# Role of Helices and Loops in the Ability of Apolipophorin-III To Interact with Native Lipoproteins and Form Discoidal Lipoprotein Complexes†

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ABSTRACT: The structure of *Locusta migratoria* apolipophorin-III consists of a five-helix bundle connected by four short loops. The role of the conformational flexibility of helices and loops on the lipid-binding activity of this apolipoprotein was investigated by disulfide mediated tethering experiments. One disulfide mutant tethering the second and fourth loops (L2-L4), and two disulfide mutants restricting the flexibility of the neighboring α-helices 3 and 4 (H3-H4) and 1 and 5 (H1-H5), were studied. The ability of the disulfide mutants to interact with phospholipid vesicles, mixed micelles of phosphatidylcholine and cholate, and in vivo with native spherical lipoprotein particles was studied. The L2-L4 mutant was active with native lipoproteins as well as being able to form discoidal lipoproteins upon incubation with either liposomes or discoidal micelles. The H3-H4 mutant was not able to interact with liposomes or native lipoproteins but interacted with discoidal micelles. The H1-H5 mutant was unable to interact with lipid in any of the three systems. Three conclusions were reached: (1) opening of the helix bundle does not require the separation of loops 2 and 4 as recently proposed by others and (2) α-helices 3 and/or 4 are involved in the insertion of apoLp-III in both phospholipid bilayers and monolayers. The conformational flexibility of helices 3 and 4 is required for the lipid-binding activity of apoLp-III. (3) Interaction of helices 1 and/or 5 with the lipid surface is required to the formation of stable lipoprotein complexes of any kind.

Apolipophorin-III (apoLp-III)[1] from the hemolymph of the locust (*Locusta migratoria*) is an exchangeable apolipoprotein with a molecular mass of ∼17 kDa (*1*). ApoLp-III participates in transporting lipids among the insect tissues (*2*, *3*). The X-ray crystal structure of *L. migratoria* apoLp-III in the lipid-free state indicates that the protein has an overall shape of a prolate ellipsoid, composed of a bundle of five amphipathic α-helices (*4*). Highly similar structures were recently inferred for *Manduca sexta* and *L. migratoria* apoLp-III by NMR spectroscopy (*5*, *6*). Both insect apoLp-III molecules share structural and physiochemical properties with the N-terminal four-helix bundle (22 kDa) of the human apolipoprotein E (apoE) (*7*).

Binding of exchangeable apolipoproteins to the lipoprotein lipid surface is a complex process, which is likely to involve several steps and changes in the protein structure as well as changes in the structural organization of the lipid molecules. Binding of human ApoA-I to lipids has been suggested to occur in a multistep process (*8*, *9*) involving an initial adsorption followed by penetration of the amphipathic α-helices into the phospholipid bilayer. On the basis of the lipid-binding activity of disulfide mutants, a similar multistep process has been suggested by Lu et al. (*10*) for human apoE. These studies indicate an initial interaction of apoE with the lipid surface without major helical rearrangements followed by a major rearrangement of constituent α-helices of apoE. Two steps in binding of apoLp-III to a planar bilayer were also distinguished by surface plasmon resonance (*11*). The interaction of apolipoproteins with lipid surfaces is dependent on the properties of both the protein and the lipid surface. The changes in lipid composition of lipoproteins that take place in circulation constitute the driving forces for binding of the apolipoproteins to the lipoprotein, as well as to their dissociation from the lipoprotein lipid surface. On the other hand, the properties of the protein define the rate of the binding process and the affinity of the bound state (*12*, *13*). One of the common features of exchangeable apolipoproteins resides in their ability to undergo conformational changes (*14*). The conformational flexibility of apolipoproteins (*13*), and membrane-binding proteins (*15*), may be required for the initial binding steps, such as the adsorption of the protein to the lipid surface, as well as for subsequent structural changes that would lead to a final lipid-bound state.

To investigate the role of the conformational flexibility of different regions of the apoLp-III molecule and its mechanism of interaction with lipid surfaces, disulfide mutants were designed on the basis of the crystal structure of the protein. Two disulfide mutants restricting the conformational flexibility of the neighboring α-helices 1 and 5 (H1-H5) and 3 and 4 (H3-H4) and one disulfide mutant tethering the second and fourth loops (L2-L4) were successfully

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[1] Abbreviations: ApoLp-III, apolipophorin-III; apoAI, apolipoprotein AI; apoE, apolipoprotein E; DMPC, dimyristoylphosphatidylcholine; DTT, dithiothreitol; MLV, multilamellar vesicles; H1-H5 mutant, W128F/T18C/A147C; H3-H4 mutant, W128F/N81C/Q112C; L2-L4 mutant, W128F/S70C/A127C.

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constructed and expressed. The ability of these mutants to form discoidal lipoproteins upon interaction with both phospholipid bilayers and preformed discoidal micelles was investigated. Moreover, the lipid-binding activity of the proteins was studied in vivo providing information on the mechanism of interaction of apoLp-III with native spherical insect lipoproteins. The comparison of the lipid-binding properties of these mutants provided information about the role of different regions of the apoLp-III molecule in the insertion of the protein into phospholipid bilayers and spherical monolayers.

