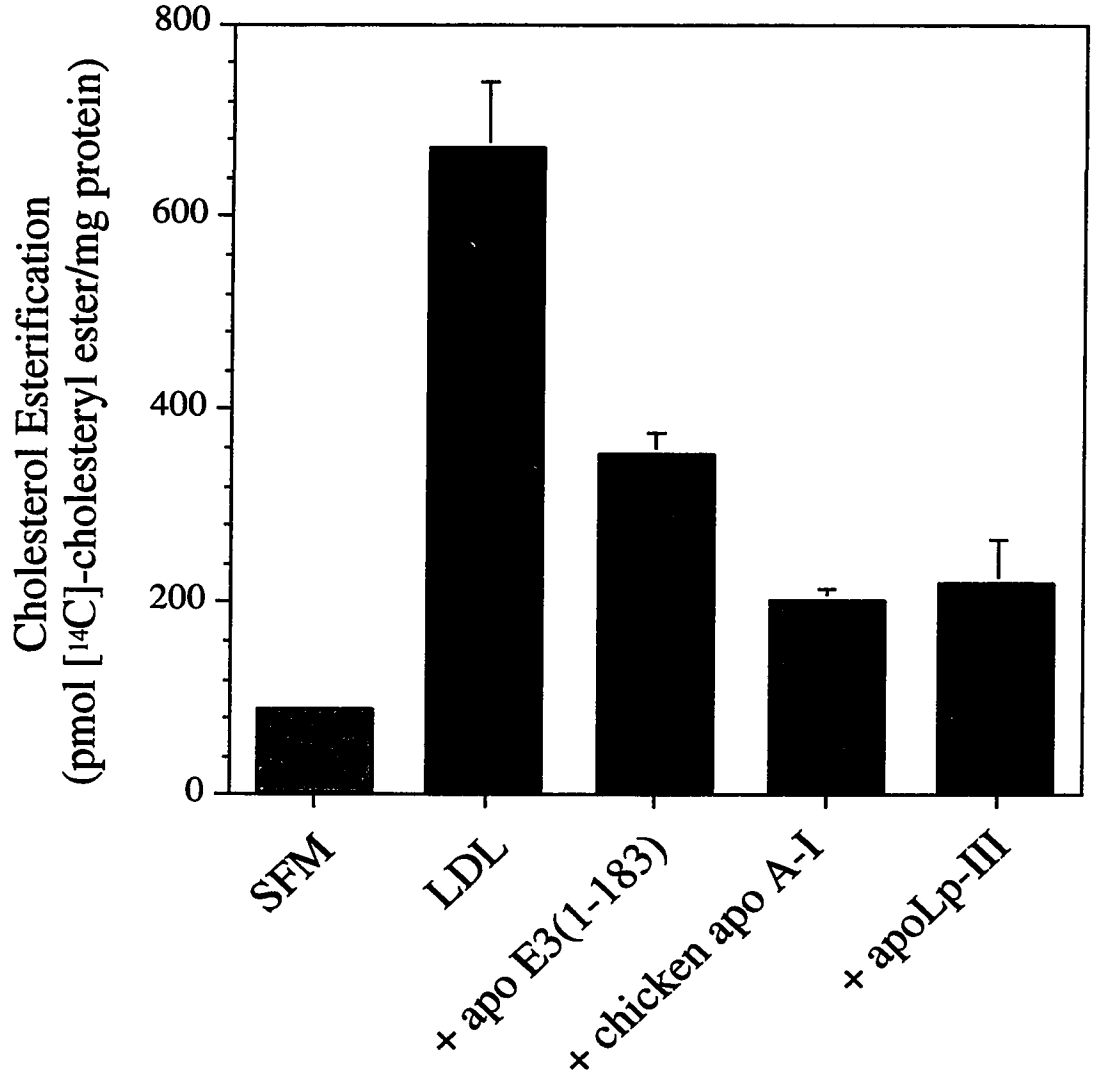

Cholesterol Esterification
(pmol [14C]-cholesteryl ester/mg protein)
800
600
400
200
0
SFM
LDL
+ apo E3(1-183)
+ chicken apo A-I
+ apoLp-III

Figure 5-6: Enhancement of cholesterol esterification by addition of human apoE3(1-183) to phospholipase C-treated LDL. Human fibroblasts were grown to confluence in lipoprotein deficient serum. Cells were then treated with DMEM with 1 mg/mL bovine serum albumin (SFM) plus either LDL (50 μg) or PLC-treated LDL (50 μg) with bound apolipoproteins for 24 h. Cells were then treated as in Figure 5-5. Results are the average of 3 determinations ± SD. Values are reported as pmols of [^{14}C]-oleate incorporated into cholesteryl ester/mg cell protein.

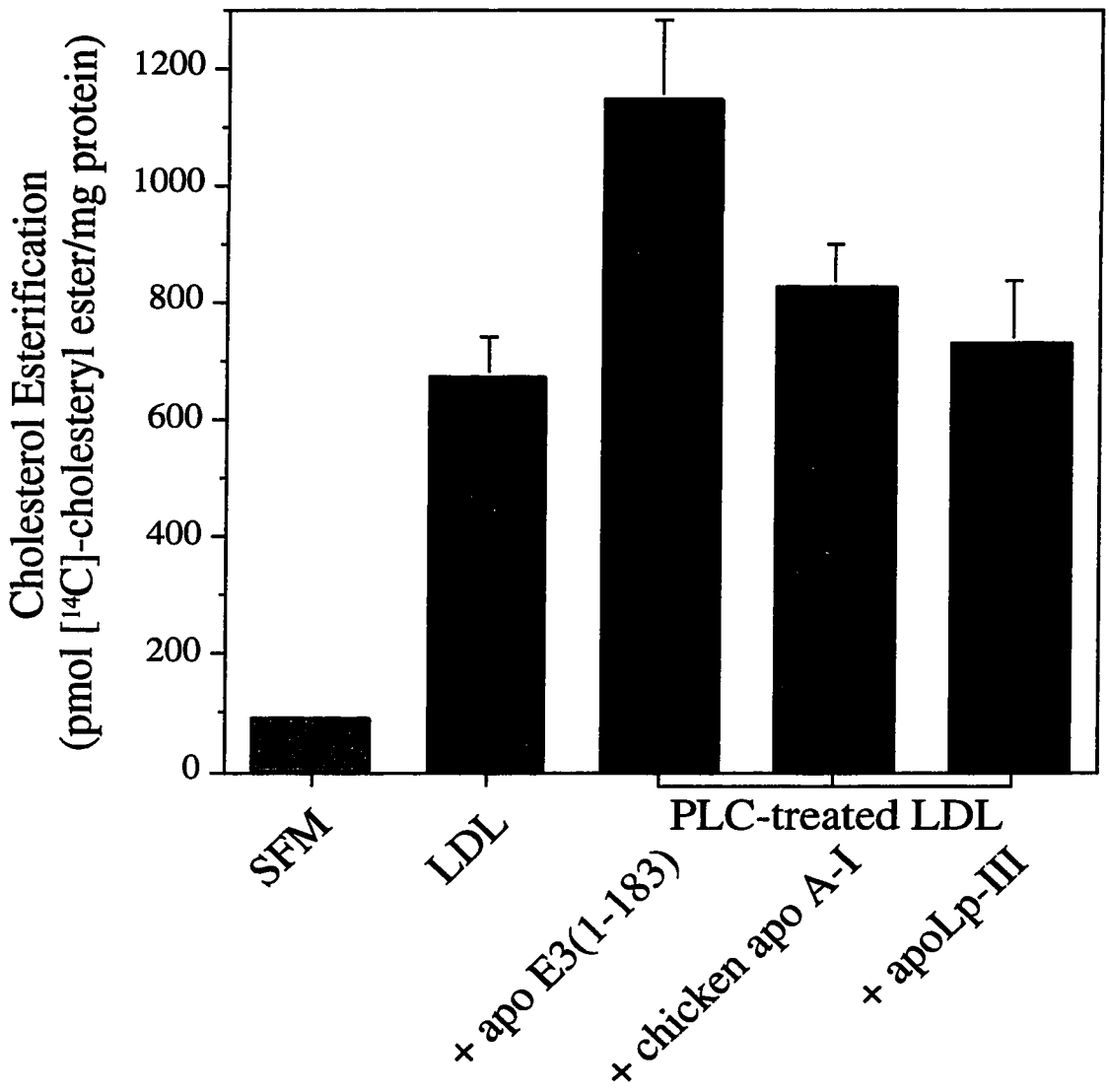

Cholesterol Esterification
(pmol [14C]-cholesteryl ester/mg protein)
1200
1000
800
600
400
200
0
SFM
LDL
PLC-treated LDL
+ apo E3(1-183)
+ chicken apo A-I
+ apoLp-III

Figure 5-7: Increased mobility of acetylated LDL by agarose gel electrophoresis. LDL was acetylated according to the Experimental Procedures. Mobility of LDL (Lane 1) compared to AcLDL (Lane 2) on an agarose gel indicates that the net charge on AcLDL has become more negative. The agarose gel was stained with Sudan Black.

