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Human apolipoprotein E expression in *Escherichia coli*: Structural and functional identity of the bacterially produced protein with plasma apolipoprotein E

(lipoprotein receptors/cholesterol transport/expression vector)

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ABSTRACT **Human apolipoprotein E (apoE) was produced in *Escherichia coli* by transforming cells with an expression vector containing a reconstructed apoE cDNA, a λ P_L promoter regulated by the thermolabile cI repressor, and a ribosomal binding site derived from the λ cII or the *E. coli* β-lactamase gene. Transformed cells induced at 42°C for short periods of time (<20 min) produced apoE, which accumulated in the cells at levels of ≈1% of the total soluble cellular protein. Longer induction periods resulted in cell lysis and the proteolytic destruction of apoE. The bacterially produced apoE was purified by heparin-Sepharose affinity chromatography, Sephacryl S-300 gel filtration, and preparative Immobiline isoelectric focusing. The final yield was ≈20% of the initial apoE present in the cells. Except for an additional methionine at the amino terminus, the bacterially produced apoE was indistinguishable from authentic human plasma apoE as determined by NaDodSO$_4$ and isoelectric focusing gel electrophoresis, amino acid composition of the total protein as well as its cyanogen bromide fragments, and partial amino acid sequence analysis (residues 1–17 and 109–164). Both the bacterially produced and authentic plasma apoE bound similarly to apolipoprotein B,E(low density lipoprotein) receptors of human fibroblasts and to hepatic apoE receptors. Intravenous injection resulted in similar rates of clearance for both the bacterially produced and authentic apoE from rabbit and rat plasma (≈50% removed in 20 min). The ability to synthesize a bacterially produced human apolipoprotein with biological properties indistinguishable from those of the native protein will allow the production of large quantities of apoE for use in further investigations of the biological and physiological properties of this apolipoprotein.**

Human apolipoprotein E (apoE) is a M_r = 34,200 (299 amino acids) component of several classes of plasma lipoproteins (1, 2). Through its ability to mediate lipoprotein binding and uptake by lipoprotein receptors (3), this apolipoprotein plays an important role in regulating plasma lipoprotein metabolism and maintaining cholesterol homeostasis. The structure of the protein has been determined by amino acid sequence analysis (4), and this structure has been confirmed by cDNA analysis of liver apoE mRNA (5, 6). ApoE is synthesized with an 18 amino acid signal peptide that is removed by cotranslational processing (6). The apoE structural gene has been mapped to chromosome 19 (7, 8), and the complete nucleotide sequence of the gene has been determined (8, 9). The protein exhibits a heterogeneity that is the result of both a genetically determined polymorphism (10, 11) and a posttranslational addition of sialic acid (11). Genetic control is exerted at a single gene locus, and three isoforms (E2, E3, and E4) are most commonly observed with isoelectric focusing (12). This results in the expression of six common phenotypes: E4/4, E3/3, E2/2, E4/3, E4/2, and E3/2; the E3 isoform is the most common in the population (11, 13–15).

The structures of numerous apoE variants have been described, several of which have a diminished ability to bind to lipoprotein receptors (4, 16–19). This defective binding ability has been determined to be an underlying cause for the familial lipoprotein disorder type III hyperlipoproteinemia (20, 21). The binding domain of apoE has been localized to amino acids in the vicinity of residues 140–160. This binding domain has been determined by mapping the amino acid substitution sites in receptor-defective apoE variants, by examining the receptor binding of apoE fragments (22, 23), and by using apoE monoclonal antibodies (24).

Because of the central role that apoE plays in lipoprotein metabolism, it is desirable to obtain large quantities of this protein for various metabolic studies. However, it is not practical to isolate large quantities of apoE from plasma sources. In addition, development of a system to produce site-specific mutants of apoE would be useful for further studies of apoE structure–function relationships. This report describes the first step toward these goals. A human apoE cDNA has been inserted into a plasmid expression vector followed by transformation and expression of apoE by *Escherichia coli*. Induced cells produce a large amount of an apoE that has structural and functional properties indistinguishable from those of the native plasma protein.

