Structural Analysis of Apolipoprotein A-I: Limited Proteolysis of Methionine-Reduced and -Oxidized Lipid-Free and Lipid-Bound Human Apo A-I†

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ABSTRACT: The domain structures of lipid-free and lipid-bound apolipoprotein A-I (apo A-I) containing reduced and oxidized methionines were analyzed by limited proteolysis. Lipid-free apo A-I is cleaved primarily in the extreme carboxy-terminus and, to a much lesser extent, in the central region of the protein between residues 115 and 136. Oxidation of methionines 112 and 148 to the corresponding sulfoxides in putative amphipathic helices 4 (P99-E120) and 6 (P143-A164), respectively, causes helices 1 (L44-G65), 2 (P66-S87), and 7 (P165-G186) to become susceptible to protease digestion. These results are consistent with a discrete, globular tertiary structure for the lipid-free protein minimally formed from amphipathic helices 1, 2, 4, 6, and 7. In distinct contrast to lipid-free apo A-I, lipid-bound apo A-I is most susceptible to cleavage in the extreme amino-terminus and, to a lesser extent, in both the central and carboxy-terminal regions. The observed cleavage pattern for the reduced lipid-bound protein supports the existence of many of the turns between helices predicted by sequence analysis of the lipid-bound protein. Methionine oxidation of lipid-bound protein results in a decreased protease susceptibility in the extreme amino-terminus and a concomitant increase in protease susceptibility in the central and carboxy-terminal regions. The results from methionine oxidation indicate the oxidation state of the protein is an important determinant in defining the conformation of both lipid-free and lipid-bound apo A-I.

Apolipoprotein A-I (apo A-I)[1] , the major protein constituent of high-density lipoprotein (HDL), is a 243 amino acid protein (Brewer et al., 1978) proposed to consist of a series of amphipathic helices (Figure 1) which bind to the periphery of lipid bilayer discs and to the surface of lipid monolayer spheres [Segrest et al., 1974; Li et al., 1988; for recent reviews, see Rosseneu (1992) and Brouillette and Anantharamaiah (1995)]. Apo A-I is thought to play an important role in the lowered risk of coronary artery disease associated with high serum levels of HDL (Rhoads et al., 1976; Gordon et al., 1977; Tyroler, 1980; Castelli et al., 1983; Miller, 1987) and has been shown to activate LCAT, the enzyme responsible for the conversion of cholesterol to cholesteryl esters in HDL particles (Fielding et al., 1972; Chung et al., 1979), an important step in reverse cholesterol transport (Glomset, 1968). An HDL receptor located on hepatic cell membranes has recently been described, providing evidence for the involvement of a specific receptor in reverse cholesterol transport (Acton et al., 1996). An understanding of apo A-I structure is crucial for understanding its role in HDL structure

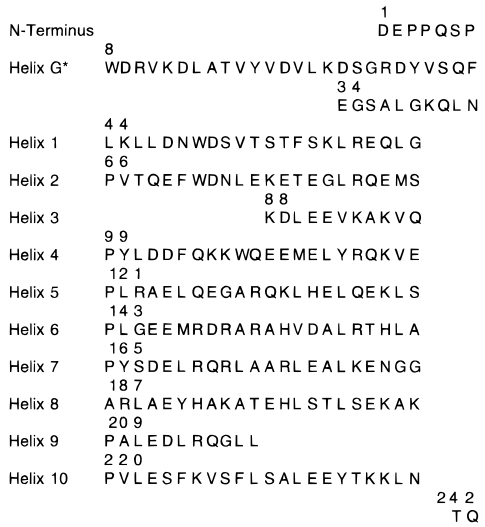

FIGURE 1: Primary sequence of apo A-I, arranged to show the location of putative amphipathic helices as suggested by DNA and protein sequence homology (Li et al. 1988; Nolte & Atkinson, 1992). Residues 8−33 comprise a G* amphipathic helix, a class of amphipathic helices similar to that found in globular proteins, whereas helices 1−10 belong to amphipathic helix classes commonly found in the exchangeable apolipoproteins (Segrest et al., 1992).

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[1] Abbreviations: apo A-I, apolipoprotein A-I; HDL, high-density lipoprotein; rLp, reconstituted lipoprotein; LCAT, lecithin-cholesterol acyl transferase; TPCK, *N*-tosyl-l-phenylalanine chloromethyl ketone; DMS, dimethylsuberimidate; HPLC, high-performance liquid chromatography; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-glycerophosphocholine; NBD-PC, 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-dodecanoyl]-*sn*-glycero-3-phosphocholine; PSC, phosphate buffered saline (0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.4); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GGE, polyacrylamide gradient gel electrophoresis.

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and function and the mechanisms of reverse cholesterol transport.

Structural models of apo A-I in which repeating amphipathic helices bind to lipid surfaces have been proposed (Segrest, et al., 1974) and refined through a number of spectroscopic studies involving CD, fluorescence, infrared, and ^{13}C NMR spectroscopy (Jonas et al., 1989; Brasseur et al., 1990; Wald et al., 1990; Jonas et al., 1990; Sparks et al., 1992a,b), antibody binding studies (Banka et al., 1991; Calabresi et al., 1993; Meng et al., 1993), mutagenesis (Sorci-Thomas et al., 1993; Schmidt et al., 1995; Rogers et al., 1997;) or "protein dissection" by proteolysis (Dalton & Swaney, 1993; Ji & Jonas, 1995), and computer analyses, such as amphipathic helix characterization (Segrest et al., 1990; Segrest et al., 1992), secondary structure predictions (Nolte & Atkinson, 1992), and molecular hydrophobicity potential assignments (Brasseur et al., 1990). These studies have helped to define regions of the protein that may be structurally distinct. For example, the amino-terminal third of the lipid-bound protein and a flexible, possibly "hinged" central pair of helices have been proposed as regions involved in accommodating size changes in rLp (Brouillette et al., 1984; Cheung et al., 1987; Jonas et al., 1989; Sparks et al., 1992b; Calabresi et al., 1993); the carboxy terminus has been identified as being important for lipid affinity (Schmidt et al., 1995; Ji & Jonas, 1995), and the central region is thought to be important for LCAT activation (Banka et al., 1991, Meng et al., 1993; Sorci-Thomas et al., 1994; Rogers et al., 1997). Most studies have focused on a functional analysis of the lipid-bound conformation, but the tertiary structure of apo A-I, particularly in the lipid-free state, remains poorly understood.

