Normalization of Receptor Binding of Apolipoprotein E2

EVIDENCE FOR MODULATION OF THE BINDING SITE CONFORMATION*

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Apolipoprotein (apo-) E3, when combined with the phospholipid dimyristoylphosphatidylcholine (DMPC), binds avidly to apo-B,E (low density lipoprotein) receptors on human fibroblasts. Apolipoprotein E2 isolated from type III hyperlipoproteinemic subjects, which differs from apo-E3 by the presence of cysteine instead of arginine at residue 158, possesses only about 1% of the receptor binding activity of apo-E3. Modification of apo-E2 with cysteamine, which converts the cysteine at position 158 to a positively charged lysine analogue, activates receptor binding -13-fold. In the present experiments, thrombin was used to cleave apo-E2 into two fragments (M₁ = 22,000 and M₂ = 10,000). The larger fragment, which has been shown to possess the receptor binding domain, displayed binding activity up to 12-fold greater than intact apo-E2 or equivalent to apo-E2 treated with cysteamine. When the M₁ = 22,000 fragment was modified with cysteamine and combined with DMPC, receptor binding was further enhanced, attaining the level of activity of normal apo-E3•DMPC, a 100-fold increase over apo-E2•DMPC binding. When the cysteamine modification was reversed by incubation with β-mercaptoethanol, the M₁ = 22,000 fragment retained most of its binding activity. However, when the same sample was tested 24 h later, the level of binding activity dropped significantly. The receptor binding of apo-E2-containing β-very low density lipoproteins could also be activated by cysteamine treatment, with the same retention of enhanced binding activity occurring after the reversal of the modification. These results indicate that apo-E2 can attain full binding activity by the removal of the carboxyl-terminal one-third of the molecule and the addition of a positive charge at residue 158 of the molecule. The retention of enhanced binding after the reversal of the cysteamine modification indicates that the enhanced binding is probably due to conformational changes induced in the binding domain (and maintained by the phospholipid) and not merely to the presence of the positive charge at residue 158.

Apolipoprotein E is a protein constituent of several different lipoproteins. It plays an important role in mediating the interaction of the lipoproteins with specific lipoprotein receptors on cell surfaces (for review see Ref. 1). One of these cell surface lipoprotein receptors, the apo₁B,E (LDL) receptor, is present on both hepatic and extrahepatic tissues (1,2) and mediates the catabolism of apo-E- and apo-B-containing lipoproteins. The liver also possesses a unique receptor, the apo-E receptor (3), which recognizes apo-E-containing lipoproteins (but not those containing apo-B). Apolipoprotein E appears to be the major, if not the exclusive, protein determinant responsible for the hepatic clearance of both chylomicron remnants (4) and the cholesterol-rich HDL-with apo-E (HDL₃) (5).

There are three major isoforms of apo-E that are now known to be genetically determined (6). They are referred to as apo-E₂, apo-E₃, and apo-E₄. Amino acid sequence analysis has established that the three major forms differ by single amino acid substitutions (7). Population studies have established that apo-E₃ is the most commonly occurring form of apo-E and thus appears to be the parent form. Apolipoprotein E₄ differs from apo-E₃ at residue 112, where arginine replaces the normally occurring cysteine. The most commonly occurring form of apo-E₂ differs from apo-E₃ at residue 158. The apo-E₂ possesses cysteine at this site instead of arginine. This mutant form is designated apo-E₂(Arg₁₅₈→Cys). In addition, other variant forms of apo-E have now been described: E₃(Cys₁₁₂→Arg, Arg₄₄₄→Cys), E₂(Asp₁₄₂→Gln), E₂(Asp₁₄₂→Thr, Ala₁₆₀→Pro), and E₁(Glu₂₈→Asp, Arg₄₅₅→Cys) (8–11).

