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CHARACTERIZATION OF AN APOLIPOPROTEIN C-III MUTANT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND TIME-OF-FLIGHT SECONDARY ION MASS SPECTROMETRY*

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SUMMARY

Apolipoprotein (apo) C-III isoforms from a patient with a mutant apo C-III and from controls were isolated to homogeneity by isoelectric focusing and subjected to proteolytic digestion. The peptides obtained were separated by reversed-phase high-performance liquid chromatography, and their molecular masses were determined by time-of-flight secondary ion mass spectrometry. Molecular masses of peptides derived from apo $C\text{-}III_0$, $C\text{-}III_1$ and $C\text{-}III_2$ were indistinguishable from control preparations, whereas the mutant apo C-III contained a COOH-terminal, carbohydrate-containing peptide with an abnormal retention time in high-performance liquid chromatography and a molecular mass higher by 291 daltons owing to oversialation at position 74 of the amino acid sequence (apo $C\text{-}III_3$).

INTRODUCTION

The principal lipolytic enzymes in the catabolism of triglyceride-rich lipoproteins are lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL). Apolipoprotein (apo) C-II activates LPL, whereas apo C-III has been proposed to inhibit LPL activity and to retard the clearance of chylomicron remnants by the liver [1, 2]. Apo C-III is composed of 79 amino acid residues and exists in three major isoforms in human plasma (apo $C\text{-}III_0$, apo $C\text{-}III_1$ and apo $C\text{-}III_2$), which

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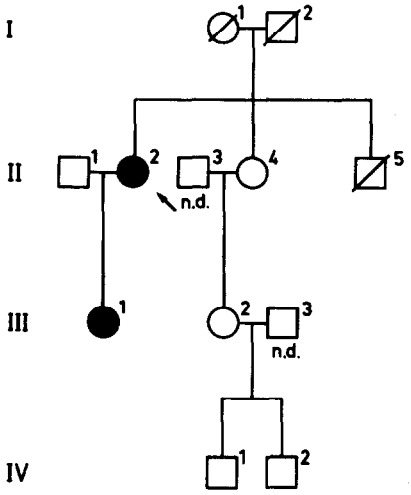

Fig. 1. Pedigree of a family with apo C-III$_3$. Individuals with normal apo C-III isoforms are denoted by open symbols and those individuals with apo C-III$_3$ by filled symbols. The arrow denotes the propositus; n.d. = not determined.

differ by one charge unit on isoelectric focusing gels [3]. In order to identify structural mutants of apolipoproteins and to investigate their potential influence on the development of hyperlipoproteinaemia, screening studies using isoelectric focusing of apo very-low-density lipoproteins (VLDL) were performed. We here report a mutant apo C-III, which was discovered in a proband affected by severe hypertriglyceridaemia. The mutant apo C-III was structurally characterized using high-performance liquid chromatography (HPLC) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) [4].

EXPERIMENTAL

Patients

In about 1000 coronary angiography patients the apo E polymorphism was determined using isoelectric focusing of apo VLDL. In this group of subjects, one proband was detected that had an additional band on isoelectric focusing gels one charge unit more acidic than apo C-III$_2$. Family studies revealed that the apo C-III mutant was inherited (Fig. 1). The proband, a 60-year-old woman, had a plasma triglyceride concentration of 1012 mg/dl (cholesterol: 267 mg/dl) and had suffered from angina since she was 30 years old. Starting in her fourth decennium she suffered from diabetes mellitus type II. Her 31-year-old daughter was not a diabetic and had a plasma triglyceride concentration of 205 mg/dl (cholesterol: 196 mg/dl).

Lipoprotein and apolipoprotein isolation

VLDL were isolated from 20 ml of human plasma by sequential ultracentrifugation [5]. After delipidation of VLDL with ethanol–diethyl ether (3:1, v/v),

the precipitated apolipoproteins were solubilized in 0.01 *M* Tris–HCl (pH 8.2, 2% decylsulphate). For the separation of cysteine-containing apolipoproteins, apo VLDL were modified by β-mercaptoethylamine (Sigma, Deisenhofen, F.R.G.): 100 μl of apo VLDL solution were incubated with 1 mg of β-mercaptoethylamine at 37°C overnight.

