# Genetic polymorphism of apolipoprotein E: a variant form of apolipoprotein E2 distinguished by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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**Abstract** The apolipoprotein E2 (apoE2) variant that possesses a cysteine substituted for an arginine at residue 158 in the amino acid sequence E2($Arg_{158}$ → Cys) can be distinguished by sodium dodecyl sulfate–polyacrylamide gel electrophoresis from other forms of apoE, including E3 (the parent form), E4($Cys_{112}$ → Arg), E2($Arg_{145}$ → Cys), and E2($Lys_{146}$ → Gln). The E2($Arg_{158}$ → Cys) migrates as a distinctly separable band with a higher apparent molecular weight than the other forms. Chemical modification of apoE2($Arg_{158}$ → Cys) with sulfhydryl reagents (2-bromoethyl)-trimethylammonium bromide or cysteamine, which convert cysteine to arginyl or lysyl analogues, respectively, abolishes the difference in apparent molecular weight and results in the co-electrophoresis of E2($Arg_{158}$ → Cys) with other apoE forms. The mobilities of the other apoE variants are not affected by these modifications. These results suggest that the substitution site at residue 158 is a key location, important in modifying the behavior of apoE and in modulating its apparent molecular weight on sodium dodecyl sulfate–polyacrylamide gels. Furthermore, the technique used in this study may be very helpful in distinguishing specific mutant forms of apoE2.—**Utermann, G., K. H. Weisgraber, W. Weber, and R. W. Mahley.** Genetic polymorphism of apolipoprotein E: a variant form of apolipoprotein E2 distinguished by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *J. Lipid Res.* 1984. **25:** 378–382.

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Three common genetically determined isoforms of human apolipoprotein E (apoE) designated E2, E3, and E4, can be distinguished by isoelectric focusing or two-dimensional gel electrophoresis (1–3). These isoforms are controlled by the alleles $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ at the apoE structural gene locus (2–4). The most common form of apoE2 and the only form of apoE4 thus far observed differ from the parent apoE3 isoform by single cysteine for arginine interchanges at one of two positions in the protein: residues 112 and 158. These two variants are designated E4($Cys_{112}$ → Arg) and E2($Arg_{158}$ → Cys) (4, 5). Recently, two additional E2 mutants have been described: E2($Arg_{145}$ → Cys) and E2($Lys_{146}$ → Gln) (6, 7).

All three apoE2 variants are functionally defective in that they display reduced binding activity to LDL (apoB, E) receptors (6–9). Homozygotes and compound heterozygotes for these mutant apoE forms have primary dysbetalipoproteinemia, either with or without hyperlipidemia (type III hyperlipoproteinemia).

Due to their identical charge, the three E2 variants cannot be distinguished by isoelectric focusing. However, it has been demonstrated in this report that apoE2($Arg_{158}$ → Cys) differs from the other apoE2 variants in its mobility on SDS–polyacrylamide gels, and that the difference is due to the cysteine for arginine interchange at position 158 in its amino acid sequence.

## MATERIALS AND METHODS

The SDS (research grade 98%) was purchased from Serva (Heidelberg, West Germany) and was recrystallized twice from ethanol. Acrylamide (recrystallized four times) was obtained from Roth KG (Karlsruhe, West Germany) and N,N′-methylenbisacrylamide (recrystallized twice) from Serva (Heidelberg, West Germany). The BETAB was purchased from Aldrich (Milwaukee, WI), and the cysteamine from Sigma Chemicals (St. Louis, MO).

### Preparation of apoVLDL and isolation of apoE

The VLDL were prepared by ultracentrifugation (10) from the plasma of individual human donors of defined apoE phenotypes. The VLDL were delipidated by drop-

Abbreviations: apo, apolipoprotein; BETAB, (2-bromoethyl)-trimethylammonium bromide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTE, dithioerythritol; VLDL, very low density lipoproteins.

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wise addition into at least 50 volumes of acetone–ethanol 1:1 (v/v) that had been precooled to −20°C. The mixture was kept at −20°C for 2 hr. The precipitate that formed was pelleted by low speed centrifugation at 4°C. The supernatant was decanted, and the apolipoprotein pellet was re-extracted twice with acetone–ethanol 1:1 (v/v) at −20°C and finally dried with a stream of nitrogen.

Apolipoprotein E was isolated from apoVLDL by preparative SDS–PAGE (11). The apoE preparations from San Francisco have been used in sequence studies, and the primary structure of these proteins has been reported (4–7, 12). These apoE preparations were from patients E.T., E3; D.R. and A.G., E2($Arg_{158}$ → Cys); W.M., E2($Arg_{145}$ → Cys); J.T., E2($Arg_{158}$ → Cys) and E2($Arg_{145}$ → Cys); and D.F., E3/E2($Lys_{146}$ → Gln). The E2($Lys_{146}$ → Gln) isoform was isolated from the apoE of subject D.F. by preparative isoelectric focusing as previously described (4).