The lipid disorder type III hyperlipoproteinemia is associated with the occurrence of apo-E₂ and is characterized by the accumulation of cholesterol-rich chylomicron remnants and hepatic β-VLDL in the plasma (for review see Ref. 12). The defective receptor binding of apo-E₂ is required for the expression of this genetic disease (13,14), although its presence is not necessarily sufficient to cause the full expression of the lipid abnormality, which is the result of the increase in plasma levels of the lipoproteins that are usually cleared by the liver via apo-E. Studies of the abnormal receptor binding mutants of apo-E have focused attention on the central region of the 299-amino acid apo-E molecule, including residues 142, 145, 146, and 158. These studies have emphasized the importance of the amino acids in this region in mediating receptor binding and, in particular, the positively charged amino acids arginine and lysine (8–10,14).

Studies of apo-E using fragments of the molecule generated

1 The abbreviations used are: apo-, apolipoprotein; apo-E₂(T-22K), the thrombolytic, M₁ = 22,000 fragment of apo-E2•DMPC; apo-E₂(CysNH₂), apo-E₂ modified with cysteamine; apo-E₂(T-22K)-CysNH₂, the thrombolytic, M₁ = 22,000 fragment of apo-E₂ modified with cysteamine; β-VLDL, β-migrating very low density lipoproteins; DMPC, dimyristoylphosphatidylcholine; HDL₃, high density lipoproteins; LDL, low density lipoproteins.

2 Stanley C. Rall, Jr., unpublished observations.

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by enzymatic and chemical cleavage have indicated that the receptor binding domain of apo-E exists in the region between residues 126 and 191 (15). Thrombolytic cleavage of apo-E generates two major fragments: a $M_r = 22,000$ fragment comprising the NH$_2$-terminal region (residues 1 through 113) and a $M_r = 10,000$ fragment (residues 216 through 299). The $M_r = 22,000$ fragment was shown to possess full receptor binding activity, whereas the $M_r = 10,000$ fragment lacked detectable binding activity. Cyanogen bromide cleavage further narrowed the potential receptor binding region to a fragment including residues 226 to 181 (15).

The use of monoclonal antibodies, which react with different regions of the apo-E molecule, has also indicated that the middle of the apo-E molecule contains the receptor binding domain (16). The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150. The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150. The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150. The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150. The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150. The apo-E monoclonal antibody 1D7, which inhibits receptor binding, has been shown to react specifically with the apo-E molecule in the vicinity of residues 140 to 150.

The importance of a positive charge at residue 158 for receptor binding was established by treating apo-E2(Arg$_{158}$→Cys) with the reagent cysteamine. Cysteamine (β-mercaptoethylamine) forms a reversible mixed disulfide linkage with cysteiny1 residues and converts the cysteine to a positively charged analogue of lysine (17). This chemical modification has been shown to activate the receptor binding activity of apo-E2-phospholipid complexes 10- to 20-fold (14). Moreover, cysteamine treatment enhances the receptor binding of apo-E2 β-VLDL for both the apo-E and the apo-B,E receptors (18).

In the present study, we describe modifications that increase the receptor binding activity of apo-E2(Arg$_{158}$→Cys) to normal values. The evidence gathered in the study indicates that the cysteine for arginine substitution at residue 158 disrupts receptor binding by altering the conformation of the binding domain of this mutant apo-E.

MATERIALS AND METHODS

Human LDL (d = 1.02 to 1.05) were isolated from the plasma of normal fasting subjects by sequential ultracentrifugation (19). Apo-protein E3 was isolated from two subjects with type V hyperlipoproteinemia; apo-E2(Arg$_{158}$→Cys) was isolated from two subjects with type III hyperlipoproteinemia. The β-VLDL from type III hyperlipoproteinemia patients were isolated and washed once by centrifugation at $d = 1.006$ g/ml for 16 h at 50,000 rpm in a 60 Ti rotor. The β-VLDL were separated from the pre-β-VLDL by Pevikon block electrophoresis as described (18). Apoprotein E was isolated from the d < 1.02 ultracentrifugal fraction by Sephadex G-200 column chromatography of the delipidated d < 1.02 lipoproteins (17). The purity of the apoproteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20).