METHODS

Construction of Human ApoE-Expressing Plasmids. An apoE cDNA clone, designated pHAE178, encoding the entire mature apoE sequence and contained in the *Pst* I site of pBR322 (5), was used for construction of the cDNA to be inserted into the expression vector. This plasmid was cleaved with *Hin*fI, and the cDNA fragment was isolated, made blunt-ended by incubation with the Klenow fragment of *E. coli* DNA polymerase I, ligated to *Eco*RI linkers, and inserted into the *Eco*RI site of pBR322 (25). The subcloned apoE cDNA was cleaved with *Ava* I (34th nucleotide of mature apoE, corresponding to proline-12) and *Nde* I (2296th nucleotide of pBR322). A 38-base-pair double-stranded synthetic oligonucleotide linker (T ATG AAG GTT GAA CAG GCT

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Abbreviations: apoE, apolipoprotein E; Myr_2-PtdCho (also known as DMPC), dimyristoyl phosphatidylcholine; LDL, low density lipoproteins.

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GTT GAA ACT GAA CCG GAA C) that contained *Ava* I and *Nde* I ends was ligated to the 5′ end of the apoE cDNA fragment. This oligomer reconstructed a nucleotide sequence that codes for the authentic amino terminus of apoE and added a methionine translation initiation codon. The codons in this linker were chosen to reflect the codon preference in *E. coli* (26). The reconstructed apoE cDNA was cleaved with *Eco*RI, made blunt-ended by the Klenow fragment, ligated to *Nde* I linkers, and cleaved with *Nde* I. The purified apoE *Nde* I fragment (1040 base pairs) was ligated to the *Nde* I-linearized expression vector generating the recombinant plasmid pTV170 (Fig. 1). A second plasmid, pTV194, was constructed from pTV170 by removing the *c*II ribosomal binding site and replacing it with the β-lactamase ribosomal binding site (Fig. 1). The correct orientation of the apoE cDNA insert in transformed cells was determined by immunochemical screening of colony lysates (27).

Bacterial Growth and Induction Protocol. Human apoE-expressing plasmids were propagated in an *E. coli* strain derivative of C600 cells [*c600 r*$^-$ *m*$^+$ *gal*$^+$ *thr*$^-$ *leu*$^-$ *lacz*$^-$ (*c*I857 ΔH1 ΔBam N$^+$)]. Cultures were grown overnight at 30°C in broth (1 liter) supplemented with ampicillin (100 μg/ml). The cultures were diluted to 0.05% with fresh medium and grown in either flasks (laboratory scale, up to 250 ml) or fermenters (fermenter scale, up to 75 liters). Induction was commenced when the medium containing the cells reached an OD_{660} of about 0.7 for flasks or 13 for fermenters, respectively. The temperature was raised to and maintained at 42°C until induction was terminated by rapidly cooling the cells to 4°C by adding crushed ice. Bacterial cells were harvested by centrifugation and stored at −20°C either as a frozen cake or a lyophilized dried cake.

Analysis of Bacterial Extracts. Bacterial cells were harvested by centrifugation and suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride. Aliquots were lysed in 1.5× concentrated sample buffer (15% glycerol/4.5% $NaDodSO_4$/1 mM 2-mercaptoethanol/93.5 mM Tris·HCl/0.25% bromophenol blue, pH 6.8) and heated at 100°C for 10 min; the proteins were analyzed on 10% $NaDodSO_4$/polyacrylamide gels (28). Proteins separated on polyacrylamide gels either were stained with Coomassie brilliant blue or were electrophoretically transferred to nitrocellulose sheets (29) and allowed to react with anti-human apoE monoclonal or polyclonal immunoglobulin G that was iodinated (^{125}I). The immunoblots were washed, air dried, and exposed to Agfa Gevaert Curix RPZ x-ray film. Radioimmunoassay of apoE was performed by using a solid-phase modification of the assay of Havel *et al.* (30).

