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An Approach to the Functional Analysis of Lecithin-Cholesterol Acyltransferase

Activation by Recombinant Normal and Mutagenized Apolipoprotein AI

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Summary: Apolipoprotein AI (apo AI) of human serum high-density lipoprotein functions as an activator of lecithin-cholesterol acyltransferase (LCAT) and therefore plays an important role in reversed cholesterol transport. The mechanism of the acyltransfer, the activating polypeptide domains of apo AI and the active site of LCAT in this transesterification are not yet known. Synthetic peptides of the apo AI sequence have been designed to determine the activating structure, but did not yet lead to conclusive results. This also applies to spontaneous apo AI mutants.

We therefore used the method of site-directed mutagenesis of apo AI cDNAs using the overlap extension approach by the polymerase chain reaction. These constructs were cloned into the procaryotic vector pET8c and expressed under the inducible T7 promoter. The engineered apo AI polypeptides were isolated and purified by affinity chromatography and assayed for their activator activity.

The essentials of this approach to the structure and function of activators in general have successfully been exemplified for the LCAT activation by engineering apo AI mutant polypeptides a) by the deletion of two adjacent amphipathic helices (amino acid residues 146–186) and b) by introducing a point mutation ($Glu^{111} \rightarrow Gln$).

Ein molekularbiologischer Zugang zur Funktionsanalyse der Lecithin-Cholesterin-Acyltransferase-Aktivierung durch rekombinantes normales und mutagenisiertes Apolipoprotein AI

Zusammenfassung: Apoliporotein AI (Apo AI) des menschlichen High-Density-Lipoproteins wirkt als Aktivator der Lecithin-Cholesterin-Acyltransferase (LCAT). Es übernimmt damit eine wichtige Rolle im reversen Cholesterintransport. Der Mechanismus des Acyltransfers sowie die aktivierenden Polypeptiddomänen des Apo AI und das Reaktionszentrum der LCAT sind bisher noch unbekannt. Versuche mit synthetischen Peptiden aus der Apo-AI-Sequenz führten genausowenig zu schlüssigen Ergebnissen wie das Auftreten spontaner Apo-AI-Mutanten.

Enzymes:
Deoxyribonuclease I (DNAse), EC 3.1.21.1;
Klenow enzyme, DNAse I large fragment, recommended name: DNA-directed DNA polymerase, deoxyribonucleoside-triphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7;
Lecithin-cholesterol acyltransferase, recommended name: phosphatidylcholine:sterol *O*-acyltransferase, EC 2.3.1.43;
Lysozyme (muramidase), peptidoglycan *N*-acetylmuramoylhydrolase, EC 3.2.1.17;
Mungbean nuclease; T7 RNA polymerase; Taq polymerase.

Abbreviations:
apo AI, apolipoprotein AI of human serum high density lipoprotein; bp, base pairs; C^7dGTP, 7-deaza-2′-deoxyguanosine triphosphate; cDNA, complementary DNA; DEAE, diethylaminoethyl-; EDTA, ethylene-diamine tetraacetic acid; HDL, high-density lipoprotein; HSA, human serum albumin; HPLC, high-performance liquid chromatography; kb, kilo base pairs; LCAT, lecithin:cholesterol acyltransferase, LDL, low-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCR, polymerase chain reaction; PEG, polyethyleneglycol; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; Tris, Tris(hydroxymethyl)aminomethane; VLDL, very-low-density lipoprotein.

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Deshalb wandten wir die Methode der gezielten Mutagenese der Apo-AI-cDNA mittels der Methode der überlappenden Sequenzen durch die Polymerase-Kettenreaktion an. Die dabei entstehenden Konstrukte wurden in den prokaryontischen Vektor pET8c kloniert und unter dem T7-Promoter exprimiert. Die so gewonnenen Apo-AI-Polypeptide wurden durch Affinitätschromatographie isoliert und auf ihre aktivierende Wirkung hin untersucht.

Die Grundlagen dieses experimentellen Ansatzes zur Aufklärung der Wechselwirkung zwischen Struktur und Funktion von Aktivatorproteinen wurde durch Proteinengineering des das LCAT-Enzym aktivierenden Apolipoproteins AI mittels a) der Deletion zweier benachbarter amphipathischer Helices (Aminosäuren 146–188) und b) mittels einer Punktmutation ($Glu^{111} \rightarrow Gln$) im Apo AI erfolgreich angewandt.

Key terms: Apolipoprotein AI, amphipathic helices, deletion and point mutations, overlapping extension polymerase chain reaction, recombinant apoAI-proteins, functional assay.

In homeostasis of cholesterol metabolism serum HDL play the important function of reversed cholesterol transport from peripheral tissue to the liver. The main apolipoprotein constituents of HDL subclasses are apo AI and apo AII. They guarantee the integrity of the HDL particle by covering the apolar lipid core and binding phospholipids, mainly phosphatidylcholine, and cholesterol in a monomolecular surface layer[1]. The prevailing secondary structure of the apoproteins is the amphipathic helix, the hydrophobic side chains of which face the hydrophobic core of the HDL particle whereas the hydrophilic side chains together with the polar head groups of the amphiphilic lipids form the surface and interphase to the aqueous environment. In addition the main apolipoprotein component of HDL, apo AI, activates the key enzyme in the homeostasis of cholesterol, lecithin-cholesterol acyltransferase (LCAT), a 69-kDa glycoprotein which transfers the acyl group from position 2 of phosphatidylcholine to cholesterol in the phospholipid surface monolayer of the HDL particles. In this acyl transfer no intermediate binding of the acyl residue to apo AI has been observed[2,3], instead an activation of the phospholipid molecule has been postulated[4]. The apo AI polypeptide is folded in eight 22 amino-acid residues containing α-amphipathic helices which bind the phosphatidylcholine and cholesterol and present these as substrates for LCAT. The activator function of apo AI has been studied with synthetic amphipathic peptides of the apo AI amino-acid sequence[4,5]. They activate the LCAT only by one third compared to the complete sequence of apo AI. It is conceivable that beyond the lipid binding additional specific protein-protein interactions between apo AI and LCAT are required for optimal activation.