The apo-E2 was hydrolyzed with thrombin and the fragments were isolated by chromatography on a Sephadex G-100 column as previously described for apo-E3 (15). The apo-E2(T-22K) fragment possessed the correct amino acid composition and the purity was confirmed by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21). The LDL were radiolabeled by the iodine monochloride method as described by Bilheimer et al. (22) and the apo-E and apo-B(T-22K) were iodinated by the Bolton-Hunter technique as described (23, 24), after the protein lipid complexes were isolated and dialyzed against 0.15 M NaCl, 0.01% EDTA. Cysteamine modification of β-VLDL, apo-E2, and the $M_r = 22,000$ fragment was performed as described (17, 18). The β-VLDL (600 μg of protein) or the apoproteins (150 μg of protein) in 0.1 M NH$_4$HCO$_3$ were modified by incubation with 10 μl of a cysteamine solution (100 mg/ml) per 150 μg of protein for 4 h at 37°C. Control (unmodified) β-VLDL were incubated similarly without cysteamine for 4 h at 37°C. The control and cysteamine-treated intact apo-E2 and the control and cysteamine-treated thrombolytic fragments of apo-E2 were not dialyzed. They were complexed with DMPC as described below.

The DMPC vesicles were prepared by the procedure of Roth et al. (25). An aliquot of 1 ml (20 mg) of DMPC (Avanti Polar Lipids, Catalogue No. 850345), stored at −20°C in a chloroform solution, was dried in tissue culture dishes. The lipid was dissolved in benzene (1 to 2 ml), frozen, and lyophilized. Two ml of a 0.15 M NaCl, 10 mM Na$_2$EDTA, 1 mM Tris-HCl buffer (pH 7.6) was added to the dried DMPC and the DMPC was allowed to hydrate for 30 to 60 min at room temperature. The 15-ml glass conical tube containing the DMPC was placed in a beaker of H$_2$O at 30°C and sonicated for 15 min using a Branson sonifier No. 200 with a microtip and a No. 4 setting. Titanium particles from the sonifier were removed by low speed centrifugation. Routinely, 150 μg of apoprotein (0.5 to 1 mg/ml) in 0.1 M NH$_4$HCO$_3$ (pH 7.9) was complexed with 56 μl of DMPC vesicles. All apo-E samples were reduced with 0.5 μl of β-mercaptoethanol/100 μg of apoprotein unless specified otherwise. The complexes were cycled through the transition temperature of 23.3°C by cooling to 15°C and warming to 30°C several times, then left at room temperature for 30 min. The complexes were isolated on a 1.006 to 1.21 KBr density gradient. After layering the sample on the top of the gradient, the samples were centrifuged at 15°C in a SW5 rotor for 20 h at 55,000 rpm (369,000 x g). The apo-E-DMPC complex was isolated at a density of 1.09 to 1.10 and dialyzed against 0.15 M NaCl and 0.01% EDTA before use in tissue culture experiments. The apo-E2(T-22K)-DMPC complex was similar in size, morphology, and lipid composition to the apo-E-DMPC complexes previously described (24, 26).

The reversal of the cysteamine modification was performed on the isolated protein-lipid complexes or the β-VLDL after they had been dialyzed against saline-EDTA. The β-mercaptoethanol was added to the β-VLDL or protein complexes at a final concentration of 0.1% or 0.2% and the samples were incubated for 1 h at room temperature before they were used for the tissue culture experiments. Samples for isoelectric focusing were dialyzed against 0.01% EDTA, lyophilized, and delipidated with chloroform/methanol (2:1, v/v). Isoelectric focusing on polyacrylamide gels was used to determine the apo-E isofrom pattern and to monitor the extent of the cysteamine modification and the reversal of the modification (17). The isoelectric focusing gels from a typical experiment illustrate that the modification of the apo-E2(T-22K) fragment with cysteamine and the reversal of the modification with 0.2% β-mercaptoethanol were essentially complete (Fig. 1).

