Effect of oxidation on the properties of apolipoproteins A-I and A-II

G. M. Anantharamaiah,* Thomas A. Hughes,[1] M. Iqbal,* Ali Gawish,* Peter J. Neame,* Mark F. Medley,* and Jere P. Segrest†

Departments of Medicine* and Biochemistry,† and the Atherosclerosis Research Unit, University of Alabama at Birmingham, Birmingham, AL 35294

Abstract Purified apolipoprotein A-I has been separated by reversed-phase high performance liquid chromatography (HPLC) into multiple peaks and these peaks have been characterized. One peak, apoA-Ib had a relatively longer retention time on HPLC but its retention time could be shortened by treatment by hydrogen peroxide. CNBr cleavage studies indicated that the differences in apoA-Ib and in its oxidation product, apoA-Ia, were due to the different oxidation states of methionine. This phenomenon was also observed in apoA-II, where methionine oxidation produced two more forms of this apolipoprotein in addition to the native form. These isomers were found to have different secondary structures and affinities for lipid. Model peptide analogs of the amphipathic helix with the same sequence but with methionine and methionine sulfoxide at the nonpolar face of the amphipathic helix were synthesized and studied. It was found that the lipid affinities of these synthetic peptide isomers were very different. They also differed in their secondary structures as studied by circular dichroism (CD). We propose that methionine oxidation introduces hydrophilic residues at the nonpolar face of the amphipathic helical domains of these apolipoproteins and, therefore, alters their secondary structure and lipid affinity. —**Anantharamaiah, G. M., T. A. Hughes, M. Iqbal, A. Gawish, P. J. Neame, M. F. Medley, and J. P. Segrest.** Effect of oxidation on the properties of apolipoproteins A-I and A-II. *J. Lipid Res.* 1988. **29:** 309–318.

Supplementary key words apoA-I • apoA-II • methionine oxidation • synthetic peptides • amphipathic helix • apolipoprotein isomers • HPLC

High density lipoproteins (HDL) are protein-lipid complexes found in plasma with a density range of 1.063 to 1.21 g/ml. There is now substantial evidence that HDL cholesterol concentration is inversely related to the incidence of coronary artery disease (1, 2). HDL is composed of approximately 50% protein of which apolipoproteins A-I and A-II make up about 90% of the total protein. The primary structures of these apolipoproteins are known (3). Although extensive investigations have been carried out on the behaviors of these apolipoproteins, particularly apoA-I, conflicting reports still exist on their molecular properties (4). It is known that apoA-I and A-II contain repetitive helical domains with opposing nonpolar/polar faces (amphipathic helices). This structure is believed to contribute to the ability of these apolipoproteins to solubilize lipid in an aqueous environment (5). Recently, apoA-I has been reported to exist in different polymorphic forms (6–9). Some investigators have suggested that these forms arise due to a sequential deamidation process which gives rise to successively acidic isomers (8). It has also been suggested that differences in the polymorphic forms of apoA-I are due to oxidation of the protein (10). There are no reports in the literature on the characterization of apoA-II isoforms.

This laboratory has recently developed a reversed-phase HPLC methodology for analyzing the apolipoprotein composition of plasma lipoproteins (11). Using this technique, we have isolated two forms of apoA-I (apoA-Ia and apoA-Ib) and three forms of apoA-II (apoA-IIa, apoA-IIb, and apoA-IIc). In this report, we will present data that indicates that these forms of apoA-I and A-II (as identified by HPLC) are due to the oxidation of methionine in these proteins. Two model peptide analogs of the amphipathic helix (with the same amino acid sequence but with methionine or methionine sulfoxide on the nonpolar face of the amphipathic helix) have been synthesized and studied. Results from these studies support our contention that methionine oxidation of peptides with identical primary structures leads to differences in HPLC retention times. Furthermore, these peptides have very different lipid binding characteristics and secondary structural features indicating that methionine oxidation of apolipoproteins is likely to significantly alter their interactions with lipids.

Abbreviations are in accordance with IUPAC-IUB nomenclature. Others used are: Met(O), methionine sulfoxide; HDL, high density lipoproteins; DMPC, dimyristoylphosphatidylcholine; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; HPLC, high performance liquid chromatography.

[1]Present address: Department of Medicine, University of Tennessee, Memphis, TN 38103.

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METHODS

Lipoprotein isolation

HDL (d 1.063–1.21 g/ml) was isolated by gradient ultracentrifugation. Ten ml of plasma was raised to a density of 1.35 g/ml with 5.94 of KBr. A second layer of buffer (10 ml) (NaCl 0.9%, EDTA 1.0 mM, Tris 10 mM, sodium azide 0.1%, pH 8.5, containing protease inhibitors 6-aminohexanoic acid (100 mM), benzamidine-HCl (5 mM), and phenylmethylsulfonylfluoride (1.0 mM)) raised to a density of 1.20 g/ml with KBr, was added to the tube and, finally, the tube was filled with the above buffer (d 1.006 g/ml) to a final volume of approximately 40 ml. The tubes were sealed and spun to 8.0×10^{11} $rad^2 \cdot sec^{-1}$ at 70,000 rpm at 15°C in a 70 Ti rotor (approximately 4 hr and 15 min). HDL was dialyzed at 4°C against 0.1 M ammonium bicarbonate buffer, 0.01% NaN_3, pH 8.0. The lipoproteins were delipidated with five volumes of hexane isopropanol 3:2. The aqueous layer was dried and the proteins were solubilized in 3 M guanidine-HCl, and then analyzed by HPLC.

Analytical HPLC procedure

A Perkin Elmer Series 4 pump was used with an LC-55 or LC-75 UV detector at 214 nm. Peak areas were quantitated by a Shimadzu data station. Analytical reversed-phase HPLC was done on a Vydac C-18 column (4.6 × 250 mm) as a gradient of acetonitrile in water (containing 0.1% TFA) from 25 to 58% at 1.0%/min, column temperature, 50°C and a flow rate of 1.2 ml/min. **Fig. 1** shows typical chromatograms of apoHDL and apoA-I. These peaks were isolated and identified by amino acid analysis (**Table 1**), N-terminal analysis, and SDS polyacrylamide gel electrophoresis. Apolipoprotein isomers with the following retention times on the analytical HPLC were named as follows: apoA-Ia, 26.4 min; apoA-Ib, 28.2 min; apoA-IIc, 29.4 min; apoA-IIa, 30.7 min; apoA-IIb, 31.8 min.

