High performance liquid chromatography and time-of-flight secondary ion mass spectrometry: a new dimension in structural analysis of apolipoproteins

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Abstract We report the isolation and characterization of an apolipoprotein A-I mutant using a new technique for structural analysis of apolipoproteins based upon the combined techniques of protein isolation by isoelectric focusing in immobilized pH-gradients, reversed-phase HPLC of tryptic peptides, and subsequent molecular weight analysis of isolated peptides by time-of-flight secondary ion mass spectrometry (TOF-SIMS). The particular advantages of the TOF-SIMS procedure in the characterization of proteolytic peptides are the detection limits in the picomole range, the accuracy of molecular weight determinaton (up to 3000 ± 1 D), the speed of analysis, and the wide range of applications for involatile biomolecules. The described procedure for the analysis of apolipoproteins requires only 2 ml of serum as starting material. This method can be used to monitor for genetic polymorphisms and posttranslational modifications on a microscale basis. Applying these techniques, we characterized a new apolipoprotein A-I mutant with an amino acid exchange arginine$_{177}$ by histidine. —**Jabs, H-U., G. Assmann, D. Greifendorf, and A. Benninghoven.** High performance liquid chromatography and time-of-flight secondary ion mass spectrometry: a new dimension in structural analysis of apolipoproteins. *J. Lipid Res.* 1986. **27:** 613–621.

Supplementary key words apolipoprotein mutants • structural analysis • focusing in immobilized pH-gradients • apoA-I

Lipoproteins are complexes composed of apolipoproteins and non-covalently bound lipids. A major structural component of high density lipoproteins (HDL) is apolipoprotein A-I (apoA-I). This apolipoprotein binds lipids, serves as an activator for the cholesterol-esterifying enzyme lecithin:cholesterol acyltransferase (LCAT), and interacts in the cellular recognition of HDL with plasma membrane receptors (1–3). The study of apolipoprotein mutants is of great interest for the further understanding of the metabolic functions of apolipoproteins. In the past, several structural variants of apoA-I have been detected through the screening analysis of human serum by isoelectric focusing procedures (4–7). These variants have the same immunologic characteristics and about the same molecular weights as the normal apoA-I, but differ in charge due to amino acid substitutions at various sites of the molecule. However, non-charged mutants (exchange of an uncharged by another uncharged amino acid or of a charged by another equally charged amino acid) are not detectable with isoelectric focusing techniques. Therefore, a new strategy based upon time-of-flight secondary ion mass spectrometry (TOF-SIMS) of distinct apolipoprotein fragments generated by proteolytic digestion has been developed. Apolipoprotein A-I is isolated from serum or HDL by focusing in immobilized pH-gradients and subsequently cleaved by the protease trypsin. Isolation of the tryptic fragments is done by reverse-phase high performance liquid chromatography (HPLC).

The molecular weights of the isolated peptides are then determined by mass spectrometry and compared with the calculated molecular masses as deduced from the primary structure of apoA-I. TOF-SIMS allows the molecular weight analysis of peptides in the picomole range with a sensitivity of one dalton for molecular weight differences (8). Applying these techniques, an apoA-I mutant with an exchange of arginine$_{177}$ by histidine has been characterized.

MATERIALS AND METHODS

Patients

In about 1,000 coronary angiography patients the apoE polymorphism was determined using isoelectric focusing

Abbreviations: TOF-SIMS, time-of-flight secondary ion mass spectrometry; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; HPLC, high performance liquid chromatography.

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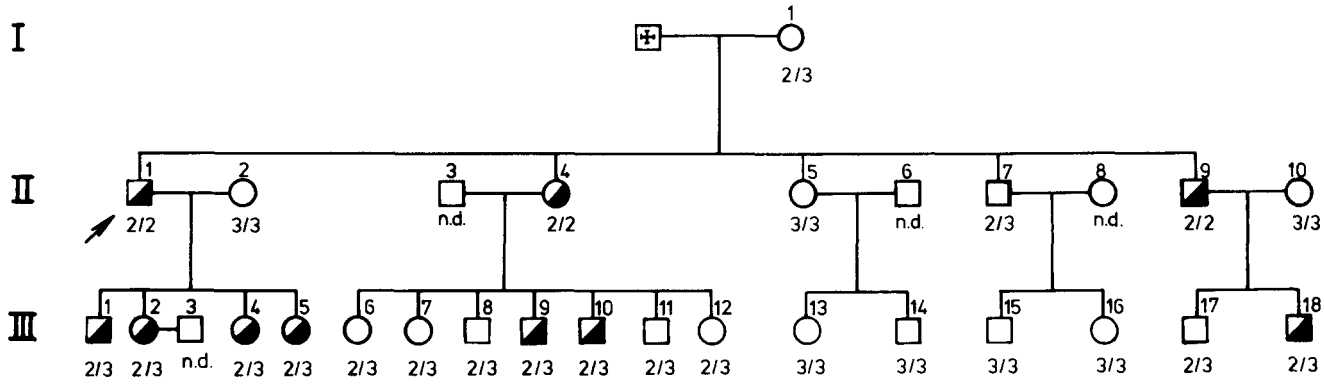

Fig. 1. Pedigree of a family with apoA-$I_{Münster-1}$. Individuals with normal apoA-I (□, ○); individuals with both normal and apoA-$I_{Münster-1}$ (◪, ◒). The arrow denotes the propositus. Homozygosity for apoE-2 (2,2); and for apoE-3 (3,3); Heterozygosity for apoE (2,3); n.d., not determined.

