# Interaction of the α-Helices of Apolipophorin III with the Phospholipid Acyl Chains in Discoidal Lipoprotein Particles: A Fluorescence Quenching Study†

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*Received May 9, 2001*

ABSTRACT: Quenching of tryptophan fluorescence by nitroxide-labeled phospholipids and nitroxide-labeled fatty acids was used to investigate the lipid-binding domains of apolipophorin III. The location of the Trp residues relative to the lipid bilayer was investigated in discoidal lipoprotein particles made with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and five different single-Trp mutants of apoLp-III. A comparison of the quenching efficiencies of phospholipids containing nitroxide groups at the polar head, and at positions 5 and 16 of the *sn*-2 acyl chain, indicated that the protein is interacting with the acyl chains of the phospholipid along the periphery of the bilayer of the discoidal lipoprotein. *N*-Bromosuccinimide readily abolished 100% of the fluorescence of all Trp residues in the lipid-bound state. Larger quenching rates were observed for the Trp residues in helices 1, 4, and 5 than for those located in helices 2 and 3, suggesting differences between the interaction of these two groups of helices. However, the extent of Trp fluorescence quenching observed in lipoproteins made with any of the mutants was comparable to that reported for deeply embedded Trp residues, suggesting that all Trp residues interact with the phospholipid acyl chains. This study provides the first experimental evidence of a massive interaction of the α-helices of apoLp-III with the phospholipid acyl chains in discoidal lipoproteins. The extent of interaction deduced is consistent with the apolipoprotein adopting a highly extended conformation.

Exchangeable apolipoproteins represent a group of proteins characterized by their ability to reversibly bind to lipoprotein lipid surfaces. These apolipoproteins play a key role in the transport of lipids among tissues as well as in the regulation of lipid metabolism (*1*). Apolipophorin III[1] is a typical exchangeable apolipoprotein that is found in the hemolymph of insects in a lipid-free state or bound to the major lipoprotein, lipophorin (*2*, *3*). The study of the structure−function relationship of *Locusta migratoria* apoLp-III is of interest because the knowledge of the crystal structure of apoLp-III offers the possibility of relating the structure of an apolipoprotein to its function. ApoLp-III shares many physical−chemical properties with exchangeable apolipoproteins of humans and other vertebrates (*2*, *4*). The similarity between the properties of insect and vertebrate exchangeable apolipoproteins suggests that elucidation of the mechanism of interaction of apoLp-III with lipids would be useful to the understanding of the function of apolipoproteins in general. ApoLp-III from *Locusta migratoria* is the only full-length exchangeable apolipoprotein whose structure was elucidated by X-ray crystallography (*5*). An almost identical folding pattern was found for *Manduca sexta* apoLp-III, whose structure has been solved by NMR (*6*). The structure apoLp-III is represented as a bundle of five amphipathic α-helices. A complete understanding of the relationship between the structure and function of exchangeable apolipoproteins requires knowledge of the structure of both the lipid-free and the lipid-bound apolipoprotein. Contrarily to the case of lipid-free apoLp-III, the structure of this protein in the lipid-bound state is unknown, and elucidation of the topology and structure of exchangeable apolipoproteins in the lipid-bound state represents a major challenge. One of the common properties of exchangeable apolipoproteins resides in their ability to form discoidal lipoprotein particles. On the basis of the size and stoichiometry of discoidal lipoproteins, and the surface properties of the apolipoproteins, it has been postulated, and generally accepted, that the apolipoproteins spread along the periphery of the lipid disks, interacting with the phospholipid acyl chains. This model was first described for apoA-I (*7*) and later extended to apoE (*8*), and apoLp-III (*9*). This model is consistent with the properties of the proteins and the lipoprotein complexes. However, it has not been supported by experimental evidence showing the interaction of specific helices with specific domains of the lipid structure. This study represents an attempt to characterize further the structure of an apolipoprotein in discoidal lipoproteins by a combination of site-directed mutagenesis and Trp fluorescence quenching.

A suitable and highly used approach to study the location of fluorophores in membranes resides in analyzing fluores-

† This research was supported by an ARA Award to J.L.S., by NIH Grant GM 55622, and by OSU Oklahoma Agricultural Experiment Station.