Isolation of ApoE. Authentic apoE was isolated from the $\rho < 1.02$ plasma lipoproteins of a hypertriglyceridemic subject (E3/3 phenotype) by Sephacryl S-300 column chromatography as described (16). The E3 isoform was obtained by preparative Immobiline isoelectric focusing (LKB, Bromma, Sweden, pH range 4.9–5.9) (31).

The bacterially produced apoE was isolated from 33 g of lyophilized cells, which represented the cell mass from a 5-liter fermentation. The cells were ground to a fine powder with the aid of 22 g of alumina (Buehler, Evanston, IL) in a chilled (4°C) mortar and pestle. The ground cells were extracted with 300 ml of 6 M urea (freshly deionized) containing 0.1 M NH_4HCO_3, 2 mM phenylmethylsulfonyl fluoride, and 0.1% Trasylol (Mobay, New York, NY) (pH 7.8). The insoluble cellular residue was sedimented by ultracentrifugation at 4°C in a Beckman SW 28 rotor (25,000 rpm for 50 min) and reextracted with 200 ml of 6 M urea buffer. The combined supernatant fractions were dialyzed against three changes of 2 M urea containing 25 mM NH_4HCO_3, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 0.1% Trasylol, and 0.1% 2-mercaptoethanol (pH 7.4).

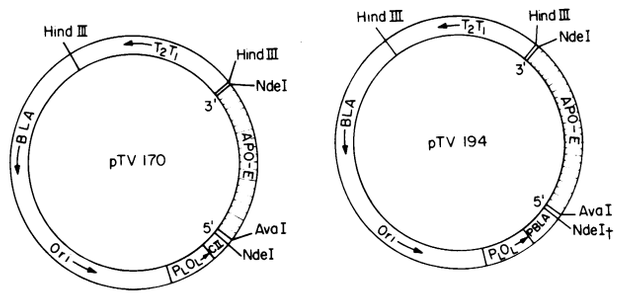

FIG. 1. Schematic representation of expression vectors containing human apoE cDNA. Both the pTV170 (*Left*) and pTV194 (*Right*) clones are derivatives of a λ P_L expression vector (37) and contain the T_1T_2 ribosomal RNA transcription termination signals at the 3′ end of the apoE cDNA. The plasmids contain either the λ *c*II (pTV170) or the β-lactamase (PBLA, pTV194) ribosomal binding sites. In both constructs, the methionine initiation codon is located 11 base pairs from the ribosomal binding site. Other elements contained in the plasmids are the β-lactamase gene (BLA) and the λ phage temperature-sensitive regulatory region (P_LO_L). *Nde* I+: In the construction of pTV194 from pTV170, the *Nde* I end was filled in by the Klenow fragment and then ligated to an *Alu* I fragment containing the β-lactamase ribosomal binding site.

The dialyzed supernatant was added to ≈200 ml of heparin-Sepharose (prepared as described in ref. 32) and equilibrated with the 2 M urea buffer. The gel/supernatant mixture was incubated overnight at 4°C on a rotating platform and then packed into a glass column (4.0 × 3.5 cm, Kontes). The material not bound to the heparin-Sepharose was washed from the column by pumping ≈300 ml of 2 M urea buffer at a rate of 25 ml/hr through the column. The bound material was eluted from the column with 50 ml of 1.0 M NH_4HCO_3 in 2 M urea and then dialyzed against 5 mM NH_4HCO_3 and lyophilized. This semipurified apoE was solubilized in 15 ml of 6 M guanidine/0.1 M Tris·HCl/1 mM EDTA/1.0% 2-mercaptoethanol, pH 7.4, applied to a Sephacryl S-300 column (2.5 × 300 cm; Pharmacia, Uppsala, Sweden), and equilibrated with 4 M guanidine/0.1 M Tris·HCl/1 mM EDTA/0.1% 2-mercaptoethanol, pH 7.4. The fractions containing apoE were pooled, dialyzed exhaustively against 5 mM NH_4HCO_3, and lyophilized. Final purification was accomplished by preparative isoelectric focusing on an Immobiline gel (31).

