Site-specific methionine sulfoxide formation is the structural basis of chromatographic heterogeneity of apolipoproteins A-I, C-II, and C-III

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Abstract ApoA-I and apoC-II are eluted in two isoforms and apoC-III_2 is eluted in three isoforms by reversed phase high performance liquid chromatography (HPLC). The structural basis of these nongenetic heterogeneities was unravelled using HPLC of proteolytic peptides and time-of-flight secondary ion mass spectrometry (TOF-SIMS). In apoA-I, the chromatographic microheterogeneity was caused by the formation of methionine sulfoxides (MetSO). However, only residues Met_{112} and Met_{148} were found oxidized, whereas Met_{86} was unaffected and also resistant towards artificial oxidation. To assess whether and to what extent amino acid substitutions in apoA-I might affect methionine sulfoxidation, the tryptic peptides of 13 different mutant apoA-I proteins from 24 heterozygous apoA-I variant carriers were analyzed by HPLC. In normal apoA-I, the ratios $MetSO_{112}/Met_{112}$ and $MetSO_{148}/Met_{148}$ were highly variable. By contrast, the relative ratio of oxidation of methionine residues 112 and 148 was constant. The amino acid changes $Lys_{107}\rightarrow Met$, $Lys_{107}\rightarrow O$, $Glu_{139}\rightarrow Gly$, $Glu_{147}\rightarrow Val$, and $Pro_{165}\rightarrow Arg$ resulted in the preferential oxidation of Met_{112}, and $Asp_{103}\rightarrow Asn$ resulted in a preferential oxidation of Met_{148}; whereas $Pro_3\rightarrow Arg$, $Pro_3\rightarrow His$, $Pro_4\rightarrow Arg$, $Asp_{89}\rightarrow Glu$, $Ala_{158}\rightarrow Asp$, $Glu_{198}\rightarrow Lys$, and $Asp_{213}\rightarrow Gly$ had no impact. ApoC-II and apoC-III isoforms differed by the oxidation of the two methionine residues in these proteins. Whereas in apoC-II both methionine residues were oxidized in parallel, in apoC-III the two methionine residues differed in their susceptibility towards oxidation. We conclude that the formation of MetSO depends on the molecular microenvironment within a protein. —**von Eckardstein, A., M. Walter, H. Holz, A. Benninghoven, and G. Assman.** Site-specific methionine sulfoxide formation is the structural basis of chromatographic heterogeneity of apolipoproteins A-I, C-II, and C-III. *J. Lipid Res.* 1991. **32:** 1465–1476.

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Apolipoproteins undergo various posttranslational modifications, e.g., proteolytic cleavage, glycosylation, phosphorylation, acylation, deamidation, and oxidation (reviewed in 1). Some of these modifications cause electrophoretic or chromatographic heterogeneities, which have been traced back to their structural origin, e.g., to the presence of propeptides or of glycosylation (1). Other nongenetic apolipoprotein polymorphisms are not entirely understood, e.g., that of apoA-I upon isoelectric focusing (IEF). By this technique apoA-I is separated into five isoforms. The most cathodic isoform corresponds to proapoA-I_1 representing the secretion form of apoA-I (2, 3). Intraplasmatic removal of the aminoterminal hexapeptide by a yet unidentified proapoA-I-propeptidase results in mature apoA-I. The corresponding isoform, apoA-I_1, focuses two relative charge units anodic from proapoA-I_1. The other three isoforms, proapoA-I_2, apoA-I_2, and apoA-I_3, are due to nonenzymatic degradation of apoA-I_1 and proapoA-I_2, respectively, putatively either by deamidation (4) or oxidation (5). Other nongenetic protein polymorphisms have been demonstrated for apolipoproteins A-I, A-II, C-II, and C-III (6–8) by reversed phase high performance liquid chromatography (HPLC). Using this technique, apoA-I and apoC-II were eluted in two fractions, and apoA-II and apoC-III in three fractions. Based upon the results of cyanobromide cleavage studies, the chromatographic apoA-I and apoA-II isoforms were suggested to differ by their relative content of reduced and oxidized methionine residues (6).

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; Met, methionine; MetSO, methionine sulfoxide; TOF-SIMS, time-of-flight secondary ion mass spectrometry; VLDL, very low density lipoprotein.

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The functional implications of the posttranslational modifications demonstrated in apolipoproteins by IEF or HPLC are not entirely understood. Storage, alkaline treatment, and lipid peroxidation of HDL were reported to change the expression of specific apoA-I epitopes towards monoclonal antibodies (9, 10). In synthetic peptides imitating the apoA-I primary structure, the oxidation of methionine to methionine sulfoxide (MetSO) has been shown to interfere with the formation of amphipathic α-helices, the central characteristic of apoA-I necessary for its lipid binding and LCAT activation properties (6). Thus, irrespective of whether or not these nongenetic polymorphisms do occur in vivo, their structural elucidation will be important for our understanding of apolipoprotein functions at least in in vitro tests.

Recently, our laboratory reported a simple and rapid method for the structural analysis of mutant apolipoproteins using HPLC and time-of-flight secondary ion mass spectrometry (TOF-SIMS) (11). TOF-SIMS has made it possible to determine the molecular mass of peptides up to 3,000 Da with an accuracy of ± 1 Da, thus allowing precise identification of amino acid substitutions in several variants of apoA-I and apoC-III without sequencing the whole variant protein (11–15). Since certain modifications of amino acids, e.g., oxidation of methionine, cannot be unravelled by sequence analysis, we also used this technique to elucidate the structural bases of the chromatographic heterogeneities in apolipoproteins A-I, C-II, and C-III. They could be related to the presence or absence of MetSO. Detailed analysis revealed that in apoA-I only two of three methionine residues are oxidized, thus suggesting that the intramolecular microenvironment of proteins influences the formation of MetSO. This hypothesis was tested by the evaluation of 13 different natural apoA-I variants with known defects (11–13, 16–18).

MATERIALS AND METHODS

Preparation of apolipoproteins A-I, C-II, and C-III$_2$

Isoproteins of apolipoprotein A-I, C-II, and C-III were prepared from EDTA-plasma as previously described by our laboratory (11–15). ApoA-I variants were prepared after a maximum storage of 12 weeks at – 20°C. In detail, VLDL (for apoC-II and apoC-III$_2$) and HDL (for apoA-I) were isolated from plasma by sequential ultracentrifugation and delipidated with ethanol–ether 3:1 v/v. After solubilization in buffer (containing 5 mM sodium dihydrogen phosphate, 1% decylsulfate (w/w), 100 mM dithiothreitol, and 6 M urea pH 8.0), apolipoproteins were separated by IEF in preparative gels with immobilized pH gradients (Pharmacia-LKB, Bromma, Sweden) ranging from pH 3.75 to 5.25 (apoC-II and apoC-III$_2$) or from pH 4.5 to 5.5 (apoA-I) which, after washing, were rehydrated in a solution with 6 M urea and 15% glycerol (w/v). After overnight separation at 2000 V, 15 mA, 5 W and 10°C and for 4 additional hours at 3000 V, gel strips containing the isoproteins were cut out without prior staining. After electroelution for 24 h at 4°C in 0.05 M Tris-HCl buffer (pH 9.0) and dialysis against 0.01 M ammonium hydrogen carbonate (pH 7.8) for an addition 24 h the protein solutions were lyophilized and stored at – 20°C.