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[1] Abbreviations: Apo, apolipoprotein; apoLp-III, apolipophorin III; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Tempo-PC, 1,2-dioleoyl-*sn*-glycero-3-[4-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl; 5-doxyl-PC, 1-palmitoyl-2-(5-doxyl)stearoylphosphatidylcholine; 16-doxyl-PC, 1-palmitoyl-2-(16-doxyl)-stearoylphosphatidylcholine; NBS, *N*-bromosuccinimide; TOE, tryptophan octyl ester.

10.1021/bi010949d CCC: $20.00 
Published on Web 11/01/2001

cence quenching of the fluorophores with quenchers attached at different depths along the acyl chains of the phospholipids (*10*−*12*). This approach has been used to locate Trp residues in membrane-inserted acetylcholine receptor (*13*), diphtheria toxin (*14*), and several other proteins (*15*−*17*), including human apolipoprotein A-I (*18*).

Five single-Trp mutants of apoLp-III, each of them containing a Trp residue in a different α-helix, were used in this study. The Trp residues were located on the nonpolar side of the amphipathic α-helices. Several spectroscopic properties of these mutants in the lipid-free state (*19*) and bound to discoidal lipoproteins particles of DMPC (*20*) have been recently reported, including the accessibility of the Trp residues to the aqueous quencher acrylamide. In the present study we extended our investigation of the interaction of the helical domains of apoLp-III with the lipid surface by determining the accessibility of different Trp residues to different lipid-soluble quenchers. For this purpose we determined and compared the quenching efficiencies of a series of nitroxide-labeled phospholipids and doxyl fatty acids.

## EXPERIMENTAL PROCEDURES

*Materials.* Spin-labeled PCs (Tempo-PC, 5SLPC, and 16SLPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Acrylamide was purchased from Bio-Rad Laboratories (Richmond, CA). Recombinant enterokinase and chromatographic supplies for protein purification were purchased from Novagen (La Jolla, CA). DE52 was obtained from Whatman. BCA protein determination kit was purchased from Pierce (Piscataway, NJ). *N*-Bromosuccinimide was from Sigma (St. Louis, MO).

*Site-Directed Mutagenesis, Protein Expression, and Purification of apoLp-III.* Site-directed mutagenesis was carried out as previously described (*19*) with the commercial kit QuickChange (Stratagene) and a pET-32a plasmid (Novagen, Madison, WI) containing the insert of *Locusta migratoria* apoLp-III. Thioredoxin−apoLp-III fusion protein was isolated from the bacteria and purified by Ni affinity chromatography (*21*). The fusion protein was cleaved with enterokinase, and apoLp-III was purified from the cleavage reaction by Ni affinity chromatography and anion-exchange chromatography in DE52.

*Preparation of Lipoproteins*. Discoidal lipoproteins were obtained by the method of Jonas et al. (*22*). The initial amount of protein to be used in the preparation of the lipoproteins was quantitated from the absorbance at 280 nm in the presence of 2 M GdnHCl ($\epsilon = 6000\ M^{-1}\ cm^{-1}$). A thin film of POPC, or a mixture of POPC and a given lipid quencher, was solubilized by addition of 50 mM Tris buffer, pH 7.8, containing cholate, and an amount of protein such that the final lipid:protein molar ratio was 260:1. To remove the cholate and obtain the lipoproteins, the mixture was extensively dialyzed during 48 h against 16 L (4 × 4 L) of buffer containing no detergent. Using this proportions, we obtained a lipoprotein preparation that showed no aggregates as judged by UV-absorption spectroscopy and FPLC gel-filtration chromatography on Superose 6 (Pharmacia). Analysis of the lipoprotein samples by gel-filtration chromatography also indicated the absence of lipid-free protein. After dialysis the concentration of protein was determined by the bicinchoninic acid method (BCA).

*Gel-Filtration Chromatography*. The size of the lipoprotein particles was estimated by gel-filtration chromatography on Superose 6. The column was calibrated with blue dextran 2000, DL-tryptophan, and the following standard proteins with their corresponding hydrated diameters: albumin, 7.1 nm; aldolase, 8.2 nm; catalase, 10.4 nm; ferritin, 12.2 nm; and thyroglobulin, 17.0 nm. The chromatography was run in 50 mM potassium phosphate buffer, pH 7.4, containing 250 mM NaCl at a flow rate of 0.5 mL/min.

