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Expression of the C-terminal domain of human apolipoprotein A-I using a chimeric apolipoprotein

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ABSTRACT

Human apolipoprotein A-I (apoA-I) is the most abundant protein in high-density lipoprotein, an anti-atherogenic lipid-protein complex responsible for reverse cholesterol transport. The protein is composed of an N-terminal helix bundle domain, and a small C-terminal (CT) domain. To facilitate study of CT-apoA-I, a novel strategy was employed to produce this small domain in a bacterial expression system. A protein construct was designed of insect apolipophorin III (apoLp-III) and residues 179–243 of apoA-I, with a unique methionine residue positioned between the two proteins and an N-terminal His-tag to facilitate purification. The chimera was expressed in *E. coli*, purified by Ni-affinity chromatography, and cleaved by cyanogen bromide. SDS-PAGE revealed the presence of three proteins with masses of 7 kDa (CT-apoA-I), 18 kDa (apoLp-III), and a minor 26 kDa band of uncleaved chimera. The digest was reloaded on the Ni-affinity column to bind apoLp-III and uncleaved chimera, while CT-apoA-I was washed from the column and collected. Alternatively, CT-apoA-I was isolated from the digest by reversed-phase HPLC. CT-apoA-I was α-helical, highly effective in solubilizing phospholipid vesicles and disaggregating LPS micelles. However, CT-apoA-I was less active compared to full-length apoA-I in protecting lipolyzed low density lipoproteins from aggregating, and disrupting phosphatidylglycerol bilayer vesicles. Thus the novel expression system produced mg quantities of functional CT-apoA-I, facilitating structural and functional studies of this critical domain of apoA-I.

1. Introduction

Human apolipoprotein A-I (apoA-I) is the main protein associated with high-density lipoprotein (HDL) [1–3]. ApoA-I exhibits remarkable structural flexibility existing in a lipid free form or bound to HDL of various sizes, which requires structural adaptations accommodating changes in lipoprotein size and shape [4]. The protein is a major player in heart disease and has been well documented for its roles in reverse cholesterol transport, inflammation, and innate immunity [5]. ApoA-I is a 243 residue protein with residues 1–178 folded into a helix bundle forming the N-terminal (NT) domain [6,7]. This domain contains several amino acid residues responsible for lecithin cholesterol acyl transferase activity [2]. The C-terminal (CT) tail, residues 179–243, is referred to as the CT domain, and is relatively unstructured in its monomeric form but can adopt helical structure upon self-association or lipid binding [8]. When the CT domain was removed from apoA-I, this resulted in significant changes in apoA-I function. The remaining NT helix bundle was less effective in solubilizing phospholipid vesicles, and binding to macrophages was impaired resulting in decreased cholesterol and phospholipid efflux [9]. Moreover, other CT deletion variants showed reduced capability of ABCA-I driven lipid efflux in J774 macrophages and preference for smaller HDL, and increased plasma clearance [10,11]. It has been reported that the CT domain of apoA-I initiates contact with the lipid surface, bringing the NT domain in close proximity to lipids. A conformational change then provides access of hydrophobic residues of the NT domain for a stable binding interaction with the lipid environment to form HDL [1,12].

Abbreviations: apoA-I, apolipoprotein A-I; apoLp-III, apolipophorin III; CT, C-terminal; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; HDL, high density lipoprotein; LPS, lipopolysaccharides; LDL, low density lipoprotein; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; NT, N-terminal; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PG, phosphatidylglycerol; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles.

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To gain more insight in the properties of the CT domain of apoA-I, it would be beneficial to have access to sufficient quantities of this protein domain. De novo peptide synthesis has been employed to produce CT-apoA-I [8], but this can be expensive, especially due to the large number of amino acid residues. Furthermore, purchase of the peptide may have to be done successively, and while changes can be made in the amino acid sequence upon synthesis, this can become rather costly. Normally, over-expression in *Escherichia coli* is the preferred method for producing small proteins, which also permits changes at the amino acid level by site-directed mutagenesis. While full-length apoA-I and the NT helix bundle can be expressed in large quantities in *E. coli* [13–16], the CT-domain may be too small for efficient recombinant over-expression. Recently, we engineered a chimeric protein made of insect apolipophorin III (apoLp-III) and CT-apoA-I, which was produced in large quantities in *E. coli* [17]. This apolipoprotein construct provided an opportunity to isolate CT-apoA-I, by expression and purification as a full-length chimeric protein followed by removal of apoLp-III. To facilitate this, a unique methionine residue was introduced between apoLp-III and CT-apoA-I. This allowed for cyanogen bromide cleavage producing apoLp-III and CT-apoA-I. This would have been much more difficult to accomplish in full-length apoA-I as it includes four methionine residues in its NT domain. Since the cyanogen bromide reaction may not be complete, the cleavage reaction produces many fragments, making isolation of CT-apoA-I challenging. In the present report we demonstrated that purification of CT-apoA-I from the cyanogen bromide digest of the chimera yielded a high purity protein preparation with sufficient quantities required for functional studies of this critical part of apoA-I.