Anantharamaiah et al.[5] synthesized a 44 residues polypeptide which resembled the consensus sequence of all amphipathic helices of apo AI and found a 100% activation as compered with native apo AI. This peptide contained glutamic acid in position 13 in the hydrophobic domain of helix 2 of the 8 repeats which are located between residues 66 and 121 of apo AI. This domain has been proposed to be responsible for LCAT activation due to a specific ionic interaction between Glu^{111} of apo AI and LCAT and the substrate.

A method for the characterization of functional domains of a protein is the site-directed change of the primary structure and the subsequent functional analysis of the mutated protein.

We describe here the essential steps of apo AI engineering by site-directed mutagenesis of apo AI-encoding cDNA by overlap extension using the polymerase chain reaction and subsequent expression. This approach has been exemplified with wild type apo AI cDNA, the deletion mutant of apo AI cDNA in which the coding sequence of two amphipathic helices of apo AI (amino acids 146–188) is missing and a point mutation in which Glu^{111} is substituted by glutamine.

The apo AI wild type and mutant cDNA constructs were cloned into the procaryotic expression vector pET8c and expressed in *Escherichia coli*. The recombinant apoproteins were isolated and purified by affinity chromatography and used in activation studies with purified serum LCAT. Our experiments demonstrate that the apo AI domains investigated so far with synthetic peptides are not responsible for the activation of LCAT.

The approach outlined here allows to pinpoint the structural requirements for the functional domains of apolipoproteins.

Results

Expression of recombinant apo AI in E. coli. *The expression system* E. coli *BL21 (DE 3) pLys/pET8c*

We chose *E. coli* BL21 (DE 3) [6] as expression system. It guarantees a highly selective and efficient expression due to the T7 RNA polymerase gene which is lysogenic in the BL21 genome. Transcription is under

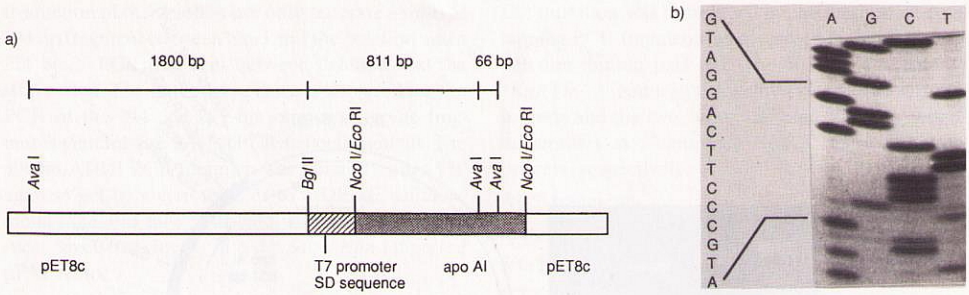

Fig. 1. a) Structure of the pET8c-AI = pPAI vector. The fragments at the top were used to determine the orientation of the apo AI insert.
b) Nucleotide sequence around the 5′ ligation site with the start codon ATG.

the strong control of the isopropyl thiogalactoside-inducible lac UV5 promoter.

The pET8c vector contains the strong T7 Φ10 promoter, the ribosome-binding site, the translation initiation codon within the *Nco* I restriction sequence (CCATGG) and downstream of this cloning site the transcription terminator TΦ. The *E. coli* strain also harbors the plasmid pLys S with the gene of T7 lysozyme, which acts as inhibitor of the T7 RNA polymerase. The gene is under the control of the weak T7 promoter Φ3.8 and inhibits the transcription of foreign DNA unless the strong Φ10 promoter is induced. Lysozyme also facilitates the isolation of the gene product.

A) Construction of expression vectors with modified preproapo AI cDNA insert

We first cloned the human preproapo AI cDNA isolated previously from a human liver cDNA library in this laboratory as an 893-bp *Eco* RI fragment[7] into the *Nco* I linearized pET8c vector[6]: the 5′-overhanging ends of the apo AI fragments were digested with mungbean nuclease and the *Nco* I site of the vector, filled in with Klenow enzyme and dephosphorylated. Blunt end ligation yielded the construct shown in Fig. 1a. The sequence around the 5′ ligation site is shown in the autoradiogram of Fig. 1b. The preproapo AI cDNA is extended at the 5′ end by four codons starting with an additional ATG. The restriction analysis of the vector pPAI and its organization is given in Fig. 2.

BL21 (DE 3) pLys S cells were transformed with this pET8c AI vector (= pPAI), the expression was induced and the total protein extract analysed by 15% SDS-polyacrylamide gel electrophoresis. Neither the translation product preproapo AI nor proapo AI or mature apo AI could be detected by Western blot analysis.

