Bacterial expression and site-directed mutagenesis of a functional recombinant apolipoprotein

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Abstract To facilitate structure-function studies of *Manduca sexta* apolipophorin III (apoLp-III), its nucleotide coding sequence was cloned from a fat body cDNA library by in vitro DNA amplification. The amplification product was cloned in the pET expression vector and introduced into *E. coli*. After induction, cultures were screened for apoLp-III protein production by immunoblotting with anti-apoLp-III serum. Data obtained indicated the presence of apoLp-III in both cell lysates and media of cell cultures harboring the apoLp-III-pET construct but not in cells containing the parent vector. The protein was isolated from the cell-free supernatant of cultures grown in minimal media 4 h after induction. Verification that the recombinant protein produced was indeed apoLp-III was obtained by electrospray mass spectrometric analysis. Circular dichroism (CD) spectroscopy of the isolated recombinant protein revealed a characteristic content of α-helical secondary structure with a further induction of helix upon addition of 50% trifluoroethanol. In urea denaturation studies, monitored by CD, evidence was obtained that recombinant and natural apoLp-III possess indistinguishable thermodynamic properties. In addition, lipid binding assays revealed that recombinant apoLp-III formed stable complexes with phospholipids and was capable of associating with lipoprotein surfaces. Examination of the fluorescence properties of recombinant apoLp-III revealed the presence of a noncovalently associated fluorescent contaminant that was effectively removed by reverse phase HPLC. Subsequent fluorescence characterization revealed the expected intrinsic quenching of tyrosine fluorescence in buffer. Furthermore, in a manner very similar to the native protein, tyrosine fluorescence in recombinant apoLp-III was greatly enhanced upon interaction with detergent micelles, indicating an ability to undergo characteristic conformational changes upon lipid interaction. An F148L mutant was isolated whose tyrosine fluorescence quantum yield was considerably higher than that observed for natural apoLp-III. In urea denaturation studies, the F148L apoLp-III was found to be less stable than wild type apoLp-III. Bacterial expression of full length, soluble and functional apoLp-III offers a useful system to probe the structure-function relationship of this unique apolipoprotein.—**Ryan, R. O., D. Schieve, M. Wientzek, V. Narayanaswami, K. Oikawa, C. M. Kay, and L. B. Agellon.** Bacterial expression and site-directed mutagenesis of a functional recombinant apolipoprotein. *J. Lipid Res.* 1995. **36:** 1066–1072.

Supplementary key words apolipophorin III • lipid • bacteria • insect • *Manduca sexta*

Apolipophorin III (apoLp-III) is an exchangeable amphipathic apolipoprotein present in hemolymph of the Sphinx moth, *Manduca sexta,* that represents a useful model for studies of protein-lipid interaction. An intriguing aspect of this protein is its apparent dual existence as a water-soluble, monomeric plasma protein and lipid surface binding apolipoprotein (1). Determination of the X-ray crystal structure of the homologous apoLp-III from *Locusta migratoria* in the lipid-free state revealed that the protein exists as an up and down five helix bundle wherein the amphipathic α-helices are connected by short loops (2). The implication from the crystal structure is that the protein undergoes a significant conformational change upon lipid interaction and it has been proposed that it may open about putative hinge regions located in the loops between helices 2 and 3 and 4 and 5 (2). Recent spectroscopic evidence provides further support for such an opening upon lipid interaction (3).

One aspect that must be considered with respect to *L. migratoria* apoLp-III is the presence of unusual carbohydrate moieties bound to Asn^{16} and Asn^{83} (4). These structures contain fucose, mannose, and N-acetylglucosamine (5). Interestingly, some mannose and N-acetylglucosamine residues have covalently bound 2-aminoethylphosphonate moieties (6). It has recently been shown that enzymatic removal of the carbohydrate moieties of *L. migratoria* apoLp-III induces structural changes that lead to denaturation of the protein (3). Thus, expression of recombinant *L. migratoria* apoLp-III that lacks, or possesses altered, carbohydrate structures is unlikely to be suitable for structure-function studies. On the other hand, apoLp-III from *Manduca sexta* is functionally in-

Abbreviations: CD, circular dichroism; apoLp-III, apolipophorin III; DMPC, dimyristoylphosphatidylcholine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LDL, low density lipoprotein; IPTG, isopropyl β-D thiogalactopyranoside; HPLC, high performance liquid chromatography.