Demonstration of the chromatographic heterogeneity of apoA-I by reversed phase HPLC

One hundred μg apoA-I dissolved in 0.01 M ammonium hydrogen carbonate was separated by reversed phase HPLC using a C18 wide-pore column (300 Å) with a length of 250 mm and a diameter of 4 mm (Baker, Phillipsburg, NJ). The gradient was formed by two solutions with 0.1% trifluoroacetic acid (eluent A) and 100% acetonitrile in 0.1% trifluoroacetic acid (eluent B). The flow rate was 1 ml/min. The gradient was started with 40% eluent B. The acetonitrile content was linearily increased to 90% within 50 min and subsequently decreased to 40% within 10 min.

The same HPLC method was used to separate apolipoproteins of 100 μg undelipidated HDL or proteins that were precipitated from 100 μl plasma by incubation/delipidation with chloroform–methanol 2:1 for 24 h at 20°C.

Demonstration of the chromatographic heterogeneity of apoC-II and apoC-III$_2$

Chromatographic isoforms of apoC-II and C-III were separated by a procedure similar to that described above but using a different HPLC column (LiChrospher CH18, Merck, Darmstadt, Germany; length, 250 mm; diameter, 4 mm; pore width, 100 Å). The acetonitrile content was increased to 30% within the first 10 min, to 45% within the following 15 min, and to 100% within the last 10 min.

Oxidation of apoA-I

ApoA-I was oxidized using either *i*) hydrogen peroxide or *ii*) chloramine T. *i*) One mg apoA-I was dissolved in 100 mM ammonium bicarbonate and 0.5 mM EDTA (pH 8.0) to a final protein concentration of 2.5 mg/ml. Hydrogen peroxide was added to a final concentration of 200 mM and the mixture was incubated overnight at 4°C. The reaction was completed by the addition of 465 U catalase (Sigma, St. Louis, MO) for 1 h. Subsequently the mixture was lyophilized and purified by reversed phase HPLC using the 300 Å column as described above for apoA-I. Oxidation of apoA-I with hydrogen peroxide was performed in the absence and in the presence of 6 M guanidinium hydrochloride. *ii*) For oxidation with chloramine T, 1 mg apo-I or HDL (protein) was incubated with 20 μl PBS buffer containing 1 mg/ml chloramine T overnight at 37°C.

Reduction of apoA-I

For reduction studies, 1 mg apoA-I or HDL was incubated at 37°C either for 48 h in a buffer containing 0.1 M acetic acid and 5% mercaptoethanol (v/v) or, subsequent to oxidation with chloramine T, for 3 h in a buffer containing 10% dimethyl sulfide (v/v).

Proteolytic digestion of apolipoproteins

Five hundred μg lyophilized apoA-I, apoC-II, or apo-C-III_2 isoproteins were solubilized in 0.01 M ammonium hydrogen carbonate (pH 7.8) and digested either with trypsin (Cooper Biomedical, Wiesbaden, F.R.G.; sp act: 212 U/mg protein) at a ratio of 40:1 (w/w) with endoproteinase lysine C (Boehringer Mannheim GmbH, F.R.G.; sp act: 150 U/mg protein) at a ratio of 125:1 (w/w). The solutions were incubated for 24 h at 37°C.

HPLC separation of proteolytic peptides

The proteolytic digests were separated by reversed phase HPLC using a LiChrospher CH18 HPLC column (Merck, Darmstadt, F.R.G.; length, 250 mm; diameter, 4 mm; pore width, 100 Å). Conditions of eluents and flow were as described before. The acetonitrile content was increased to 40% within 55 min and to 100% in the following 2 min. A peak detector monitoring at 215 nm and a fraction collector (Pharmacia-LKB, Bromma, Sweden) were used to separately collect the peptides. Data from the peak detector were processed using a personal computer and commercially available software (Wavescan, Pharmacia-LKB, Bromma, Sweden). Peak areas of methionine as well as of MetSO-containing peptides were integrated using a subdirectory of this program (Nelson).

Characterization of proteolytic peptides by mass spectrometry and sequence analysis

The molecular masses of the peptides contained in HPLC fractions were analyzed by TOF-SIMS (11). Sequence analyses were performed by automated gas-phase protein sequencing (Applied Biosystems, Foster City, CA). The peptides were aligned to the sequences of apoA-I (19), apoC-II (20), and apoC-III (21). In the following, Tn (n = 1,2,3,...) designates tryptic peptides and Ln (n = 1,2,3,...) endoproteinase lysine C proteolytic peptides (**Table 1**, **Table 2**, **Table 3**). By the enumeration, we anticipated complete digestion of the protein at the typical digestion sites (i.e., carboxyl terminal of lysine and arginine in the case of trypsin and carboxyl terminal of lysine in the case of endoproteinase lysine C, respectively). Actually, digestion was not always complete. In these cases, the peptides were termed as Tn-(n + k) or Ln-(n + k), where n represents the amino terminal proteolytic peptide and k gives the number of consecutive potential cleavage sites.

RESULTS

Demonstration of the chromatographic heterogeneity of apoA-I

Reversed phase HPLC of isolated apoA-I, of undelipidated HDL, or of proteins obtained subsequent to a 24-h chloroform–methanol delipidation of 100 μl plasma resulted in the separation of two apoA-I isoforms, apoA-I_A and apoA-I_B (**Fig. 1**). Both isoforms exhibited a typical apoA-I banding pattern upon isoelectric focusing. By immunoblotting, both bands were shown to contain apoA-I (not shown). Proteolysis of these two chromatographic isoforms either with trypsin (**Fig. 2**) or with endoproteinase lysine C (not shown) and subsequent HPLC of the digests resulted in distinct chromatograms. They differed by the retention times and peak areas of those fractions containing tryptic peptides T16 and T22 or the endoproteinase lysine C peptides L11-12 and L15 (see Figs. 2 and 5). Upon TOF-SIMS analysis of peptides originating from isoform apoA-I_B, molecular masses of $T16_b$, $T22_b$, and L11-12_b corresponded to those that were predicted from the sequence analyses: 1283 Da, 1031 Da, and 1668 Da, respectively. By contrast, the respective apoA-I_A peptides $T16_a$, $T22_a$, and L11-12_a exceeded the molecular masses that were expected from the sequences by 16 Da. The calculated mass of L15 (4823 Da) was too high to be determined by TOF-SIMS.

Demonstration of the chromatographic heterogeneities of apolipoproteins C-II and C-III

Freshly isolated apoC-II was separated in two isoforms A and B (**Fig. 3**). Proteolysis of the two apoC-II isoforms with trypsin and subsequent HPLC resulted in chromatograms that differed by the retention times of those fractions containing peptides T1 and T7. Peptides $T1_b$ (m = 2202 Da) and $T7_b$ (m = 2232 Da) from isoform B were eluted with longer retention times and exhibited molecular masses upon TOF-SIMS that corresponded to those calculated from the sequence, whereas $T1_a$ (m = 2218 Da) and $T7_a$ (m = 2248 Da) from isoform A were eluted earlier and exhibited molecular masses exceeding the calculated values by 16 Da.