Normal human fibroblasts were grown in Dulbecco's modified Eagle's medium (GIBCO, Catalogue No. 4320-2100) with 10% fetal calf serum (Sterile Systems, Logan, UT) at 37°C in a humified incubator at a CO$_2$ concentration of 7.5%. One week before an experiment, the fibroblasts were plated at a density of 3.5 x 10$^5$ cells/dish. The cells were switched to medium containing 19% lipoprotein-deficient serum 48 h before the binding experiment. The binding assays at 4°C were performed as described previously (24) (see also the figure legends). The binding results were analyzed by the method of Scatchard (27).

The apo-E monoclonal antibody 1D7 was kindly provided by Drs. R. W. Milne and Y. L. Marcel of Montreal, Canada. The ability of this antibody to block receptor binding activity of apo-E-DMPC (16)
and apo-E-containing lipoproteins (18) has been described previously. Highly purified thrombin (specific activity, 2800 units/ml) was a generous gift from Dr. J. W. Fenton II of the New York Department of Health, Albany, NY.

**RESULTS**

Apolipoprotein E2(Arg158→Cys), when combined with the phospholipid DMPC, typically demonstrated only ~1% of the receptor binding activity of apo-E3-DMPC. As shown in Table I, 50% displacement of 125I-LDL from the apo-B,E (LDL) receptors on human fibroblasts occurred at an apo-E2(Arg158→Cys) concentration of 5.1 μg of protein/ml, compared to an apo-E3 concentration of 0.044 μg of protein/ml required for similar displacement of 125I-LDL. In agreement with a previous report (14), cysteamine treatment of the E2(Arg158→Cys) activated receptor binding activity ~12-fold (Table I, 50% displacement at an apo-E2-CysNH2 concentration of 0.42 μg of protein). Cysteamine treatment of LDL or apo-E3 did not increase their receptor binding activity (data not shown) (14). Cysteamine modification of the apo-E was monitored by changes in the isoelectric focusing pattern on polyacrylamide gels (17). For each cysteiny1 residue modified, an additional positive charge was added to the apoprotein. Thus, because apo-E2 possesses two cysteines when modified with cysteamine, its migration on isoelectric focusing gels differs from untreated apo-E2 by two charge units.

To explore other treatments that might modulate receptor binding, the apo-E2(Arg158→Cys) was hydrolyzed with thrombin and the fragments were recombined with DMPC and tested for binding activity. Previously, Innerarity et al. (15) and Gianturco et al. (28) showed that two major fragments of apo-E3 are generated by thrombin cleavage. Likewise, when apo-E2 was cleaved with thrombin, a Mr = 22,000 fragment, apo-E2(T-22K), and a Mr = 10,000 fragment, apo-E2(T-10K), were obtained. Since the Mr = 22,000 fragment of apo-E3 demonstrated full receptor binding (15), the same fragment of apo-E2(Arg158→Cys) was recombined with DMPC and tested for its ability to bind to fibroblast receptors. As shown in Fig. 2, the receptor binding activity of the Mr = 22,000 fragment of apo-E2 was enhanced about 10-fold compared to that of intact apo-E2. The results from four preparations used in five separate studies are summarized in Table I.

It was then determined if cysteamine treatment of the Mr = 22,000 fragment would further activate receptor binding. Following treatment with cysteamine, the Mr = 22,000 thrombolytic fragment of apo-E2 complexed with DMPC was as effective as intact apo-E3-DMPC in competing with 125I-LDL for apo-B,E receptors (Fig. 3A). The results obtained with the apo-E2(T-22K)-CysNH2 in a number of experiments, summarized in Table I, revealed that the combined treatment normalized the receptor binding activity of the apo-E2 so that it reached the level of that of apo-E3-DMPC.

In direct binding studies, the cysteamine-treated, Mr = 22,000 fragment of apo-E2 gave results identical with those obtained for apo-E3 (Fig. 3B). When the results from the binding experiments were plotted by the method of Scatchard, the data from both curves fit straight lines. In addition, both lipid-protein complexes bound with high affinity and almost identical binding parameters. The equilibrium dissociation constants for the binding of the cysteamine-treated, Mr = 22,000 fragment and the apo-E3 were 9.0 × 10−7 μg of protein/ml and 7.4 × 10−7 μg of protein/ml, respectively. Thus, nearly normal binding activity could be obtained by removing residues 192 to 290 (the carboxyl-terminal one-third of the apo-E2 molecule), followed by treating the Mr = 22,000 fragment with cysteamine (addition of a positive charge via cysteine modification).