Purification of apolipoprotein A-I and A-II isomers

The preparative HPLC method used to isolate these proteins in large amounts (5–10 mg) was similar to the analytical method described above, except that a larger column (10 mm diameter) and a slower gradient (0.5%/min) were used. Peaks were identified by analytical HPLC analysis.

Circular dichroism

The CD spectra were taken using a JASCO spectropolarimeter coupled to a DPN-500 integrator. ApoA-Ia

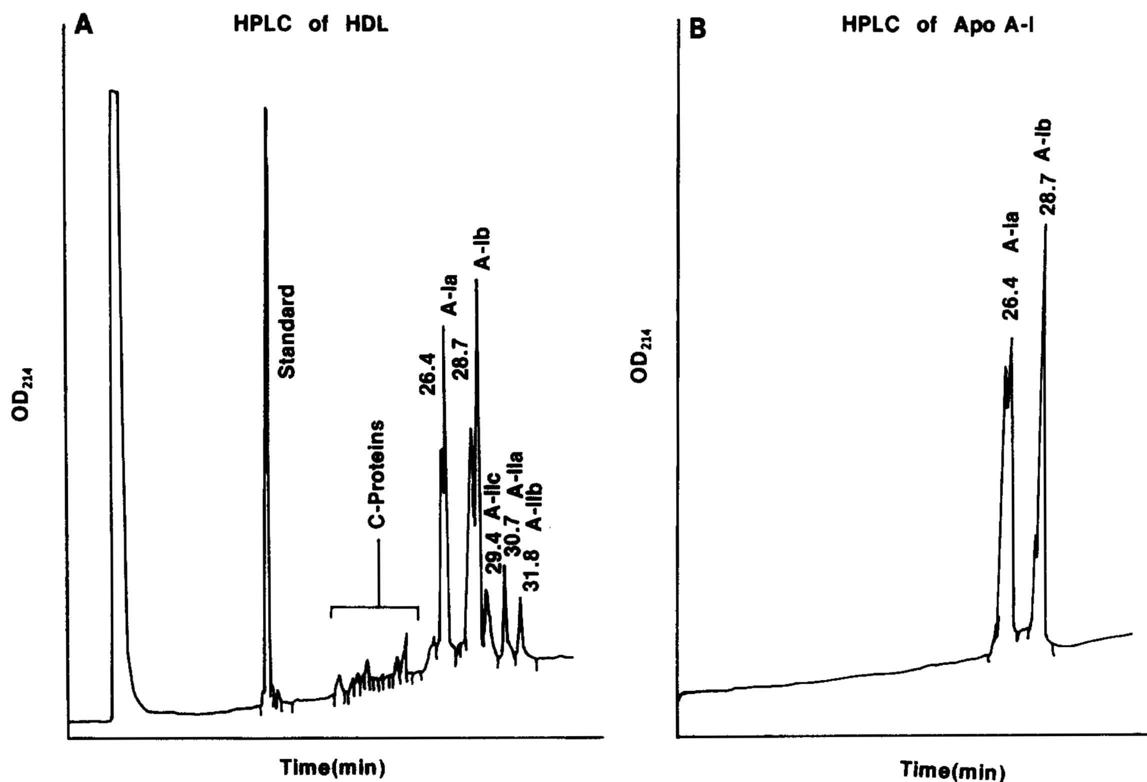

Fig. 1. HPLC profile of apoHDL and apoA-I. Panel A: human apoHDL was analyzed by HPLC as described in Methods. The isomers of apoA-I and apoA-II are indicated. Panel B: apoA-I, purified by S-200 gel chromatography, was analyzed by HPLC. Isomers of apoA-I are identified.

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TABLE 1. Amino acid composition ratios and values for amino acids/molecule used to identify apoA-I and apoA-II

	Expected ApoA-I	Obtained A-Ia	Obtained A-Ib	Expected ApoA-II	Obtained A-IIa	Obtained A-IIb	Obtained A-IIc
His	5.0	4.8	5.7	0.0	0.0	0.2	0.2
Met	3.0	2.9	3.7	1.0	2.2	2.0	2.0
Ile	0.0	0.0	0.0	2.0	1.8	2.0	1.8
Asx/Glx	0.46	0.42	0.41	0.19	0.17	0.20	0.18
Thr/Ala	0.53	0.50	0.48	1.20	1.14	1.00	1.12
Leu/Phe	6.17	5.92	5.12	2.00	1.91	2.03	1.91

Values for His, Met, and Ile are residues per molecule of apoA-I and per molecule of apoA-II dimer. Hydrolyses were performed at 110°C for 20 hr in 6 M HCl in the presence of dithiothreitol to reduce methionine sulfoxide to methionine. Amino acids were analyzed on a Durrum amino acid analyzer using post column derivatization with O-phthalaldehyde.

and apoA-Ib (50 μg/ml) were dissolved in 10 mM phosphate buffer, 150 mM NaCl, pH 8.0. Synthetic peptide analogs of apolipoproteins were also studied under the same conditions.

Electron microscopy

The apolipoprotein or synthetic peptides complexed with unilamellar vesicles of DMPC at 1:1 or 1:2 weight ratio were stained with 2% potassium phosphotungstate, pH 5.9, and examined with a Philips EM400 microscope on carbon-coated Formvar grids.

Gradient gel electrophoresis

A 10–25% linear acrylamide gradient gel was formed. Electrophoresis was performed at 4°C with 300 V/62.5 mA in the stacking gel and 500 V/80 mA in the running gel. The chamber buffer was Tris-glycine with 0.1% SDS at pH 8.5. The gel was stained in 0.25% Coomassie Blue.