ApoC-III_2 was separated in three isoforms A, B, and C (**Fig. 4**). HPLC-chromatograms of the respective tryptic digests differed by the retention time of the fractions containing T1. Upon TOF-SIMS analysis, peptide $T1_c$ from isoform C exhibited the expected molecular mass of 1907 Da, whereas the molecular masses of $T1_a$ from isoform A (m = 1939 Da) and $T1_b$ from isoform B (m = 1923 Da) were 32 Da ($T1_a$) and 16 Da ($T1_a$) and 16 Da ($T1_b$) higher than calculated from the sequence.

TABLE 1. Tryptic and endoproteinase lysine proteolytic peptides of apoA-I

Tryptic Peptide	Lysine Peptide	Endoproteinase Sequence
T1	L1	Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-
T2		Val-Lys-
T3	L2	Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-Val-Leu-Lys-
T4	L3	Asp-Ser-Gly-Arg-
T5		Asp-Tyr-Val-Ser-Gln-Phe-Gln-Gly-Ser-Ala-Leu-Gly-Lys-
T6	L4	Gln-Leu-Asn-Leu-Lys-
T7	L5	Leu-Leu-Asp-Asn-Try-Asp-Ser-Val-Thr-Ser-Thr-Phe-Ser-Lys-
T8	L6	Leu-Arg-
T9		Glu-Gln-Leu-Gly-Pro-Val-Thr-Gln-Glu-Phe-Try-Asp-Asn-Leu-Glu-Lys-
T10	L7	Glu-Thr-Glu-Gly-Leu-Arg-
T11		Gln-Glu-Met-Ser-Lys-
T12	L8	Asp-Leu-Glu-Glu-Val-Lys-
T13	L9	Ala-Lys-
T14	L10	Val-Gln-Pro-Tyr-Leu-Asp-Asp-Phe-Gln-Lys-
T15	L11	Lys-
T16	L12	Try-Gln-Glu-Glu-Met-Glu-Leu-Tyr-Arg-
T17		Gln-Lys-
T18	L13	Val-Glu-Pro-Leu-Arg-
T19		Ala-Glu-Leu-Gln-Glu-Gly-Ala-Arg-
T20		Gln-Lys-
T21	L14	Leu-His-Glu-Leu-Gln-Glu-Lys-
T22	L15	Leu-Ser-Pro-Leu-Gly-Gln-Gln-Met-Arg-
T23		Asp-Arg-
T24		Ala-Arg-
T25		Ala-His-Val-Asp-Ala-Leu-Arg-
T26		Thr-His-Leu-Ala-Pro-Tyr-Ser-Asp-Glu-Leu-Arg-
T27		Gln-Arg-
T28		Leu-Ala-Ala-Arg-
T29		Leu-Glu-Ala-Leu-Lys-
T30	L16	Glu-Asn-Gly-Gly-Ala-Arg-
T31		Leu-Ala-Glu-Tyr-His-Ala-Lys-
T32	L17	Ala-Thr-Glu-His-Leu-Ser-Thr-Leu-Ser-Glu-Lys-
T33	L18	Ala-Lys-
T34	L19	Pro-Ala-Leu-Glu-Asp-Leu-Arg-
T35		Gln-Gly-Leu-Leu-Pro-Val-Leu-Glu-Ser-Phe-Lys-
T36	L20	Val-Ser-Phe-Leu-Ser-Ala-Leu-Glu-Glu-Tyr-Thr-Lys-
T37	L21	Lys-
T38	L22	Leu-Asn-Thr-Gln-

The apoA-I sequence is from reference 19. Methionine residues (Met_{86}, Met_{112}, and Met_{148}) are underlined.

Impact of oxidation and reduction on the chromatographic heterogeneity of apoA-I

Peptides T16, T22, L11-12, and L15 from apoA-I, and peptides T1 and T7 from apoC-II contain one methionine residue, and peptide T1 from apoC-III_2 contains two methionines. Thus, the discrepancy between the results from molecular mass and sequence analyses could be explained if these methionine residues were assumed to be present either in the reduced or in the oxidized state, as the presence of MetSO would add a molecular mass of 16

TABLE 2. Tryptic peptides of apoC-II

Peptide	Sequence
T1	Thr-Gln-Gln-Pro-Gln-Gln-Asp-Glu-Met-Pro-Ser-Pro-Thr-Phe-Leu-Thr-Gln-Val-Ly-
T2	Glu-Ser-Leu-Ser-Ser-Tyr-Trp-Glu-Ser-Ala-Lys-
T3	Thr-Ala-Ala-Gln-Asn-Leu-Tyr-Glu-Lys-
T4	Thr-Tyr-Leu-Pro-Ala-Val-Asp-Glu-Lys-
T5	Leu-Arg-
T6	Asp-Leu-Tyr-Ser-Lys-
T7	Ser-Thr-Ala-Ala-Ala-Met-Ser-Thr-Tyr-Thr-Gly-Ile-Phe-Thr-Asp-Gln-Val-Leu-Ser-Leu-Lys-
T8	Gly-Glu-Glu-

The apoC-II sequence is from reference 20. Methionine residues ($Met_{\cdot}$ and Met_{60}) are underlined.

TABLE 3. Tryptic peptides of apoC-III

Peptide	Sequence
T1	Ser-Glu-Ala-Glu-Asp-Ala-Ser-Leu-Leu-Ser-Phe-Met-Gln-Gly-Tyr-Met-Lys-
T2	His-Ala-Thr-Lys-
T3	Thr-Ala-Lys-
T4	Asp-Ala-Leu-Ser-Ser-Val-Gln-Ser-Gln-Gln-Val-Ala-Ala-Gln-Gln-Arg-
T5	Gly-Trp-Val-Thr-Asp-Gly-Phe-Ser-Ser-Leu-Lys-
T6	Asp-Tyr-Trp-Ser-Thr-Val-Lys-
T7	Asp-Lys-
T8	Phe-Ser-Glu-Phe-Trp-Asp-Leu-Asp-Pro-Glu-Val-Arg-
T9	Pro-Thr*-Ser-Ala-Val-Ala-Ala-

The apoC-III sequence is from reference 21. Methionine residues (Met_{12} and Met_{16}) are underlined. The glycosylation site is marked by an asterisk (Thr_{64}).

Da. To verify oxidation of methionine as the basis of its chromatographic microheterogeneity, apoA-I was oxidized by the addition of hydrogen peroxide. Theoretically, methionines in positions 86, 112, and 148 of apoA-I should be subject to oxidation, and therefore an attempt was made to analyze the chromatographic behavior and molecular masses of peptides encompassing these residues. After extensive oxidation of either isolated apoA-I or HDL with hydrogen peroxide, apoA-I was eluted by HPLC in one single fraction that exhibited the retention time of apoA-I_A (see inset of Fig. 5 bottom and Fig. 6b). Oxidation of apoA-I or HDL with chloramine T was milder than with hydrogen peroxide and did lead to an increase of apoA-I_A but not to the complete disappearance of apoA-I_B.