Limited proteolysis is a valuable tool for probing the tertiary structure of apo A-I, although previous studies using this method have reported somewhat conflicting results. Proteolytic cleavage of lipid-bound apo A-I has been reported to occur primarily in either the amino-terminal (Lins et al., 1993), the central (Kunitake et al., 1990; Dalton & Swaney, 1993), and/or the carboxy-terminal region (Dalton & Swaney, 1993; Ji & Jonas, 1995), while no systematic studies of the susceptibility of lipid-free apo A-I to proteolytic cleavage have been reported. In this paper, results are presented from the most extensive analysis to date of the products from the limited proteolysis of both lipid-free and lipid-bound apo A-I. These studies demonstrate that methionine oxidation significantly affects the protease susceptibility of the protein in both environments, providing insights into the structure of the reduced protein in the two conformational states. Furthermore, these results provide an alternative explanation in addition to the size, method of preparation and lipid composition of the reconstituted lipoproteins, for the apparent discrepancies among previous reports involving limited proteolysis of the lipid-bound protein.

MATERIALS AND METHODS

Chemicals, Enzymes, and Lipids. Chymotrypsin and *Staphylococcus aureus* V_8 protease (endoproteinase Glu-C) were purchased from Calbiochem (La Jolla, CA) or Sigma Chemical Company (St. Louis, MO); chymotrypsin was treated with *N*-tosyl-*l*-phenylalanine chloromethyl ketone (TPCK) to inhibit possible trypsin activity before use. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), sodium cholate was purchased from Calbiochem, and DMS and 3,4-dichloroisocoumarin were purchased from Sigma. All other materials were purchased from Pierce Chemicals.

Preparation of Protein. Apo A-I containing reduced methionines was purified from delipidated HDL using reversed-phase HPLC as previously described (Hughes et al., 1988; Anantharamaiah et al., 1988) and stored lyophilized at −25 °C; apo A-I prepared in this manner contains greater than 95% reduced methionines, migrates as a single band on SDS−PAGE, and exhibits a single peak by reversed-phase HPLC. Lyophilized protein was dissolved in 6 M guanidine-HCl immediately prior to dialysis into PSC buffer [0.02 M phosphate, 0.15 M NaCl, 0.02% (w/v) NaN_3, pH 7.4] and used within 2 weeks. The protein was routinely analyzed using reverse-phase HPLC just prior to reconstitution into lipoprotein particles to insure that the methionine residues had not become oxidized during storage; only reduced protein was used in the formation of lipoprotein particles except when oxidized protein was specifically desired. Protein in lipoprotein particles was also analyzed using HPLC following reconstitution to insure that oxidation did not occur during the reconstitution process. To prepare oxidized apo A-I containing sulfoxides at methionines 112 and 148, the protein was treated with 0.45% (v/v) hydrogen peroxide for 5 min at room temperature in 3 M guanidine-HCl, pH 7.4, and then purified using reverse-phase HPLC (Hughes et al., 1988) as previously characterized (Anantharamaiah et al., 1988; von Eckardstein et al., 1991). It has been shown by mass spectral analysis of oxidized fragments that this procedure results in the oxidation of M112 and M148, but not M86 (von Eckardstein et al., 1991).

Preparation of rLp. POPC in ethanol was dried on the surface of a small test tube with a stream of argon and then kept under vacuum in the dark for at least 2 h. The fluorescent lipid NBD-PC was added with the POPC (1:20 mol/mol); addition of NBD-PC did not change the size of the resulting particles as compared to particles prepared without NBD-PC. Sodium cholate in PSC buffer was added to the dried lipid (4:1 mol/mol), and the mixture was incubated with gentle shaking at room temperature for 2−4 h. Apo A-I in PSC buffer was added at 100:1 lipid to protein mole ratio, and the mixture was incubated with gentle shaking for a minimum of 4 h at room temperature before exhaustive dialysis against PSC buffer at 4 °C. Dialyzed rLp was loaded onto 70 mm × 80 mm 4−20% nondenaturing polyacrylamide gradient gels and electrophoresed for 20 h at 120 V in 25 mM Tris, 192 mM glycine, pH 8.3; under these conditions, complexes run to the pore limit of the gel. Three major, discrete rLps are formed. Each homogeneously sized rLp was isolated from the gel by cutting out the respective band, mincing it and incubating it at 5 °C with PSC for a minimum of 24 h. From 1 to 4 mg of the rLp mixture was loaded on a single gel from which about 50−600 μg of each purified rLp could be obtained (depending which of the three rLp classes, which were not produced in equal proportions, was isolated). Purified rLps were usually used within 2 weeks of preparation. Proteolysis was conducted on all three purified rLps containing reduced protein but was performed on mixtures of rLp containing oxidized protein; this decision was based on two observations. First, the three rLp classes containing reduced protein were shown to give very similar proteolysis results, indicating similar protein conformations (the explanation for this result will be detailed in the Discussion). Second, the size classes,

distribution of particles, and helicity of protein for rLp containing oxidized protein were very similar to those containing reduced protein, as previously observed by others (Jonas et al., 1993).

Proteolysis Experiments. Monomeric apo A-I free in solution or complexed with lipid (about 0.1 mg/mL in either case) was treated with chymotrypsin or *S. aureus* V_8 protease (obtained from either Sigma or Calbiochem) at 37 °C using a 2000:1 (protein:protease, w/w) for experiments with lipid-free apo A-I and either 50:1 (chymotrypsin) or 20:1 (V_8 protease) for experiments with lipid-bound apo A-I. An additional equivalent aliquot of protease was added after 8 h to the samples that were incubated overnight. Proteolysis was stopped using a 200 mol excess (relative to protease) of 3,4-dichloroisocoumarin. Proteolytic digests of rLp were delipidated using hexane/isopropyl alcohol (3:2) followed by several hexane washes and then lyophilized (Radin, 1981).

Proteolytic fragments from soluble apo A-I and delipidated complexes were separated on polyacrylamide gels using either a tricine buffer system (Schagger & von Jagow, 1987) or the standard Laemmli system (Laemmli, 1970). In Laemmli gels, the top third of the separating gel contained 12% acrylamide (w/v) and the bottom two thirds contained 20% acrylamide (w/v) to enhance separation of small peptides. Alternatively, fragments were separated by reversed-phase HPLC by resuspending the hydrolyzed peptides in 6 M guanidine-HCl followed by injection onto either a C18 or C4 column (Vydac) equilibrated with 10% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water. Samples were eluted with a 30 min linear gradient of 10 to 70% acetonitrile. Fractions containing selected peaks were collected and lyophilized before peptide sequencing.

Peptide Sequencing. Proteolytic fragments separated on SDS gels were blotted onto a polyvinylidene difluoride membrane using a Bio-Rad Transblot semidry system. The membrane was stained with Coomassie Blue, and the appropriate band was carefully cut from the membrane for sequencing. Sequencing of HPLC column fractions and membrane blots was performed by the Glycoprotein Analysis Core Facility at University of Alabama at Birmingham on a Porton Instruments Inc. (Tarzana, CA) Model PI 2090E gas-phase microsequencing system equipped with an online PTH-amino acid analyzer.