Oxidation with hydrogen peroxide

Purified apolipoprotein isomers (70 μg of apoA-Ib or 24 μg of apoA-IIb) were treated with hydrogen peroxide solution (10 μl of 3% in the case of apoA-Ib and 1, 3, and 20 μl of 0.3% in the case of apoA-IIb) at room temperature for 5 min. The oxidation products were studied by analytical HPLC.

Treatment with mercaptoethanol

Apolipoproteins A-Ia, A-IIa, A-IIb, and A-IIc (50 μg) were treated with mercaptoethanol (3 μl) and heated in boiling water for 20 sec. The reduction products were examined by analytical HPLC.

CNBr treatment

One mg of each protein was dissolved in 80% HCOOH (0.5 ml) and treated with the same amount of CNBr at room temperature for 24 hr. The reaction mixtures were diluted with 5 ml of H_2O and lyophilized (12).

Peptide synthesis

Two peptide analogs of 18A ([Met^3]18A and [$Met(O)^3$]18A) were synthesized and purified following our procedure described elsewhere (13). These peptide analogs differ in having Met or Met(O) at the third position from the N-terminus of the 18A peptide with the following sequence: Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe, which has been well characterized by us (13).

RESULTS

Apolipoprotein isolation and characterization

ApoA-I has three methionines at positions 86, 112, and 148 and apoA-II has one methionine at position 26 from the N-terminus.

Based on the amino acid analysis and N-terminal analysis of individual peaks, the peaks seen on the HPLC chromatograph of apoHDL were designated as shown in Fig. 1A. Fig. 1B shows that apoA-I, isolated by conventional gel filtration (on S-200 sephacryl in 3 M guanidine-HCl) is a mixture of two isomers (apoA-Ia and apoA-Ib). These isomers were subjected to SDS-PAGE, amino acid analyses, and N-terminal sequencing to three residues (Table 1). These studies formally identified the peaks observed by reversed-phase HPLC. Similar studies of the apoA-II isomers (Table 1) again identified all peaks as being apoA-II. No N-terminal residue for peaks due to apoA-II was found as they contain pyroglutamic acid which does not react with PITC used in the Edman degradation procedure. Trypsin digestion of the apolipoprotein isomers also produced HPLC peptide maps with similar fragments (data not shown).

Oxidation of apolipoproteins

ApoA-Ib, apoA-Ia, apoHDL, and apoA-IIb were treated with hydrogen peroxide, and subjected to HPLC analysis.

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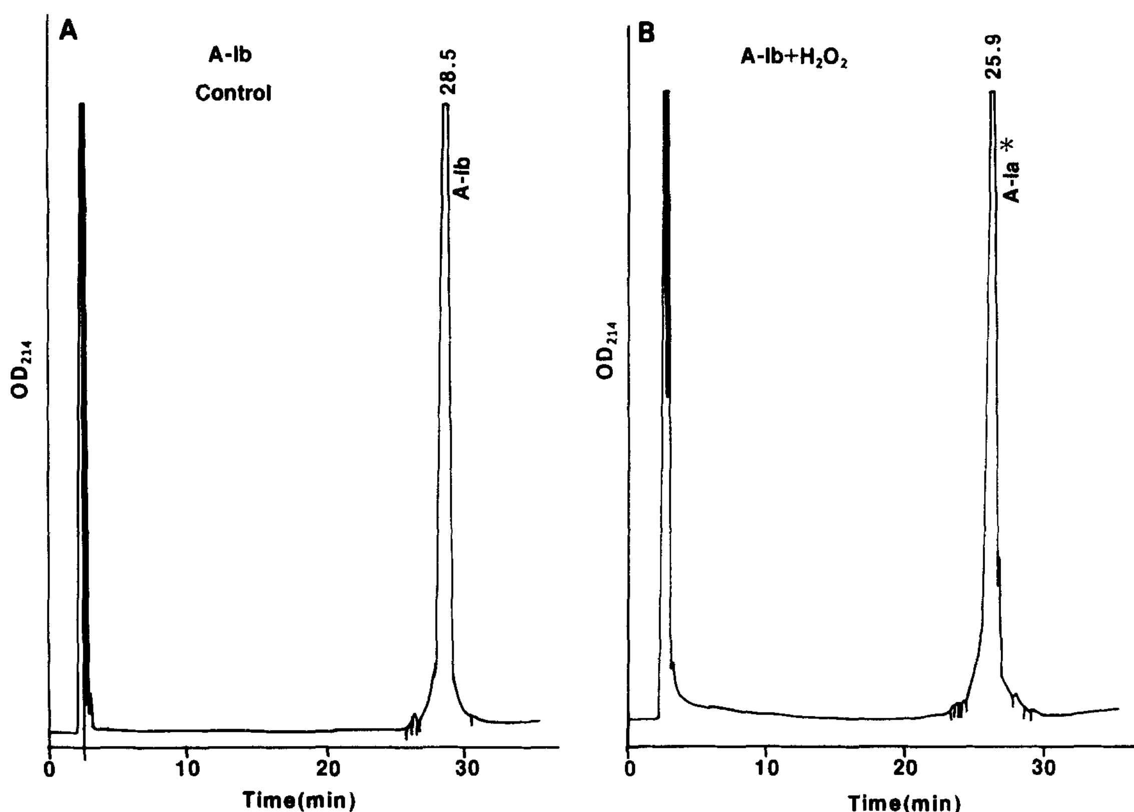

Fig. 2. HPLC chromatogram of apoA-Ib and apoA-Ib treated with hydrogen peroxide. Panel A: HPLC of untreated apoA-Ib. Panel B: HPLC of apoA-Ib after treatment.

The following results were observed. ApoA-Ib completely disappeared, and a new peak (with the same retention time as A-Ia in Fig. 1B) appeared (**Fig. 2**). The new peak is a chemically prepared apoA-Ia and is designated as apoA-Ia*. There was no change in the retention time of apoA-Ia following treatment with hydrogen peroxide (results not shown). ApoA-IIb was progressively converted to apoA-IIa and then to apoA-IIc with increasing amounts of hydrogen peroxide (**Fig. 3**). Similar changes were observed when whole HDL was treated with hydrogen peroxide. These results indicated that apoA-Ia, apoA-IIa, and apoA-IIc are the oxidized forms of these apolipoproteins.

