Dynamics and Hydration of the α-Helices of Apolipophorin III*

Jose L. Soulages† and Estela L. Arrese
From the Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078

Apolipophorin III (apoLp-III) is an exchangeable apolipoprotein whose structure is represented as a bundle of five amphipathic α-helices. In order to study the properties of the helical domains of apolipophorin III, we designed and obtained five single-tryptophan mutants of Locusta migratoria apoLp-III. The proteins were studied by UV absorption spectroscopy, time-resolved and steady-state fluorescence spectroscopy, and circular dichroism. Fluorescence anisotropy, near-UV CD, and solute fluorescence quenching studies indicated that the Trp residues in helices 1 (N-terminal) and 5 (C-terminal) have the highest conformational flexibility. These two residues also showed the highest degree of hydration. Trp residues in helices 3 and 4 display the lowest mobility, as assessed by fluorescence anisotropy and near UV CD. The Trp residue in helix 2 is protected from the solvent but shows high mobility. As inferred from the properties of the Trp residues, helices 1 and 5 appear to have the highest conformational flexibility. Helix 2 has an intermediate mobility, whereas helices 3 and 4 appear to constitute a highly ordered domain. From the configuration of the helices in the tertiary structure of the protein, we estimated the relative strength of the five interhelical interactions of apoLp-III. These interactions can be ordered according to their apparent stabilizing strengths: helix 3-helix 4 > helix 2-helix 3 > helix 4-helix 1 = helix 2-helix 5 > helix 1-helix 5. A new model for the conformational change that is expected to occur upon binding of the apolipoprotein to lipid is proposed. This model is significantly different from the currently accepted model (Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., and Holden, M. (1991) Biochemistry 30, 603–608). The model presented here predicts that the relaxation of the tertiary structure and the concomitant exposure of the hydrophobic core take place through the disruption of the weak interhelical contacts between helices 1 and 5. To some extent, the weakness of the helix 1-helix 5 interaction would be due to the parallel arrangement of these helices.

Exchangeable apolipoproteins belong to a class of proteins rich in amphipathic α-helical domains (1, 2). These proteins regulate the metabolism of lipids and lipoproteins in animals (3–5) and can be found associated with lipoproteins or in a lipid-free state. Several contributions to the understanding of the relationship between structure and function of exchangeable apolipoproteins have been made through the study of the insect exchangeable apolipoprotein, apolipophorin III (apoLp-III). Apolipophorin III is an exchangeable apolipoprotein (17 kDa) that is present as a free water-soluble protein, or bound to the lipid surface of the major insect lipoprotein, lipophorin (6–8). The structure of Locusta migratoria apoLp-III has been determined by x-ray crystallography (9). Its structure is described as a bundle of five amphipathic α-helices, where the nonpolar faces of the helices are oriented toward the protein core.

The binding of exchangeable apolipoproteins to the lipoprotein surface responds to changes in physical chemical properties of the lipoprotein lipid surface domain. The physiological role of apoLp-III is binding to a lipoprotein surface that has been destabilized by an excess of diacylglycerol (10–12). The hydrophobic moment and the distribution of charged residues on the polar face of the amphipathic α-helices of the apolipoproteins constitute a key structural element of the exchangeable apolipoproteins (1, 2). Moreover, the thermodynamics and kinetics of the binding process are also dependent on the structure and dynamics of the apolipoprotein molecule. Because major conformational changes are expected to occur when the soluble form of the apolipoprotein binds to a lipid surface, the conformational flexibility and the hydration of the protein in the lipid-free state appear as important properties to study. We have recently shown that the hydration and the tertiary interactions of Manduca sexta apoLp-III play a major role on the kinetics of association of this apolipoprotein with lipids (13). However, much work remains to be done to fully understand the role of the molecular properties of the lipid-unbound form of apolipoproteins on the kinetics and thermodynamics of the lipid-binding process.

In order to study the properties of the five α-helical domains of apoLp-III, we have constructed and expressed five single-Trp mutants. These mutants were studied by several spectroscopic techniques that provided information about the dynamics and hydration of the domains. The spectroscopic observations made in this study revealed that significant differences exist among the properties of the helical domains of the apoLp-III molecule. Particularly interesting are the properties of helices 1 and 5. The high mobility and hydration of the Trp residues located in these helices has prompted us to postulate a new potential mechanism for the conformational change that leads to the exposure of hydrophobic core of the apolipoprotein.