The regulation of the translation in procaryotic cells is not well known. The secondary structure of the 5′ region of mRNA for the initiation of protein biosynthesis seems to be important, the Shine-Dalgarno-Sequence may not be part of a hairpin structure[8] and a freely accessible initiation codon is required. Indeed, pET8c AI (pPAI) does not fulfill these requirements as deduced from the predicted mRNA structure of the pET8c vector[9]. We therefore modified the 5′ region of the mRNA. The calculation of the mRNA structure of the resulting proapo AI after the deletion of 18 codons of the signal sequence indicated that the ATG starting codon is still inaccessible. The insertion of a GGC (Gly) triplett following ATG exposed the translation start and generated an *Nco* I restriction site, most suitable for cloning into the pET8c vector. The 5′ sense oligonucleotide had the following sequence:

```
          Met Gly Arg His Phe Trp Gln Gln Asp
5' GGTCC ATG GGC CGG CAT TTC TGG CAG CAA GAT 3'
     Nco I       prosequence
```

The 3′-untranslated region has no regulatory function in procaryotic expression systems. We therefore deleted this region which reduced the size of the apo AI sequence to be amplified in the PCR reaction and yielded construct pPAI. The 3′ antisense oligonucleotide had the sequence:

```
              Stop
5' GCACCATGG TCA CTG GCT GTT GAG CTT CTT 3'
     Nco I  ***
```

The two synthetic oligonucleotide primers for the PCR have estimated melting points of 52 and 58 °C due to the *Nco* I restriction sequence added to their 5′ ends. A continuous temperature gradient over the

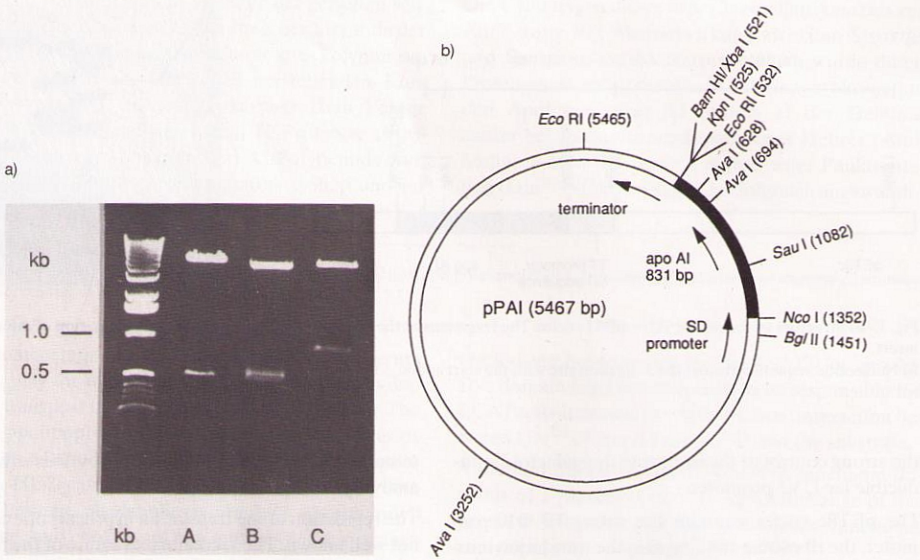

Fig. 2. a) Restriction enzyme analysis of pPAI. A) *Eco* RI, 520 bp fragment; B) *Eco* RI/*Sau* I, 520 and 550 bp fragments; C) *Nco* I/*Kpn* I, 831 bp fragment.
b) Vector pPAI with selected restriction sites.

first 10 cycles from 45° to 65 °C in the hybridization step was chosen.

The *Nco* I/*Kpn* I-restricted PCR-fragment (831 bp) was cloned into the *Nco* I-linearized and dephosphorylated pET8c vector resulting in the vector pPAI, which was used for the transformation of competent *E.coli* DH5α cells. Positive clones were selected by colony hybridization with a ^{32}P-labelled apo AI probe (*Sau* I-*Xba* I 561-bp fragment). Double strand sequencing of the construct pPAI confirmed the correct sequence and excluded errors introduced during the Taq polymerase reaction, Fig. 2.

Apo AI: deletion mutant pAI d41

The deletion of extended polypeptide domains should induce no or minimal perturbation of the secondary structure of apo AI and thus allows the direct correlation of structure and function such as lipid binding and activation of LCAT. Among the eight 22 amino-acid residues long amphipathic α-helices the helices 6 and 7 between amino-acid residues 146 and 186 were chosen because they are part of the C-terminal cyanogen bromide fragment which has been suggested to contain the LCAT activating domain.

We applied the site-directed mutagenesis using two independent PCRs and sets of primer oligonucleotides which yield DNA fragments with overlapping ends

5' fragment

Outer primer "*Sau* I s" sense

5' CAG AGG GCC TGA GGC AAG AGA TGA GCA AGG 3'

Sau I

Deletion primer "D41 as" antisense

5' GGC CAG TCT GGC GCC CAG TGG GCT CAG CTT CTC TTG C 3'

3' fragment

Outer primer "*Kpn* I as" antisense

5' ATC GGT ACC TCA CTG GGT GTT GAG CTT CTT AGT GTA 3'

Kpn I

Deletion primer "D41s"

5' AGC CCA CTG GGC GCC AGA CTG GCC GAG TAC CAC GCC A 3'

Scheme: Primer oligonucleotides.