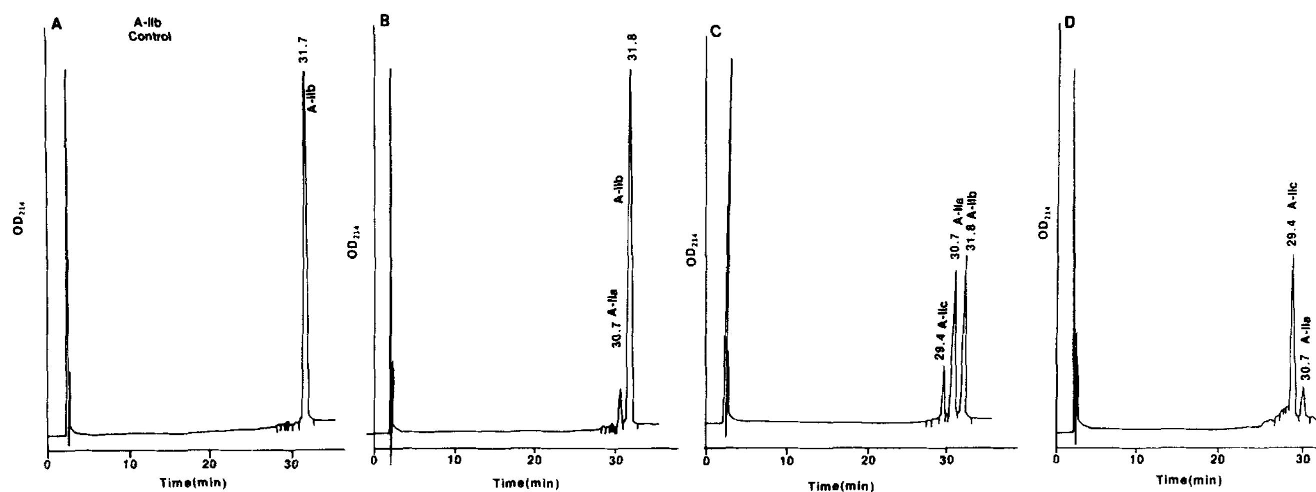

Fig. 3. HPLC chromatogram of apoA-IIb and its conversion products after treatment with increasing amounts of hydrogen peroxide. Panel A: apoA-IIb before treatment. Panel B: apoA-IIb after treatment with 1 μl of 0.3% hydrogen peroxide. Panel C: apoA-IIb after treatment with 3 μl of 0.3% hydrogen peroxide. Panel D: apoA-IIb after treatment with 20 μl of 0.3% hydrogen peroxide.

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CNBr studies

The results of CNBr digestion of apoA-Ia and apoA-Ib are shown in **Fig. 4.** CNBr specifically cleaves peptides at the methionine residues (12) but when methionine is oxidized, the protein becomes resistant to CNBr digestion. As can be seen in Fig. 4, apoA-Ia* was almost completely resistant to CNBr, while apoA-Ib underwent substantial fragmentation. These results indicate that apoA-Ia* contains almost entirely oxidized methionine residues while apoA-Ib contains reduced methionine residues. Since the CNBr/amino acid analysis methodology is unlikely to give complete results in either direction, the data are relative rather than absolute. That is, A-Ia is more oxidized than A-Ib. ApoA-I and A-II isomers were treated with CNBr for 1 hr under acidic conditions in order to convert the methionine residues to homoserine. This reaction does not alter oxidized methionine residues. Acid hydrolysis of each of the isomers was then performed under reducing conditions (which convert oxidized methionine to reduced methionine) and the hydrolyzate was analyzed for its amino acid composition. The amount of methionine measured, therefore, reflects the amount of oxidized methionine in the original sample. **Table 2** shows that there were highly significant differences in the amount of oxidized methionine in the apolipoprotein isomers and that the shorter the HPLC retention time the greater the degree of methionine oxidation. One sample of apoA-Ia* was analyzed and it was found to contain 95% oxidized methionine. This is consistent with the high degree of resistance to CNBr digestion noted above.

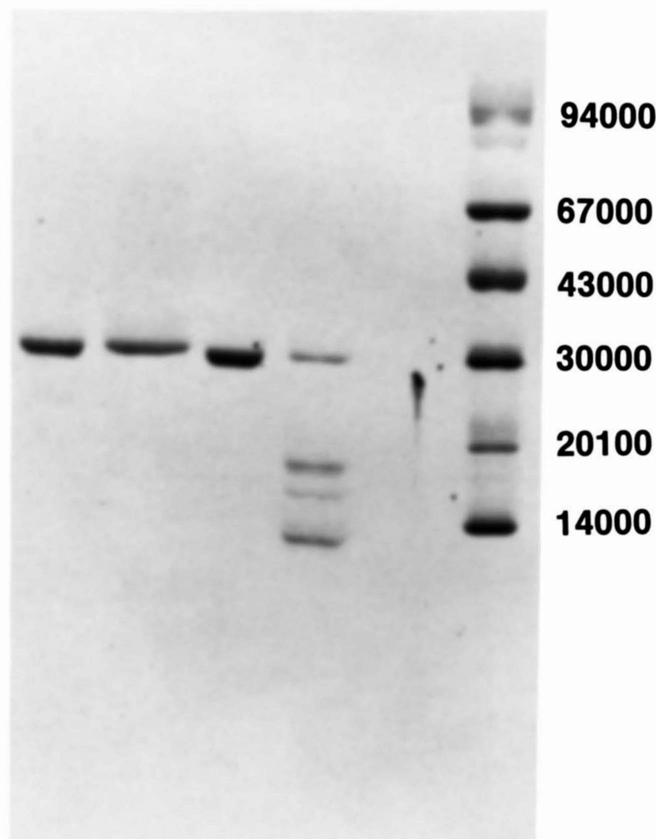

Fig. 4. SDS-polyacrylamide gel electrophoresis (10–25% gradient) of apoA-Ia, apoA-Ib, and their CNBr fragments. Lanes 1 and 3: apoA-Ia* and apoA-Ib, respectively, prior to CNBr digestion. Lanes 2 and 4: apoA-Ia* and apoA-Ib, respectively, after CNBr digestion. Lane 5: molecular weight standards.