Fig. 5 shows the chromatograms obtained from apoA-I by endoproteinase lysine C digestion before (top) and after (bottom) oxidation with hydrogen peroxide. After oxidation, the peak areas of L11-12_a (m = 1686 Da) and $L15_a$ had increased and L11-12_b (m = 1668 Da) and $L15_b$ had disappeared. The molecular mass of the Met_{148}-containing fraction L15 (m = 4823 Da) exceeded the range of TOF-SIMS and was therefore searched by sequence analysis. Before and after oxidation, the Met_{86}-containing peptide L7 was eluted in single fractions exhibiting identical retention times and molecular masses upon TOF-SIMS (m = 1307 Da). This molecular mass was also predicted from the sequence and suggested that Met_{86} was not oxidized. In principal, identical observations were made for apoA-I trypsinized before and after oxidation: before oxidation, T16 and T22 were eluted in two fractions differing by moelcular masses of 16 Da. After oxidation, $T16_b$ (m = 1283 Da) and $T22_b$ (m = 1031 Da) disappeared, whereas the peak areas of $T16_a$ (m = 1299 Da) and $T22_a$ (m = 1047 Da) increased. Before and after oxidation T11 (m = 621 Da) was eluted in one single fraction exhibiting the identical retention time and molecular mass, which was predicted

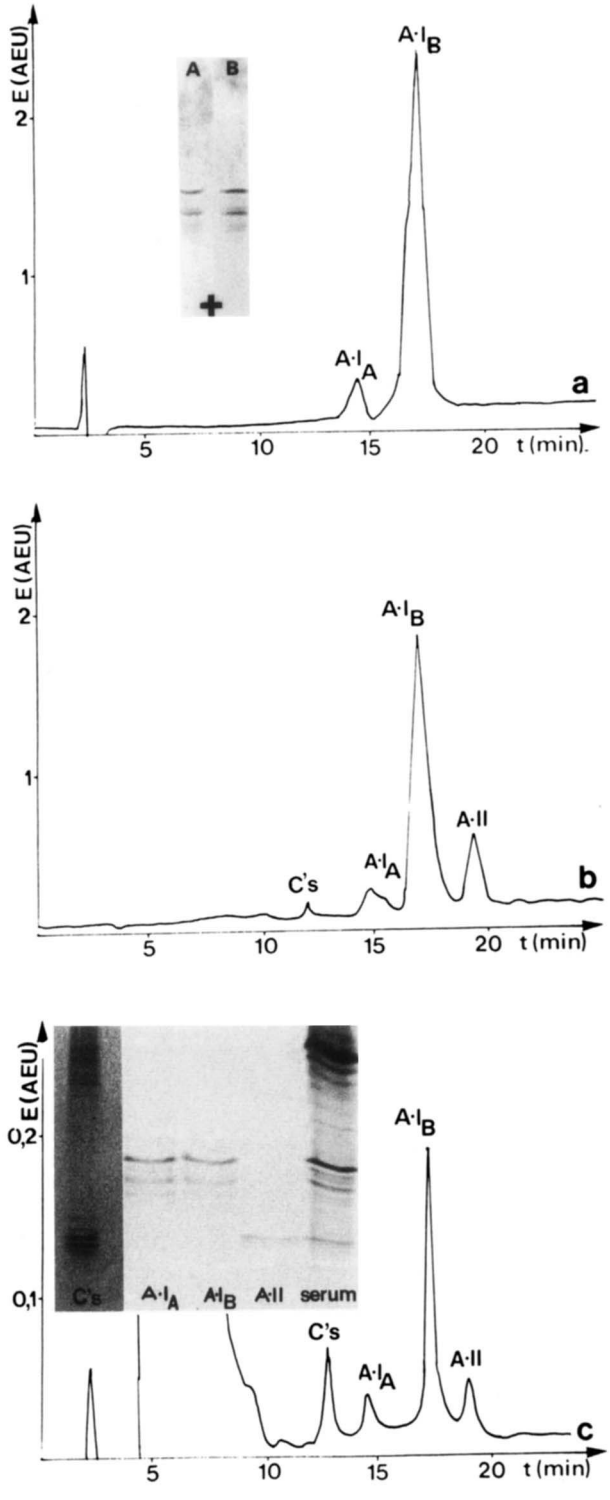

Fig. 1. Demonstration of the chromatographic isoforms of apoA-I by reversed phase HPLC of purified apoA-I (a), HDL (b) and immunoprecipitated plasma (c). Insets in a and b show IEF patterns from the various fractions. For the description of methods, see text. Shoulders on either side of peaks B may reflect the presence of additional hitherto unknown posttranslational modifications of apoA-I.

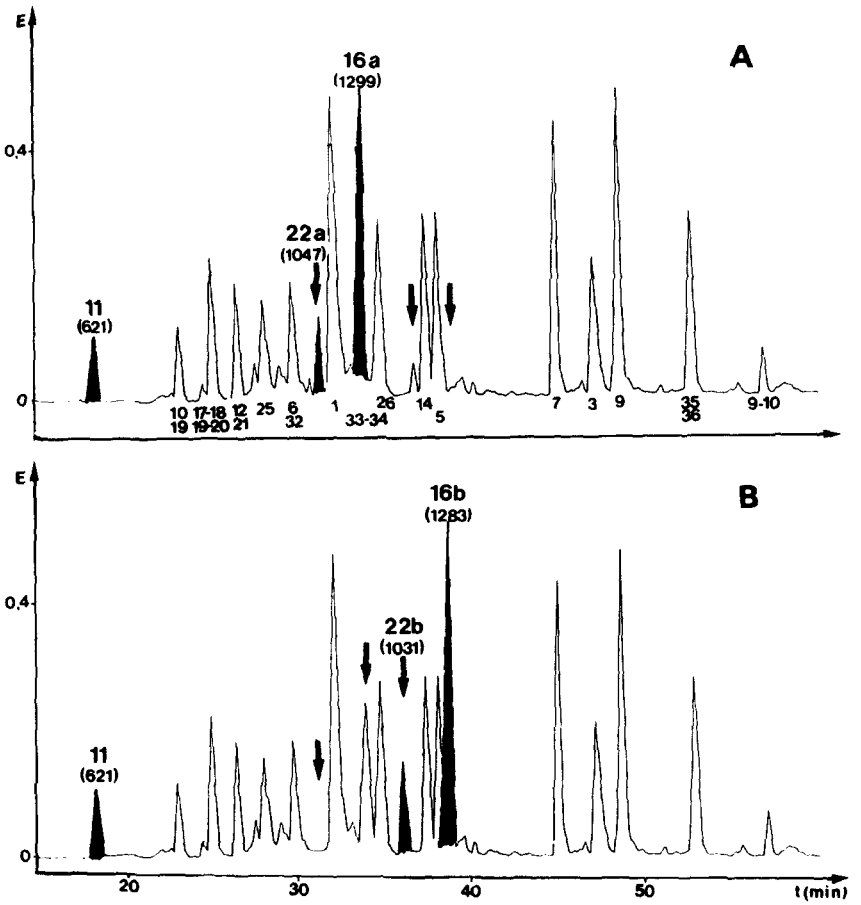

Fig. 2. HPLC separation of peptides obtained from tryptic digestion of the chromatographic apoA-I isoforms. For the digestion and the chromatographic conditions see Materials and Methods. Chromatogram A corresponds to isoform apoA-I_A, and chromatogram B to isoform apoA-I_B in Fig. 1a. Note that the chromatograms differ by the retention times and peak areas of the methionine-containing fractions T16 and T22 (hatched) and that the molecular masses of these peptides differ by 16 Da (numbers in parentheses) as measured by TOF-SIMS. The enumeration (n = 1,2,3...36) in the bottom of A refers to the enumeration of tryptic peptides as described in Table 1.

from its sequence. Thus, analysis of the three methionines present in normal apoA-I revealed that Met_{112} and Met_{148} can be oxidized whereas Met_{86} is inert also towards artificial oxidation.