(see Scheme). The two fragments were combined, their overlapping ends hybridized and the hybridization product PCR amplified with the outer primers.

Among the four PCR primers for the deletion mutant pAI d41 the inner primers contained the deletion of 122 bp between 543 and 665 of apo AIcDNA (pPAI). The outer 5′ PCR primer "*Sau* I s" applied to the amplification of the 5′ fragment of the deletion mutant pAI d41 contains the *Sau* I restriction site of pPAI and the outer 3′ primer "*Kpn* I as" eliminates the 3′-noncoding region of apo AI. These primers for the in-

troduction of the deletion not only generate a short 5′ 194 bp fragment between Sau I and the deletion and a 221 bp 3′ PCR fragment between deletion and the stop codon. The outer primers were also used for the PCR of the 194 and 221 bp long overlapping fragments yielding the 390-bp PCR fusion fragment. The 390 bp-AI d41 PCR fragment was isolated from a 1% agarose gel by electrotransfer to a DEAE cellulose membrane and then restricted with *Sau* I and *Kpn* I successively for cloning into the *Sau* I-*Kpn* I digested pPAI vector.

We used a randomly labelled 79-bp *Hpa* II fragment of the 3′-untranslated region of the apo AI gene (bp 1928–2636[10])which detected the negative clones in the screening procedure (wild type pPAI) but not the pAI d41 DNA which ends at the stop codon.

The deletion mutant pAI d41 was further characterized by restriction enzyme analysis (Fig. 3a and double strand sequencing around the deletion, Fig. 3b).

Apo AI: Point mutation AI Glu^{111}

The acidic glutamate residues in position 78 and 111 of the mature apo AI polypeptide have been suggested to be essential residues for the specific activation of LCAT on the basis of experiments with synthetic peptides. We chose the most direct method for testing these results by modifying the primary structure of the complete apo AI by site-directed substitution mutation. The negatively charged side chain in position 111 was replaced by the neutral, indifferent glutamine: CAG→GAG (bp 418 of apo AI cDNA).

The mutation was introduced by the method of overlapping PCR fragments as described in detail for the deletion mutant pAI d41. The 5′ "*Nco* I s" and 3′ "*Kpn* I as" oligonucleotide primers were used as outer primers and the two "Glu^{111} as" and "Gln^{111} s" oligonucleotides as 5′ antisense and 3′ sense mutation primers, respectively:

A) 5′ fragment

mutation primer, antisense "Glu^{111} as"

5′GTA GAG CTC CAT CTG CTC CTG CCA CTT CTT 3′

B) 3′ fragment

mutation primer "Glu^{111} s"

5′ AAG AAG TGG CAG GAG CAG ATG GAG CTC TAC 3′

The expected 372-kb 5′ fragment was obtained and isolated from 1% low melting agarose gel. However, the 3′ fragment of the mutant required the introduction of C^{7}dGTP into the amplification reaction to suppress secondary structures of the single-stranded DNA due to Hogsteen base pairing[11]. The 424-bp 3′ fragment was obtained and introduced together with the 5′ fragment into the combined PCR which yielded the fused 781-bp fragment after *Kpn* I and *Nco* I restriction. The fragment was ligated into the *Nco* I and *Kpn* I-linearized pPAI vector. Transformation of competent DH5α cells, colony screening, restriction enzyme analysis (Fig. 4a) and DNA sequence analysis (Fig. 4b) confirmed the C→G base exchange introduced by the overlapping PCR.

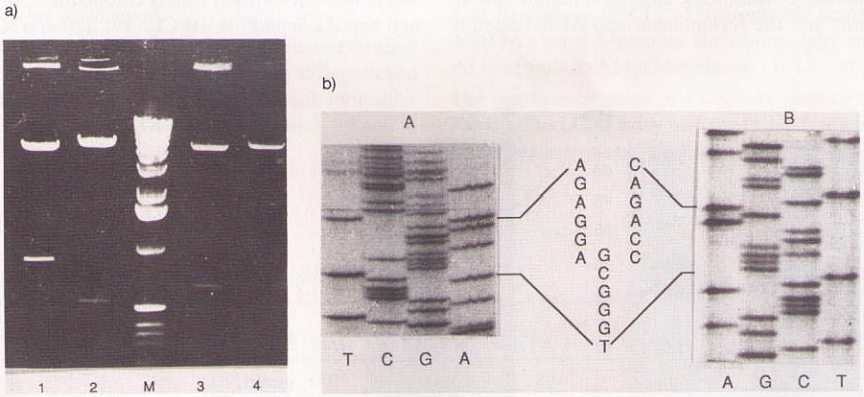

Fig. 3. a) Restriction enzyme analysis of pAId41. 1) pPAI *NcoI*/*KpnI*, 831 bp; 2) pPAI *Eco* RI/SauI, 550-bp; 3) pAId41 *NcoI*/*KpnI*, 651 bp; 4) pAId41 *Eco* RI/*Sau*I 550 bp; M) kb-ladder.
b) Nucleotide sequence. A) pPAI and B) pAId41 around the deletion site in pAId41.