Analytical Methods. rLp Stokes radii were determined from a standard curve obtained by least squares regression analysis of the R_f of protein standards having known Stokes radii (Pharmacia): thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.3 nm). Molecular masses of proteolytic fragments were obtained from a similar analysis using SDS−PAGE protein standards. Protein content of the purified rLp was determined using the Lowry protein assay (Lowry et al., 1951) modified by addition of SDS to a final concentration of 1% (w/v). Phospholipid content was determined using an enzymatic assay (Warnick, 1986). Cross-linking to determine the apo A-I stoichiometry per particle was performed using DMS (Swaney, 1980) and cross-linked protein was analyzed by SDS−PAGE on a linear gradient of 8 to 25% acrylamide in the resolving gel.

Circular Dichroism. CD spectra were collected on solutions of 0.06−0.25 mg/mL apo A-I in PSC buffer with a 0.01 cm path-length cell using an AVIV 62DS spectropolarimeter. Data were collected every nanometer from 190 to 260 nm with 1.0 s averaging per point and a 2 nm band width. Spectra of at least five scans were signal averaged and baseline corrected by subtracting an averaged buffer spectrum.

Table 1: Identity of Fragments Obtained by Proteolysis of Lipid-free Apo A-I

SDS−PAGE band[a]	molecular mass (kDa)	amino-terminal sequence	location of cleavage
methionine-reduced apo A-1			
1c	26.3	DEPPQ	F225[b]
2c*	22.4	DEPPQ	Y192[b]
3c	13.1	RQKVE	Y115, F229[c]
4c	1.6	HAKAT	Y192, L211[c]
5c	0.7	HAKAT	Y192, L200[c]
1v	27.4	DEPPQ	E235[b]
2v	26.1	DEPPQ	E223[b]
3v	25.6	DEPPQ	E212[b]
4v	14.4	DEPPQ	E125[b]
5v	11.9	LQEKL	E136
6v	11.2	YHAKA	E191[d]
		SFKVS	E223[d]
methionine-oxidized apo A-I			
6c	25.3	DEPPQ	L219[b]
7c*	20.3	DEPPQ	L174[b]
		SKLRE	F57[d]
8c	13.2	SKLRE	F57, L174[c]
9c*	10.3	RQKVE	Y115, L203[c]
10c	8.7	SKLRE	F57, L134[c]
11c	5.1	SKLRE	F57, Y100[c]
		SDELR	Y166, L21[c]
12c	4.8	SDELR	Y166, L211[c]
13c	4.0	RQKVE	Y115, L144[c]
7v	5.1	GSALG	E34, E78[c]
8v	4.0	DEPPQ	E34[b]

[a] Labels of fragments refer to their corresponding band position on SDS−PAGE (#c are chymotrypsin-derived fragments and #v are V_8 derived fragments) which can be found in Figure 2. Major sites of cleavage deduced from SDS−PAGE are marked with an asterisk (*); these sites are marked with an arrow in Figure 3. [b] Inferred cleavage site from the apparent molecular mass of the fragment. [c] A second cleavage nearer to the C-terminus is inferred from the apparent molecular mass of the fragment. [d] In cases where more than one sequence is present, comigration of fragments is assumed due to nonoptimal resolution on SDS−PAGE.

RESULTS

Proteolysis of lipid-free Apo A-I. To examine the protease susceptibility of lipid-free apo A-I with all methionines reduced, two proteases with different specificities, chymotrypsin and *S. aureus* V_8 protease, were used, which allows for potential cleavage in all regions of the protein. Chymotrypsin cleaves at the carboxy-terminal side of aromatic and bulky, hydrophobic residues, which are likely to be located in nonpolar regions such as the hydrophobic face of amphipathic helices, whereas V_8 protease cleaves at the carboxy-terminal side of glutamic acid residues (under the conditions used here), which are likely to be located primarily in polar regions such as the hydrophilic face of amphipathic helices.

Treatment of monomeric reduced apo A-I with chymotrypsin results in the appearance of two large peptides by SDS−PAGE (Figure 2, upper panel), both of which yielded an intact amino-terminus upon sequencing (complete cleavage results are given in Table 1). The product closest in size to apo A-I (26.3 kDa, band 1c) is not discernible until about 15 or 20 min after addition of protease; this product may be generated earlier but is not visible due to overlap

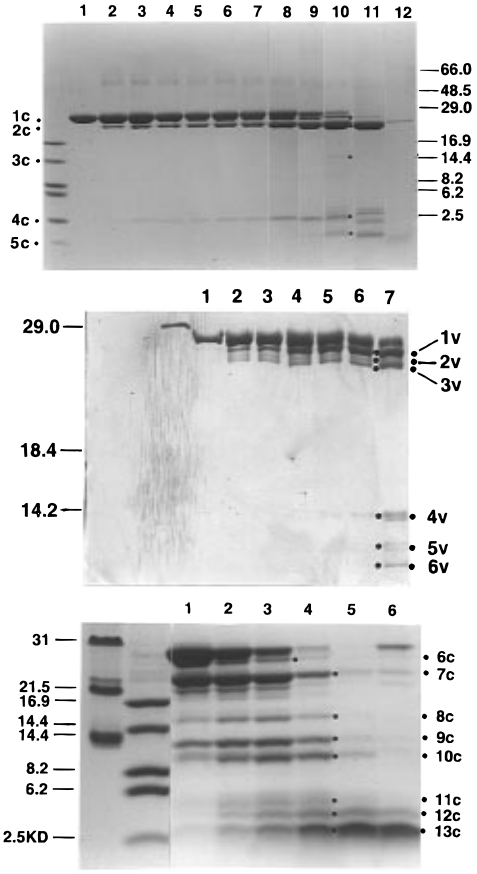

FIGURE 2: SDS−PAGE analysis of the proteolysis of methionine-reduced (upper and middle panels) and methionine-oxidized (lower panel) lipid-free apo A-I. The positions and molecular masses, in kilodaltons, of protein standards are indicated on one side of the gel and the positions and labels of the products 1c−13c (chymotrypsin-derived products) and 1v−8v (*S. aureus* V_8-derived products) are on the other side. The sequenced bands are marked with a small closed circle in each gel. The amino-terminal sequences of the products can be found in Table 1. Upper Panel. The time course of chymotrypsin treatment. Lanes 1−12, respectively, represent 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h of proteolysis. Middle Panel. The time course of *S. aureus* V_8 protease treatment. Lanes 1−7, respectively, represent 0, 1, 5, 10, 20, 60, and 90 min of proteolysis. Lower Panel. The time course of chymotrypsin treatment. Lanes 1−6, respectively, represent 5 min, 15 min, 30 min, 1 h, 2 h, and 4 h of proteolysis.

with uncleaved apo A-I. The molecular mass of this peptide is most consistent with cleavage at F225 in helix 10, yielding the fragment D1-F225 (Table 1). The numbering of helices is shown in Figure 1. The smaller peptide (22.4 kDa, band 2c) is discernible at all of the time points and increases at each time point until it is the major product 8 h after the addition of protease; it is the only product in this size range remaining after 24 h of proteolysis. Molecular mass and sequence analysis indicate the 22.8 kDa fragment is most likely D1-Y192.

