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Expressed protein ligation-mediated template protein extension

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Abstract

Expressed protein ligation (EPL) was performed to investigate sequence requirements for a variant human apolipoprotein A-I (apoA-I) to adopt a folded structure. A C-terminal truncated apoA-I, corresponding to residues 1–172, was expressed and isolated from *E. coli*. Compared to full length apoA-I (243 amino acids), apoA-I(1-172) displayed less α -helix secondary structure and lower stability in solution. To determine if extension of this polypeptide would confer secondary structure content and/or stability, 20 residues were added to the C-terminus of apoA-I(1-172) by EPL, creating apoA-I_{Milano}(1-192). The EPL product displayed biophysical properties similar to full-length apoA-I_{Milano}. The results provide a general protein engineering strategy to modify the length of a recombinant template polypeptide using synthetic peptides as well as a convenient, cost effective way to investigate the structure / function relations in apolipoprotein fragments or domains of different size.

Keywords

Expressed protein ligation; apolipoprotein A-I; Milano; Protein stability; Intein

Introduction

Apolipoprotein A-I (apoA-I), the predominant protein component of high-density lipoprotein (HDL), plays a central role in reverse cholesterol transport (RCT), the pathway by which cholesterol is removed from peripheral tissues and returned to the liver [1]. The 2.2-Å crystal structure of a C-terminal truncated apoA-I, (Δ 185-243)apoA-I, reveals a half-circle dimer wherein the N-terminus adopts a helix bundle conformation that is stabilized by inter-helical hydrophobic interactions [2]. While this structure provides information about the organization of wild type apoA-I, questions remain about a rare mutant form of apoA-I that is associated with decreased incidence of heart disease. ApoA-I_{Milano} (i.e. Arg173Cys apoA-I) carriers display a unique phenotype of markedly reduced plasma HDL, very low prevalence of atherosclerosis and increased lifespan compared to non-carriers [3]. The mechanistic link between apoA-I_{Milano} and athero-protection remains unclear despite many *in vitro* and *in vivo* experiments examining this protein and its effects on cardiovascular disease.

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One of the key features of apoA-I_{Milano} is a propensity to form disulfide-linked homodimers. Dimerization of apoA-I_{Milano} limits cholesterol ester accumulation in plasma HDL resulting in smaller particles. ApoA-I_{Milano} present in reconstituted HDL preferentially exists in a dimeric state [4] leading to the suggestion that dimer formation is causally linked to the reduction in cholesterol esterification and HDL particle size observed in mice and humans expressing this protein.

Given its beneficial properties, efforts have been made to use apoA-I_{Milano} as a therapeutic agent. Indeed, *in vivo* administration of apoA-I_{Milano} reconstituted HDL reduced the severity of intimal plaque deposits [5,6]. In the present study we report a facile method to generate size variants of apoA-I_{Milano} using apoA-I(1-172) as a template for expressed protein ligation (EPL) [7]. Characterization studies reveal this approach may be useful to evaluate the effect of polypeptide size on apoA-I_{Milano} structural properties, dimerization propensity and biological effects.

Material and Methods

Preparation of apoA-I(1-172)

ApoA-I(1-172) was cloned and expressed as a *S. cerevisiae* derived vacuolar ATPase intein domain and chitin binding domain (CBD) fusion protein using the pTYB1 vector (New England Biolabs) as previously reported [8]. Expression and purification procedures for apoA-I(1-172) followed a standardized protocol previously established for generating thioester-adducted protein templates for EPL [9]. Briefly, saturated overnight cultures of ER2566 *E. coli* cells harboring a plasmid vector encoding the apoA-I(1-172)-intein-CBD fusion protein were inoculated into 2 liters 2xYT media containing 50 µg/ml ampicillin, grown to OD₆₀₀ = 0.6 and induced with 1 mM isopropyl thiogalactopyranoside. After 6 h at 30 °C the cells were pelleted by centrifugation (8000 g for 15 min), solubilized in 50 ml buffer A (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing 1% Triton X-100 and stored at -20 °C. Resuspended cell pellets were combined, passed through a microfluidizer, sonicated and centrifuged at 12000 g for 20 min. The supernatant obtained was passed over a chitin bead column pre-equilibrated with buffer A containing 1% Triton X-100. The column was washed with 10 column volumes of detergent-free buffer A and fusion protein cleavage was induced by addition of buffer A containing 60 mM 2-mercaptoethanesulfonic acid (MESNA). Flow was arrested for 16 – 24 h at 22 °C followed by elution with 2 bed volumes of buffer A containing 5 mM MESNA. The sample was dialyzed against deionized H₂O, lyophilized and stored at -20 °C. ApoA-I(1-172)-MESNA was further purified by semi-preparative C₈ reversed-phase high performance liquid chromatography on a Perkin-Elmer Series 200 HPLC.

