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Protein Expression and Purification 25 (2002) 353–361

Protein Expression & Purification

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Bacterial expression and characterization of mature apolipoprotein A-I

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Received 20 November 2001; and in revised form 18 February 2002

Abstract

Plasma levels of apolipoprotein A-I (apoA-I) are correlated with reduced incidence of heart disease due to the critical role of this protein in reverse cholesterol transport. Because of its diversity of function and poorly understood structure, much research has sought to understand how the structure of apoA-I facilitates its function. A popular approach has been the use of site-directed mutagenesis followed by structural and functional studies. There are a wide variety of expression systems available to produce these mutant proteins including eukaryotic cell lines and prokaryotic cells such as *Escherichia coli*. Expression in a bacterial system is generally favorable because it can produce large amounts of pure protein quickly and economically through the use of affinity tags on the expressed protein. Unfortunately, many of these systems are not ideal for the production of apolipoproteins because, in many cases, the proteolytic digestion required to remove the affinity tag also cleaves the target protein. Here we describe a method that produces large amounts of recombinant protein that is easily purified using a histidine (His) affinity tag that is cleaved with IgA protease from *Neisseria gonorrhoeae*. This enzyme does not cleave the wild type apoA-I sequence, leaving intact, mature apoA-I (containing a Thr–Pro- on the N-terminus). We show that this recombinant protein is similar to wild type protein in structure and function using circular dichroism analysis, lipid clearance assays, recombinant particle formation and cholesterol efflux assays. This system is particularly useful for the bacterial production of apolipoproteins because of the extreme specificity of IgA protease for its target cleavage site.

Despite years of study, atherosclerosis remains one of the leading causes of death in developed countries [1]. It has been repeatedly demonstrated that, due to their key role in reverse cholesterol transport, levels of high-density lipoprotein (HDL)[1] and its protein constituent apolipoprotein A-I (apoA-I) are inversely correlated with the incidence of heart disease [2]. During the process of reverse cholesterol transport excess cholesterol is removed from tissues, such as the arterial wall, and returned to the liver for catabolism. ApoA-I likely forms nascent HDL particles as a result of ATP Binding Cassette 1 (ABCA1) activity [3]. Once particles are formed, apoA-I interacts with lecithin-cholesterol acyltransferase (LCAT) to esterify cholesterol on the surface of the HDL particle [4]. Unfortunately, the molecular details of apoA-I interactions with a variety of plasma enzymes and cell surface proteins remain unknown. Mutant forms of apoA-I have proven useful for both structural and functional characterizations and hold great promise as a tool for understanding the structure/function relationships of this protein.

In humans, apoA-I is synthesized in liver and intestinal cells as a non-glycosylated pre-pro-protein [5]. The 18 amino acid pre-segment is removed before the protein leaves the cell and the 6 amino acid pro-segment is

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[1] *Abbreviations used:* ABCAI, ATP binding cassette AI; apoA-I, apolipoprotein A-I; apoA-IV, apolipoprotein A-IV; apoE, apolipoprotein E; CD, circular dichroism; CHO, Chinese hamster ovary; HDL, high-density lipoprotein; His-tagged apoA-I, apoA-I containing a 6 amino acid histidine tag sequence; Igase, IgA protease from *Neisseria gonorrhoeae*; K_{sv}, Stern–Volmer constant; LCAT, lecithin-cholesterol acyltransferase; LB, Luria–Bertani bacterial media; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; rHDL, reconstituted HDL; IPTG, isopropyl-β-D-thiogalactoside; PAGE, polyacrylamide gradient gel electrophoresis; POPC, 1-palmitoyl, 2-oleoyl phosphatidylcholine; STB, standard Tris buffer; TrisN/S, standard Tris buffer without the NaCl.

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PII: S1046-5928(02)00020-7

cleaved post secretion by an unknown protease in the plasma, leaving the mature 243 amino acid protein [6]. In vitro, several eukaryotic strategies have been used for apoA-I production including the baculovirus–insect cell system [7] and the Chinese hamster ovary cell system (CHO) [8,9]. However, these cell types are generally more difficult to transfect than bacteria. In the baculovirus system, once the cells are successfully transfected, there is an in-depth screening process before cells with the correct construct can be used for expression. Similarly, CHO cell colonies must undergo a screening process to find stably transfected, high expressing colonies. The extended length of time required for transfection reduces the number of different mutants that can be produced quickly. Additionally, both of these cell types require a longer period of time for expression (days versus hours) and a much higher level of maintenance than bacteria. Since the protein is non-glycosylated, a eukaryotic system is not required for mature protein production and the simpler option of expression in a bacterial system appears to be the most efficient way to produce large amounts of protein.