Analytical isoelectric focusing in ampholyte gels

Isoelectric focusing was done according to the method of Haglund [6], with certain modifications. We used the slab gel system Model 220 (Bio-Rad Labs., Richmond, CA, U.S.A.) with ampholytes pH 3–5 (Serva, Heidelberg, F.R.G.), pH 5–7 (Serva) and pH 5–7 (LKB, Bromma, Sweden), mixed 2:1:1 by volume. The electrophoresis was run overnight at 200 V, starting with a power limited to 3 W per plate, and at 600 V in the morning for 1 h. Second-dimension sodium dodecylsulphate (SDS) gel electrophoresis was done in 20% acrylamide slab gels according to Neville [7].

Preparative isoelectric focusing in immobilized pH gradients

VLDL apolipoproteins were separated by preparative isoelectric focusing in immobilized pH gradients (Immobiline®, LKB). The focusing was done in a pH range from 3.75 to 5.25 [8]. After focusing, the different apo C-III isoforms were cut out of the gel without prior staining. After electroelution in a 0.02 *M* Tris–HCl pH 9.0 buffer, the proteins were dialysed against 0.01 *M* ammonium hydrogen carbonate (pH 7.4).

Proteolytic digestion of apo C-III

The lyophilized apo C-III proteins were solubilized in 0.01 *M* ammonium hydrogen carbonate–0.01 *M* calcium chloride (pH 7.4) and digested with trypsin (Worthington, Freehold, NJ, U.S.A.; TPCK★-treated, activity 212 U/mg of protein) in a ratio of 70:1 (w/w). The solution was incubated at 37°C overnight and the digestion was stopped by lyophilization.

After HPLC separation, the peptides T7,8,9 from the apo C-III isoforms, as well as from the apo C-III mutant, were lyophilized. They were then solubilized in ammonium hydrogen carbonate buffer and further digested with *Staphylococcus aureus* protease (Worthington; activity 690 U/mg of protein) in a ratio of 70:1 (w/w). The solution was incubated at 37°C for 12 h. The digestion was stopped by lyophilization.

Neuraminidase treatment of VLDL

A 100-μl volume of VLDL (0.5 mg protein) was digested with 10 μl of neuraminidase solution (0.01 U neuraminidase in 0.05 *M* sodium acetate, 0.154 *M* sodium chloride, 4 m*M* calcium chloride, pH 5.5; neuraminidase from *Clostridium perfringens*, Worthington) for 12 h at 37°C. The digestion was stopped by delipidation of VLDL with ethanol–diethyl ether (3:1, v/v).

★TPCK = L-tosylamido-2-phenyl) ethyl chloromethyl ketone.

HPLC separation of proteolytic peptides

The lyophilized proteolytic peptides were solubilized in 0.1% trifluoroacetic acid at a concentration of 10 mg/ml and were separated using a TSK-ODS-120T HPLC column (LKB). The peptides were eluted isocratically for 10 min and then a gradient of 0 to 50% acetonitrile in 50 min was started. The trifluoroacetic acid buffer was purified on a preparative reversed-phase column (Lobar, Merck, Darmstadt, F.R.G.). The gradient was formed by two HPLC pumps controlled by an HPLC controller (LKB).

The separated fractions were analysed by a diode array detector (LKB) connected to an IBM XT computer for data reduction and calculation of chromatograms at different wavelengths. The fractions were collected by a fraction collector equipped with a peak detector (Superrac, LKB).

Manual microsequence analysis of tryptic peptides

Microsequence analysis of HPLC-separated peptides was done by using the 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC)-phenyl isothiocyanate double coupling method [9].

Time-of-flight secondary ion mass spectrometry

The molecular masses of HPLC-separated tryptic peptides were determined by TOF-SIMS. The mass spectrometer used in our experiments is a non-commercial instrument, developed at the Physikalisches Institut of the University of Münster [10]. A single fraction corresponding to one HPLC peak contains ca. 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 a silver foil and lyophilized in the air-lock system of the mass spectrometer. Typically, less than 1 mm^2 of this sample was bombarded by a pulsed 12-keV argon ion beam, corresponding to an average current of $2.5 \cdot 10^{-10}$ A for 60 s. This corresponds to a total primary ion dose of ca. 10^{10} ions. This procedure results in the desorption (sputtering) of so-called parent ions of the general composition $(M+H)^+$, $(M-H)^-$ and $(M+\text{cation})^+$. By mass analysis of these secondary ions, the molecular mass M can be determined from each of these parent ions [11].