Dramatic conformational changes are expected to occur when these apolipoproteins switch between the lipid-free and

* This work was supported by an Atorvastatin Research Award (to J. L. S.), by National Institutes of Health Grant GM 55622, and by Project ORLO2598, Oklahoma State University, College of Agriculture.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, 355 Noble Research Center, Oklahoma State University, Stillwater, OK 74078. Tel.: 405-744-6212; Fax: 405-744-7799; E-mail: joes@biochem.okstate.edu.

‡ The abbreviations used are: apoLp-III, apolipoporphin III; apoE, apolipoprotein E; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; GdnHCl, guanidine hydrochloride; $K_s$, Stern-Volmer constant.
lipid-bound states (9, 14). On the basis of the crystal structure of the protein, Breiter et al. (9) postulated that the five-helix bundle of apoLp-III undergoes a lipid-triggered opening at putative hinges domains located between helices 2 and 3 and between helices 4 and 5. This opening would expose a large extent of the hydrophobic interior of the protein allowing the lipid-protein interaction. A similar mechanism was postulated for apoE (14). On the basis of the experimental results obtained with five single-Trp mutants in the lipid-free state, we present an alternative mechanism to explain the required exposure of the hydrophobic core of the protein. The proposed mechanism is consistent with the properties of the helices and the inferred interhelical interactions. This mechanism would also explain why tethering of the helix bundle by means a disulfide bond between the loops connecting helices 1 and 2 and 3 and 4 did not prevent the interaction of apoLp-III with DMPC (15).

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification of L. migratoria ApoLp-III**—Site-directed mutagenesis was carried out using the commercial kit, QuickChange, manufactured by Stratagene. A PET-32a plasmid containing the insert of the L. migratoria apoLp-III was used for mutagenesis. The polymerase chain reaction containing the plasmid and the primers was performed according to the manufacturer’s instructions using PuO DNA polymerase. Competent cells, Epicurian (Stratagene), were transformed with the polymerase chain reaction. The plasmid DNA of the transformed bacteria was sequenced from the S tag and T7 terminator sites with S tag and T7 terminator primers. AD494 expression host cells were transformed with the PET-32a-apoLp-III plasmids containing the correct sequences. All sequencing reactions were carried out at the DNA sequencing Service of the University of Arizona. Expression of the protein was induced by isopropyl-1-thio-β-β-galactopyranoside and the protein isolated from the bacteria and purified as reported previously (16). Briefly, the fusion protein was purified by nickel affinity chromatography. Recombinant locust apoLp-III was cleaved from the rest of the fusion protein with enterokinase (Novagen, Madison, WI). ApoLp-III does not have any cleavage sites for enterokinase. ApoLp-III was purified from the cleavage reaction by nickel affinity chromatography. Several passages through the column were necessary to achieve a complete purification. The quality of the purified protein was checked by N-terminal sequencing and electrospray ionization mass spectrometry. Edman degradation analysis gave the sequence of the first six amino acids of apoLp-III (DAAGHV..), confirming the specificity of the enterokinase cleavage.

**Circular Dichroism**—CD spectra were acquired in a Jasco-715 CD instrument using a 0.1-cm path-length cell over the 180–250-nm range and a 1-cm path-length cell in the near-UV range (250–315 nm). The CD spectra were acquired at 25 °C, every 1 nm with a 2-s averaging time/point and a 1-nm band pass. Quadruplicate average spectra were corrected for the blank and smoothed.

GdnHCl was used as unfolding reagent to study the conformational stability of the apoLp-III molecules. Data were fitted by nonlinear regression to Equation 1.

\[ Y = \frac{(a + bD^c + (c + dD)^e)^{\frac{m-nD}{m}}}{1 + (c + dD)^e} \]  

\( D \) is the denaturant concentration, and \( \Delta G \) is the free energy of unfolding in the absence of denaturant.