Previously, we demonstrated that the apo-E monoclonal antibody 1D7 prevents apo-E3 from binding to apo-B,E receptors (16). The epitope for this antibody was shown to be located in the vicinity of residues 140 to 150. Using this same

| Protein and treatment | Concentration of the apoprotein: DMPC complex that displaced 50% of the 125I-LDL from the apo-B,E receptors on cultured human fibroblasts | Enhancement of binding activity compared to intact E2 |
|-----------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------|
| Apo-E2(Arg158→Cys)    | 5.1 ± 1.7, N = 6 (N = 4)                                                                        | 70                                             |
| Apo-E2-CysNH2          | 0.42 ± 0.12, N = 4 (N = 5)                                                                       | 12                                             |
| Apo-E2(T-22K)          | 0.38 ± 0.13, N = 5 (N = 4)                                                                       | 13                                             |
| Apo-E2(T-22K)-CysNH2   | 0.058 ± 0.038, N = 7 (N = 5)                                                                     | 100                                            |
| Apo-E3                 | 0.044 ± 0.014, N = 6 (N = 6)                                                                      | 65                                             |

*In contrast to these results, two preparations used in three experiments did not display this marked activation in receptor binding activity (50% displacement at 3.0 ± 1.7 μg of protein/ml). There were no discernable differences in the preparations or the densities in which the lipid-protein complexes were isolated after density gradient centrifugation. The reason for the inactivity of these two preparations cannot be explained.

**Fig. 2.** Comparison of the ability of apo-E2-DMPC (A), apo-E2-DMPC treated with cysteamine (B), and the thrombolytic Mr = 22,000 fragment of apo-E2-DMPC (C) to compete with 125I-LDL for binding to the LDL (apo-B,E) receptors on normal human fibroblasts. One ml of medium containing 10% lipo-protein-deficient serum, 2 μg/ml of human 125I-LDL, and the indicated concentration of apoproteins were added to the 30-mm dishes of cells. After 2-h incubation at 0°C, the cells were washed extensively with cold phosphate-buffered saline containing 2 mg/ml of bovine serum albumin and then solubilized in 0.1 M NaOH prior to counting radioactivity. The 100% control value for the 125I-LDL bound was 82 ng of LDL protein/mg of cellular protein.
Fig. 3. Comparison of the ability of apo-E3-DMPC (■) and apo-E2(T-22K) treated with cysteamine (○) to bind to LDL receptors on cultured human fibroblasts as shown by a competitive binding assay (A) and a direct binding assay (B). In the competitive binding assay (A), the indicated concentrations of apo-E lipid complexes were added in 1 ml of medium containing 10% serum and 2 μg/ml of human 125I-LDL. Other experimental conditions are described in Fig. 2. In the direct binding experiment (B), 125I-apo-E3-DMPC (■) and 125I-apo-E2(T-22K)-CysNH2-DMPC (○) in 1 ml of medium were added to cultured human fibroblasts. After a 5-h incubation at 0 °C, the cells were harvested as described in Fig. 2. The results shown are of specific binding, which was calculated by subtracting the nonspecific binding from the total binding. The nonspecific binding was the amount of 125I-labeled apoprotein bound to the cells in the presence of the d = 1.02 to 1.063 ultracentrifugal fraction (300 μg/ml) from a hypercholesterolemic dog. The average cell protein per 35-mm Petri dish was 302 μg.