If the pro-segment of apoA-I is removed, expression levels in *Escherichia coli* are generally very low unless one modifies the existing DNA sequence for the first 8 amino acids to a sequence in which bacterial codons are more readily available [10]. One might also express the protein with the pro-segment [11] or alternative leader sequence [12]. In fact, several different types of fusion proteins have been created for the purpose of expression and purification [13,14]. Fusion proteins containing a histidine tag are particularly advantageous because the protein can be purified over a nickel chelating column. However, in order to use these expression products in experiments, one must either accept a form of the protein with the fusion protein left on, which runs a significant risk of adversely affecting both structure and function, or adopt a strategy to proteolytically cleave the tag sequence away from the target sequence. Several common proteolytic systems have been used for this purpose including enteropeptidase [11], factor Xa [14], and thrombin [15]. Although these systems work well for many proteins, their use for apolipoprotein systems has been limited by the fact that these proteases often cut at locations within the target protein in addition to the intended site engineered between the fusion and target proteins [16]. In the case of several apolipoproteins, this problem has been circumvented by lipidating the protein before cleavage, presumably masking the secondary cleavage sites through a conformational change [16]. However, these lipidation and subsequent delipidation steps cost extra time and can have significant impacts on final protein yield. Therefore, we searched for an enzyme that would not cleave lipid-free apoA-I and found that IgA protease (Igase) from *Neisseria gonorrhoeae* did not cleave the protein. We then added an Igase site to the pET30 expression vector containing the apoA-I cDNA.

This paper describes the structural and functional characterizations of recombinant mature apoA-I that is expressed as a soluble fusion protein containing an N-terminal histidine tag (His-tag) that is removed post purification with IgA protease (Ig-apoA-I). This construct expresses in *E. coli* at high levels. We show that IgA protease cleaves the His-tag without affecting the target apoA-I product and without the need for protein lipidation before the cleavage. Most importantly, we show that the final protein has similar structural and functional characteristics as plasma apoA-I including similar far UV circular dichroism spectra, fluorescence properties, guanidine denaturation curves, reconstituted HDL particle formation, DMPC clearance and induction of apolipoprotein-mediated cholesterol efflux from RAW264.7 mouse macrophages. Therefore this system allows easy and efficient expression of recombinant mature apoA-I that performs closely to plasma apoA-I in structural and functional studies.

Materials and methods

Materials

SDS–PAGE gels were obtained from Bio-Rad (Hercules, CA) or Amersham-Pharmacia (Piscataway, NJ). Primer synthesis and DNA sequencing were performed by University of Cincinnati DNA Core (Cincinnati, OH). IgA protease (Igase) was purchased from Mobitech (Marco Island, FL). BL-21(DE3) *E. coli* and the pET30 vector were from Novagen (Madison, WI). Isopropyl-β-D-thiogalactoside (IPTG) was from Fisher Scientific (Pittsburgh, PA). 1-Palmitoyl, 2-oleoyl phosphotidylcholine (POPC) was acquired from Avanti Polar Lipids (Birmingham, AL). All chemical reagents were of the highest quality available.

Methods

Construction of expression vector

The manipulations to the DNA sequence of the pET30 vector containing the apoA-I cDNA [17] were accomplished by PCR. The forward primer included a clamp region complimentary to the gene, a flap region with the desired changes and an *Nco*I cleavage site for ligation of the PCR product into the pET30 expression vector (see Fig. 3). The reverse primer was the M13 reverse primer designed for the pET30 vector downstream from the gene (5′-CAG GAA ACA GCT ATG ACC-3′). PCR amplification was carried out using a Perkin–Elmer thermocycler: 1 min hold at 94 °C; 30 rounds of 94 °C for 1.5 min, 55 °C for 1.5 min, 72 °C for 1.5 min; followed by a hold for 10 min at 72 °C to ensure full

extension. Platinum Pfx polymerase (Gibco, Gaithersburg MD) was utilized for the reactions. The PCR product was cut with *Nco*I (New England Biolabs, Beverly, MA) and *Hin*dIII (Gibco) and ligated into the pET30 vector cut with the same enzymes. Correct sequence was confirmed by DNA sequencing.

Protein expression and purification

The pET30 vectors containing the apoA-I constructs (pro-apoA-I; apoA-I with no pro-segment (Δpro-apoA-I); pro-apoA-I with the Igase cleavage site (Ig-pro-apoA-I); and apoA-I with the Igase cleavage site (Ig-apoA-I)) were transfected into BL-21 *E. coli* cells. Cells were grown on Luria–Bertani (LB) agar plates with 30 μg/ml kanamycin (Calbiochem, San Diego, CA) at 37 °C. After approximately 16 h, a single colony was picked from each plate to inoculate 10 ml LB liquid cultures containing 30 μg/ml kanamycin. These cultures were grown overnight in a 37 °C shaking incubator (225 rpm). Then 100 ml LB cultures with 30 μg/ml kanamycin were inoculated with 800 μl of the 10 ml cultures. The 100 ml cultures were grown for about 3 h at 37 °C, with shaking at 225 rpm, to an A_{600} of 0.9. The cultures were then induced with 50 μl of 1 M IPTG and were grown for 2 h under the same conditions.

Cells were pelleted at 9700*g* in a Sorvall SLA1500 for 10 min, frozen overnight, then resuspended in 10 mM ammonium bicarbonate (1 ml/20 ml of original culture media). Cells were lysed using sonication with a Model 550 sonic dismembrator at level 5 (Fisher Pittsburgh, PA) three times for 1.5 min each, with 1.5 min on ice between sonication periods. The lysed cells were then spun at 28,384*g* in a Sorvall SA600 for 30 min. The supernatant containing the soluble protein fraction, which included the apoA-I [11], was collected, and the pellet was discarded.