**Fluorescence Spectroscopy**—Lifetime studies were performed using a multifrequency phase and modulation fluorometer model K-2 (ISS, Urbana, IL) using a 300-watt xenon lamp as excitation source. The light was modulated between 2 and 200 MHz by Pockels cell modulator. The excitation was set up at 295 nm and polarized at 35° by a Glan-Thompson prism polarizer. The emission was collected through a 335WG filter (Schott Inc.). A solution of p-terphenyl in absolute ethanol was used as lifetime reference (τ = 1 ns). The fluorescence intensities of the samples were matched to the intensity of the reference by changing the concentration of p-terphenyl. Data were collected at 15 different frequencies and analyzed by nonlinear least squares using the ISS software. Different decay models were fitted assuming a multi-exponential decay law. For each lifetime model, the best-fitting parameters were obtained by minimization of the reduced \( \chi^2 \) value. All measurements were carried out at 28 °C, and the samples were in 20 mM Tris buffer, pH 7.8. These experiments were carried out in 0.4-cm path-length cells.

**RESULTS**

The selective replacement of amino acid residues by tryptophan, which is an excellent and versatile probe, constitutes one of the less perturbing methods for the incorporation of spectroscopic probes in proteins. To simplify the experiments and the interpretation of the results, it is preferable to have only one Trp residue per molecule of apoLp-III. On the basis of the crystal structure of the L. migratoria protein and attempting to introduce the least number of perturbations in the protein, we designed a set of five single-Trp mutants according to the following criteria. (a) The amino acid residue replaced by Trp was hydrophobic and resided on the hydrophobic face of the α-helices; (b) the volume of the replaced residue was similar to the volume of the Trp. The order of priority was Phe > Ile > Leu. Several residues of apoLp-III met the conditions indicated above. The substitutions that were carried out are the following: Ile19 → Trp (reporter of helix 1), Phe49 → Trp (helix 2), Phe78 → Trp (helix 3), and Leu113 → Trp (helix 5). The wild type apoLp-III contains Trp113 in helix 4 and Trp129 in the loop connecting helices 4 and 5. Therefore, apoLp-III molecules containing Trp in helices 1, 2, 3, and 5 were constructed by mutating the double mutant Trp129 → Phe/Trp113 → Phe, which contains no Trp. The mutant containing a reporter Trp in helix 4, at position 113, was obtained with a single mutation (Trp129 → Phe). Fig. 1 shows the location of the residues that were replaced by Trp.

The proteins were expressed in very high yields and purified until any detectable contaminant was removed. The purified proteins showed a single and sharp band by SDS-polyacrylamide gel electrophoresis (data not shown).

The secondary structure of the mutants was studied by circular dichroism. The far-UV CD-spectra of the five Trp mutants are shown in Fig. 2. All proteins have typical spectra of α-helical peptides with minima around 208 and 222 nm. The structural composition of the mutants as deduced from the analysis carried out with the program Selcon 3 is shown in Table I. The α-helical contents estimated from the spectra (Table I) are similar to those reported for the wild type protein, which range between 40% and 59% (16). The mutants containing Trp residues in helices 1 and 5 have a lower α-helical content than the mutants with Trp residues in helices 2, 3, and 4. However, the content of unordered structure is similar for all mutants.

The stability of the proteins was determined by denaturation with GdnHCl (Table I). The unfolding parameters are similar for all mutants as well as similar to the values reported for the wild type apoLp-III (18, 19). Similar results were obtained when the denaturation was monitored by fluorescence inten-
sity, suggesting that unfolding proceeded through a two-state unfolding process (20). The functionality of all mutants used in this study was tested by means of their ability to interact with DMPC, leading to the formation of discoidal lipoprotein particles. In these assays, all mutants were functional and showed highly similar kinetics of interaction with DMPC. These data will be discussed elsewhere.2

The properties of the Trp residues located in each of the five helical domains were studied by several spectroscopic methods directed to determine the polarity of the environment of the Trp residues, their hydration, and mobility.