Structural Characterization of Bacterially Produced ApoE. Protein or peptide samples for amino acid analysis were hydrolyzed in 6 M HCl for 20 hr at 110°C in sealed, evacuated tubes. Analyses were performed on a Beckman 121MB Analyzer equipped with a model 126 data system.

Peptides for amino acid and sequence analyses were generated by digesting 3 mg of Sephacryl S-300 column-purified apoE with 90 mg of CNBr in 600 μl of 70% HCOOH for 30 hr at room temperature. Resultant peptides were separated on a Sephadex G-50 column as described for authentic apoE (4).

Sequence analyses were performed on an updated Beckman 890C Sequencer by using a standard 0.1 M Quadrol program. The intact protein was degraded in the presence of 3 mg of Polybrene/0.5% $NaDodSO_4$; peptides were degraded in the presence of Polybrene only. Phenylthiohydantoin amino acids were identified and quantified by high-performance liquid chromatography as described (16).

Analytical isoelectric focusing and $NaDodSO_4$/polyacrylamide gel electrophoresis were performed as described (16). Charge modification with cysteamine (2-mercaptoethylamine) was conducted as described (16).

Biological Characterization of Bacterially Produced ApoE. Phospholipid complexes of apoE and dimyristoyl phosphatidylcholine (Myr_2-PtdCho) were prepared and isolated as described (33). Lipoprotein receptor binding assays were

performed as described for fibroblasts (34) and hepatic membranes (35). Iodinations of apoE were performed in 0.10 M NH_4HCO_3 with Iodo-Beads (Pierce) according to manufacturer's directions.

For rabbit and rat *in vivo* studies, iodinated authentic and bacterially produced apoE (45 μg each) were incubated for 30 min at room temperature with 1 ml of rabbit or rat plasma prior to injection into male New Zealand White rabbits or Sprague–Dawley rats, respectively. The apoE is readily incorporated into the plasma lipoproteins during the incubation. Plasma radioactivity is reported as trichloroacetic acid-precipitable protein by using the precipitation method described previously (36). Calculation of the percent of the injected dose remaining in plasma at the various time intervals was based on a plasma volume estimate of 4.5% of the body mass.

RESULTS AND DISCUSSION

Expression of Human ApoE. Expression of human apoE was achieved by using an expression vector containing the λ phage P_LO_L regulatory region, the N utilization site (Nut L), and either the λ *c*II ribosomal binding site (pTV170, Fig. 1) or the β-lactamase ribosomal binding site (pTV194, Fig. 1). Both expression plasmids contained the T_1T_2 ribosomal RNA transcription termination signals (38) at the 3′ end of the apoE cDNA to reduce read-through transcription of the β-lactamase gene downstream from the apoE cDNA. After induction, the pTV194 vector produced approximately three times more apoE than did the pTV170 vector and thus was used for subsequent studies. As shown in Fig. 2, after cells transformed with pTV194 were raised to 42°C, a protein with an apparent molecular weight identical to that of apoE was specifically induced. This induced protein reacted with anti-human apoE antibodies (not shown).

Induction periods of 30 min or longer caused cell lysis, whereas noninduced cells maintained at 30°C were stable. The cell lysis, which was associated with the intracellular accumulation of apoE, was followed by the proteolytic destruction of apoE. This cellular lysis was not a general feature of this expression system because the same expression system containing a human growth hormone cDNA did not show this effect (39). The problem of cell lysis caused by apoE accumulation was overcome by inducing the cells for short periods of time (≈20 min) and then cooling the cells by adding ice to the fermenter. Similar observations relating to cell lysis induced by expression of lipid binding proteins have been reported (40, 41).

As determined by solid-phase radioimmunoassay, the apoE levels in cells induced for short periods of time were ≈1% of the total soluble cellular protein. The apoE was isolated and purified from cell extracts by heparin-Sepharose and Sephacryl S-300 chromatography. This two-step process resulted in an apoE preparation that was >90% pure, with a yield representing ≈20% of the initial apoE present in the cell extract. Final purification for characterization was accomplished by preparative Immobiline isoelectric focusing (Fig. 3). The purification scheme used in these studies was not optimized for total recovery but, rather, was designed to obtain pure material for characterization.