Fig. 4. Ability of the apo-E monoclonal antibody 1D7 to inhibit the binding of 125I-apo-E3-DMPC (■) and 125I-apo-E2(T-22K)-CysNH2-DMPC (○) to normal human fibroblasts. One ml of medium containing 10% lipoprotein-deficient serum and either 125I-apo-E3-DMPC or 125I-apo-E2(T-22K)-CysNH2-DMPC (both at 0.05 μg of protein/ml) were preincubated with anti-apo-E (1D7) for 1.5 h at 23 °C and then cooled to 4 °C for 30 min. The mixture was then added to the cultured fibroblasts. The 100% control values for the specific binding of 125I-apo-E3-DMPC and 125I-apo-E2(T-22K)-CysNH2-DMPC were 2.9 and 3.3 μg/mg of cellular protein, respectively. More detailed experiments using 1D7 to inhibit apo-E binding to receptors can be found in Refs. 16 and 18.

antibody, we were able to show that the cysteamine-treated, 

Mₐ = 22,000 fragment of apo-E2 was binding to receptors at the same region of the molecule. As shown in Fig. 4, the apo-

E monoclonal antibody 1D7 was equally effective in inhibiting the receptor binding activity of the intact apo-E3-DMPC and the apo-E2(T-22K)-CysNH2-DMPC.

Consideration was given to the possibility that the positive charge at residue 158 is not directly involved in mediating the interaction of apo-E with cell surface receptors, but is instead important in attaining the proper conformation of the binding domain. It was possible to test this hypothesis by reversing the cysteamine modification of the apo-E2(T-22K)-CysNH2-

DMPC. The Mₐ = 22,000 fragment was treated with cysteamine, combined with DMPC, isolated by centrifugation, and dialyzed. It was then treated with β-mercaptoethanol. While cysteamine treatment increased the positive charge of the Mₐ = 22,000 fragment by two charge units, the addition of β-mercaptoethanol reversed this modification. The Mₐ = 22,000 fragment that had undergone reversal of cysteamine modification was as active as the cysteamine-modified fragment in competing with 125I-LDL for binding to LDL receptors on cultured fibroblasts. The samples that had undergone reversal of cysteamine modification were tested immediately after a 1-h incubation with β-mercaptoethanol at room temperature. The 100% control value for the amount of 125I-LDL bound was 115 ng of LDL protein/mg of cellular protein.

Fig. 5. Comparison of the ability of apo-E2(T-22K)-CysNH2-DMPC (■) and apo-E2(T-22K)+DMPC treated with cysteamine and then with β-mercaptoethanol to reverse cysteamine modification (○) to compete with 125I-LDL for binding to LDL receptors on cultured fibroblasts. The samples that had undergone reversal of cysteamine modification were tested immediately after a 1-h incubation with β-mercaptoethanol at room temperature. The 100% control value for the amount of 125I-LDL bound was 115 ng of LDL protein/mg of cellular protein.
were tested 24 h later, the cysteamine-treated fragment that had been subjected to reversal of cysteamine modification with β-mercaptopoethanol showed a substantial decrease in receptor binding activity (Fig. 6). These results demonstrate that the presence of the positive charge was not essential in the direct interaction with the receptor. Once the normal binding conformation was attained, the positive charge could be removed and, at least temporarily, the normal binding conformation of the apo-E2-phospholipid complex could be maintained. A diagram of this model is shown in Fig. 7.

Other insights were also gained by these experiments. For example, the receptor binding activity of the Mr = 22,000 fragment was only partially enhanced by cysteamine if the fragment was combined with DMPC prior to cysteamine treatment (data not shown). Furthermore, if the cysteamine treatment of the Mr = 22,000 fragment of apo-E2 was reversed with β-mercaptopoethanol and the fragment was recombined with DMPC, it was no more active in regard to receptor binding than the Mr = 22,000 fragment that had never been modified with cysteamine. Normal receptor binding activity was attained only when the fragment was treated with cysteamine and then recombined with DMPC.

