DIFFERENT MOBILITY IN SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS OF APOLIPOPROTEIN E FROM PHENOTYPES Apo E-N AND Apo E-D

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1. Introduction

Apolipoprotein E from human plasma lipoproteins exhibits a genetic polymorphism that is under the control of two allelic autosomal genes designated *Apo* E^n and *Apo* E^d. These alleles determine the three phenotypes Apo E-N, Apo E-ND and Apo E-D [1,2]. The Apo E-ND polymorphism possibly is of high biological significance. Both the mean plasma cholesterol levels and the distribution of cholesterol among the lipoprotein classes are different in the three phenotypic groups [3].

The Apo E-phenotypes are demonstrated by isoelectric focusing and are defined by the ratio of the polymorphic forms Apo E-II/Apo E-III [1,4]. In the phenotype Apo E-D the Apo E-III form is deficient. Individuals with Apo E-III deficiency that represent about 1% of the German population have low plasma cholesterol levels and primary dysbetalipoproteinaemia (type III dyslipoproteinaemia) [1–3]. Coinheritance however of the gene *Apo* E^d in double dose and genes for familial hyperlipidaemia results in the clinical disorder hyperlipoproteinaemia type III [2,5].

The chemical differences between the polymorphic forms of Apo E are not yet known. We show here that apolipoproteins E from phenotypes Apo E-N and Apo E-D differ in M_r according to their mobility in SDS–PAGE and that this reflects a difference in size of Apo E-II and Apo E-III, that are the major Apo E polymorphic forms of the two phenotypes, respectively.

Abbrevations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing

2. Materials and methods

Blood was obtained from fasting individuals with hyperlipoproteinaemia types III, IV and V. Very low density lipoproteins (VLDL) were isolated at +4°C by centrifugation of sera at 59 000 rev./min for 20 h in the 60 Ti rotor of the L2-65B ultracentrifuge (Beckman Inst.) and purified by recentrifugation in 0.85% NaCl–0.05% EDTA (pH 7.4). Lipids were extracted from VLDL by acetone–aethanol 1:1 (v/v) at −20°C. Apo E was purified from apo VLDL by preparative SDS–PAGE in the discontinuous system [6] as outlined in [7]. Analytical SDS–PAGE [6] was performed in slab gels either at a continuous main gel concentration (10% or 15% acrylamide) or in a linear gradient from 15–30% acrylamide. Apoproteins were reduced and alkylated before electrophoresis [8]. For radioactive labelling apo VLDL (2.5 mg) was solubilised in 0.5 ml 0.2 M Tris–HCl (pH 8.6), 6 M guanidine–HCl and reduced by addition of 620 nM dithioerythritol (DTE) for 60 min at 37°C and then reacted with 10 μCi iodo-[1-^{14}C]acetamide (Amersham Buchler, spec. act. 57 mCi/mmol) for 30 min at room temperature in the dark. A 3-fold molar excess over DTE of cold iodo-acetamide (0.37 mg) were then added and the reaction continued for a further 30 min period. Alkylation was stopped by addition of 2-mercaptoaethanol to 5% final conc. The sample was dialysed against H_2O and lyophilised.

The polymorphic forms of Apo E were isolated by preparative IEF in the presence of 6 M urea, in granulated gels [9] containing a 2:1 (v/v) mixture of ampholines (pH 4–6; pH 5–7) (LKB productor, Bromma). Reduced Apo E was focused for 18 h at

20 W using the LKB multiphor equipment. Protein zones were detected by a paper print technique [9]. The pI values were determined in aliquots of the gel diluted in H_2O with a pH electrode. Ampholines were removed from protein by chromatography on Sephadex G-50. Analytical IEF was performed in polyacrylamide gels in a gradient of pH 3.5–10 as outlined [1,4]. Reduced and alkylated proteins were dialysed extensively against 0.02 M aethylmorpholine–HCl (pH 8.6) before focusing. CNBr fragmentation of 0.2–1.0 mg Apo E in 70% HCOOH was performed for 24 h at room temperature with a 400-fold molar excess of CNBr. CNBr peptides were analysed on polyacrylamide gel gradients as outlined [10].