In order to analyze whether Met_{86} would be oxidized in addition to methionine residues 112 and 148 under denaturing conditions, apoA-I was also oxidized in the presence of 6 M guanidinium hydrochloride (**Fig. 6**) or 4 M urea (not shown). The oxidation of three instead of two methionine residues theoretically should result in the formation of an HPLC fraction which is eluted slightly earlier than apoA-I_A. This was not the case. Instead, oxidation under denaturing conditions led to the disappearance of peaks A and B and the very fast elution of a single peak (Fig. 6D).

In order to analyze whether methionine-sulfoxidized apoA-I could be reduced, apoA-I was incubated with 5% mercaptoethanol for 48 h. Subsequent trypsinization resulted in a chromatogram that was identical to that without mercaptoethanol (Fig. 1a). Methionine-containing peptides T16 and T22 were eluted in two fractions exhibiting the described molecular mass differences of 16 Da. These results indicated that mercaptoethanol cannot revert methionine sulfoxidation in apoA-I. By contrast, as is also the case for other proteins (22), methionine sulfoxides of apoA-I could be reduced by dimethyl sulfide. Incubation with dimethyl sulfide and subsequent HPLC of apoA-I or HDL that were previously oxidized by chloramine T resulted in the elution of apoA-I in one single peak that exhibited the retention time of apoA-I_B.

Consequence of amino acid substitutions for the formation of methionine sulfoxides in apoA-I

Interestingly, Met_{112} and Met_{148} exhibited identical properties towards oxidation: either none (apoA-I_B) or both methionines (apoA-I_A) were oxidized. This finding

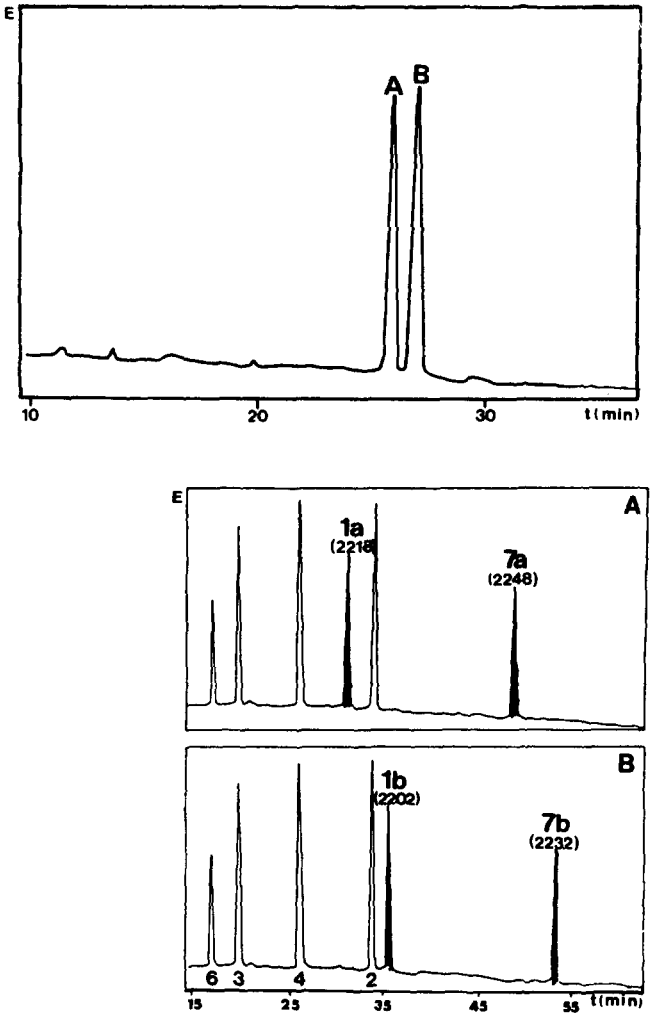

Fig. 3. Demonstration of the chromatographic microheterogeneity of apoC-II (top) and the chromatograms obtained after trypsinization of the chromatographic apoC-II isoforms A and B. For experimental details see text. Note the different retention times and molecular masses of peptides T1 and T7 obtained from the two isoforms. The enumeration (n = 1,2,3...7) below the tryptic peptides refers to the enumeration of tryptic apoC-II peptides as described in Table 2.

as well as the missing oxidation of Met_{86} suggested that methionine sulfoxide formation in apolipoproteins is a function of the protein's primary and secondary structure. Therefore, in order to study the impact of amino acid changes within apoA-I on the oxidation pattern of methionine residues, we evaluated the chromatograms of trypic peptides derived from normal and mutant apoA-I proteins (11-13, 16-18):$Pro_3 \rightarrow Arg$ (n = 1), $Pro_3 \rightarrow His$ (n = 1), $Pro_4 \rightarrow Arg$ (n = 2), $Asp_{89} \rightarrow Glu$ (n = 1), $Asp_{103} \rightarrow Asn$ (n = 1), $Lys_{107} \rightarrow O$ (n = 5), $Lys_{107} \rightarrow Met$ (n = 1), $Glu_{139} \rightarrow Gly$ (n = 2), $Glu_{147} \rightarrow Val$ (n = 1), $Ala_{158} \rightarrow Asp$ (n = 1), $Pro_{165} \rightarrow Arg$ (n = 4), $Glu_{198} \rightarrow Lys$ (n = 2), and $Asp_{213} \rightarrow Gly$ (n = 1) (**Table 4**).

In apoA-I ($Lys_{107} \rightarrow Met$), two methionines are present in the mutated peptide T16*, Met_{107} and Met_{112}. This peptide was eluted in two fractions exhibiting molecular masses of 1430 and 1446 Da, both exceeding the molecular mass calculated from the sequence of this peptide (1414 Da). This suggested that one of the two methionines present in T16* is only present in its oxidized form. In contrast to normal apoA-I, peptide T22 from apoA-I($Lys_{107} \rightarrow Met$) containing Met_{148} was detected only in its reduced form. A similar absolute change in the oxidation/reduction of methionine residues 112 and 148 was observed in apoA-I($Glu_{147} \rightarrow Val$): the mutated peptide T22* containing Met_{148} was detected only in its reduced form with m = 1001 Da, whereas peptide T16

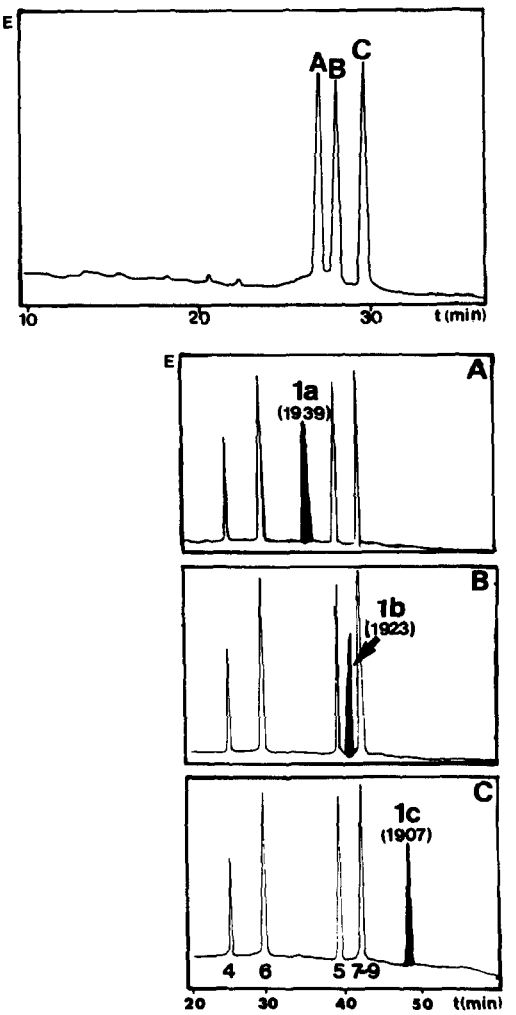

Fig. 4. Demonstration of the chromatographic microheterogeneity of apoC-III_2 (top) and the chromatograms obtained after trypsinization of the chromatographic apoC-III isoforms A, B, and C. For experimental details see text. Note the different retention times and molecular masses of peptide T1 from the three isoforms. The enumeration (n = 1,2,3...9) below the tryptic peptides refers to the enumeration of tryptic apoC-III_2 peptides as described in Table 3.