**UV Absorption Spectroscopy**—A qualitative estimation of the polarity of the environment of the Trp residues and/or their hydration can be obtained by the comparison of the UV absorption spectra with the spectra obtained under conditions of full-hydration, such as those achieved when the protein is unfolded. Because apoLp-III from *L. migratoria* has no Tyr residues, the spectroscopic properties of the proteins in the near-UV (250–300 nm) are dominated by the absorption and emission properties of the single Trp residue. Fig. 3A shows the UV absorption spectra of the five single-Trp mutants under native conditions. In the presence of 3 M GdnHCl, and over the range of 270–300 nm, the UV absorption spectra of the mutants were indistinguishable. Distinctive features are observed among the mutants in the region of the 1Lb band near 290 nm. The second derivative spectra were included in Fig. 3B because they allow a better comparison of the spectral features than the zero order spectra. The position and the intensity of the trough at 290 nm are indicative of the polarity. It is clear from previous studies (21) that an increase in the intensity of the minimum at 290 nm, or a red shift in its position, are indicative of a decreased polarity in the environment of the Trp. Compared with the fully hydrated residue, this is to the spectra of the unfolded protein, Trp78 (helix 3) displays the largest red shift of all mutants with a maximum around 292 nm, indicating a highly nonpolar en-

---

2 J. L. Soulages and E. L. Arrese, submitted for publication.
Conformational Change upon Binding to Lipid

TABLE I  Secondary structure and stability of the single-Trp mutants

|             | Trp-H1  | Trp-H2  | Trp-H3  | Trp-H4  | Trp-H5  |
|-------------|---------|---------|---------|---------|---------|
| α-Helix (%) | 50.6    | 59.1    | 55.8    | 54.8    | 49.8    |
| Turn (%)    | 18.6    | 16.9    | 13.6    | 13.9    | 21.2    |
| Unordered (%) | 23.3 | 22.3    | 27.3    | 24.8    | 24.7    |
| β-Structure (%) | 7.5  | 4.9      | 4.9      | 6.0      | 5.7      |
| ΔG^ΔΔ (cal/mol) | 1,742 ± 158 | 1,936 ± 87 | 2,030 ± 107 | 2,051 ± 146 | 1,563 ± 281 |
| m-Value (s⁻¹ cal/mol) | 5,469 ± 326 | 5,684 ± 111 | 5,944 ± 108 | 6,724 ± 146 | 6,506 ± 281 |

*Trp-H1, Trp-H2, Trp-H3, Trp-H4, and Trp-H5 refer to the mutants containing a single Trp residue in helices 1, 2, 3, 4, and 5, respectively.

The secondary structure was estimated from the analysis of the CD spectra (185–260 nm) using the SELCON method (27).

Unfolding was induced by GdnHCl and monitored by CD at 220 nm. The free energy of unfolding, ΔG^ΔΔ ± 95% confidence interval, and the m-values ± 95% confidence interval were estimated by nonlinear regression of the unfolding data.

---

Steady-state Fluorescence Spectra—The polarity of the environment of a given Trp residue in a protein can be inferred from the position of the maximum in the fluorescence emission spectrum (22). Because the mutants were designed by replacing residues that, according to the crystal structure, were on the nonpolar side of the amphipathic α-helices, we expected that all residues would show a significant spectral blue shift relative to the spectrum of an exposed Trp residue, which in our fluorometer has a maximum at 356 nm (Fig. 4, inset). The second derivative spectra is even lower than that observed in the unfolded protein. Contrasting with the spectral features of the Trp in helix 3, the Trp residues in helices 2 and 4 show a small red shift. However, the large increase in the intensity of the second derivatives at 290 nm also indicates a nonpolar environment for the Trp in helices 2 and 4. On the other hand, the spectra of the Trp residues in helices 1 and 5 show high similarities with the spectrum of a hydrated tryptophan residue, indicating that these residues reside in a medium of relatively high polarity.

Steady-state Fluorescence Quenching—To study the exposure of the Trp residues or its accessibility to the aqueous medium, we carried out solute fluorescence quenching studies using acrylamide. Acrylamide does not interact with proteins (23). Quenching studies with uncharged quenchers, such as acrylamide, avoid charge effects and have the potential advantage of partitioning into the protein core. The steady-state quenching plots were linear within the concentration range used in these studies (Fig. 5). Therefore, the data were analyzed on the basis of the simple Stern-Volmer equation. The apparent Stern-Volmer constants (Ksv) for the unfolded proteins showed an average value of 13 s⁻¹ M⁻¹ (data not shown). The apparent Ksv for the mutants are shown in Table III. The Ksv values obtained for the Trp residues located in helices 1 and 5 indicate that these two Trp are significantly exposed. In consistency with the position of the fluorescence maximum, the Trp located in helix 4 shows the lowest apparent accessibility to the quencher. The Trp residues located in helices 2 and 3 appear to have similar and low accessibilities.