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distinguishable from *L. migratoria* apoLp-III (7) and possesses >50% sequence similarity, yet lacks glycosylation, or any other post-translational modification. Physical and spectroscopic studies of *M. sexta* apoLp-III suggest that the protein adopts a helix bundle structure in the absence of lipid and undergoes a conformational change similar to that proposed for *L. migratoria* apoLp-III upon lipid interaction (8–10). The present report describes the high level expression of full length, functional *M. sexta* apoLp-III in bacteria, as well as the fluorescence and stability properties of F148L apoLp-III.

MATERIALS AND METHODS

Materials

Natural apoLp-III was isolated from adult *M. sexta* according to Wells et al. (11). Low density lipoprotein (LDL) was isolated from human plasma between the density limits 1.006 and 1.063 g/ml by sequential density ultracentrifugation (12).

cDNA cloning, vector construction and recombinant protein identification

An *M. sexta* adult fat body cDNA library was constructed. The full-length apoLp-III cDNA was cloned directly from the library by DNA in vitro amplification using primers complementary to the 5' and 3' ends of the apoLp-III nucleotide sequence (13). A 500 base pair amplification product was cloned and the sequence was verified (14). The apoLp-III cDNA was re-engineered and transferred into the pET expression vector (Novagen), immediately downstream to the pelB leader sequence. ApoLp-III expression in *E. coli* BL21 cells harboring the plasmid were induced with 2 mM isopropyl β-D thiogalactopyranoside (IPTG) and the recombinant protein was visualized by immunoblotting.

Site-directed mutagenesis

Site-directed mutagenesis was performed according to the method of Kunkel (15). Phe^{148} was converted to Leu by preparing a synthetic oligonucleotide with a single base substitution. The sequence of the mutated cDNA was confirmed, introduced into *E. coli* BL21 for expression, and the isolated mutant protein was analyzed by electrospray mass spectrometry.

Isolation of recombinant apoLp-III

Saturated overnight cultures of *E. coli* cells (grown in 2XYT media) harboring the apoLp-III-pET plasmid were diluted 1:50 with minimal media and grown at 37°C to a 0.5 OD_{600}. IPTG was then added to a final concentration of 2 mM and the culture was incubated at 37°C for a further 4 h. The media collected was concentrated by ultrafiltration and exhaustively dialyzed against deionized H_2O. Dialysis resulted in the appearance of a precipitate that was removed by centrifugation. The clear supernatant containing the recombinant apoLp-III was quick-frozen and lyophilized. The typical yield was 0.1 mg/ml of culture grown in minimal media. At this stage the recombinant apoLp-III preparations were electrophoretically pure. However, samples were observed to have a slight yellow coloration that interfered with fluorescence measurements. It was surmised that a host-derived small hydrophobic molecule associated with the recombinant protein. This contaminant was removed by reverse phase HPLC with a linear AB gradient of 2% B/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. The fractions were monitored at 220 nm and those containing the pure recombinant protein (as analyzed by analytical HPLC) were pooled and lyophilized.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 computer, controlled by Jasco software. The thermostatted cell holder was maintained at 25°C with a Lauda RMS circulating water bath (Lauda, Westbury, NY). The instrument was routinely calibrated with ammonium d-(+)-10 camphor sulfonate at 290.5 nm and 192 nm, and with d-(−)-pantoyllactone at 219 nm. Each sample was scanned 10 times and noise reduction was applied to remove the high frequency before calculating molar ellipticities. The voltage of the photomultiplier was kept below 500 V to prevent distortion of the CD spectrum. The cell used for the region below 250 nm was 0.02 cm (calibrated for path length). Protein concentration was between 0.5 to 1.0 mg/ml for far UV spectra. CD spectra were analyzed for secondary structure content using the Contin program (version 1.0) of Provencher and Glöckner (16). Stock protein concentrations were established in the ultracentrifuge using the Rayleigh interference optical system, assuming 41 fringes equal to 10 mg/ml (17).