A 1.6 kDa peptide (band 4c) also appears at the earliest time points and remains until after 4 h of digestion, at which point several new small fragments appear, including one with a molecular mass of about 0.7 kDa (band 5c). The amino-terminal sequence of both the 1.6 and the 0.7 kDa peptides is H_{193}AKAT, corresponding to cleavage at Y192. Since the molecular mass of H193-N243 is approximately 6.0 kDa, these bands must be the product of two cleavages, one at Y192 and the other closer to the carboxy-terminus. A minor product (band 3c), not observed until 4 h after treatment, was produced by cleavage in the central region of the protein and has the amino-terminal sequence R_{116}QKVE.

Proteolysis of lipid-free apo A-I with V_8 protease resulted in cleavages in the same regions of the protein as those observed using chymotrypsin (SDS−PAGE shown in Figure 2, middle panel. While there are many potential V_8 cleavage sites distributed throughout the protein, analysis of fragments revealed that cleavage occurred exclusively in two regions: the carboxy-terminus from E191 to the end of the protein (bands 1v, 2v, 3v, and 6v) and the central region encompassing helices 4 and 5 (bands 4v and 5v).

Thus, treatment of reduced apo A-I in solution with both chymotrypsin and V_8 protease resulted in cleavage primarily in the carboxy-terminal region, with a lesser amount of cleavage occurring in the central region of the protein. Figure 3A shows a linear map of the apo A-I sequence which summarizes the positions of all observed cleavages determined by amino-terminal sequencing and inferred by SDS−PAGE molecular mass analysis.

To examine the protease susceptibility of apo A-I with M112 and M148 oxidized to the corresponding sulfoxides, lipid-free apo A-I was treated with hydrogen peroxide and then purified using reverse-phase HPLC as previously described (Anantharamaiah et al., 1988; Rogers et al., 1997). This procedure has been shown by mass spectral analysis of proteolytic fragments to oxidize only M112 and M148 (von Eckardstein et al., 1991). The retention time of apo A-I is exquisitely sensitive to the oxidation state of the protein, which facilitates purification of both forms (Hughes et al., 1988; Rogers et al., 1997). Proteolysis of oxidized apo A-I with chymotrypsin (SDS−PAGE shown in Figure

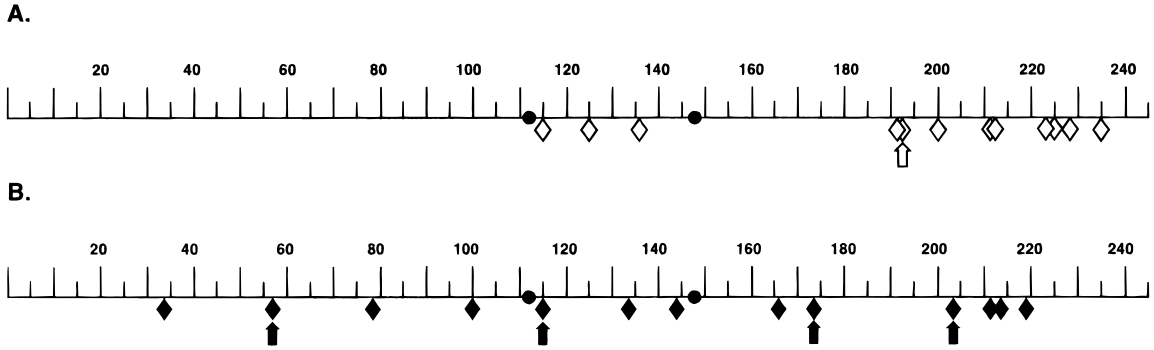

FIGURE 3: Linear sequence maps of apo A-I showing the observed proteolytic cleavage sites in methionine-reduced (panel A, open symbols) and methionine-oxidized (panel B, closed symbols) lipid-free apo A-I. Diamonds and arrows indicate observed cleavage sites; arrows indicate major cleavage sites. The position of methionines 112 and 148 are indicated on each line with a closed circle. All fragments listed in Table 1 are represented.

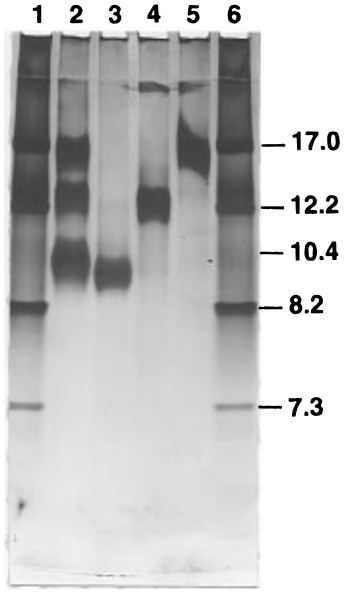

FIGURE 4: Nondenaturing GGE of rLp containing methionine-reduced apo A-I. Lanes 1 and 6 contain native protein standards with the Stokes diameters (nanometers) shown. Lane 2 is the mixture of complexes before purification. Lanes 3, 4, and 5 are purified rLp2A-I, rLp3A-I, and rLp4A-I, respectively.

2, lower panel) and V_8 (not shown) resulted in the appearance of fragments not observed in proteolysis of the reduced protein. In particular, new cleavages occurred in helix 1 (bands 7c, 8c, 10c, 11c, 7v, and 8v) and helix 7 (7c, 11c, and 12c). Neither of these helices was cleaved under the same conditions in the reduced form of the protein, thus, oxidation of the protein at M112 and M148 resulted in exposure of these regions of the protein to protease. The cleavages observed in oxidized lipid-free apo A-I are summarized in the linear sequence map shown in Figure 3B.