For neuraminidase treatment 300 μg apo VLDL was solubilised in 300 μl 0.05 M Na-acetate, 0.068 M $CaCl_2$, 0.154 M NaCl (pH 5.5). The buffer was either 1 mM in SDS or 0.5 mM in Triton X-100. Neuraminidase (*V. cholerae*, 500 u/ml, Behringwerke, Marburg) 30 μl, was added and the mixture incubated for 20 h at 37°C. Controls were incubated without neuraminidase. Samples were dialysed against 0.02 M aethylmorpholine–HCl (pH 8.6). Then 300 μl of 12 M urea in H_2O were added and 200 μl aliquots were subjected to IEF.

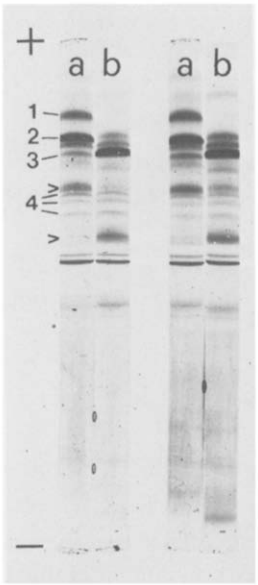

Fig.1. Isoelectric focusing in PAGE of control apo VLDL (a) and neuraminidase treated apo VLDL (b) of phenotype Apo E-ND in a gradient of pH 3.5–10. Apo VLDL was treated with neuraminidase in the presence of SDS or Triton X-100 (right). Numbers label apolipoprotein C-III-2 (1), C-III-1 (2), C-III-0 (3) and apo E (4). Arrow indicates an unidentified apolipoprotein affected by the neuraminidase treatment.

3. Results and discussion

The polymorphism of Apo E demonstrated by IEF might be a result of different degrees of sialylation of the Apo E protein. Therefore apo VLDL was treated with neuraminidase in the presence of detergent (Triton X-100 and SDS) and the Apo E patterns were analysed by IEF. No changes in the focusing properties of the Apo E bands were observed (fig.1). The polymorphic forms of Apo C-III in VLDL however that are known to contain 2 (Apo C-III-2) and 1 (Apo C-III-1) mol sialic acid/mol protein [11,12] were almost quantitatively converted to the asialoprotein (C-III-0). This strongly suggests that sialic acid is not responsible for the Apo E polymorphism.

Reduced and alkylated samples of apo VLDL from individuals of phenotypes Apo E-N, Apo E-ND and Apo E-D were subjected to SDS–PAGE. This demonstrated a slower mobility of the major Apo E component from phenotype Apo E-D compared to Apo E-N. The heterozygous phenotype Apo E-ND exhibited two clearly separated Apo E bands (fig.2). SDS–PAGE in a gradient gel from 15–30% acrylamide confirmed this finding (fig.3). This indicated that Apo E from phenotypes Apo E-N and Apo E-D is of different molecular size. Apo E-N was M_r ~33 000 and Apo E-D M_r ~34 500.

In IEF Apo E-III is the major constituent of Apo E-N and Apo E-II is the main constituent of Apo E-D. This suggests that the different mobilities of Apo E in SDS–PAGE reflect size differences in the Apo E-III and Apo E-II forms of the protein. These forms as well as Apo E-I were partially purified by preparative IEF in granulated gels (not shown). SDS–PAGE of the reduced and alkylated apoproteins shows that Apo E-II and Apo E-III in fact have different mobilities (fig.2) and that the Apo E-I protein has the lowest mobility. This difference in mobilities cannot be explained by the charge differences of the proteins, since according to charge Apo E-II should be of higher mobility than Apo E-III. Also anomalous binding of SDS by one of the proteins seems unlikely