Fluorescence spectroscopy

Fluorescence spectra were obtained with a Perkin-Elmer MFP-44B Spectrofluorometer, with an attached thermostatted cell holder. Temperature was controlled by a Lauda RMS water bath at 20°C, and a microprocessor differential corrected spectral unit (DSCU2). The samples were measured in a semimicro 1-cm cell, with a bandwidth of 5 nm used for both the excitation and emission monochromators. The samples were excited at 277 nm and emission was monitored at 300 nm to record either the excitation or emission spectrum with the blank mode set on the DSCU2. Fluorescence spectra of apoLp-III samples were obtained in phosphate buffer alone or in buffer containing 1% dodecylphosphocholine (Avanti Polar Lipids, Alabaster, AL).

Lipid binding assays

Routine lipoprotein binding assays (18) were conducted at 37°C in microtiter plates containing 150 μg human LDL in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM Ca^{2+} in the presence or absence of native and recombinant apoLp-III. Reactions were initiated by the addition of 400 milliunits *Bacillus cereus* phospholipase C (Boerhinger) and stopped by the addition of an aliquot of 50 mM EDTA. Absorbances were measured at 340 nm on a SLT Labinstruments microtiter plate reader at specified intervals. For phospholipid binding experiments dimyristoylphosphatidylcholine (DMPC; obtained from Sigma Chemical Co.) vesicles were prepared by bath sonication according to Wientzek et al. (10). ApoLp-III · DMPC complexes were then prepared by mixing DMPC vesicles with apoLp-III at a DMPC:apoLp-III weight ratio of 2.5:1 (67:1 molar ratio) and incubating the sample for 18 h at 24°C in buffer (0.2 M Tris, pH 7.2, 8.5% KBr, 0.01% EDTA). After incubation, the complexes were isolated by density gradient ultracentrifugation. Native PAGE analysis was performed on 4–20% acrylamide gradient slab gels run at a constant 150 V for 24 h (19). Protein standards (Pharmacia) with the following Stoke's diameters were used for calibration (20): thyroglobulin (17.0 nm), ferritin (12.2 nm), catalase (9.2 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm).

Analytical procedures

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (21) and stained with Coomassie Brilliant Blue R-250. In some cases samples separated by SDS-PAGE were electrophoretically transferred to polyvinylidene (PVDF) membrane and probed with anti apoLp-III serum (1:2,500 fold dilution) (22). A peroxidase-linked goat anti-rabbit IgG together with the chemiluminescence (ECL) reagents (Amersham) were used to detect antigen-antibody complexes. Mass spectrometric analysis was carried out on a VG Biotech (Fisons Instruments) spectrometer by platform electrospray ionization.

RESULTS AND DISCUSSION

Identification and isolation of recombinant wild type apoLp-III

The apoLp-III cDNA was cloned into the pET vector to produce a chimeric protein containing the pelB leader sequence joined in frame to apoLp-III residues 1 to 166. Immunoblots of proteins derived from *E. coli* cultures harboring the apoLp-III-pET plasmid revealed an approximate equal distribution of immunoreactive material in the cell pellet and the media. To facilitate downstream processing we focussed on the material secreted into the media. **Figure 1** shows the results of an immunoblot of culture supernatants from cells grown with the apoLp-III containing pET construct, control parent pET vector, and apoLp-III standard. The results reveal that immunoreactive material appears only in *E. coli* harboring the apoLp-III-pET plasmid, thereby verifying that the immunoreactive material is not a product of *E. coli* or the vector itself. The immunoblot also reveals the presence of a slower migrating immunoreactive band of lesser intensity. This band is much more abundant in immunoblots of the cell lysates (not shown) and we speculated that it corresponds to translated apoLp-III that still possesses the pelB leader sequence attached. This is consistent with the size difference between the bands as well as the greater abundance of this band in the cell pellet versus the culture supernatant. Subsequently, we set out to optimize the expression conditions. Preliminary results indicated that culture supernatants from cells grown in minimal media contained few contaminating proteins. An apoLp-III expression time course, monitored by SDS-PAGE, revealed that >95% of the protein present corresponded to apoLp-III (**Fig. 2**). The data showed that, after induction, there was a time-dependent increase in the amount of apoLp-III recovered in the media.