of apoVLDL. In this group of subjects, one proband was detected who was homozygote for apoE-2 and heterozygote for an apoA-I mutant. The mutant apoA-I had a relative charge of −0.3 as compared with normal apoA-I on isoelectric focusing gels. Family studies revealed that the apoA-I mutant was inherited (**Fig. 1**). The proband, his brother and his sister (generation II) were homozygote for apoE-2 and heterozygote for the apoA-I mutant. They were hyperlipidemic (serum cholesterol and triglyceride concentrations were between 300 and 500 mg/dl), ex-

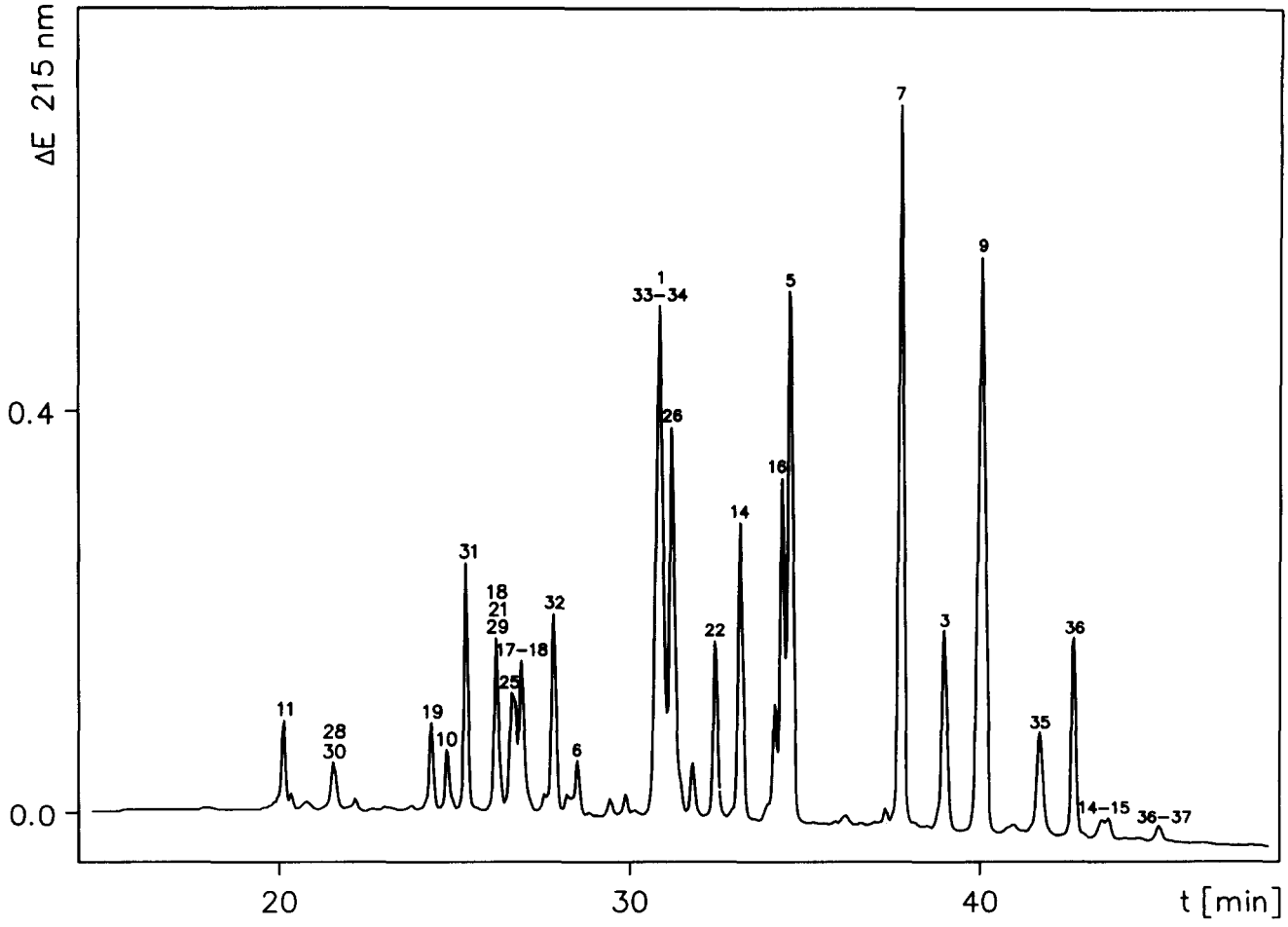

Fig. 2. HPLC chromatogram of tryptic peptides obtained from normal apo A-I. ApoA-I was digested with trypsin in a ratio of 70 to 1 (w/w) at 37°C for 12 hr. Tryptic peptides were separated on a TSK-ODS-120-T HPLC column: 10 min isocratically with 0.1% trifluoroacetic acid, then with a gradient from 0 to 40% acetonitrile in 50 min. The numbers of tryptic peptides are indicated. Uncleaved peptides are indicated as peptide numbers separated by a hyphen (e.g., T17-18).