Lifetimes of Fluorescence and Quenching by Acrylamide—Further study the accessibility of the Trp residues through the effect of acrylamide on the fluorescence lifetime. This study provides an independent evaluation of the dynamic quenching. Table II shows the lifetime components for the decays of fluorescence of the five mutants. In general, the decays were well described by a major contribution to the intensity of a long-lifetime, 4-ns component, and a minor contribution from a...
rapidly decaying component of about 1 ns. In the presence of guanidine, the fully unfolded protein showed an average decay described by a major contribution of a 5-ns lifetime and a minor contribution of a 1.2-ns lifetime. Among the mutants, only that containing a Trp residue in helix 5 showed some similarity to the fluorescence lifetime obtained in the unfolded protein. Frequency-domain intensity decays of the mutants are shown in Fig. 6. From left to right, these data are for the indicated mutants in the presence of increasing concentrations of acrylamide, ranging from 0 to 0.3 M. For all proteins, quenching by acrylamide results in progressive shifting of the frequency response to higher frequencies, which indicates a decrease of the mean decay time. In none of the proteins could the decay data be analyzed through a single lifetime. In general, the fluorescence decay was well represented by a multiple exponential decay model, even in the absence of acrylamide.

The fluorescence maximum and the accessibility to quenchers of the Trp residue located in helix 3 indicate an environment of intermediate polarity and an intermediate degree of exposure for this residue. Surprisingly, this residue has the lowest mobility as assessed by fluorescence anisotropy and/or near-UV CD. The higher accessibility Trp in helix 3 and the intermediate polarity of its environment could be related to the fact that this helix is connected to helix 4 through a highly deformed helical segment (residues 87–94). This segment could provide the access of some solvent molecules and quenchers without altering the packing around the indole group. A similar flexible domain called helix 3’ was observed in M. sexta apoLp-III (26).

On the other hand, the spectra of Trp residues located in helices 1, 2, and 5 indicate that these residues can reside in different packing arrangements or, otherwise, are highly mobile. The results of fluorescence anisotropy suggest that the mobility of the residues is an important contribution to the low intensity of the CD bands in the near UV.

**DISCUSSION**

In order to gain insight into the physicochemical properties of individual α-helices of the exchangeable apolipoprotein, apoLp-III, we designed five single-Trp mutants, each of them containing a Trp residue in a different α-helix. These mutants were constructed on the basis of the crystal structure of apoLp-III and designed such that the indole groups would reside on the nonpolar side of the amphipathic α-helices.

The characterization of the mutants indicated that the amino acid replacements performed produced minor changes in the structure and functionality of the protein. According to the far-UV CD study, the secondary structure of the mutants is similar to the structure of the wild type proteins, and the
fraction of unordered structure is nearly the same for all mutants. The similar conformational stability of the proteins, as determined by GdnHCl unfolding studies, also indicates that the mutations did not introduce major structural changes. Moreover, all mutants were functional and showed highly similar kinetics of interaction with DMPC. These results allow us to assume that the properties of the indole groups determined in this study are due to the local properties of the protein domains containing the Trp residues rather than to mutagenesis-induced, global structural changes in the protein molecule.

The rationale behind this study resided on the assumption that, to a certain extent, the properties of the indole groups would reflect the properties of the helix where they are residing. We have used the properties of one residue to infer the properties of helices containing an average of 20 residues. Because about half of the residues are buried in the protein core, we are inferring the properties of the helix from a study that samples ~10% of the helix’s extent. Because the properties of a residue are highly influenced by its interactions with the neighboring residues, it is reasonable to assume that with one residue we are sampling considerably more than 10% of the helix’s extent. Consequently, there is a considerably high probability that the properties of a given Trp residue be correlated with the properties of the helix and its immediate environment.