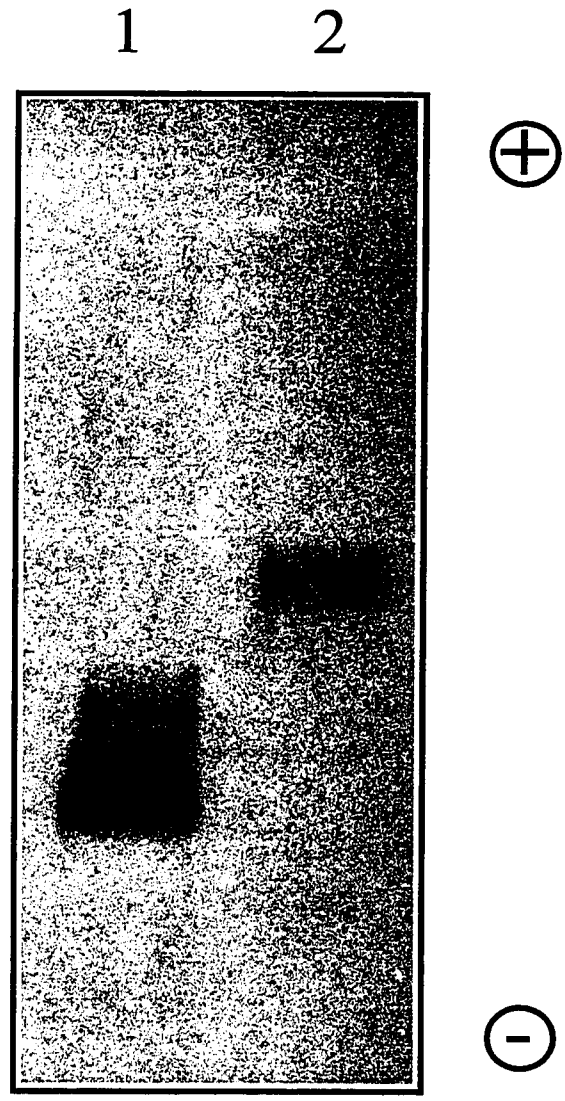
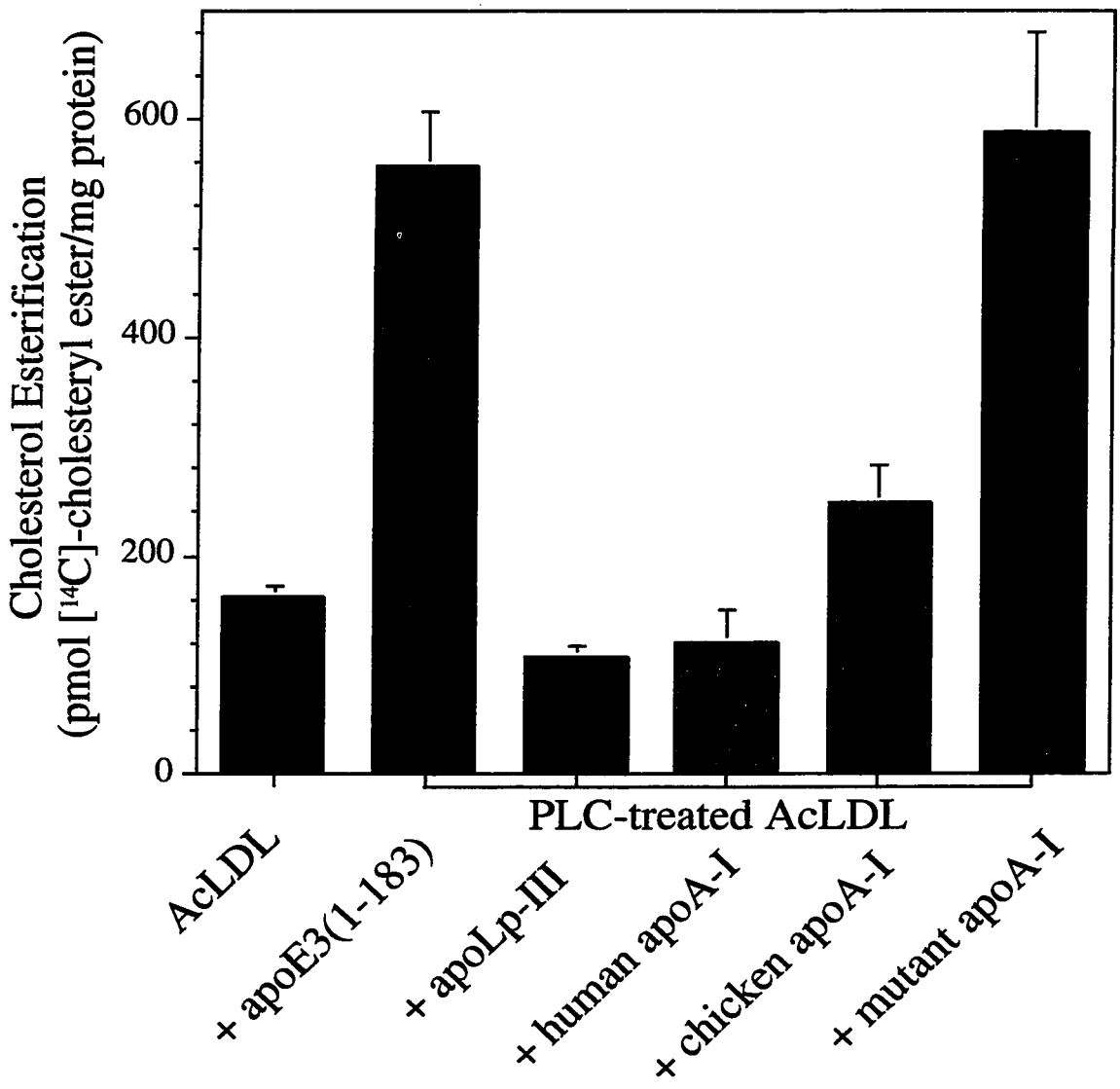

1
2
⊕
⊖

Figure 5-8: Enhancement of cholesterol esterification by addition of apoE3(1-183), chicken apoA-I and double mutant chicken apoA-I to phospholipase C-treated AcLDL. Fibroblasts grown in lipoprotein deficient serum were incubated in DMEM with 1 mg/mL bovine serum albumin plus AcLDL (50 μg) or PLC-treated AcLDL (50 μg) with bound apolipoproteins for 24 h. Cells were then treated as in Figure 5-5. Results are the average of 3 determinations ± SD. Values are reported as pmols of [^{14}C]-oleate incorporated into cholesteryl ester/mg cell protein.


Cholesterol Esterification
(pmol [14C]-cholesteryl ester/mg protein)
600
400
200
0
PLC-treated AcLDL
AcLDL
+ apoE3(1-183)
+ apoLp-III
+ human apoA-I
+ chicken apoA-I
+ mutant apoA-I

Chapter 6

Residues 131-151 of human apolipoprotein E confer receptor binding activity to apolipophorin III, a helix bundle apolipoprotein

6.1 Introduction

Apolipoprotein (apo) E is a 299 amino acid glycosylated protein which plays a major role in lipid metabolism and cholesterol homeostasis (1, 2). Knockout of the apoE gene in mice resulted in severe premature atherosclerosis, stressing the importance of apoE in proper regulation of lipoprotein metabolism (3, 4). ApoE is found in plasma and many other body tissues including the liver, intestine, and nervous tissue (5, 6). ApoE is a ligand for the low density lipoprotein (LDL) receptor and the LDL receptor related protein (LRP) and thereby mediates the uptake of lipoprotein. The ability of apoE to redistribute among lipoproteins in response to fat metabolism and bind to multiple lipoprotein receptors is crucial for its role in lipoprotein metabolism.

ApoE is composed of two independently folded domains (7). Residues 1-191 form an N-terminal domain which has been shown to be responsible for the receptor binding function (8), while residues 210-299 form the C-terminal domain which has a high lipid binding affinity (9). The three-dimensional crystal structure of the N-terminal domain showed that it is a globular bundle consisting of four amphipathic α-helices (10). Helix 4 of the N-terminal domain has been implicated in containing the residues responsible for receptor binding (11).

To partially address the question of what constitutes the receptor binding region, a peptide of residues 129-169 (with a lipophilic anchor attached) was shown to bind the LDL receptor when bound to an LDL substrate lipoprotein but a peptide lacking lipid modification could not (12). Another study showed that a dimer consisting of two polypeptides of residues 141-155 could bind the LDL receptor with about 1 % of the affinity as LDL

(13). In this context, as long as the peptide was highly helical, bound to a lipid surface, and contained residues 140-150, the peptide was receptor active. By natural mutation or site-directed mutagenesis, Arg 136, His 140, Arg 142, Lys 143, Arg 145, Lys 146, Arg 150 were all shown to be involved in receptor binding (14, 15). However, the conformation of the receptor binding region is also crucial in order for apoE to be in the receptor-active state. This aspect is illustrated by mutations that affect the amphipathic nature of the helix bearing the receptor binding region but not the positive charges. Substitution of Leu 144 and Ala 152 with prolines diminished receptor binding (14). As well, lipid-free apoE was shown to be a poor ligand for receptor binding in comparison to lipid-bound apoE (11). The lipid composition of a lipoprotein can also affect the conformation of apoE. It was demonstrated that apoE on the surface of large very low density lipoproteins (VLDL) had a higher affinity for the LDL receptor than small VLDL particles (16). From receptor binding studies of the three most common naturally-occurring mutations of apoE (apoE2, apoE3, apoE4), it is believed that other regions of the protein may influence receptor binding and lipoprotein preference. Mutation of Arg 158 (substitution of Cys for Arg in apoE2), a residue which lies outside of the receptor binding region, results in disruption of a salt bridge between Arg 158 and Asp 154 and is replaced with a new salt bridge between Asp 154 and Arg 150. Arg 150 is shifted away from the receptor binding region resulting in an inability of apoE2 to bind to the receptor (17). ApoE4 (substitution of Arg for Cys112) shows a preference for binding to very low density lipoprotein (VLDL). Arg 112 was shown to form a new salt bridge with Glu 109, disrupting the salt bridge between Glu 109 and Arg 61 (18). It was shown that Arg 61 and Glu

255 interact via a salt bridge to determine the preference of apoE for VLDL or high density lipoprotein (HDL) (19). Preferential binding of apoE4 to VLDL over HDL will alter the apoE-mediated metabolism of lipoproteins. Therefore, single point mutation within and outside of the receptor binding region, as well as changes in tertiary structural conformations of the protein, affect its ability to bind to the receptor. It would seem more relevant to understand the restrictions of the receptor binding region in the context of an α-helix bundle apolipoprotein rather than as a peptide.

Therefore, a chimeric protein was constructed and synthesized where the 21 terminal residues of the fifth helix of *M. sexta* apolipophorin-III (apoLp-III) have been substituted with residues 131-151 of human apoE. This construct (apoIIIE) exhibits physicochemical and functional characteristics of apoLp-III and receptor binding characteristics of apoE. Therefore, residues 131-151 are capable of receptor binding in the context of a functional helix bundle exchangeable apolipoprotein.

6.2 Experimental Procedures

Materials: Trifluoroethanol (TFE), bovine serum albumin (BSA), dimyristoylphosphatidylcholine (DMPC), penicillin-streptomycin solution and *Bacillus cereus* phospholipase C (PLC) were all obtained from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Bio-Whittaker (Walkersville, MD), and fetal bovine serum (FBS) and lipoprotein deficient serum (LPDS) were obtained from Hyclone (Logan, UT). Whatman PE SIL-G thin layer chromotography plates were obtained from Fisher Scientific (Edmonton, AB). Human LDL was isolated from the blood of

healthy male volunteers by sequential ultracentrifugation between the density ranges of 1.019-1.063. LDL was radioiodinated according to Langer *et al.* (20). The specific activity of the ^{125}I-LDL was approximately 100 cpm/ng LDL protein. ApoE3(1-183) was obtained in our laboratory as described previously (21).