TABLE 1. Amino acid sequence of normal apolipoprotein A-I (Ref. 17)

Sequence of Human Apolipoprotein A-I		
Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-	T-1	(1226)
Val-Lys-	T-2	
Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-Val-Leu-Lys-	T-3	(1235)
Asp-Ser-Gly-Arg-	T-4	(433)
Asp-Tyr-Val-Ser-Gln-Phe-Glu-Gly-Ser-Ala-Leu-Gly-Lys-	T-5	(1400)
Gln-Leu-Asn-Leu-Lys-	T-6	(614)
Leu-Leu-Asp-Asn-Trp-Asp-Ser-Val-Thr-Ser-Thr-Phe-Ser-Lys-	T-7	(1612)
Leu-Arg-	T-8	
Glu-Gln-Leu-Gly-Pro-Val-Thr-Gln-Glu-Phe-Trp-Asp-Asn-Leu-Glu-Lys-	T-9	(1932)
Glu-Thr-Glu-Gly-Leu-Arg-	T-10	(703)
Gln-Glu-Met-Ser-Lys-	T-11	(621)
Asp-Leu-Glu-Glu-Val-Lys-	T-12	(731)
Ala-Lys-	T-13	
Val-Gln-Pro-Tyr-Leu-Asp-Asp-Phe-Gln-Lys-	T-14	(1252)
Lys-	T-15	
Trp-Gln-Glu-Glu-Met-Glu-Leu-Tyr-Arg-	T-16	(1283)
Gln-Lys-	T-17	
Val-Glu-Pro-Leu-Arg-	T-18	(612)
Ala-Glu-Leu-Gln-Glu-Gly-Ala-Arg-	T-19	(872)
Gln-Lys-	T-20	
Leu-His-Glu-Leu-Gln-Glu-Lys-	T-21	(895)
Leu-Ser-Pro-Leu-Gly-Glu-Glu-Met-Arg-	T-22	(1031)
Asp-Arg-	T-23	
Ala-Arg-	T-24	
Ala-His-Val-Asp-Ala-Leu-Arg-	T-25	(780)
Thr-His-Leu-Ala-Pro-Tyr-Ser-Asp-Glu-Leu-Arg-	T-26	(1301)
Gln-Arg-	T-27	
Leu-Ala-Ala-Arg-	T-28	(429)
Leu-Glu-Ala-Leu-Lys-	T-29	(572)
Glu-Asn-Gly-Gly-Ala-Arg-	T-30	(602)
Leu-Ala-Glu-Tyr-His-Ala-Lys-	T-31	(830)
Ala-Thr-Glu-His-Leu-Ser-Thr-Leu-Ser-Glu-Lys-	T-32	(1215)
Ala-Lys-	T-33	
Pro-Ala-Leu-Glu-Asp-Leu-Arg-	T-34	(812)
Gln-Gly-Leu-Leu-Pro-Val-Leu-Glu-Ser-Phe-Lys-	T-35	(1230)
Val-Ser-Phe-Leu-Ser-Ala-Leu-Glu-Glu-Tyr-Thr-Lys-	T-36	(1386)
Lys-	T-37	
Leu-Asn-Thr-Gln-	T-38	(474)

Sequence position of tryptic peptides (T1–T38) and molecular weights as determined by TOF-SIMS are indicated.

hibited β-migrating VLDL upon agarose electrophoresis of d < 1.006 g/ml lipoproteins, and suffered from premature coronary heart disease. Their normolipidemic children (generation III) ranged in age from 10 to 25 years. Serum HDL cholesterol and apoA-I concentrations in affected and nonaffected family members were between 35–55 mg/dl and 110–140 mg/dl, respectively.

Lipoprotein and apolipoprotein isolation

High density lipoproteins were isolated from 2 ml of human serum by sequential ultracentrifugation by the method of Havel, Eder, and Bragdon (9). After delipidation of HDL with ethanol–ether 3:1 (v/v) (10), the precipitated apolipoproteins were solubilized in 0.01 mol/l Tris-HCl, pH 8.2, 2% decylsulfate, 5% β-mercaptoethanol.

Isoelectric focusing

A preparative gel was prepared by mixing an acidic and a basic buffer solution with a gradient mixer. The buffer solutions containing acrylamide derivates (Immobiline®, LKB Bromma) with covalently bound buffering groups (11). The buffers were prepared by mixing the calculated amounts of Immobiline with an acrylamide/bis solution, TEMED, and ammonium persulfate. For isoelectric focusing of apoA-I in the pH range 5–6, the acidic solution contained 316 μl of Immobiline pK 3.6, 1726 μl of Immobiline pK 4.6, 1726 μl of Immobiline pK 6.2, and 6.00 ml of 29.1% acrylamide/0.9% bis solution. The solution was made up to 35 ml with 30% glycerol. The basic buffering solution contained 1726 μl of Immobiline pK 4.6, 1606 μl of Immobiline pK 6.2, 676 μl of Immobiline pK 9.3, and 6.00 ml 29.1% acrylamide/0.9% bis solution. The mixture was diluted to 35 ml with distilled water.

To each solution 100 μl of 10% TEMED and 100 μl of 10% ammonium persulfate were added. The solutions were used to generate a linear gradient in a gradient mixer; the mixture was added to an 11 × 11 × 0.5 cm chamber prepared from two glass plates and a 0.5