## EXPERIMENTAL PROCEDURES

*Site-Directed Mutagenesis and Protein Expression.* Wild-type apoLp-III was cloned in a pET32a expression vector (Novagen, Inc., Madison, WI) and used as template for site-directed mutagenesis. Mutagenesis was carried out using the QuickChange kit from Qiagen Sciences, MD. The procedures followed for the expression and purification of wild-type and cysteine mutants of apoLp-III are the same as reported earlier (*16*, *17*). The proteins were expressed in bacteria as thioredoxin-apoLp-III fusion proteins, cleaved with enterokinase, and purified by affinity chromatography. To facilitate the spectroscopic characterization of the proteins, the disulfide mutants were constructed after obtaining the single Trp mutant W128F, which has structural and lipid-binding properties similar to the wild-type protein (*18*). The location of the residues potentially suitable for the formation of a disulfide bond was selected by visual inspection of the crystal structure (1aep PDB) and replaced by Cys using the molecular modeling program Insight-II. The distances between the S-atoms were measured for different combinations of rotamers of the Cys side chains. Those residues that provided the shortest distance of separation between the sulfur atoms were selected for mutagenesis. The mutant H3-H4, which tethers the centers of α-helices 3 and 4, has the following substitutions: W128F, N81C (helix-3), and Q112C (helix-4). The mutant L2-L4, which tethers the loops connecting helices 2−3 and 3−4, has the following substitutions: W128F/S70C/A127C. The construction and expression of T18C/A147C has been reported earlier (*17*).

*Alkylation of Cysteine Mutants.* The purified double cysteine mutants were fully oxidized (disulfide state). To study the reduced form of the mutants, it was necessary to alkylate the Cys residues. Alkylation was carried out with iodoacetamide as described previously (*17*).

*Circular Dichroism.* Far and near UV CD spectra were obtained in a Jasco-715 CD instrument, as described previously (*17*). The concentration of the free apoLp-III mutant and wild-type were determined by UV absorption spectroscopy in the presence of 3 M Gdm-HCl.

*Kinetics of the Spontaneous Formation of Discoidal Lipoproteins.* The kinetics of formation of discoidal lipoproteins was studied using multilamellar vesicles of DMPC, as described by Pownall et al. (*19*). The reaction was performed at 23.9 °C, in 50 mM potassium phosphate buffer pH 6.5, with continuous magnetic stirring in a Hewlett-Packard 8453 diode array spectrophotometer equipped with a temperature-controlled cell holder at a protein to lipid molar ratio of 1:20. The reaction was started by the addition of 25 μL of 2.5 mg/mL DMPC (MLV), and data acquisition was started within 15 s after the addition of liposomes. Data were acquired continuously over a 30 min time period.

To determine the sizes of lipoprotein disks formed by spontaneous interaction, a concentrated sample of apoLp-III and MLV of DMPC (47.5 μM protein in a final volume of 40 μL) was prepared using a 1:20 protein to lipid molar ratio. ApoLp-III was incubated for 24 h with MLV of DMPC at 23.9 °C, and then, the reaction mixture was subjected to nondenaturing gradient PAGE in 4−20% gels for 12 h at 120 V.

*Preparation of ApoLp-III-DMPC Discoidal Complexes by Detergent Dialysis.* Discoidal complexes of apoLp-III-mutants and DMPC were prepared by a cholate dialysis method (*20*) using a protein/DMPC/cholate molar ratio of 1:60:60. Cholate was removed from the mixture by extensive dialysis against 25 mM potassium phosphate buffer, 125 mM NaCl, pH 7.3. After dialysis, the apoLp-III/DMPC complexes were characterized by nondenaturing gradient gel electrophoresis and KBr density gradient ultracentrifugation. The sizes of the lipoprotein particles were calculated from the $R_f$ values and calibration curve of the high range molecular weight markers (Pharmacia).

*Ultracentrifugation.* The lipoprotein complexes formed by cholate dialysis were characterized by ultracentrifugation in a KBr density gradient (*21*). The samples (0.1 mL, 100 μg of protein) were mixed with 2.0 mL of KBr solution (0.5 g/mL), prepared in buffer 0.15 M NaCl, 0.001% EDTA, pH 7.5, and loaded in OptiSeal polyallomer tubes (Beckman Instruments, Inc. Palo Alto, CA). The samples were over layered with 2.9 mL of saline and centrifuged at 328 000*g* in a VTi-65.2 vertical rotor. After centrifugation, each tube was fractionated, and the density of the fractions was estimated by refractometry. The protein concentration was estimated from the second derivative spectra of the fractions.

*In Vivo Binding of Recombinant L. migratoria apoLp-III to M. sexta Lipophorin.* Binding of the disulfide mutants or wild-type locust apoLp-III to native spherical lipoproteins was studied in vivo as recently described (*22*). Adult male *M. sexta* insects were injected with 20 μg of locust apoLp-III mutant. Hemolymph was collected 120 min after the injection of the protein and subjected to fractionation by ultracentrifugation in a KBr gradient. The fractions were dialyzed against 25 mM phosphate buffer, pH 6.5, for 2 h at room temperature and used for SDS−PAGE and Western blot analysis. The relative amounts of locust apoLp-III associated with each fraction were determined by Western blotting using a rabbit anti-locust apoLp-III antibody. The blot was developed by chemiluminescence using horseradish peroxidase tagged goat anti rabbit IgG.