### Modification of apoE with BETAB and cysteamine

The apoVLDL were solubilized in 0.02 M N-ethylmorpholine-HCl in 8 M urea, pH 8.5. The insoluble apoproteins (apoB) were removed by centrifugation in an Eppendorf laboratory centrifuge. To the urea-soluble apoproteins (protein concentration of 0.25–1.0 mg/ml), DTE was added to a final concentration of 10 mM, and the samples were incubated at 37°C for 1 hr. Then BETAB was added at a concentration of 40-fold over that of DTE, and the reaction was allowed to proceed at 37°C for 4 hr. Cysteamine modification of the cysteine residues of apoE was performed as previously described (4).

### Electrophoretic procedures

Genetic apoE phenotypes were determined by isoelectric focusing or by two-dimensional electrophoresis of apoVLDL essentially as described (1, 3). The SDS–PAGE of apoVLDL or apoE was performed on slab gels using the Havanna gel electrophoresis equipment (Desage, Heidelberg, West Germany). The discontinuous system of Neville (13) was performed as described using 13% acrylamide in the lower gel. Modification of the procedure by including 0.1% SDS in the upper and lower gel solutions and replacing the upper reservoir buffer containing 75 mM Tris-borate buffer, pH 8.6, with a 75 mM Tris-glycine buffer, pH 8.64, gave identical results. Samples were reduced with DTE or reduced and alkylated (14) before electrophoresis.

## RESULTS AND DISCUSSION

The three major isoforms of apoE are all $M_r$ = 34,000 polypeptides composed of 299 amino acids (5, 6, 15). These major isoforms, as distinguished by isoelectric focusing, are referred to as apoE2, E3, and E4. They differ from one another by single amino acid substitutions (5–7). Both E3 and E4 have been shown to vary in primary structure at residue 112; apoE3 possesses cysteine at this site, whereas E4 possesses an arginyl residue (5). As shown in **Fig. 1,** apoE3 and E4 migrate to an identical position on SDS–polyacrylamide gels. Apparently, the cysteine for arginine interchange does not alter the electrophoretic mobility of these isoforms on SDS–PAGE. As previously described (14), however, and as confirmed in this study, the reduced and alkylated E2 from certain dysbetalipoproteinemic E2/2 subjects exhibits a higher apparent molecular weight than apoE3 and E4 on SDS–polyacrylamide gels (by approximately 1,500 daltons) (Fig. 1).

The primary structure of apoE2 from two different E2/2 homozygous type III hyperlipoproteinemic patients (D.R. and A.G.) has been shown to differ from apoE3 at residue 158 (5, 12). Whereas apoE3 possesses arginine at this site, the E2 from D.R. and A.G. possesses cysteine. Their apoE2($Arg_{158}$ → Cys) displays abnormal electrophoretic migration compared to apoE3 and certain other apoE2s (**Fig. 2A and B**). A cysteine for arginine interchange has been shown to have a similar effect on a mutant, histidine-binding protein of *Salmonella typhimurium* (16).

Recently, other apoE variants that focus in the E2 position on isoelectric focusing gels have been described: E2($Arg_{145}$ → Cys) and E2 ($Lys_{146}$ → Gln). It is significant that both of these variants exhibit mobility on SDS–poly-

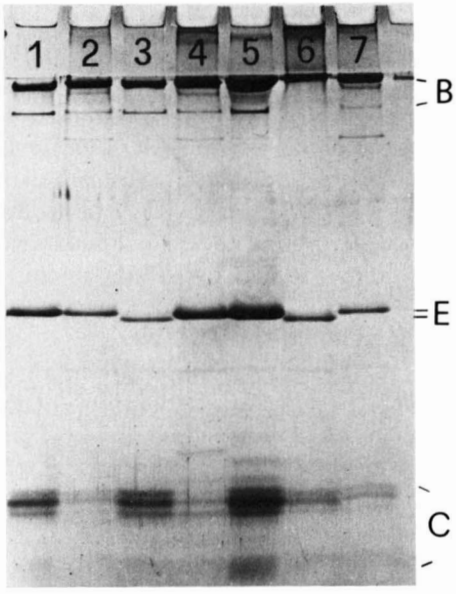


**Fig. 1.** SDS–polyacrylamide gels of individual apoVLDL preparations from probands with different apoE phenotypes. Lanes 1, 2, 4, 5, and 7: apoE2/2; lane 3: apoE3/3; lane 6; apoE4/4. The letters B, E, and C denote positions of apolipoproteins B, E, and C, respectively.