Purification and Characterization of Discoidal rLp. Reconstituted lipoproteins (rLp) formed from a 100:1 molar ratio of lipid to reduced apo A-I by the cholate dialysis method (Jonas et al., 1989) produced several discrete products upon analysis by GGE (Figure 4). Most preparations exhibited three major products (rLp2A-I, rLp3A-I, and rLp4A-I), each of which were isolated from preparative native GGE; these rLps were homogeneous in size and stable for at least 2 weeks as indicated by analytical native GGE (Figure 4, lanes 3−5). The stoichiometry of apo A-I in the isolated rLp was determined by chemical cross-linking (Table 2) using DMS (Swaney, 1980). Negative stain electron microscopy confirmed that these rLps have a discoidal morphology, and CD spectroscopy indicated that the amount of helix in apo A-I increases from about 55%, when not bound to lipid, to between 70 and 80% in the rLp (data not shown), in agreement with results from other laboratories (Jonas et al., 1989, 1990, Sparks et al., 1992a). Lipid:protein ratios determined for the purified complexes agree well with the calculated values based on the size and apo A-I stoichiometry of each particle (Table 2). In general, complexes produced by this method are comparable with respect to size, composition, and apo A-I particle stoichiometry to those produced by other methods in other laboratories (Jonas et al., 1989, Calabresi et al., 1993). rLps prepared with methionine-oxidized apo A-I using the same procedure were found to be very similar in terms of size and distribution of major species and amount of helix to those prepared with reduced apo A-I as observed by others (Jonas et al., 1993).

Table 2: Characteristics of Homogeneous rLp

rLp	molar ratio of POPC:A-I meas	molar ratio of POPC:A-I calc[a]	diameter (nm)[b]	no. apo A-I per particle	no. helices, per apo-A-I[c]
rLp2A-I	72 ± 2:1[d]	40−60:1	8.5 ± 0.3[d]	2	7
rLp3A-I	100 ± 1:1	70−104:1	11.9 ± 0.4	3	7
rLp4A-I	142 ± 3:1	101−151:1	15.4 ± 0.6	4	7

[a] The number of PC molecules per discoidal complex, *n*, is estimated from the calculated surface area, i.e., $n = [\pi(r - 1.5)^2/(4.0-7.5\ \text{nm}^2)]2$. The diameter of the apo A-1 helix, approximated to be 1.5 nm, is subtracted from the measured radius of the disk, *r* [(column 4)/2], in order to calculate just the surface area contributed by the lipid component. The reported range in the calculated molar ratio comes from the range observed in the cross-sectional area of PC molecules, 4.0−7.5 nm (Small, 1967). [b] From nondenaturing GGE. [c] The calculated number of helices per particle, *n*, is determined from the circumference of the disk (after correcting for the radius of a helix, ∼0.75 nm), and the apo A-I stoichiometry of the particle, #, found in column 5 is $n = [2\pi(r - 0.75)/1.5]/\#$, where $2p(r - 0.75)/1.5$ is the circumference of the disk divided by the average diameter of a helix which gives the number of helices around the perimeter of the disk. [d] ±standard deviation.

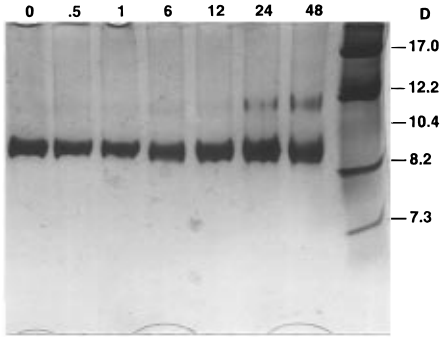

FIGURE 5: Nondenaturing GGE of rLp2A-I containing methionine-reduced apo A-I treated with *S. aureus* V_8 protease for 0 to 48 h. Lanes are labeled with times (hours) of protease treatment. The far right lane contains native protein standards with the Stokes diameter (nanometers) shown. Proteolysis conditions are the same as for samples depicted in Figure 5, middle panel.

Proteolysis of Lipid-Bound Apo A-I. Forty (chymotrypsin) or 100 (V_8 protease) times more protease and longer incubation times (several hours *versus* several minutes) were required to achieve the same level of cleavage in reduced lipid-bound apo A-I compared to reduced lipid-free apo A-I (as judged by the loss of native apo A-I upon SDS−PAGE), indicating the much greater stability of apo A-I to protease when bound to lipid, as has been previously observed (Kunitake et al., 1990; Lins et al., 1993; Dalton & Swaney, 1993). Particle integrity over the time course of the proteolysis experiment was assessed by nondenaturing GGE. All rLps containing reduced apo A-I were found to be stable, in terms of size, over at least the first 12 h of proteolysis (Figure 5).

Each of the three rLps containing reduced apo A-I were subjected to proteolysis, and the resulting cleavage patterns were remarkably similar. Several fragments are observed that are greater than 20 kDa in mass and similar in size to those observed in the proteolysis of lipid-free protein (SDS−PAGE is shown in Figure 6, upper panel). However, in contrast to reduced, lipid-free apo A-I, amino-terminal sequencing of these fragments revealed that substantial

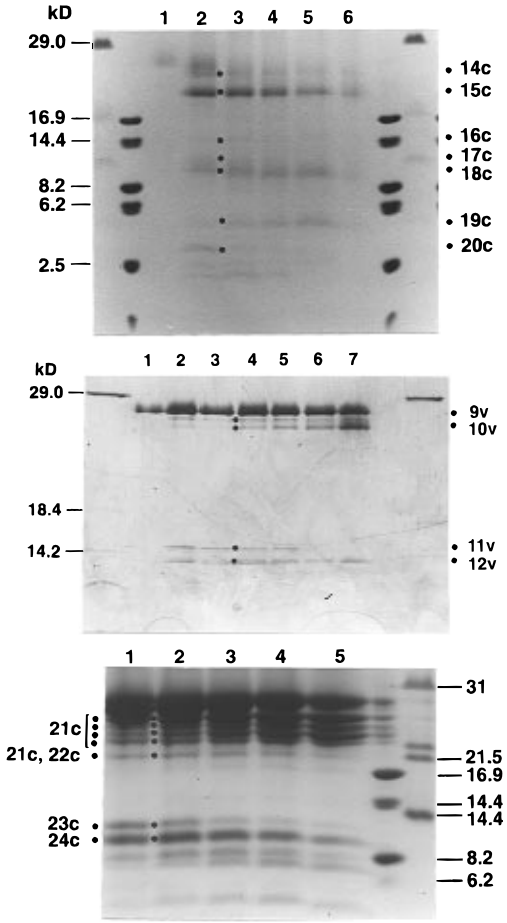

FIGURE 6: SDS−PAGE analysis of the proteolysis of rLp containing methionine-reduced (upper and middle panels) and methionine-oxidized (lower panel) apo A-I. The positions and molecular masses, in kilodaltons, of protein standards are indicated on one side of the gel and the positions and labels of the products 14c−24c (chymotrypsin-derived products) and 9v−12v (*S. aureus* V_8-derived products) are on the other side. The amino-terminal sequences of the products are reported in Table 3. Upper panel. The time course of chymotrypsin treatment of purified rLp3A-I. Lanes 1−6, respectively, represent 0 min, 30 min, 2 h, 4 h, 8 h, and 24 h of proteolysis. Middle panel. The time course of *S. aureus* V_8 treatment of purified rLp3A-I. Lanes 1−7, respectively, represent 0 min, 30 min, 1 h, 6 h, 12 h, 24 h, and 48 h of proteolysis. Lower Panel. The time course of chymotrypsin treatment of rLp mixture. Lanes 1−5, respectively, represent 30 min, 1 h, 2 h, 3 h, and 4 h of proteolysis. The amino-terminal sequences of the five products labeled 21c were analyzed together; the smallest in this group, 22c, was also analyzed separately.

cleavage occurred in the amino-terminal region of the protein (the complete cleavage results are given in Table 3). Fragments closest in size to intact apo A-I produced by chymotrypsin reflect cleavage in the amino-terminal 60 amino acids, at Y18 (band 14c), L42 (band 15c′), and F57 (band 15c), although early in proteolysis, one of these fragments (band 15c′, see Table 3) consisted of a transient mixture of products.