To separate recombinant apoLp-III from contaminating proteins, including unprocessed apoLp-III (with an intact pelB leader sequence), we took advantage of the solubility characteristics of the protein. Culture supernatants from induced cells were concentrated by ultrafiltration and dialyzed against deionized H_2O. After 3 days of dialysis with several changes of H_2O, a precipitate formed in the solution. The sample was then centrifuged and the pellet and supernatant were analyzed by SDS-PAGE and immunoblotting. The data revealed that while apoLp-III remained in solution, nearly all contaminating proteins,

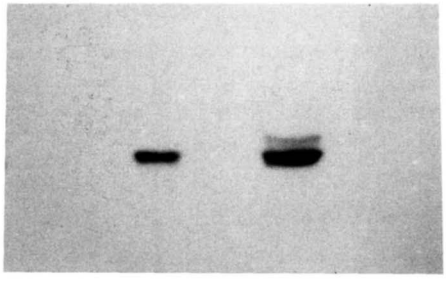

Fig. 1. Immunoblot of recombinant apoLp-III. Induced *E. coli* cell culture supernatants were separated by SDS-PAGE and electrophoretically transferred to a PVDF membrane. Non-specific sites on the membrane were blocked by incubation with 5% BSA. Incubation with anti-apoLp-III serum was followed by peroxidase-linked goat anti-rabbit IgG and detection by chemiluminescence (ECL). Lane 1, apoLp-III standard (2 μg); lane 2, culture supernatant from *E. coli* harboring the parent pET vector; lane 3, culture supernatant from *E. coli* harboring the apoLp-III-pET vector.

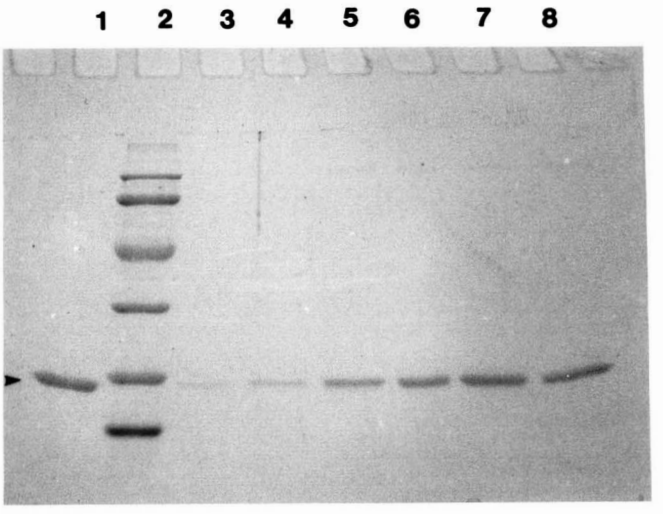

Fig. 2. Time course of recombinant apoLp-III production. *E. coli* cells harboring the apoLp-III-pET construct were induced with IPTG (2 mM). At specified times, 30 μl of culture supernatant was applied to a 15% acrylamide slab gel and electrophoresed. The gel was stained with Coomassie Brilliant Blue. Lane 1, apoLp-III standard (5 μg); lane 2, molecular weight markers; lanes 3–8, culture supernatants at times 0, 1, 2, 3, 4, and 5 h, respectively.

including the immunoreactive species of higher M_r than wild type apoLp-III, precipitated. Following this protocol, a typical 500-ml cell culture in minimal media yields approximately 50 mg purified recombinant apoLp-III.

Characterization of the recombinant protein

Electro-spray mass spectrometry of apoLp-III revealed a molecular mass = 18,379, similar to that predicted from the amino acid sequence (molecular mass = 18,380). Circular dichroism spectroscopy of recombinant apoLp-III revealed that, like natural protein isolated from *M. sexta* hemolymph, recombinant apoLp-III possesses considerable α-helical structure (**Fig. 3**). Further, comparison of CD spectra of natural and recombinant apoLp-III in the presence of 50% TFE revealed a similar relative induction of helical content. Likewise, CD aromatic spectra of native and recombinant apoLp-III revealed similar spectral properties. Provencher-Glöckner analysis of the far UV spectra indicated 65% and 69% α-helix for native and recombinant apoLp-III, respectively. An approximate 20% increase in α-helix content was observed for both native and recombinant apoLp-III in the presence of 50% trifluoroethanol. In experiments designed to determine the stability properties of natural versus recombinant apoLp-III, urea denaturation experiments were performed with the effect of increasing urea concentration monitored by CD. It was observed that above 2.5 M urea nearly all the helical structure was lost. Recombinant apoLp-III displayed a midpoint of urea-induced denaturation = 1.08 M urea while the corresponding value for native apoLp-III was 1.01 M. On the assumption that denaturation of apoLp-III is a two-state process representing a reversible equilibrium between the native and denatured states, the free energy of unfolding of recombinant and native apoLp-III were determined from the urea denaturation data (**Table 1**). The values obtained provide further support that recombinant and natural apoLp-IIIs assume similar structures.

