methods

A proteolytic method for distinguishing between lipid-free and lipid-bound apolipoprotein A-I

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Abstract **Recent studies indicate that certain lipid-poor forms of apolipoprotein (apo)A-I may be particularly important in promoting cholesterol release from overburdened cells in the periphery. However, a detailed understanding of the physiological relevance of these species has been hampered by the difficulty in measuring them. As part of a search for a rapid assay for these forms of apoA-I, we have observed that the protease enteropeptidase can specifically cleave human lipid-free apoA-I but not its lipid-bound form. Enteropeptidase cleaved lipid-free apoA-I at a single site at amino acid 188, resulting in an N-terminal fragment of 22 kDa. However, apoA-I was not susceptible to enteropeptidase when present in reconstituted high-density lipoprotein (rHDL) particles as small as 6 nm in diameter or in human HDL_3 particles, even at extremely high enzyme-to-protein ratios and extended reaction times. We capitalized on this observation to develop an assay for the measurement of lipid-poor apoA-I in in vitro systems. Densitometry was used to generate a standard curve from sodium dodecyl sulfate polyacrylamide gels to determine theamounts of the N-terminal proteolytic fragment in unknown samples treated with enteropeptidase. This system could accurately quantify apoA-I that had been displaced from rHDL particles and human HDL_3 with purified apoA-II. On the basis of the results, a system of nomenclature is proposed for "lipid-free," "lipid-poor," and "lipid bound" apoA-I. The reported method distinguishes forms of apoA-I by a conformational parameter without previous separation of the species. This simple and inexpensive method will be useful for understanding the characteristics of plasma HDL that are favorable for the dissociation of apoA-I.**—Safi, W., J. N. Maiorano, and W. S. Davidson. **A proteolytic method for distinguishing between lipid-free and lipid-bound apolipoprotein A-I.** ***J. Lipid Res.*** **2001.** 42: **864-872.**

Supplementary key words enteropeptidase • lipid-poor apoA-I • pre-β HDL • lipid-free apoA-I • lipoprotein • cholesterol

It is well established that plasma levels of high-density lipoprotein (HDL) are inversely correlated with the risk of cardiovascular diseases. HDL is thought to accomplish this by removing excess cholesterol from cells in the periphery and returning it to the liver or steroidogenic tissues for catabolism. Although human HDL is generally thought of as an amalgam of lipid-containing particles of varying size and composition, a small fraction of so-called "lipid-poor" forms of apoA-I are detectable in plasma and interstitial fluid (1–3). These subspecies adopt a distinct conformation from the bulk of plasma apoA-I (4, 5) and have been shown to be particularly effective at promoting cholesterol efflux (6). This is despite the fact that estimated levels in normal fasting plasma range from only 2–10% of total plasma apoA-I (6–9).

Recent work has demonstrated that lipid-poor forms of apoA-I likely promote cholesterol efflux through interactions with an ATP-binding cassette protein (ABC-AI) on the cell surface (10). This unidirectional and energy-dependent pathway appears to be a key step for the lipidation of apoA-I during the first step of reverse cholesterol transport and is important for the maintenance of plasma HDL levels in general (11–13). This discovery has focused significant attention on the role of lipid-poor forms of apoA-I. Numerous studies have previously characterized these particles by physically separating them from the bulk of plasma HDL and subjecting them to compositional and structural analyses. These include various preparative gel electrophoresis techniques (2, 6, 14, 15), size exclusion chromatography (16), and isotachophoresis methods (17). In addition, several analytical techniques have been developed for quantitating levels of these species in human plasma or lymph. The most utilized method has been two-dimensional electrophoresis (one dimension in charge, the other in size) (2, 6, 14, 15), but several one-dimensional agarose electrophoresis methods (4, 8) and analytical isotachophoresis methods (18) have also been used. Unfortunately, many of these separation/detection methods have proven too laborious and/or

Abbreviations: a.a., amino acid residue; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; CVD, cardiovascular disease; FC, unesterified (free) cholesterol; HDL, high-density lipoprotein; His-tagged apoA-I, apoA-I containing a 6 amino acid histidine tag sequence; rHDL, reconstituted HDL; PAGGE, polyacrylamide gradient gel electrophoresis; PL, phospholipid; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; STB, standard Tris buffer.

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prone to artifacts inherent to the separation to be useful for convenient analysis of lipid-poor apoA-I in a clinical setting (2).

While searching for new methodologies that distinguish lipid-poor forms of apoA-I without the need for separation, we observed that the protease enteropeptidase could cleave lipid-free apoA-I but does not cleave any form that is bound to lipids. In the present article, we capitalized on this observation to develop a novel assay for measuring levels of lipid-free apoA-I in the presence of lipid-bound apoA-I. The assay is simple, inexpensive, and distinguishes between apoA-I species by a *conformational* parameter rather than a *physicochemical* one. Although the current method is limited to in vitro studies, the principles underlying the method may allow the development of an assay suitable for more complex samples such as human plasma or lymph.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl, 2-oleoyl phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL). The atomic phosphorus standard and free cholesterol were obtained from Sigma (St. Louis, MO). The recombinant form of the bovine enteropeptidase (EC 3.4.21.9) catalytic domain (light chain) was obtained from Novagen (Madison, WI). Enteropeptidase purified from porcine intestine was purchased from Sigma (Cat. no. E 0885). All other reagents were analytical grade.

Methods

Purification of apolipoproteins. Purified human plasma apoA-I and human apoA-II were generous gifts from Dr. Sissel Lund-Katz and Dr. Michael Phillips at the Children's Hospital of Philadelphia (19). The preparations of apoA-I have been demonstrated to contain >90% of the methionine-reduced form of apoA-I (Sissel Lund-Katz, personal communication). Human HDL_3 (1.21 < density > 1.125 g/ml) samples were isolated by well-established ultracentrifugation methods (19). Recombinant human proapoA-I containing the 6 amino acid residue (a.a.) pro segment was overexpressed in *Escherichia coli* using the pET 32 expression system (Novagen) as described previously (20). The exact sequence from the N-terminus was as follows: N-Ala-His-Phe-Trp-Gln-Gln-Asp-etc. After harvesting, the cells were resuspended in 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.0) containing 0.15 M NaCl and 1 mM EDTA (standard Tris buffer, STB). The cells were lysed by probe sonication, and the soluble cell contents were applied to a His-bind column (Novagen) and eluted according to the manufacturer's instructions. The His-tagged proteins were dialyzed into 10 mM ammonium bicarbonate buffer (pH 8.0) and lyophilized until used. All apolipoproteins in this study were solubilized in 6 M guanidine HCl for 2 h and then dialyzed into STB to minimize aggregation of the protein. The site-directed mutagenesis was accomplished using the Quick-Change mutagenesis kit (Stratagene). The sequence of each construct was verified by DNA sequencing. Proteins prepared by this method were >95% pure as visualized by sodium dodecyl sulfate (SDS) electrophoresis stained with Coomassie blue, and the yields were about 5 mg protein per 100 ml of original culture.