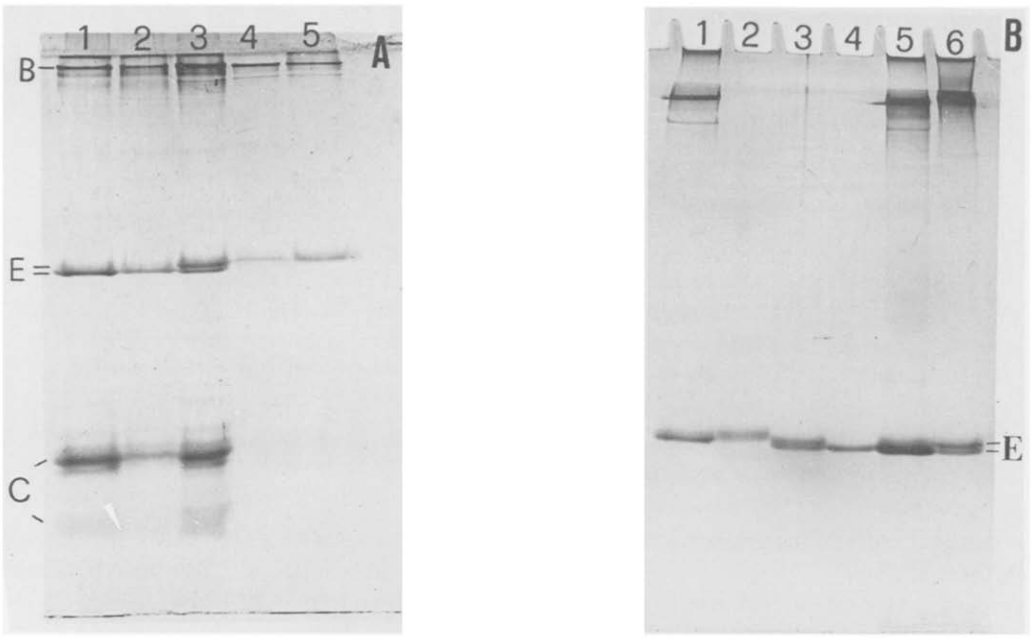

Fig.2. SDS–PAGE in 15% acrylamide gels (A) and 10% acrylamide gels (B) of apo VLDL and Apo E preparations. (A) apo VLDL of phenotypes Apo E-N [1,2], Apo E-ND [3] and Apo E-D [4,5]. (B) apo VLDL of phenotypes Apo E-D [1], Apo E-ND [6] and Apo E-N [5] and of Apo E-I [2], Apo E-II + E-III [3] and Apo E-III [4]. In the 10% gels Apo C has run out of the gels.