Preparation of apoA-I_{Milano}(173-192)

A twenty amino acid peptide corresponding to apoA-I_{Milano} residues 173–192 was generated by solid phase peptide synthesis and isolated by reversed phase HPLC. The peptide [NH₂-CLAAARLEALKENGARLAIEY-COOH] was soluble in ligation buffer.

Analytical methods

Protein purity and/or ligation reaction progress was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 10–20% acrylamide gradient or fixed 18% acrylamide slab gels. Gels were stained with Coomassie Blue G-250 or R-250.

Expressed protein ligation

EPL reactions contained apoA-I(1-172)-MESNA and apoA-I_{Milano}(173-192). Typical reactions were carried out in 150-250 µl incubations containing 1-1.5 mM apoA-I(1-172)

and 2-3 mM peptide. Protein fragments were dissolved in 20 mM NaH₂PO₄, 150 mM NaCl, 2M urea supplemented with 5 % (w/v) MESNA and 10 mM Tris(2-carboxyethyl)phosphine (final pH 8.5). Samples were incubated at 37 °C for 96 h with gentle agitation. Ligation reaction progress was assessed by SDS-PAGE and quantified by densitometry of the stained gel using ImageJ software for Macintosh [10,11]. Residual peptide, urea and MESNA were removed from samples by dialysis against 20 mM NaH₂PO₄, 150 mM NaCl, pH 7.2 using a 12,000–14,000 molecular weight cutoff membrane.

Fluorescence Spectroscopy

Spectra were recorded on a Perkin-Elmer LS55 fluorescence spectrometer. Emission was scanned from 300 to 400 nm (excitation 280 nm; slit width 2.0 nm for the excitation and emission monochromators). All spectra were recorded in 20 mM NaH₂PO₄, 150 mM NaCl, pH 7.2.

Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectroscopy was performed on a Jasco 810 spectropolarimeter. Scans were recorded between 185 and 260 nm in 20 mM sodium phosphate, 20 mM DTT, pH 7.4, at a protein concentration of 0.5 mg/mL in a 0.2 mm path length cuvette. Secondary structure content was calculated as previously described [12]. For studies of guanidine HCl induced denaturation, protein samples (0.2 mg/mL) were incubated overnight at a given denaturant concentration to attain equilibrium, and ellipticity was measured at 222 nm in a 1-mm path length.

Results

Bacterial expression of recombinant apoA-I (1-172)

As the sole cysteine in apoA-I_{Milano}, Cys173 provides a site for protein engineering via EPL. To pursue this, a bacterial expression system for recombinant apoA-I(1-172) was established. This N-terminal fragment of apoA-I was expressed as a fusion protein with a) a modified intein and b) a chitin binding domain. This design affords single step purification of a thioester adducted apoA-I(1-172) that is EPL reactive. Application of the supernatant fraction obtained from a solubilized bacterial cell pellet to a chitin affinity column results in specific retention of the fusion protein (Figure 1). After washing the column of unbound protein, application of buffer containing MESNA induces intein-mediated cleavage of apoA-I(1-172) at the intein - apoA-I(1-172) junction, with MESNA remaining bound to C-terminal residue of the apoA-I(1-172) product. Upon washing, apoA-I(1-172)-MESNA elutes from the column. The final yield was ~ 2.5 mg protein per L bacterial cell culture.