Preparation and characterization of the lipid-containing particles. Discoidal rHDL particles with diameters of 78 and 96 Å were prepared via the sodium cholate dialysis technique (20) using POPC and plasma or recombinant apoA-I. The particles were isolated by gel filtration chromatography on a Superdex 200 gel filtration column (Amersham) to remove any unreacted protein and vesicular structures. The phosphorus assay of Sokolof and Rothblat (21) and the Markwell modification of the Lowry protein assay (22) determined the final lipid and protein concentrations after column purification. Particle hydrodynamic diameters were measured by gradient native polyacrylamide electrophoresis (23). The lipid-poor particle with a diameter of 60 Å was generated as reported by Sparks et al. (24) by cosonication of POPC, free cholesterol (FC), and human apoA-I. Briefly, the amounts of starting lipids for a PL/FC/apoA-I molar ratio of 5:10:1 were dried under nitrogen in glass test tubes. STB (800 ml) was added, and the mixture was sonicated for 1 min at 158C. After a 30-min incubation at 378C, the mixture was sonicated again for 5 min. apoA-I (2 mg) was then added and sonicated again for four periods of 1 min, punctuated by cooling on ice. The resulting particles were filtered through a 0.22-mm syringe filter. A Superdex 200 gel filtration column was used to separate the particles from the unreacted lipid. We also attempted to reconstitute complexes with starting ratios of 5:10:1 and 10:15:1 PL/FC/apoA-I (mol/mol), but, unlike Sparks et al. (24), we were unable to recover complexes that were distinguishable from lipid free apoA-I by nondenaturing gel electrophoresis.

Miscellaneous analyses. N-terminal sequencing and matrix-assisted laser desorption ionization (MALDI)-mass spectroscopy were performed on purified mature apoA-I and the purified enteropeptidase cleavage product by the University of Illinois Biotechnology Center. The extent of enteropeptidase cleavage was determined by SDS-PAGE analysis on 12% mini-gels (Bio-Rad), stained with Coomassie blue. The specifics of the reaction conditions are outlined as appropriate in the figure legends. Densitometric quantitation of the band intensity was measured as previously reported (23). Western blot analyses were carried out using an ECL kit (Amersham) performed according to the manufacturer's instructions. The primary antibody was a polyclonal anti-apoA-I (Calbiochem) produced in rabbit.

RESULTS

Initial observations

The observations that formed the foundation of these studies arose from serendipitous results. During attempts to simplify the bacterial expression and purification of human plasma apoA-I, we had modified our previously published method (25) to include the attachment of a 13-kDa histidine tag, followed by an enteropeptidase cleavage site, on the apoA-I N-terminus. In theory, this should allow for rapid purification of the product on a nickel affinity column followed by cleavage of the tag by enteropeptidase. However, during our initial expression experiments, it was apparent that the protease cleaved within the apoA-I sequence in addition to the His-tag sequence. **Figure 1** shows the difference in molecular weight between recombinant apoA-I containing the His-tag (lane 2) with mature apoA-I purified from human plasma (lane 4). When the lipid-free, His-tagged protein was incubated with enteropeptidase, the resulting product was slightly smaller (about 22 kDa) than mature apoA-I (28 kDa, compare lanes 1 and 4), indicating that the peptidase cleaved somewhere within the apoA-I sequence. To confirm this, we

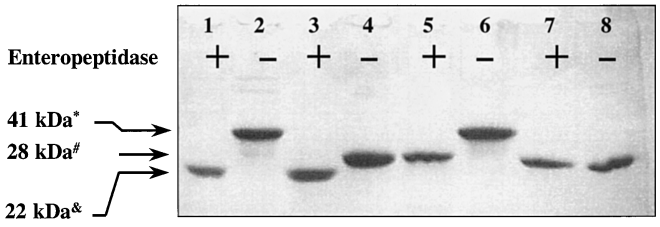

Fig. 1. Enteropeptidase cleaves lipid-free apoA-I within its sequence in addition to cleaving the Histidine tag from apoA-I. The figure shows a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of various apoA-I samples after a 16-h incubation at 37°C with or without enteropeptidase (stained by Coomassie blue). Samples that are indicated as lipid bound were present as rHDL particles made with POPC with diameters of 98 Å (see Table 2). Where indicated by a plus sign, enteropeptidase was present at 0.13 U per μg apoA-I. Lanes 1 and 2: Lipid-free recombinant His-tag apoA-I. Lanes 3 and 4: Lipid free human plasma apoA-I. Lanes 5 and 6: Lipid-bound recombinant His-tag apoA-I. Lanes 7 and 8: Lipid-bound human plasma apoA-I. The molecular weight of each band is shown on the left. The symbols denote the following: (*) Molecular weight (MW) of recombinant apoA-I containing the 13 kDa histidine tag sequence; (#) MW of proapoA-I (and mature apoA-I) in the absence of the histidine tag; (&) MW of the overdigestion product of the enteropeptidase cleavage.

treated mature human plasma apoA-I with enteropeptidase under the same conditions and also observed an identical 22-kDa fragment (compare lanes 3 and 4). These results were surprising because we had previously searched the sequence of apoA-I but were unable to locate a locus that even resembled the known enteropeptidase cleavage site [-Asp_4-Lys↓- (26)]. The observed cleavage could not be attributed to contaminating proteases within the enteropeptidase stock for two reasons. First, both enteropeptidase isolated from porcine intestine and highly purified recombinant bovine enterokinase were equally capable of the cleavage (data not shown). The chance of having the same contaminant in two such differently prepared stocks of enteropeptidase is unlikely. Second, the recombinant enteropeptidase was incapable of cleaving a fluorescent casein derivative in an assay that is specifically designed for detecting contaminating proteases within a sample (EnzChek; Molecular Probes, Eugene, OR).