*Nondenaturing Gel Electrophoresis*. Electrophoresis under nondenaturing conditions was carried out in 4−20% polyacrylamide gels with Pharmacia high molecular weight markers (*23*).

*Steady-State Fluorescence Studies*. Tryptophan fluorescence spectra were recorded with an ISS K2 fluorescence spectrophotometer. The emission spectra of the lipoprotein samples were acquired at 26 °C in 4 mm path-length cells. Quenching by nitroxide-labeled phospholipids, or fatty acids, was determined with the integrated emission intensity of Trp in the 300−450 nm range. Samples were excited at 280 nm with 0.5 mm slits for excitation and emission monochromators (8 nm bandwidth). Because apoLp-III does not have Tyr residues, the spectra of the lipoprotein particles contained no contributions from Tyr fluorescence. The optical density at the excitation wavelength (280 nm) was similar and relatively low for all samples ($OD^{1cm} \sim 0.04$). Therefore, the correction for inner filter effect was not necessary. Data were corrected for scattering as indicated under Results.

TOE quenching experiments were carried out under conditions identical to those reported by Abrams and London (*16*). A 1.5 μM solution of TOE was titrated with multilamellar liposomes of POPC containing 15% either Tempo-PC, 5-doxyl-PC, or 16-doxyl-PC. The concentration of lipid was increased until the fluorescence signal leveled off (∼300 μM lipid). The intensities of the plateaus measured at 336 nm were used to estimate the quenching efficiencies of the nitroxide phospholipids.

*Reaction with N-Bromosuccinimide.* A microliter aliquot of a 1 mM solution of NBS was directly added to the fluorescence cell containing the lipoprotein particles. The molar ratio of NBS to protein was 5:1. Fluorescence spectra were recorded immediately (1 min spectrum) and 10 min after the addition of NBS.

*Quenching Studies with Doxylstearic Acids.* Lipoproteins samples were titrated by addition of microliter aliquots of a 1 mM ethanolic solution of either 5-doxylstearic acid or 16-doxylstearic acid. Before the acquisition of the spectra, the samples were stirred and incubated for 10 min. Longer incubation times did not change the fluorescence spectrum or quenching efficiency. The quenching data were obtained in samples of nearly identical concentration of phospholipid (0.24 mg of POPC/mL). The potential effect of ethanol and/or the fatty acids on the structure of the protein and/or lipoprotein was controlled by circular dichroism. No changes in the CD spectrum were observed between the samples containing no additions or 7.5 mol % doxyl fatty acid. The acquisition parameters of the fluorescence studies were indicated above. The data were fitted to a monoexponential decay model with the assumption that a fraction of the Trp residues were not accessible to the quencher. This model is suitable if we consider that different conformations of binding are possible, and some of them will not be quenchable at