RESULTS

Fig. 2 shows the isoelectric focusing gel and the two-dimensional SDS gel electrophoresis of apo VLDL isolated from a proband affected by hypertriglyceridaemia. The additional protein focusing one charge unit more acidic than apo C-III$_2$ is labelled apo C-III$_x$. This protein migrates in the two-dimensional SDS gel electrophoresis in the same molecular mass region as the other apo C-III isoforms.

To characterize further the structural defect of this abnormal protein, control tryptic peptides derived from normal isoforms of apo C-III were first subjected to HPLC–TOF-SIMS analysis. The corresponding HPLC chromatograms are shown in Fig. 3. The assignment of the tryptic peptides to the sequence position was achieved by manual microsequencing (three or four amino acid residues) and by TOF-SIMS mass analysis (Fig. 4). The fourth peak in the chromatograms of apo

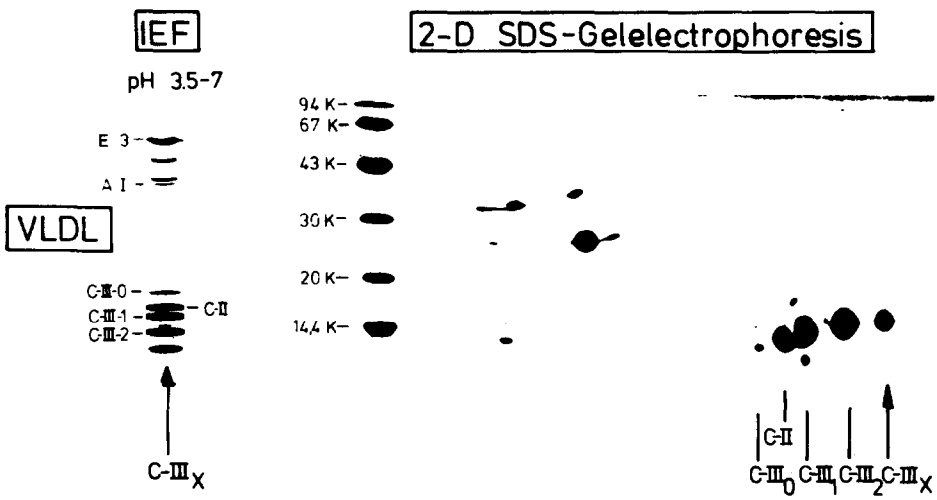

Fig. 2. Isoelectric focusing gel and two-dimensional SDS gel electrophoresis of apo VLDL of the patient with the mutant apo C-III. The mutant apo C-III is indicated as C-III$_x$.

C-III$_0$ and of apo C-III$_1$ is splitted (T7,8,9; T8,9 in Fig. 3). Peptide T8,9 apparently is completely protected from tryptic cleavage at position 72 in all apo C-III isoforms; in apo C-III$_0$ and apo C-III$_1$ partial cleavage at position 60 could be achieved, resulting in the formation of peptides T7,8,9 and T8,9. With apo C-III$_2$ only the uncleaved peptide T7,8,9 was generated. Fig. 5 shows four representative mass spectra of peptides T1, T4, T5 and T6 derived from apo C-III. The characteristic three peaks for each molecular mass represent the various peptides with which a proton, a sodium ion and a silver ion, respectively, are associated.

It is noteworthy that the molecular masses of peptides T7,8,9 and T8,9 derived from apo C-III$_0$ were determined as 2381 and 2138, representing non-glycosylated, non-sialylated peptides (Fig. 6). By contrast, neuraminidase treatment of apo C-III$_1$ and subsequent tryptic cleavage resulted in peptides T7,8,9 and T8,9 with molecular masses of 2746 and 2503, respectively, representing non-sialylated, but glycosylated peptides (D-galactosyl-(1–3)-N-acetyl-D-galactosamine linked to Thr-74 of apo C-III).