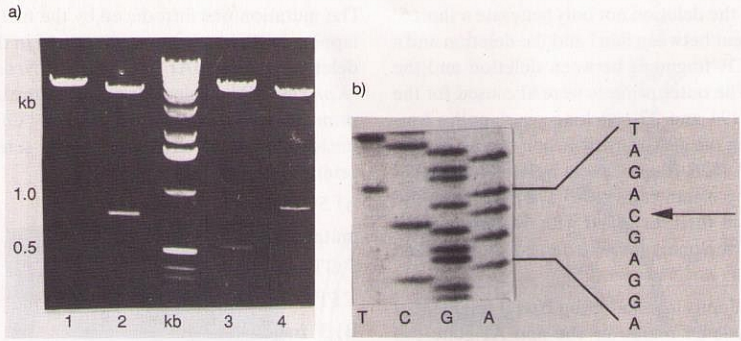

Fig. 4. Restriction enzyme analysis of pAI Glu[111]. 1) pAIGlu[111], *Eco* RI; 2) pAIGlu[111], *Nco* I/*Kpn*I, 831 bp; 3) pPAI, *Eco* RI/*Sau*I, 550 bp; 4) pPAI, *Nco*I/*Kpn*I, 831 bp.
b) pAIGlu[111] partial sequence, the point mutation is marked.

B) Expression of apo AI wild type and mutant genes and isolation of gene products

Competent BL21 (DE3) pLys S cells were transformed with the vector DNAs of pAI, pPAI and pAId41 and pAI Glu[111]. The expression was induced with isopropyl thiogalactoside (0.4mM) at a cell density A_{600} of 0.8 to 1.0. Cells were collected 3 h after the induction, washed, lysed with SDS (2%) and centrifuged again. A 50-μl aliquot of the total extract was separated by 15% SDS-PAGE and the gel either stained with Coomassie Blue (Fig. 5a) or the apo AI band visualized in Western blot analysis (Fig. 5b).

The size difference between the protein band of mature apo AI and the recombinant apo AI is due to the extended 1.03 kDa long prosequence of the pPAI expression product.

Comparative densitometric analysis of mature apo AI standards and the recombinant apo AI indicated a yield of 4.8 mg/l culture (A_{600} = 1.5) and 9.5% of the solubilized host cell proteins. This improved yield was obtained with M9ZB medium and induction at a cell density of A_{600} = 0.8. Cells were lysed by freezing (−70 °C), thawing (25 °C), and incubation with lysozyme in the presence of protease inhibitors.

Insoluble cellular residues were removed by high-speed centrifugation. The supernatant was cleared through a 0.4-μm filter and passed over an anti apo AI Sepharose 4B-CL affinity column. Non-specifically absorbed proteins were eluted with 0.1% Tween/PBS, apo AI with 0.2M glycine, pH 2.8 (Fig. 6).

Similarly competent BL21 (DE3) pLys S cells were transformed with the vectors pAI d41 and pAI Gln[111] as described for pPAI and the respective apoproteins were isolated from cells (2 and 4 *l* cultures) by cell lysis and immunoadsorption affinity chromatography over anti apo AI Sepharose 4B-CL. 350 $\mu g/l$ apo AI d41

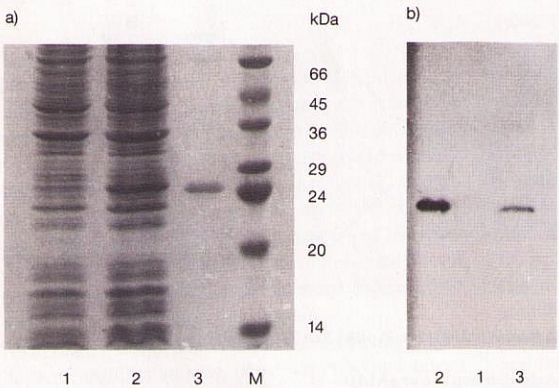

Fig. 5. SDS-Polyacrylamide gel electrophoresis (a) and Western blot (b) of the *E. coli* cell extract.

E. coli cells had been transformed with: 1) BL21 (pET8c) wild type and 2) BL21 (pPAI), 29 kDa; 3) apo AI standard, 28.4 kDa; M) marker.

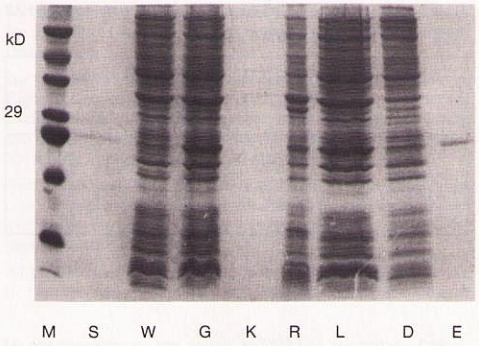

Fig. 6. SDS-Polyacrylamide gel electrophoresis of proteins in the isolation steps of apo AI.
M) marker proteins; S) apo AI standard; W) BL21 (pET8c) wild type; G) total expression extract; K) culture medium; R) residual cell detritus; L) lysate; D) solution for affinity column; E) apo AI (29 kDa) desorbed with 0.2M glycine, pH 2.8 from anti apo AI-Sepharose affinity column.

