Disulfide Bond Engineering to Monitor Conformational Opening of Apolipopophorin III During Lipid Binding*

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Apolipopohrin III (apoLp-III) from the Sphinx moth, Manduca sexta, is an exchangeable, amphipathic apolipoprotein that alternately exists in water-soluble and lipid-bound forms. It is organized as a five-helix bundle in solution, which has been postulated to open at putative hinge domains to expose the hydrophobic interior, thereby facilitating interaction with the lipoprotein surface (Breiter, D. R., Kanost, M. R., Benning, M. M., Wessenberg, G., Law, J. H., Wells, M. A., Raymond, J. L., and Holden, H. M. (1991) Biochemistry 30, 603–608). To test this hypothesis, we engineered two cysteine residues in apoLp-III, which otherwise lacks cysteine, by site-directed mutagenesis at Asn-40 and Leu-90. Under oxidizing conditions the two cysteines spontaneously form a disulfide bond, which should tether the helix bundle and thereby prevent opening and concomitant lipid interaction. N40C/L90C apoLp-III was overexpressed in Escherichia coli and characterized for disulfide bond formation, secondary structure content, and stability, under both oxidizing and reducing conditions. Functional characterization was carried out by comparing the abilities of the oxidized and reduced protein to associate with modified lipoproteins in vitro. While the reduced form behaved like wild type apoLp-III, the oxidized form was unable to associate with lipoproteins. These results suggest that opening of the helix bundle is required for interaction with lipoproteins and provide a molecular basis for the dual existence of water-soluble and lipid-bound forms of apoLp-III. However, in phospholipid bilayer association assays, wild type, reduced, and oxidized N40C/L90C apoLp-III exhibited similar abilities to transform dimeristoylphosphatidylcholine multilamellar vesicles to disc-like complexes, as judged by electron microscopy. These data emphasize that underlying differences exist in initiating or maintaining a stable interaction of apoLp-III with phospholipid disc complexes versus spherical lipoprotein surfaces.

Exchangeable apolipoproteins belong to a class of amphipathic α-helical proteins that regulate the metabolism and dynamics of lipoprotein interconversions. These proteins reversibly associate with the surface of lipoprotein particles in response to hydrophobic surface availability or their intrinsic ability to displace pre-existing apolipoproteins. This functional property implies an ability to exist in both lipid-free and lipid-bound forms in plasma, and it has been proposed that a dramatic conformational change is required for initiation and maintenance of interaction with lipid surfaces (Breiter et al., 1991; Weisgraber, 1994). The sole exchangeable apolipoprotein found in insect hemolymph, apolipopohrin III (apoLp-III),1 provides an excellent model to study lipid association-induced conformational changes of amphipathic exchangeable apolipoproteins. ApoLp-III is well characterized in terms of physicochemical and functional properties (see Van der Horst, 1990; Blacklock and Ryan, 1994; Soulasses and Wells, 1994, for reviews). Structural information at 2.5-Å resolution is available for apoLp-III from Locusta migratoria (Breiter et al., 1991) and represents the only x-ray structure of a full-length apolipoprotein available. ApoLp-III is readily isolated in large quantities for structural studies, and an efficient bacterial expression system is available for production of recombinant Manduca sexta apoLp-III (Ryan et al., 1995), which shares a high degree of structural and functional homology with apoLp-III from L. migratoria (Van der Horst et al., 1988; Smith et al., 1994). In resting insects, apoLp-III is found as a monomeric lipid-free hemolymph protein that is recruited onto lipophorin particles upon adipokinetic hormone-induced enhancement in particle diacylglycerol content (Beenakkers et al., 1985; Wells et al., 1987). The stabilizing effect of apoLp-III likely facilitates further diacylglycerol loading of lipophorin particles for delivery to muscle tissues during sustained flight (Van der Horst, 1990; Blacklock and Ryan, 1994).

The crystal structure of L. migratoria apoLp-III (Breiter et al., 1991) reveals a compact, globular five-helix bundle, made up of long, anti-parallel amphipathic α-helices connected by short loops. The hydrophobic faces of the helices are oriented toward the interior of the molecule, whereas hydrophilic side chains are directed toward the aqueous environment. Amphipathic α-helices in apoLp-III appear to be the fundamental structural motif required for lipid association. There is considerable similarity between the tertiary structural organization of apoLp-III and the 22-kDa N-terminal fragment of human apolipoprotein E (apoE), the x-ray structure of which has also been elucidated (Wilson et al., 1991).