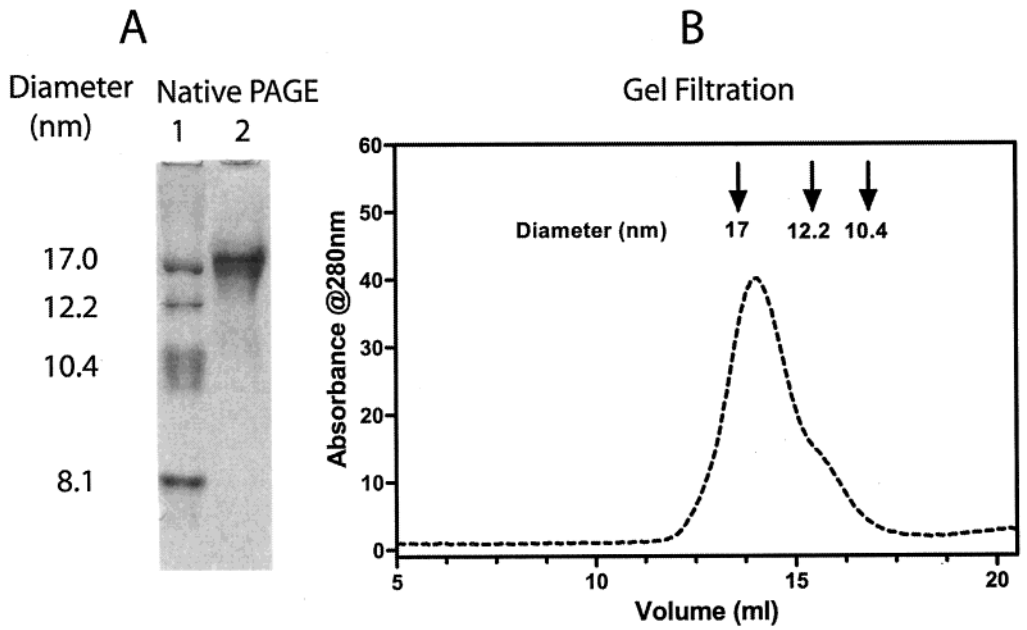


FIGURE 1: Lipoprotein homogeneity and size. (A) Coomassie Blue-stained polyacrylamide gel (4−20%) of discoidal lipoproteins containing 260 molecules of phospholipid per molecule of apoLp-III (lane 2). The electrophoresis was carried out under nondenaturing conditions. Lane 1 shows the diameters of the molecular size markers (thyroglobulin, ferritin, catalase, and lactate dehydrogenase). (B) Typical Superose 6 chromatographic profile of the lipoprotein particles used in this study. The arrows indicate the elution volumes of the molecular markers and their corresponding hydrated diameters (thyroglobulin, ferritin, and catalase).

all. Under this assumption, the magnitude of the quenching constant is inversely related to the distance of maximal approach of the quenchable residues, and the data can be fitted to an equation of the form

$$\Delta F/\Delta F_{max} = (F - F_{min})/(F_0 - F_{min}) = e^{-(K_i C)}$$

where $K_i$ is the quenching constant, whose magnitude depends on the concentration units used for the concentration of quencher, $C$. We expressed the concentration in moles of quencher added per mole of PC.

*Circular Dichroism.* The CD spectra of the samples used in the fluorescence study were acquired in a Jasco-715 CD instrument with a 0.1 cm path-length cell. The spectra were acquired at 24 °C every 1 nm, with 1 s averaging time per point and a 1 nm band-pass.

## RESULTS

Five single-Trp mutants were designed and expressed to study the interaction of the α-helices of apoLp-III with the lipid molecules in reconstituted lipoproteins. Each of these mutants contains a single Trp residue in a different α-helix, and in every case the Trp residue resides in the nonpolar face of the amphipathic helix. We have named each of these mutants according to the helix where the Trp residues are located. Thus, Trp-H1(W@19), Trp-H2 (W@49), Trp-H3 (W@78), Trp-H4 (W@113), and Trp-H5 (W@135) will refer to the mutants containing single Trp residues in helices 1, 2, 3, 4, and 5, respectively. Trp fluorescence was used to probe the interaction of the amphipathic α-helices of apolipophorin III with the lipid surface in a model lipoprotein system. The ability of water-soluble (NBS) and lipid-soluble molecules (nitroxide-labeled phosphatidylcholines and stearic acid) to quench the Trp fluorescence was studied and analyzed to gather information about the location of individual α-helices in discoidal particles and their interaction with the lipid structure.

*Characterization of the Lipoprotein Particles.* The homogeneity and size of the lipoprotein particles made with different mutants and POPC were studied by nondenaturing gradient gel electrophoresis and gel-filtration chromatography (Figure 1). The estimated size of the particles was different depending on the method used. The apparent size of the lipoproteins was always smaller by gel-filtration chromatography (diameter 16 nm) than by electrophoresis (diameter 17−18 nm). However, both methods showed that the lipoprotein preparations were reasonably homogeneous, containing one major population of particles (Figure 1). Similar results were obtained with lipoprotein preparations made with any of the five single-Trp mutants used in this study. The size of the lipoprotein particles was similar to the size reported by other research groups for discoidal lipoproteins made with apoLp-III and phosphatidylcholine (*9*, *24*). These lipoproteins contain five or six molecules of apoLp-III per particle (*9*, *24*).