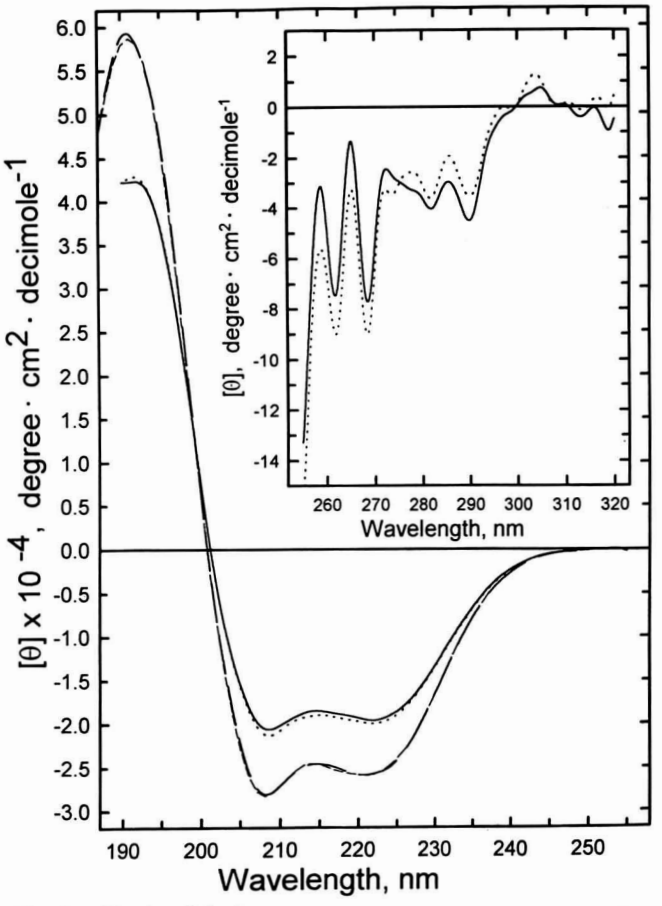

Fig. 3. Circular dichroism spectra of native and recombinant apoLp-III. Far UV (190–250 nm) spectra of recombinant (·····) and native (——) apoLp-III (0.5–1.0 mg/ml) in 50 mM phosphate, pH 7.0. Far UV spectra of recombinant (----) and native (— — —) apoLp-III were also recorded in buffer containing 50% TFE. Inset: near UV spectra of recombinant and natural apoLp-III in buffer.

TABLE 1. Thermodynamic properties for the reversible denaturation of apoLp-III

Sample	$[Urea]_{½}$[a]	$\Delta G_D H_2O$[b]
	M	*kcal/mol*
Natural apoLp-III	1.01	2.59
Recombinant apoLp-III	1.08	2.73
F148L apoLp-III	0.51	1.47

[a] $[Urea]_{½}$ is the transition midpoint, the molar concentration of urea required to give a 50% decrease in negative ellipticity at 221 nm.
[b] $\Delta G_D H_2O$ is the free energy of unfolding in the absence of urea, determined according to Pace (23).

Interaction of recombinant apoLp-III with lipid

Previous studies have revealed that *M. sexta* apoLp-III can prevent turbidity development induced by incubation of human LDL with phospholipase C (18). As this prevention is directly correlated to phospholipase C activity-dependent association of apoLp-III with the lipoprotein surface, this method provides an opportunity to examine the function of recombinant apoLp-III in lipoprotein interactions. **Figure 4** reveals that, in the absence of apoLp-III, phospholipase C induces an increase in LDL sample turbidity. However, inclusion of recombinant or natural apoLp-III prevents turbidity development, indicating that a stable binding interaction has occurred. The similar profiles obtained for the two proteins indicates that recombinant apoLp-III is functional as an apolipoprotein. The interaction of apoLp-III with lipids was further studied by characterizing protein-phospholipid complexes created upon incubation of apoLp-III with multilamellar vesicles of DMPC. It has been shown previously that *M. sexta* apoLp-III is capable of spontaneously transforming bilayer vesicles of DMPC into uniform disk-like particles (10). When wild type and recombinant apoLp-III were compared with respect to their interaction with DMPC vesicles, both created disk-like structures of comparable size, as judged by native gradient gel electrophoresis (**Fig. 5**).