The protein fraction was then lyophilized and subjected to a chloroform/methanol delipidation to remove bacterial lipopolysaccharide (LPS) from the proteins. The lyophilized protein was resuspended in 15 ml of a 2:1 mixture of chroroform:methanol. After 30 min on ice, 12 ml methanol was added and the mixture was spun at 8100*g* for 30 min. The supernatant was removed and the protein was washed by resuspending in 27 ml methanol and incubating for 20 min on ice. The suspension was centrifuged at 8100*g* for 30 min and the supernatant was removed. The remaining protein was dried under nitrogen for 10 min then was resuspended in 3 M guanidine HCl and dialyzed into 5 mM ammonium bicarbonate. Any insoluble protein was removed by centrifugation at 3200*g* in a Sorvall RT-6000 for 20 min. The supernatant protein was filtered through a 0.45 μm filter. The sample was then run over a 5 ml Hi Trap Metal chelating column (Amersham-Pharmacia, Piscataway, NJ) charged with nickel (50 mM $NiSO_4$) on an FPLC AKTA system. The His-tagged protein was eluted with high imidizole buffer (1 M imidizole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). Fractions containing the apoA-I were dialyzed into 1× standard Tris buffer (STB: 10 mM Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 0.2% NaN_3, pH 8.2). Total protein was determined with the Lowry assay [18] and the protein was incubated 16–24 h with Igase (1:5000, enzyme:protein, w:w). After cleavage with Igase, the protein was brought to 1 M ammonium sulfate with solid ammonium sulfate. The apoA-I was then separated from the tag using a HI Trap Phenyl HP column (Amersham-Pharmacia, Piscataway, NJ) starting with Tris buffer with no salt (TrisN/S: 10 mM Tris–HCl, 1 mM EDTA, 0.2% NaN_3, pH 8.2) containing 1 M ammonium sulfate, followed by a linear gradient elution with TrisN/S (5 column volumes). Fractions containing apoA-I were combined, concentrated by ultrafiltration and dialyzed into STB. The final yield of protein was about 20 mg of pure protein per liter of culture medium.

Cleavage with proteases

Lipid-free plasma apoA-I was incubated with enterokinase and factor Xa at the manufacturer recommended concentrations for 16 h at 37 °C. Lipid-free plasma apoA-I was incubated with Igase at a mass to mass concentration of 1:5000 (enzyme:protein) 16 h at 37 °C. Plasma apoA-I was incubated for 16 h at 37 °C without any enzyme as a control for degradation due to heat.

Structural studies

Fluorescence. Wavelength of maximum fluorescence and Stern–Volmer constants (K_{sv}) were obtained using a Photon Technology International spectrofluorometer (Monmouth Junction, NJ) in emission scan mode. The excitation and emission band passes were at 1 nm. The samples were excited at 295 nm to avoid tyrosine fluorescence and the spectra were scanned over 300–400 nm to find the wavelength of maximum fluorescence [19]. All samples were diluted to 0.1 mg/ml in Tris salt buffer prior to fluorescence analysis. For the quenching studies, the samples were then quenched with increasing concentrations of 5 M acrylamide (Sigma, St. Louis, MO). Acrylamide was added in aliquots so that the final concentration was 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 M (adjusted for volume change). K_{sv} and F_a were calculated by the Lehrer modified method of Stern–Volmer [20].

Circular dichroism. Samples were diluted to 0.1 mg/ml on 20 mM phosphate buffer. Spectra were taken in a Jasco S-720 spectropolarimeter using a 0.1 cm pathlength quartz cuvette. Spectra were read over wavelengths 250 nm to 185 nm. Percent alpha helix was calculated from mean residual ellipticity at $\lambda = 222$ nm [21]. For guanidine denaturation studies, samples were prepared at 0.075 mg/ml in phosphate buffer with increasing amounts of guanidine (ranging from 0 to

5.85 M) for guanidine denaturation curves and incubated for 3 days at 4 °C [22].

Functional studies

Preparation of rHDL particles. Reconstituted HDL (rHDL) particles were prepared using 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL) at lipid to protein molar ratios of 110:1 and 40:1 according to the method of Matz and Jonas [23]. Lipids were dried down under nitrogen and resuspended in Tris salt buffer. Deoxycholate (1.3:1, w:w, deoxycholate:lipid) was added and incubated at 37 °C for 1.5 h with mild vortexing every 15 min. The protein was then added and incubated a 37 °C for 1 h. The cholate was dialyzed away against STB. The formation and the size of the subsequent particles were analyzed on a non-denaturing, native polyacrylamide gel using the Phast gel electrophoresis system (Amersham-Pharmacia, Piscataway, NJ).

DMPC clearance. DMPC in chloroform (Avanti Polar Lipids, Alabaster, AL) was dried down in a glass tube and brought up in 1 ml of STB at a concentration of 5 mg/ml. Multilamellar liposomes were formed by sonication for 1 min with a Model 550 sonic dismembrator at level 5 with a microtip (Fisher Pittsburgh, PA). Experiments were performed in a Pharmacia Biotech Ultraspec 4000 at a constant temperature of 25 °C by adding the liposomes to the acceptor apolipoproteins (in STB) at a mass ratio of 3:1 (DMPC:protein). The decrease in light scattering was analyzed over 30 min at 325 nm.