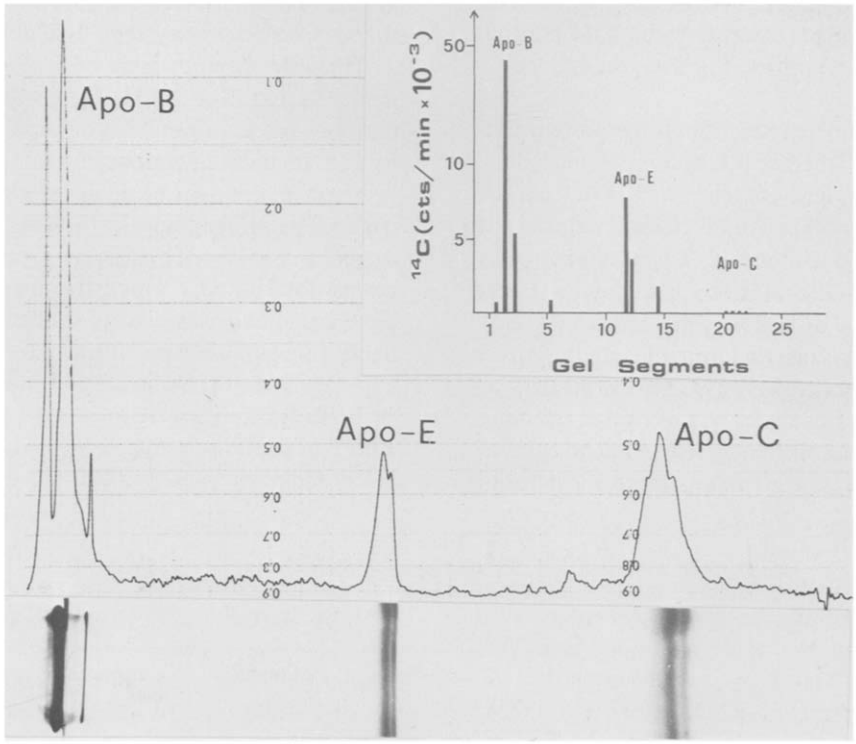

Fig.3. SDS–PAGE of apo VLDL (phenotype Apo E-ND) in a gel gradient from 15–30% acrylamide concentration. Apo VLDL was reduced with DTE and alkylated with iodo-[1-^{14}C]acetamide. The Coomassie brilliant blue-stained gel and the evaluation by scanning densitometry is shown. In the insert the distribution of ^{14}C radioactivity in the same gel is shown.

Table 1
Distribution of Apo E-proteins in unreduced and reduced and alkylated apo VLDL (% densitometric area)

Apoprotein	Phenotype					
	Apo E-N		Apo E-ND		Apo E-D	
	unred.	red.	unred.	red.	unred.	red.
E-I	29	16	43	31	49	30
E-II	25	25	30.5	35.5	51	59
E-III	46	59	26.5	33.5	7	11

unred., unreduced; red., reduced and alkylated

in view of the similarities of the primary structure of the proteins (see below).

Apoprotein E does contain cysteine [13] and does form complexes with Apo A-II by disulfide bridge formation [14]. Also the quantitative distribution of Apo E isoproteins in IEF is affected by reduction and alkylation (table 1) indicating inter- and/or intrachain disulfide bridge formation. In unreduced apo VLDL comparatively more protein does focus in the position of Apo E-I and in a position of minor bands anodic of Apo E-I.

To ensure complete reduction of the protein and also to determine whether the Apo E polymorphic forms differ in cysteine content apo VLDL from the heterozygous phenotype Apo E-ND was reduced with DTE and alkylated with iodo-[^{14}C]acetamide.

After SDS–PAGE and IEF of aliquots the stained gels were evaluated by scanning densitometry. They were then sliced and analysed for radioactivity. Apo B (in SDS–PAGE) and Apo E were heavily labelled whereas no ^{14}C radioactivity was recovered from the Apo C proteins, that are known to contain no cysteine [11]. Hence there is little, if any, unspecific labelling of the proteins under our experimental conditions.

The distribution of ^{14}C radioactivity in the Apo E bands from SDS–PAGE corresponds closely to the distribution of protein from scanning densitometry. The data from SDS–PAGE also agree well with those obtained from the IEF gels (table 2). The experiment thus shows that Apo E is in a reduced state in both systems and that the Apo E forms probably contain the same number of cysteine residues per chain.

The polymorphic nature of human Apo E has been demonstrated [1–5,7,15]. There was however no direct evidence showing that the Apo E bands detected by IEF are indeed polymorphic forms of one protein rather than representing nonidentical polypeptide chains. Crossed immunoelectrofocusing of apo VLDL against a monospecific anti-Apo E serum (raised against total Apo E) demonstrated a reaction of complete immunological identity of the Apo E focusing bands (fig.4). This was confirmed by double diffusion experiments of the isolated Apo E bands (not shown).