## RESULTS

The conformational flexibility of different helical and loop regions of apoLp-III was restricted by means of disulfide bonds, and the effect of the disulfide bonds on the ability of apoLp-III to form lipoprotein complexes was investigated in three different lipid-binding systems. In the first group of experiments, the ability of apoLp-III mutants to form lipoprotein complexes upon spontaneous interaction with liposomes of phosphatidylcholine was studied. In a second group of experiments, the ability of apoLp-III mutants to form discoidal lipoproteins upon interaction with discoidal

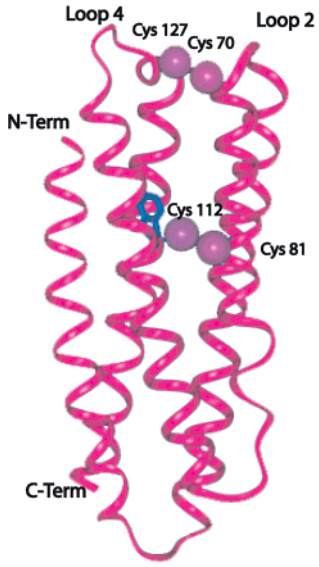


FIGURE 1: Position of the disulfide bonds tethering α-helices 3 and 4 (H3-H4 mutant) and loops 2 and 4 (L2-L4 mutant). The location of the single Trp residue, W113, of these mutants is also shown.

mixed micelles of phosphatidylcholine and $Na^+$-cholate was investigated. Finally, the ability of apoLp-III mutants to interact with native spherical insect lipoproteins was studied in a novel in vivo lipid-binding assay.

*Characterization of the Disulfide Mutants.* The location of the Cys residues was selected on the basis of the coordinates reported with the crystal structure of apoLp-III (*4*), taking into account the predicted distances and angles of orientation between the sulfur atoms of the Cys residues. The disulfide bonds were designed to reside on the protein surface to minimize potential perturbations in the internal packing of the protein. Three disulfide mutants of apoLp-III were designed and expressed: the H3-H4 (N81C/Q112C) and H1-H5 (T18C/A147C) mutants have a disulfide bond tethering the centers of helices 3 and 4 and 1 and 5, respectively. The L2-L4 mutant (S70C/A127C) has a disulfide bond linking the second and fourth loops of the five-helix bundle of apoLp-III. The position of the Cys residues in the H3-H4 mutant and L2-L4 mutant (S70C/A127C) is illustrated in Figure 1. The location of the disulfide bond on the H1-H5 mutant has been reported already (*17*).

ApoLp-III mutants were expressed and purified to homogeneity. The difference between the electrophoretic mobility of oxidized and reduced disulfide mutants was used to investigate the formation of the disulfide bonds (*10*, *17*, *23*). The formation of disulfide bonds was complete for all mutants included in this study (data not shown).

The far UV CD spectra of oxidized and reduced (alkylated) forms of the H3-H4 and L2-L4 mutants (Figure 2) showed that the proteins are predominantly α-helical. The wild-type protein and the disulfide mutants H3-H4 and L2-L4 have α-helical contents of 61, 61, and 55%, respectively. Reduction of the disulfide bonds by alkylation decreased the α-helical content of both the H3-H4 and the L2-L4 mutants to 41%. As reported before (*17*), the estimated α-helical content of the oxidized H1-H5 is nearly identical to that of the wild-type protein. The structure of the mutants was also studied by fluorescence spectroscopy.The disulfide mutants used in this study contain a common single Trp residue in

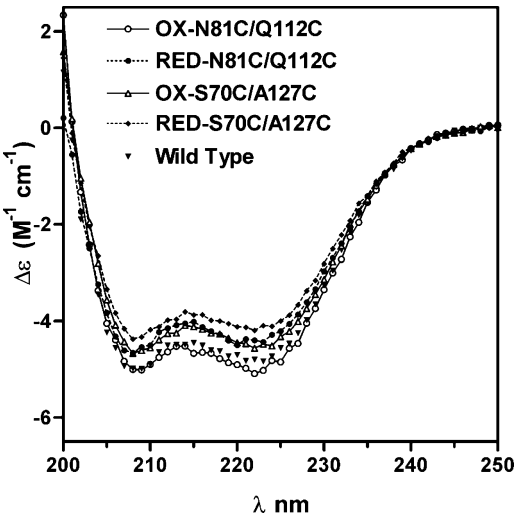


FIGURE 2: Secondary structure of the ApoLp-III mutants. The figure shows the far-UV CD spectra of the proteins used in this study.

helix 4 (W113, Figure 1). For all mutants, the fluorescence maximum was located at 314−315 nm, which is consistent with the nonpolar environment expected for the single Trp residue (*24*, *25*).

*Spontaneous Interaction of Disulfide Mutants with Multilamellar Vesicles (MLV) of DMPC.* The kinetics of the formation of discoidal lipoproteins upon the spontaneous interaction of apoLp-III with MLV of DMPC was monitored spectrophotometrically. The transformation of the large MLV into small discoidal lipoproteins is accompanied by a nearly complete clearance of the turbidity of the reaction mixture. Figure 3A shows the turbidity decays obtained for the disulfide mutants in both reduced and disulfide states as well as the decay obtained with the wild-type protein. In the reduced state, the mutants H3-H4 and L2-L4 clear the liposomal turbidity completely and at a rate comparable to that of the wild-type protein. However, both H3-H4 and L2-L4 mutants appear to be inactive in the oxidized state. It has been previously shown that the mutant H1-H5 is unable to interact with MLV of DMPC in the disulfide state but associates normally upon reduction (*17*).