Plasmid vector construction: The plasmid encoding the cDNA of apoE3(1-183) (22) served as a template for amplification of the coding sequence of residues 131-151 of human apoE3. Oligonucleotides containing nonannealing unique *Stu*I and *Hind*III restriction sites served as primers for polymerase chain reaction (PCR)-amplification of the apoE cDNA fragment. ApoLp-III/pET (22) and the apoE cDNA fragment were digested with *Stu*I and *Hind*III restriction enzymes, and the apoE cDNA fragment was religated in place of the 21 terminal codons of the apoLp-III coding sequence. The sequence of the apoIIIE/pET construct was verified by the chain termination sequencing procedure (Dyenamic ET terminator cycle sequencing kit, Amersham Pharmacia Biotech). Nonannealing oligonucleotides containing unique *Nde*I and *Sma*I restriction enzyme sites served as primers of the PCR amplification of the entire apoIIIE coding sequence. The amplified product was ligated into the pTYB2 vector (New England Biolabs, Mississauga, ON) at unique *Nde*I and *Sma*I restriction enzyme sites, downstream of the T7 promoter, in frame with the translation start codon and the vector-encoded intein/chitin binding domain sequence, producing the apoIIIE/pTYB2 vector. The sequence of the apoIIIE/pTYB2 construct was verified by the chain termination sequencing

procedure (Dyenamic ET terminator cycle sequencing kit, Amersham Pharmacia Biotech).

Recombinant protein synthesis and purification: Escherichia coli BL-21 (DE3) cells were transformed with the apoIIIE/pTYB2 vector. Saturated overnight cultures of *E. coli* cells (grown in 2xYT media with ampicillin (50 μg/mL of culture)) harbouring the apoIIIE/pTYB2 plasmid were diluted 30-fold in fresh 2xYT media (with 50 μg ampicillin/mL of culture) and grown at 37 °C until the optical density of the culture at 600 nm was 0.6. Following induction with 2 mM isopropyl-β-D-thiogalactopyranoside, the cells were incubated for 4 h at 37 °C. Cells were collected by centrifugation. Cell pellets in buffer A (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA) were sonicated in the presence of 0.1 % Triton X-100. After sonication, the soluble fraction of the cell pellet was loaded onto a column containing chitin beads. Recombinant protein synthesis utilized IMPACT™ (Intein-Mediated Purification with an Affinity Chitin-binding Tag) technology, in which the protein of interest was fused with an autocatalytic intein and a high affinity chitin-binding domain at the C-terminus. Only the recombinant protein was bound to a chitin bead column by means of the chitin-binding domain. Overnight incubation of the column with 30 mM dithiothreitol at room temperature resulted in intein-mediated self-cleavage, liberating apoIIIE protein and leaving the intein/chitin-binding domain bound to the column. The apoIIIE protein was then eluted from the column with buffer A and checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23). ApoIIIE protein was further purified by reversed phase high-pressure liquid chromatography

using a RXC-8 Zorbax 300SB column with a linear AB gradient of 2% B/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions were monitored at 210 nm, and pure fractions were pooled, lyophilized and stored at -20 °C until use. Recombinant apoIIIE contained a vector-encoded glycine at the C-terminus, which was necessary for efficient intein-mediated cleavage.

Physical characterization of apoIIIE: Immunoblot analysis was performed by transferring the separated proteins from SDS-polyacrylamide gels onto a polyvinylidene difluoride membrane (Millipore Corporation, Nepean, ON) and probing the membrane with specific antibodies. Presence of apoLp-III epitopes were detected using a rabbit polyclonal antibody to apoLp-III at a dilution of 1:5000. Monoclonal antibodies specific for the receptor binding region of human apoE3 (1D7 provided by Dr. Ross Milne and 2E8 provided by Dr. Karl Weisgraber) were used to probe apoIIIE for the presence of residues 131-151 of human apoE, both at dilutions of 1:5000. Electrospray ionization mass spectrometric analyses were performed using a VG quattro electrospray mass spectrometer (Fisons Instruments, Manchester, UK). Protein masses were determined as the mean value of several multiply charged ions within a coherent series. The instrument was calibrated with horse heart myoglobin (16, 951 Da). Isoelectric focussing (IEF) gel electrophoresis was performed using the PHAST gel apparatus (Amersham Pharmacia Biotech, Piscataway, NJ) with IEF standards (Pharmacia). Circular dichroism (CD) experiments were performed using a Jasco J-720 spectrapolarimeter (Jasco Inc., Easton, MD) interfaced with a Epson Equity 386/25 computer with Jasco

software. Near- and far-ultraviolet (UV) CD spectra were obtained as described in Chapter 2. Denaturation experiments were performed by observing the effect of increasing concentrations of urea on the molar ellipticity at 222 nm. The denaturation curve was fit to a sigmoidal shape to approximate the midpoint of urea denaturation. Sedimentation equilibrium experiments were carried out at 20 °C and 50, 000 rpm using a XLI Analytical Ultracentrifuge and absorbance optics as described in the instruction manual by Spinco Business Center of Beckman Instruments, Inc., Palo Alto, CA (1997). Sedimentation equilibrium runs were performed over 4 hours during which 20 scans were taken. A partial specific volume of 0.7297 for apoIIIE was calculated from the sequence and was used to determine the molecular weight and sedimentation calculations (24).

Lipid-binding assays: Human LDL (150 μg) in 10 mM sodium phosphate buffer (pH 7.5) was incubated with 80 milliunits of *Bacillus cereus* PLC at 37 °C in the absence or presence of 30 μg of apoLp-III or apoIIIE. Over a time course, turbidity development was measured at 340 nm in a Spectromax 250 microtiter plate reader (Molecular Devices, Sunnyvale, CA). DMPC disc complexes were prepared by the sonication method. DMPC (5 mg) was dissolved in a small volume of chloroform:methanol (3:1, v:v) and dried under a $N_{2\,(g)}$ stream. The thin film was dispersed into 10 mM sodium phosphate (pH 7.5) by vortexing and then sonicated until the solution was clear. ApoIIIE (2 mg) was added at a lipid:protein ratio of 2.5:1 (w/w) and incubated for 16 h at 24 °C. The discs were isolated by density gradient ultracentrifugation at 65,000 rpm for 75 min at 4 °C in a VTi65.2 rotor. After centrifugation, fractions

containing discs were pooled and dialyzed against 10 mM sodium phosphate (pH 7.5) before use. DMPC disc complexes were evaluated by non-denaturing PAGE.

LDL receptor binding assay: Human skin fibroblasts were grown to approximately 60% confluence in the presence of DMEM with 10% FBS. Fibroblasts were then grown to 100% confluence in DMEM with 10% LPDS. At confluence, cells were cooled on ice for 30 min, washed two times with phosphate-buffered saline (PBS) with 1 mg/mL fatty acid-free albumin (FAFA), then incubated with DMEM containing 1 mg/mL FAFA, 2 μg/mL ^{125}I-LDL and different amounts of receptor binding competitor for 2 h at 4 °C. The medium was removed, and the cells were washed five times with chilled PBS-FAFA and two times with chilled PBS. Incubation of the cells with 0.1 N NaOH for 1 h at 24 °C resulted in release of cells from the surface of the dishes. The relative ability of DMPC:apolipoprotein complexes to compete with ^{125}I-LDL for LDL receptor binding sites was determined by measuring the cell-associated ^{125}I in a scintillation counter. Cell protein was quantitated by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

6.3 Results

6.3.1 Design of apoIIIE

The three dimensional structures of *M. sexta* apoLp-III (25, 26) and the N-terminal domain of human apoE3(residues 1-191) (10) have been previously determined and were utilized to design an apoLp-III/apoE (apoIIIE) chimera.

ApoLp-III is a 166 amino acid protein composed of five amphipathic α-helices forming a globular bundle. ApoLp-III has been well characterized in terms of its physical and functional properties (27), and possesses no receptor binding properties. The chimeric protein was created by replacing the C-terminal 21 amino acid residues of apoLp-III (part of the fifth helix) with 21 residues from human apoE that form the receptor binding region (**Figure 6-1**). The chimeric protein was carefully designed to assure that the amphipathic α-helical nature of the fifth helix and the overall tertiary fold of the apoLp-III helix bundle in the chimeric construct would be maintained.

6.3.2 Synthesis and purification of apoIIIE

Overlapping PCR primers were used to prepare a chimeric DNA construct of codons corresponding to residues 1-145 from *M. sexta* apoLp-III and residues 131-151 from the receptor binding region of human apoE. The apoIIIE construct was ligated into the pTYB2 vector and bacterially-synthesized recombinant protein was produced. Recombinant apoIIIE protein was purified taking advantage of the vector-encoded chitin binding domain and intein system.

6.3.3 Identification of apoIIIE

Purified recombinant protein was analyzed by SDS-PAGE (**Figure 6-2**). As expected, apoIIIE (Lane 2) had about the same eletrophoretic mobility as apoLp-III (Lane 1), indicating that these proteins are of similar size. Electrospray ionization mass spectrometric analysis of apoIIIE gave an average mass of 18, 777 ± 6.67 Da, in very good agreement with the predicted mass of

18,775 Da. To confirm the identity of the purified protein, an immunoblot was performed with a polyclonal apoLp-III-specific antibody (**Figure 6-3**). ApoLp-III (Lane 1) and apoIIIE (Lane 2) both crossreacted with the antibody, but apoE3(1-183) (Lane 3) did not. Other immunoblots, using two monoclonal antibodies (1D7, 2E8), specific for the receptor binding region of apoE, were used. ApoLp-III and apoIIIE failed to crossreact, while apoE3(1-183) did crossreact. ApoIIIE was further investigated by amino acid analysis. ApoIIIE showed a large increase in the number of moles of arginine residues present per mole of protein, as compared to apoLp-III. This is as expected since apoIIIE possesses the receptor binding region of apoE (residues 131-151) which contains 6 arginines. ApoIIIE was evaluated by isoelectric focusing gel electrophoresis. The apparent pI, or isoelectric point, of apoIIIE increased to approximately 9, compared to apoLp-III, the pI of which is approximately 6 (27). The shift in pI corresponds to the increase in the number of positively charged residues in the receptor binding region of apoE. Therefore, the recombinant apoIIIE protein was confirmed to be of appropriate size and composition.

6.3.4 Characterization of apoIIIE

Several physical characterization techniques were performed to confirm that apoIIIE adopts a folded structure in solution. A far-UV CD spectrum of apoIIIE (**Figure 6-4**) indicated a high content of α-helix (61 %). Addition of 50 % trifluoroethanol (TFE), a lipid mimetic co-solvent, resulted in an induction of α-helix (77 %). Near-UV CD analysis (**Figure 6-4, inset**) demonstrated a pattern of peaks and troughs representing the environments of the aromatic

residues (7 phenylalanines and one tyrosine) in apoIIIE. The CD spectra demonstrated that apoIIIE is folded in solution with a high α-helical content. To determine the relative stability of apoIIIE, we examined the effect of urea on the molar ellipticity of apoIIIE at 222 nm. ApoIIIE has a single denaturation event with a midpoint of denaturation of approximately 1.05 M urea (**Figure 6-5**). Finally, analysis of sedimentation equilibrium experiments suggested that the protein was not monomeric and may display self-association properties in solution.

