Molecular Characterization of Native and Recombinant Apolipoprotein A-I_{Milano} Dimer

THE INTRODUCTION OF AN INTERCHAIN DISULFIDE BRIDGE REMARKABLY ALTERS THE PHYSICOCHEMICAL PROPERTIES OF APOLIPOPROTEIN A-I*

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The disulfide-linked dimer of apolipoprotein A-I_{Milano} (A-I_{Milano}/A-I_{Milano}), a natural Arg^{113} → Cys variant of apoA-I, was purified from carriers' plasma and produced in Escherichia coli. The recombinant A-I_{Milano}/A-I_{Milano} is identical to native A-I_{Milano}/A-I_{Milano}, by mass spectrometry, SDS-polyacrylamide gel electrophoresis, and isoelectric focusing. Lipid-free A-I_{Milano}/A-I_{Milano} undergoes concentration-dependent self-association similar to apoA-I, but at all concentrations apoA-I_{Milano} is more self-associated than A-I_{Milano}/A-I_{Milano}. Far-ultraviolet CD spectra of A-I_{Milano}/A-I_{Milano} reveal a highly α-helical structure predicted to be ~65% in the lipid-free and ~78% in the lipid-associated states, versus 43 and 78% for apoA-I. A significant loss of α-helix occurs below pH 3.5 and above pH 10 in both apoA-I and A-I_{Milano}/A-I_{Milano}; A-I_{Milano}/A-I_{Milano} constantly shows a higher α-helical content than apoA-I over the entire pH range (1.7–12.8), suggesting that hydrophobic forces stabilize the interaction between the two A-I_{Milano} chains. Indeed, and differently from apoA-I, the α-helical content of A-I_{Milano}/A-I_{Milano} is minimally affected by solvent ionic strength. The aromatic side chains in both lipid-free and lipid-bound A-I_{Milano}/A-I_{Milano} are immobilized in a more asymmetric and hydrophobic environment than in lipid-free apoA-I, the conformation of A-I_{Milano}/A-I_{Milano} being instead similar to that achieved by apoA-I following interaction with lipids. The present findings prove that A-I_{Milano}/A-I_{Milano} is structurally identical to the native protein; the conformation of A-I_{Milano}/A-I_{Milano} is remarkably different from that of apoA-I, thus possibly explaining some of the peculiar functional properties of the apoA-I_{Milano} dimer.

High density lipoproteins (HDL) and its major protein component, apolipoprotein A-I (apoA-I), play crucial roles in cholesterol homeostasis, primarily acting as a vehicle of cholesterol in the "reverse cholesterol transport," the process by which excess cholesterol is removed from peripheral tissues and transported to the liver for final elimination.

ApoA-I actively modulates the reverse cholesterol transport by acting at various stages in the process. Reconstituted HDL, or naturally occurring pre-β-migrating HDL, containing apoA-I as the only protein component, are more efficient acceptors of cell cholesterol than those containing other exchangeable apolipoproteins (2, 3), and lipid-free apoA-I can mediate cellular cholesterol efflux by forming pre-β-HDL-like particles in the extracellular space. Esterification of cell-derived cholesterol, the second step in reverse transport, is also remarkably dependent on the ability of apoA-I to activate the lecithin:cholesterol acyltransferase enzyme (5). Finally, HDL can directly transport the lecithin:cholesterol acyltransferase-devoid cholesteryl esters to the liver, in a process possibly mediated by recognition of apoA-I (6).