When the HPLC profiles of tryptic peptides derived from control apo C-III$_2$ and mutant apo C-III$_x$ were compared, identical migration positions, partial amino acid sequences and molecular masses were observed for peptides T1, T4, T5 and T6. However a small difference in the retention time of peak T7,8,9 was detected (Fig. 7). With manual microsequencing the following three amino acids of this peptide were found: Asp-Lys-Phe. The calculated molecular mass of peptide T7,8,9 (3329) derived from normal apo C-III$_2$, however, is at the upper detection limit of the TOF-SIMS procedure. Therefore it was chosen to generate lower-molecular-mass peptides from apo C-III isoforms, as well as from the apo C-III mutant by further digestion of the tryptic peptides T7,8,9 utilizing *S. aureus* protease. This protease specifically cleaves peptides after the amino acid residues glutamic acid and aspartic acid. Molecular masses of these peptides are shown in Fig. 8. After cleavage of peptide T7,8,9 from the mutant apo C-III, a peptide with a

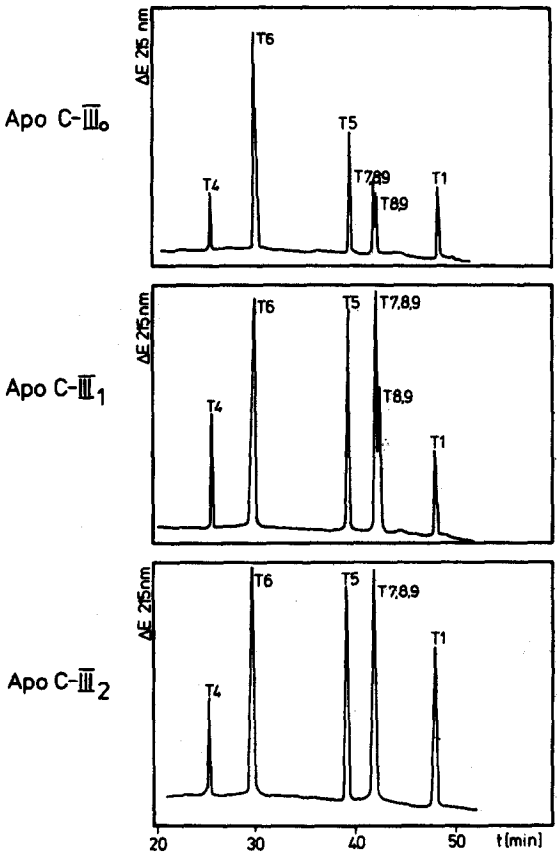

Fig. 3. HPLC of tryptic peptides obtained from normal apo C-III isoforms. Apo C-III was digested with trypsin in a ratio of 70:1 (w/w) at 37°C for 12 h. Tryptic peptides (500 μg) were separated on a TSK ODS-120T HPLC column: 10 min isocratically with 0.1% trifluoroacetic acid, then with a gradient from 0 to 50% acetonitrile in 50 min. The numbers of the tryptic peptides are indicated. Uncleaved peptides are indicated as peptide numbers separated by a comma (e.g., T7,8,9).

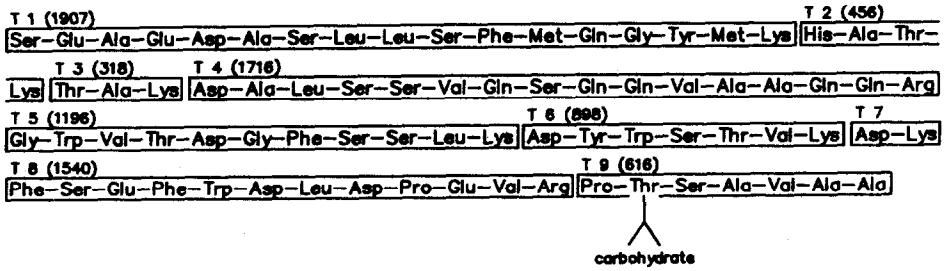

Fig. 4. Amino acid sequence of apo C-III [12]. The number and the molecular mass of each tryptic peptide are indicated. The molecular mass given for peptide T9 does not include the molecular mass of the carbohydrate chain.