Cholesterol efflux studies. The transformed mouse macrophage cell line, RAW264.7 (ATCC, Manassas, VA), was maintained in Dulbecco's modified Eagle media (DMEM, Gibco, Gaithersburg, MD) with 10% FBS and 50 μg/ml gentamycin. Cells were grown to confluence in a 48-well plate and then growth media were removed and cholesterol labeling media were added for 24 h (DMEM, 10% FBS, 50 μg/ml gentamycin, [^{3}H]cholesterol (Amersham-Pharmacia), 1.0 μCi/ml; 0.5 ml per well). After 24 h, labeling media were removed and cells were washed twice with PBS, 0.2% BSA and once with DMEM, 0.2% BSA. Efflux media were added (DMEM, 0.2% BSA, 10 μg/ml apoA-I, with or with out 0.3 mM cAMP). Efflux media were removed after 24 h and filtered through a 0.45 μm filter to remove any cells. The amount of [^{3}H]cholesterol in the media was then measured by liquid scintillation counting. Percentage of efflux was calculated by dividing the counts in the media by total counts in the cells at time 0 h [24]. T_0 cells were washed three times with 1 ml of PBS. Lipids were then extracted by adding 1 ml of isopropanol to each well and incubating overnight. The lipid extract was removed and the wells were washed with 0.5 ml isopropanol to assure full removal of lipids. The wash was added to the lipid extract, and the isopropanol was removed under a stream of air. Lipids were solubilized in 1 ml toluene. Scintillation counting was performed on 100 μl of the toluene mixture.

Results

As outlined above, non-specific proteolytic cleavage within the target protein during the removal of fusion proteins from apoA-I requires additional lipidation and delipidation steps that cost time and yield. We approached this problem by: (a) finding a protease system that did not cleave lipid-free apoA-I, and (b) engineering the recognition site for that protease between the N-terminus of apoA-I and a His-tag utilized for rapid purification of the expressed protein from the bacterial lysate.

We first screened the popular protease systems, enterokinase and Factor Xa, and another commercially available option, Igase, to find one that does not cleave lipid-free apoA-I. Fig. 1 shows that both enterokinase (lane 2) and factor Xa (lane 3) cleaved the lipid-free protein to various extents. Enterokinase completely cleaved the intact protein under these conditions re-

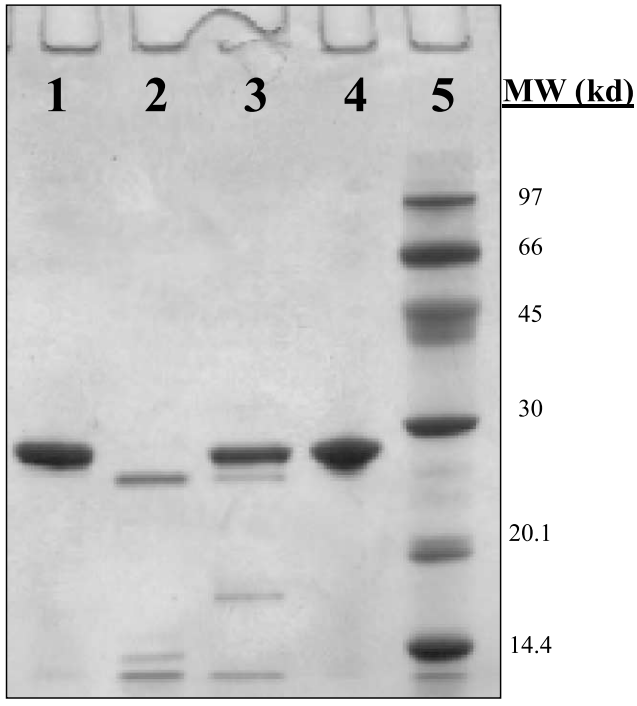

Fig. 1. Cleavage of lipid-free apoA-I with various proteases. Twelve percent SDS–PAGE gel stained with Coomassie blue. Lane 1: Uncut plasma apoA-I (incubated as a control for degradation due to temperature for 16 h at 37 °C). Lane 2: Enterokinase cut plasma apoA-I (0.5 U/4 μg protein for 16 h at 37 °C). Lane 3: Factor Xa cut plasma apoA-I (0.5 U/4 μg protein for 16 h at 37 °C). Lane 4: Igase cut plasma apoA-I (4 μg protein, 1:5000, w:w, enzyme:protein for 16 h at 37 °C). Lane 5: Low molecular weight marker (Amersham-Pharmacia, cat. #17-0446-01).

sulting in a 22 kDa major fragment [16]. Factor Xa did not cleave the intact protein as extensively, but several degradation products were visible. By contrast, Igase showed no evidence of cleaving apoA-I (lane 4). Therefore, we added an Igase cleavage site to our existing vector system (pET30) to develop a new apoA-I expression system.

We have previously shown that apoA-I expresses at high levels when it has the pro-segment on its N-terminus [11]. However, in the absence of an affinity tag, purification of these proteins was labor intensive. Since the pET30 vector already had an enterokinase site built into the vector, we first placed the pro-apoA-I sequence after this cleavage site. Fig. 2 shows that this construct was highly expressed by the cells (lane 1). We then replaced the enterokinase site with an Igase site (Ig-proapoA-I), which also showed high levels of expression in the system (Fig. 2, lane 3). Interestingly, when we attempted to produce mature apoA-I by simply deleting the pro-segment (Δ-pro apoA-I), the protein product was not expressed (Fig. 2, lane 2). We hypothesized that the bacteria would express the protein as long as the pro-segment was present close to the beginning of the mature protein. Therefore, we added the Igase site between the pro-segment and the beginning of the mature gene so that the pro-segment would be removed with the histidine tag upon cleavage with the enzyme (Ig-apoA-I, see Fig. 3). We found that the bacteria expressed Ig-apoA-I at levels equal to the Ig-proapoA-I construct (Fig. 2, lane 4).