ApoA-I is a single polypeptide chain, composed of 243 amino acids (7). Studies of apoA-I structure have mainly focused on secondary structure because of the high percentage of α-helix and the motif of amphipathic helices, which is believed to mediate protein-lipid interaction in the lipoproteins (8). The most striking feature of the apoA-I molecule is the presence of internal repeat units of 11 or 22 amino acids, with the periodicity of an amphipathic α-helix (9). This structure allows for the main biological activities of apoA-I, i.e. lipid-binding (8), lecithin:cholesterol acyltransferase activation (5), stimulation of cholesterol efflux from lipid-loaded cells (10), and interaction with the putative HDL receptor (11). The amino-terminal end of the protein has significantly less α-helix than the COOH-terminal fragment (residues 149–243), which is organized in highly packed antiparallel amphipathic helices, and has the highest affinity for lipids (12, 13). Less is known about the tertiary structure of apoA-I; a structural model has been recently proposed, with a domain of interacting α-helical segments in the carboxyl terminus and a globular domain in the amino terminus (14–15). Physicochemical (14, 16), monoclonal antibody (17, 18), and limited proteolysis studies (13) suggest that a hinge domain may exist in the apoA-I molecule (9), possibly involved in lecithin:cholesterol acyltransferase activation (5, 19, 20) and promotion of cell cholesterol efflux (21).

Apolipoprotein A-I_{Milano} (A-I_{Milano}) is the first described molecular variant of human apolipoproteins (22) and seems to confer to the carriers an increased protection against vascular disease (23). ApoA-I_{Milano} is characterized by an Arg^{113} → Cys substitution (24), resulting in the formation of disulfide-linked homodimers (A-I_{Milano}/A-I_{Milano}) and heterodimers with apoA-II. The presence of these covalently linked apoA-I molecules is likely responsible for most of the structural/metabolic abnormalities of carriers'
HDL. Indeed, in an in vitro system, the interconversion of small, A-I/A-I-containing HDL is impaired (25), while in vivo the A-I/A-I dimers are catabolized more slowly than monomeric A-I/A-I or normal apoA-I (26).

The Arg772 → Cys replacement in monomeric apoA-I significantly alters the structural and lipid-binding properties of apoA-I (27). To identify alterations induced in apoA-I structure by the introduction of an interchain disulfide bond, the present study compared the spectroscopic properties of A-I/A-I and apoA-I. A-I/A-I was either purified from carriers' plasma or expressed in Escherichia coli; the recombinant A-I/A-I(A-I/A-I) proved to be structurally identical to native A-I/A-I, both showing physicochemical properties distinct from that of normal apoA-I.

**EXPERIMENTAL PROCEDURES**

**Purification of Plasma A-I/A-I (HDL).** (d = 1.063–1.21 g/ml) were isolated from A-I/A-I plasma by sequential ultracentrifugation in a 50.2 Ti rotor (Beckman) and washed at 40,000 rpm, 4°C for 48 h. HDL were extensively dialyzed against 5 mM NH₄HCO₃, containing 0.01% EDTA, pH 7.4, lyophilized, and delipidated with diethyl ether/ethanol (3/1, v/v). Ti rotor (Beckman) and washed at 40,000 rpm, 4°C for 48 h. HDL were then reconstituted into buffer, pH 7.4, containing 0.01% NaN₃, pH 7.4, containing 4 mM GdnHCl. Fractions corresponding to A-I/A-I were concentrated and reapplied to the same column.

**Expression and Purification of rA-I/A-I.** rA-I/A-I was expressed in E. coli K12 derivative strain BC0050 (xyl-7, ara-14, T4RZ) and excreted to the growth medium. The expression vector was derived from pTrc99A (28). The rA-I/A-I cDNA was prepared by site-directed mutagenesis of apoA-I cDNA (29) and placed immediately downstream of the OmprA signal sequence derivative, where the second to last amino acid was changed from Gln to Asn. Two transcription termination codons were placed downstream of the A-I/A-I gene to reduce expression of the ampicillin resistance gene. The expression vector was transformed into E. coli strain, and colonies were selected on agar plates containing 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, and the temperature was raised to 37°C. Six hours after induction, the concentration of A-I/A-I in the supernatant, determined by radioimmunoassay (Apolipoprotein A-I RIA, Pharmacia Biotech Inc.), was 2 g/liter.