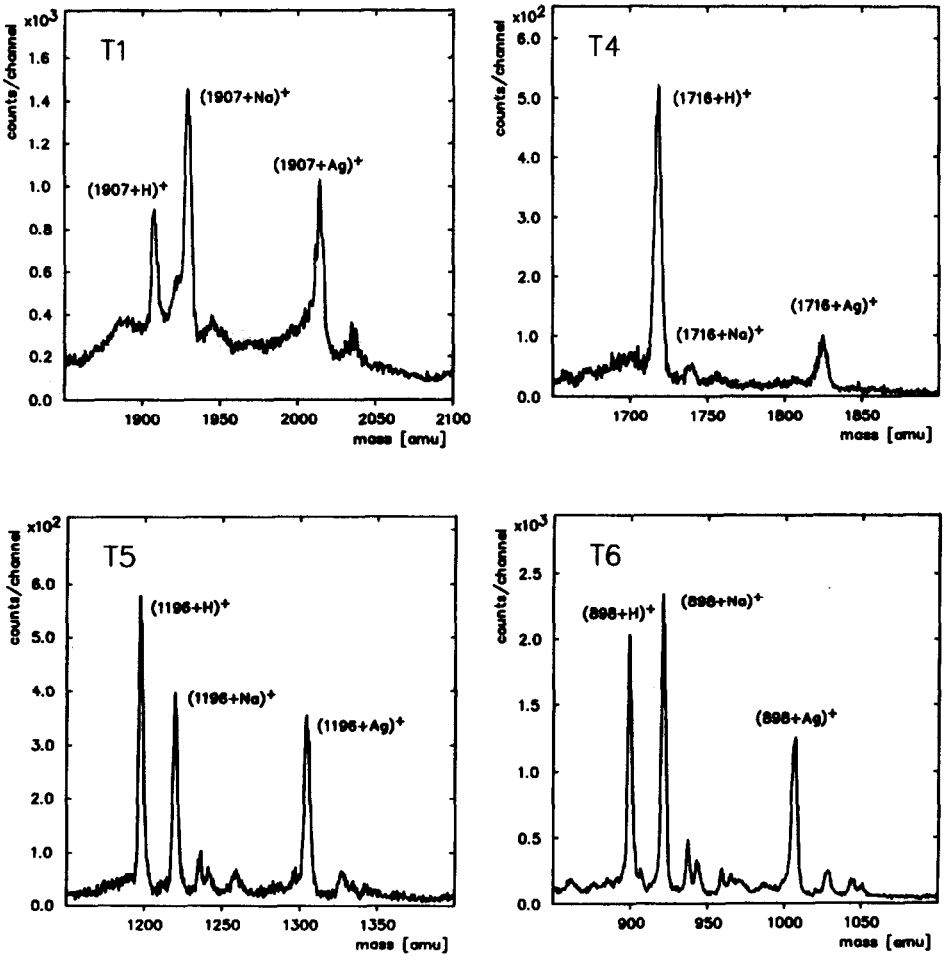

Fig. 5. Molecular mass determinations of peptides derived from apo C-III by TOF-SIMS analysis. Molecular ion region in the positive secondary ion spectrum obtained from peptides T1, T4, T5 and T6. Primary ion bombardment: $2.5 \cdot 10^{-10}$ A, 12 keV in 60 s. From this ion bombardment ca. 1% of the deposited sample material is sputtered. The peptides were identified by partial amino acid sequence analysis.

molecular mass of 2110 was detected. This mass is compatible with an additional sialic acid residue linked to the carbohydrate chain of the mutant apo C-III (Figs. 8 and 9). Treatment of the patient's VLDL with neuraminidase resulted in the complete removal of the sialic acid residues, as evidenced from the disappearance of the apo $C\text{-}III_1$, apo $C\text{-}III_2$ and apo $C\text{-}III_x$ bands in isoelectric focusing gels and the appearance of the digested proteins in the apo $C\text{-}III_0$ position.