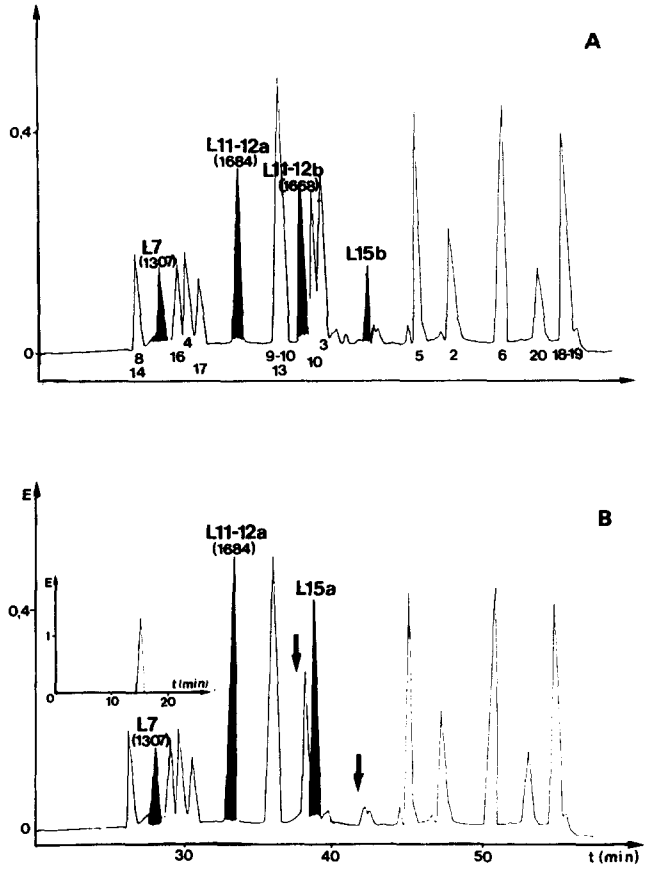

Fig. 5. Demonstration of the effect of oxidation on the chromatographic microheterogeneity of apoA-I. Chromatographic conditions were similar to those in Fig. 2. Oxidation of 1 mg apoA-I was performed overnight at 4°C in a solution containing 2.5 mg/ml apoA-I, 200 mM H_2O_2, and 0.5 mM EDTA (pH 8.0). A: Chromatogram of apoA-I digested with endoproteinase lysine C before oxidation. B: Chromatogram of apoA-I after oxidation (inset) and after subsequent digestion with endoproteinase lysine C. Oxidation yields one single fraction with apoA-I exhibiting the retention time of the chromatographic isoform apoA-I_A in Fig. 1. Note that only L11-12_a (m = 1684 Da) is present in B and that L15_a (in B and A) exhibits a shortened retention compared to L15b in A. Also note that in both chromatograms L7 exhibits identical retention times and molecular masses (m = 1307 Da). The enumeration (n = 1,2,3...20) in the bottom of figure A refers to the enumeration of endoproteinase lysine proteolytic peptides as described in Table 1.

containing Met_{112} was found only in its oxidized form (m = 1299 Da).

In order to study the degree of methionine oxidation in positions 112 and 148 in other apoA-I variants, peak areas of the MetSO-containing tryptic peptides $T16_a$ (m = 1299 Da) and $T22_a$ (m = 1047 Da) were related to the peak areas of the corresponding reduced methionine-containing peptides $T16_b$ (m = 1283 Da) and $T22_b$ (m = 1031 Da), respectively (Table 4). In normal apoA-I (n = 24), these two ratios $Ox_{T16} = T16_a/T16_b = 0.51 \pm 0.50$ and $Ox_{T22} = T22_a/T22_b = 0.38 \pm 0.31$ were characterized by high standard deviations, implying that the oxidative state of apoA-I exhibits a high variability. However, the ratio $Ox_{rel} = Ox_{T16}/Ox_{T22}$ reflecting the relative oxidation of these two methionines was found constant (1.06 ± 0.11, n = 24). Table 4 summarizes the evaluations of 13 different apoA-I variants from 24 different apoA-I variant carriers. The substitutions $Pro_3 \rightarrow$ Arg, $Pro_3 \rightarrow$His, $Pro_4 \rightarrow$Arg, $Asp_{89} \rightarrow$Glu, $Ala_{158} \rightarrow$Asp, $Glu_{198} \rightarrow$Lys, and $Asp_{213} \rightarrow$Gly did not result in any preferential oxidation of one of the two methionines. The amino acid changes $Lys_{107} \rightarrow$O ($P < 0.001$, n = 5, two-tailed t-test), $Glu_{139} \rightarrow$Gly ($P < 0.05$, n = 2), and $Pro_{165} \rightarrow$Arg ($P < 0.001$, n = 4), however, resulted in the preferential oxidation of Met_{148}. The substitution of Asp_{103} by Asn resulted in a preferential oxidation of Met_{112} ($P < 0.01$, n = 1).

To analyze whether the electrophoretic heterogeneity of apoA-I is accompanied by changes in the relative content of MetSO, the electrophoretic isoforms (apoA-I_1 and apoA-I_2) were isolated from normal individuals by preparative IEF and digested with trypsin. Chromatograms of both isoforms were identical and did not reveal any differences in Ox_{T16}, Ox_{T22}, and Ox_{rel}.

DISCUSSION

Apolipoproteins A-I, A-II, C-II, and C-III were previously reported to exhibit nongenetic polymorphisms upon reversed phase HPLC (6–8): apoA-I and apoC-II were eluted in two fractions, and apoA-I and apoC-III in three fractions (Figs. 1, 3, and 4). Anantharamaiah and colleagues (6) suggested that the chromatographic heterogeneity of apoA-I and apoA-II results from the formation of methionine sulfoxides in these proteins, as the isoforms with the shorter retention time were not fragmented by CNBr in contrast to those with the longer retention time. In this report, we elucidated the structural basis of the chromatographic polymorphism of apoA-I, apoC-II, and apoC-III using reversed phase HPLC of proteolytic peptides and a highly sensitive mass-spectrometric technique (TOF-SIMS) (11). Thus, our data gave more direct and more differentiating evidence for the assumption that methionine sulfoxide formation is the structural basis for the chromatographic polymorphism of apoA-I, but also of apoC-II and apoC-III; methionine-containing proteolytic peptides from the faster eluted isoforms apoA-I_A, apoC-II_A, and apoC-III_A exhibited shorter retention times and molecular masses 16 Da (apoA-I_A, apoC-II_A, and apoC-III_B) or 32 Da (apoC-III_A) higher than those from the slower eluted isoforms apoA-I_B, apoC-II_B, or apoC-III_C, respectively. Only peptides from apoA-I_B, apoC-II_B, and apoC-III_C exhibited molecular masses on TOF-SIMS that were predicted from the primary structure. The molecular mass difference of 16 Da corresponds to that of one oxygen atom, which alters methionine to methionine sulfoxide. Artificially oxidated apoA-I was eluted in one