Several smaller products were produced from all three proteolyzed complexes upon treatment with chymotrypsin (Table 3). Sites of cleavage identified by amino-terminal sequencing of the products obtained after 1 h of cleavage include F57 (bands 16c, 19c, and 20c), Y115 (bands 16c and 17c), and L122 (bands 19c and 20c). The existence of these smaller products confirms that substantial cleavage occurs in the amino-terminal region of apo A-I in rLp and also indicates cleavage in the central region of the protein.

Table 3: Identity of Fragments Obtained by Proteolysis of Lipid-Bound Apo A-I

SDS−PAGE band[a]	molecular mass (kDa)	amino-terminal sequence	location of cleavage
methionine-reduced apo A-I			
14c	26.7	VDVLK	Y18
15c′*	23.6 (0.5 h)[b]	DEPPQ	E205[c]
	(1 h)	NLKLL	L42
15c*	(8 h)	SKLRE	F57
16c	13.0	SKLRE	F57,[e] L174[d]
		RQKVE	Y115,[e] L229[d]
17c	10.0	DEPPQ	L90[c,e]
		RQKVE	Y115,[e] L203[d]
18c*	9.0	DEPPQ	L42[c]
19c	4.5	SKLRE	F57,[e] Y100[d]
		RAELQ	L122,[e] L163[d]
20c	3.4	SKLRE	F57,[e] L190[d]
		RAELQ	L122,[e] L144[d]
9v	27.4	DEPPQ	E235[c]
10v*	26.2	GSALG	E34
11v	14.5	DEPPQ	E125[c]
12v	13.5	GSALG	E34, E147[d]
methionine-oxidized apo A-I			
21c	25.0	DEPPQ	L214[c,f]
21c	23.6	DEPPQ	L200[c,f]
21c	22.0	DEPPQ	Y192[c,f]
21c	20.8	DEPPQ	L178[c,f]
21c, 22c	19.1	DEPPQ	Y166[c,f]
23c	11.4	DEPPQ	Y100[c,e]
		RAELQ	L122,[e] L214[d]
24c	10.0	DEPPQ	L90[c,e]
		RQKVE	Y115,[e] L122[d]
		RAELQ	L122,[e] L134[d]

[a] Labels of fragments refer to their corresponding band position on SDS−PAGE which can be found in Figure 6. Major sites of cleavage deduced from SDS−PAGE (#c are chymotrypsin-derived fragments and #v are V_8 derived fragments) are marked with an asterisk (*); these sites are marked with an arrow in Figure 7. Tabulated sequences were obtained from cleavage experiments on all three rLp as shown in Table 2. [b] At 0.5 h, the N-terminal sequence of the major fragment migrating at position 15c′ is DEPPQ; at 1 h, the N-terminal sequence of the major fragment migrating at position 15c′ is NLKLL, and so forth. [c] Inferred cleavage site from the apparent molecular mass of the fragment. [d] A second cleavage nearer to the C-terminus is inferred from the apparent molecular mass of the fragment. [e] In cases where more than one sequence is present, comigration of fragments is assumed due to nonoptimal resolution on SDS−PAGE. [f] All bands labeled 21c were sequenced together in one sample. The only sequence present was DEPPQ. Band 22c was also isolated as a single band and sequenced individually; the only sequence present was DEPPQ.

Although no sequences were obtained for the carboxy-terminal region of the protein, it can be inferred from the sizes of several proteolytic fragments that cleavage also occurred within this region (Table 3). For example, a 10 kDa peptide (band 17c) with an amino-terminal sequence corresponding to cleavage at Y115 indicates cleavage occurred at or near L203.

Proteolysis of rLp containing reduced apo A-I with V_8 protease yielded results similar to those obtained using chymotrypsin (Figure 6, middle panel). Two large fragments were observed, indicating cleavage in both the amino-terminus at E34 (band 10v) and in the carboxy-terminus (band 9v); additional cleavage was observed in the central region of the protein, at residues E125 and E147 (bands 11v and 12v). Thus, as summarized in the linear sequence map shown in Figure 7A, rLpA-Is containing reduced protein are proteolyzed at a number of locations throughout the protein with significant cleavage occurring in the amino-terminal third of the protein.

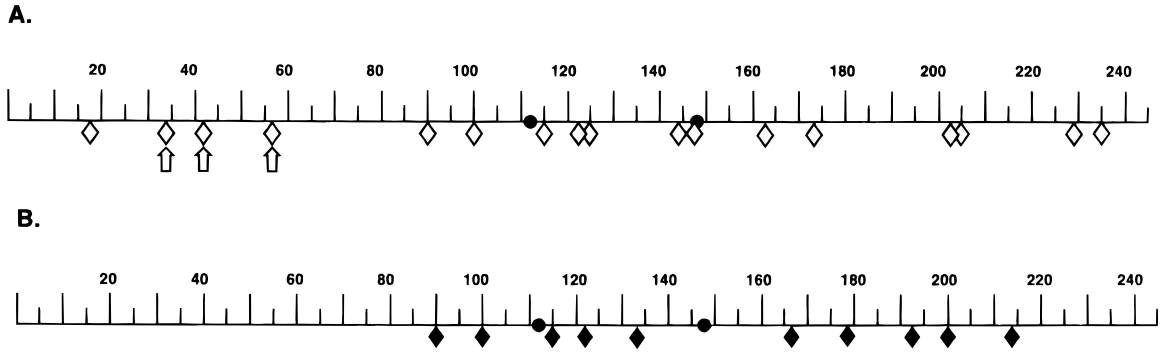

FIGURE 7: Linear sequence maps of apo A-I showing the observed proteolytic cleavage sites in methionine-reduced (panel A, open symbols) and methionine-oxidized (panel B, closed symbols) lipid-bound apo A-I. Diamonds and arrows indicate observed cleavage sites; arrows indicate major cleavage sites. The position of methionines 112 and 148 are indicated on each line with a closed circle. All fragments listed in Table 3 are represented.