Fluorescence studies

A hallmark property of *M. sexta* apoLp-III in the lipid-free, helix bundle conformation is a virtual lack of fluorescence emission from its single tyrosine residue (9). Interestingly, interaction with lipid surfaces results in a large fluorescence enhancement which is correlated with putative protein conformational changes (10). We speculated that if recombinant apoLp-III adopted the same tertiary fold as that of wild type apoLp-III, it would display similar fluorescence properties. Preliminary fluorescence studies, however, revealed the presence of a contaminating fluorophore in preparations of recombinant apoLp-III. The contaminating substance, which was apparently noncovalently associated to the protein, interfered with fluorescence measurements. To circumvent this problem, recombinant apoLp-III was subjected to reverse phase HPLC. This step allowed for separation of the contaminant permitting further spectroscopic analysis. It was found that recombinant apoLp-III underwent a 7.9-fold enhancement of tyrosine fluorescence upon interaction with dodecylphosphocholine compared to a corresponding 10.2-fold increase for the natural, insect-derived protein. The observed similar extents of tyrosine fluorescence enhancement strongly suggest that the lipid environment-induced conformational adaptability of recombinant apoLp-III is indistinguishable from that displayed by wild type apoLp-III, thereby supporting the conclusion that

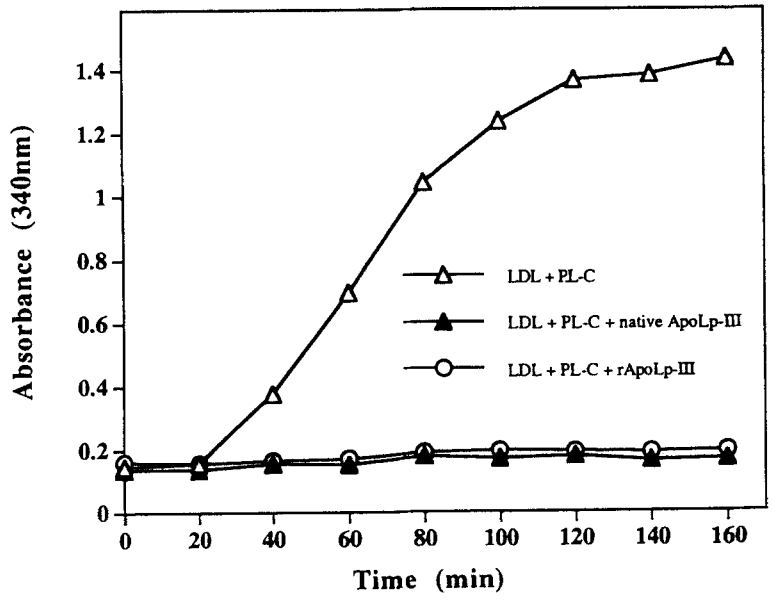

Fig. 4. The effect of apoLp-III on the stability of phospholipase C-treated human low density lipoprotein. Human LDL (100 μg protein) was incubated with phospholipase C (0.2 units) in the presence and absence of recombinant or native apoLp-III (100 μg). Incubations were carried out at 37°C in a microtiter plate and the absorbance was determined on a microtiter plate reader (340 nm) at specified intervals.