These findings were further examined in studies of the effects of cysteamine treatment on the apo-E2 associated with plasma β-VLDL. Previously, we demonstrated that cysteamine treatment of the β-VLDL from a subject homozygous for apo-E2(Arg15ε-Cys) markedly activated the binding of these particles to both apo-B,E and apo-E receptors (18). In addition, we showed that even though the apo-E2 in the native β-VLDL of these subjects was defective, it was still responsible for residual receptor binding activity (18). Therefore, it was of interest to determine whether reversal of the cysteamine modification of the β-VLDL would result in sustained enhancement of receptor binding activity. As shown in Table II, cysteamine-treated β-VLDL subjected to reversal of cysteamine modification demonstrated sustained, enhanced binding to the apo-B,E receptors of fibroblasts. The level of this binding activity was similar to that of β-VLDL treated with cysteamine.

**DISCUSSION**

Three lines of evidence have implicated the same region of the 299-amino acid apo-E molecule as the receptor binding domain of the molecule: 1) binding studies using portions of apo-E (which were fragmented enzymatically or chemically) focused attention on the central region of the molecule (15), 2) monoclonal antibody studies showed that the epitope of an apo-E monoclonal antibody that inhibited receptor binding activity was located in the region of residues 140 to 150 (16), and 3) structure-function studies identified a number of mutant forms of apo-E that have diminished ability to bind to apo-B,E (LDL) and apo-E receptors (8-10, 14, 18). The amino acid substitutions responsible for these mutants are all clustered in the middle of the apo-E molecule and involve the substitution of a neutral amino acid for the basic amino acid arginine or lysine. These mutants include: E3(Cys117→Arg, Arg156→Cys), E2(Arg156→Cys), E2(Lys15ε→Gln), and E2(Arg15ε→Cys) (7, 9, 10).

The first three apo-E variants all display slightly or moderately defective receptor binding activity, while the apo-E2 with a substitution at residue 158, E2(Arg15θ→Cys), has greatly diminished receptor binding. An inspection of the amino acid sequence and a space-filling model of the predicted

![Fig. 6. Comparison of the ability of apo-E2(T-22K)-CysNH2*DMPC (●) and apo-E2(T-22K)+DMPC treated with cysteamine and then with β-mercaptopoethanol to reverse cysteamine modification (●) to inhibit the binding of 125I-LDL to cultured fibroblasts 24 h after the second sample had been treated with β-mercaptopoethanol. The 100% control value for the amount of 125I-LDL bound was 145 ng of LDL protein/mg of cellular protein.](image)

![Fig. 7. Diagram summarizing the alterations in receptor binding activity of apo-E2 after treatment with cysteamine and/or thrombin. Because apo-E2 possesses two cysteiny1 residues, cysteamine modifies both of them. Previous studies have indicated that the second cysteiny1 residue (position 112) is probably not involved directly or indirectly in receptor binding.](image)

**Table II**

Comparison of the ability of control 125I-β-VLDL, cysteamine-modified 125I-β-VLDL, and 125I-β-VLDL that had undergone modification of cysteamine modification to bind to cultured human fibroblasts

| Treatment | ng/mg cellular protein |
|-----------|------------------------|
| Control 125I-β-VLDL | 0.2 μg/ml | 23 |
| Control 125I-β-VLDL | 0.4 μg/ml | 38 |
| Cysteamine-modified 125I-β-VLDL | 0.2 μg/ml | 42 |
| Cysteamine-modified 125I-β-VLDL | 0.4 μg/ml | 91 |
| Reversal of cysteamine modification of 125I-β-VLDL | 0.2 μg/ml | 41 |
| Reversal of cysteamine modification of 125I-β-VLDL | 0.4 μg/ml | 87 |

One ml of Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient serum and the indicated concentrations of lipoproteins were added to the 35-mm dishes of cultured fibroblasts. After a 3-h incubation at 0°C, the cells were harvested as described in the caption to Fig. 2. The results represent specific binding.
secondary structure of the binding domain affords a possible explanation (Fig. 8). The region that extends from residues 130 to 150 is rich in basic amino acids. Previously, it has been shown that the basic amino acids arginine and lysine are necessary for the receptor binding of apo-E (29, 30). Based on the Chou-Fasman rules (31, 32), this region (residues 130 to 150) would exist as an α-helix followed by a β-turn and then a β-sheet structure that includes residue 158 (7). The amino acid substitutions at residues 142, 145, and 146 are all part of this predicted α-helical structure and lie on the same face of the helix. As shown, steric considerations do not preclude the positioning of residue 158 in close spatial proximity. It is possible that all of these residues may be involved directly in mediating receptor binding. However, consideration was also given to the possibility that residue 158 might be involved only indirectly in receptor binding by modulating the conformation of the protein.