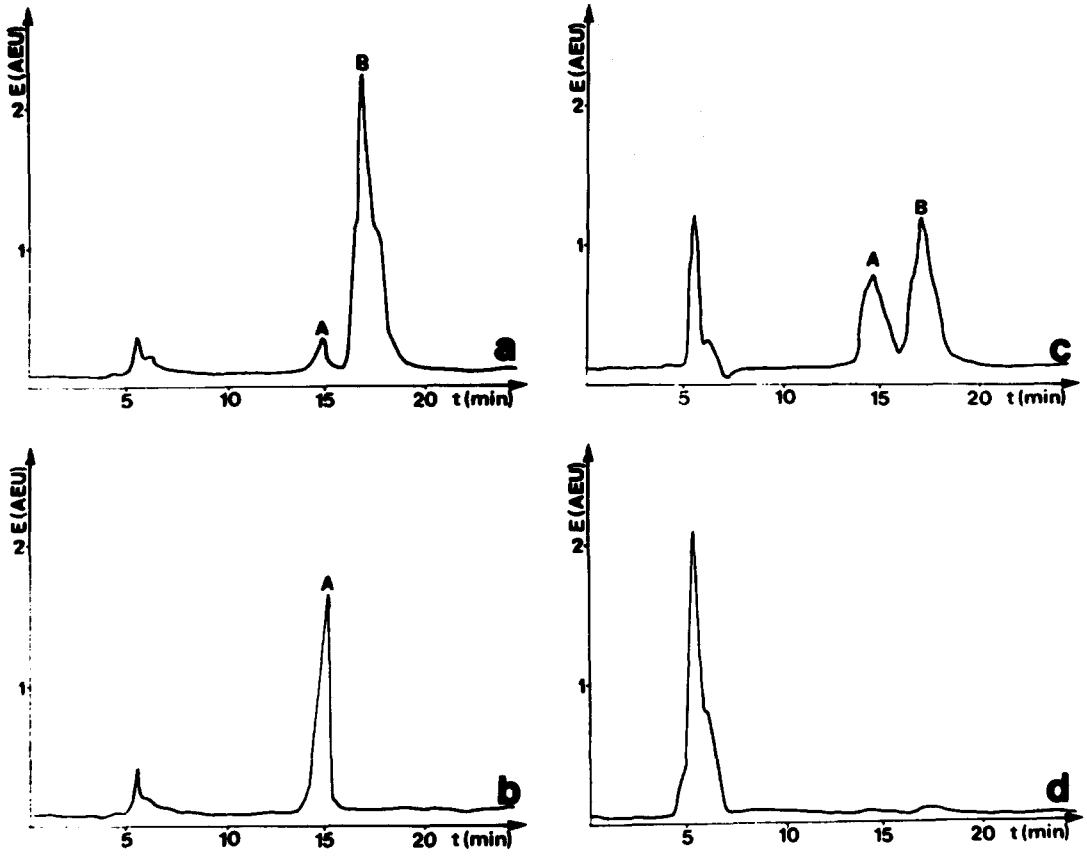

Fig. 6. Impact of oxidation under nondenaturing and denaturing conditions on the chromatographic heterogeneity of apoA-I. HPLC separation of freshly prepared apoA-I before (a) and after oxidation with hydrogen peroxide (b), after 16 h incubation with 6 M guanidine hydrochloride plus hydrogen peroxide.

single isoform exhibiting the retention time of apoA-I_A. This isoform, subsequent to proteolysis with either trypsin or endoproteinase lysine C, yields peptide patterns characteristic of native apoA-I_A (Fig. 5). Thus, there is convicing evidence that the chromatographic isoforms of apoA-I, apoC-II, and apoC-III differ by the presence and absence of methionine sulfoxides.

Theoretically, successive oxidation of the two methionine residues present in apoC-II and apoC-III and of the three methionine residues present in apoA-I should

TABLE 4. Impact of amino acid substitutions on the formation of MetSO in apoA-I

Phenotype	n	Ox_{T16}[a]	Ox_{T22}	Ox_{rel}[b]	P[c]
Normal apoA-I	24	0.51 ± 0.50	0.38 ± 0.31	1.06 ± 0.11	
$Pro_3 \rightarrow Arg$	1	0.51	0.53	0.96	n.s.
$Pro_3 \rightarrow His$	1	0.81	0.84	0.96	n.s.
$Pro_3 \rightarrow Arg$	2	0.35 ± 0.11	0.45 ± 0.25	0.91 ± 0.28	n.s.
$Asp_{89} \rightarrow Glu$	1	0.96	0.81	1.14	n.s.
$Asp_{103} \rightarrow Asn$	1	0.62	0.78	0.79	<0.05
$Lys_{107} \rightarrow 0$	5	0.96 ± 0.42	0.47 ± 0.20	2.04 ± 0.22	<0.001
$Glu_{139} \rightarrow Gly$	2	0.96 ± 0.42	0.77 ± 0.30	1.84 ± 0.01	<0.001
$Ala_{158} \rightarrow Asp$	1	0.47	0.45	1.04	n.s.
$Pro_{165} \rightarrow Arg$	4	0.72 ± 0.20	0.36 ± 0.17	1.92 ± 0.41	<0.001
$Glu_{198} \rightarrow Lys$	2	0.50 ± 0.04	0.46 ± 0.06	1.08 ± 0.03	n.s.
$Asp_{213} \rightarrow Gly$	1	0.77	0.68	1.14	n.s.

In apoA-I($Lys_{107} \rightarrow Met$) (n = 2. and apoA-I($Glu_{147} \rightarrow Val$) (n = 1) the amino acid changes led to gross changes in the oxidation of methionine residues 112 and 148. For details see text.

[a] $Ox_{T16} = T16_a/T16_b$, $Ox_T = T22_a/T22_b$.

[b] $Ox_{rel} = Ox_{T16}/Ox_{T22}$.

[c] P was determined by two tailed *t*-test comparing Ox_{rel} of normal and variant apoA-I; n.s., not significant. The variants were reported in references 22–26.

give rise to three (apoC-II and apoC-III) and four chromatographic isoforms (apoA-I), respectively. However, this was the case only for apoC-III (Fig. 4), whereas apoA-I and apoC-II were eluted in two fractions (Figs. 1 and 3). Detailed analyses of chromatograms of the proteolytic peptides of apoC-II and apoA-I revealed that methionine residues 112 and 148 in apoA-I and methionine residues 9 and 60 in apoC-II are only oxidized in parallel (Figs. 2 and 3). In apoA-I, residue Met_{86} remained unoxidized even under such artificial oxidative conditions that led to the complete oxidation of Met_{112} and Met_{148} (Fig. 5). These findings suggested that the intramolecular microenvironment within the apolipoproteins influences the formation of MetSO in apolipoproteins. Further confirmation for this hypothesis was derived from the analyses of MetSO formation in naturally occurring apoA-I variants. In normal apoA-I, the ratios $MetSO_{112}/Met_{112}$ and $MetSO_{148}/Met_{148}$ were found to be highly variable. By contrast, the relative ratio of oxidated methionines 112 and 148 was constant, implying that both residues are exposed to oxidants in a direct proportional degree (Table 4). Some naturally occurring single amino acid changes ($Asp_{103}\rightarrow$Asn, $Lys_{107}\rightarrow$O, $Lys_{107}\rightarrow$Met, $Glu_{139}\rightarrow$Gly, $Glu_{147}\rightarrow$Val, and $Pro_{165}\rightarrow$Arg) influenced this relative ratio $MetSO_{112}/MetSO_{148}$ significantly, suggesting that these changes altered the exposition of methionine residues 112 and 148 towards oxidants.