DISCUSSION

Molecular defects in patients with dyslipoproteinaemias may be categorized into defects of receptors, lipolytic enzymes and apolipoproteins. Several structural mutants of apolipoproteins have been described [13, 14]. They may affect

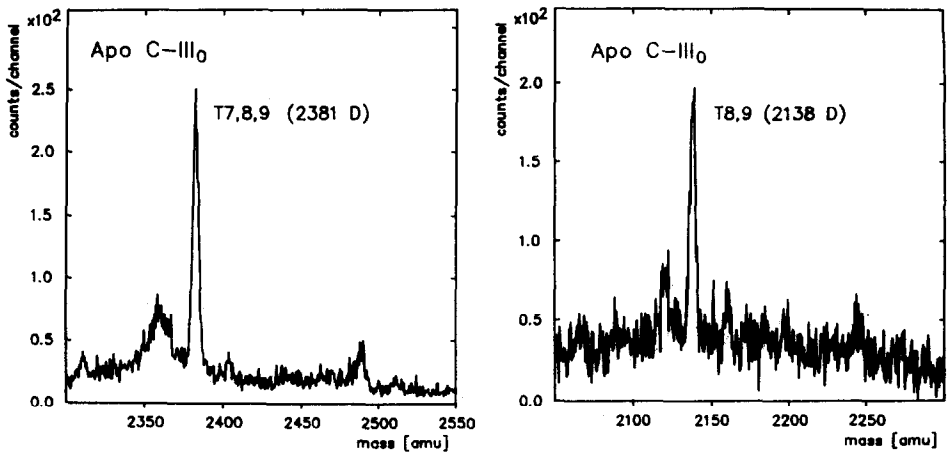

Fig. 6. Molecular mass determination of tryptic peptides T7,8,9 and T8,9 obtained from apo C-III_0 (for details see Fig. 5).

regular metabolism and concentration of plasma lipoproteins. As previously discussed, the combined techniques of isoelectric focusing, HPLC and TOF-SIMS analysis of proteolytic peptides constitute a fast and sensitive tool to identify and to characterize such mutants [4]. The above techniques were here applied to the structural elucidation of an apo C-III mutant, which was discovered in a proband affected by severe hypertriglyceridaemia. The following three observations were made.

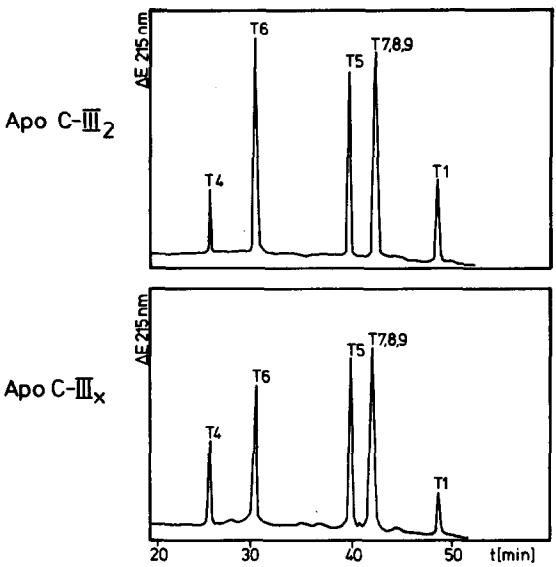

Fig. 7. HPLC of tryptic peptides obtained from apo C-III_2 and from the mutant apo C-III. The assignment of the tryptic peptides to the sequence position was achieved by partial amino acid sequencing and by TOF-SIMS mass analysis.