**Cells were separated by centrifugation at 3,000 rpm for 20 min, and the medium was diluted with distilled water to a conductivity below 10 mS/cm prior to loading onto a Q-Sepharose FF (Pharmacia Biotech Inc.) column, equilibrated with 0.02 mM sodium phosphate, pH 8.0. The column was washed and then eluted with 0.4 NaCl in the same buffer. A-I/A-I-containing fractions were applied to a phenyl-Sepharose FF gel column, equilibrated with 0.02 mM phosphate buffer, pH 7.5, containing 0.75% ammonium sulfate. After washing with 0.02 mM phosphate buffer, pH 7.5, containing 0.04 mM ammonium sulfate, 0.02 mM phosphate, A-I/A-I was eluted with distilled water, and aliquots of this material were loaded onto a Superdex 200 10/300 column, equilibrated with 5 mM NH₄HCO₃, 0.01% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanol and eluted at a flow rate of 25 ml/min. Fractions containing rA-I/A-I were pooled, and the buffer was changed to 20 mM sodium phosphate, pH 8.0, by dialfiltration on Omega 8k filter cassettes. The concentrated rA-I/A-I was applied to a Q-Sepharose FF column, equilibrated with 0.02 mM sodium phosphate, pH 7.5, containing 0.05 M NaCl and was eluted with a linear gradient of sodium chloride (0–1 M). Pooled fractions were concentrated and dialyzed against 0.2 M phosphate buffer, pH 7.8, containing 1% mannitol and lyophilized. Before use, rA-I/A-I was dissolved in 20 mM phosphate buffer, pH 7.4, containing 6 M GdnHCl and extensively dialyzed against 20 mM phosphate buffer, pH 7.4.

**Characterization of rA-I/A-I.** rA-I/A-I (350 μg) was digested with the protease Lys-C (Boehringer Mannheim) (2 μg in 5 μl of water) for 18 h at 37°C in 200 μl of 25 mM Tris-HCl, pH 8.5, 1 mM EDTA. The resulting peptides were separated by reverse-phase HPLC, using a Brownlee Aquapore butyl column developed at 40°C with a gradient of 5–40% acetonitrile in water, containing 0.25% pentafluorophosphonic acid. Each peptide was sequenced by automated Edman degradation, and the molecular mass was determined by a Biodesk 2000. The desorption time-of-flight mass spectrometer (Applied Biosystems). A 59-residue COOH-terminal fragment (H2: residues 152–203) was prepared by cleavage with cyanogen bromide. Lyophilized (rA-I/A-I (480 μg) was dissolved in 1 ml of cleavage solution containing 20 mM hydroxyamine, 3 mM GdnHCl, 0.2 M NaCl, and 2 mM EDTA, pH 8.4, and incubated for 5 h at 40°C. The COOH-terminal fragment was purified by reverse-phase HPLC, using a YM-30 Protein column, eluted with a gradient of 10–60% acetonitrile in water, containing 0.25% pentafluorophosphonic acid.

**For determination of COOH-terminal amino acids, rA-I/A-I or its COOH-terminal H2 fragment (4 nmol) was dissolved in 85 μl of 0.2 M acetic acid, containing 0.04 M ammonium sulfate, pH 7.5, containing 35 and 6 nmol of the same standard. Carboxypeptidase P Sigma) (15 μg in 15 μl of water) was added, and the solution was incubated at 20°C. Samples (25 μl) were withdrawn after 0, 1, 2, and 4 min, and the digestion was stopped by adding 10 μl of 50% trifluoroacetic acid. The released amino acids were analyzed by the Ficotag method (Waters). The NH₂-terminal amino acid sequence was determined using a Hewlett-Packard G1000A protein sequenator or a Milligen Biosearch Prosequencer type 6600, with Sequelon AA membranes (Millipore/Miligen).

The molecular mass of the intact protein was determined using positive electrospray mass spectrometry (VG Quattro, Fisons Instruments). The samples were applied in 60% methanol, 1% acetic acid at a flow rate of 5 μl/min.

**Amino Acid Analysis and Protein Concentration.—**Protein concentration of stock solutions was assayed by amino acid analysis, performed on a Beckman 6300 amino acid analyzer after acid hydrolysis of samples in 6 M HCl for 45 min at 115°C.