In order to explain the lipid-binding capability of apoLp-III, it was postulated that the five-helix bundle undergoes a lipid-triggered opening (Fig. 1) at putative “hinge domains” located between helices 2 and 3 and between helices 4 and 5, thereby exposing the elongated contiguous hydrophobic interior that

1 The abbreviations used are: apoLp-III, apolipopohrin-III; apoE, apolipoprotein E; DAG, diacylglycerol; DMPC, dimeristoylphosphatidylcholine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; HDLp, high density lipophorin; LDL, low density lipoprotein; LTP, lipid transport particle; PAGE, polyacrylamide gel electrophoresis; PL-C, phospholipase C; HPLC, high pressure liquid chromatography; WT, wild type.
facilitates lipid interaction (Breiter et al., 1991). Evidence in support of hinge opening has been obtained from apoLp-III monolayer studies at the air-water interface (Kawooya et al., 1986) and surface plasmon resonance spectroscopy (Soulages et al., 1995), although the location of the hinge domain(s) and the mode of protein-lipid interaction remain unknown. We report here studies designed to test the validity of the hinge opening hypothesis by using a strategy that tethers the helix bundle by means of a disulfide bond and is hypothesized to prevent its proposed opening during lipoprotein interaction (Breiter et al., 1991). Site-directed introduction of non-native intramolecular disulfide bonds has proven to be a valuable tool for obtaining information about protein conformational changes (Duchêne et al., 1994; Chang and Cronan, 1995), folding (Cardamone et al., 1995), stability (Wetzel et al., 1988; Matsumara et al., 1989), and proximity relationships (Wolf-Lang et al., 1985). We have introduced two cysteines into M. sexta apoLp-III (which otherwise lacks cysteine) by site-directed mutagenesis at strategic locations postulated to facilitate and maximize the probability of intramolecular disulfide bond formation. Disulfide-linked apoLp-III was unable to associate with modified lipoprotein surfaces, but this ability was restored upon treatment with reducing agents.

The implications of such lipid-triggered hinge opening of exchangeable apolipoproteins in the dynamics of lipoprotein interconversions are discussed. Our results also underscore fundamental differences in the mode of interaction of apolipoproteins with lipoprotein surfaces versus phospholipid bilayers, the latter being the commonly used assay system for studying apolipoprotein/lipid interaction.

EXPERIMENTAL PROCEDURES

Materials—Dimyristoylphosphatidylcholine, dithiothreitol, and phospholipase C from Bacillus cereus were purchased from Sigma; HinIII was from USB (Cleveland, OH); XbaI from Life Technologies, Inc.; AflII from New England Biolabs (Beverly, MA); Taq DNA polymerase from Promega (Madison, WI); dNTPs from Pharmacia (Uppsala, Sweden); TA Cloning Kit from Invitrogen (San Diego, CA), pET expression vector from Novagen (Madison, WI); Prep-A-Gene matrix from Bio-Rad; and isopropyl-β-thiogalactopyranoside from Chemica Alfa (Edmonton, Canada). All other chemicals and media were reagent grade.

Disulfide Bond Engineering—Potential locations for site-specific introduction of cysteine residues were identified based on a structure of M. sexta apoLp-III generated by homology model building. The modeling strategy utilized the sequence alignment of apoLp-IIIIs from four different insect species (Cole et al., 1987; Smith et al., 1994; Narayanaswami et al., 1995) and the x-ray crystal coordinates of L. migratoria apoLp-III (Breiter et al., 1991). M. sexta apoLp-III is proposed to have a five-helix bundle architecture similar to that of L. migratoria apoLp-III, with small differences in the locations of each of the helices. The model allowed us to identify the spatial locations for possible disulfide sites and to measure the distance (Cβ-Cβ) and dihedral angles (γ) between them. Asn-40 and Leu-90, predicted to be located in (or very close to) the loops between helices 1 and 2 and between helices 3 and 4, respectively, were identified as candidates for point mutation. Upon mutagenesis to cysteine (N40C and L90C) and subsequent disulfide bond formation, it was predicted that the helix bundle would be tethered and unable to open (Fig. 1).