6.3.5 Functional properties of apoIIIE

A hallmark property of exchangeable apolipoproteins is the ability to bind to lipoproteins. A functional assay of apolipoprotein binding has been developed taking advantage of the ability of exchangeable apolipoproteins to bind to the surface of PLC-treated LDL (28). PLC hydrolyzes the phosphocholine headgroups from LDL surface phospholipids, thus creating a DAG hydrophobic surface. Exposure of the hydrophobic surface is destabilizing to the LDL particles, and aggregation occurs. Exchangeable apolipoproteins can bind to the hydrophobic surfaces as they are being generated by PLC, thereby preventing LDL aggregation (Chapter 3). The extent of aggregation can be measured conveniently in a turbidimetric assay over a period of time. Treatment of LDL with PLC resulted in a rapid increase in turbidity development, as measured by absorbance at 340 nm (**Figure 6-6**). Addition of apoLp-III or apoIIIE was effective at preventing PLC-induced LDL aggregation. This indicated that apoIIIE was functional and capable of binding to lipoprotein particles. Another assay of apolipoprotein lipid binding

deals with the ability of exchangeable apolipoproteins to transform phospholipid vesicles into disc complexes. DMPC:ApoIIIE disc complexes were analyzed by nondenaturing PAGE, and produced disc complexes of approximately 600 kDa, a size similar to those reported for DMPC:apoLp-III disc complexes (29). Although no further characterization of these disc complexes was performed, it is clear that apoIIIE was effective at transforming DMPC vesicles into disc complexes.

6.3.6 Receptor binding activity of apoIIIE

Finally, to test if apoIIIE is a functional ligand for receptor binding, an ^{125}I-LDL competition assay was performed. Human skin fibroblasts were grown to confluence in lipoprotein deficient serum to increase LDL receptor activity on the surface of the fibroblasts. Upon confluence, cells were placed at 4 °C so that no receptor-mediated uptake would take place. The cells were then treated with ^{125}I-LDL in the absence or presence of competitor. DMPC:apoE3(1-183) discs were shown to be effective competitors in the ^{125}I-LDL competition assay (21). In the absence of any competitor (referred to as serum free medium or SFM), ^{125}I-LDL binds to the receptors on the cell surface and this value is taken as 100% binding (**Figure 6-7**). Addition of a 50-fold excess of unlabeled LDL (cold LDL; 100 μg) resulted in a marked decrease in ^{125}I-LDL binding, serving as a positive control. Free apoIIIE protein was capable of a slight decrease in ^{125}I-LDL binding. Addition of DMPC:apoIIIE discs resulted in a concentration dependent reduction in ^{125}I-LDL binding, indicating that DMPC:apoIIIE discs were an effective and specific competitor of ^{125}I-LDL binding. In comparison, DMPC:apoLp-III discs were a poor

competitor. Taken together, these data indicate that lipid-bound apoIIIE is a functional receptor binding apolipoprotein.

6.4 Discussion

The goal was to understand the necessary sequence and structural requirements for apoE receptor binding in the context of another exchangeable apolipoprotein. We created a chimeric construct (apoIIIE) where the receptor binding region of apoE (residues 131-151) replaced the 21 C-terminal residues of apoLp-III, a known helix bundle exchangeable apolipoprotein. The scaffold of a helix-bundle apolipoprotein should allow the proper presentation of the receptor binding region to receptors, by maintaining the stability and conformation of the receptor binding region. As well, the proper alignment and orientation of the hydrophobic face is necessary for maintaining and stabilizing helix-lipid and helix-helix interactions (30).

ApoIIIE was synthesized using the pTYB2 plasmid, and purified by means of the intein/chitin-binding domain. Recombinant apoIIIE, as predicted, was of slightly larger molecular mass (18, 775 Da) compared to apoLp-III (18, 380 Da), as determined by mass spectrometry. Identity of the chimeric protein was confirmed by amino acid analysis. Immunoblots showed that apoLp-III antibodies crossreacted with apoIIIE, but monoclonal antibodies specific for the receptor binding region of apoE did not crossreact with apoIIIE. Although the epitopes of 1D7 and 2E8 include the sequence in apoIIIE, the epitopes may also include sequences not in apoIIIE (15). Antibody 2E8 showed reduced cross-reactivity with the human apoE2 isoform, where arginine 158 had been substituted with a cysteine (15), and 1D7 was also shown to crossreact with

residues 150-160 of human apoE (Dr. Ross Milne, personal communication). Perhaps then, it is not too surprising that these apoE antibodies did not crossreact with apoIIIE.

Far-UV CD spectra showed distinct troughs at 208 nm and 222 nm, indicative of the presence of predominantly α-helical structure (31). The α-helix content, as predicted from Provencher-Glöckner analysis (using the Contin Program), gave values of 61 % α-helix, which is similar to that reported for apoLp-III (60 %; (32)). Addition of 50% TFE, a lipid mimetic cosolvent, resulted in an increase in the predicted α-helical content (77 % α-helix), which is consistent with apoLp-III and other helix-bundle forming exchangeable apolipoproteins (21, 22, 33, 34). Near-UV CD spectra indicated troughs corresponding to discrete environments of the aromatic residues of apoIIIE. The occurrence of such troughs indicates a folded tertiary structure. Comparison of the near-UV CD spectra of apoIIIE and apoLp-III (apoIIIE has only one less phenylalanine than apoLp-III) showed similar patterns (22, 32), indicating that the overall environment of the aromatic residues was maintained, presumably in the helix bundle conformation. Denaturation studies of apoIIIE gave a midpoint of denaturation of 1.05 M urea. In comparison to the urea denaturation value of apoLp-III (1.01 M; (22)), apoIIIE and apoLp-III seem to have similar overall stability, suggesting that the introduction of the apoE segment to apoLp-III did not affect the stability of the helix bundle. Sedimentation equilibrium experiments demonstrated that apoIIIE was not monomeric in solution in the lipid-free state, unlike apoLp-III. Although the stability was not affected by the apoE introduction, exposure of some hydrophobic surface may initiate self-association. In summary, the

physicochemical properties of apoLp-III and apoIIIE, despite the introduction of the apoE sequence, are very similar and did not result in a major disruption of the conformation of the apoLp-III helix bundle structure.

To assure that introduction of the apoE sequence did not disrupt the ability of apoLp-III to interact with lipid, lipid binding assays were performed. Treatment of LDL with PLC destroys surface components of LDL creating a hydrophobic surface. Exchangeable apolipoproteins are capable of binding to the surface and preventing LDL aggregation. ApoLp-III has been shown to be fully functional in this regard (28). ApoIIIE was effective at preventing turbidity development (Figure 6-5). Thus, apoIIIE was capable of binding to the LDL substrate lipoprotein. Exchangeable apolipoproteins are also capable of forming disc complexes with phospholipids. DMPC:apoIIIE discs were formed using the sonication method. In comparison to DMPC:apoLp-III discs (29), DMPC:apoIIIE discs were of similar size as estimated from nondenaturing PAGE. Further characterization of the apoIIIE disc complexes is necessary, but initial results demonstrate that apoIIIE can form disc complexes with DMPC, similar to apoLp-III. These two lipid binding assays provide initial evidence that the apoIIIE chimeric protein retains the functional lipid binding properties of apoLp-III.

Receptor binding properties of apoIIIE were examined using a ^{125}I-LDL competition assay. DMPC:ApoE3(1-183) discs were studied extensively for receptor binding properties using this assay (21). The basis of the assay relies on the ability of competitor to displace ^{125}I-labeled LDL from LDL receptors on the surface of human skin fibroblasts. Addition of a large excess of unlabeled LDL effectively displaced bound ^{125}I-LDL. It was shown that

apoE must be bound to a lipid surface to effectively compete for LDL receptor binding (11). Lipid-free apoIIIE was not capable of significant displacement. However, when apoIIIE was bound to DMPC discs, apoIIIE became an effective competitor for LDL receptor binding. Increasing protein concentrations of DMPC:apoIIIE discs were capable of increasing displacement of ^{125}I-LDL. DMPC:ApoIIIE discs were not as effective a competitor as DMPC:apoE3(1-183) discs (21), but still demonstrate functional receptor binding. Although the overall structure of the apoIIIE helix bundle was not disturbed, indicating that residues 131-151 of apoE were folded properly and formed part of the helix bundle, the conformation of residues 131-151 of apoE was not optimal for receptor binding. This demonstrates the unique conformation of the apoE receptor binding region in the whole apoE protein, and that the apoIIIE chimera allows for evaluation of this. Future experiments using chimeras of apoLp-III and residues 140-150 or 130-160 may further dilineate conformation and sequence requirements of receptor binding.

The residues of human apoE3 that are involved in receptor interaction have been elucidated by a series of experiments. Natural mutations and site-directed mutagenesis demonstrated the importance of the positive charges on the hydrophilic face of helix 4 of the N-terminus of apoE, but also highlighted the necessity of a proper conformation of the receptor binding region (14, 15). Elucidation of the crystal structure of the the N-terminus of apoE confirmed these findings (10). More recently, peptide models further demonstrated the precondition of an amphipathic α-helical conformation and the necessity of binding to lipid, although their receptor binding affinity was very low (13). These studies may prove beneficial in developing

pharmaceutical agents to prevent atherosclerosis. However, the peptide models failed to address the issue of the conformation of the receptor binding region in the context of the entire protein. Other residues outside of the receptor binding region can affect receptor binding and clearly there are interactions between the N- and C-terminal domains that confer preference for binding to certain lipoproteins (13, 19). Therefore, understanding the conformation and sequence requirements of the receptor binding region of apoE in the context of another helix bundle exchangeable apolipoprotein would address these issues. In conclusion, apoIIIE, a chimeric protein of apoLp-III and the apoE receptor binding region, is physically and functionally similar to apoLp-III, but is also capable of binding the LDL receptor. Therefore, in the context of an exchangeable apolipoprotein, residues 131-151 of human apoE are sufficient for confering receptor binding activity to another apolipoprotein.

Acknowledgement

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Figure 6-1: Schematic representation of the construction of the apoIIIE chimera. The C-terminal 21 amino acids of apoLp-III were replaced with residues 131-151 of human apoE to produce the apoIIIE chimeric protein.