Because of the short time span of the turbidimetric assay, a mutant characterized by a very slow rate of formation of discoidal lipoproteins could be seen as an inactive protein. To investigate this possibility, the ability of the mutants to form discoidal complexes was also studied using longer incubation times (24 h) and a higher protein and lipid concentration (45 $\mu$M protein) than that used in the turbidity assay (∼4 $\mu$M). After incubation, an aliquot of the samples was used to determine the turbidity, and the remaining solution was analyzed by nondenaturing PAGE to determine the size of the lipoproteins. No discoidal lipoprotein complexes were detected by electrophoresis for oxidized H1-H5 and H3-H4 mutants (Figure 3B), confirming the lack of activity of the mutants H1-H5 and H3-H4 observed in the kinetics. However, after 24 h of incubation, a nearly complete clearance of liposomal turbidity was observed with the disulfide mutant L2-L4, and analysis of the samples by gradient gel electrophoresis confirmed the ability of the L2-L4 mutant to form lipoprotein complexes. The lipoproteins obtained with this mutant were slightly larger than those obtained with the wild-type protein (Figure 3B, lanes 7 and 8).

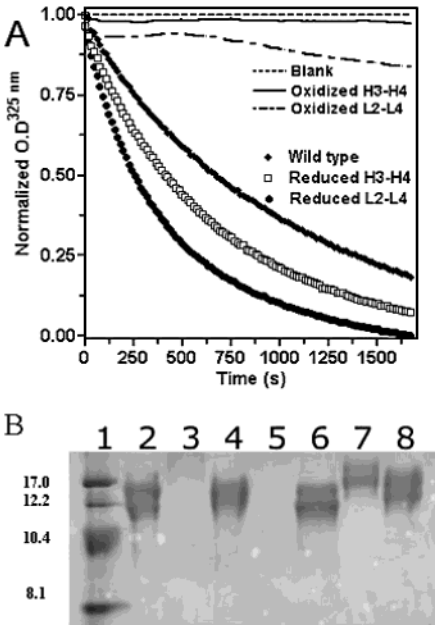


FIGURE 3: Spontaneous interaction of apoLp-III with multilamellar liposomes. (A) The kinetics of formation of discoidal lipoproteins was studied using a protein to DMPC molar ratio of 1:20. The assay was performed at 23.9 °C for 30 min time period with 4 $\mu$M apoLp-III; (B) Nondenaturing 4−20% gradient PAGE of lipoprotein complexes obtained upon 24 h incubation of apoLp-III mutants (45 $\mu$M) with DMPC: lane 1, high range molecular weight markers (Pharmacia); lane 2, wild-type; lane 3, oxidized H1-H5 mutant; lane 4, reduced H1-H5 mutant; lane 5, oxidized H3-H4; lane 6, reduced H3-H4; lane 7, oxidized L2-L4; and lane 8, reduced L2-L4. The numbers on the left side of the figure indicate the apparent diameters, in nanometers, of the markers loaded in lane 1.

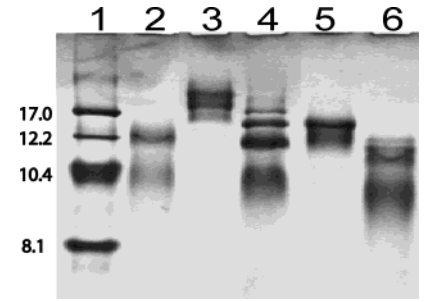


FIGURE 4: Interaction of ApoLp-III disulfide mutants with Cholate/DMPC mixed micelles. Nondenaturing PAGE of the lipoproteins prepared using a 1:60:60 protein/DMPC/cholate molar ratio: lane 1, high range molecular weight markers; lane 2, wild-type; lane 3, oxidized H3-H4; lane 4, reduced H3-H4; lane 5, oxidized L2-L4; and lane 6, reduced L2-L4. The numbers on the left side of the figure indicate the apparent diameters, in nanometers, of the markers loaded in lane 1.

*Interaction of ApoLp-III Disulfide Mutants with Preformed Discoidal DMPC Micelles.* ApoLp-III mutants were allowed to interact with preformed mixed micelles of DMPC and $Na^+$-cholate, and their ability to lead to the formation of discoidal lipoproteins upon removal of the detergent was investigated. The H1-H5 mutant was the only inactive protein in the disulfide state. The disulfide mutants H3-H4 and L2-L4 promoted the formation of lipoprotein particles. The lipoproteins formed were characterized by their size (Figure 4) and density (Figure 5). The comparison of the sizes of the lipoprotein complexes obtained with the mutants H3-H4 and L2-L4 in both oxidized and reduced states shows that in the oxidized state the mutants produce larger

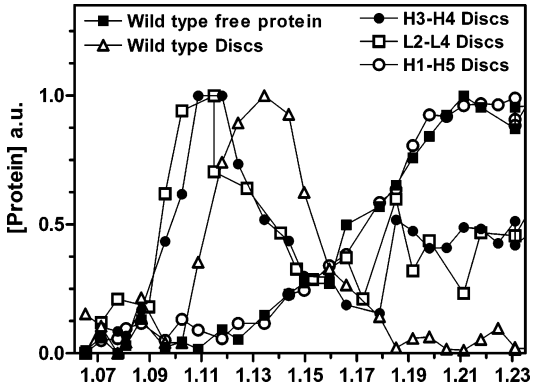


FIGURE 5: Interaction of ApoLp-III disulfide mutants with Cholate/DMPC mixed micelles. The density of the lipoprotein complexes and the presence of lipid-free (unbound) apoLp-III were studied by KBr density gradient ultracentrifugation. The lipoproteins were obtained using an initial protein/DMPC/cholate molar ratio of 1:60:60.

lipoprotein particles than the wild-type protein. In the reduced state, these two mutants and the wild-type protein formed similar lipoproteins (Figure 4).