TABLE 2. Determination of the degree of methionine oxidation in apoA-I and A-II

Apolipoprotein	Average Number of Methionine Residues/Molecule[a]
A-Ib	1.47 ± 0.12
A-Ia	1.95 ± 0.16[b]
A-IIb	0.40 ± 0.05
A-IIa	1.14 ± 0.02[b]
A-IIc	1.64 ± 0.05[b]

Apolipoproteins (in triplicate) were incubated with CNBr for 1 hr under acidic conditions in order to convert methionine residues to homoserine. Methionine sulfoxide is not altered during this reaction. The protein was then hydrolyzed under reducing conditions to convert methionine sulfoxide to methionine. An amino acid analysis was then performed. The amount of methionine measured gives an estimate of the amount of methionine sulfoxide in the original protein.

[a] The values for apoA-II reflect for the dimer.

[b] $P < 0.01$ versus value immediately above.

Treatment of apolipoprotein isomers with mercaptoethanol

ApoA-II is a dimer with two identical peptide monomers connected by a disulfide bond. Each monomer contains a single methionine residue at position 26 from the N-terminus. **Fig. 5** shows that reduction of apoA-IIa produced two peaks of almost equal size while reduction of apoA-IIb resulted in a single peak with a retention time identical to the second peak from apoA-IIa (Fig. 5). ApoA-IIc produced a single peak with the retention time identical to the first peak produced from apoA-IIa. These results indicate that apoA-IIb and apoA-IIc are made up of identical monomers and apoA-IIa is a mixed dimer. Since apoA-I does not contain disulfide bonds and it is unlikely that the mild reducing conditions used in these experiments would be able to reduce methionine residues, we would not expect mercaptoethanol to have any effect on apoA-Ia or A-Ib retention times. This is what was observed.

Synthetic peptide analogs of the amphipathic helix

In our previous studies, we have shown that our model synthetic amphipathic peptide analog (18A) with a sequence Asp-Trp-Leu-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Leu-Lys-Glu-Ala-Phe, mimics the properties of apoA-I (13). In order to investigate the effects of methionine oxidation on

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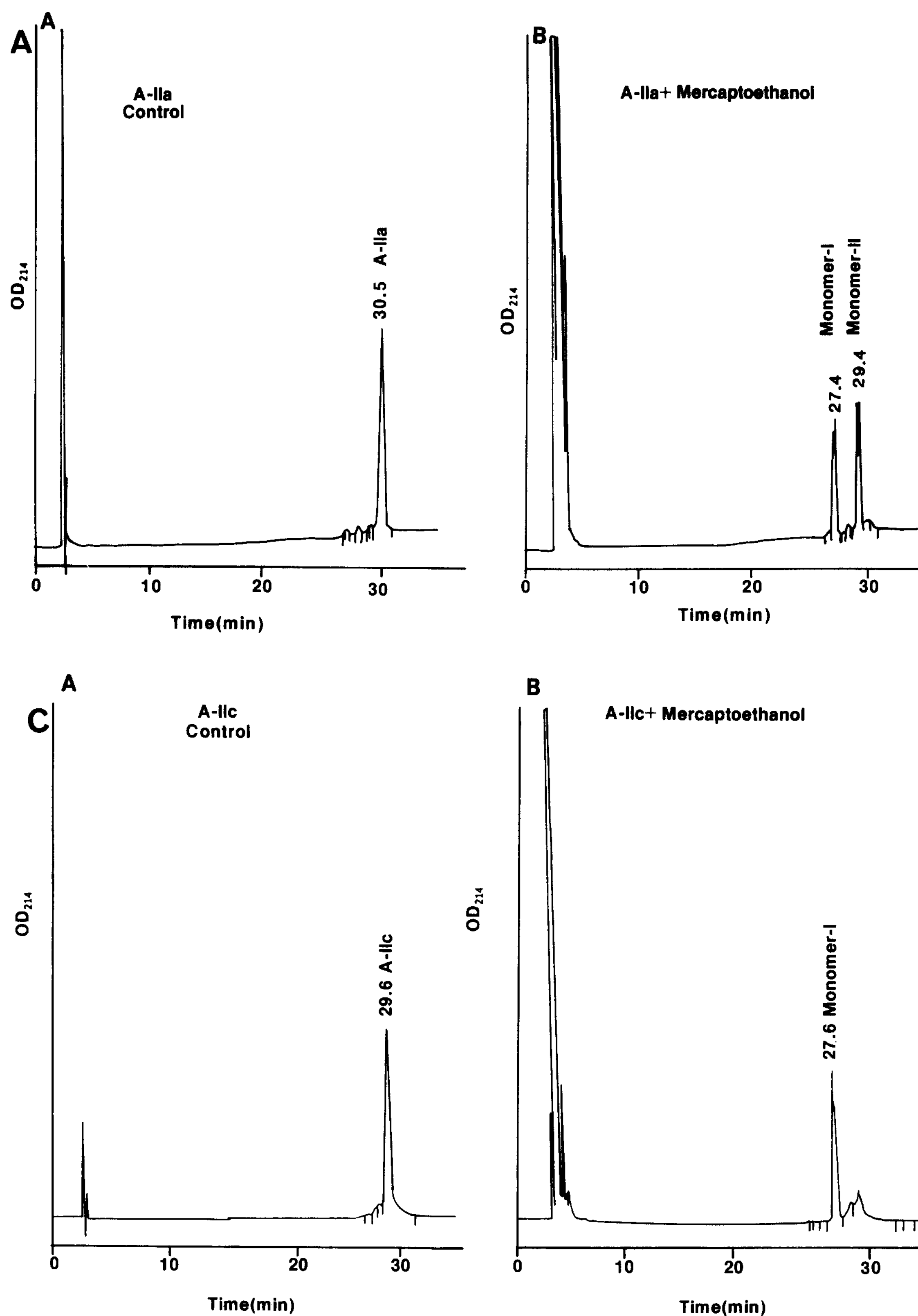

Fig. 5. A: HPLC chromatogram of apoA-IIa and its conversion products after treatment with mercaptoethanol. Panel A: apoA-IIa before treatment. Panel B: apoA-IIa after treatment with mercaptoethanol. B: HPLC chromatogram of apoA-IIb and its conversion products after treatment with mercaptoethanol. Panel A: apoA-IIb before treatment. Panel B: apoA-IIb after treatment with mercaptoethanol. C: HPLC chromatogram of apoA-IIc and its conversion products after treatment with mercaptoethanol. Panel A: apoA-IIc before treatment. Panel B: apoA-IIc after treatment with mercaptoethanol.