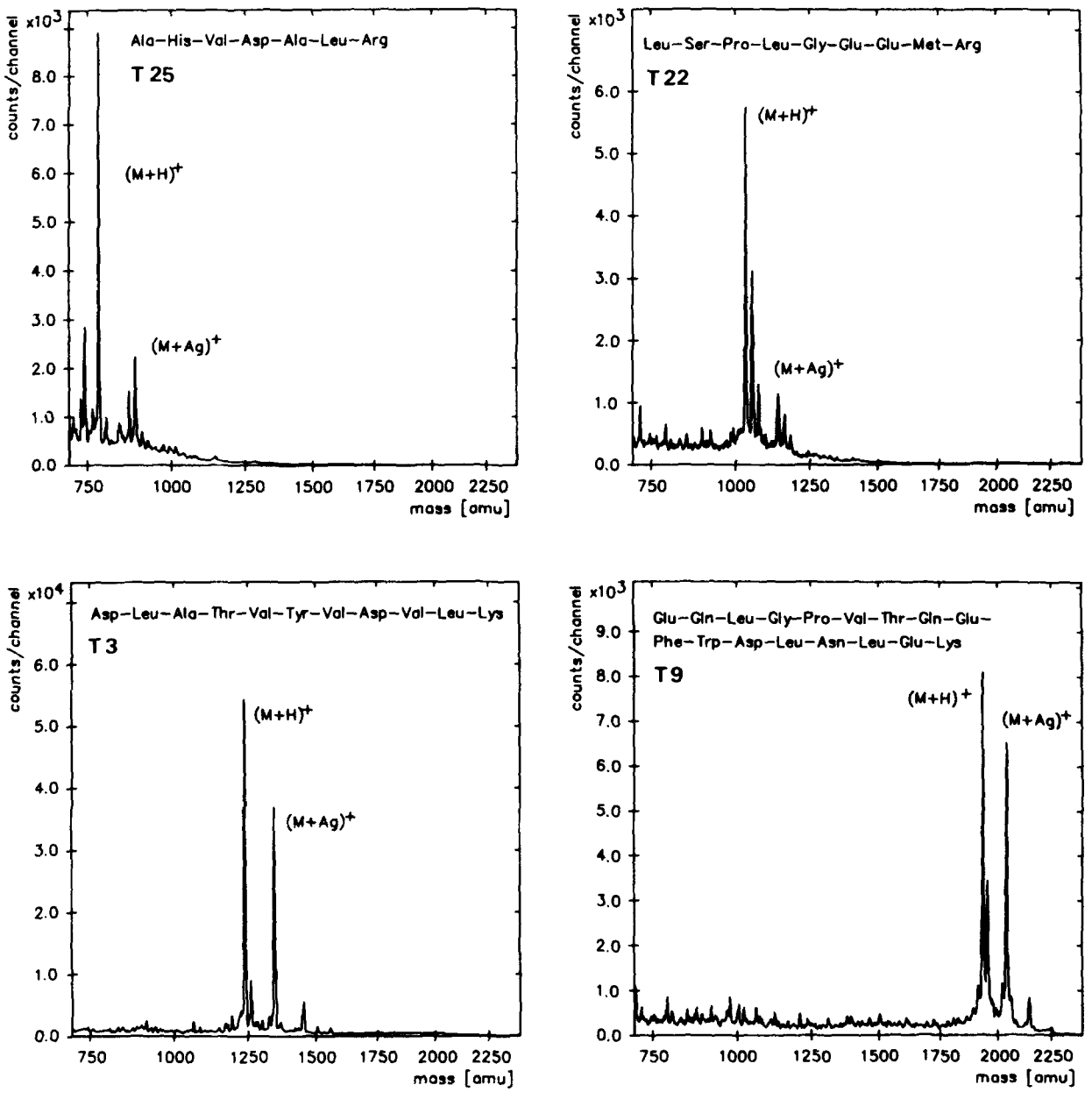

Fig. 3. Molecular weight determinations of peptides derived from apoA-I by TOF-SIMS analysis. Molecular ion region in the positive secondary ion spectrum obtained from 1 nmol of peptides T3, T9, T22, T25 (see Fig. 2 and Table 1) deposited on a 100-mm^2 silver target. Primary ion bombardment: 2.5 × 10^{-10} A^+, 12 KeV in 60 sec. From this ion bombardment about 1% of the deposited sample material is sputtered. The peptides were identified by partial amino acid sequence analysis.

cm-thick silicon rubber gasket. A Gelbond® film was placed on one glass plate and on the opposite glass plate a 10 × 0.9 × 0.4 cm sample slot was fixed. The gel was polymerized in 90 min at 50°C. After polymerization, the gel was incubated for 12 hr in 6 mol/l deionized urea containing 30% glycerol. After drying the gel at room temperature for 4 hr, 2 ml (approx. 1 mg of protein) of apoHDL was applied in the sample slot of the gel. The electrode strips were soaked in 0.01 mol/l NaOH at the cathodic side and in 0.01 mol/l glutamic acid at the anodic side. To prevent drying of the electrode strips, a Whatman 3MM paper soaked in distilled water was put under the strips and placed in the buffer chamber of the Multiphor II (LKB, Bromma) filled with distilled water. The apolipoproteins were focused at 4°C overnight with a setting of 10 W, 5000 V, and 150 mA. After focusing, the gel was washed in distilled water for 15 min. The apolipoproteins appeared as opalescent lines. These lines were cut out of the gel without staining.

Electroelution

The protein was separated from the gel by electroelution. Briefly, the gel strip was placed in a dialysis tube (molecular weight exclusion limit: 3,500 D) containing