Structural Characterization of Bacterially Produced ApoE. The Immobiline-purified, bacterially produced apoE migrated as a single band on $NaDodSO_4$ gels with an apparent molecular weight identical to that of authentic apoE (Fig. 3). On isoelectric focusing gels, the bacterially produced apoE focused as one major band with an isoelectric point identical to that of Immobiline-purified apoE3 (Fig. 3). Consistent with the presence of one residue of cysteine was the finding that the bacterially produced apoE was shifted one charge unit toward the anode (Fig. 3) after cysteamine modification. In the example shown in Fig. 3, the modification was not complete, which resulted in a shift of ≈60% of the apoE. Amino acid analysis of the Immobiline-purified product was compared to authentic human apoE3 purified by the same method. As shown in Table 1, the amino acid compositions of both proteins were nearly identical to each other as well as to the theoretical composition derived from previous sequence analysis of human apoE3. However, the analyses showed that the bacterially produced product contained an additional residue of methionine compared to the authentic apoE. In addition, the presence of one cysteine residue, demonstrated by cysteamine treatment, was confirmed.

Amino acid sequence analysis (18 cycles) of the intact bacterially produced apoE (≈6 nmol) demonstrated that the extra methionine residue was at the amino terminus of the protein and yielded a single sequence of Met-Lys-Val-Glu-

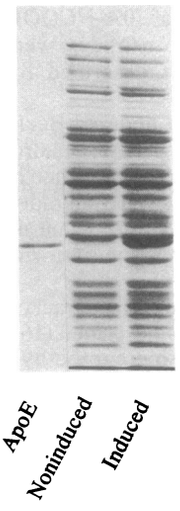

FIG. 2. Synthesis of human apoE in transformed *E. coli*. After the C600 cells were either incubated at 30°C for 20 min (Noninduced lane) or induced at 42°C for 20 min (Induced lane), the cell extracts containing the expression plasmid pTV194 were analyzed on a $NaDodSO_4$/polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Lane 1 contained 1 μg of purified human plasma apoE.

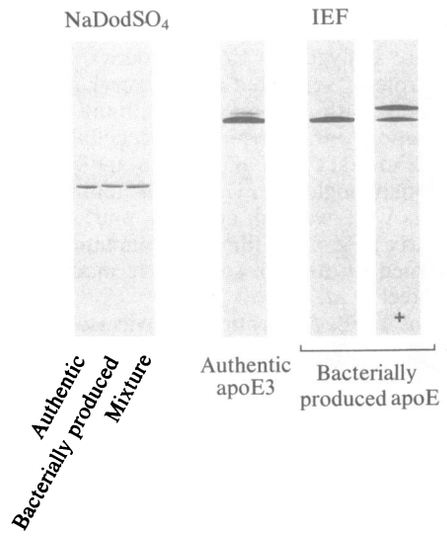

FIG. 3. Gel electrophoresis of isolated bacterially produced apoE and authentic apoE3. (*Left*) $NaDodSO_4$ gel electrophoresis on a 10–20% gradient polyacrylamide gel of bacterially produced and authentic apoE. Both proteins were purified by preparative Immobiline isoelectric focusing. Approximately 2 μg of each protein was subjected to electrophoresis; the mixture of the two proteins contained 1 μg of each protein. Gels were stained with Coomassie brilliant blue R-250. (*Right*) Isoelectric focusing (IEF) (pH 4–6) on polyacrylamide gels of Immobiline-purified bacterially produced and authentic apoE. Approximately 5 μg of each protein was focused. A sample of the bacterially produced apoE in 100 μl of incubation buffer was charge-modified by treatment with 1 mg of cysteamine (+) for 2 hr at 37°C.