Previously, two apoA-I variants were assumed to have undergone conformational changes in their α-helical domain: apoA-I($Lys_{107}\rightarrow$O) and apoA-I($Pro_{165}\rightarrow$Arg). *i*) The amino acid deleted in apoA-I($Lys_{107}\rightarrow$O) is located within a 22-amino acid repeat which presumably forms an amphipathic α-helix. The $lysine_{107}$ deletion would alter the orientation of hydrophilic and hydrophobic faces by approximately 100° (17). This change was responsible for the impaired lipid binding and LCAT activating properties of this mutant (17, 23, 24). Further, recent studies revealed alterations in the intrinsic tryptophan fluorescence properties of reconstituted HDL containing apoA-I($Lys_{107}\rightarrow$O) indicative of conformational changes in the α-helix containing lysine residue 107 and tryptophan residue 108 (24). The change in the relative oxidation pattern of Met_{112} and Met_{148} is one further indication that conformation changes have taken place in apoA-I($Lys_{107}\rightarrow$O). *ii*) Pro_{165} putatively plays a crucial role in the formation of a β-turn between two neighboring α-helices. Application of the Chou-Fasman algorithm suggested that the Pro→Arg replacement makes the formation of a β-turn more unlikely (12). The elimination of a β-turn, however, should alter the relative orientation of the two neighboring helices. As a consequence, several functional implications were found: HDL-deficiency in apoA-I($Pro_{165}\rightarrow$Arg) heterozygotes (12), diminished LCAT cofactor activity of the mutant apoA-I (24), and in these studies, an altered $MetSO_{112}/MetSO_{148}$ratio.

From our data we cannot conclude whether sulfoxidation of methionine residues 112 and 148 in apoA-I has occurred in vivo or in vitro during the preparation procedure. However, as Met_{86} was only observed in its reduced state, it is clear that this residue is protected from oxidation in any case. Site-specific oxidation of methionine residues has also been observed in other proteins, e.g., in human chorionic somatomammotropin, in human growth hormone (25), or in the human β-globin chain (26). It has been hypothesized that the accessibility of a methionine residue within a protein to oxidation depends on its exposure towards oxidizing agents in its environment (25). Likely, methionine residues buried in the protein are not available, whereas those on the protein's surface are accessible to oxidative modification. Because the detailed conformation of apoA-I is as yet unknown, the site-specific oxidation of methionine residues could be relevant to explore whether defined methionine residues are exposed at the lipoprotein surface or buried within the core. Due to secondary structure models, apoA-I is supposed to consist of several amphipathic α-helices, with their hydrophilic sites exposed to the plasma and their hydrophobic sites exposed to the lipid moiety of lipoproteins (1). Because of their hydrophobicity, methionine residues in apoA-I are assumed to be confronted with the lipid core. Typical amphipathic apoA-I α-helices are composed of homologous tandem repeats of 11 amino acids and punctuated by proline residues that form β-turns between two adjacent helices, thus optimizing their interactions with lipids. Interestingly, the α-helix encompassing residues 66→87 and containing the protected Met_{86} is different from other α-helices including those containing the unprotected Met_{112} and Met_{148}. *i*) In contrast to other 22-amino acid repeats, its primary structure is highly conserved interspecies (13). *ii*) Acidic residues are normally located in the center of the polar faces of the helices, but in helix 66→87, Glu_{78} is located on the hydrophobic site. *iii*) In contrast to other helices, helix 66→87 is not separated from the following by a proline residue. *iv*) The following repeat 88→98 encompasses only 11 amino acids. Therefore, and because it contains a methionine residue that is inaccessible to oxidation, α-helix 88→98 appears to exhibit a conformation and orientation that is different from other α-helices in apoA-I. Recently, the existence of such an atypical α-helix in apoA-I has been proposed by Cheung and colleagues (27). This so-called hinged domain is thought to be formed by two adjacent α-helices, to be reversibly associated with HDL lipids, to interact with other apolipoproteins, and to regulate the interaction of apoA-I with LCAT (27-29). Interestingly, the highly conserved helix 66→87 contains Glu_{78}, which has been postulated to play a crucial role in LCAT activation (29), as well as Met_{86}, which is stable towards oxidation. Further research is necessary to establish whether these coincidences are caused by the existence of a hinged domain.

In this report we did not unravel whether methionine sulfoxide formation in apolipoproteins occurs in vivo or in vitro and, if it does occur in vivo, whether it is of physiological importance. The demonstration of the chromatographic heterogeneity in isolated apoA-I, in HDL, and in freshly delipidated plasma may account for the occurrence of this posttranslational modification in vivo. This view is furthermore supported by our observation that, in fresh plasma samples, the ratio oxidized/unoxidized apoA-I (apoA-I_A/apoA-I_B) exhibits considerable interindividual variability (A. von Eckardstein and G. Assmann, unpublished observation). In several other proteins, oxidation of methionine residues was shown to occur both in vivo and in vitro and to impair their functions (30, 31). For example, sulfoxidation of Met_{358} in α_1-antitrypsin results in the complete loss of the protein's activity to inactivate elastase. This is considered of key importance in the pathogenesis of lung emphysema and rheumatoid arthritis (31). In lipoprotein metabolism, lipid peroxidation in LDL and the resulting structural changes in apoB (e.g., lysine modification) appear to be a central step for the recognition of modified LDL by macrophage scavenger receptors and thereby for atherogenesis (reviewed in 32). HDL was shown to inhibit LDL oxidation (33). This raises the question whether this process leads to oxidation of lipids and proteins in HDL. The impact of lipid peroxidation in HDL on apoA-I structure is reflected by the altered expression of specific epitopes for monoclonal antibodies against apoA-I (9, 10). It remains to be established whether or not this modification of apoA-I is due to methionine sulfoxide formation. In model peptide analogs of apoA-I, methionine sulfoxidation changed the secondary structure and lipid binding properties (6). Although the physiological importance of methionine sulfoxidation in apolipoproteins A-I, C-II, and C-III remains to be established, our finding of MetSO in isolated apoC-II, apoC-III, and apoA-I is important in two ways. *i*) The knowledge might be important for the interpretation of functional assays performed with isolated apolipoproteins. *ii*) Our observation of site-specific methionine sulfoxidation in apoA-I, which can be influenced by amino acid substitutions, is important for the general understanding of oxidation of amino acids in proteins (e.g., of methionines). Oxidation of a protein is not only a function of the concentrations of oxidants and antioxidants in its environment and of its time of exposure towards this environment (i.e., biological life time), but also of its structure.

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