Site-directed Mutagenesis—The first mutation, N40C, was introduced by cassette mutagenesis using oligonucleotide-directed DNA amplification with mismatched primers (Cass et al., 1988) as template (Ryan et al., 1995). The amplified segment was subcloned into a pCR™II vector using the TA Cloning Kit (Invitrogen), digested with XbaI and AflII, isolated using the Prep-A-Gene matrix, and ligated into the wild type (WT) apoLp-III/pET vector cut with the same restriction enzymes and then transformed into E. coli BL21 cells. The second mutation, L90C, was introduced by the recombinant polymerase chain reaction protocol (Higuchi et al., 1988) using mismatched primers bearing the L90C mutation. The amplified product was treated as described above, except in the final step, where the pCR™II was digested with AflII and HinIII and the fragment bearing the L90C mutation was ligated into the apoLp-III/pET vector containing the N40C mutation, and transformed into E. coli. The resulting plasmid, N40C/L90C apoLp-III/pET, was sequenced by the dideoxynucleotide chain termination method on the double-stranded template (Sanger et al., 1977). Sequencing confirmed the presence of the two desired mutations and further revealed a CAC → CGC mutation that introduced an amino acid substitution from His-94 to Arg, located in a flexible loop region between helices 3 and 4. Since this mutation was present in both the oxidized and reduced forms of the expressed protein, any differences observed between the two were solely due to the oxidized or reduced state of the disulfide bond.

Protein Expression, Purification, and Characterization—N40C/L90C apoLp-III was expressed by E. coli cultured in M9 minimal medium (Sambrook et al., 1989) supplemented with 2 mM MgSO4 and 0.1 mM CaCl2, followed by induction with 1 mM isopropyl-1-thio-β-galactopyranoside (Ryan et al., 1995). The medium containing the expressed protein was concentrated about 20-fold by ultrafiltration, dialyzed against deionized water, and lyophilized. ApoLp-III is the only major protein that accumulates in the medium, and typical yields of N40C/L90C apoLp-III were approximately 150 mg/liter culture medium.

The protein was purified on a preparative reversed-phase HPLC column (Zorbax) with a linear AB gradient of 0.25% B/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions containing the pure protein were pooled after assessing the purity on an analytical reversed phase C-8 HPLC column (Zorbax, 2.1 × 15 mm) using a linear gradient of 2% B/min.

Disulfide Bond Formation—Recombinant N40C/L90C apoLp-III contained no free sulfhydryl groups, as judged by the lack of reactivity of both native and denatured protein toward 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959), suggesting the existence of disulfide bonded cysteines. Reduction of the oxidized form was accomplished by treatment with dithiothreitol (DTT) at a 100-fold molar excess for 2 h at 37 °C. Reduction of the disulfide bond in N40C/L90C apoLp-III was deduced by a shift in retention time upon reversed-phase HPLC (Matsumara and Matthews, 1989) and by a shift in mobility upon SDS-PAGE (Scheele and Jacoby, 1982) under reducing and nonreducing conditions.

Structural Characterization—Circular dichroism (CD) spectroscopy was used to determine the secondary structural characteristics of N40C/L90C apoLp-III in the oxidized and reduced states, and these data were compared with those obtained for WT apoLp-III in both 0.1 mM phosphate buffer (pH 7.0) and in 50% trifluoroethanol (Weintae et al.,...
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1994). Samples of the oxidized and reduced forms of N40C/L90C apoLP-III, as well as WT apoLP-III, were treated with varying concentrations of guanidine hydrochloride (GdnHCl) and left overnight. The ellipticity ([θ]) in deg cm$^2$ dmol$^{-1}$ was measured at 222 nm. Temperature denaturation was carried out by monitoring the ellipticity at 222 nm as a function of varying temperatures between 5 and 80 °C in a thermostat cell holder. The reversibility of the denaturation process was evaluated by cooling to 25 °C samples that were heated to 80 °C. CD aro-
matic spectra were recorded between 250 and 320 nm.

**Lipoprotein Binding Assays**—The functional competence of N40C/L90C apoLP-III was evaluated by comparing the relative abilities of the oxidized and reduced forms to interact with lipoprotein particles in two independent lipoprotein interaction assays. The first assay involved incubation of human low density lipoprotein (LDL) with phospholipid vesicles containing N40C/L90C apoLP-III (Ryan et al., 1995). LDL was isolated from fresh human plasma by sequential dens-
ity ultracentrifugation (Schumaker and Puppione, 1986). The assay medium consisted of 35 μg of human LDL protein in 50 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and 2 mM CaCl$_2$ in a final volume of 250 μl and was carried out at 37 °C with absorbance measured at 340 nm at the indicated times. The reaction was initiated by the addition of 180 milliunits of PL-C, which hydrolyzes the polar head groups of LDL phosphatidylincholine, yielding DAG. Control incubations, lacking added apolipoproteins or PL-C, as well as incubations with WT apoLP-III (100 μg) were included. Reduced N40C/L90C apoLP-III was prepared by treating the oxidized form with a 100-fold molar excess of DTt for 2 h at 37 °C, followed by dialysis against buffer containing a 2-fold molar excess of DTt. To control experiments, under the conditions employed, DTt alone had no discernible effect on the assay. Assays were performed in triplicate with three different preparations of the protein.