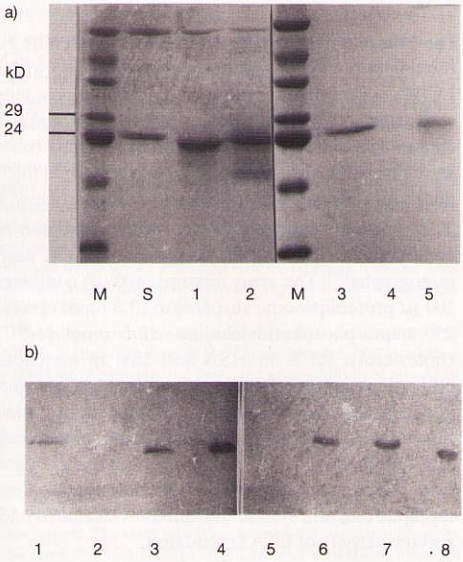

Fig. 7. a) SDS-Polyacrylamide gel electrophoresis of isolated recombinant apo AI.
M) marker proteins; S) apo AI standard (28.4 kDa); expression products of: 1) pAId41 (24.6 kDa); 2) pAI (29 kDa); 3) pAI-Glu^{111} (29 kDa); 4) BL21 (pET8c); 5) pAI control (29 kDa).
b) Western blot analysis of mature HDL apo AI (1 and 8); expression product of BL21 (pET8c) vector (2 and 5), pAId41 (3), pPAI (r-apoAI) (4 and 7) and pAIGlu^{111} (6).

and 650 $\mu g/l$ apo AI Gln^{111} as compared to 290 $\mu g/l$ apo AI pPAI wild type were isolated.

Fig. 7a presents the SDS-PAGE (15% acrylamide) of the purified mutated apo AI proteins stained with Coomassie Blue. Densitometry indicated that 75% of the recombinant apo AI was present in the soluble eluate, 25% in the insoluble residue. The Western blot analysis of normal and mutant recombinant apo AI proteins is given in Fig. 7b.

C) LCAT activation studies with recombinant apo AI and mutagenized apo AI d41 and apo AI Gln^{111}

1) Enzyme purification

Following the purification cascade of the established procedure[12] a LCAT preparation with 55% enriched enzyme and contaminants of apparent molecular masses 75 kDa, 66 kDa (serum albumin), 45 and < 25 kDa was obtained.

Serum albumin and apo AI are the most strongly interferring proteins. We therefore developed a purification procedure consisting of dextrane sulfate/$Mg^{2\oplus}$ precipitation of VLDL and LDL, removal of serum albumin by affinity chromatography over anti HSA-IgG Sepharose 4B-CL, of hydrophobic serum proteins by phenyl-Sepharose chromatography and apo AI over antiapo AI IgG-Sepharose 4B-CL.

The purification steps of LCAT are summarized in Table 1. The LCAT assay with the highest enriched enzyme fraction was negative in the absence of apo AI.

Table 1. Purification steps of LCAT from human serum.

	Volume	Protein		Activity			Purity
	[ml]	[mg]	[mg/ml]	[U]	[U/mg]	[%]	factor
Serum	110	17820	162.0	14924	0.84	100	1
Dextrane sulfate	100	13000	130.0	13282	1.0	89	1.2
Anti HSA Sepharose	120	724	6.0	12000	16	80	19
Phenyl-Sepharose	10	15	1.5	2141	143	14	170
Anti AI Sepharose	5	2.5	0.5	491	196	3.3	233

2) LCAT assay

The comparative activity studies with different activating apo AI polypeptides were established with a LCAT assay of high reproducibility. Proteoliposomes, consisting of [4-^{14}C]cholesterol, soy-phosphatidylcholine and apo AI in a molar ratio of 12.5:250:0.8[13] were prepared with the cholate-dialysis method introduced for the reassembling of lipoprotein particles[14].

The proteoliposomes were separated from non-recombined components by Sepharose 4B-CL chromatography[14]. The assay mixture (400 μl) contained 100 μl proteoliposome suspension (0.8 nmol apo AI, 250 nmol phosphatidylcholine, 12.5 nmol [4-^{14}C]-cholesterol), 0.5% in HSA and 5nM in mercaptoethanol. Aliquots of the enzyme preparation were added and the incubation stopped after 1 h by extraction with chloroform/methanol. The ratio of the integrated radioactivity in the cholesterol and cholesterol ester bands was determined after thin-layer chromatography (solvent system: hexane/ethylacetate 85:15) and the activity of LCAT calculated.

The activator function of the recombinant apolipoprotein AI was tested in two different systems: 1) small unilamellar vesicles (egg PC/cholesterol 20:1) to which the activator protein spontaneously binds upon incubation at 37 °C for 30 min, and 2) discoidal proteoliposomes reconstituted from the substrates cholesterol, PC and the activator apo AI by the cholate method.

3) Activation results

1) With the small unilamellar vesicle system native apo AI, recombinant apo AI and the two mutant apo AI (0.8 nmol each) activated LCAT to the same degree linearly over a period of three hours with 1.25 nmol cholesterol ester formation per hour.

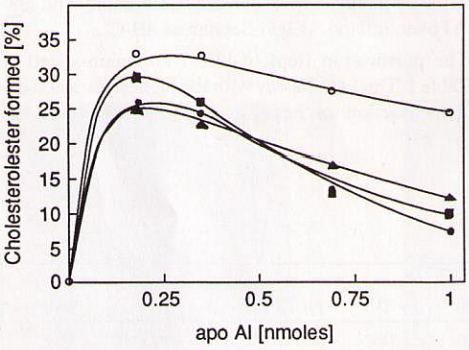

Fig. 8. Esterification of cholesterol in relation to activator concentration.