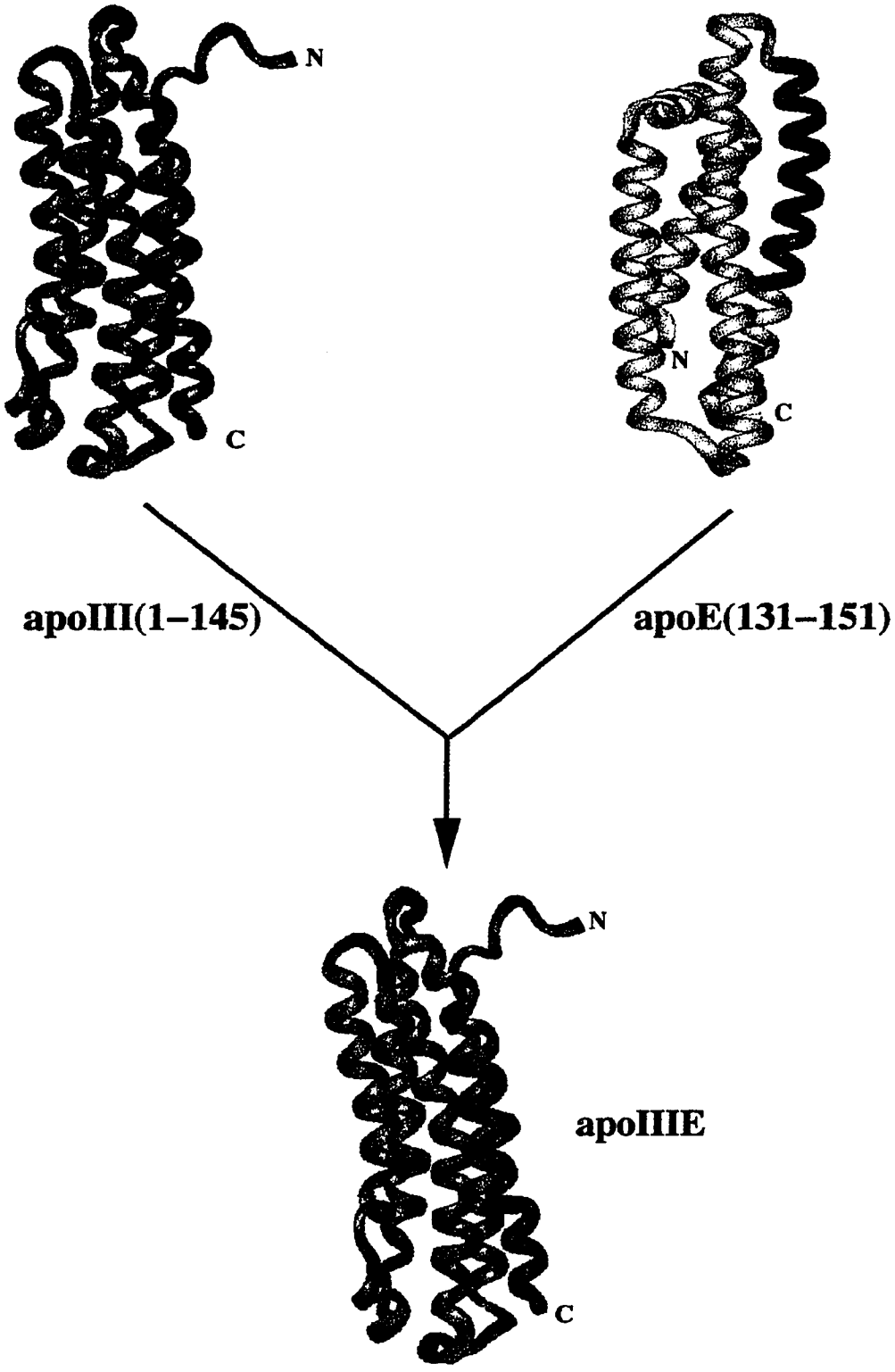

N
C
N
C
apoIII(1–145)
apoE(131–151)
N
C
apoIIIE

Figure 6-2: ApoLp-III and apoIIIE are of similar size as determined by SDS-PAGE. ApoLp-III (Lane 1) and purified recombinant apoIIIE (Lane 2) were analyzed by 8 - 25 % SDS-PAGE using the PHAST system (Amersham Pharmacia Biotech) for 100 AmpVolt hours.

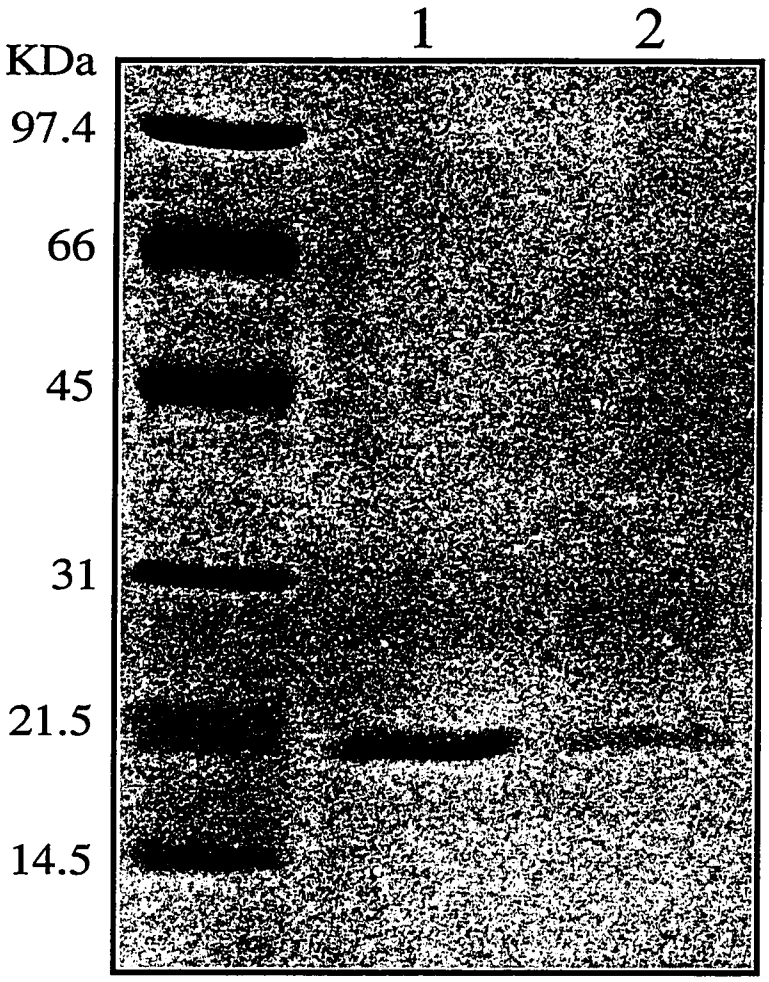

1
2
KDa
97.4
66
45
31
21.5
14.5

Figure 6-3: ApoLp-III antibody crossreacts with apoIIIE. Immunoblotting of apoIIIE with an apoLp-III specific antibody showed crossreactivity. Lane 1, apoLp-III; Lane 2, apoIIIE; Lane 3, apoE3(1-183).

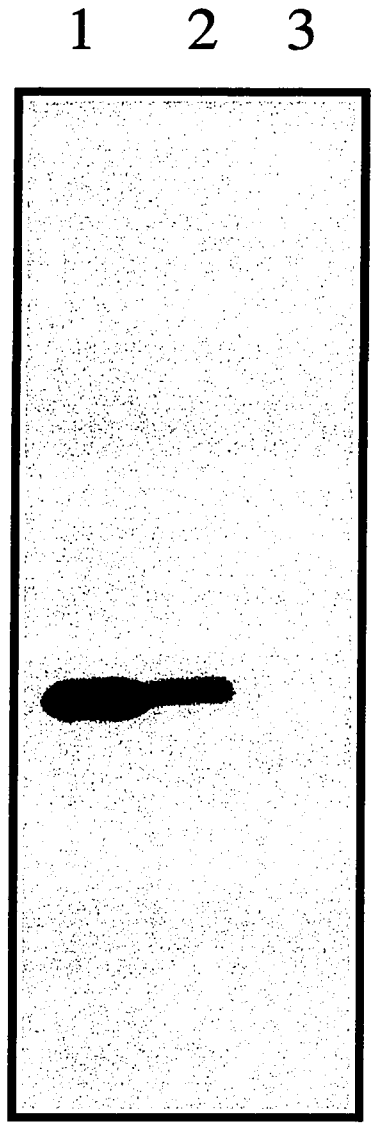

1 2 3

Figure 6-4: Far-UV CD spectrum of apoIIIE (in 25 mM sodium phosphate, pH 7.4) scanned from 180 to 255 nm: (—) apoIIIE in buffer; (- - -) apoIIIE in buffer containing 50 % trifluoroethanol. Near-UV CD spectrum (*inset*) of apoIIIE (in 25 mM sodium phosphate, pH 7.4) scanned from 250 to 320 nm, demonstrates discrete environments of aromatic residues in apoIIIE.