The second assay was based on the ability of M. sexta lipid transfer protein (LTP) to mediate transfer of diacylglycerol from insect high density lipoprotein (HDLp) to human LDL (Ryan et al., 1990; Singh et al., 1992). LTP was isolated from larval hemolymph according to the procedure of Ryan et al. (1988). The assay medium consisted of HDLp and LDL in a ratio of 5:1 (250 μg of HDLp protein and 50 μg of LDL protein) in phosphate-buffered saline (0.1 mM sodium phosphate (pH 7.0), 0.15 mM NaCl, 5 mM EDTA). Assays performed in the presence of 75 μg of WT apoLP-III served as a positive control and were compared directly with oxidized and reduced N40C/L90C apoLP-III. DTt had no effect on LTP activity nor did it alter the results of experiments with WT apoLP-III. The reaction was initiated by the addition of 2 μg of LTP, incubated at 37 °C for the indicated times, and the absorbance measured at 340 nm.

**Interaction with Dimyristoylphosphatidylcholine Bilayers**—The ability of oxidized and reduced forms of N40C/L90C apoLP-III to transform pre-formed multilamellar vesicular structures to disc-like complexes was determined by right angle scattering measurements, native PAGE, and electron microscopy. DMPC vesicles were prepared as described previously (Wientzek et al., 1994) and incubated with WT, oxidized, or reduced N40C/L90C apoLP-III (lipid/protein ratio of 2.5:1, w/w) at 24 °C for 18 h. Right angle scattering measurements were carried out in triplicate on a spectrophotometer by setting the excitation and emission at 400 nm and determining the light scattering of a portion of the sample (Epand et al., 1987). Values were expressed as fraction of control DMPC vesicles alone with no added apolipoprotein. Native PAGE of the different samples was performed at 150 V for 24 h at 4 °C, followed by staining with Amido Black 10B. Electron micros-
copy was performed in a Philips EM420 as described earlier (Wientzek et al., 1994).

**RESULTS**

**Evaluation of Disulfide Bond Formation and Structural Characterization**

Disulfide bond formation was evaluated by subjecting WT and N40C/L90C apoLP-III to 18% SDS-PAGE under reducing and nonreducing conditions (Fig. 2, panel A). Under nonreducing conditions N40C/L90C apoLP-III has an increased mobility compared with the WT protein, whereas under reducing conditions its mobility corresponds directly to that of WT apoLP-III. Under nonreducing conditions no evidence of dimer forma-
tion was observed, indicating that intermolecular disulfide bond formation does not occur. Intramolecular disulfide bond formation was confirmed by analytical reversed-phase HPLC. The HPLC profiles (Fig. 2, panel B) of the WT and N40C/L90C apoLP-III, under oxidizing and reducing conditions, reveal a 2.5-min decrease in retention time for oxidized N40C/L90C apoLP-III versus that of its reduced counterpart or WT apoLP-III. Evidence for disulfide bond formation in N40C/L90C apoLP-III under oxidizing conditions was also shown by the lack of reactivity with DTNB under native and denaturing conditions (data not shown) indicating that there were no free sulphydrys in the protein. Near UV CD spectra of N40C/L90C apoLP-III (Fig. 3, inset) revealed a relatively flexible microen-
vironment of the 8 Phe and 1 Tyr residues (all located on the hydrophobic face of the 5 amphipathic α-helices). In the ox-
dized state, the reduction in ellipticity at 304 nm relative to the reduced protein may be assigned to the disulfide bond (Jirgensons, 1976). Also, in the oxidized disulfide bonded state, where the protein is less flexible, a decreased mobility in the side chains of aromatic residues is observed, reflected by an increase in negative ellipticity between 250 and 280 nm (Bailey et al., 1982). All of these criteria confirm the spatial proximity of N40C and L90C and their ability to form a disulfide bond.