Once it was ascertained that we could easily express high levels of both mature apoA-I and proapoA-I with an Igase site to conveniently remove the His-tag, we developed the purification scheme described in Methods to generate pure protein. An SDS gel showing the results at each major stage of the purification is shown in Fig. 4. After purification of the His-tagged protein on a chelating affinity column (Fig. 4, lane 4), the expressed protein was cleaved with varying amounts of Igase (Fig. 4, lane 5). The optimal conditions were found to be a ratio of 1:5000 (mg Igase:mg apoA-I) at 37 °C for 16 h. After verification of complete cleavage of the His-tag by SDS–PAGE, the enzyme and cleaved tag were separated

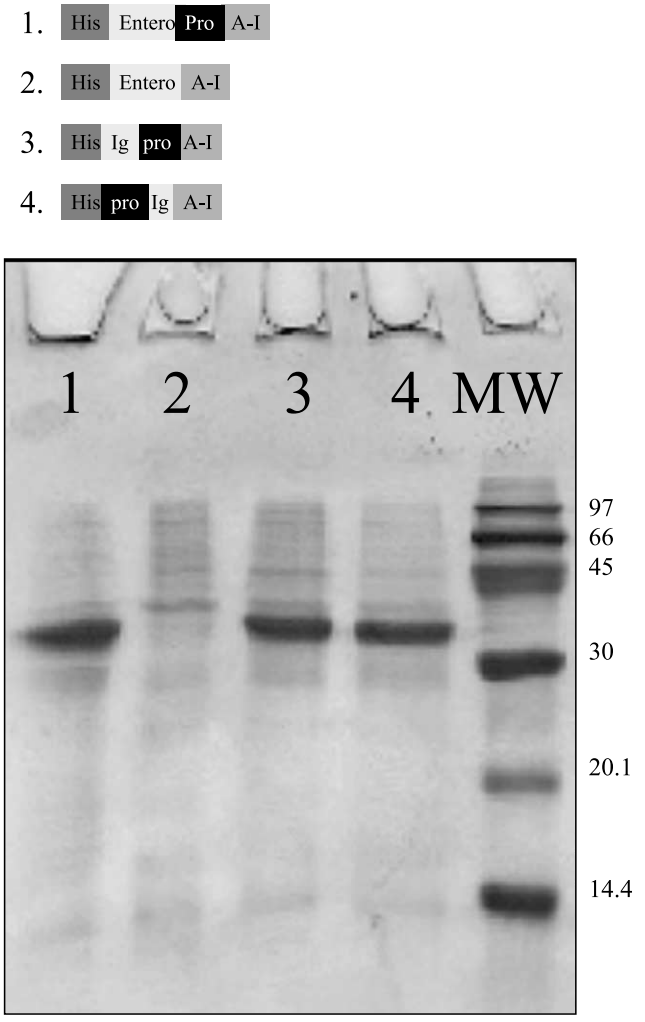

Fig. 2. Expression of various constructs of apoA-I in the pET30 vector. Eighteen percent SDS–PAGE gel stained with Coomassie blue. Lanes 1–4 show crude cell extract solublized in SDS-running buffer and boiled for 5 min: lane 1: pro-apoA-I with enterokinase cleavage site; lane 2: Δpro apoA-I (pro-segment deleted via PCR) with enterokinase site; lane 3: Igase site in front of pro-apo-A-I (Ig-proapoA-I); lane 4: Igase site after pro-segment directly in front of mature gene (Ig-apoA-I). Lane 5 contains low molecular weight markers (Amersham-Pharmacia, cat. #:17-0446-01).

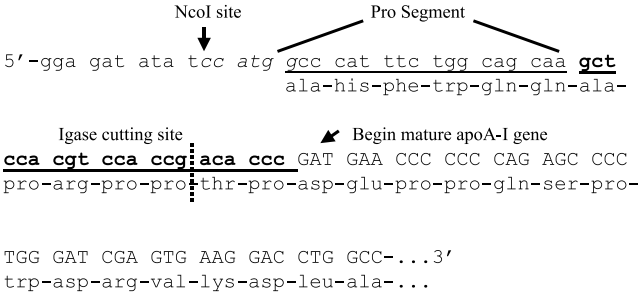

Fig. 3. Forward oligonucleotide primer sequence. This primer was used to add the IgA protease site to the pET30 vector containing apoA-I cDNA. The same general strategy was used to create all the constructs used in Fig. 3. The amino acid sequence is indicated under each codon. The primer contains a clamp region that corresponds to the cDNA of mature apoA-I, indicated by capital letters. The Igase cleavage site is directly in front of the sequence for the mature form of the gene and is indicated by bold, underlined, lower-case letters. The pro-segment is upstream of the Igase cleavage site, indicated by underlined, lower-case letters. The *Nco*I site used to cut the resulting PCR product and ligate it into the pET30 expression vector is indicated by lower-case italicized letters with an arrow at the cleavage site. The pro-segment is removed with the His-tag when the protein is cleaved with Igase between the Pro and Thr indicated by the dotted vertical line. Cleavage at the Igase recognition site, Pro–Pro–Thr–Pro, leaves an N-terminal Thr–Pro– on the protein.