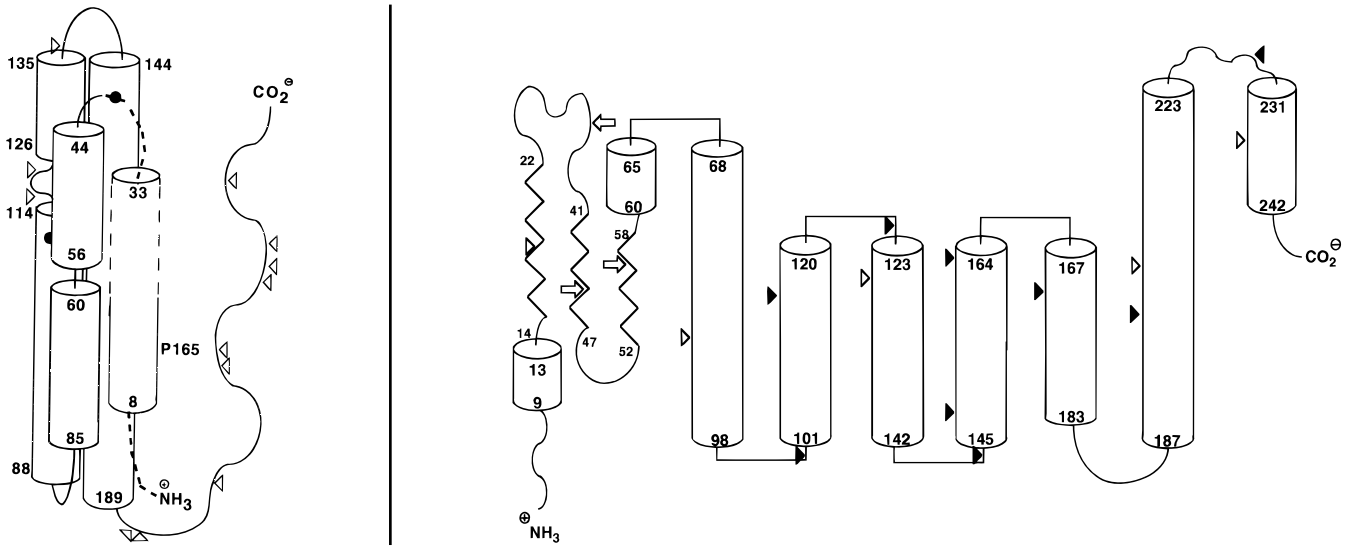

FIGURE 8: Structural model of lipid-free (left side) and lipid-bound apo A-I (right side, adapted from Nolte and Atkinson, 1992). Position of cleavage sites in methionine-reduced protein are indicated; primary cleavage sites (▽), secondary cleavage sites (▼) and, in the lipid-bound structure, major cleavage sites (⟹). Cylinders represent helical structure. Dashed lines represent regions of probable, but not necessarily helical, secondary structure; prolines occurring within helical stretches are also indicated by dashed lines.

rLp made with methionine-oxidized apo A-I were similar in size, distribution, and protein secondary structure to those made with reduced protein. In spite of these similarities, rLp made with oxidized apo A-I were considerably less stable to proteolysis, as indicated by a noticeable change in size of the rLp observed by nondenaturing GGE, which occurred after 2 h of exposure to protease (data not shown). Consequently, sequencing was obtained on bands obtained before observable changes in rLp size. Figure 6 (lower panel) shows the SDS−PAGE of fragments produced by chymotrypsin cleavage of rLp containing oxidized apo A-I. In contrast to rLp containing reduced apo A-I, where the major products are greater than 19 kDa, two major products of cleavage isolated from proteolyzed rLp containing oxidized protein are in the 10−12 kDa size range. Amino-terminal sequencing of these fragments indicate that they are produced by cleavage in the central region of the protein encompassing helices 4 and 5 (bands 23c and 24c).

In contrast to reduced, lipid-bound protein, all of the proteolytic fragments within the 19+ kDa size range observed in oxidized, lipid-bound protein were produced by cleavage in the carboxy-terminal region of the protein (bands 21c and 22c); no fragments produced solely by cleavage in the amino-terminal third of the protein were observed. The cleavage sites for oxidized, lipid-bound protein are summarized in Figure 7B.

DISCUSSION

Limited proteolysis has been widely used to study the domain structure of proteins (Wilson, 1991). The basis for its success rests on the correspondence between accessibility to proteolytic attack and the lack of a stable secondary structure in highly mobile or flexible regions of the protein (Fontana et al., 1986). It is notable that an early report of the proteolytic susceptibility of lipid-free apolipoprotein E (Wetterau et al., 1988) led to a structural model of the amino-terminal domain that was largely confirmed by the X-ray crystal structure (Wilson et al., 1991). To gain a better understanding of the domain structure of both lipid-bound and lipid-free apo A-I, we have expanded on earlier proteolysis studies (Kunitake et al., 1990; Lins et al., 1992; Dalton & Swaney, 1993; Leroy & Jonas, 1994; Ji & Jonas, 1995), by conducting an exhaustive analysis of the major and minor proteolytic fragments produced early in the reaction time course in lipid-bound and lipid-free apo A-I with two of the protein's three methionines (M112 and M148) in either the reduced or oxidized states. These

results are discussed with reference to structural models for lipid-free and lipid-bound apo A-I (Figure 8).

Lipid-Free Apo A-I Structure. Taken together with CD data and secondary structural analyses of apo A-I, the proteolysis results are consistent with a structure for lipid-free apo A-I in which the amino-terminal approximately 190 amino acids are folded into a largely helical domain, while the carboxy-terminal 53 amino acids are highly flexible and contain little stable structure. There is considerable evidence for the existence of a folded helical domain; for the purpose of discussion, one possible model is shown in Figure 8. First, CD measurements of lipid-free apo A-I indicate a high percentage of helix, ranging from 50 to 66% (Nolte & Atkinson, 1992; Sparks et al., 1992a, Leroy & Jonas 1994); the model shown contains 61% helix. Second, lipid-free apo A-I exhibits a cooperative unfolding transition consistent with a globular, folded structure (Reijngould & Phillips, 1982; Leroy & Jonas 1994; Rogers et al., 1997). Third, a helix bundle motif is consistent with the crystal structures of apolipophorin (Briter et al., 1991) and the amino-terminal domain of apolipoprotein E (Wilson et al., 1991); these structures suggest that a helix bundle motif may be common among apolipoproteins crystallized in the absence of lipid. In fact, a model of apo A-I derived from the apolipophorin III structure has previously been proposed (Brown et al., 1994). Fourth, in the present studies, and elsewhere (Ji & Jonas 1995), the amino-terminal 190 residues have been found to be relatively resistant to protease digestion, suggesting a compact structure for this region of the protein. Our proteolysis studies were instrumental in suggesting the design of an amino-terminal deletion mutant, the solution properties of which we recently reported (Rogers et al., 1997). This mutant, missing the amino-terminal 43 amino acids, exhibits a tertiary structure distinctly different from native apo A-I, indicating residues 1−43 are involved in stabilizing the folded structure of the native protein. In contrast, we have shown here that the carboxy-terminus of lipid-free apo A-I is extremely susceptible to protease digestion, suggesting that this region of the protein is very flexible. It is, therefore, unlikely to play a significant role in stabilizing the domain composed of the amino-terminal two-thirds of the protein. The carboxy-terminus may play a role in HDL assembly by initiating binding to lipid (Ji & Jonas 1995; Gursky & Atkinson 1996; Rogers, D. P., Roberts, L. M., Lebowitz, J., Engler, J. A., & Brouillette, C. G., unpublished observations).