Secondary structure characterization was performed by far UV CD measurements of N40C/L90C apoLP-III in buffer and in the presence of 50% trifluoroethanol, a solvent-induced agent (Fig. 3). In buffer, the extent of α-helical structure for both the oxidized and reduced forms of the protein was similar to that of WT apoLP-III (−60% α-helix). Likewise, there was a similar induction of α-helical content upon addition of trifluoroethanol, as observed for WT apoLP-III (−20%) (Ryan et al., 1993). Thus, the CD data indicate that neither the point mutations nor the formation of a disulfide bond alter the α-helical content or the overall folding of the helix bundle. In order to assess the effect of the two substitutions on protein stability, denaturation studies were carried out using guanidine hydrochloride (GdnHCl) (Fig. 4). The ellipticity at 222 nm, reflecting the α-helicity of the protein, was followed as a function of increasing GdnHCl concentration. Transition midpoints (i.e. the concentration of GdnHCl required to give a 50% decrease in ellipticity at 222 nm) were determined for WT apoLP-III as well as oxidized and reduced N40C/L90C apoLP-III. Consistent with the known lability of exchangeable amphiphilic apolipoproteins (Edelstein and Scania, 1980; Weinberg and Spector, 1985), the observed transition midpoints of reduced and oxidized N40C/L90C apoLP-III were 0.25 and 0.43 M GdnHCl, respectively. These values are comparable with those of 0.36 M for WT M. sexta apoLP-III (Ryan et al., 1993) and 0.6 M for L. migratoriaapoLP-III (Weers et al., 1994) and suggest that no major structural perturbations in N40C/L90C apoLP-III resulted from the mutations and disulfide bond formation.

**Effect of Disulfide Bond Formation on Lipid Binding**

**Interaction with PL-C-treated Lipoprotein Surfaces**—This assay measures the ability of exchangeable apolipoproteins to associate with human LDL particles that have been treated with PL-C. PL-C hydrolyzes the polar head groups of phos-
phatidylcholine moieties resulting in the exposure of product DAG on the lipoprotein surface, which in turn causes particle aggregation (Suits et al., 1989). Aggregation results in sample turbidity development that can be monitored at 340 nm as a function of time. Exchangeable apolipoproteins are able to prevent this aggregation by associating with the lipolyzed surface (Liu et al., 1993; Singh et al., 1994). The ability of oxidized N40C/L90C apoLP-III to interact with the lipoprotein surface was evaluated in comparison with the reduced form and WT apoLP-III under oxidizing and reducing conditions (Fig. 5). In control experiments, the onset of turbidity was prevented by the presence of WT apoLP-III. Reduced N40C/L90C apoLP-III also had the ability to stabilize the lipolyzed particle. However, oxidized disulfide bonded apoLP-III
was unable to protect against aggregation, reflecting its inability to bind to the lipoprotein surface.

**Interaction with a Swelling Lipoprotein Particle**—In the aforementioned system, apoLp-III binds to the lipoprotein surface as phospholipid is converted enzymatically to DAG, whereas the core of the lipoprotein particle remains the same. In an independent apolipoprotein binding assay, isolated human LDL was co-incubated with high density lipophorin (HDLp) in the presence of catalytic amounts of LTP, which facilitates net vectorial transfer of DAG from HDLp to LDL (Ryan *et al.*. 1990; Singh *et al.*. 1992). DAG enrichment results in an increased LDL particle size, which is accompanied by surface exposure of DAG. This, in turn, causes irreversible aggregation of LDL that can be monitored turbidimetrically. The aggregation phenomenon and subsequent turbidity development, however, are prevented when apoLp-III or other exchangeable amphipathic apolipoproteins are present in the incubation due to the formation of a stable binding interaction with the modified lipoprotein (Singh *et al.*. 1992). We have employed this assay to evaluate the relative binding capabilities of the disulfide bonded and reduced N40C/L90C apoLp-III compared with WT apoLp-III (Fig. 6). WT apoLp-III prevented aggregation of LDL as seen by the low turbidity level over the 3-h time period. In the presence of oxidized disulfide bonded N40C/L90C apoLp-III aggregation of LDL was not prevented, reflecting the inability of the tethered protein to bind to lipid-enriched LDL. By contrast, reduced N40C/L90C apoLp-III afforded protection from aggregation in a manner that was indistinguishable from that of WT apoLp-III.