We hypothesized that a specific region within lipid-free apoA-I could adopt a conformation that appeared to the enteropeptidase as a usable recognition site, despite containing little sequence homology to the known site. Indeed, this phenomenon has been documented previously for enteropeptidase cleavage of cholecystokinin (27), lysozyme (28), and even other proteases used for apoA-I fusion protein constructs (29). To test this, we determined the ability of enteropeptidase to cleave lipid-bound apoA-I present in reconstituted HDL (rHDL) particles. We reasoned that the conformation of the enteropeptidase recognition site might change in response to lipid binding because multiple regions within lipid-free apoA-I are known to change conformation in rHDL (reviewed in 30). The His-tagged apoA-I was perfectly capable of forming rHDL complexes with POPC, as was the human plasma apoA-I. The particle compositions were found to be similar, but the His-tagged apoA-I rHDL exhibited a slightly increased diameter as a result of the extra protein sequence contained within the His-tag leader. It is clear from Fig. 1 that the His-tag was still efficiently cleaved from apoA-I in rHDL (compare lanes 6 and 5). However, in contrast to the case of the lipid-free protein, the resulting apoA-I fragment from the rHDL/enteropeptidase incubation was of an identical size to full-length human plasma apoA-I (compare lanes 5 and 4). Enteropeptidase was also unable to cleave human plasma apoA-I in rHDL (lanes 7 and 8). These results indicated that there was an enteropeptidase-sensitive site within the sequence of lipid-free apoA-I and that lipid binding changed the conformation such that the recognition site was completely masked.

Characterization of the cleavage reaction

It is clear from Fig. 1 that enteropeptidase cleaves lipid-free apoA-I to form a major fragment of about 22 kDa. This could result from a cleavage site that is about 5–6 kDa into the sequence either from the N-terminus or from the C-terminus. To distinguish between these, we purified the enteropeptidase cleavage fragment by gel filtration chromatography and sequenced the N-terminus by Edman degradation analysis. **Table 1** shows that the N-terminal sequence of the major enteropeptidase fragment corresponded exactly to the N-terminal sequence of intact human plasma apoA-I, indicating that the enteropeptidase cleavage site was located in the C-terminal end of the protein. MALDI mass spectroscopy analysis on the fragment determined that its molecular weight (MW) was 21,912 Da. This corresponds to an N-terminal apoA-I fragment encompassing amino acids 1–188 (theoretical MW of 21,914 Da). To confirm the calculated cleavage site, a mutant form of apoA-I was generated in which Arg 188 was mutated to a Glu. Enteropeptidase was unable to cleave this mutant (data not shown). Table 1 shows the five amino acids preceding Arg 188 in the apoA-I sequence and compares this stretch to the known enteropeptidase recognition site in trypsinogen (26). The sites are quite different in terms of sequence and hydrophobicity/charge characteristics, but two general similarities can be pointed out: *1*) The cleavage site in both sequences occurs after a positively charged residue, and *2*) both sequences have a run of four amino acids preceding the positively charged residue that are relatively low in side-chain volume.

TABLE 1. Analysis of the enteropeptidase cleavage fragment of lipid-free human plasma apoA-I

N-terminal sequencing	
Human plasma apoA-I	N-Asp-Glu-Pro-Pro-Thr-Gln . . .
Major enteropeptidase cleavage fragment	N-Asp-Glu-Pro-Pro-Thr
Mass spectroscopy	
Human plasma apoA-I	28,016 Da
Major enteropeptidase cleavage fragment	21,912 Da
Calculated cleavage site in apoA-I	N- . . . -Asn-Gly-Gly-Ala-Arg^{188} ↓
Consensus enteropeptidase site (26)	N- . . . -Asp-Asp-Asp-Asp-Lys ↓

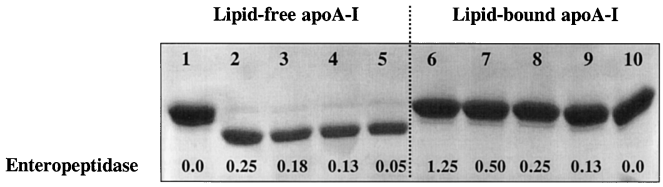

Fig. 2. Concentration dependence of enteropeptidase cleavage of lipid-free apoA-I. Lipid-free apoA-I (4 μg) was incubated for 90 min at 37°C with the indicated amount of enteropeptidase (in units of activity per μg apoA-I) in a volume of 10-μl STB. The samples were run on a 12% sodium dodectl sulfate (SDS) gel stained with Coomassie blue. Lane 1: Lipid-free plasma apoA-I incubated without enteropeptidase. Lanes 2–5: Lipid-free plasma apoA-I. Lanes 6–10: Lipid-bound plasma apoA-I in the form of 98 Å rHDL particles (see Table 2).

To characterize the cleavage reaction, we ran a time course for the cleavage of lipid-free apoA-I at 0.125 U enteropeptidase/μg apoA-I at 37°C. Under these conditions, the cleavage was fully complete by 60 min (data not shown). On the basis of these data, we chose to use a consistent incubation time of 90 min for all further studies. We next varied the amount of enteropeptidase to determine the optimal enzyme-protein ratio for future assays. **Figure 2** shows that an enzyme-protein ratio as low as 0.05 U/μg apoA-I was sufficient for complete cleavage of lipid-free apoA-I for 90 min at 37°C. In contrast, we observed no cleavage of lipid-bound apoA-I even with excessively high amounts of enteropeptidase (lane 6). This was also the case when the reaction was allowed to proceed for longer than 24 h at 37°C (data not shown). These results underscore the sensitivity of lipid-free apoA-I to enteropeptidase cleavage and indicate that the observations made in Fig. 1 were not due simply to using suboptimal conditions for cleavage of lipid-bound apoA-I. From these results, we selected the following reaction conditions for subsequent experiments: 0.13 U enteropeptidase/μg apoA-I incubated for 90 min at 37°C in STB.