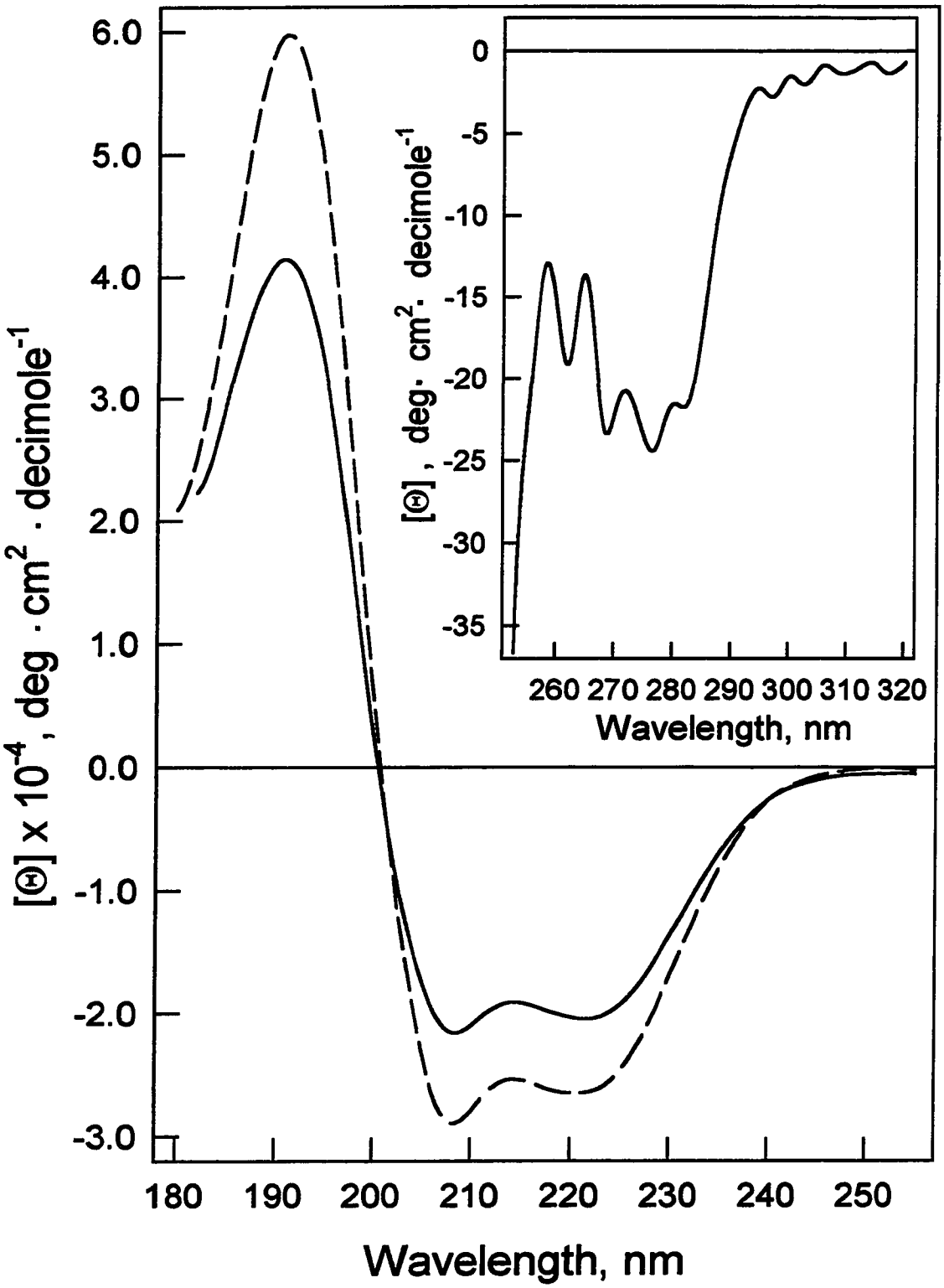

6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
[Θ] x 10^-4, deg · cm^2 · decimole^-1
180 190 200 210 220 230 240 250
Wavelength, nm
0
-5
-10
-15
-20
-25
-30
-35
[Θ], deg· cm^2· decimole^-1
260 270 280 290 300 310 320
Wavelength, nm

Figure 6-5: Effect of urea on the molar ellipticity $[\Theta]_{222}$ of lipid-free apoIIIE. Urea was added to apoIIIE (in 25 mM sodium phosphate, pH 7.4), and at each concentration $[\Theta]_{222}$ was determined.

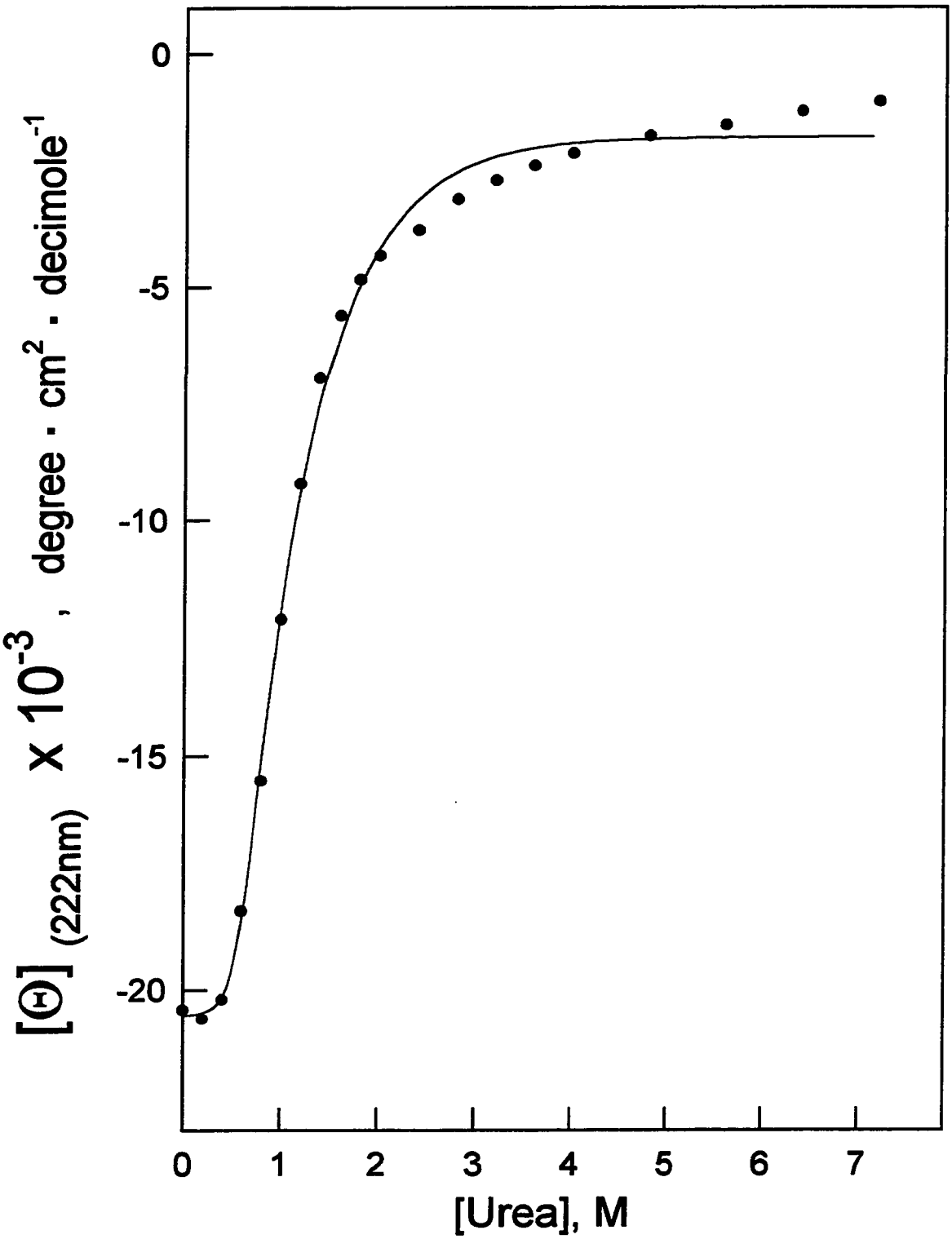

0
-5
-10
-15
-20
$[\Theta]_{(222nm)} \times 10^{-3}$, degree · cm^2 · decimole^{-1}
0
1
2
3
4
5
6
7
[Urea], M

Figure 6-6: ApoIIIE can prevent PLC-induced LDL aggregation. Human LDL (150 μg) in 10 mM sodium phosphate buffer (pH 7.5) was incubated with 80 milliunits of PLC in the absence or presence of 30 μg of apoLp-III or apoIIIE. The absorbance at 340 nm was determined at indicated time intervals. Open squares, LDL alone; closed squares, LDL plus PLC; open circles, LDL, PLC plus apoLp-III; closed circles, LDL, PLC plus apoIIIE.

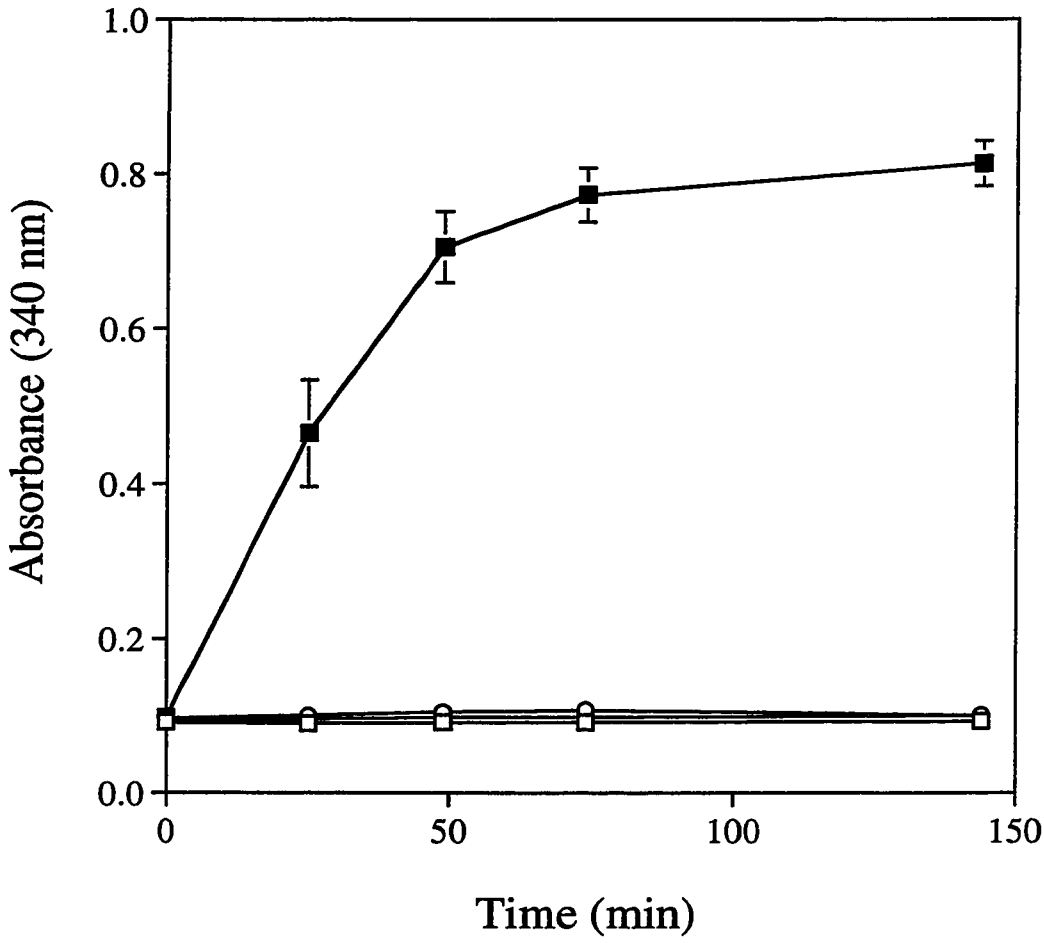
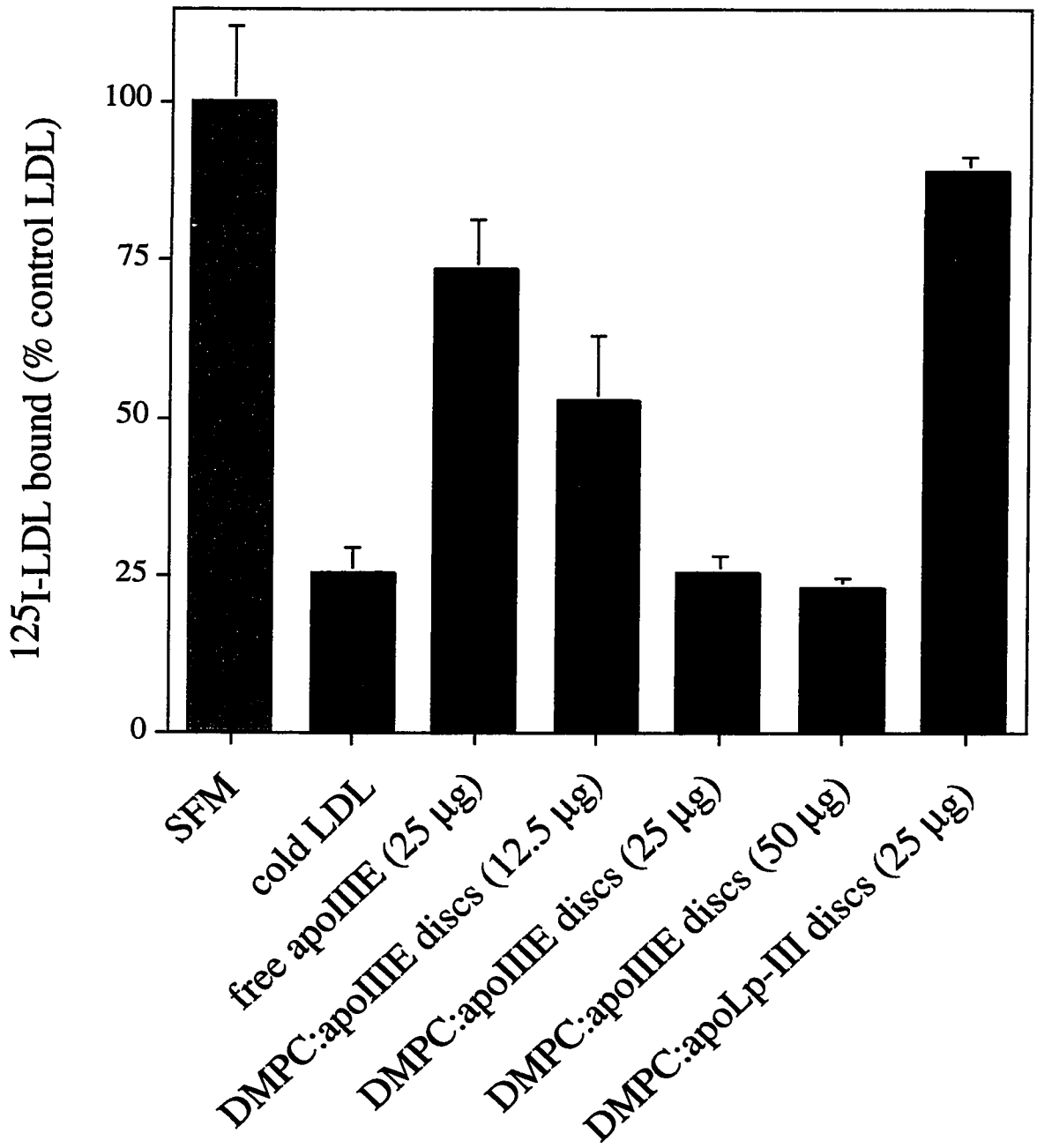