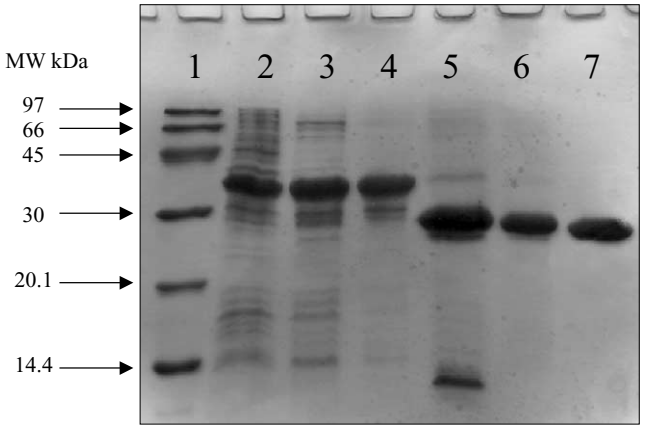

Fig. 4. SDS–PAGE gel showing steps of purification. Eighteen percent SDS–PAGE gel stained with Coomassie blue. Lane 1 contains Amersham-Pharmacia low molecular weight markers (17-0446-01). Lane 2 contains whole cell lysate of bacteria expressing apoA-I. The cells were solublized in SDS containing loading buffer (under reducing conditions) and boiled for 5 min. Lane 3 contains the soluble protein fraction after the delipidation step. Lane 4 shows the His-tagged protein after it has been run over the His-binding columns. Lane 5 shows the His-tagged protein after the His-tag has been cleaved by Igase. Note the change in size to the correct 28 kDa molecular weight and the presence of the cleaved tag at the bottom of the gel. Lane 6 contains the pure apoA-I after purification over the HI Trap HIC column and demonstrates protein purity of >95%. Lane 7 contains human plasma apoA-I as a comparison to show that the recombinant protein is the same size as the naturally occurring protein.

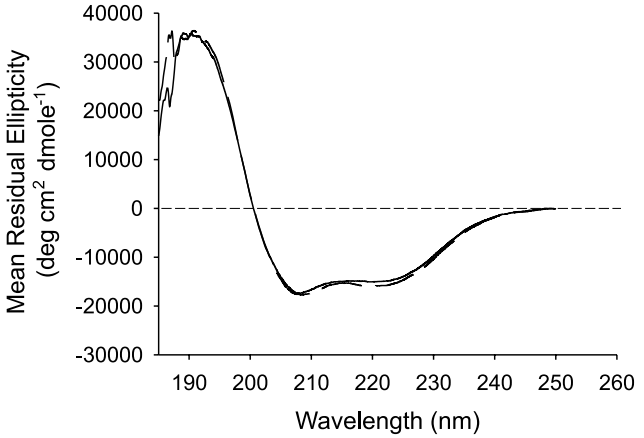

Fig. 5. Far UV circular dichroism spectra of recombinant and human plasma apoA-I. The spectra of lipid-free recombinant apoA-I (solid line) and lipid-free plasma apoA-I (dotted line) were recorded on a Jasco J-720 spectropolarimeter from 250 to 185 nm. All samples were at 0.1 mg/ml.

from the target protein by hydrophobic interaction chromatography using a Hi-Trap phenyl sepharose column (Fig. 4, lane 6) (Amersham-Pharmacia, Piscataway, NJ). The resulting proteins were routinely >95% pure as determined from Coomassie stained gels (Fig. 4, lane 6). The pure, recombinant apoA-I and purified human plasma apoA-I exhibited an identical apparent molecular weight on 18% SDS–PAGE gels (Fig. 4, lanes 6 and 7). We then characterized the structural and functional properties of the mature form of apoA-I to show that this expression system produces protein that is comparable to plasma apoA-I.

Structural studies

Circular dichroism. Circular dichroism (CD) was used to calculate the average alpha helical content of plasma apoA-I versus Igase cut mature apoA-I to assure that secondary structure was similar for both forms of the protein. Both recombinant Ig-apoA-I and plasma apoA-I had alpha helical contents of about 50%, which is consistent with previous studies (Table 1) [22]. Fig. 5 demonstrates that the CD spectra for each protein were similar both in shape and magnitude of the minima at 208 and 222 nm. The relative stability of the lipid-free forms of plasma apoA-I and IgA protease cut apoA-I was determined by isothermal denaturation studies using increasing concentrations of guanidine HCl. The molar ellipticity of each protein was observed at 222 nm at each concentration to create denaturation curves. The overall stability of the two proteins was not significantly different (data not shown).

Fluorescent studies. To further compare the conformation of the recombinant protein with plasma apoA-I, we studied the fluorescent properties of the proteins. The comparable wavelengths of maximum fluorescence (Table 1) indicated that the Trp residues in each of the two proteins were in similar environments. Likewise, quenching studies were performed by adding increasing concentrations of 5 M acrylamide. The Stern–Volmer constant (K_{sv}) indicates that the relative accessibility of the Trp residues to solvent was similar in both proteins.