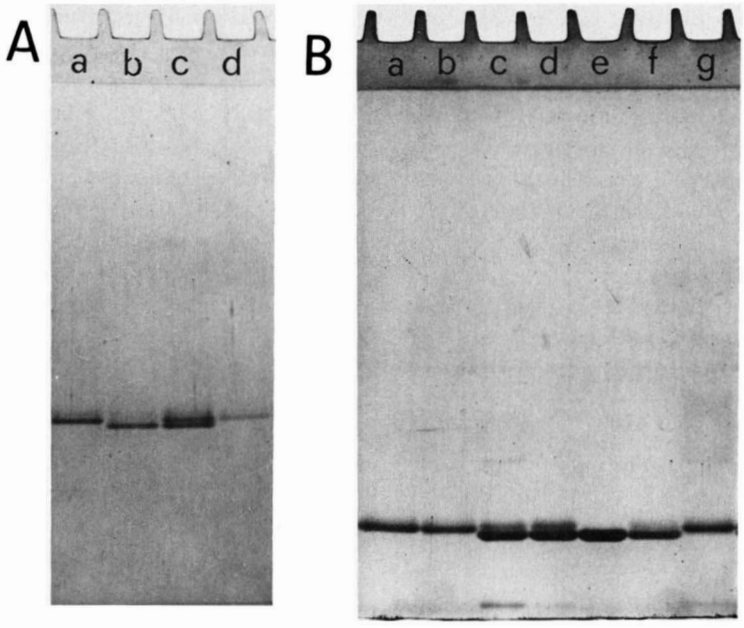


**Fig. 2.** SDS–polyacrylamide gels of the apoE from different patients with type III hyperlipoproteinemia and control subjects. A: Lane a, apoE2($Arg_{158}$ → Cys) from patient D.R. (identical results obtained with apoE2($Arg_{158}$ → Cys) from patient A.G.); lane b, apoE2($Arg_{145}$ → Cys) from patient W.M.; lane c, apoE2 from patient J.T., who is heterozygous for E2($Arg_{158}$ → Cys) and E2($Arg_{145}$ → Cys); lane d, apoE2/2 (Marburg, patient K.G.). B: Lanes a and b, apoE2/2 (Marburg); lane c, apoE3/3 (San Francisco); lane d, apoE3 and E2($Lys_{146}$ → Gln) from patient D.F., who is heterozygous (E3/2); lane e, isolated apoE2 from patient D.F.; lane f, apoE2($Arg_{145}$ → Cys) from patient W.M.; lane g, apoE2($Arg_{158}$ → Cys) from patient D.R. The diffuse higher molecular weight bands in lanes c and d are due to the presence of sialylated derivatives, which occur in a higher proportion than usual in these two apoE preparations.

acrylamide gels identical to apoE3 and E4 (Figs. 1 and 2). The E2($Arg_{145}$ → Cys), like E2($Arg_{158}$ → Cys), differs from the parent E3 by a single cysteine for arginine interchange, but in a different position of the sequence (6). Even though the amino acid substitution in E2($Arg_{145}$ → Cys) is only 13 residues from the substitution site in E2($Arg_{158}$ → Cys), it does not result in the same change of apparent molecular weight. It is of particular interest that the apoE2 from patient J.T. displays two bands on SDS–polyacrylamide gels (Fig. 2A, lane c). Subject J.T. has been shown to be genotypically heterozygous, with one allele coding for E2 ($Arg_{158}$ → Cys) and the other coding for E2($Arg_{145}$ → Cys) (6). Thus, the J.T. apoE2 forms a split band, one corresponding to the position of apoE3, E4, E2($Lys_{146}$ → Gln), and E2($Arg_{145}$ → Cys), and the other corresponding to the apoE2($Arg_{158}$ → Cys) with the apparent higher molecular weight. Consequently, the apoE2 with the cysteine for arginine interchange at position 158, which is thus far the most commonly described apoE2 variant (17), can be distinguished from the other known E2 variants. The simultaneous application of isoelectric focusing and SDS–PAGE permits the distinction of different apoE electrophoretic patterns.

It was of interest to determine whether the chemical modification of the cysteine residues of apoE2 variants