The incubation time was 1.5 h. Empty circles: serum apo AI; filled circles: r-apo AI; squares: AI Glu^{111}; triangles: AI d41.

Table 2. Activation of LCAT by native and recombinant apo AI proteins in unilamellar vesicles.

Activator protein	Cholesterolesters formed [nmol]*	Activation [%]
Mature, native apo AI	2.69	100
r-apo AI	2.65	98
AI d41	2.25	85
AI Gln^{111}	2.24	84

* during 90 minutes.

Table 3. Activation of LCAT by native and recombinant apo AI proteins in proteoliposomes.

Activator protein	Cholesterolester formed [nmol]*	Activation [%]
apo AI	2.57	100
r-apo AI	2.55	100
AI d41	2.15	90
AI Gln^{111}	1.93	87

* During 90 minutes; 0.3 nmol activator was used in the LCAT assay.

The enzyme reaction was then carried out with increasing apo AI concentrations over a period of 1.5 h. Fig. 8 summarizes the cholesterol esterification dependent on increasing activator concentrations. The optimal activator concentration in the assay system was 5 μg or 0.15 nmol apo AI.

Table 2 compares the yields of the transacylation activated by native apo AI and the different recombinant activator apo AI proteins.

2) Proteoliposomes were assembled by the cholate dialysis method using PC, cholesterol and the activator apo AI in the same molar ratio as for the small unilamellar vesicles (250:12.5:0.8) and separated by gel filtration over Sepharose CL 6B from free components. Human apo AI and the recombinant apo AI activated LCAT equally well, the deletion mutant AI d41 approximately 90% and the point mutation AI Gln^{111} 87% of the normal apo AI (Table 3).

Methods and Material

Radioisotopes were purchased from Amersham Buchler, W-3300 Braunschweig, Germany, enzymes, the random-primed labelling kit and the DNA III marker from Boehringer, W-6800 Mannheim, Taq polymerase and kb ladder marker from Gico-BRL Life

Technologies, W-7514 Eggenstein, the Gene clean kit from Bio 101 Inc., La Jolla, CA 92037, USA, SDS-7 marker from Sigma, W-8024 Deisenhofen.

For quantitative radio thin-layer chromatography the Scanner of Raytest-Isotopenmeßgeräte was used, for PCR the DNA Thermal Cycler, Perkin-Elmer-Cetus Corporation, Emeryville, CA 94608, and for oligonucleotide synthesis the DNA Synthesizer model 380 A from Applied Biosystems, W-6108 Weiterstadt.

Anti AI and anti HSA Sepharose 4B-CL for affinity chromatography were prepared from monospecific sheep anti human apo AI and antihuman serum albumin and cyanogen-bromide activated Sepharose 4B-CL.

A1 recombinant DNA techniques not described in detail were carried out according to Maniatis et al.[15], Davis et al.[16] or Ausubel et al.[17].

Polymerase Chain Reaction (PCR)

The Gene Amp. Taq Polymerase kit (Perkin-Elmer-Cetus Corp.) was used for the polymerase chain reaction[18]. The reaction was run for 30 cycles in the DNA Thermal Cycler (Perkin-Elmer-Cetus Corp.) with the following condition: denaturation 95 °C for 1 min, annealing at 45 to 60 °C for 2 min, extension 72 °C for 3 min. The primers used and outlined in the text were synthezised on a model 380 A Oligonucleotide Synthesizer (Applied Biosystems Inc.); the amplified reaction mixture was subjected to agarose gel electrophoresis. The DNA migrating at the expected size was eluted, purified by Gene clean, phosphorylated and subcloned by standard methods[15] into the vectors described in the text.

DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method[19] on the double-stranded DNA. Synthetic primers were used as primers in the sequencing reactions.

Electrophoresis and Immunoblotting

Electrophoresis on SDS-polyacrylamide (15%) slab gels was performed as described before[20] and immunoblotting according to Towbin et al.[21]. Affinity-purified sheep anti-human apo AI was used for immunoblots.

LCAT purification

The enzyme was purified from 1 *l* human serum by a combination of dextrane sulfate/$Mg^{2\oplus}$ precipitation and column chromatographies according to Chen and Albers[12]. This included a phenyl-Sepharose, Blue Sepharose, DEAE Sepharose 6B-CL and hydroxylapatite chromatography. Human serum albumin was eliminated by affinity chromatography with anti HSA Sepharose 4B CL, AI by anti AI IgG Sepharose 4B CL chromatography.

Isolation of recombinant apo AI

A 2-*l* culture of pPAI-transformed bacteria in M9ZB-medium was grown to $A_{260} = 0.8$ and then expression-induced with 0.4mM isopropyl thiogalactoside. Three hours after induction cells were sedimented at 4000 × *g*, washed with PBS/1mM phenylmethanesulfonyl fluoride and resuspended in 20 μl of the same buffer. Cells were lysed by freezing (−80 °C) and thawing, completed by the addition of 20 μl (10 mg/ml) lysozyme. DNA was digested with 2 μl (10 mg/ml) DNAse I. Cell detritus was sedimented at 15000 × *g* for 30 min and the supernatant filtered through 0.4 μm filter. Apo AI was adsorbed to anti-apo AI IgG Sepharose 4B-CL (10 ml bed volume); unspecifically bound proteins were washed out with 150 ml PBS, 0.1% Tween 20 and again 100 ml PBS. Apo AI was desorbed with 0.2M glycine, pH 2.8, the apo AI-containing fraction neutralized with 0.2M NaOH, dialysed against water and lyophilized if stored.