The lipid-bound form of apoA-I studied in Fig. 1 and 2 was a discoidal rHDL particle that is ~98 Å in diameter and contained 2 molecules of apoA-I (see **Table 2**). To determine the lower limit of lipid content as well as the particle shape characteristics that were required to mask the enteropeptidase cleavage site, other lipid-containing complexes were tested, which varied in size and conformational characteristics. The first was a smaller rHDL, about 78 Å in diameter, which also contained two molecules of apoA-I per complex. The second was a much smaller complex formed by sonication techniques originally reported by Sparks et al. (24). This is one of the first techniques that has proven capable of generating stable rHDL particles that contain less than ~30 molecules of phospholipid per molecule of apoA-I. These complexes exhibited diameters of about 60 Å with α-helical contents that are comparable to lipid-free apoA-I but that express significantly different monoclonal antibody epitope patterns. In our hands, the final molar composition of these particles was 7:1:1 (PC/FC/apoA-I), whereas Sparks et al. reported 9:4:1. Nevertheless, the particles eluted in a distinct peak on the gel filtration column and exhibited a band on native PAGE that was clearly distinguished from that of lipid-free apoA-I (data not shown). The third particle was spherical human HDL_3 isolated by density ultracentrifugation in the range 1.125–1.21 g/ml. Table 2 shows that none of these lipid-containing particles was significantly cleaved by enteropeptidase, despite large differences in apoA-I conformation and lipid composition. Therefore, the lower limit of lipidation required to mask the enteropeptidase recognition site is somewhere below 7–8 molecules of lipid per molecule of apoA-I.

An assay for lipid-free apoA-I

To determine if the specificity of enteropeptidase for lipid-free apoA-I could be used in an assay to distinguish between lipid-free and lipid-bound apoA-I, we used a simple densitometric method for quantitating the enteropeptidase cleavage of intact apoA-I. The approach was to generate a set of standards containing known amounts of both fully intact apoA-I and the proteolytic fragment. These were run on SDS-PAGE gels along with unknown

TABLE 2. Summary of previously published physical characteristics of various apoA-I–containing particles and their susceptibility to enteropeptidase cleavage compared with pure, lipid-free apoA-I

Particle	Morphology	Composition PC/FC/apoA-I	Hydrodynamic Diameter	α-helix	Reference	Enteropeptidase Sensitivity[a]
		mol/mol/mol	*Å*	%		%
Lipid-free apoA-I	nd[b]	0:0:1	70	49–55	(24, 25)	100
Large disc	Discoidal	170:0:2	98	70–75	(20, 25)	Not detectable
Small disc	Discoidal	114:0:2	79	63–67	(20, 25)	Not detectable
Human HDL_3[c]	Spherical	51:13:3	~78	—	(47)	0–4, varying between preps[c]
Lp1A-I complex[d]	nd	9:4:1	60	52	(24)	Not detectable

[a] Enteropeptidase cleavage results are presented as the percentage of initial intact apoA-I that had been converted to the cleavage fragment under the standard assay conditions listed in the text.

[b] nd Signifies that the particle morphology is not determinable by electron microscopy.

[c] HDL_3, having been isolated from human plasma, obviously has numerous additional lipid and protein components from those listed in this table [see (47) for more information]. Some HDL_3 preparations exhibited slight levels of enteropeptidase sensitive apoA-I. We believe that this resulted from a small amount of lipid-free protein shed from the particles during storage.

[d] In our hands, the Lp1A-I complex differed slightly from that reported by Sparks et al. (24) in that it had a composition of 7 ± 2:1:1 (mole PC–mole FC–mole apoA-I).

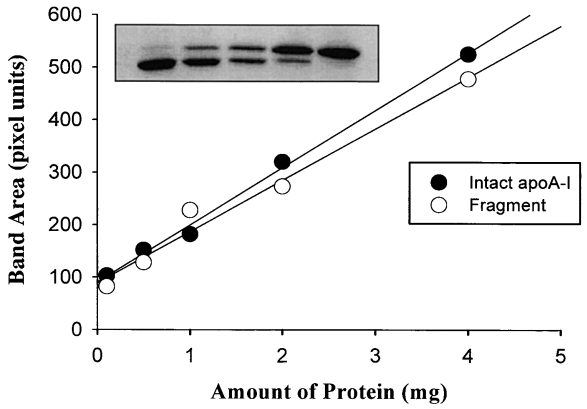

Fig. 3. Densitometric scans of SDS gel standards generated from intact apoA-I and the purified enteropeptidase cleavage fragment. A set of five standard solutions was prepared by combining intact apoA-I and the purified (by gel filtration) fragment in various ratios. Each protein was quantitated by Lowry assay prior to mixing. For example, standard 1 contained 4.0 μg per lane of intact apoA-I and 0.1 μg per lane of the purified cleavage fragment; standard 2 contained 2.0 μg per lane of intact apoA-I and 0.5 μg per lane of the purified cleavage fragment, and so on. Both standards ranged from 4 μg per lane to 0.1 μg per lane across all five standards. The insert shows the Coomassie blue stained gel that was scanned to get the data shown in the graph.