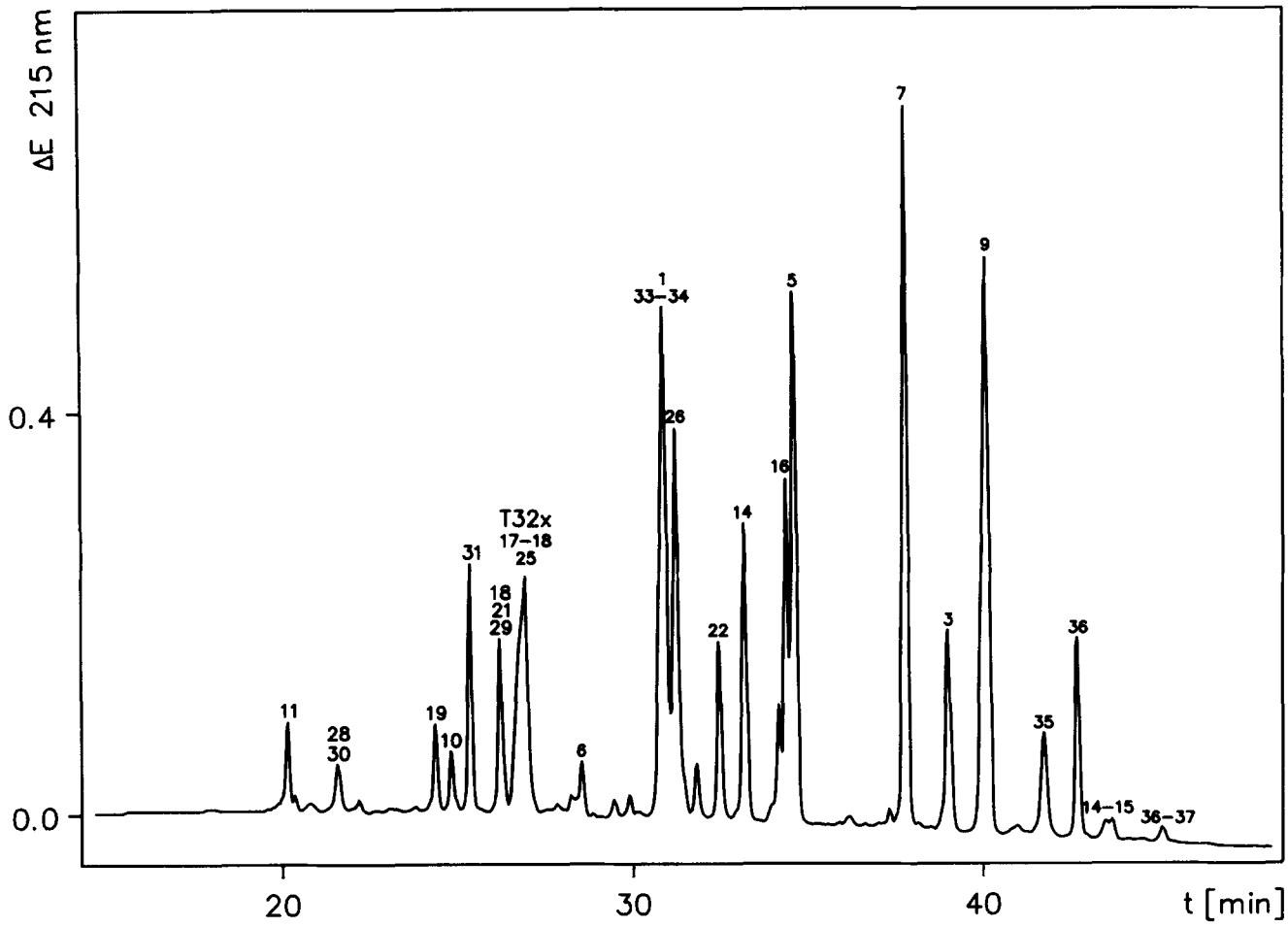

Fig. 4. HPLC chromatogram of tryptic peptides obtained from mutant apoA-$I_{Münster-4}$. The newly appearing peptide is indicated as T32x (for details see Fig. 2).

0.02 mol/l Tris-glycine, pH 9.3. The protein was eluted at 4°C for 12 hr at 125 mA in a Western blot chamber filled with the same buffer. The tube was dialyzed against 0.01 mol/l ammonium bicarbonate. Then the apoA-I solution was lyophilized. Recovery of apoA-I (determined by radioimmunoassay (12) of serum apoA-I and gel-eluted apoA-I) was between 80 and 90%.

Tryptic digestion of apoA-I

The lyophilized apoA-I was solubilized in 0.01 mol/l ammonium bicarbonate, 0.01 mol/l calcium chloride, pH 7.4. The protein concentration was determined at E = 280 nm (mg/dl = $E_{280\,nm}$ × dilution × 0.88; molar extinction coefficient of apoA-I at $E_{280\,nm}$ = 32.350 $cm^{-1}mol^{-1}l$).

The trypsin concentration was determined at E = 280 nm (mg/dl = $E_{280\,nm}$ × dilution × 0.7) (Worthington Trypsin TPCK, 238 U/mg protein). ApoA-I was digested with trypsin in a ratio of 70 to 1 (w/w). The solution was incubated at 37°C for 12 hr. The digestion was stopped by lyophilization.

HPLC separation of tryptic peptides

The lyophilized tryptic peptides of apoA-I were solubilized in 0.1% trifluoroacetic acid in a concentration of 10 mg/ml. Three hundred μg (10.5 nmol) of the tryptic digest was injected on a TSK ODS-120-T 5 μm, 4.6 × 250 mm HPLC column (LKB, Bromma) and eluted isocratically with 0.1% trifluoroacetic acid for 10 min. Then a gradient of 0 to 40% acetonitrile in 50 min was started. The trifluoroacetic acid buffer was purified on a preparative reverse-phase column (Lobar, Merck, Darmstadt). The gradient was formed by two HPLC pumps (LKB, Bromma) controlled by an HPLC controller (LKB, Bromma).