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (340 nm)
0
50
100
150
Time (min)

Figure 6-7: Human skin fibroblasts were incubated with DMEM containing 1 mg/mL FAFA, 2 μg/mL ^{125}I-LDL and indicated concentrations of receptor binding competitor for 2 h at 4 °C. Cold LDL was added in a 50 fold excess (100 μg) to radiolabeled LDL. 100 % binding corresponded to 46 285 cpm of ^{125}I-LDL/mg cell protein in serum free medium treated cells. Each result is the average of 3 determinations ± SD.


125I-LDL bound (% control LDL)
100
75
50
25
0
SFM
cold LDL
free apoIIIE (25 µg)
DMPC:apoIIIE discs (12.5 µg)
DMPC:apoIIIE discs (25 µg)
DMPC:apoIIIE discs (50 µg)
DMPC:apoLp-III discs (25 µg)

Chapter 7

Discussion and future directions

Chicken apoA-I was characterized by its physicochemical, functional and receptor binding properties. Chicken apoA-I is monomeric in the lipid-free state, unlike human apoA-I which oligomerizes in the absence of lipid (1, 2). In order to stabilize itself, chicken apoA-I was found to have a higher content of α-helix than human apoA-I (3; Chapter 2). Spectroscopic analysis of chicken apoA-I led to the conclusion that chicken apoA-I was a monomeric amphipathic α-helix bundle (Chapter 2). Because of the physical similarities between chicken and human apoA-I, with the exception of the tendency to self-associate, it may be possible that human apoA-I also forms a helix bundle. Lipid binding assays with different lipoprotein substrates revealed that chicken and human apoA-I possess similar lipid binding affinities (Chapter 3). The relative ability to make phospholipid bilayer disc complexes with chicken and human apoA-I was deemed similar, as was the ability to promote cholesterol mobilization in human skin fibroblasts. However, in mouse peritoneal macrophages, human apoA-I was more efficient at promoting cholesterol efflux than chicken apoA-I. Chicken apoA-I was cloned into a bacterial plasmid and synthesized by bacteria. Recombinant chicken apoA-I was shown to be physically and functionally indistinguishable from plasma-derived chicken apoA-I (Chapter 4). Site-directed mutagenesis was used to create a double mutant chicken apoA-I (Glu157Arg/Glu158Lys) to assess the enhancement of the receptor binding ability of chicken apoA-I by enhancing the similarity of the apoE-like region of chicken apoA-I to the receptor binding region of apoE. By two independent assays, a LDL receptor competition assay and a cholesterol mobilization assay, chicken apoA-I was shown to have significant receptor binding activity (Chapter 5). The double mutant chicken apoA-I had

enhanced receptor binding ability showing that substitution of the negative charges of Glu157 and Glu158 in the apoE-like region of chicken apoA-I with Arg and Lys residues, respectively, resulted in enhanced receptor binding. Investigation of the apoE-like region of chicken apoA-I as a potential receptor binding region lead to the questioning of what actually composes the receptor binding region of apoE. A chimeric protein, fusing residues 131-151 of human apoE to another helix bundle exchangeable apolipoprotein, was created to address the sequence and structural requirements of a receptor binding region. In the context of an exchangeable apolipoprotein, residues 131-151 of human apoE are sufficient for confering receptor binding activity to another apolipoprotein.

Previously, our laboratory took advantage of the pET vector and successfully synthesized recombinant *M. sexta* apoLp-III, *L. migratoria* apoLp-III, and human apoE3(1-183) (4-6). In these cases, recombinant protein was synthesized at high levels (greater than 50 mg/L of culture) and was found in the supernatant, thereby facilitating purification. Using the pET vector, recombinant chicken apoA-I was synthesized at lower levels (less than 50 mg/L of culture) and was found intracellularly. A more complicated procedure of cell lysis, signal peptide cleavage, and HPLC purification was necessary to obtain recombinant chicken apoA-I. It was unfortunate that chicken apoA-I was not as amenable to bacterial synthesis as the other monomeric apolipoproteins. More recently, new bacterial synthesis vectors have adopted technology for high production and ease of purification. One such vector, pTYB2 (see Chapter 6), combines a chitin-binding domain with a self-cleaving intein. The recombinant protein is synthesized as a fusion protein of the

intein/chitin binding domain, whereby the fusion protein can be isolated from other cellular proteins by virtue of the chitin-binding domain binding to a chitin column. Addition of dithiothreitol causes functional activity of the intein to cleave itself and the chitin binding domain from the recombinant protein, and the recombinant protein can be eluted from the column essentially pure. It would be worthwhile to take advantage of these new bacterial vectors for recombinant protein synthesis in the future.

Chicken apoA-I was shown to have receptor binding activity. The physiological and functional significance of this result is not clear. To date, apoA-I has not been shown to bind to an LDL receptor, VLDL receptor or LRP in humans or chickens. Human apoA-I has been shown to bind to some HDL receptors including SR-BI (7). The assays described here used human skin fibroblasts with high amounts of LDL receptors on the surface of the cells. The LDL receptor competition assay dealt with the hybrid LDLp particles' ability to displace ^{125}I-LDL on LDL receptors. The cholesterol mobilization assay involved uptake of the phospholipase C-treated acetylated LDL particles by the LDL receptor. The results of these assays showed that chicken apoA-I could mediate uptake of lipoprotein particles by the LDL receptor and could compete for binding to the LDL receptor. Chicken apoA-I receptor binding could merely be a functional artifact, but this is not supported by the similarities between chicken apoA-I and mammalian apoE. Rather, it is more likely that evolution has maintained a common apolipoprotein ligand receptor interaction and a rudimentary receptor binding motif still exists in chicken apoA-I. Human apoA-I is a saturable, high affinity ligand for SR-BI receptor binding (8). The specific region of apoA-I responsible for receptor binding has

not yet been elucidated. Further characterization of chicken apoA-I receptor binding in chicken fibroblasts, as well as further characterization of apoA-I/HDL receptors will aid in understanding the physiological role of chicken apoA-I in chickens.

In summary, chicken apoA-I was physically and functionally similar to human apoA-I. The result of these analyses could allow the use of chicken apoA-I as a model of apoA-I to determine the mechanism of apoA-I binding to lipid surfaces. Different monomeric helix bundle exchangeable apolipoproteins were evaluated for their structural adaptations upon binding to lipid surfaces. Using site-directed mutagenesis based on computer modeling of *M. sexta* apoLp-III, Narayanaswami *et al.* (9) engineered a double cysteine mutant which could form a disulfide bond in the lipid-free helix bundle conformation. Upon introduction of lipid, the helices remained tethered and could not expose the hydrophobic interior of the helix bundle, thus preventing lipid binding. This experiment provided strong support for the open conformation model for helix bundle apolipoproteins binding to lipid surfaces. With the evidence that chicken apoA-I is a monomeric helix bundle, similar experiments can be planned for apoA-I. Although a three dimensional structure for chicken apoA-I does not exist, Borhani et al. (10) have determined the three dimensional structure for an N-terminal truncation mutant of human apoA-I ((Δ1-43)apoA-I) in which the limits of the helical segments are clearly defined. A series of double cysteine mutations at strategic loop regions between helices could allow us to map out disulfide bond formation within the apoA-I structure and hence, investigate helix-helix interactions. This may be especially useful in identifying the putative disc bound structure of apoA-I. Based on the picket-

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efficiency

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relative pc

be examir

cysteine r

lipid-free

with the r

evidence c

experimen

apolipopr

monomeri

directed r

protein w

conformations of apoA-I and the structural changes that accompany interconversion of apoA-I-lipid complexes.

In the beginning, there was one apolipoprotein. Through a series of gene duplication events, internal repeat duplications and deletions, and random mutations over hundreds of millions of years, apolipoproteins have evolved into many multifunctional proteins involved in lipid metabolism and homeostasis.