Table 1
Conformational and fluorescent properties of Ig-apoA-I and plasma apoA-I

Sample	Alpha helix content[a] (±5%)	λ max[b]	K_{sv}[c]	F_a[d]
Igase-apoA-I	49	334 ± 0.5	8.2 ± 2.4	0.71 ± 0.11
Plasma apoA-1	47	334 ± 0.5	7.3 ± 3.1	0.78 ± 0.18

[a] Determined from mean residual ellipticity at 222 nm. Percent error value is an average standard deviation from repeated measurements.
[b] Wavelength of maximum fluorescence measured at 295 nm excitation. The Trps at positions 8, 50, 72, and 108 are equivalent in both proteins.
[c] Lehrer modification of the Stern–Volmer constant showing the relative exposure of Trp to the quenching agent (5 M acrylamide).
[d] Fraction of Trp signal affected by the quenching agent.

The F_a term indicates the relative exposure of Trp signal to the quencher was also similar between the two forms. The K_{sv} values obtained (plasma apoA-I 7.28 ± 3.1 and Ig-apoA-I 8.18 ± 2.4) as well as the F_a values (plasma 0.78 ± 0.18 and Ig-apoA-I 0.71 ± 0.11) were consistent with other studies and with the two proteins being in similar conformations to each other [11].

Functional studies

Lipid binding studies. The structural studies showed that the two proteins had nearly identical structural features and it follows that the two proteins should behave at similar levels in functional assays. One of the most critical functions of apoA-I is to arrange phospholipid into discoidal particles that eventually form the spherical HDL particles that deliver cholesterol to the liver for catabolism. ApoA-I was reconstituted into rHDL particles using starting ratios of 110:1 and 40:1 phospholipid to protein using the sodium cholate dialysis method. These starting ratios were selected because they were found to generate a range of rHDL particles that vary in diameter from 76 to 98 Å for the 40:1 ratio, and 104 to 130 Å for the 110:1 ratio. The resulting particles were analyzed by native PAGE to determine the extent of particle formation and the distribution of protein within each sized particle for both the Ig-apoA-I and plasma apoA-I control. The two types of apoA-I formed identical particle size classes and distributions for the 110:1 rHDL particles (data not shown). The 40:1 reconstitution exhibited similar size classes between the two protein types, but showed the protein distribution was slightly different with the Ig-apoA-I being more distributed to the smaller bands. The reason for this slight difference was not immediately clear.

We also measured the ability of Ig-apoA-I to rearrange and clear phospholipid using a DMPC turbidity clearance assay. ApoA-I is known to bind to packing defects on the liposome surface and rearrange the lipids into small discoidal complexes that do not scatter light [25]. Fig. 6 shows that the recombinant apoA-I was able to clear lipid at a rate equal to that of plasma apoA-I, implying a similar lipid affinity.

Cholesterol efflux studies. The discovery of ABCA1 as a key player in apolipoprotein-mediated cholesterol efflux has provoked much research on the interaction of these two molecules. To determine if mutant forms of apoA-I produced in this expression system can be used to study apolipoprotein-mediated cholesterol efflux we tested them in the RAW264.7 macrophage efflux assay [24]. In this system, the ABCA1 transporter is up-regulated by adding 0.3 mM cAMP and efflux promoted by the transporter can be distinguished from basal levels of efflux (samples not containing cAMP) [3]. Fig. 7 shows that Ig-apoA-I performs equally to plasma apoA-I in this assay with both proteins inducing about 18% efflux in the presence of cAMP over a 24-h period. This result indicates that this method of expression will be valid for creating mutant proteins to analyze the ABCA1/apoA-I interaction.

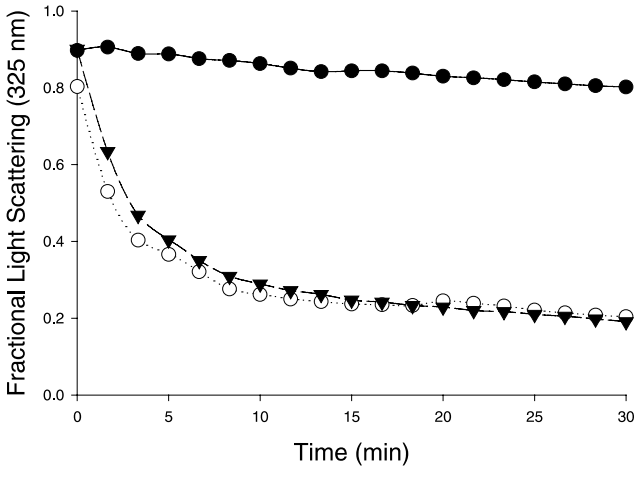

Fig. 6. Interaction of plasma versus recombinant Igase mature apoA-I with DMPC liposomes. Multilamellar DMPC liposomes suspended in STB were added to protein in a 3:1 mass ratio of DMPC:protein. Absorbance at 325 nm was recorded as a function of time at a constant temperature of 24.5 °C as maintained by circulating water bath. Symbols represent DMPC alone (filled circle), plasma apoA-I (open circle), and recombinant Igase apoA-I (filled triangle).