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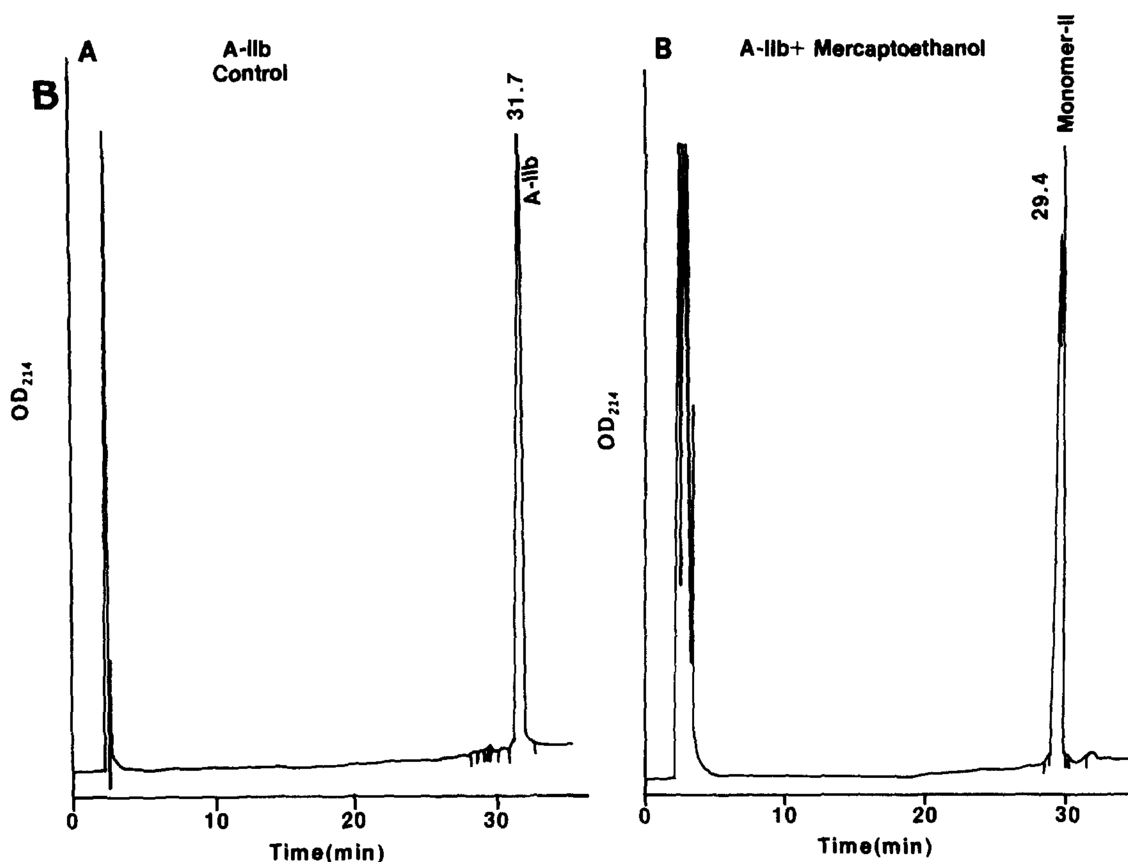

the properties of the lipid binding domains of apolipoproteins, we synthesized two analogs of the 18A amphipathic peptide ([Met[3]]18A and [Met(O)[3]]18A). Position three of these analogs is in the center of the nonpolar face of the amphipathic helix (13). Peptide [Met(O)[3]]18A was found to have a shorter retention time on reversed-phase HPLC than [Met[3]]18A (**Fig. 6**). This was similar to the oxidized apolipoprotein isomers which also had a shorter retention time than the reduced isomers.

Circular dichroism

CD of the [Met(O)[3]]18A and [Met[3]]18A peptides in solution and in the presence of DMPC demonstrated that the oxidized peptide analog had less helicity than the reduced analog. Similar results were obtained with the apoA-I and A-II isomers (Table 1) except that apoA-IIa (with one oxidized methionine) had slightly greater helicity than apoA-IIb (no oxidized methionines). The fully oxidized apoA-II (A-IIc) had significantly less helicity than either of the other isomers. These studies indicate that an alteration in the hydrophobicity of a single amino acid residue on the nonpolar face of the amphipathic helix can cause changes in the secondary structural features.

Apolipoprotein-lipid complexes

Peptide analogs and apolipoprotein isomers were mixed with DMPC at 1:1 weight ratio. While [Met[3]]18A clarified unilamellar vesicles of DMPC instantly, [Met(O)[3]]18A had little effect. Even at a 2:1 peptide–lipid ratio, the analog with oxidized methionine only slowly clarified DMPC vesicles. Electron microscopy of the complexes (**Fig. 7**) showed that even at a higher peptide–lipid ratio, particles formed with [Met(O)[3]]18A were larger (diameter 16.8 ± 1.7 nm) than those formed by [Met[3]]18A at a 1:1 weight ratio (diameter 11.7 ± 2.4 nm, $P < 0.001$).