**Interaction with Phospholipid Bilayer Vesicles**—To determine the effect of disulfide bond formation on the ability of apoLp-III to interact with phospholipid bilayer vesicles, the oxidized and reduced forms of N40C/L90C apoLp-III were tested for their ability to transform bilayer vesicles into disc-like structures. This is a well-documented characteristic of exchangeable apolipoproteins (Segrest *et al.*. 1994), although

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**Fig. 2. Disulfide bond formation in N40C/L90C apoLp-III.** Panel A, SDS-PAGE of WT and N40C/L90C apoLp-III under reducing (lanes 1 and 2) and oxidizing (lanes 4 and 5) conditions. Lane 1, WT apoLp-III; lane 2, N40C/L90C-apoLp-III. Lane 4, WT apoLp-III; lane 5, N40C/L90C-apoLp-III. Lanes 3 and 6, molecular mass standards. Panel B, reversed-phase HPLC trace of oxidized (dashed line) and reduced (solid line) N40C/L90C-apoLp-III. The samples were separated on a Zorbax C-8 column eluted at 0.25 ml/min with a linear AB gradient of 2% B/min, where A is 0.05% aqueous trifluoroacetic acid and B is acetonitrile containing 0.05% trifluoroacetic acid.
there are subtle variations in the sizes of the discs and preference for certain phospholipids. When WT apoLp-III reacts with DMPC vesicles it transforms them into disc-like complexes (Fig. 7, panel A), with a stoichiometry of about 6 molecules of apoLp-III per disc (Wientzek et al., 1994). Surprisingly, both oxidized (panel B) and reduced (panel C) N40C/L90C apoLp-III displayed an ability to transform DMPC vesicles into discs, indicating association of both proteins with DMPC. Panel D shows the vesicular structure of DMPC starting material. The transformation of vesicular to disc-like structures is also accompanied by a reduction of suspension turbidity which can be followed by right angle light scattering. In comparison to the light scattering caused by DMPC vesicles alone, DMPC-WT apoLp-III complexes decreased the scatter by about 80% (data not shown). Both oxidized and reduced N40C/L90C apoLp-III caused a similar decrease, indicative of complex formation with DMPC. Furthermore, native gradient PAGE revealed that the sizes of the disc complexes formed from DMPC-oxidized N40C/L90C apoLp-III were about the same as those of the DMPC-WT apoLp-III (data not shown). In order to confirm that during the process of disc formation the oxidized protein did not undergo a reduction and thereby attain the capacity to undergo an opening, SDS-PAGE of the disc complexes was performed. This revealed the shift in mobility characteristic of disulfide bond formation. Finally, GdnHCl denaturation of DMPC/N40C/L90C-oxidized apoLp-III complexes could be fit to two separate sigmoid curves, corresponding to mid-points of denaturation of about 1.7 and 4.1 m GdnHCl, which corresponds well to that displayed by DMPC-WT apoLp-III complexes, 2.2 and 3.7 m (Wientzek et al., 1994). This indicates that the nature of the protein-lipid interaction is similar for WT and N40C/L90C apoLp-III.

**DISCUSSION**

Insect apoLp-III provides an excellent model system for exploring the molecular basis of the interactions of water-soluble exchangeable apolipoproteins with lipid surfaces. Lipid binding represents a common functional property shared by amphipathic exchangeable apolipoproteins. Physiologically, apoLp-III exists in equimolar N40C/L90C apoLp-III and (up)apoLp-III-bound forms, as shown by biochemical and functional studies with the two best characterized homologues, *L. migratoria* and *M. sexta* apoLp-III (Van der Horst et al., 1988; Smith et al., 1994). Based on sequence alignment and the X-ray crystal structure of *L. migratoria* apoLp-III, a model structure of *M. sexta* apoLp-III was generated and used in the design of site-specific mutants of *M. sexta* apoLp-III. Unlike *L. migratoria* apoLp-III, the *M. sexta* protein is nonglycosylated and has been overexpressed as a recombinant in *E. coli* (Ryan et al., 1995). The engineered mutations were designed to evaluate the proposed lipid-triggered conformational opening of the protein and other aspects of protein-lipid interaction.