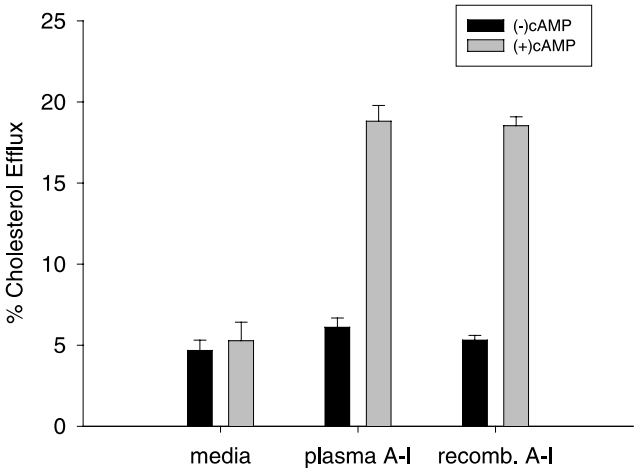

Fig. 7. Efflux of [^{3}H]cholesterol from RAW264 cells to apolipoprotein acceptors. RAW264 cells were labelled for 24 h with [^{3}H]cholesterol as described in Experimental procedures. After labeling media were removed and cells were washed three times, DMEM with 0.2% BSA containing 10 μg/ml acceptor apoA-I $\pm$ 0.3 mM cyclic AMP was added to the cells. Plasma apoA-I and recombinant Igase apoA-I served as acceptor proteins as indicated under the bar graphs. Media alone served as control indicating basal efflux without acceptors. Black bars represent samples without cAMP while gray bars indicate samples with 0.3 mM cAMP. The efflux data are expressed as percentage of total cell cholesterol for cells whose lipids were extracted immediately after the washes that followed labeling (t_0).

Discussion

This study shows that recombinant, mature apo-AI can be produced relatively quickly (within two weeks) with a high yield in a bacterial system by putting the pro-segment before a cleavage site intended to remove the histidine tag. This protein can be easily purified using nickel columns after which the histidine tag, along with the pro-segment, can be cleaved with IgA protease to leave the mature protein intact without the time-consuming lipidation steps [16]. This enzyme is unique in its ability to cleave the His-tag from lipid-free apoA-I without damaging the rest of the protein. One drawback of this system is that the resulting protein has a threonine and proline on the N-terminus. Nevertheless, the protein performs much like plasma apoA-I in the various structural and functional assays that were performed in this study. In addition, previous studies have shown that the N-terminal 43 amino acids are not involved in lipid association and function [26]. However, if one is concerned about the two additional N-terminal amino acids in a particular application, the enzyme dipeptidylpeptidase IV that cleaves these two N-terminal amino acids is available (Sigma, product #D7052).

An important finding in this study is that Ig-apoA-I performs as well as plasma apoA-I in RAW264.7 macrophage cholesterol efflux assays. If one wants to compare mutant forms of apoA-I expressed in any recombinant system to plasma apoA-I, it is *critical* that the wild type apoA-I expressed in these systems can perform at the same level in efflux assays as plasma apoA-I. This control is commonly omitted in published cholesterol efflux studies using recombinant apolipoproteins. If the recombinant protein does perform at the same level as plasma apoA-I, one can more safely justify that any changes in levels of efflux are from the intended mutation rather than the expression system. For this reason, as well as ease of expression and high yield production, this method is ideal for studying a variety of mutants that can be utilized in characterizing the ABCA1–apoA-I interaction.

Another interesting finding in this study was the observation that when the apoA-I pro-segment is completely removed from the construct, protein expression is almost non-existent. However, when the pro-segment is present near the N-terminus of the protein, even if it is not directly attached to apoA-I, the protein is expressed at similar levels to pro-apoA-I. As suggested by Isacchi et al. [10], possible explanations for this observation are that the presence of the pro-sequence may increase the stability of the transcribed mRNA or may prevent the formation of secondary structure that prevents translation in this system. We have noted that numerous mutants including large deletion and insertion mutants of apoA-I created in this system all seem to express at very similar levels to WT as long as the pro-segment is present near the N-terminus of the construct. The latter feature of this expression system is another reason it is exceedingly useful for structure/function studies of apoA-I.

One other feature of the Igase expression strategy is that it can be used for a variety of other apolipoproteins. To date, we have developed two other expression systems for rat apoA-IV and human apoE-3 (in collaboration with Drs. Getz and Reardon at the University of Chicago) using the same strategy. Igase appears to have extremely high specificity for its consensus cleavage site and does not appear to be "fooled" by conformational effects within the target protein as can happen for other proteases [16]. Therefore, the dynamic nature of the exchangeable apolipoproteins may preclude the use of many other proteolytic systems for fusion protein cleavage.

In summary, the expression of apoA-I using an Igase cleavage site is ideal for many reasons. It allows for high expression levels but also for removal of the pro-segment. The resulting recombinant protein is equal to apoA-I in structural and functional characteristics allowing for comparison between mutant proteins expressed in this system and plasma apoA-I. It is also easy to make mutants of apoA-I in the pET30 expression vector through site-directed mutagenesis [17]. These mutants can be used to find the functional domains involved in the wide variety of apoA-I functions such as LCAT activation, lipid binding, and interaction with the ABCA1 transporter.

Acknowledgments

This work was supported in part by a RO1 (HL62542) grant from the National Heart Lung and Blood Institute (WSD). Dr. Davidson is an Established Investigator of the American Heart Association. Ms. Panagotopulos received stipend support for this project from the Graduate Research Council of the University of Cincinnati.

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