Our lipid-free apo A-I model will be used to suggest the design of additional apo A-I mutants. However, it should be noted that other arrangements of the residues are possible and are not excluded by our data. For example, others have predicted a β-sheet for the extreme amino-terminal region (Nolte & Atkinson, 1992). Inclusion of a β-sheet in the amino-terminus requires some helical structure to be added to the carboxy-terminus of the protein to maintain a helix content of at least 60%. While studies on the 1−190 residue proteolytic fragment (Ji & Jonas, 1995) and a 1−187 residue recombinant apo A-I mutant (Rogers, D. P., Roberts, L. M., Lebowitz, J., Engler, J.A., & Brouillette, C. G., unpublished observations) are consistent with the presence of some helicity in the carboxy-terminus, the proteolysis data are inconsistent with extensive stable secondary structure in this region of the protein.

Effects of Methionine Oxidation on Lipid-Free Apo A-I. Further support for the proposed structure comes from the observation that oxidation of the hydrophobic residues M112 and M148 to hydrophilic sulfoxides results in a dramatic increase in the protease susceptibility of the amino-terminal and central regions of the protein. Since the side chains of M112 and M148 are on the hydrophobic faces of the amphipathic helices, we have modeled them to project toward the interior of the bundle and, hence, oxidation is expected to cause, at a minimum, local perturbations in the helical packing in the vicinity of each methionine. The major products formed early in proteolysis are produced by cleavage at E34, Y115 (which is also observed as a minor cleavage product in methionine-reduced apo A-I), L174, and L203. As can be seen from the model (Figure 8), with the exception of L203, cleavage at these residues can be rationalized based on their proximity to either M112 or M148 in the proposed structure.

Lipid-Bound Apo A-I Structure. Several structural models for lipid-bound apo A-I have been proposed [Sparks et al., 1992a; Nolte & Atkinson, 1992; Calabresi et al., 1993; reviewed in Brouillette and Anantharamaiah (1995)]. Of these, the model presented by Nolte and Atkinson, which is based on an extensive structural analysis of the protein sequence plus experimental data, fits reasonably well to the major cleavage sites we observe by limited proteolysis (Figure 8). Nearly all cleavage sites occur near breaks in secondary structure. However, two primary cleavages [defined by fragments produced by a single cleavage (open symbols in Figure 8)], at L90 and L205, occur in the two 33-mer helices proposed in the structure, suggesting that these long helices may have breaks near the observed cleavage sites.

A large number of secondary cleavages occur in the lipid-bound protein. (Secondary cleavage is defined from fragments produced by two cleavages, one of which has previously been identified as a primary site.) These are almost exclusively located in the central region of the protein, from residue 100 to residue 203. Apparently, cleavage in the amino-terminus exposes the central region of the protein to additional cleavage. This result does not intuitively follow from an extended structural model such as depicted in Figure 8 and may suggest the two regions interact. Alternatively, an extended structure may be able to cooperatively transmit a structural change through the lipid milieu to which it is bound. Similar concepts have been discussed in the context of monoclonal antibody reactivity of rLp (Collet et al., 1991; Calabresi et al., 1993; Meng et al., 1993).

Effects of Methionine Oxidation on Lipid-Bound Apo A-I. Oxidation of M112 and M148 causes a dramatic increase in protease susceptibility in the central and carboxy-terminal region of the protein, with a concomitant decrease in susceptibility in the amino-terminal region of the protein. The three major cleavages occurring at E34, L42, and F57 in the reduced protein are not observed at all in proteolysis of the oxidized protein. Oxidation of M112 presumably causes a significant change in the lipid-binding affinity of the protein near M112 which, somehow, causes residues located at least two helices away in the amino terminus to become more tightly bound to lipid. Alternatively, a decrease in lipid-binding near M112 may cause the amino-terminal region of the protein to adopt a more globular protease-resistant structure. In either case, the change in

protease susceptibility of the amino terminus indicates the lipid-bound conformation cooperatively responds to local changes in structure.

Interestingly, while many cleavages are observed in the central region, there is a conspicuous lack of cleavage in the vicinity of M148, even though two cleavages occur nearby in the reduced protein. This region, encompassing residues 135−166, may form an alternative structure as a consequence of M148 oxidation which renders it inaccessible to protease.

Concluding Remarks. While several earlier reports involving limited proteolysis of lipid-bound apo A-I have been published, characterization of select proteolytic fragments in these earlier reports led to different interpretations of the protein's protease susceptibility, alternately suggesting that the amino-terminal (Lins et al., 1992), central (Kunitake et al., 1990; Dalton & Swaney, 1993), or carboxy-terminal (Dalton & Swaney, 1993; Ji & Jonas, 1995) region of apo A-I is particularly susceptible to cleavage in the lipid-bound conformation. The discrepancies may be explained by such factors as the method of rLp preparation, rLp heterogeneity, or type of lipid used [as previously suggested by Ji and Jonas (1995)]. These factors, when responsible, give testimony to the conformational plasticity of apo A-I. On the other hand, it is highly unlikely that the use of different proteases could account for the different results since we and others have shown essentially identical domain susceptibilities to cleavage using multiple proteases with very different sequence specificities. The fact that oxidation of two methionines switches the protease susceptibility from the amino-terminus to the central and carboxy-terminal regions of the protein suggests that the oxidation state of the protein is also an important determinant in defining the conformation of both lipid-free and lipid-bound apo A-I and may provide an additional explanation for the differences seen.

The studies reported here combining limited proteolysis with chemical modification have allowed for the construction of a structural model for lipid-free apo A-I while providing support for some aspects of the generally accepted extended structural model for lipid-bound apo A-I. The oxidation of only two residues caused a structural perturbation that could be observed using limited proteolysis. This suggests that the use of chemically modified protein and single-site mutants in combination with limited proteolysis is an effective approach for probing the structures of lipid-free and lipid-bound apo A-I. Future studies employing this approach will allow for a more complete understanding of apo A-I structure and, consequently, its role in lipid metabolism.

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