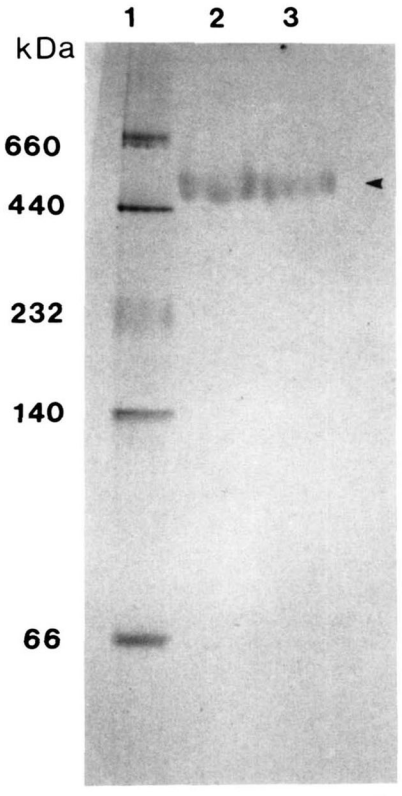

Fig. 5. Interaction of recombinant and native apoLp-III with phospholipid vesicles. Recombinant and native apoLp-III were incubated with DMPC vesicles at a molar ratio of 67:1 for 18 h at 24°C. After incubation the sample was subjected to density gradient ultracentrifugation, fractionated, and analyzed for protein. Protein-containing fractions were subjected to electrophoresis on a 4–20% acrylamide gradient slab gel for 27 h at 4°C. The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular weight standards; lane 2, native apoLp-III · DMPC complexes; lane 3, recombinant apoLp-III · DMPC complexes.

recombinant apoLp-III adopts a helix fold similar to that of the natural protein.

F148L mutant apoLp-III

An important question with regard to the fluorescence properties of apoLp-III centers on the structural reason for the observed quench in the lipid-free, helix bundle conformation. Previously, we postulated that Phe[148] may play a role in quenching of Tyr[145] in an α-helical conformation as the aromatic side chains of these residues could conceivably orient to form a hydrophobic stacking interaction. Such an interaction could stabilize the orientation of Tyr[145] toward the putative quenching moiety resident on a separate helix in the five helix bundle (10, 24). To address the potential role of Phe[148] in the observed tyrosine fluorescence quenching of lipid free apoLp-III, this residue was mutated to Leu by oligonucleotide directed site specific mutagenesis. The mutant protein was purified from induced *E. coli* cultures and characterized. Electrospray mass spectrometry of the mutant apoLp-III revealed a molecular mass of 18,349, which corresponds closely to that predicted for the specific change made in the protein sequence (18,347). Further confirmation that the mutation has been introduced was obtained by DNA sequencing. Characterization of the mutant protein by CD spectroscopy indicated a high α-helix content, similar to that observed for the native protein. To assess the effect of the mutation on the overall stability of apoLp-III, denaturation studies were performed. The data revealed that F148L apoLp-III displayed a urea-induced denaturation midpoint and $\Delta G_D H_2O$ that were considerably lower than the corresponding values for the natural insect-derived protein or recombinant wild type apoLp-III (Table 1). These data suggest that this mutation has produced an alteration in the tertiary organization of the protein that is reflected in a decreased inherent stability. In addition, fluorescence studies revealed that lipid-free F148L apoLp-III displays a tyrosine quantum yield that is considerably higher than that observed for wild type apoLp-III (0.055 versus 0.015). The data suggest that Phe[148] may play a structural role in maintaining the helix bundle conformation of apoLp-III. It is conceivable that the aromatic side chain of Phe[148] may interact with other residues in the helix bundle, including Tyr[145], through aromatic-aromatic interactions (25), stabilizing a protein conformation that maintains Tyr[145] in an orientation optimal for interaction with the quenching moiety. Based on these considerations it is not possible to draw conclusions about the putative role of Phe[148] in the fluorescence properties of apoLp-III. Further site-directed mutagenesis studies using the expression system described in this report will be directed at understanding the role of specific amino acids in maintenance of helix bundle stability as well as contributions to the unusual fluorescence properties displayed by this protein.

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REFERENCES

1. Ryan, R. O. 1990. Dynamics of insect lipophorin metabolism. *J. Lipid Res.* **31:** 1725–1739.
2. Breiter, D. R., M. R. Kanost, M. M. Benning, G. Wesenberg, J. H. Law, M. A. Wells, I. Rayment, and H. M. Holden. 1991. Molecular structure of an apolipoprotein determined at 2.5-Å resolution. *Biochemistry.* **30:** 603–608.
3. Weers, P. M. M., C. M. Kay, K. Oikawa, M. Wientzek,