**Spectroscopy.—**Circular dichroism (CD) spectra were recorded with a Jasco J500A spectropolarimeter at the constant temperature of 25°C. Cells were 0.01 and 0.1 cm for the region below 250 nm, and 1.0 cm above 250 nm. Molar mean residue ellipticity (θ) was expressed in degrees cm²dmol⁻¹, and calculated as:

\[ \theta = \theta_{220}/110 \text{ (Eq. 1)} \]

where θ is the observed ellipticity in degrees, 115 is the mean residue molecular weight of the proteins, l is the optical path length in centimeters, and c the protein concentration in grams/ml. All of the spectra were base line-corrected. The α-helical content was calculated by the method of Chong et al. (31).

The ultraviolet absorption spectra were measured with a Jasco Uvidec-610 spectrophotometer equipped with on-line digital data processor. Differential absorption spectra were calculated by subtraction of the base-line spectrum stored in memory. Topographical location of tyrosyl residues was investigated by second-derivative analysis, according to Bagone et al. (32). The a and b values were calculated as the peak to trough distances between the minima at 283 and 290.5 nm, and the maxima at 287 and 295 nm, respectively. The fractional tyrosine exposure was then expressed as θ = (r – r_{min})/r_{max}, where r is the ratio a/b, measured from the second-derivative absorption spectra. r_{min} is the ratio for the protein dissolved in 6 M GdnHCl, and r_{max} is the ratio calculated for a model protein solution containing the same 6 M mol of noreacine as acid dissolved in ethylene glycol to simulate residues completely buried in the protein interior. For apoA-I, r_{min} and r_{max} were 1.20 and 0.35, respectively.

**Reconstitution Procedures.—**Weighed amounts of dimyristoylphosphatidylcholine (DMPC) were dissolved in chloroform and the solvent dried under vacuum for 2 h. Dispersions of DMPC in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 8.5% KBr, were mixed with apoA-I, A-I/A-I, or rA-I/A-I at 2.5:1, DMPC:protein weight ratio and incubated for 20 h at 25°C. The complexed lipoprotein was then dialyzed against 20 mM phosphate buffer, pH 7.4, before use.

**Electrophoresis.—**SDS-PAGE was performed on 10% acrylamide gels using the Tris-Tricine buffer system of Schagger and Von Jagou (33) in...
a Mini Protean slab minigel apparatus (Bio-Rad). In selected samples, reduction of disulfide bonds was accomplished by addition of dithiothreitol to the sample buffer, to give a final concentration of 84 mM. The gels were scanned at 627 nm with an Ultroscan XL laser densitometer (Pharmacia Biotech Inc.). Two-dimensional charge-size separations were achieved with a linear 4–10 immobilized pH gradient in the SDS-PAGE (34). Cross-linking was performed by the addition of 1 part di-methylsuberimidate (DMS) solution, 10 mg/ml in 0.3 M triethanolamine, to 10 parts protein solution (35). Cross-linked samples were analyzed by SDS-PAGE on 4–10% acrylamide gradient slab gels.

RESULTS AND DISCUSSION

Characterization of rA-IM/A-IM—The HPLC profile of purified rA-IM/A-IM showed three major peaks (Fig. 1). Mass spectrometry analysis revealed that the peak eluting at 21 min corresponds to the full-length product, while the other two peaks are disulfide-linked A-IM dimers containing one (peak at 19 min) or two (peak at 17 min) NH₂-terminally truncated monomers (see below). The full-length protein was further purified by reverse-phase HPLC, eliminating most of the truncated monomers (Fig. 2, lane C); this product was used in following experiments.

The rA-IM/A-IM co-migrated with the native protein on SDS-PAGE in both non-reduced and reduced conditions (Fig. 2). No protein thiols were detected by the Ellman’s (36) assay in preparations of either native or recombinant A-IM/A-IM, indicating that the purified proteins were entirely in the disulfide form. Two-dimensional gel electrophoretic separation of apoA-I revealed three major and three minor isoproteins (Fig. 3) (37). Reduced rA-IM/A-IM, as well as native A-IM (38), showed the same isoproteins, but with one less positive charge compared with normal apoA-I, as expected from the amino acid substitution in the mutant apolipoprotein.