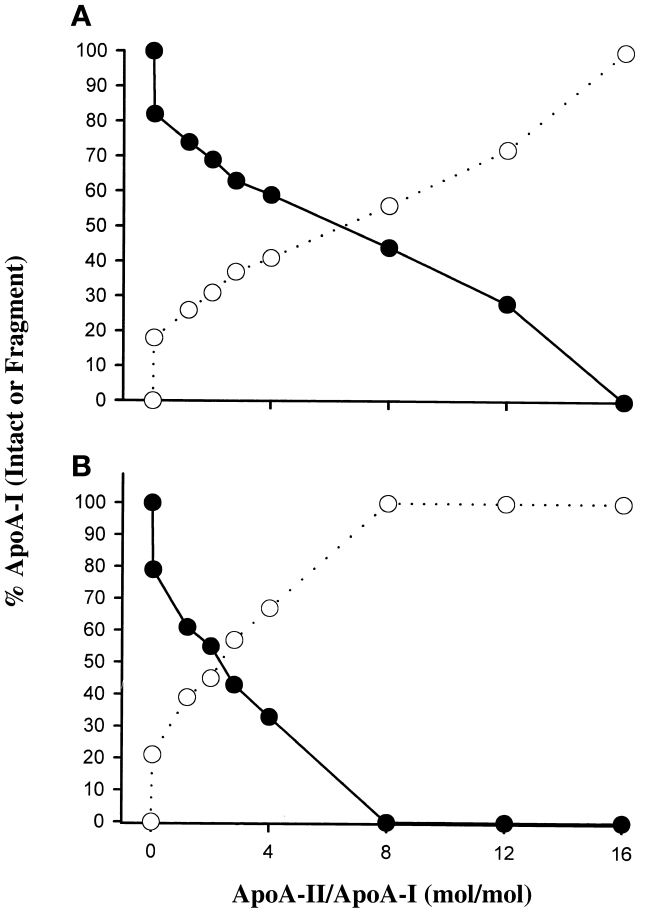

Fig. 4. The susceptibility of lipid-bound apoA-I to enteropeptidase cleavage after displacement by apoA-II.rHDL (98 Å) (A) and human HDL_3 (B) were incubated with human apoA-II at the indicated ratio at 25°C for 16 h. Then the samples were treated with 0.13 U of enteropeptidase per μg of original apoA-I content for 90 min at 37°C. Each sample was analyzed by SDS–PAGE, and the amount of intact apoA-I versus fragment was quantitated by densitometric analysis using a standard curve generated from the panel of five standards run on the same gel. Solid circles represent intact apoA-I at each concentration of apoA-II, whereas the open circles represent the appearance of the enteropeptidase cleavage fragment.

samples that had previously been treated with enteropeptidase. Laser densitometry was then used to compare the intensity of the unknown sample with a standard curve generated from the standards run on the same gel. **Figure 3** shows a typical standard curve run with the set of standards. It is clear that the band intensity of both the intact protein and the proteolytic fragment respond linearly with protein concentration and with a similar slope when visualized by Coomassie staining. We have also verified a similar response if the proteins are visualized by Western blot analysis using a polyclonal antibody to apoA-I.

We next tested the ability of the assay to detect lipid-poor apoA-I that had been liberated from human HDL_3 and from a reconstituted HDL disc (98 Å in diameter). Each lipoprotein was incubated with increasing amounts of purified human apoA-II for 16 h at room temperature. It is well known that apoA-II has a higher lipid affinity than apoA-I and can displace it from the surface of lipoproteins (31, 32) under these conditions. After the incubation with apoA-II, the sample was treated with enteropeptidase under the standard assay conditions, and the relative proportions of intact apoA-I and proteolytic fragment were quantitated by the densitometric analysis. **Figure 4** confirms the result in the previous experiments showing that lipid-bound apoA-I was completely insensitive to enteropeptidase in both types of lipoproteins in the absence of apoA-II. However, as apoA-II was added, a fraction of the apoA-I originally associated with both lipoproteins became sensitive to enteropeptidase cleavage. This sensitive component increased with increasing amounts of apoA-II competitor to a point where essentially all of the apoA-I was enteropeptidase sensitive. This occurred at a much lower apoA-II concentration for the HDL_3 sample than for the rHDL sample. The mole ratio of apoA-II to apoA-I at which half of the originally present apoA-I had become enteropeptidase sensitive was 7.2 for the rHDL particle and 3.8 for human total HDL. The simplest explanation for these data is that apoA-II displaced apoA-I from the surface of both types of particles, allowing it to be detected by the enteropeptidase sensitivity assay. However, it was also possible that the addition of apoA-II to the complexes simply caused a conformational change within apoA-I, allowing it to be detected by enteropeptidase even if still bound to the particle surface. Indeed, conformational effects on apoA-I in response to apoA-II addition have been reported by Durbin et al. (33). To rule out this possibility, we checked whether apoA-I had in fact been displaced from the surface of the lipoprotein. We performed an apoA-II competition experiment on human HDL_3 particle as shown in Fig. 4 under conditions in

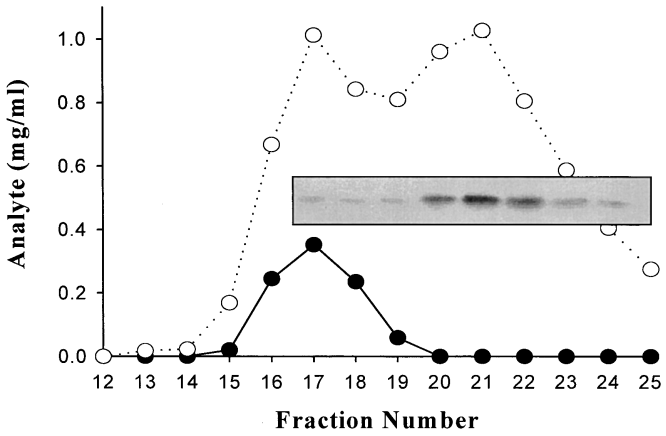

Fig. 5. Verification of displaced apoA-I from HDL_3 by gel filtration chromatography. Human HDL_3 that had been incubated with apoA-II at a 16:1 (mole apoA-II–mole apoA-I) ratio under the same conditions as in Fig. 4 was analyzed on a Superdex 200 gel filtration column. The filled circles indicate phospholipid content in each fraction and the open circles show the total protein content measured by Lowry. The insert shows a Western blot for each fraction using a polyclonal antibody for human apoA-I.

which all of the apoA-I was found to be enteropeptidase sensitive after the incubation. Instead of performing an enteropeptidase assay, the sample was analyzed by gel filtration chromatography on the Superdex gel filtration column. **Figure 5** shows the elution profile of the total protein as well as the particle phospholipid. The peak on the phospholipid profile corresponded to the typical elution position of control HDL_3. The protein profile, on the other hand, showed two peaks. The first corresponded to the phospholipid peak, and the second exhibited a longer column retention time, indicating that the protein in this peak was no longer associated with the HDL phospholipid. The Western blot analysis on the inset shows the distribution of human apoA-I in the fractions. It is clear that the second protein peak was composed mostly of apoA-I that had been displaced from the HDL phospholipid. This peak appeared to be extremely phospholipid poor because no phospholipid was detectable by our assay within these fractions. SDS-PAGE analysis demonstrated that the HDL peak contained predominantly apoA-II and only trace amounts of apoA-I. This was in contrast to the untreated control HDL sample that contained predominantly apoA-I and apoA-II with trace levels of other apolipoproteins. This experiment confirmed that apoA-II displaced apoA-I from the lipoprotein, indicating it was the lipid-free form of the protein that was recognized by enteropeptidase in the experiments shown in Fig. 4.