The separated fractions were analyzed by a diode array detector (LKB, Bromma) connected to an IBM XT computer for data reduction and calculation of chro-

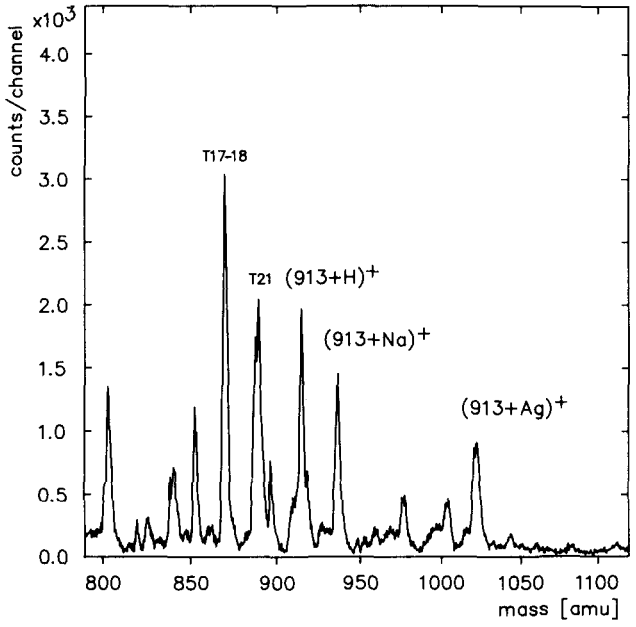

Fig. 5. Molecular weight determination of peptide T32x by TOF-SIMS analysis. Molecular ion region of the new peptide T32x found in mutant apoA-$I_{Münster-4}$. The masses of peptides T17–18 and T21, which coelute with the peptide T32x, are indicated.

matograms at different wavelengths. The fractions were collected by a fraction collector equipped with a peak detector (LKB, Bromma).

Manual microsequence analysis of tryptic peptides

Microsequence analysis of HPLC separated peptides was done by using the 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC)/phenylisothiocyanate double coupling method (13). The peptides of one HPLC peak were lyophilized, solubilized in 100 μl of 50% aqueous pyridine, and treated with 40 μl of freshly prepared DABITC solution (2.82 mg/ml pyridine). The mixture was flushed with nitrogen for 10 sec and incubated for 50 min at 56°C. Then 10 μl of phenylisothiocyanate was added, the tube was flushed with nitrogen and was then incubated for an additional 30 min at 56°C. Excess reagents and byproducts were extracted with three portions of 0.5 ml heptane–ethyl acetate 2:1 (v/v). After mixing and centrifugation, the organic phase was discarded. For cleavage, the dried remainder was dissolved in 50 μl of anhydrous trifluoroacetic acid, flushed with nitrogen, and incubated for 15 min at 56°C. The sample was lyophilized, dissolved in 50 μl of water, and mixed with 200 μl of butyl acetate. After centrifugation and removal of the butyl acetate extract, the aqueous phase was lyophilized for the next sequencing cycle. The butyl acetate extract was lyophilized and redissolved in 50% aqueous trifluoroacetic acid. Conversion was carried out for 20 min at 56°C.

The sample was dried and solubilized in 2μl of ethanol for thin-layer chromatography identification. Chromatography was performed on polyamide sheets (Schleicher & Schüll, 2 × 2 cm) as follows: first dimension, acetic acid–water 1:2 (v/v); second dimension, toluene–n-hexane–acetic acid 2:1:1 (v/v/v). The DABITH amino acids were identified after exposure to HCl vapor.

Time-of-flight secondary ion mass spectrometry

The molecular weights of HPLC separated tryptic peptides were determined by secondary ion mass spectrometry. This technique is based on the fact that keV-bombardment of even thermally labile, involatile organic compounds results in the desorption (sputtering) of so-called parent ions of the general composition $(M + H)^+$, $(M - H)^-$ and $(M + \text{cation})^+$ (refer to Fig. 3) (14). By mass analysis of these secondary ions, the molecular weight M can be determined from each of these parent ions (15, 16). The high performance mass spectrometer used in our experiments is a noncommercial instrument, developed at the Physikalisches Institut of the University of Münster. It is equipped with a mass selecting pulsed primary ion source, an angle and energy focusing time-of-flight analyzer, and a high efficiency single ion counting detector (16). A single fraction corresponding to one HPLC peak contains about 10 nmol of a single tryptic peptide, dissolved in 100 μl of the eluate. From such a fraction, less than 10% was deposited on a 100-mm^2 area of silver foil and lyophilized in the air-lock system of the mass spectrometer. Before applying the sample, the silver target had been cleaned and prepared by etching in 20% nitric acid at 40°C for about 3 min and rinsed in distilled water. The deposition procedure results in the formation of a monomolecular layer of the corresponding tryptic peptide covering 100 mm^2 of the silver target.

Typically, less than 1 mm^2 of this sample, covered by less than 10 pmol of peptides, was bombarded by a pulsed 12 keV argon ion beam, corresponding to an average current of 2.5×10^{-10} A for about 60 sec. This corresponds to a total primary ion dose of about 10^{10} ions.

The secondary ions of the tryptic peptides were detected with a typical number of about 10^5 under the preparation and bombardment conditions mentioned above. The total primary ion dose of 10^{10} primary ions results in sputtering of 10% of the sample material deposited in the bombarded area. Thus, only 1 pmol of the sample material was sputtered during the registration of a spectrum. The total procedure resulting in a complete secondary ion spectrum was carried out in less than 6 min.