### Table II

#### Summary of spectroscopic properties of the single-Trp mutants of *L. migratoria* apoLp-III in the lipid-free state

|                  | Trp-H1 | Trp-H2 | Trp-H3 | Trp-H4 | Trp-H5 |
|------------------|--------|--------|--------|--------|--------|
| λ<sub>max</sub> (nm) | 349    | 317    | 333    | 311    | 333    |
| Quantum yield ratio<sup>a</sup> | 1.82   | 1.87   | 1.83   | 3.9    | 1.40   |
| Fluorescence anisotropy<sup>b</sup> | 0.0706 ± 0.0025 | 0.0761 ± 0.0037 | 0.1265 ± 0.0024 | 0.0971 ± 0.0026 | 0.0717 ± 0.0046 |
| Near-UV CD λ<sub>c</sub> (m<sup>-1</sup> cm<sup>-1</sup>) | 291    | 289.5  | 290.5  | 290    |
| λ<sub>max</sub> (nm) | 349.0  | 317.0  | 333.0  | 311.0  | 333.0  |
| Lifetimes (ns)<sup>c</sup> | 3.99   | 0.91   | 4.08   | 0.94   | 4.11   |
| Fractional Intensity | 0.86   | 0.14   | 0.82   | 0.18   | 0.75   |
| χ<sup>d</sup> | 19     | 11.9   | 2      | 1      | 0.14   |

<sup>a</sup> Trp-H1, Trp-H2, Trp-H3, Trp-H4, and Trp-H5 refer to the mutants containing a single Trp residue in helices 1, 2, 3, 4, and 5, respectively.

<sup>b</sup> The quantum yield ratio was calculated as the ratio between the fluorescence intensity of the protein in 20 mM sodium phosphate buffer, pH 7.4, and the fluorescence intensity of the unfolded protein (3 M GdnHCl). The emission maxima and the apparent quantum yields were determined with an excitation wavelength of 280 nm.

<sup>c</sup> The anisotropy of fluorescence was determined using a reduced chi-squared value. The errors for the phase and modulation data used in the fitting were 0.2° and 0.004, respectively.

<sup>d</sup> τ<sub>1</sub> and τ<sub>2</sub> refer to the long and short lifetime components, respectively.

#### Table III

#### Steady-state and time-resolved fluorescence quenching

|                  | Trp-H1 | Trp-H2 | Trp-H3 | Trp-H4 | Trp-H5 |
|------------------|--------|--------|--------|--------|--------|
| Apparent K<sub>q</sub>, steady state (M<sup>-1</sup>)<sup>a</sup> | 9.01 ± 0.35 | 3.84 ± 0.25 | 4.31 ± 0.27 | 2.80 ± 0.28 | 8.68 ± 0.34 |
| K<sub>q</sub> (M<sup>-1</sup>) lifetimes<sup>a</sup> | 4.93 ± 0.46 | 2.76 ± 0.50 | 2.67 ± 0.21 | 1.17 ± 0.35 | 4.28 ± 0.25 |
| ⟨τ⟩ mean lifetime<sup>a</sup> | 3.56   | 3.52   | 3.43   | 3.90   | 3.26   |
| k<sub>q</sub> (s<sup>-1</sup>) steady-state<sup>a</sup> | 2.53   | 1.09   | 1.26   | 0.71   | 2.66   |
| k<sub>q</sub> (s<sup>-1</sup>) lifetime<sup>a</sup> | 1.38   | 0.78   | 0.78   | 0.3    | 1.31   |

<sup>a</sup> K<sub>q</sub> was determined from the equation 

\[ F_0/F = 1 + K_{q}[\text{acrylamide}] \]

<sup>b</sup> K<sub>q</sub> was determined from the equation 

\[ τ_1/τ = 1 + K_{q}[\text{acrylamide}] \]

τ<sub>0</sub> and τ are the mean lifetimes in the absence and in the presence of quencher, respectively.

The mean lifetime, ⟨τ⟩, was calculated from: 

\[ ⟨τ⟩ = f_1τ_1 + f_2τ_2 \]

where f<sub>1</sub> and f<sub>2</sub> represent the fractional contributions of the intensities.

The apparent coalitional quenching constants were obtained from the K<sub>q</sub> values and the mean lifetimes in the absence of quencher.
As discussed under “Helix-Helix Interaction” below, the overall consistency of the data obtained with five independent Trp mutants supports this assumption.

Properties of the Helical Domains—The spectroscopic results obtained with the mutants containing Trp in helices 1 and 5 indicate that the N-terminal and C-terminal helices of *L. migratoria* apoLp-III are characterized by a high hydration and/or conformational flexibility. This conclusion is amply supported by the UV absorption data, the accessibility of the Trp residues to acrylamide, the near UV CD, and the fluorescence anisotropy data.