CNBr fragments were prepared from purified Apo E of phenotype Apo E-N (predominantly Apo E-III) and phenotype Apo E-D (predominantly Apo

Table 2
Distribution of reduced and alkylated Apo E-proteins in phenotype Apo E-ND

Apoprotein	IEF				SDS–PAGE	
	Densitometric area (%)		^{14}C radioactivity (%)		Densitometric area (%)	^{14}C radioactivity (%)
E-I	31.0	72.0	29.5	73.1	70.5	68.6
E-II	41.0		43.6			
E-III	28.0		26.9		29.5	31.4

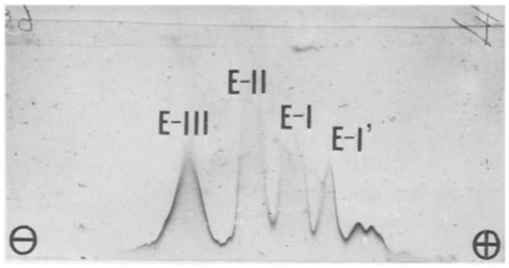

Fig.4. Crossed immunoelectrofocusing of urea soluble apo VLDL (phenotype Apo E-ND) against anti-Apo E. Focusing was performed in a gradient of pH 3.5–6. Staining: Coomassie brilliant blue. E-I′ labels a minor band (pI ~5.2) anodic of Apo E-I.

E-II, fig.2) and the resulting peptides were analysed by SDS–PAGE gradient electrophoresis (fig.5). The peptide patterns from both proteins were indistinguishable and exhibited a major band of M_r ~11 500. This confirms the structural similarities of the Apo E-II and Apo E-III forms. The difference in the M_r of the intact proteins thus is not reflected in the peptide pattern. This however possibly is due to the poor staining and resolution of the smaller peptides in the electrophoretic system used.

It is not yet clear by what mechanism the deficiency of the E-III protein does result in the dysbetalipoproteinaemia. In normal individuals VLDL is degraded in a continous process to lipoproteins of intermediate density (IDL or remnants) and finally to LDL (α_2-pathway, [16]). The typical dyslipoproteinaemia in individuals of phenotype Apo E-D is best explained assuming a metabolic block in the α_2-pathway at the IDL → LDL interconversion step. The finding that also heterozygous individuals (phenotype Apo E-ND) that contain relatively less Apo E-III in VLDL particles than the usual phenotype Apo E-N, tend to a mild form of dysbetalipoproteinaemia [3] suggests that the number of Apo E-III copies/particle is critical for the catabolism of VLDL. The slightly but definitely higher M_r of the E-II form in connection with the quantitative character of the Apo E-ND polymorphism might then indicate that Apo E-II is an inactive precursor that is only partially converted to the active E-III form in phenotype Apo E-ND and not at all in phenotype Apo E-D.

In summary our data show that the Apo E bands

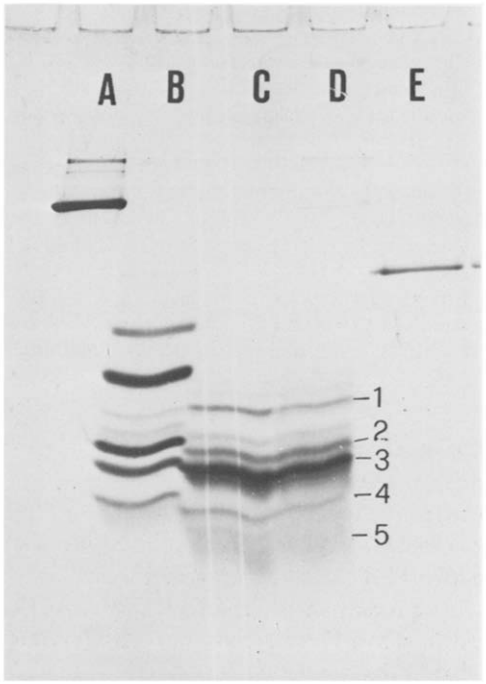

Fig.5. SDS–polyacrylamide gel gradient electrophoresis of CNBr peptides from Apo E of phenotypes Apo E-N (C) and Apo E-D (D): A, HSA; B, CNBr peptides from HSA; E, Apo E. Numbers label fragments of M_r ~16 500 (1), ~12 500 (2), ~11 500 (3), ~8000 (4) and ~6000 (5).

separated by IEF are structurally closely related and represent polymorphic forms of one protein. There does however exist a difference in the M_r of the Apo E-I, Apo E-II and E-III proteins.

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