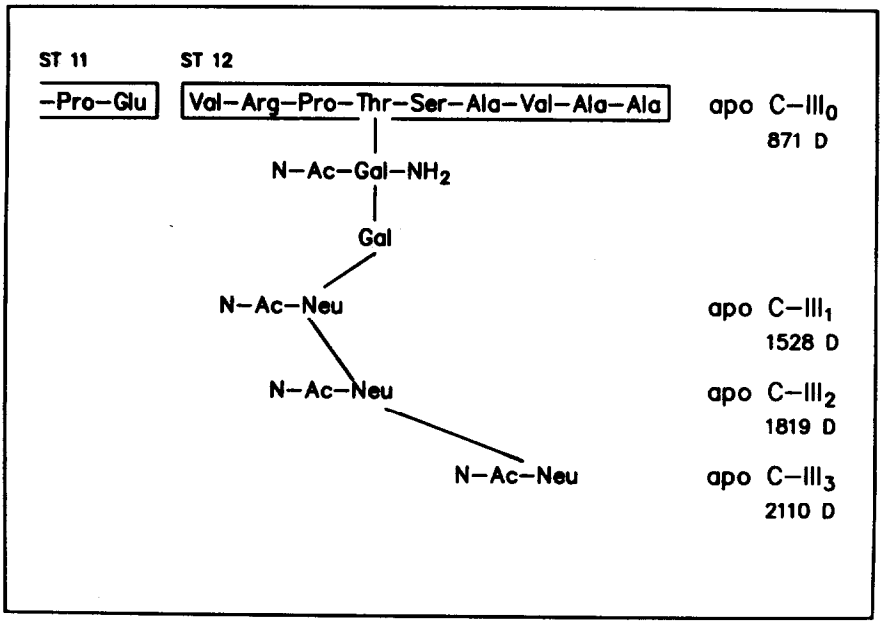

Fig. 8. The carbohydrate chain of apo C-III isoforms and of the mutant apo C-III. The apo C-III isoforms as well as the apo C-III mutant were cleaved with trypsin. The tryptic peptides were characterized by partial amino acid sequence analysis and by TOF-SIMS mass analysis. After separation of the tryptic peptides with HPLC, the peptides T7,8,9 were cleaved with *S. aureus* protease and the molecular masses of these peptides were determined by TOF-SIMS mass analysis.

(1) Normal apo C-III$_0$ lacks any carbohydrate chain. This demonstrated by molecular mass analysis of the carboxy-terminal tryptic peptide of apo C-III$_0$. A similar finding has been previously made for apo E. The major isoform of this

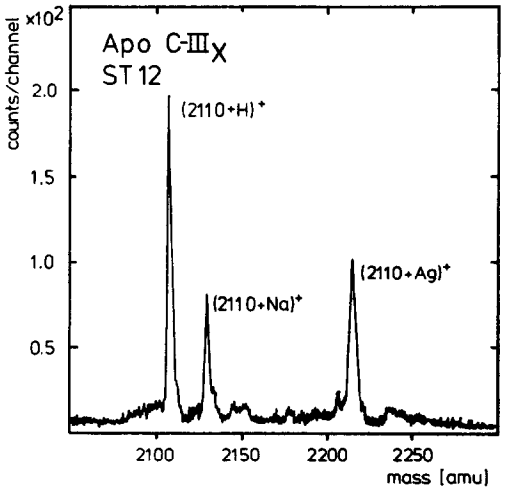

Fig. 9. Molecular mass analysis of peptide T7,8,9 after *S. aureus* protease cleavage (for details see Experimental and Fig. 5).

apolipoprotein is non-glycosylated, in contrast to several minor isoforms. Comparing the biosynthesis of apo C-III and apo E it is noteworthy that both apo E and apo C-III are translated as pre-apolipoproteins. In both proteins no propeptide is found. The pre-peptide of apo C-III contains twenty amino acid residues, and the pre-peptide of apo E has eighteen. Both pre-peptides begin with methionine, have a series of hydrophobic residues in the middle segment and terminate with alanine. After the intracellular cleavage of the signal peptide, apo E is glycosylated with carbohydrate chains containing sialic acid, secreted as sialoapo E, and subsequently desialylated in plasma [15]. Assuming similar posttranslational events for both apo C-III and apo E, it is reasonable to speculate that the mutant apo C-III_3 is a result of incomplete desialylation. Whether this occurs in the cellular compartment (Golgi apparatus) or extracellularly remains to be investigated.

(2) The tryptic peptide T8,9 is completely protected from cleavage in all apo C-III isoforms. Since proline disturbs the helix structure of proteins, the cleavage site Arg-72-Pro is not recognized by the enzyme trypsin. The cleavage site Lys-60-Phe is partially protected in apo C-III_0 and apo C-III_1 and completely protected in apo C-III_2. Thus, the carbohydrate chain of apo C-III seems to interfere in the proteolytic digestion of this cleavage site.

(3) The mutant apo C-III was associated with hypertriglyceridaemia. A family study revealed that the mutant was inherited (Fig. 1). Whether or not hypertriglyceridaemia in the affected patients has a causal relationship to the presence of oversialylated apo C-III remains at present speculative. In vitro studies are currently being performed in our laboratory to study the possible inhibitory effect of apo C-III_3 on lipoprotein lipase.

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