The density of the lipoproteins formed and the fraction of unbound apolipoprotein were studied by ultracentrifugation in a KBr density gradient. At a protein to DMPC molar ratio of 1:60, wild-type apoLp-III forms lipoprotein complexes with an average density of 1.135 g/cm$^3$ (Figure 5). The samples prepared with oxidized H3-H4 and L2-L4 mutants form lipoprotein complexes with average densities of 1.105 g/cm$^3$. The lower density of the lipoprotein complexes obtained with the disulfide mutants is consistent with the fact that 30% of the protein remains in the lipid-free state accumulating at the bottom of the gradient. On the other hand, ultracentrifugation of the samples prepared with the H1-H5 mutant (oxidized state) indicated that 100% of the apolipoprotein is in the lipid-free state and found in the bottom half of the gradient (Figure 5).

On reducing the disulfide bonds, the density of the disks formed by the mutants is restored to the wild-type density of 1.13 g/mL (data not shown).

*In Vivo Lipid-Binding Activity of ApoLp-III Disulfide Mutants.* The hemolymph of adult *M. sexta* insects contains 1−1.5 mg of apoLp-III, which is found in the lipid-free form and bound to lipophorin particles of different density. Ultracentrifugation of the hemolymph in KBr density gradient readily separates lipid-free apoLp-III, which accumulates at the bottom of the gradient, from lipophorin-bound apoLp-III, which is distributed between HDLp and LDLp subspecies and found in the top-half of the gradient (*26*). ApoLp-III molecules from *M. sexta* and *L. migratoria* insects share identical functions (*27*) and structures (*28*). We have recently shown that wild-type *L. migratoria* apoLp-III injected into the circulation of *M. sexta* insects binds to the insect lipoproteins with an affinity similar to that of the endogenous, native, apoLp-III (*22*). To study the in vivo lipid-binding activity of the disulfide mutants, we have compared the distribution of apoLp-III mutants between the lipid-free and the lipoprotein-bound fractions of the hemolymph with the distribution of wild-type locust apoLp-III. Figure 6 shows the distribution of wild-type *L. migratoria* apoLp-III and the disulfide mutants H1-H5, H3-H4, and L2-L4 among the lipoprotein-bound and lipid-free fractions of the *M. sexta*

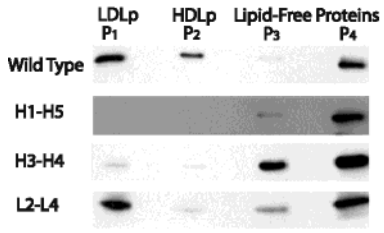


FIGURE 6: In vivo binding of *Locusta migratoria* ApoLp-III disulfide mutants to *Manduca sexta* lipophorin.

Table 1: Summary of Lipid-Binding Activity of the Disulfide Mutants of Apolipophorin-III

| assay\mutant | WT | H1-H5 | H3-H4 | L2-L4 |
|---|---|---|---|---|
| spontaneous interaction with liposomes | yes | no | no | yes |
| interaction with discoidal micelles | yes | no | yes | yes |
| in vivo binding to lipophorin | yes | no | no | yes |

hemolymph. A simple inspection of Figure 6 shows major differences between the distribution of the wild-type protein, 45% of which is associated to lipoproteins (LDLp + HDLp fractions), and the distributions of the disulfide mutants H1-H5 and H3-H4, for which the fraction of lipoprotein bound apoLp-III represents only ~5%. These results indicate that the disulfide bonds tethering α-helices 1 and 5, or 3 and 4, inactivate apoLp-III. On the other hand, the L2-L4 mutant is distributed among the lipoprotein bound (36%) and lipid-free forms in a fashion similar to the wild-type protein indicating that tethering of the loops 2 and 4 has a minor impact on the lipid-binding activity of apoLp-III.

The distribution of *L. migratoria* apoLp-III mutants among the density fractions of *M. sexta* hemolymph was determined by Western blotting as indicated in the Experimental Procedures. The hemolymph was separated into four density fractions by ultracentrifugation in a KBr gradient; pool 1($P_1$): LDLp (low-density lipophorin, $\delta < 1.06$ g/cm$^3$); pool 2 ($P_2$): HDLp ($1.06 < \delta < 1.12$ g/cm$^3$); pool 3 ($P_3$) and pool 4 ($P_4$) contain lipid-free proteins ($\delta > 1.12$ g/cm$^3$).

For the sake of clarity, a summary of the activity of the disulfide mutants of apoLp-III observed in different lipid-binding assays is shown in Table 1.

## DISCUSSION

The association of apolipoproteins with the lipid surface of lipoproteins and membranes may involve several steps. Among the possible steps, we could distinguish adsorption of the protein to the lipoprotein lipid surface, insertion into the phospholipid monolayer, or bilayer, and one or more conformational changes that would be required to achieve the extended protein conformation of the final lipid bound state. The protein conformational changes required in different steps along the binding process are not known. Some of the conformational fluctuations available to the protein in the lipid-free state could be particularly important for the lipid-binding activity of the protein. For instance, concerted conformational fluctuations of one or more α-helices could be necessary for the initial interaction of the protein with the lipid surface or for the insertion of the protein into a phospholipid bilayer or monolayer. Other conformational changes may occur after an initial association of the apolipoprotein with the lipid surface. The role of the conformational flexibility in the lipid-binding activity of apoLp-III was investigated using three disulfide mutants, which introduced well-defined structural restrictions in different regions of the apoLp-III molecule.

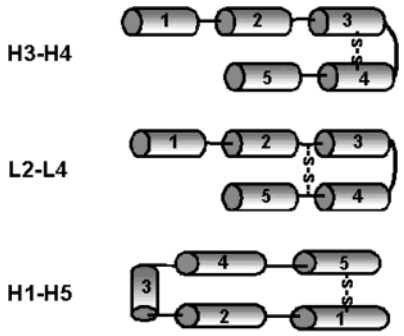


FIGURE 7: Sketched representation of the extended conformations that could be adopted by different disulfide mutants in the lipid-bound state.