Preparation of proteoliposomes

The substrates for the LCAT assay were proteoliposomes consisting of apo AI-, dioleoyl-*sn*-glycerophosphocholine or soy-PC and cholesterol in a molar ratio of 0.8:250:12.5.

a) *Cholate dialysis method*[14]: Toluene solutions of [4-^{14}C] cholesterol (0.5 μCi) and PC were dried in a stream of nitrogen and dissolved in 0.3 ml (0.725mM) sodium cholate in TBS/1mM EDTA. 0.8 ml apo AI solution (1.1 mg/ml) in the same buffer was added and adjusted to 3.5 ml with TBS/EDTA. The mixture was vortexed and dialysed against TBS/1mM EDTA for 48 h at 4 °C to eliminate the detergent. Proteoliposomes were filtered through a 2 × 30 cm Sepharose 6B-CL column, equilibrated with PBS to separate the unbound components. Proteoliposome-containing fractions were pooled and concentrated by dialysis against 20% PEG 6000 in PBS/1mM EDTA.

b) Proteoliposomes were also prepared by *ultrasonication* (3 × 15 s at 20W). Toluene solutions of [4-^{14}C]cholesterol and PC were dried in a stream of nitrogen and sonicated after addition of apo AI in TBS/5mM mercaptoethanol in the same molar ratio as described above. The proteoliposome preparations were analysed for [4-^{14}C]cholesterol protein and lipid-phosphorus.

LCAT assay

The activity of the enzyme was assayed via the labelled cholesterol ester formed. The assay mixture contained 100 μl proteoliposomes (250 nmol PC, 12.5 nmol [4-^{14}C]cholesterol (11000 dpm) and 0.8 nmol apo AI, 100 μl 2% HSA, 20 μl 100mM 2-mercaptoethanol and 165 μl TBS/1mM EDTA. After a preincubation of 20 min at 37 °C, 15 μl enzyme solution was added and incubated for 1 h at 37 °C. The reaction was stopped with 4 volumes of chloroform/methanol 2:1 (v/v), vortexed and the organic phase carefully separated and concentrated in a stream of nitrogen. The residue was dissolved in 50 μl chloroform and applied to a thin-layer chromatography plate (HPTLC Alu-Folien, Kieselgel 60 F_{254}, Merck, W-6100 Darmstadt) and developed in the solvent system hexane/ethylacetate 55:15 (v/v). The radioactivity in the cholesterol and cholesterol ester bands was quantified with the thin-layer radioactivity scanner (Raytest-Isotopengeräte).

Discussion

The expression system for the eucaryotic gene of apo AI in its wild type and mutated cDNA used in the experiments described here constitutes the prerequisite of a selective and high yield expression. The vector pET8c fulfills this requirement: it has a strongly inducible T7 promoter, a high transcription efficiency due to its specific T7 polymerase. The presence of T7 lysozyme inhibits the T7 polymerase in the non-induced state[6].

Despite these favorable conditions apo AI cDNAs comprising the prepro and mature sequence were not expressed. The reason could have been the presence of rare codons in the foreign gene with a reduced transcription level. Four of these, CTA for Leu, CGG and CGA for Ala and GGA for Gly, were found in the apo AI cDNA. However, it is more likely that the secondary structure of the mRNA of the pPAI construct is responsible: four out of five bases of the Shine-Dalgano sequence and two of the initiation codon hyb-

ridize with complementary sequences of the signal sequence.

Deletion of the signal sequence renders the Shine-Dalgano sequence and the translation initiation codon accessible only after the insertion of a trinucleotide (GGC) encoding Gly following the initiation codon. The final apo AI cDNA construct encoded Met-Gly followed by the prosequence, a hexapeptide, and the sequence of mature apo AI.

This construct was strongly expressed with a yield of 10% of the soluble proteins of *E. coli*. The point mutation apo AI Glu^{111} was expressed with the same yield; however, the expression of the deletion mutant apo AI d41 was reduced by 40%. Northern blot analyses and pulse chase experiments might give further information whether the deletion of 126 bp causes reduced mRNA stability or protein stability. The activator activity of apo AI has been assigned to the carboxy-terminal cyanogen-bromide fragment (position 149–243)[22]. The expression of the apo AI d41 construct yielded an apo AI activator which is deprived of helices 6 and 7 of this fragment. We eliminated these two α-helices conserving the secondary structure of the activator. The deletion, however, reduced the activator activity only by 10%. It is conceivable that the amphipathic helices of the isolated carboxy-terminal cyanogen-bromide fragment non-specifically activate the LCAT.

Similarly our experiments with the apo AI Gln^{111} mutant protein as activator showed only a minor reduction in the LCAT assay (13–16%). This is at variance with the postulated essential role of this Glu^{111} for the LCAT activation. However, further mutation experiments of Glu^{78} and Glu^{111} modified simultaneously to Gln will prove the role of these acidic side chains in these conserved positions.

The approach described here allows the directed engineering of apo AI activator proteins which, unlike activation studies of the LCAT with synthetic peptides or peptide fragments, conserve their secondary structure and should give insight into the structural requirements of the activator polypeptide generated as recombinant apolipoproteins.

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