EPL mediated fragment extension

The overall strategy for EPL mediated extension of apoA-I(1-172)-MESNA with peptides corresponding to apoA-I_{Milano} is shown in Figure 2. The semisynthesis reaction employs apoA-I(1-172)-MESNA as a template for C-terminal extension using a synthetic apoA-I_{Milano} peptide. Since EPL requires an N-terminal cysteine nucleophile to initiate protein ligation (Xu and Evans, 2001, Muir, 2003) and apoA-I_{Milano} contains a cysteine at position 173, peptide bond formation between the C-terminal residue of apoA-I(1-172)-MESNA and the N-terminal Cys of the substrate peptide is expected to occur via a rate-limiting transthioesterification reaction [13]. The choice to use apoA-I(1-172)-MESNA eliminates the need for mutagenic introduction of a potentially disruptive cysteine elsewhere in the protein and utilizes an apoA-I fragment that can be generated in high yield.

Ligation product analysis

The appearance of a ligation product is readily detected by an ~2000 Da increment in molecular weight of the template polypeptide (Figure 3). Under the conditions employed, ligation efficiencies of >90 % were achieved. Whereas apoA-I(1-172), apoA-I_{Milano}(1-192), full length apoA-I_{Milano} and wild type apoA-I migrate as single bands when subjected to SDS-PAGE under reducing conditions, apoA-I_{Milano} forms dimers under non-reducing conditions. By contrast, dimer formation was not observed in the case of apoA-I_{Milano}(1-192), despite the presence of Cys at position 173. This result suggests that apoA-I self-association induced by C-terminal domain residues 193–243 may be necessary for disulfide bond formation.

Fluorescence properties of apoA-I proteins and truncated variants

ApoA-I possesses 4 tryptophan residues that are located within the first 108 residues of the protein. As such, Trp fluorescence emission spectroscopy can be used to evaluate the global fold of full-length apoA-I and truncated variants (Figure 4). Compared to wild type apoA-I ($\lambda_{\text{max}} = 349.5$ nm), the wavelength of maximum Trp fluorescence emission for full-length apoA-I_{Milano} (350.5 nm) was very similar. By contrast, the Trp fluorescence emission maximum of apoA-I(1-172) was red shifted to 356.5 nm, consistent with a partially folded structure that results in greater solvent exposure of Trp residues. When this polypeptide was extended by 20 residues to form apoA-I_{Milano}(1-192), however, the Trp fluorescence emission maximum was blue shifted to 351.5 nm, resembling the behavior of full-length apoA-I_{Milano}.

Stability studies

The secondary structure stability of protein samples were evaluated by far UV CD spectroscopy following incubation with increasing concentrations of guanidine HCl (Figure 5). Wild type apoA-I gave rise to a midpoint of guanidine HCl induced unfolding of 1.0, in agreement with values reported elsewhere [14]. ApoA-I (1-172) was much less resistant to denaturation, giving rise to a guanidine HCl denaturation midpoint of 0.5 M. Under reducing conditions, full-length apoA-I_{Milano} and apoA-I_{Milano} (1-192) had midpoints of guanidine HCl induced unfolding of 1.0 and 0.9 M, respectively.

Discussion

An objective of the present study was to develop a general strategy to study apolipoprotein fragments of different length. The general approach employs a template polypeptide that, when subjected to EPL using different length synthetic peptide substrates, provides a means to extend the size of the template polypeptide at will. For this purpose we employed a fragment of apoA-I comprising the N-terminal 172 amino acids. EPL was performed using a 20 amino acid synthetic peptide corresponding to the sequence a rare apoA-I variant, apoA-I_{Milano}. Characterization of the ligation product, apoA-I_{Milano}(1-192), revealed it adopts a stable conformation, similar to full-length apoA-I_{Milano} yet does not display the intramolecular disulfide bond formation property that is characteristic of full length apoA-I_{Milano}. Further refinement of peptide substrate length will permit evaluation of the structural requirements for disulfide bond formation and the biological properties of apoA-I_{Milano} variants, with a view to identifying an optimal sequence for *in vivo* therapeutic purposes [6].