D. J. Van der Horst, and R. O. Ryan. 1994. Factors affecting the stability and conformation of *Locusta migratoria* apolipophorin III. *Biochemistry.* **33:** 3617–3624.
4. Kanost, M. R., M. S. Boguski, M. Freeman, J. I. Gordon, G. R. Wyatt, and M. A. Wells. 1988. Primary structure of apolipophorin-III from the migratory locust, *Locusta migratoria. J. Biol. Chem.* **263:** 10568–10573.
5. Ryan, R. O., R. Ziegler, D. J. Van der Horst, and J. H. Law. 1990. Characterization of apolipophorin III from *Barytettix psolus* and *Melanoplus differentialis. Insect Biochem.* **20:** 127–133.
6. Hård, K., J. M. Van Doorn, J. E. Thomas-Oates, J. P. Kamerling, and D. J. Van der Horst. 1993. Structure of the Asn-linked oligosaccharides of apolipophorin III from the insect *Locusta migratoria.* Carbohydrate-linked 2-aminoethylphosphonate as a constituent of a glycoprotein. *Biochemistry.* **32:** 766–775.
7. Van der Horst, D. J., R. O. Ryan, M. C. Van Heusden, T. K. F. Schulz, J. M. Van Doorn, J. H. Law, and A. M. T. Beenakkers. 1988. An insect lipoprotein hybrid helps to define the role of apolipophorin III. *J. Biol. Chem.* **263:** 2027–2033.
8. Kawooya, J. K., S. C. Meredith, M. A. Wells, F. J. Kézdy, and J. H. Law. 1986. Physical and surface properties of insect apolipophorin III. *J. Biol. Chem.* **261:** 13588–13591.
9. Ryan, R. O., K. Oikawa, and C. Kay. 1993. Conformational, thermodynamic and stability properties of *Manduca sexta* apolipophorin III. *J. Biol. Chem.* **268:** 1525–1530.
10. Wientzek, M., C. M. Kay, K. Oikawa, and R. O. Ryan. 1994. Binding of insect apolipophorin III to dimyristoylphosphatidylcholine vesicles: evidence for a conformational change. *J. Biol. Chem.* **269:** 4605–4612.
11. Wells, M. A., R. O. Ryan, S. V. Prasad, and J. H. Law. 1985. A novel procedure for the purification of apolipophorin III. *Insect Biochem.* **15:** 565–571.
12. Schumaker, V. N., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
13. Cole, K. D., G. J. P. Fernando-Warnakulasuriya, M. S. Boguski, M. Freeman, J. I. Gordon, W. A. Clark, J. H. Law, and M. A. Wells. 1987. Primary structure and comparative sequence analysis of an insect apolipoprotein: apolipophorin III from *Manduca sexta. J. Biol. Chem.* **262:** 11794–11800.
14. Price, H. M., R. O. Ryan, and N. H. Haunerland. 1992. Primary structure of locust flight muscle fatty acid binding protein. *Arch. Biochem. Biophys.* **297:** 285–290.
15. Kunkel, T. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* **82:** 488–492.
16. Provencher, S. W., and J. Glöckner. 1981. Estimation of globular protein secondary structure from circular diochroism. *Biochemistry.* **20:** 33–37.
17. Babul, G., and E. Stellwagen. 1969. Measurement of protein concentration with interference optics. *Anal. Biochem.* **28:** 216–221.
18. Liu, H., D. G. Scraba, and R. O. Ryan. 1993. Prevention of phospholipase-C induced aggregation of low density lipoprotein by amphipathic apolipoproteins. *FEBS Lett.* **316:** 27–33.
19. Ryan, R. O., P. S. Keim, M. A. Wells, and J. H. Law. 1985. Purification and properties of a predominantly female specific protein from the hemolymph of the larva of the tobacco hornworm, *Manduca sexta. J. Biol. Chem.* **260:** 782–787.
20. Nichols, A. V., R. M. Krauss, and T. A. Muslinger. 1986. Non-denaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol.* **128:** 417–431.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
22. Kawooya, J. K., P. S. Keim, R. O. Ryan, J. P. Shapiro, P. Samaraweera, and J. H. Law. 1984. Insect apolipophorin III. Purification and properties. *J. Biol. Chem.* **259:** 10733–10737.
23. Pace, C. N. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Methods Enzymol.* **131:** 266–279.
24. Narayanaswami, V., C. M. Kay, K. Oikawa, and R. O. Ryan. 1994. Structural and binding characteristics of the carboxyl terminal fragment of apolipophorin III from *Manduca sexta. Biochemistry.* **33:** 13312–13320.
25. Burley, S. K., and G. A. Petsko. 1985. Aromatic-aromatic interaction: a mechanism of protein structure stabilization. *Science.* **229:** 23–28.