RESULTS

The HPLC chromatogram of tryptic peptides of normal apoA-I is presented in **Fig. 2.** Molecular weights of individual peptides as determined by TOF-SIMS as well as the position of the tryptic peptides in the primary

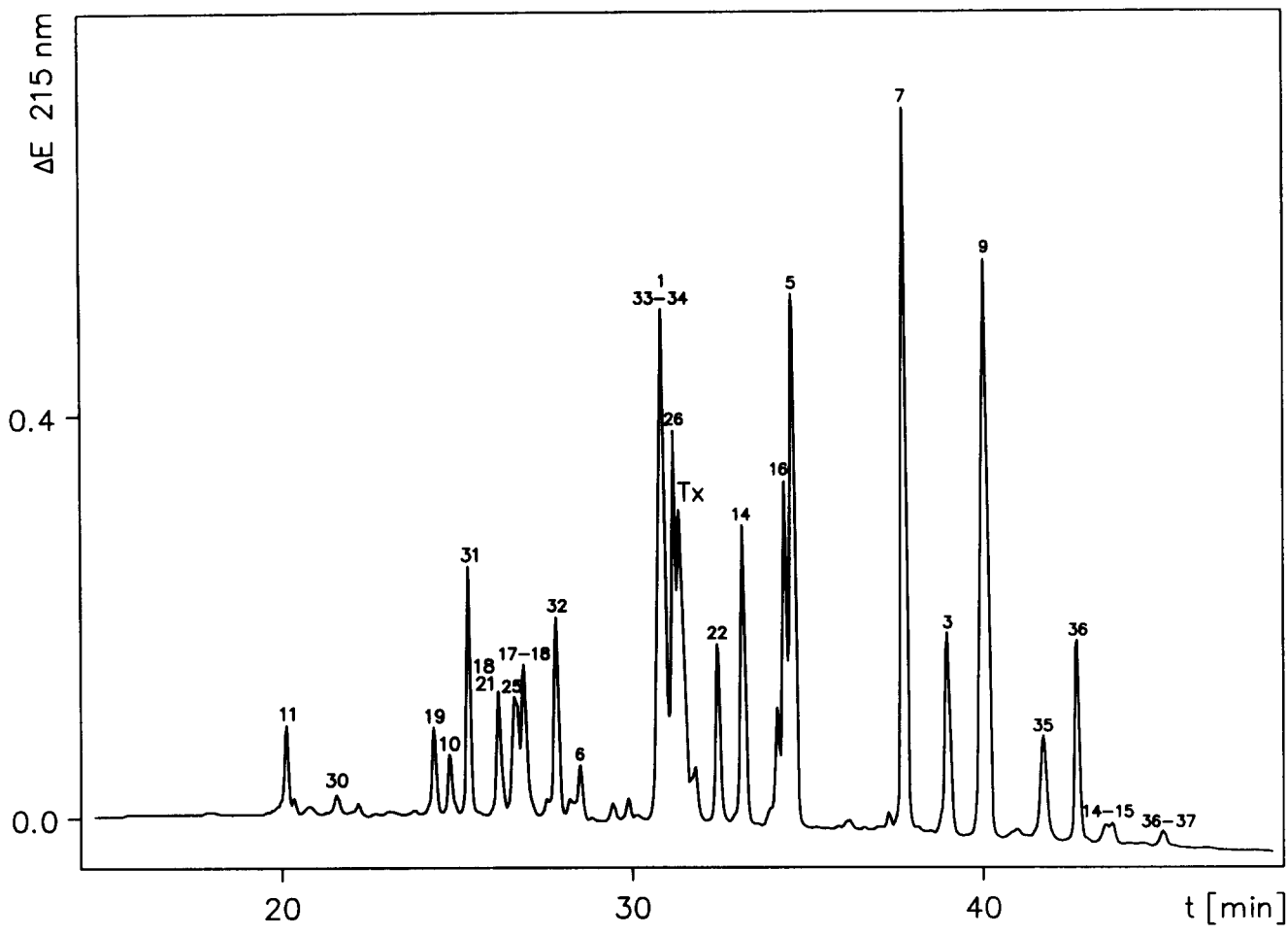

Fig. 6. HPLC chromatogram of tryptic peptides obtained from mutant apoA-$I_{Münster-1}$. The newly appearing peptide is indicated as Tx (for details see Fig. 2).

structure of apoA-I (T1–T38, **Table 1**) are indicated (17). Representative mass spectra of four different tryptic apoA-I peptides are shown in **Fig. 3.** Correct assignment of the apoA-I peptides as predicted by TOF-SIMS molecular weight analyses was confirmed by partial sequencing using the manual DABITC method and by amino acid analyses. To check the applicability of this new technique in the structural elucidation of apoA-I mutants, we re-investigated one of the known charged apoA-I mutants whose amino acid exchange had been previously determined by EDMAN degradation [mutation at the apoA-I sequence position $Glu_{198} \rightarrow$ Lys, apoA-$I_{Münster-4}$ (4)]. This mutant apoA-I had a charge difference of +2 compared to the normal isoforms as seen in analytical isoelectric focusing gels. The HPLC chromatogram of the tryptic peptides of the mutant apoA-I (**Fig. 4**) differed from the HPLC chromatogram of the tryptic peptides of the normal apoA-I isoform by the complete loss of a peptide (T32) with a molecular weight of 1215. Instead, a newly generated peptide (T32x) (Fig. 4) with altered migration properties in HPLC (comigrates with T25, T17–18) and a molecular weight of 913 as detected by TOF-SIMS (**Fig. 5**) was present. The absence of peptide T32 and the formation of peptide T32x are readily explained by a new tryptic cleavage site (Lys) at position 198 in the primary sequence:

T32 Ala-Thr-Glu-His-Leu-Ser-Thr-Leu-Ser-Glu-Lys (1215 D)
T32x Ala-Thr-Lys|His-Leu-Ser-Thr-Leu-Ser-Glu-Lys (913 D)

Thus, the information derived from HPLC/TOF-SIMS analysis is compatible with a Glu → Lys substitution at position 198 causing a charge difference of +2 and a new tryptic cleavage site comparing the native and mutant apoA-I.