The experiments described above were performed in clean in vitro systems. To determine if the enteropeptidase assay would be useful for detecting lipid-free forms of apoA-I in plasma, we performed several experiments to measure the activity of enteropeptidase in human plasma. Freshly isolated plasma was doped with His-tag recombinant apoA-I to a concentration of 1 mg/ml. An aliquot of doped plasma was then treated with various concentrations of enteropeptidase under the standard conditions of the assay. Even at high ratios of enteropeptidase to apoA-I, we were unable to detect significant levels of enteropeptidase cleavage of the His-tag from apoA-I by Western analysis (data not shown). This lack of cleavage was not due to absorption of the apoA-I to lipoproteins in the plasma because lipid binding does not impede the cleavage of the His-tag from apoA-I (see Fig. 1). The most likely explanation is that the proteolytic activity of the enzyme is compromised in plasma. This result is perhaps not unexpected given the high levels of proteolysis inhibitors in plasma and demonstrates that the current form of the assay is restricted to well-defined in vitro systems.

DISCUSSION

Taken together, these experiments indicate that enteropeptidase can recognize a particular conformation of the sequence near a.a. 188 within lipid-free apoA-I but is completely incapable of recognizing the same sequence in forms of apoA-I that contain even 7–8 molecules of lipid per molecule of apoA-I. This observation led us to develop a simple in vitro assay for measuring the generation of lipid-free apoA-I from lipid-containing particles. The assay is inexpensive, easy to perform, and requires equipment found in most laboratories.

The benefits of the enteropeptidase assay versus existing approaches

As summarized above, several preparative and analytical methods have been developed for distinguishing between lipid-bound and lipid-poor apoA-I. Each of these strategies has various advantages and disadvantages, but all rely on some physicochemical parameter (difference in charge or size, etc.) as a basis for physically separating the lipid-poor species from the bulk of plasma lipoproteins. Thus, these highly unstable species must first endure a separation procedure before they are quantitated. As a consequence, many of these methods are technically complicated and/or prone to artifacts inherent to the separation. We suggest that the assays that will prove most useful in the future will depend on a *conformational* method of distinction without the need to separate the particles before the analysis. The obvious candidate for this would be a conformationally specific antibody capable of differentiating between the species in an in situ immunoassay. Several laboratories have reported monoclonal antibodies that have various degrees of preference for lipid-free or lipid-bound forms of apoA-I (5, 36–38), but we are unaware of any such antibodies that are absolutely specific for one form of apoA-I and completely lack the ability to recognize the other.

In the absence of an antibody, the specificity of proteases offers an attractive alternative for the conformational differentiation of various forms of apoA-I. Several limited proteolysis studies have been used to probe the exposed sites in lipid-free apoA-I versus lipid-bound apoA-I (39–42), with the most complete study performed by Roberts et al. (43). Whereas these proteases have provided a

wealth of information on the structure of apoA-I, they do not lend themselves well to an assay designed to distinguish lipid-free and lipid-bound apoA-I. Chymotrypsin (42, 43), V8 protease (41, 43), trypsin (39–42), elastase (41, 42), and subtillisin (42) all rapidly digest lipid-free apoA-I into a complex set of peptide products. However, they also are capable of digesting lipid-bound apoA-I into a separate complex set of peptides, albeit at a significantly slower rate. Enteropeptidase, on the other hand, rapidly cleaves lipid-free apoA-I at only a single, easily visualized site. In addition, it cannot cleave lipid-bound or even lipid-poor forms of apoA-I at high levels of enzyme and/or extended incubation times. These unique cleavage characteristics of enteropeptidase for apoA-I make it ideal for an assay.

Implications for the assay of lipid-free apoA-I

At this point, we have not yet successfully adapted the enteropeptidase assay for detecting lipid-free apoA-I in complex samples such as plasma or lymph. However, if enteropeptidase itself cannot be used, it is conceivable that an antibody can be generated to the exposed region around residue 188 in lipid-free apoA-I. If such an antibody were sufficiently specific for lipid-free apoA-I (and/or certain lipid-poor forms), it would allow for a convenient enzyme-linked immunoassay capable of analyzing plasma samples. Such an in vivo assay would be useful for answering such important questions as whether or not truly lipid-free apoA-I can occur in plasma and if so, whether plasma levels of these species track in a meaningful way with the propensity of a given individual for cardiovascular disease. Therefore, ways to translate the underlying principle of conformational detection to such an in vivo assay are under active investigation in our laboratory.

Although limited to well-defined in vitro systems, the enteropeptidase sensitivity assay reported here will be extremely useful for basic studies designed to determine the mechanisms behind the generation of lipid-free apoA-I from lipoproteins. There is strong evidence that lipid-free and/or lipid-poor apoA-I may be interconverted between various lipid-bound states through the action of LCAT and CETP (34, 44, 45) and that the lipid composition of intact lipoproteins may modulate the generation of these species (46). This assay lends itself well to experiments in which the lipid content of various particles can be manipulated and the effects on apoA-I liberation measured. As an example, Fig. 4 and 5 show that the assay is capable of analyzing the displacement of apoA-I from both reconstituted and native HDL particles. It is interesting that less apoA-II is required to fully displace apoA-I from a spherical HDL_3 particle than from a discoidal rHDL complex. This may indicate that apoA-I is less stable on HDL particles of different morphology or lipid composition. The in vitro assay described here will be useful for studying such processes in defined systems.

In closing, we propose a convention for defining the terms, "lipid-free," "lipid-poor," and "lipid-bound" apoA-I. In practice, the former two terms are frequently used interchangeably or have been called other names including "pre-β" and "free apoA-I like," among others. To date, there is no widely accepted nomenclature that relates what these terms actually mean at the molecular level. To address this problem, we propose that *Lipid-free apoA-I* be defined as apoA-I that contains *no* measurable associated lipid and exhibits an α-helical content indistinguishable from apoA-I delipidated by organic solvents. Functionally, we would suggest that lipid-free apoA-I is any form of apoA-I that is cleavable by enteropeptidase. *Lipid-poor apoA-I* is defined as any particle that contains only 1 molecule of apoA-I and greater than 1 but less than 30 molecules of lipid (phospholipid/cholesterol etc.) per molecule of apoA-I. These include the particles studied by Castro and Fielding (6), Ishida, Albee, and Paigen (34), and others (15). For the most part, these particles have been difficult to isolate from plasma in a stable form. *Lipid-bound apoA-I* refers to stable particles that can be isolated and studied, including traditional plasma HDL and reconstituted forms that contain 2 or more molecules of apoA-I per particle and >30 molecules of lipid per molecule of apoA-I. Generally, these particles are easily isolated by density ultracentrifugation. Neither lipid-poor nor lipid-bound apoA-I is cleavable by enteropeptidase. It should be noted that the term "pre-β HDL" refers only to particle charge characteristics and, in certain instances, can describe all three types of particles listed above (35). Because the discovery of the ABC-AI pathway will undoubtedly increase interest in these particles, we believe that this convention will be useful for clarifying and standardizing the meanings of these terms for future work in the field.