Previously, we have shown that apo-E3, devoid of phospholipid, displays little or no receptor binding activity (21). Upon the addition of DMPC, the helical content of rabbit apo-E (25) and human apo-E3 increases from 45% to 65% and total restoration of receptor binding activity is achieved. Evidently, the DMPC plays a role in orienting the apo-E3 into the necessary configuration for receptor recognition and binding. In addition to increasing the percentage of the α-helical structure of the protein, the recombination with phospholipids undoubtedly causes major alterations in tertiary structure. It is reasonable to speculate that the increased α-helical content and its probable effect on tertiary structure are important for the binding domain to exist in the correct conformation. The importance of secondary structure (α-helix) in the lipid binding and receptor binding processes is underscored by the observation that the binding domain contains strong α-helical-forming amino acids and that the carboxyl-terminal one-third of the protein is probably the major lipid-binding domain, containing regions of predicted amphiphilic helical structure (7).

The present studies also suggest that an amino acid substitution proximal to the binding domain, or the removal of a segment of the molecule distal to the binding region, can modulate the conformation of the binding domain and greatly alter the binding of apo-E to the receptor. Removal of the carboxyl-terminal one-third of the apo-E2(Arg158→Cys) can result in a 13-fold enhancement of receptor binding. The removal of the carboxyl-terminal portion of apo-E most likely releases a structural restraint on the molecule, allowing the binding domain of the mutant apo-E to achieve a partially normal binding conformation upon the addition of DMPC (Fig. 7).

In addition to the distal modulation of the binding site conformation caused by the removal of the carboxyl-terminal fragment, the charge effect of the cysteine for arginine substitution at residue 158 in the E2(Arg158→Cys) may cause a more proximal alteration of the conformation of the binding domain. When the cysteine at 158 is modified by cysteamine and the thrombolytic M₉ = 22,000 fragment (with the positive

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Virginia Gordon, unpublished observations.
charge) is complexed with phospholipid, the apo-E fragment can achieve a completely normal binding conformation. The cysteamine-treated, $M_f = 22,000$ fragment of E2(Arg$_{158}$→Cys) then attains a level of binding activity nearly identical with that of the normal apo-E$_3$ (Fig. 7).

The role of the cysteine for arginine substitution at residue 158 in the disruption of receptor binding has been clearly outlined in the series of experiments demonstrating that the positive charge at residue 158 is not essential for normal binding. However, its presence is apparently essential in establishing and maintaining the correct conformation necessary for normal binding. The reversal of the cysteamine modification with β-mercaptoethanol (and the subsequent loss of the positive charge at that site) of the apo-E$_2$(T-22K). DMPC does not result in an immediate loss of normal binding activity. Once the binding conformation is established, other forces (most likely restraints imposed on the secondary structure of the protein by the phospholipid) help maintain the structure of the lipoprotein particle. For example, the interaction of apo-E with other apoproteins residing near apo-E on the lipoprotein increases the apparent molecular weight and results in the co-electrophoresis of the E2(Arg$_{158}$→Cys) with the other variant forms of apo-E. These results suggest that the substitution at residue 158 plays a key role in determining the apparent molecular weight of the protein, most likely by modifying its conformation.

It is reasonable to speculate that the binding activity of apo-E may be modulated by the environment within the lipoprotein particle. For example, the interaction of apo-E with other apoproteins residing near apo-E on the lipoprotein surface may alter its conformation and affect binding activity. Likewise, the lipid composition of the particles could theoretically affect the binding domain and modulate receptor interaction. A determination of the effects of various perturbations on the secondary and tertiary structure of apo-E is now required for further evaluation of these hypotheses.

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