We further attempted to identify the formerly uncharacterized apoA-I mutant (Münster-1). The familial nature of this mutant protein was established by pedigree analysis. As shown in Fig. 1, the proband (II, 1), his sister (II, 4), and his brother (II, 9), and several children were heterozygous for apoA-$I_{Münster-1}$. The high-resolution

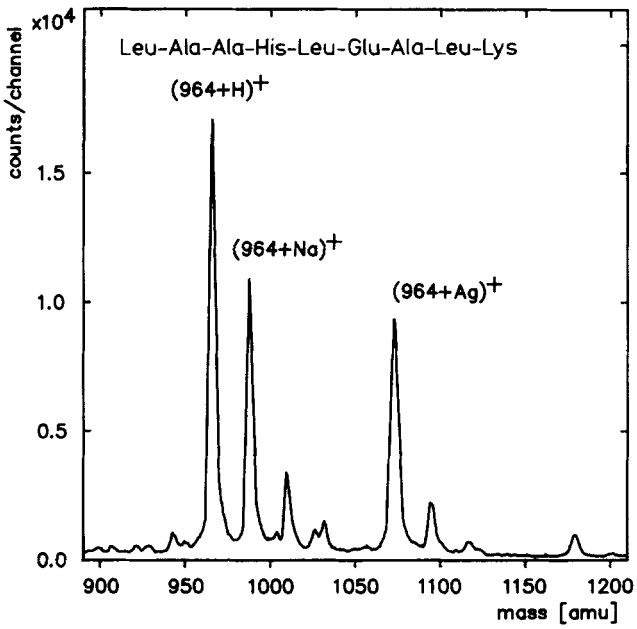

Fig. 7. Molecular weight determination of peptide Tx by TOF-SIMS analysis. Molecular ion region of the new peptide Tx found in mutant apoA-$I_{Münster-1}$. For details see Fig. 3.

power of the Immobiline gels permitted the separation and preparative isolation of the normal and mutant proteins. The HPLC chromatogram of the mutant apoA-I is presented in **Fig. 6.** The HPLC chromatogram differs from the chromatogram of the control preparation (Fig. 2) by the appearance of a newly generated peptide with a molecular weight of 964 (Tx) and by decreased fractions normally containing unseparated peptides T28 and T30 as well as unseparated peptides T18, T21, T29, respectively. Upon TOF-SIMS analysis of these fractions, peptides T28 and T29 were undetectable.

The mass spectrum of the newly generated peptide Tx is shown in **Fig. 7.** With manual microsequencing the following five amino acids of peptide Tx were found: Leu-Ala-Ala-His-Leu..... Assuming a point mutation, the combined results (molecular weight and the sequence analysis of peptide Tx) can only be explained by an exchange of $arginine_{177}$ by histidine:

T28 Leu-Ala-Ala-Arg_{177} (429 D); **T29** Leu-Glu-Ala-Leu-Lys (572 D)
Tx Leu-Ala-Ala-His-Leu-Glu-Ala-Leu-Lys (964 D)

This amino acid exchange resulted in a more acidic isoelectric point of the mutant apoA-I.

DISCUSSION

Combining the techniques of isoelectric focusing, reverse-phase HPLC separation of proteolytic peptides, and subsequent molecular weight analysis by TOF-SIMS constitutes a new tool in the structural analysis of protein mutants and offers the following advantages.

1.) Isoelectric focusing in immobilized pH-gradients allows the generation of very narrow pH-gradients in which proteins could be separated with a pI difference of only 0.01 pH. Preparative Immobiline® gels have a high capacity (up to 5 mg/band) and an improved resolution compared to preparative Ampholyte-Sepharose® gels. There are no contaminations with ampholytes and no protein/ampholyte complexes. The identification of the separated bands can be done without staining. With preparative Immobiline gels, normal and mutant apolipoproteins with minor charge differences can be separated and electroeluted out of the gels.

2.) The specificity of the protease trypsin allows a reproducible cleavage of apoA-I so that, after HPLC separation, a mutation can be identified by newly generated as well as disappearing peaks in the HPLC chromatograms comparing the normal and mutant protein. Since only 20% of all theoretical point mutations are charged mutations, 80% of all possible mutations cannot be detected by isoelectric focusing alone.

3.) The TOF-SIMS procedure permits accurate molecular weight determination of peptides (up to 3000 ± 1 D), detection limits in the sub-nanomole range, rapid analysis (minutes), and a wide range of applications for involatile biomolecules. Theoretically, it is possible to monitor for genetic polymorphisms of proteins and post-translational modifications on a microscale basis. Time-of-flight secondary ion mass spectrometry was applied here for the first time for the detection of protein mutants by molecular weight determination of derived peptides. Until now, this technique was only used for the detection of nucleotides, PTH-amino acids, and drugs.

Using these combined techniques, we characterized the mutant apoA-$I_{Münster-1}$ as an Arg-His substitution in position 177. Pedigree analysis shows that the proband, his brother, and his sister were heterozygous for this mutation, but also homozygous for apoE-2. This fact makes it difficult to ascertain whether the mutant apoA-I or apoE-2 or the combined anomalies are causally related to hyperlipidemia and/or coronary heart disease in these patients. Seven children who were all heterozygous for the apoA-I mutant as well as for apoE-2 had no hyperlipidemia and were free of signs of coronary heart disease (Fig. 1). Anomalies in serum HDL cholesterol or apoA-I concentrations were not apparent in affected family members. Thus, despite extensive analyses of lipoproteins and apolipoproteins, it could not be established whether or not the apoA-$I_{Münster-1}$ mutant causes alterations in lipoprotein metabolism. In vitro studies to ascertain the reassociation characteristics of the mutant protein with phospholipid vesicles, as well as its LCAT activation properties, are now in progress.

Manuscript received 25 July 1985.

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