The amino acid composition of both A-IM/A-IM and rA-IM/A-IM was in close agreement with that deduced from the DNA sequence (39). The primary structure of rA-IM, as well as the sequencing strategy, is presented in Fig. 4. Determination of the NH₂-terminal sequence showed, besides the correct translation product, shorter NH₂-terminal sequences in preparation containing truncated rA-IM. The molecular mass (6,530 ± 3 Da), amino acid composition and sequence of the COOH-terminal H2 fragment, together with the direct determination of the COOH-terminal Gln by carboxypeptidase P, confirmed that the COOH terminus of rA-IM was correct. The rA-IM primary sequence was deduced from direct sequencing of the peptides generated by specific cleavage with lysyl and aspartyl endoproteinases. Their molecular mass deviated from theoretical values by less than 0.2%. The direct estimation of the rA-IM/A-IM mass by electrospray ionization mass spectrometry gave a value of 56,053 ± 28 Da, i.e. in good agreement with the calculated molecular mass deduced from the amino acid sequence (56,049 Da). This finding argues against the occurrence of post-
translational modifications in rA-l_{H/A-I,}.

Cross-linking Studies—Cross-linking with DMS has been repeatedly used to evaluate the self-association of soluble apolipoproteins (35, 40). Cross-linking of apoA-I gives five prominent bands, identified as monomer (M_{r}, 28,000) through pentamer (M_{r}, 140,000) (35). The cross-linking pattern is sensitive to protein concentration, monomers and dimers predominating at the lower values (<0.5 mg/ml), and tetramers and pentamers becoming major species at higher concentrations (35). Cross-linking experiments with both native and recombinant A-I_{H}/A-I_{M} gave superimposable patterns and showed a similar formation of high molecular weight forms (Fig. 5A). Cross-linked A-I_{H}/A-I_{M} gave four prominent bands; some high molecular weight material was seen at high protein concentration, but was negligible compared to the total area of the prominent bands (Fig. 5A). A-I_{H}/A-I_{M} co-migrated, as expected, with the apoA-I dimer (M_{r}, 56,000). The band co-migrating with the apoA-I tetramer (M_{r}, 112,000) was identified as a cross-linked species made of two A-I_{H}/A-I_{M} molecules. Notably, no bands co-migrating with the apoA-I trimer and pentamer were present in cross-linked A-I_{H}/A-I_{M}, while the two high molecular weight bands were tentatively identified as cross-linked species containing three and four A-I_{H}/A-I_{M} molecules. As previously shown for apoA-I (35), the A-I_{H}/A-I_{M} cross-linking pattern was sensitive to protein concentration (Fig. 5A). The percentage of apoA-I and A-I_{H}/A-I_{M} oligomers, calculated from the SDS-PAGE scans and reported as a function of protein concentration in the reaction mixture.

Fig. 5. Cross-linking of A-I_{H}/A-I_{M}. Panel A, SDS-PAGE of cross-linked rA-I_{H}/A-I_{M}. Equal amounts of rA-I_{H}/A-I_{M} were diluted with water and cross-linked with DMS before separation on a 4–10% acrylamide gradient gel. Cross-linked apoA-I was used as molecular weight standards (lanes). The concentrations of rA-I_{H}/A-I_{M} present in the reaction mixture were: A, 0.0625 mg/ml; B, 0.125 mg/ml; C, 0.25 mg/ml; D, 0.5 mg/ml; E, 1.0 mg/ml; F and G, 2.0 mg/ml. Panel B, concentration dependence of the self-association of apoA-I (squares) and rA-I_{H}/A-I_{M} (circles). Proteins were cross-linked at various concentrations and electrophoresed on gradient gels; the percentage of oligomers was calculated from the SDS-PAGE scans and reported as a function of protein concentration.