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REFERENCES

1. Asztalos, B. F., C. H. Sloop, L. Wong, and P. S. Roheim. 1993. Comparison of apo A-I-containing subpopulations of dog plasma and prenodal peripheral lymph: evidence for alteration in subpopulations in the interstitial space. *Biochim. Biophys. Acta.* **1169:** 301–304.
2. Asztalos, B. F., and P. S. Roheim. 1995. Presence and formation of 'free apolipoprotein A-I-like' particles in human plasma. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1419–1423.
3. Lefevre, M., C. H. Sloop, and P. S. Roheim. 1988. Characterization of dog prenodal peripheral lymph lipoproteins. Evidence for the peripheral formation of lipoprotein-unassociated apoA-I with slow pre-beta electrophoretic mobility. *J. Lipid Res.* **29:** 1139–1148.
4. Kunitake, S. T., K. J. La Sala, and J. P. Kane. 1985. Apolipoprotein A-I-containing lipoproteins with pre-beta electrophoretic mobility. *J. Lipid Res.* **26:** 549–555.
5. Fielding, P. E., M. Kawano, A. L. Catapano, A. Zoppo, S. Marcovina, and C. J. Fielding. 1994. Unique epitope of apolipoprotein A-I expressed in pre-beta-1 high-density lipoprotein and its role in the catalyzed efflux of cellular cholesterol. *Biochemistry.* **33:** 6981–6985.

6. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. *Biochemistry.* **27:** 25–29.
7. Asztalos, B., W. Zhang, P. S. Roheim, and L. Wong. 1997. Role of free apolipoprotein A-I in cholesterol efflux. Formation of pre-alpha-migrating high-density lipoprotein particles. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1630–1636.
8. O'Kane, M. J., G. B. Wisdom, J. McEneny, N. V. McFerran, and E. R. Trimble. 1992. Pre-beta high-density lipoprotein determined by immunoblotting with chemiluminescent detection. *Clin. Chem.* **38:** 2273–2277.
9. O'Connor, P. M., B. R. Zysow, S. A. Schoenhaus, B. Y. Ishida, S. T. Kunitake, J. M. Naya-Vigne, P. N. Duchateau, R. F. Redberg, S. J. Spencer, S. Mark, M. Mazur, D. C. Heilbron, R. B. Jaffe, M. J. Malloy, and J. P. Kane. 1998. Prebeta-1 HDL in plasma of normolipidemic individuals: influences of plasma lipoproteins, age, and gender. *J. Lipid Res.* **39:** 670–678.
10. Oram, J. F., and A. M. Vaughan. 2000. ABCA1-mediated transport of cellular cholesterol and phospholipids to HDL apolipoproteins. *Curr. Opin. Lipidol.* **11:** 253–260.
11. Rust, S., M. Rosier, H. Funke, J. Real, Z. Amoura, J. C. Piette, J. F. Deleuze, H. B. Brewer, N. Duverger, P. Denefle, and G. Assmann. 1999. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat. Genet.* **22:** 352–355.
12. Bodzioch, M., E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, W. E. Kaminski, H. W. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K. J. Lackner, and G. Schmitz. 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat. Genet.* **22:** 347–351.
13. Brooks-Wilson, A., M. Marcil, S.M. Clee, L. H. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. A. Collins, H. O. Molhuizen, O. Loubser, B. F. Ouelette, K. Fichter, K. J. Ashbourne-Excoffon, C. W. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J. J. Kastelein, and M. R. Hayden. 1999. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat. Genet.* **22:** 336–345.
14. Sasahara, T., T. Yamashita, D. Sviridov, N. Fidge, and P. Nestel. 1997. Altered properties of high density lipoprotein subfractions in obese subjects. *J. Lipid Res.* **38:** 600–611.
15. Jaspard, B., X. Collet, R. Barbaras, J. Manent, C. Vieu, J. Parinaud, H. Chap, and B. Perret. 1996. Biochemical characterization of pre-beta 1 high-density lipoprotein from human ovarian follicular fluid: evidence for the presence of a lipid core. *Biochemistry.* **35:** 1352–1357.
16. Nanjee, M. N., and E. A. Brinton. 2000. Very small apolipoprotein A-I-containing particles from human plasma: isolation and quantification by high-performance size-exclusion chromatography. *Clin. Chem.* **46:** 207–223.
17. Bottcher, A., J. Schlosser, F. Kronenberg, H. Dieplinger, G. Knipping, K. J. Lackner, and G. Schmitz. 2000. Preparative free-solution isotachophoresis for separation of human plasma lipoproteins: apolipoprotein and lipid composition of HDL subfractions. *J. Lipid Res.* **41:** 905–915.
18. Schmitz, G., C. Mollers, and V. Richter. 1997. Analytical capillary isotachophoresis of human serum lipoproteins. *Electrophoresis.* **18:** 1807–1813.
19. Lund-Katz, S., and M. C. Phillips. 1986. Packing of cholesterol molecules in human low-density lipoprotein. *Biochemistry.* **25:** 1562–1568.
20. Maiorano, J. N., and W. S. Davidson. 2000. The orientation of helix 4 in apolipoprotein A-I-containing reconstituted high density lipoproteins. *J. Biol. Chem.* **275:** 17374–17380.
21. Sokoloff, L., and G. H. Rothblat. 1974. Sterol to phospholipid molar ratios of L cells with qualitative and quantitative variations of cellular sterol. *Proc. Soc. Exp. Biol. Med.* **146:** 1166–1172.
22. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
23. Davidson, W. S., W. V. Rodrigueza, S. Lund-Katz, W. J. Johnson, G. H. Rothblat, and M. C. Phillips. 1995. Effects of acceptor particle size on the efflux of cellular free cholesterol. *J. Biol. Chem.* **270:** 17106–17113.
24. Sparks, D. L., P. G. Frank, S. Braschi, T. A. Neville, and Y. L. Marcel. 1999. Effect of apolipoprotein A-I lipidation on the formation and function of pre-beta and alpha-migrating LpA-I particles. *Biochemistry.* **38:** 1727–1735.
25. McGuire, K. A., W. S. Davidson, and A. Jonas. 1996. High yield overexpression and characterization of human recombinant proapolipoprotein A-I. *J. Lipid Res.* **37:** 1519–1528.
26. Matsushima, M., M. Ichinose, N. Yahagi, N. Kakei, S. Tsukada, K. Miki, K. Kurokawa, K. Tashiro, K. Shiokawa, and K. Shinomiya. 1994. Structural characterization of porcine enteropeptidase. *J. Biol. Chem.* **269:** 19976–19982.
27. Mikhailova, A. G., and L. D. Rumsh. 1999. Autolysis of bovine enteropeptidase heavy chain: evidence of fragment 118–465 involvement in trypsinogen activation. *FEBS Lett.* **442:** 226–230.
28. Light, A., H. S. Savithri, and J. J. Liepnieks. 1980. Specificity of bovine enterokinase toward protein substrates. *Anal. Biochem.* **106:** 199–206.
29. Rogers, D. P., L. M. Roberts, J. Lebowitz, J. A. Engler, and C. G. Brouillette. 1998. Structural analysis of apolipoprotein A-I: effects of amino- and carboxy-terminal deletions on the lipid-free structure. *Biochemistry.* **37:** 945–955.
30. Brouillette, C. G., and G. M. Anantharamaiah. 1995. Structural models of human apolipoprotein A-I. *Biochim. Biophys. Acta.* **1256:** 103–129.
31. Edelstein, C., M. Halari, and A. M. Scanu. 1982. On the mechanism of the displacement of apolipoprotein A-I by apolipoprotein A-II from the high density lipoprotein surface. Effect of concentration and molecular forms of apolipoprotein A-II. *J. Biol. Chem.* **257:** 7189–7195.
32. Labeur, C., G. Lambert, T. Van Cauteren, N. Duverger, B. Vanloo, J. Chambaz, J. Vandekerckhove, G. Castro, and M. Rosseneu. 1998. Displacement of apo A-I from HDL by apo A-II or its C-terminal helix promotes the formation of pre-beta1 migrating particles and decreases LCAT activation. *Atherosclerosis.* **139:** 351–362.
33. Durbin, D. M., and A. Jonas. 1997. The effect of apolipoprotein A-II on the structure and function of apolipoprotein A-I in a homogeneous reconstituted high density lipoprotein particle. *J. Biol. Chem.* **272:** 31333–31339.
34. Ishida, B. Y., D. Albee, and B. Paigen. 1990. Interconversion of pre-beta-migrating lipoproteins containing apolipoprotein A-I and HDL. *J. Lipid Res.* **31:** 227–236.
35. Davidson, W. S., D. L. Sparks, S. Lund-Katz, and M. C. Phillips. 1994. The molecular basis for the difference in charge between pre-beta- and alpha-migrating high density lipoproteins. *J. Biol. Chem.* **269:** 8959–8965.
36. Collet, X., B. Perret, G. Simard, E. Raffai, and Y. L. Marcel. 1991. Differential effects of lecithin and cholesterol on the immunoreactivity and conformation of apolipoprotein A-I in high density lipoproteins. *J. Biol. Chem.* **266:** 9145–9152.
37. Marcel, Y. L., P. R. Provost, H. Koa, E. Raffai, N. V. Dac, J. C. Fruchart, and E. Rassart. 1991. The epitopes of apolipoprotein A-I define distinct structural domains including a mobile middle region. *J. Biol. Chem.* **266:** 3644–3653.
38. Curtiss, L. K., and R. S. Smith. 1988. Localization of two epitopes of apolipoprotein A-I that are exposed on human high density lipoproteins using monoclonal antibodies and synthetic peptides. *J. Biol. Chem.* **263:** 13779–13785.
39. Lins, L., S. Piron, K. Conrath, B. Vanloo, R. Brasseur, M. Rosseneu, J. Baert, and J. M. Ruysschaert. 1993. Enzymatic hydrolysis of reconstituted dimyristoylphosphatidylcholine-apo A-I complexes. *Biochim. Biophys. Acta.* **1151:** 137–142.
40. Kunitake, S. T., G. C. Chen, S. F. Kung, J. W. Schilling, D. A. Hardman, and J. P. Kane. 1990. Pre-beta high density lipoprotein. Unique disposition of apolipoprotein A-I increases susceptibility to proteolysis. *Arteriosclerosis.* **10:** 25–30.
41. Dalton, M. B., and J. B. Swaney. 1993. Structural and functional domains of apolipoprotein A-I within high density lipoproteins. *J. Biol. Chem.* **268:** 19274–19283.
42. Ji, Y., and A. Jonas. 1995. Properties of an N-terminal proteolytic fragment of apolipoprotein AI in solution and in reconstituted high density lipoproteins. *J. Biol. Chem.* **270:** 11290–11297.
43. Roberts, L. M., M. J. Ray, T. W. Shih, E. Hayden, M. M. Reader, and

C. G. Brouillette. 1997. Structural analysis of apolipoprotein A-I: limited proteolysis of methionine-reduced and -oxidized lipid-free and lipid-bound human apo A-I. *Biochemistry.* **36:** 7615–7624.
44. Kunitake, S. T., C. M. Mendel, and L. K. Hennessy. 1992. Interconversion between apolipoprotein A-I-containing lipoproteins of pre-beta and alpha electrophoretic mobilities. *J. Lipid Res.* **33:** 1807–1816.
45. Liang, H. Q., K. A. Rye, and P. J. Barter. 1995. Cycling of apolipoprotein A-I between lipid-associated and lipid- free pools. *Biochim. Biophys. Acta.* **1257:** 31–37.
46. Calabresi, L., A. Lucchini, G. Vecchio, C. R. Sirtori, and G. Franceschini. 1996. Human apolipoprotein A-II inhibits the formation of pre-beta high density lipoproteins. *Biochim. Biophys. Acta.* **1304:** 32–42.
47. Shen, B. W., A. M. Scanu, and F. J. Kezdy. 1977. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA.* **74:** 837–841.