*Differences between the Lipid-Binding Assays.* Because the lipid−protein interaction is in general dependent on the physical and chemical nature of the lipid structure (*29*), the impact of the disulfide bonds on the lipid-binding activity of apoLp-III was studied in three different lipid-binding assays. The assays used imposed different restrictions to the lipid-binding activity of the protein providing additional information on the role of the conformational flexibility as well as on the mechanisms of interaction. The spontaneous formation of lipoprotein particles upon interaction of the apolipoproteins with liposomes involves multiple steps including, among others, the insertion of the apolipoprotein into the bilayer and the breakdown of the large liposomal structures into small lipoprotein particles. On the other hand, the spontaneous association of apoLp-III with native spherical lipoproteins is limited by the ability of the protein to interact and become inserted into a spherical phospholipid monolayer. Finally, the interaction with discoidal micelles of DMPC overrides the initial steps of the lipid−protein interaction, such as the penetration of the protein or some of its α-helices in the lipid bilayer. In this assay, the protein activity would be limited by the ability of the protein to form a stable lipoprotein complex upon interaction with a lipid structure that is already small, discoidal, and has a preexposed hydrophobic surface around the bilayer disk (*30*). Thus, the interaction of apoLp-III with discoidal micelles of DMPC may not be impaired by the structural restrictions imposed by disulfide bonds to the extent that it impaired the interaction of the protein with a liposomal structure or with native lipoproteins.

*Conformational Flexibility of Helices 3 and/or 4 Is Necessary for the Insertion of ApoLp-III into the Phospholipid Monolayer of Spherical Native Lipoproteins.* The role of the conformational flexibility of helices 3 and 4 in the lipid-binding activity of apoLp-III was investigated with the mutants H3-H4 and L2-L4. Both mutants effectively prevent the separation of helices 3 from 4 (Figure 7). However, the disulfide present in the L2-L4 mutant tethers the N- and C-term residues of helices 3 and 4, respectively; therefore, it is expected to reduce the conformational freedom of helices H3 and H4 to a lesser extent than the H3-H4 mutant, which tethers the centers of the helices.

The inability of the oxidized H3-H4 mutant to interact spontaneously with DMPC vesicles, or in vivo, with native

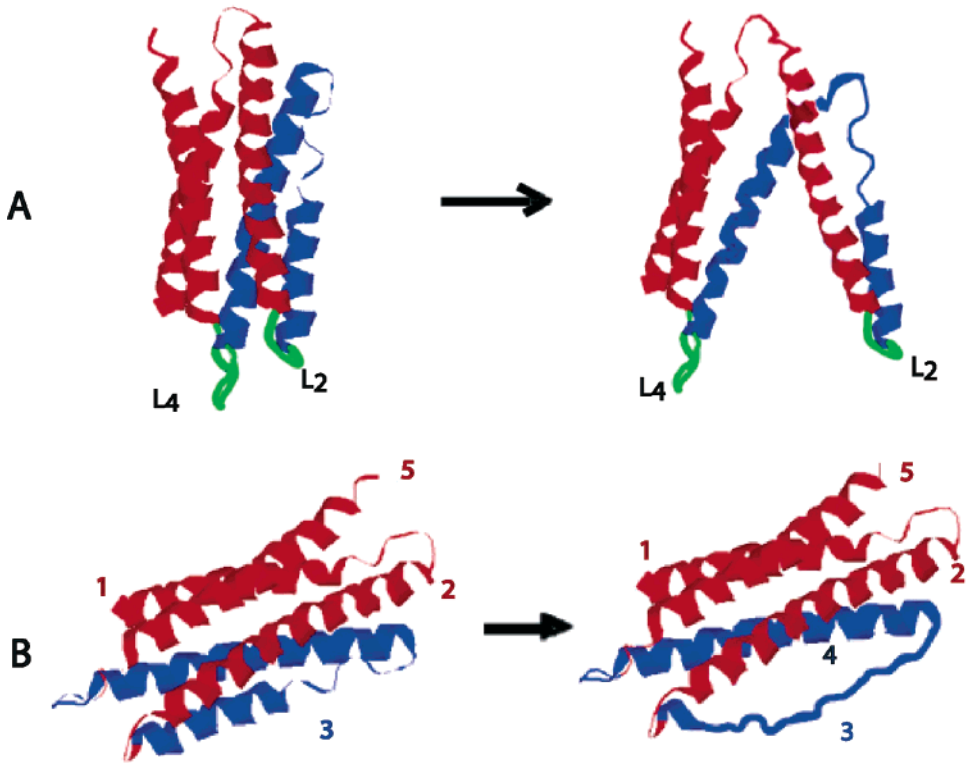


FIGURE 8: Models of interaction of ApoLp-III with lipoprotein lipid surfaces. Two models of the initial apolipoprotein−lipid interaction steps are sketched: (A) recently proposed model of initial apoLp-lipid interaction and opening of the helix bundle (Fan et al. (*6*)). In this model, the initial interaction of the loop 4 with the lipid surface would precede the opening of the helix bundle. (B) Alternative mechanism consistent with the observations made in the present study. In this model, the unfolding of helix 3 is involved in the initial insertion of the protein into the phospholipid structure. Similar models involving helix 4 or helices 3 and 4 simultaneously are also consistent with the results of this study. Helices 1 and 5 could also be involved in the initial steps of the lipid−protein interaction. However, because the disulfide mutant tethering helices 1 and 5 is inactive, our study provided no information on this regard.