2. Materials and methods

2.1. Chimeric protein, site-directed mutagenesis, protein expression and purification

The chimeric construct was custom made by Eurofins MWG Operon as described previously and inserted into the pET-20b(+) vector [17]. Asparagine 189 was replaced by methionine using the QuikChange-II site directed mutagenesis kit (Qiagen). To generate the substitution, the following primers were used: 5′-GGTTCAGAAACCGGCGATGGAAGCGCTGAAAGAAAACG-3' (forward) and 5′-CGTTTTCTTTCAGCGCTTCCATCGCCGGTTTCTGAACC-3' (reverse). The mutation was confirmed by DNA sequencing (Genewiz). The chimeric protein and apoA-I were over-expressed in *E. coli* BL21 (DE3) pLysS cells (Agilent Technologies). Overnight cultures in 2xYT broth with 50 μg/mL ampicillin and chloramphenicol were used to seed 2L cultures. Cells were grown at 37 °C until an optical density of 0.6 at 600 nm was reached, and protein over-expression was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (Gold Biotechnology). Cells were grown for another 4 h and harvested by centrifugation at 8000 g at 4 °C. Cells were resuspended in phosphate buffered saline (PBS; 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.4) and lysed using a digital sonicator (Branson) at 30% amplitude for five intervals of 30 s. Lysed cells were centrifuged 2 × at 20,000 g for 30 min to remove cell debris. The supernatant was mixed with an equal volume of sample loading buffer (2 × PBS, 6 M guanidine-HCl, pH 7.4) and loaded onto 5 mL HiTrap™ chelating columns (GE Healthcare). The column was washed with 20 mL of 40 mM imidazole, 3 M guanidine-HCl in PBS. Proteins were eluted using 20 mL elution buffer (500 mM imidazole in PBS). The eluate was dialyzed in 4 L of 10 mM ammonium bicarbonate buffer with 1 mM EDTA, pH 7.8, and three buffer changes were made over 48 h. The samples were then freeze-dried (Labconco) stored at −20 °C until use. For structural or functional analysis, dried recombinant proteins were resuspended in 6 M guanidine HCl, and refolded by dialysis for 48 h in the appropriate buffer, depending on the analysis. Protein concentrations were obtained using the bicinchoninic acid assay (Thermo Fisher Scientific) or absorbance at 280 nm. Protein purification and purity was assessed using polyacrylamide gel electrophoresis (PAGE). Protein samples (15 μg) were incubated at 70° for 10 min after adding lithium dodecyl sulfate sample loading buffer (Thermo Fisher Scientific). Samples were separated on NuPAGE 10% Bis-Tris gels at 200 V for 35 min in 2-(N-morpholino)ethanesulfonic acid, sodium dodecyl sulfate (SDS) running buffer (Thermo Fisher Scientific). Gels were stained with 0.5% (m/v) naphthol blue-black, in 10% glacial acetic acid and 45% methanol (v/v).

2.2. Chemical cleavage, peptide isolation, and circular dichroism

The chimera was cleaved with cyanogen bromide (Sigma-Aldrich) using a 1:100 M ratio of methionine to cyanogen bromide. Twenty-six mg of dry protein was dissolved in 0.5 mL of 70% formic acid, and added to 0.5 mL of 200 mM cyanogen bromide solution in 70% formic acid. The reaction was carried out for 24 h at 25 °C, after which the sample was diluted five-fold with Milli-Q water, frozen at −80 °C and freeze-dried for one day. The lyophilized digest was dissolved in 2 mL of 50 mM ammonium bicarbonate buffer and freeze-dried.

Nickel-affinity chromatograph was used to isolate the CT-apoA-I from the cyanogen digest. The lyophilized digest was dissolved in 1 mL PBS and loaded onto a 1 mL HiTrap™ chelating column (GE Healthcare). The column was washed with 1 × PBS, 40 mM imidazole, pH 7.4, releasing CT-apoA-I. ApoLp-III and uncleaved chimera were eluted with 500 mM imidazole in PBS. Column fractions were dialyzed in 2 L of 10 mM ammonium bicarbonate buffer and freeze-dried; the purity of CT-apoA-I was verified by SDS-PAGE. Alternatively, CT-apoA-I was purified from the cyanogen bromide digest with reversed-phase HPLC. Approximately 6 mg of digest was dissolved in 1 mL of 20 mM sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4, pH 7.4) and loaded on a Zorbax 300SB-C8 column (Agilent Technologies). Proteins were eluted using a linear gradient of water and acetonitrile in 0.05% trifluoroacetic acid (0.5% acetonitrile increase per min). The flow rate was set at 1 mL/min and elution was monitored at 210 nm.

CT-apoA-I was analyzed for secondary structure by circular dichroism in a Jasco 810 polarimeter. The ellipticity of the protein samples were determined from 185 to 260 nm at a concentration of 0.2 mg/mL in 20 mM sodium phosphate buffer, pH 7.4 (50 nm/min scan rate).

2.3. Phospholipid vesicle solubilization

Lipid films were prepared by dissolving 10 mg of dry lipid (Avanti Polar Lipids) in 1 mL chloroform methanol (1:3, v/v). After evaporation of solvent, the phospholipid film was further dried in a Labconco freeze-dryer for 24 h and stored at −20 °C until use. Multilamellar vesicles (MLV) were prepared from 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) or 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids). Lipid films were rehydrated with 1 mL PBS and vortexed vigorously for 1 min. The vesicles were then extruded through 200 nm membranes at 45 °C to form large unilamellar vesicles (LUV). DMPC LUV dispersions (250 μg) were solubilized by 75 μg protein. For DMPG, 250 μg MLVs were mixed with 50 μg protein. Vesicle solubilization was monitored at 325 nm in a spectrophotometer (Shimadzu UV-2401PC) at a constant temperature of 24 °C.