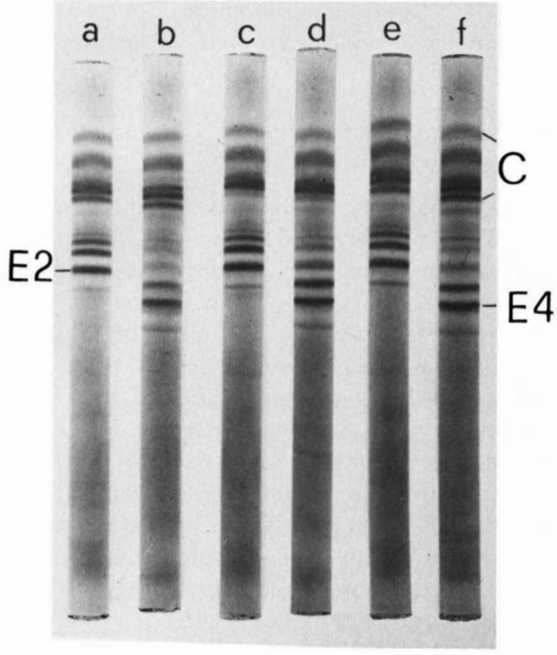


**Fig. 3.** Isoelectric focusing of urea-soluble apoVLDL from three homozygous apoE2/2 probands on a pH gradient from 3.5 to 10. Lanes a, c, and e, control samples; lanes b, d, and f, same samples modified with BETAB. Note the shift of the major isoform from the E2 to the E4 position.

alters their apparent molecular weights. The reagent cysteamine is known to react with cysteine, converting the cysteine to lysyl analogues, adding a positive charge in the protein for each residue of cysteine modified (4, 8). In the present study, the sulfhydryl reagent BETAB was used to accomplish a similar objective. This reagent modifies cysteine by generating an arginyl analogue (18). The modification is irreversible and has been shown to confer two positive charges on apoE2, which possesses cysteine at residues 112 and 158. As shown in **Fig. 3,** the VLDL isoelectric focusing patterns reveal that the BETAB-treated apoE2 shifts to the E4 position, indicating that both cysteine residues are modified.

Examination of the effects of BETAB and cysteamine treatment on the electrophoretic mobility of apoE isoforms was extended to the SDS–PAGE system. As shown in **Fig. 4,** BETAB or cysteamine modification corrects the abnormal mobility of E2($Arg_{158}$ → Cys). No change in mobility occurs when apoE3, E4($Cys_{112}$ → Arg), E2($Arg_{145}$ → Cys), or E2($Lys_{146}$ → Gln) are treated with BETAB and subjected to SDS–PAGE (data not shown). These results demonstrate that the cysteine for arginine substitution at residue 158 is responsible for the abnormal migration of apoE2($Arg_{158}$ → Cys).

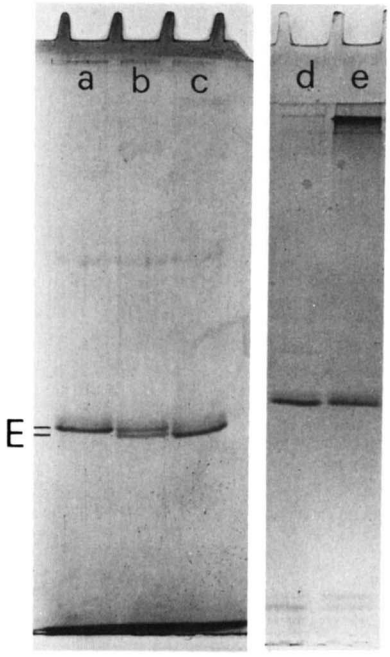


**Fig. 4.** SDS–polyacrylamide gels of control apoVLDL and apoVLDL modified with BETAB or cysteamine. The VLDL were obtained from individuals with different apoE phenotypes. Lane a, apoE2($Arg_{158}$ → Cys) homozygote; lane b, apoE3/2; lane c, apoVLDL from the E2/2 subject (lane a) after treatment with BETAB; lane d, apoE3/3 control; lane e, apoVLDL from the E2/2 subject after treatment with cysteamine. Samples in lanes d and e were run under non-reducing conditions.

The underlying factor(s) responsible for the abnormal migration of apoE2($Arg_{158}$ → Cys) is not understood. The substitution of cysteine at residue 158 may cause subtle differences in the binding of SDS or other amphiphilic substances to the protein, which in the case of SDS could alter its electrophoretic mobility. On the other hand, residue 158 may be a key determinant capable of influencing the conformation of the apoE molecule, and the absence of a positive charge at this site could have profound consequences on its behavior on SDS–PAGE. It has been shown that the apoE2($Arg_{158}$ → Cys) mutant is more severely defective in binding to apoB,E receptors (<2% of normal binding, as compared to the parent E3) than E2($Arg_{145}$ → Cys) and E2($Lys_{146}$ → Gln) (40 to 50% of normal binding) (7–9). In addition, it has also been suggested that the substitution of cysteine for arginine at residue 158 has a deleterious effect on receptor binding activity by altering the conformation of the adjacent receptor binding domain of the apoE molecule (19).

These data indicate that altered mobility on SDS–PAGE is limited to apoE2($Arg_{158}$ → Cys). However, the possibility exists that other variants may be discovered in the future, which will also show this effect. If it is confirmed that the effect is limited to a cysteine-arginine interchange at residue 158 as other mutants are described, then the SDS–PAGE system may be used as a simple screening method to identify certain "E subtypes."

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