Table 1. Amino acid composition of bacterially produced and authentic apoE3

Amino acid	Bacterially produced apoE3	Authentic apoE3	apoE3 sequence
Lys	12.1	12.0	12
His	2.0	2.0	2
Arg	33.3	33.3	34
Cys	0.8	0.8	1
Asp	12.3	12.4	12
Thr	10.5	10.5	11
Ser	12.7	12.6	14
Glu	72.0	71.9	71
Pro	8.5	8.4	8
Gly	17.3	17.3	17
Ala	35.6	35.5	35
Val	21.9	?2.3	22
Met	7.7	6.5	7
Ile	1.9	1.9	2
Leu	37.0	37.3	37
Tyr	3.8	3.9	4
Phe	3.2	3.2	3
Trp	ND	ND	7

Results are from duplicate determinations and are expressed as residues per mol. Cysteine was determined separately after performic acid oxidation. Threonine and serine values were not corrected for hydrolytic loss. Tryptophan was not determined (ND). ApoE3 sequence is from ref. 4.

Gln-Ala-Val-Glu-Thr-Glu-Pro-Glu-Pro-Glu-Leu-Arg-Gln-Gln-. The amino terminus of authentic plasma apoE is lysine, and the sequence that follows the methionine corresponds to residues 1–17 of human apoE. These results established that the synthetic oligonucleotide used to reconstruct the amino-terminal coding portion of apoE was correctly translated and that the extra methionine (the codon of which was added for bacterial translation initiation) was not removed by any processing mechanism. The initial yield in the sequence analysis was unexpectedly low (≈20%), but this was probably not due to a portion of the amino-terminal methionine being formylated. The formylated protein would have an isoelectric point distinctly different from the nonformylated polypeptide (and from authentic apoE), and this was not observed (see Fig. 3).

The amino acid compositions of several of the CNBr peptides of bacterially produced apoE were determined and found to be no different from those of authentic apoE (data not shown). In addition, sequence analysis of peptide CB4 (residues 109–125 in authentic apoE) and partial sequence analysis (39 cycles) of peptide CB5 (residues 126–218 in apoE) established that the sequence of the bacterially produced apoE was identical to authentic apoE3 in the crucial receptor binding domain. All of these data indicated that the bacterially produced apoE was identical in structure to authentic apoE3, except for the additional methionine at the amino terminus.

***In Vitro* and *in Vivo* Metabolic Characterization of Bacterially Produced ApoE.** Comparison of the receptor binding properties of apoE·Myr_2-PtdCho complexes demonstrated that the bacterially produced apoE possessed binding properties that were essentially identical to those of authentic apoE. In competition studies using human fibroblasts with apolipoprotein B,E[low density lipoprotein (LDL)] receptors, 50% displacement of ^{125}I-labeled LDL occurred at concentrations of 0.019 μg/ml and 0.024 μg/ml for bacterially produced and authentic apoE, respectively (Fig. 4). In direct binding studies using fibroblasts, both apoE preparations gave similar results (Fig. 4). Scatchard analysis of the direct binding data revealed that the dissociation constant (K_d) and