The present view of apolipoprotein (apo) evolution describes events where a primordial apolipoprotein gave birth to apoA-I, apoA-II, apoA-IV, apoE, apoC-I, apoC-II, and apoC-III (12, 13). For the purposes of this discussion, unless otherwise stated, the term "apolipoproteins" refers to the exchangeable apolipoproteins listed above. The evidence that all these apolipoproteins arose from the same ancestor apolipoprotein is as follows. All these apolipoproteins are predominantly composed of α-helix. In general, these α-helices are composed of 11, 22 or 33 codon repeats, corresponding to helical segments. These helical segments are usually preceded by a proline and are usually amphipathic α-helices. Newly synthesized apolipoproteins contain two signal sequences: one that targets apolipoproteins to the endoplasmic reticulum and the other that targets the protein for secretion. Sequence analysis of the signal sequences indicates a significant degree of sequence similarity, suggesting that these proteins may be related in evolution. In humans, chromosomal localization of the genes for the apolipoproteins show that apoA-I/apoC-III/apoA-IV genes are all localized to a small region of chromosome 11 and apoE/apoC-I and apoC-II genes are localized to chromosome 19. The apoA-II gene resides on chromosome 1. Structural

organization of the genes is remarkably similar, suggesting that the genes came from a common ancestor and arose from a series of gene duplication events.

To analyze the evolutionary relationship between these apolipoproteins, nucleotide substitution rates were determined and an evolutionary tree was modeled. **Figure 7-1** depicts events in which one apolipoprotein gene was duplicated and the two duplicates were acted upon by separate selective events to become different apolipoproteins. To put the evolutionary tree in perspective, the vertebrate-invertebrate split occurred approximately 600 million years ago, the fish-mammals split approximately 400 million years ago, and birds-mammals split 270 million years ago. Therefore, in order for a species to possess an apolipoprotein, they must have acquired it before they split from the mammalian line. In general, this premise is valid, in that higher organisms acquired more apolipoproteins with more specialized functions, and those animals that branched off possess fewer apolipoproteins with fewer specialized functions. There are, however, exceptions. For example, chickens do not possess an apoE or an apoA-II, although the duplication events that created apoE and apoA-II occurred before chickens split from mammals. On the other hand, zebrafish possess a gene for apoE (14). In another example, nucleotide sequence comparison of salmon apoA-I with human apoA-I and human apoE indicated that salmon apoA-I was more closely related to human apoE than apoA-I (15). The fact that fish apoA-I was documented to play a role in nerve regeneration (16) and possess heparin binding activity (17) (a trait formally unique to apoE) further supports that conclusion. It was shown that trout, *Xenopus* and chicken all possess apoC-II-like activity for activating lipoprotein lipase (18). As well, *C. elegans*, *Xenopus*,

fish and chicken all possess vitellogenin, a lipoprotein with receptor binding properties similar to apoB-100 or apoE. It would seem that evolutionary classification based on morphological evidence or molecular data is biased and not always correct (19). Indeed, it may be Mother Nature's little joke to watch us try to organize genes, proteins, and organisms into fancy classifications when hundreds of millions of years of divergent and convergent evolution have created the world around us. The truth may be that any vital apolipoprotein function is conserved, and the apoA-I, apoE, apoC-II, and apoB-100 functions are maintained in different forms throughout the animal kingdom.

A similar phenomenon can be observed with lipoprotein receptors. Humans are known to have many lipoprotein receptors, including the very low density lipoprotein (VLDL) receptor, low density lipoprotein (LDL) receptor, LDL receptor-related protein (LRP), ApoE receptor 2, scavenger receptors and others. The VLDL receptor, LDL receptor, and LRP belong to the LDL receptor superfamily. Identified chicken receptors resemble VLDL receptors, LDL receptors and LRP (20, 21). Chicken vitellogenin and mammalian apoE can both bind to chicken oocytic VLDL and LRP receptors (22, 23), which suggested that a similar receptor:ligand binding interaction had evolved in the chicken and mammalian species. The significant sequence similarity between *C. elegans*, *Xenopus*, fish and chicken vitellogenin (24) suggests that the lipoprotein ligand/receptor system may be common to all these organisms. In support of this assertion, rainbow trout have also been shown to possess a VLDL receptor-like protein that binds vitellogenin (25).

In the context of these evolutionary relationships, chicken apoA-I was examined in this study. Chicken apoA-I exhibits a tissue expression pattern similar to mammalian apoE (26, 27) and in the case of nerve injury, chicken apoA-I becomes highly expressed in nerve tissue, similar to mammalian apoE (28). Chickens lack apoE (29), and since apoE plays a key role in cholesterol metabolism in mammals, it is not clear how chickens cope without this apolipoprotein. It was demonstrated that a specific region of chicken apoA-I shows a high degree of similarity to the receptor binding domain of apoE (30). This striking resemblance, taken together with the similarities between chicken apoA-I and mammalian apoE, has brought about the hypothesis that chicken apoA-I may be performing the physiological role of apoA-I and apoE in the regulation of cholesterol homeostasis in the chicken. What exact role chicken apoA-I plays remains to be elucidated.

ApoA-I structure/function analysis is critical for understanding the beneficial aspects of apoA-I and HDL. ApoA-I undergoes specific conformational changes upon binding to lipid, promoting cholesterol efflux from peripheral tissues, as well as, activating LCAT. Recently, apoA-I was shown to be a ligand for the scavenger receptor, SR-BI (28). The combined functions of apoA-I as part of the reverse cholesterol transport pathway make apoA-I anti-atherogenic (31). By understanding the structural and conformational aspects that are required for apoA-I functions, the apoA-I-mediated prevention of atherosclerosis can be addressed.

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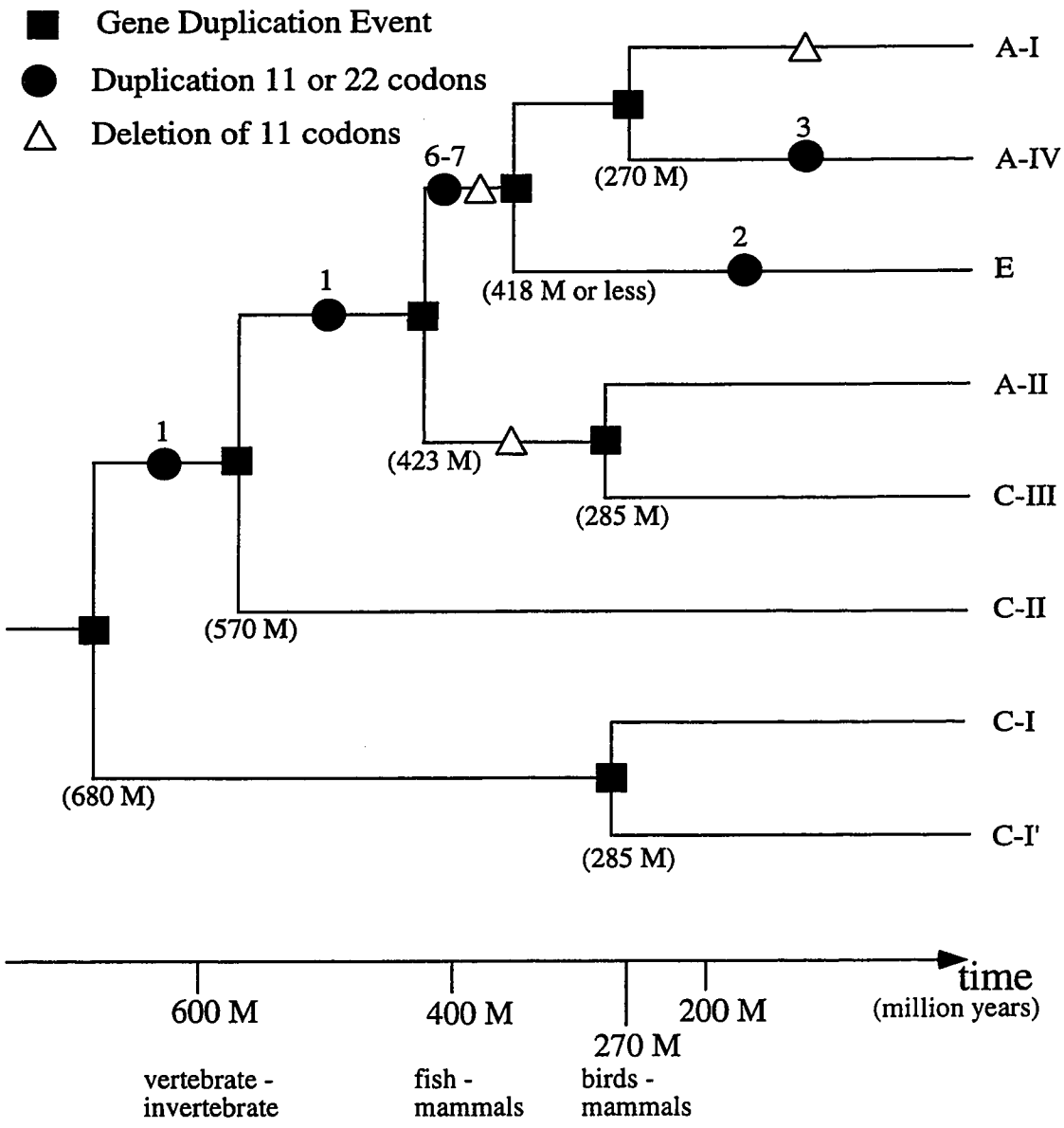
Figure 7-1: A hypothetical scheme for the evolution of the apolipoprotein gene (adapted from Luo *et al.* (13)). The figure represents an evolutionary tree based on nucleotide substitution rates of apolipoprotein genes. The timeline goes from approximately 700 million years (M) ago to the present. The proposed dates of major divergences are: vertebrate-invertebrate 600 M ago, fish-mammals 400 M ago, birds-mammals 270 M ago. Independent of these major events, the genes of apolipoproteins underwent a series of gene duplication events (closed squares), internal repeat duplications (closed circles) and deletions (open triangles), and random mutations to develop into the apolipoproteins of today. The numbers above the closed circles represent the number of internal repeat duplications that occurred during that time frame. The numbers in brackets represent the approximate date of the gene duplication events.


Gene Duplication Event
Duplication 11 or 22 codons
Deletion of 11 codons
A-I
A-IV
E
A-II
C-III
C-II
C-I
C-I'
3
6-7
(270 M)
2
(418 M or less)
1
1
(423 M)
(285 M)
(570 M)
(680 M)
(285 M)
time
(million years)
600 M
400 M
200 M
270 M
vertebrate - invertebrate
fish - mammals
birds - mammals