Circular Dichroism Studies—The secondary and tertiary structures of native A-I_{H}/A-I_{M} and A-I_{M}/A-I_{H}, in their lipid-free and lipid-associated states, were examined by CD spectroscopy. A protein concentration of 0.1 mg/ml, at which both apoA-I (35, 46) and A-I_{M}/A-I_{H}, (Fig. 5) are essentially monomeric, was initially used. In the far-ultraviolet, rA-I_{H}/A-I_{M} and the native protein gave superimposable CD spectra (Fig. 6). The CD spectra are indicative of a highly α-helical structure, exhibiting negative troughs at 208 and 222 nm, together with a positive band at 190–192 nm. ApoA-I gave a similar spectrum, but the values of negative ellipticity for the troughs at 208 and 222 nm were lower than in A-I_{H}/A-I_{M} (Fig. 6, Table I). The analysis of the CD spectra by the method of Chang et al. (31) reveals an α-helical content of 64% in rA-I_{H}/A-I_{M}, 66% in native A-I_{H}/A-I_{M}, and 43% in apoA-I, this latter being close to that previously reported by us and others (27, 47). All proteins approached a maximum molar ellipticity value (Table I) upon addition of the helix-inducing solvent trifluoroethanol (50%), the calculated
α-helical content of ~75% being close to the maximum helical potential deduced from the apo-A1 primary sequence (9).

The higher α-helical content of A-I/A-I versus apo-A-I in water may result from protein-protein interactions between the α-helices of A-I, brought in close proximity by the disulfide bond, i.e., in a way not different from that of self-associated apo-A1 (46). The CD spectrum of apo-A1 was indeed significantly affected by protein concentrations over the 0.1-1.3 mg/ml range (Table I), confirming the lower tendency of A-I/A-I, to self-assemble deduced from cross-linking studies. The α-helical content of self-associated apo-A1 was almost identical to that of covalently linked A-I/A-I, (64-66%), suggesting that a similar conformational change occurs in apo-A1 when two polypeptide chains are brought in close proximity, either by increasing the protein concentration or by introducing a covalent bond.

To monitor the influence of lipid on apolipoprotein conformation, apo-A1, A-I/A-I, and rA-I/A-I were mixed with DMPC at 25 °C. The co-incubation resulted in a complete clearing of the turbid lipid dispersion, indicating dissolution of the large phospholipid multilamellar liposomes and the formation of stable lipid-protein complexes (27). Binding to DMPC caused an increase in the α-helical content of all proteins (Fig. 6, Table I). The induction of α-helix in A-I/A-I was significantly lower compared to apo-A1, bringing the spectra of lipid-bound apo-A1 and A-I/A-I in close proximity (Fig. 6). All together, the far-UV CD data demonstrate that a maximum α-helical content is reached with both apo-A1 and A-I/A-I upon self-association, lipid-binding, or exposure to trifluoroethanol (46, 47, 48). In other studies, we investigated the effect of solvent pH on the stability of lipid-free apo-A1 and A-I/A-I solutions. Plots of [θ]222 as a function of pH are shown in Fig. 7. The ellipticity of both proteins markedly decreased at pH values below 4 and above 10. Apo-A1 and A-I/A-I, were insoluble at pH between 4.5 and 5.5, and 3.5 and 5.5, respectively. Similar pH-depend-
and a shoulder at approximately 286 nm are detectable (Fig. 8). It is of interest that these bands are reversed in sign and shifted to 283 and 290 nm following association of apoA-I with DMPC (Fig. 8). The almost identical shapes of the tryptophan and tyrosine bands in A-I/A-I and lipid-associated apoA-I indicate that comparable changes in apoA-I conformation are induced either by protein-protein interactions between two apoA-I chains, brought in close proximity by a disulfide bond as in A-I/A-I or by association with amphiphile (46, 47, 50), i.e. in good agreement with the far-ultraviolet CD data.