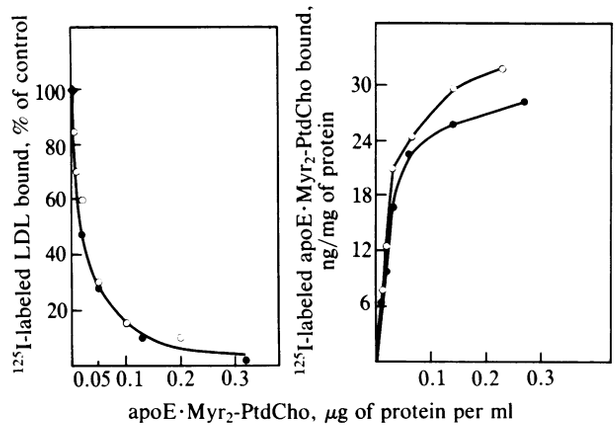

FIG. 4. Binding of apoE·Myr_2-PtdCho complexes to apolipoprotein B,E(LDL) receptors on fibroblasts. Phospholipid complexes of bacterially produced and authentic apoE were prepared by incubation of the proteins and Myr_2-PtdCho at 22°C. The complexes were separated from noncomplexed material by density-gradient ultracentrifugation as described (33). (*Left*) Ability of bacterially produced (●) and authentic (○) apoE·Myr_2-PtdCho complexes to compete with ^{125}I-labeled human LDL for binding to cultured fibroblast receptors at 4°C. The 100% value for ^{125}I-labeled LDL binding was 108 ng/mg of cell protein. (*Right*) Ability of ^{125}I-labeled bacterially produced (●) and authentic (○) apoE·Myr_2-PtdCho complexes to bind directly to cultured fibroblasts.

maximal amount bound for bacterially produced and authentic apoE were 0.93 and 0.96 × 10^{-10} M and 29.4 and 34.2 ng/mg of cell protein, respectively. In addition, both apoE preparations also bound similarly to apoE receptors on canine hepatic membranes (data not shown).

Comparison of the *in vivo* metabolic properties of bacterially produced and authentic apoE also demonstrated that both preparations behaved in an identical manner. When a mixture of ^{131}I-labeled bacterially produced and ^{125}I-labeled authentic apoE that had been incubated with normal rabbit plasma was injected into a normal rabbit, both forms of apoE

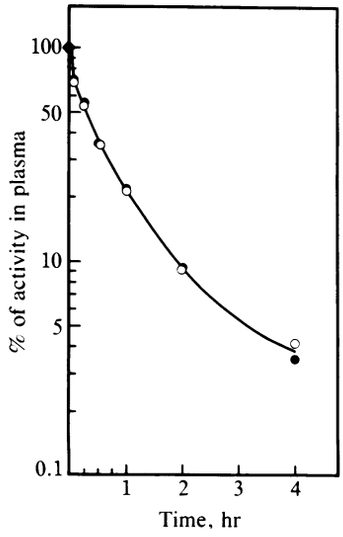

FIG. 5. Clearance of iodinated apoE from rabbit plasma. ^{131}I-labeled bacterially produced (●) and ^{125}I-labeled authentic (○) apoE (45 μg of protein) were incubated at 37°C for 30 min with 1 ml of rabbit plasma prior to injection of the mixture into the marginal ear vein of a rabbit. Blood (≈2 ml) was removed into a vial containing EDTA at the indicated time points, and the plasma radioactivity was determined. The amount of radioactivity measured was corrected for trichloroacetic acid-soluble degradation products as described (36).

were removed from the circulation with identical kinetics (Fig. 5). Clearance of 50% of the injected dose of bacterially produced and authentic apoE occurred within ≈20 min after injection. Identical results were obtained with reciprocally labeled proteins. When turnover studies were performed in rats, similar results were obtained (data not shown). Thus, in both *in vitro* and *in vivo* studies, the bacterially produced and authentic apoE exhibited properties that were essentially indistinguishable.

In summary, this report describes the insertion of a human apoE cDNA into a plasmid expression vector that subsequently was used to transform *E. coli*. Induced transformed cells synthesized apoE at relatively high levels. The apoE was purified by a three-step isolation procedure that included heparin-Sepharose affinity chromatography, Sephacryl S-300 gel filtration, and preparative isoelectric focusing. Structural characterization of the isolated apoE demonstrated that, with the exception of an additional methionine residue at the amino terminus, the structure of the bacterially produced apoE was identical to authentic plasma apoE. In addition, the *in vitro* and *in vivo* metabolic properties of the bacterially produced and authentic apoE were identical. The expression of apoE in *E. coli* represents the successful large-scale synthesis of a human apolipoprotein by bacterial cells in which the bacterially produced protein has biological properties indistinguishable from those of the native protein. Thus, studies requiring large amounts of protein are now feasible. In addition, use of this expression system will allow the production of site-specific mutants of apoE for further studies of the structure–function relationships of this important plasma apolipoprotein.

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