**Conformational Opening**—The initial concept of a lipid association-dependent conformational change in apoLp-III was based on experiments using surface monolayer techniques to study the behavior of *M. sexta* apoLp-III at an air/water interface (Kawooya et al., 1986). These experiments showed that apoLp-III forms a monolayer that can be alternately compressed and expanded. ApoLp-III occupies an area of about 4000 Å² at low surface pressures, which corresponds well with the value (4300 Å²) obtained from adsorption isotherms for binding of apoLp-III to phospholipid and DAG-coated beads. Upon compression, the protein occupies a molecular area of only about 480 Å², suggesting that a significant conformational change has taken place during the transition between these two states. With the determination of the atomic structure of *L. migratoria* apoLp-III in the lipid-free state (Breiter et al., 1991), a postulate was put forward to explain the basis of lipid interaction of this highly water-soluble, monomeric protein. As mentioned earlier, apoLp-III exists as a globular five-helix bundle in the lipid-free state, wherein the hydrophilic residues on each of the amphipathic helices are oriented toward the aqueous surface, and the hydrophobic side chains are oriented inwards. It was suggested that apoLp-III undergoes a conformational change involving a 180° opening at putative hinge domains between helices 2 and 3 and between helices 4 and 5, to expose the hydrophobic interior and facilitate lipid interaction. Because this conformational transition would involve large changes in the interhelical distances, we have devised a strategy whereby the helices can be tethered by means of a reversible intramolecular disulfide bond (Fig. 1).

**Disulfide Bond Formation**—Intramolecular disulfide bond formation occurs spontaneously in bacterially expressed recombinant N40C/L90C apoLp-III. Evidence for disulfide bond formation was shown by the following criteria: (i) increased mobility of oxidized N40C/L90C apoLp-III when compared with its reduced counterpart or WT apoLp-III, by SDS-PAGE; (ii) decreased retention time on reversed-phase HPLC for the oxidized N40C/L90C apoLp-III (26 min), compared with reduced N40C/L90C and WT apoLp-III (28.5 min); (iii) lack of reactivity of oxidized N40C/L90C apoLp-III and (iv) shift in aromatic CD spectrum of the oxidized N40C/L90C apoLp-III toward a more negative ellipticity indicative of a less flexible environment for the aromatic residues in the protein interior.
due to disulfide bond formation (Bailey et al., 1982; Jirgensons, 1976). Furthermore, GdnHCl denaturation profiles indicate that the transition midpoint of all the three variants is \( 0.3 \, M \), suggesting that the substitution mutations did not cause any major structural changes, an observation that is consistent with the CD data. It was also noted from the latter experiments that introduction of the disulfide bond in apoLp-III does not impart a significantly greater stability to this protein, in contrast to the known stabilizing effects of disulfide bonds in some other systems (Wetzel et al., 1988; Matsumara et al., 1989).

Finally, disulfide bond formation in N40C/L90C apoLp-III attests to the accuracy of the predicted locations of Asn-40 and Leu-90 and thereby the general accuracy of the model structure.

**Functional Inactivation of Disulfide Bonded ApoLp-III**—By introducing a disulfide cross-link to hold the helix bundle together, the functional ability of apoLp-III to associate with lipoprotein surfaces was abolished. This loss of function, confirmed by two independent lipoprotein binding assays, provides direct experimental evidence that conformational opening of the helix bundle is a necessary step for initiation or stable interaction with lipoprotein surfaces. These results also substantiate the existence of alternative compact, globular lipid-free and elongated, open lipid-bound conformations for apoLp-III. This concept of conformational hinge opening of α-helix bundles to expose a buried, hydrophobic interior and facilitate lipid interaction can possibly be extended to other exchangeable apolipoproteins such as apoE (Weisgraber, 1994). The reversible binding to lipoprotein surfaces appears to be the common underlying feature of this class of apolipoprotein and emphasizes their key role in facilitating the dynamic interconversions involved in lipoprotein metabolism. The exact nature of the event or molecule that triggers recruitment of these apolipoproteins to lipoprotein particles (or their removal from such particles) remains to be understood. However, on the basis of several independent experimental approaches, it is clear...
that, in the case of apoLp-III, the concentration of DAG in the surface monolayer of lipophorin particles plays a key role in recruitment and binding (Wells et al., 1987; Wang et al., 1992; Soulages and Wells, 1994; Wang et al., 1995; Soulages et al., 1996).