Spectroscopic Studies—Tyrosine exposure in apoA-I, A-I/A-I, and rA-I/A-I was determined by second-derivative ultraviolet spectroscopy, according to Ragone et al. (32). The absorption and second-derivative spectra recorded for the two A-I dimers are, again, almost identical; those of rA-I/A-I are shown in Fig. 9. In all tested conditions, i.e. proteins in the lipid-free and lipid-associated states or denatured in 6 M GdnHCl, the ultraviolet-absorption spectra revealed absorption maxima at 280 nm with a shoulder at 290.2 nm. The second-derivative spectra demonstrated characteristic peaks at 287 and 295 nm with accompanying troughs at 283 and 290.5 nm (Fig. 9). The peak r ratios calculated for apoA-I were 1.10 in phosphate buffer, 0.74 upon association with DMPC and 1.34 in 6 M GdnHCl. These values correspond to a fractional tyrosine exposure (α) of 0.88 for the native protein, 0.46 for the lipid-bound protein and 1.10 for the unfolded state, or exposure of 6, 3, and 7 tyrosine residues, respectively.

The peak r ratio determined for A-I/A-I was 0.82 and 0.85. These values correspond to a fractional tyrosine exposure (α) of 0.60 for native and lipid-bound A-I/A-I, or exposure of 8.2 tyrosine residues. This could be an overestimate of the actual number of tyrosine residues which are exposed in the native A-I/A-I molecule, because of the sensitivity of the second-derivative absorption spectrum to changes in the electronic state of buried tyrosine residues (32).

Altogether, the near-UV CD and the second-derivative UV data demonstrate that in A-I/A-I the two A-I monomers tightly fold upon themselves, mainly through apposition of the hydrophobic sites of the amphipathic helical segments. Such a folded structure would drastically reduce the flexibility of the A-I monomers thereby affecting their functional properties.

Implications for the Functional Morphology of A-I/A-I

The present data indicate that A-I/A-I possesses molecular properties that are unique compared to those of normal apoA-I and monomeric A-I. The Arg73 Cys substitution in A-I occurs at the surface of the molecule, in the middle of one of the amphipathic helical segments of apoA-I (27). By analogy with the x-ray crystal structure of the NH2-terminal domain of human apoE (45), and according to the amphipathic helix theory of apolipoprotein structure (9), the guanidinium group of Arg73 in apoA-I is salt-bridged with the oppositely charged carboxylate group of Glu169. The loss of the salt bridge in monomeric A-I (27) results in a lower a-helical content and increased flexibility in the interaction of the apolipoprotein with lipids (27). By contrast, the present findings indicate that the introduction of a disulfide bridge in A-I/A-I results in facilitated interhelix interactions, with an increased secondary structure, and a more folded tertiary structure.

The present concept of apoA-I is that of a highly flexible protein, which exists in an equilibrium between a lipid-free form and a bound lipoprotein form. By analogy with the molecular structure of other apolipoproteins (44, 45), lipid-free
apoA-I should form up and down bundles of amphipathic helices. Following binding to lipids, apoA-I spreads on the lipid surface (15, 17), the hydrophobic interactions in the interior of the lipid-free protein being substituted by interactions with lipids. In view of these unique properties, it is not surprising that the introduction of the disulfide bridge in A-I₅/A-I₅ can result in major changes in molecular organization.

While the definition of the three-dimensional structure of A-I₅/A-I₅ awaits the availability of quality crystals for structure determination by X-ray crystallographic methods, the present data suggest that the disulfide bridge in the middle of an amphipathic lipid-binding helix would drastically affect the ability of apoA-I to readily convert from a lipid-free to a lipoprotein, and the ability to desorb from lipoproteins would be increased. Indeed, a careful characterization of plasma lipoproteins in the carriers has clearly shown the presence of small, amphipathic lipid-binding helices with characteristics of A-I₅/A-I₅, which are absent in normal human plasma. Following binding to lipids, apoA-I spreads on the lipid surface (15, 17), the hydrophobic interactions in the interior of the lipid-free protein being substituted by interactions with lipids.

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