DISCUSSION

That oxidation of a single methionine changes the properties of peptides and proteins is well documented in the literature (14–16). No such studies have been reported to date in the case of apolipoproteins. Apolipoproteins A-I, A-IV, and E have been shown to contain multiple amphipathic helical domains, which are lipid binding domains (5). In the case of human apoA-I, there are eight 22mer amphipathic helical domains (3). Three methionine residues are present at positions 86, 112, and 148 (helices 2, 3, and 5, respectively) (3). Examination of the helical wheel representations of these regions indicates that all three methionines are present in the nonpolar face of amphipathic helical domains. It has been shown by studies of synthetic peptide analogs of the amphipathic helix that an alteration in the hydrophobicity of the non-

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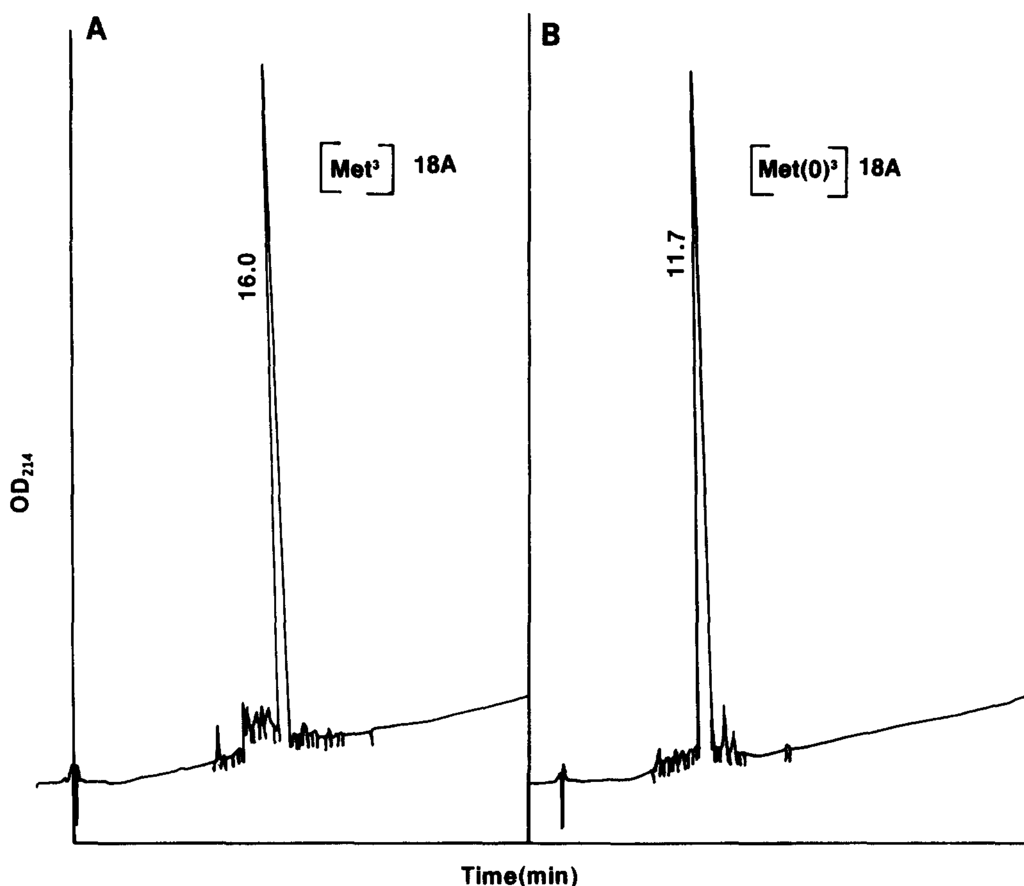

Fig. 6. HPLC chromatogram of synthetic amphipathic peptide analogs of 18A containing methionine and methionine sulfoxide. Panel A: [Met^3]18A analog. Panel B: [Met(O)^3]18A analog.

polar face directly affects lipid-associating properties (17). Oxidation of methionine to methionine sulfoxide reduces the hydrophobicity of this amino acid and, therefore, should reduce the hydrophobicity of the protein. It would be expected that a reduction in the hydrophobicity of a protein would result in a lower affinity for the reversed-phase HPLC column (shorter retention time) while a reduction in the hydrophobicity of the nonpolar face of the protein is expected to reduce the affinity for DMPC. Indeed, this was what was observed in the case of the synthetic analogs and apolipoprotein isomers. Our CD studies showed that the oxidized peptide analog, apoA-Ia, and apoA-IIc have less alpha-helicity than the corresponding reduced proteins, indicating that oxidation of methionine also alters the secondary structure of these proteins.

Since there are three methionine residues in apoA-I at positions 86, 112, and 148, which belong to three different 22mer domains of apoA-I, we could expect more than two isomeric forms on HPLC. As shown in Fig. 1, apoA-Ia and apoA-Ib are doublets which may indicate that each of these peaks contain a mixture of apoA-I isomers. It is possible that the peak designated as apoA-Ia is not completely oxidized and the peak apoA-Ib is not completely reduced. However, it is clear from the amino acid analysis and CNBr studies that A-Ia is more oxidized than A-Ib. The HPLC may not be separating pure isomeric proteins. The reason for such a difference in HPLC retention time between the oxidized and reduced forms is not clear. It is possible that certain methionine residues are more important than others for determining the HPLC retention time. This is not surprising since each of the three methionine residues is situated at the nonpolar face of three different 22mer amphipathic helical domains which are in different environments in apoA-I. The amount of oxidized and reduced apoA-I in the conventionally isolated apoA-I may vary from preparation to preparation. It may, therefore, be difficult to get consistent results in CD studies and lipid-association properties from two different batches of apoA-I.

Based on the CNBr studies (**Table 3**), we suggest that apoA-IIc is more oxidized than apoA-IIa which is more oxidized than apoA-IIb. That is, apoA-IIb has both methionines reduced, and apoA-IIc has both methionines oxidized. If this is true, then when the dimer is cleaved, apoA-IIb and apoA-IIc would be reduced to monomers with a single peak on HPLC while apoA-IIa would pro-