Attempts have been made to study lipid binding mechanisms of other apolipoproteins, e.g. human apoE and apoA-I. The N-terminal domain of apoE, the crystal structure of which has been determined to 2.5-Å resolution (Wilson et al., 1991), bears a remarkable resemblance to insect apoLp-III. In an analogous approach, using a disulfide bonded variant of apoE3 (Cys-112 and Thr-57–Cys), De Pauw et al. (1995) demonstrate that lipid binding is decreased when helices 2 and 3 of the four-helix bundle in the N-terminal domain are disulfide bonded. The disulfide bond in Thr-57–Cys apoE3, located around the central region of these helices, apparently interferes with a postulated reorientation of the helical segments to form shorter 17-residue antiparallel helices, which align parallel to the fatty acyl chains of phospholipid disc complexes. In addition, these authors reported a decrease in lipid binding affinity for the mutant protein. This observation may also be attributed to a decreased ability of the disulfide bonded protein to undergo hinge opening as proposed by Weisgraber (1994). In the case of apoA-I, it has been proposed that lipid binding involves an initial binding event by the two terminal helical domains (which have been demonstrated to have a relatively higher lipid affinity), thereby triggering a cooperative binding of the remaining six helices in the middle of the protein (Palgunachari et al., 1996).

Binding to Phospholipid Bilayers: a Caveat— Given that opening of the molecule is a necessary step in lipid binding and that disulfide bonded apoLp-III is unable to interact with lipoprotein surfaces, the location of the hinge domains is most likely to be between helices 2 and 3 and 4 and 5 as originally suggested by Breiter et al. (1991). It was therefore surprising that oxidized, disulfide bonded N40C/L90C apoLp-III was capable of binding to phospholipid bilayer vesicles. This observation suggests that there is a fundamental difference in the mode of interaction of apoLp-III with phospholipid bilayers versus lipoprotein surfaces. Binding to a lipoprotein surface and stabilizing the particle is the only known physiological function of apoLp-III. Interaction with phospholipid bilayer vesicles to form disc-like complexes (with apoLp-III binding around the perimeter of the bilayer disc), while relevant during biogenesis of nascent mammalian HDL particles, has not been reported to occur in insect hemolymph in vivo. While the nature of the specific interactions of N40C/L90C apoLp-III with discs remains to be determined, it is possible that the protein has the ability to open in an alternate manner, using the loops located between helices 1 and 2 and 3 and 4 as hinges. Such a hinge opening would be possible in disulfide bonded N40C/L90C apoLp-III.

Initial Recognition—A possible explanation for the differences in interaction of apoLp-III with phospholipid bilayers and with lipoprotein surfaces is that the initial recognition event at the surfaces encountered by apoLp-III is different. Two different postulates have been put forward to explain the nature of the initial binding interaction. The first hypothesis is that a small stretch of nonpolar residues in the loop regions between helices 1 and 2 and 3 and 4 of apoLp-III (Breiter et al., 1991; Smith et al., 1994; Soulages et al., 1995) mediates initial recognition and is followed by a binding event. The second proposal is that ionic interactions between the phospholipid head groups and charged residues on the protein exterior mediate an initial interaction and bring the protein in close proximity to the lipid surface, promoting formation of a stable interaction (Zhang et al., 1993; Upadhyaya et al., 1995). An inherent assumption in both these proposals is that a large tertiary structural reorganization is necessary for stable lipid interaction. Recruitment of apoLp-III to the surface of lipoproteins in vivo occurs synchronously with DAG loading suggesting the initial recognition event is likely to be a function of the presence of DAG in the surface monolayer of the particle (Wang et al., 1995; Soulages et al., 1996). The possibility that a combination of ionic and hydrophobic interactions takes place cannot be ruled out. In contrast to lipoprotein binding, the initial recognition event and binding interaction with phospholipid bilayers vesicles are not dependent on the presence of DAG but occur at the gel to liquid-crystal phase transition temperature of the phospholipid. A similar differential ability to interact with phospholipid bilayers and lipoprotein surfaces has been observed earlier with peptide fragments of L. migratoria apoLp-III (Narayanaswami et al., 1995) wherein the helix bundle was dissected to yield the N- and C-terminal halves (∼9 kDa each). Both fragments retain the ability to interact with phospholipid vesicles causing a transformation to disc complexes but were unable to associate with lipoproteins.

In conclusion, we have presented experimental evidence that a conformational change involving opening of the apoLp-III helix bundle is an obligatory step in stable lipoprotein association. The physiological implications of such an opening become evident if one considers the functional role of apolipoproteins in terms of reversible carriers of lipids. It is possible that similar hinge opening and closing events mediate lipid interactions of exchangeable amphipathic apolipoproteins from higher species.

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