spherical lipoproteins suggests that the conformational flexibility of helices 3 and 4 is essential in the disruption of the packing of both phospholipid monolayers and bilayers. Two facts support this interpretation; in first place, the H3-H4 mutant forms discoidal lipoproteins upon incubation with discoidal micelles of DMPC and cholate, indicating that it can interact with disk bilayers when the acyl chains are preexposed. In second place, the fact that the loop mutant interacts spontaneously with MLV of DMPC as well as with native lipoproteins indicates that the greater flexibility of the helices H3 and H4 tethered by the ends, as compared to the flexibility of the helices tethered by their centers, could be responsible of the lipid-binding activity observed in the loop mutant. Because in a fully extended conformation both mutants could adopt similar structures (Figure 7), the activity of the loop mutant could not be explained by a difference in the stability of the lipid-bound state. This is also supported by the fact that H3-H4 and L2-L4 mutants lead to the formation of lipoproteins of similar properties when incubated with discoidal micelles (Figure 4). Thus, these results suggest that the conformational flexibility of helices 3 and/or 4 is needed in the initial binding steps, including the insertion of the protein, or some of its regions, in phospholipid monolayers. For instance, as sketched in Figure 8B, unfolding of one of these helices could be required for the insertion of the protein into the phospholipid monolayer of native lipoproteins.

*Interaction of Loops 2 and 4 with the Lipid Surface Does Not Mediate Opening of the Helix Bundle of ApoLp-III.* The presence of surface exposed nonpolar residues, and the conformational flexibility of the loop regions of apoLp-III, have inspired models of apolipoprotein−lipid interaction in which the loops initiate the interaction with the lipid surface defect, and this interaction would, subsequently, trigger the opening of the helix bundle (*4*, *6*). This type of mechanism was recently proposed on the basis of the conformational dynamics of loop 4, inferred from solution NMR studies (*6*). This model of interaction is represented in Figure 8A, and it suggests that loop 4 would initiate the interaction of apoLp-III with the lipoprotein surface leading to the separation of loops 2 and 4. The importance of this mechanism was tested in the present study by means of the L2-L4 mutant, which restricts the conformational flexibility of the loops and prevents the opening of the helix bundle through the separation of the loops 2 and 4. As shown in the Results, this disulfide mutant was able to form discoidal lipoproteins upon interaction with either liposomes or discoidal micelles. Moreover, this mutant was able to interact with native spherical lipoproteins in a fashion similar to that observed with the wild-type apoLp-III. The fact that tethering of loops 2 and 4 does not affect the lipid-binding properties of apoLp-III in vivo indicates that the interaction of these loops with the lipoprotein lipid surface is not necessary for either the initial recognition of the lipoprotein lipid surface or for the opening of the helix bundle. It has also been proposed that interaction of loops 1 and 3 with the lipid surface would trigger an opening of the helix bundle (*4*). This hypothesis was tested in *M. sexta* apoLp-III, which has a similar structure to *L. migratoria* apoLp-III, by means of a disulfide mutant that was predicted to tether loops 1 and 3 (*31*).

Interestingly, tethering of loops 1 and 3 did not prevent apoLp-III from interacting spontaneously with liposomes leading to the formation of discoidal lipoproteins. Therefore, it would appear that the interaction of the loops with the lipid surface is not necessary for either the initial recognition of the lipid surface or for the opening of the helix bundle.

*Helices 1 and 5 Play a Major Role in the Stability of the Lipid-Bound State.* We have recently shown that the conformational flexibility of helices 1 and 5 is required for the spontaneous interaction of apoLp-III with liposomes of phosphatidylcholine (*17*). In the current study, we have investigated further the role of helices 1 and 5 through the study of the interaction of the H1-H5 mutant with mixed discoidal micelles of DMPC and cholate and native spherical lipoproteins in vivo. The fact that this mutant does not associate with native spherical lipoproteins in vivo suggests that helices 1 and/or 5 could play an important role in the insertion of apoLp-III into the phospholipid monolayer of spherical lipoproteins. However, the fact that this mutant cannot form discoidal lipoproteins even upon interaction with discoidal micelles indicates that the conformational freedom of helices 1 and/or 5 is essential to achieve a strong lipid−protein interaction and thus for the formation of stable lipoprotein complexes. Although helices 1and 5 play a major role in stabilizing the lipid-bound state, our results do not discard the involvement of these helices in the insertion of the protein in phospholipid monolayers and/or bilayers.

*Multiple Lipoprotein-Bound Conformations of ApoLp-III.* The preferred conformation of apoLp-III in discoidal lipoproteins appears to be one where the polypeptide chain adopts a fully extended conformation around the periphery of the disk bilayer (*32*−*34*). Because of the restriction imposed by the disulfide bond, none of the disulfide mutants used in this study could adopt such a conformation (Figure 7). Therefore, the ability of the L2-L4 and H3-H4 mutants to form discoidal lipoproteins indicates that other apolipoprotein conformations are compatible with the formation of stable discoidal lipoproteins. Recent studies of human apoA-I in discoidal lipoproteins provided evidence consistent with the presence of multiple apolipoprotein conformations, including variable apolipoprotein arrangements (*35*, *36*). Two lipid-bound conformations of apoE were also inferred in a recent study with reconstituted spherical lipoproteins (*37*). The present study provides strong evidence indicating that multiple apolipoprotein conformations, or alternative conformations, are possible not only in discoidal lipoproteins but also in spherical native lipoproteins. This is supported by the fact that the L2-L4 mutant has an in vivo lipid-binding activity similar to that of the wild-type protein.