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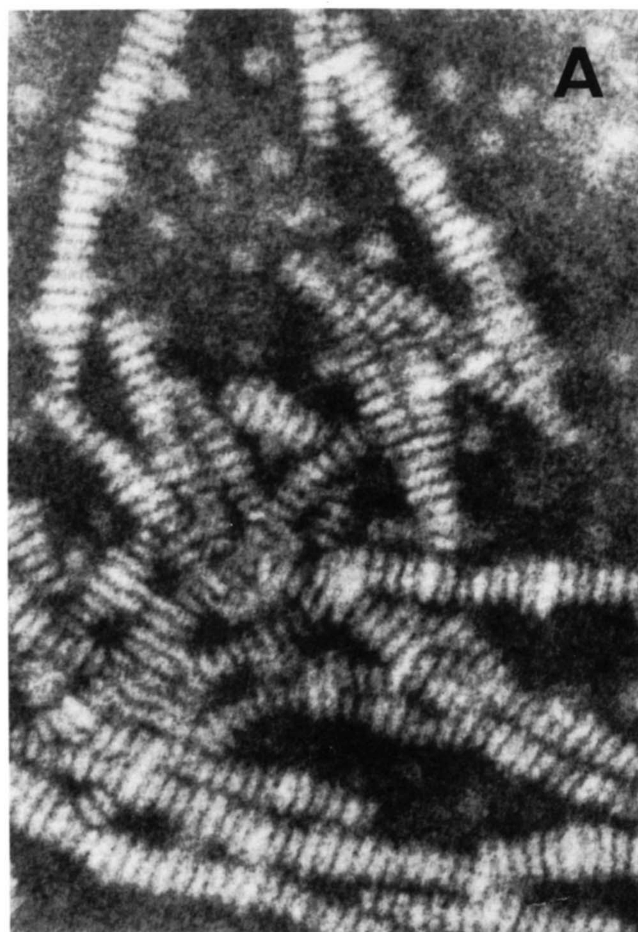

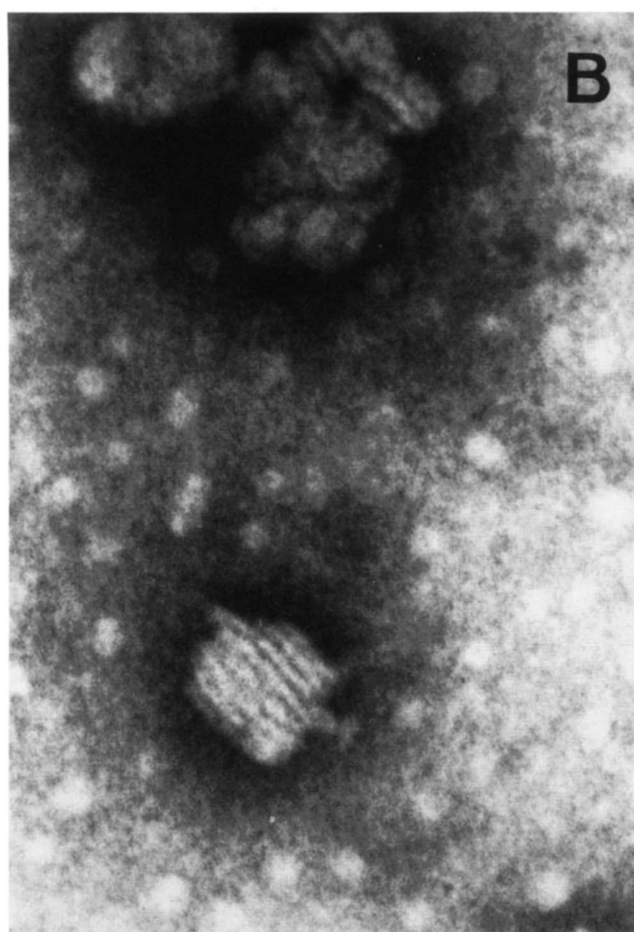

Fig. 7. Negative stain electron micrographs of complexes formed by incubation of multilamellar DMPC vesicles with peptide analogs. Panel A: [Met]18A:DMPC at a 1:1 weight ratio. Panel B: [Met(O)]18A:DMPC at a 1:1 weight ratio.

duce two peaks of equal size. This was what was observed (Fig. 5). Moreover, the first peak produced by apoA-IIa reduction with mercaptoethanol corresponds to the monomer peak produced by apoA-IIc. Similarly, the second peak produced by apoA-IIa corresponds to the monomer peak produced by apoA-IIb. Thus, our studies indicate that apoA-II exists as three isomers caused by differences in methionine oxidation. We have shown that hydrogen peroxide treatment of apoA-IIb produces the other apoA-II isomers (a and c). Since this protein has only a single methionine in each of its monomers, which would thus have identical environments, we would expect each of these residues to have an equal influence on this protein's physical properties. This appears to be the case as far as HPLC retention time is concerned. Each additional methionine oxidation reduces apoA-II retention time to a similar degree.

In summary, our data strongly suggest that methionine oxidation is a major contributor to the alteration in the HPLC retention time and lipid binding characteristics of apoA-I and apoA-II. We have successfully converted the reduced isomers to the oxidized forms with hydrogen peroxide. Since oxidized methionines are difficult to reduce with reducing agents, attempts to reduce the oxidized form have yielded mixtures that may be due to

TABLE 3. Percent helicity as determined by CD studies of the apolipoprotein isomers and peptide analogs (12)

Apolipoprotein	Buffer	DMPC 1:1
A-Ib	38.7	38.7
A-Ia	30.4	29.2
A-Ia[a]	22.6	25.0
A-IIb	35.6	46.9
A-IIa	38.5	48.6
A-IIc	29.6	39.7
[Met[3]]18A	12.9	24.7
[Met(O)[3]]18A	11.2	[a]

Protein concentrations were determined by the Coomassie Blue (Pierce Chemical Company, Rockford, IL) method and by quantitative HPLC. Fifty μg/ml of protein in 0.1 M ammonium bicarbonate buffer, pH 8.0, was used in these studies.

[a]CD could not be obtained because the solution was not clear.

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degradation of protein. We have also synthesized analogs of the amphipathic helix containing methionine or methionine sulfoxide which behave similarly to the apolipoprotein isomers on HPLC.

There are many reports in the literature that give conflicting results of apoA-I functional properties, including apoA-I self-association (4) and LCAT activation (6). We have shown that differences in the degree of methionine oxidation can alter the ability of apolipoproteins and synthetic peptides to associate with lipid. The reported differences in the properties of apoA-I may be partly due to differing amounts of apoA-Ia and A-Ib.

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