The spectroscopic properties of the Trp residues in helices 2, 3, and 4 reveal interesting differences between the α-helices probed by these Trp residues. The Trp residues probing helices 2 and 4 reside in a clearly nonpolar environment. However, the mobility of the Trp residue in helix 4 is hindered by the tertiary structure of the protein to a greater extent than the mobility of the Trp residues to acrylamide, the near UV CD, and the fluorescence anisotropy data.

The results obtained suggest that helix 3 has the lowest conformational flexibility of all helices.

Helix-Helix Interaction—We have also analyzed the results in the context of the entire molecule to find out whether or not there was a correlation between the properties of the helices and their topological relationship in the tertiary structure of the protein. This analysis provided additional and interesting information. Thus, there is an apparent correlation in the mobility of the helices as we move from helix 3 toward any of the ends of the polypeptide chain. The flexibility increases as we move away from helix 3. This profile of flexibility suggests that helices 2, 3, and 4 constitute a well packed domain, whereas helices 1 and 5 would constitute a more relaxed protein domain. The reason for the higher conformational flexibility of helices 1 and 5 is difficult to assess. However, it is possible that the parallel arrangement of these two helices, which is an
Conformational Change upon Binding to Lipid

Intrinsic consequence of the five-helix bundle structure, could contribute part of the energy of destabilization required for the conformational flexibility. Several studies have shown the importance of the helix-dipole on the stabilization of protein structure (28, 29). The high conformational flexibility of helices 1 and 5 could be due, at least in part, to the repulsion between the helical dipoles of helices 1 and 5.

The correlation found between the location and the mobility of the indole groups also indicates that the properties of the Trp residues are conditioned by the interhelical interactions. Therefore, to certain extent, the properties of the Trp residues are a measure of the interhelical interactions. Isolated α-helices are only marginally stable, and their stability in proteins is due to the tertiary interactions. In a simplified approximation, we can assume that the properties of a given α-helix are dominated by its interactions with the closest neighboring helices. Under this assumption and taking into account the flexibility profile observed, and the configuration of the helices in the up and down α-helical bundle of apoLp-III, the interhelical interactions can be ordered according to their apparent stabilizing strengths as: helix 3-helix 4 > helix 2-helix 3 > helix 4-helix 1 ~ helix 5-helix 2 > helix 1-helix 5.

Model for the Opening of the Tertiary Structure and the Exposure of the Hydrophobic Core of the Apolipoprotein—It is generally accepted that, in order to adopt the lipid-bound state, an apolipoprotein must expose its hydrophobic domains to the lipid. On the basis of the crystal structure of the protein (9), Breiter et al. postulated that the five-helix bundle undergoes a lipid-triggered opening at putative hinges domains located between helices 2 and 3 and between helices 4 and 5. This opening would expose a large extent of the hydrophobic interior of the protein allowing the lipid-protein interaction (Fig. 8).

The fact that the two most mobile helices, helices 1 and 5, are in contact induced us to speculate that this two-helix domain could constitute a gate to the hydrophobic core of the apolipoprotein molecule. Thus, it is possible that the weakly stabilized interaction between helices 1 and 5 could, in the presence of an appropriate lipid surface, lead to the exposure of the hydrophobic domains of the helices and the formation of the lipid-bound state. A schematic representation of this alternative model is presented in Fig. 8.

The mechanism of exposure of the hydrophobic core that we are suggesting for apoLp-III is substantially different to that proposed earlier by Breiter et al. (9). This former mechanism would imply a major breakdown of interactions along the interface between helices 2 and 3 as well as along the interface between helices 1 and 4. However, our study indicates that these pairs of helices would be more stabilized than the helix1-helix5 pair. Therefore, the possibility that the exposure of the protein core be preceded by the separation of the highly mobile helices 1 and 5 appears more reasonable. This type of conformational change would be more likely to occur because it would not require splitting apart the interhelical contacts between helices 2 and 3, and 1 and 4. A major separation would only affect the contacts between helices 1 and 5, which already appear to be the least stabilized α-helical domains in the lipid-free state.