## REFERENCES


1. Kawooya, J. K., Meredith, S. C., Wells, M. A., Kezdy, F. J., and Law, J. H. (1986) *J. Biol. Chem. 261*, 13588−91.
2. Soulages, J. L., and Wells, M. A. (1994) *Adv. Protein Chem. 45*, 371−415.
3. van der Horst, D. J., van Hoof, D., van Marrewijk, W. J. A., and Rodenburg, K. W. (2002) *Mol. Cell. Biochem. 239*, 113−9.
4. Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., and Holden, H. M. (1991) *Biochemistry 30*, 603−8.
5. Wang, J., Sykes, B. D., and Ryan, R. O. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 1188−93.
6. Fan, D., Zheng, Y., Yang, D., and Wang, J. (2003) *J. Biol. Chem. 278*, 21212−20.
7. Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991) *Science 252*, 1817−22.
8. Lecompte, M. F., Bras, A. C., Dousset, N., Portas, I., Salvayre, R., and Ayrault-Jarrier, M. (1998) *Biochemistry 37*, 16165−71.
9. Rogers, D. P., Roberts, L. M., Lebowitz, J., Datta, G., Anantharamaiah, G. M., Engler, J. A., and Brouillette, C. G. (1998) *Biochemistry 37*, 11714−25.
10. Lu, B., Morrow, J. A., and Weisgraber, K. H. (2000) *J. Biol. Chem. 275*, 20775−81.
11. Soulages, J. L., Salamon, Z., Wells, M. A., and Tollin, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5650−4.
12. Reijngoud, D. J., and Phillips, M. C. (1982) *Biochemistry 21*, 2969−76.
13. Soulages, J. L., and Bendavid, O. J. (1998) *Biochemistry 37*, 10203−10.
14. Brouillette, C. G., Anantharamaiah, G. M., Engler, J. A., and Borhani, D. W. (2001) *Biochim. Biophys. Acta. 1531*, 4−46.
15. Goni, F. M. (2002) *Mol. Membr. Biol. 19*, 237−45.
16. Soulages, J. L., Pennington, J., Bendavid, O., and Wells, M. A. (1998) *Biochem. Biophys. Res. Commun. 243*, 372−6.
17. Soulages, J. L., Arrese, E. L., Chetty, P. S., and Rodriguez, V. (2001) *J. Biol. Chem. 276*, 34162−6.
18. Soulages, J. L., and Arrese, E. L. (2000) *Biochemistry 39*, 10574−80.
19. Pownall, H. J., Massey, J. B., Kusserow, S. K., and Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183−8.
20. Jonas, A., Kezdy, K. E., and Wald, J. H. (1989) *J. Biol. Chem. 264*, 4818−24.
21. Chung, B. H., Segrest, J. P., Ray, M. J., Brunzell, J. D., Hokanson, J. E., Krauss, R. M., Beaudrie, K., and Cone, J. T. (1986) *Methods Enzymol. 128*, 181−209.
22. Chetty, P. S., Arrese E. L, and Soulages, J. L. (2003) *Protein Pept. Lett. 10*, 469−73.
23. Scheele, G., and Jacoby, R. (1982) *J. Biol. Chem. 257*, 12277−82.
24. Soulages, J. L., and Arrese, E. L. (2000) *J. Biol. Chem. 275*, 17501−9.
25. Weers, P. M., Prenner, E. J., Kay, C., and Ryan, R. O. (2000) *Biochemistry 39*, 6874−80.
26. Soulages, J. L., van Antwerpen, R., and Wells, M. A. (1996) *Biochemistry 35*, 5191−8.
27. Van der Horst, D. J., Ryan, R. O., Van Heusden, M. C., Schulz, T. K., Van Doorn, J. M., Law, J. H., and Beenakkers, A. M. (1988) *J. Biol. Chem. 263*, 2027−33.
28. Wang, J., Gagne, S. M., Sykes, B. D., and Ryan, R. O. (1997) *J. Biol. Chem. 272*, 17912−20.
29. Agasoster, A. V., Halskau, O., Fuglebakk, E., Froystein, N. A., Muga, A., Holmsen, H., and Martinez, A. (2003) *J. Biol. Chem. 278*, 21790−21797.
30. Muller, K. (1981) *Biochemistry 20*, 404−14.
31. Narayanaswami, V., Wang, J., Kay, C. M., Scraba, D. G., and Ryan, R. O. (1996) *J. Biol. Chem. 271*, 26855−62.
32. Sahoo, D., Weers, P. M. M., Ryan, R. O., and Narayanaswami, V. (2002) *J. Mol. Biol. 321*, 201−14.
33. Garda, H. A., Arrese, E. L., and Soulages, J. L. (2002) *J. Biol. Chem. 277*, 19773−82.
34. Soulages, J. L., and Arrese, E. L. (2001) *Biochemistry 40*, 14279−90.
35. Tricerri, M. A., Behling Agree, A. K., Sanchez, S. A., Bronski, J., and Jonas, A. (2001) *Biochemistry 40*, 5065−74.
36. Li, H. H., Lyles, D. S., Pan, W., Alexander, E., Thomas, M. J., and Sorci-Thomas, M. G. (2002) *J. Biol. Chem. 277*, 39093−101.
37. Saito, H., Dhanasekaran, P., Baldwin, F., Weisgraber, K. H., Lund-Katz, S., and Phillips, M. C. (2001) *J. Biol. Chem. 276*, 40949−54.


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