Additional versatility afforded by EPL relates to introduction of labels or modifications in specific regions or sites within a protein while other portions remains unaffected. For example, by incorporating unnatural amino acids [15,16], biophysical probes [17], post-translational modifications [18] or isotope labels [19,20] into the peptide substrate, EPL can be used to investigate structural and functional aspects of proteins that might otherwise be

difficult to address. By modifying one or both fragments prior to ligation, EPL can produce proteins that contain unique characteristics, allowing for studies of protein function as it pertains to alternative conformational state transitions [21,22], post-translational affects [23], and protein-protein interactions [17,21]. Since the only requirements for EPL are the presence of an active cysteine at position 1 of the C-terminal fragment and a leaving group moiety on the amino-terminal fragment [9], modifications made elsewhere in the fragments will be imparted to the ligation product.

Another interesting feature of EPL is the fact that the cysteine introduced into the ligation product can be reduced to alanine [24]. This affords versatility to the method and allows EPL to be used in generation of proteins that do not naturally contain cysteine. In fact, by designing the experiment to replace a natural alanine with cysteine, following reduction, an EPL product corresponding to the wild type sequence will be generated, assuming no other cysteines are present in the protein. The successful application of EPL technology to construction of a variant apoA-I_{Milano} indicates future characterization studies designed to assess its potential use as therapeutic agent in the treatment of cardiovascular disease are feasible.

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Abbreviations

EPL	expressed protein ligation
apo	apolipoprotein
RCT	reverse cholesterol transport
HDL	high density lipoprotein
CBD	chitin binding domain
CD	circular dichroism
MESNA	mercaptoethanesulfonic acid

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- Apolipoprotein structure and function
- Expressed protein ligation template extension
- Apolipoprotein A-I Milano variant as a therapeutic

Figure 1. Recombinant apoA-I(1-172)-MESNA production

SDS-PAGE (4–20 % acrylamide gradient) was performed to analyze apoA-I(1-172)-intein-CBD fusion protein expression, cleavage and apoA-I(1-172)-MESNA recovery. Lane 1: molecular weight standards. Lane 2: bacterial cell lysate of induced ER2566 *E. coli* cell cultures harboring the apoA-I(1-172)-intein-CBD fusion protein pTYB1 plasmid vector. Lane 3: chitin column wash flow through following application of cell culture preparation. Lane 4: chitin column eluate after exposure to buffer supplemented with 60 mM MESNA.

Figure 2. Design strategy for EPL-mediated apoA-I_{Milano} variant production

The template, apoA-I(1-172)-MSENA, was generated as a recombinant fusion protein, cleaved to generate the desired EPL active fragment and ligated to the apoA-I_{Milano} peptide to generate apoA-I_{Milano}(1-192). M denotes 2-mercaptoethansulfonic acid (MESNA) used to induce intein-mediated cleavage of the apoA-I(1-172)-intein-CDB fusion protein.

Figure 3. SDS-PAGE analysis of apoA-I variants

Lane 1, Molecular weight standards; lane 2, apoA-I(1-172) EPL template; Lane 3, ligation product obtained following incubation of apoA-I(1-172)-MESNA with apoA-I_{Milano} peptide; Lane 4, apoA-I_{Milano}; lane 5, wild type apoA-I. Lanes 6 - 10, same as lanes 1-5 except electrophoresis was run under non-reducing conditions.

Figure 4. Fluorescence spectroscopy of apoA-I variants

Spectra were recorded at an excitation wavelength of 280 nm (slit width 2.0 nm) in 20 mM NaH_2PO_4 , 150 mM NaCl, pH 7.4 at a protein concentration of 40 $\mu\text{g}/\text{ml}$. Dotted line, wild type apoA-I; interrupted line, apoA-I_{Milano}; solid line, apoA-I(1-172) template; dashed line, apoA-I_{Milano}(1-192) ligation product.

Figure 5. Guanidine HCl denaturation of apoA-I variants

The molar ellipticity of specified protein samples was measured at 222 nm at a protein concentration of 0.2 mg/ml in 20 mM sodium phosphate, 20 mM DTT, pH 7.4 following incubation with the indicated concentrations of guanidine HCl. Filled symbols, wild type apoA-I; open symbols, apoA-I_{Milano}; filled triangles, apoA-I(1-172) template and open triangles, apoA-I_{Milano}(1-192) ligation product.