An indirect support to our model is provided by the fact that tethering the helix bundle by cross-linking of the loops connecting helices 1 and 2 and helices 3 and 4 did not prevent the apolipoprotein molecule from interacting with DMPC and forming recombinant lipoprotein particles (15). This result is consistent with the model presented here. Thus, if the opening of the tertiary structure, and the exposure of hydrophobic core of the protein, occurs through the helix1-helix5 interface, the disulfide bond engineered by Narayanaswami et al. (15), would not prevent the required conformational change.

Acknowledgment—We thank Dr. Steve White for preparation of Fig. 1.

REFERENCES
1. Segrest, J. P., de Loof, H., Dohlman, J. G., Brouillette, C. G., and Anantharamaiah, G. M. (1990) Proteins 8, 103–117.
2. Segrest, J. P., Jones, M. K., de Loof, H., Brouillette, C. G., Venkatachalapathi, Y. V., and Anantharamaiah, G. M. (1992) J. Lipid Res. 33, 141–166.
3. Atkinson, D., and Small, D. M. (1986) Annu. Rev. Biophys. Chem. 15, 403–456.
4. Eisenberg, S. (1990) Curr. Opin. Lipidol. 1, 205–215.
5. Pownall, H. J., and Goto, A. M., Jr. (1992) in Structure and Function of Apolipoproteins (Rosenrue M., ed.) pp. 1–32, CRC Press, Boca Raton, FL.
6. Kawooya, J. K., Meredith, S. C., Wells, M. A., Kezby, F. J., and Law, J. H. (1986) J. Biol. Chem. 261, 13588–13591.
7. Blacklock, B. J., and Ryan, R. O. (1994) Insect Biochem. Mol. Biol. 24, 855–873.
8. Soulages, J. L., and Wells, M. A. (1994) Adv. Protein Chem. 45, 371–415.
9. Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., and Holden, M. (1991) Biochemistry 30, 603–608.
10. Soulages, J. L., and Wells, M. A. (1994) Biochemistry 33, 3245–3251.
11. Soulages, J. L., Salamon, Z., Wells, M. A., and Tollin, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5650–5654.
12. Soulages, J. L., van Antwerpen, R., and Wells, M. A. (1996) Biochemistry 35, 5191–5198.
13. Soulages, J. L., and Bendavid, O. (1998) Biochemistry 37, 10203–10210.
14. Weisgraber, K. H. (1994) Adv. Protein Chem. 45, 249–302.
15. Narayanaswami, V., Wang, J., Kay, C. M., Scara, D. G., and Ryan, R. O. (1990) J. Biol. Chem. 271, 26855–26862.
16. Soulages, J. L., Pennington, J., Bendavid, O., and Wells, M. A. (1998) Biochem. Biophys. Res. Commun. 243, 272–276.
17. Elwell, M. L., and Schellman, J. A. (1977) Biochim. Biophys. Acts 494, 367–383.
Conformational Change upon Binding to Lipid

18. Weers, P. M. M., Kay, C. M., Oikawa, K., Wientzek, M., van der Horst, D. J., and Ryan, R. O. (1994) Biochemistry 33, 3617–3624
19. Weers, P. M., Wang, J., Van der Horst, D. J., Kay, C. M., Sykes, B. D., Ryan, R. O. (1998) Biochim. Biophys. Acta 1393, 99–107
20. Soulages, J. L. (1998) Biophys. J. 75, 484–492
21. Demchenko, A. P. (1986) Ultraviolet Spectroscopy of Proteins, Springer-Verlag, Berlin
22. Burstein, E. A., Vedenkina, N. S., and Ivkova, M. N. (1973) Photochem. Photobiol. 18, 263–279
23. Efkin, M. R., and Ghiron, C. A. (1987) Biochim. Biophys. Acta 911, 343–349
24. Lakowicz, J. R., Zelent, B., Gryczynski, I, Kusba, J., Johnson, M. L. (1994) Photochem. Photobiol. 60, 205–214
25. Strickland, E. H. (1974) CRC Crit. Rev. Biochem. 2, 113–175
26. Wang, J., Gagné, S. M., Sykes, B. D., and Ryan, R. O. (1997) J. Biol. Chem. 272, 17912–17920
27. Sreerama, N., Venyaminov, S. Y., and Woody, R. W. (1999) Protein Sci. 8, 370–380
28. Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., and Baldwin, R. L. (1987) Nature 326, 563–567
29. Sali, D., Bycroft, M., and Fersht, A. R. (1988) Nature 335, 740–743