*Effect of N-Bromosuccinimide on the Trp Fluorescence Spectra of ApoLp-III in Discoidal Particles of POPC.* NBS is a reagent used to probe the accessibility of a Trp residue in soluble (*25*) and membrane-bound protein preparations (*17*, *26*). We used NBS to probe the accessibility of the Trp residues in the lipid-bound state of apoLp-III. The effect of NBS on the fluorescence intensity of each single-Trp mutant was determined in reconstituted lipoprotein particles made with POPC. Figure 2A shows typical fluorescence spectra of the lipoproteins particles made with one of the single-Trp mutants in the absence and in the presence of NBS. It can be observed that NBS abolished completely the fluorescence spectrum. No differences were found between the spectra obtained 2 or 10 min after the addition of NBS, indicating that the reaction with NBS was fast and complete

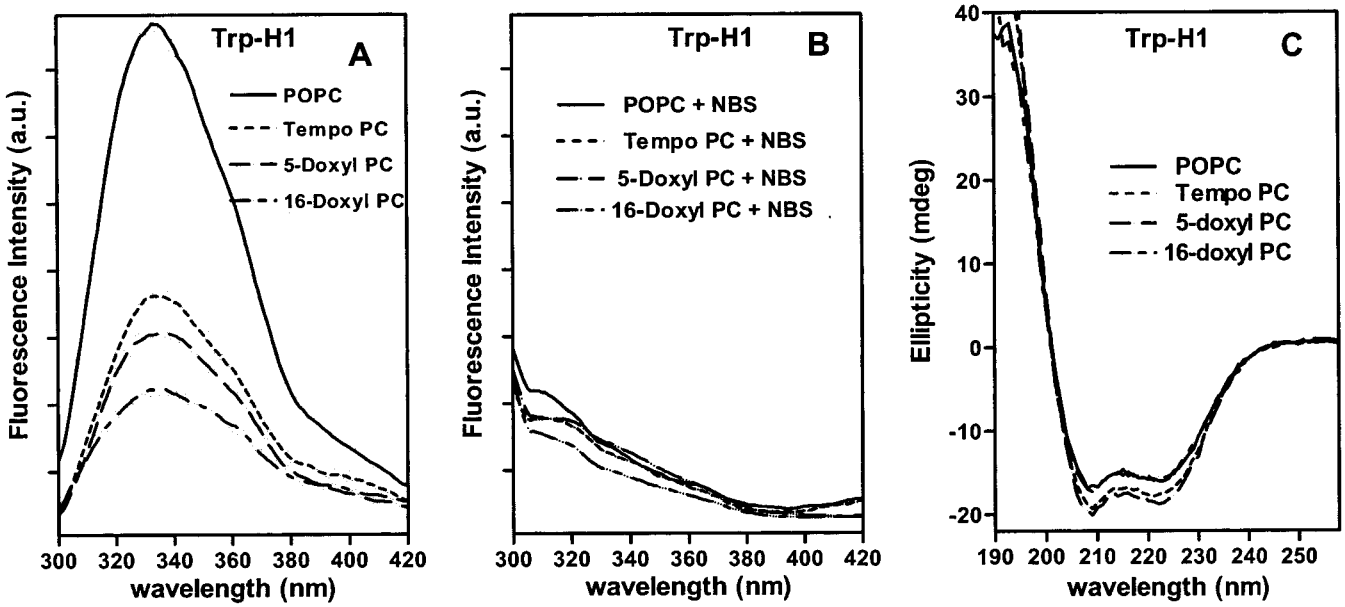


FIGURE 2: Fluorescence spectra of Trp-H1 in the presence of nitroxide-labeled phospholipids and NBS. Panel A shows the scattering-corrected fluorescence spectra of the Trp-H1 mutant in lipoprotein particles made with 100% POPC (labeled POPC) or with 85% POPC and 15% of the nitroxide labeled lipids (labeled Tempo-PC, %-doxyl-PC, and 16-doxyl-PC). The symbols corresponding to each sample are indicated in the figure. Panel B shows the spectra obtained after the addition of a 5-fold molar excess of NBS to protein to the samples shown in panel A. The *y*-axis has the same scale as in panel A. These spectra obtain the scattering- corrected spectra shown in panel A. Panel C shows the CD spectra of the same samples shown in panel A. The figure shows the raw data obtained in a 0.1 cm path-length cell. The concentration of protein, ~0.1 mg/mL, was slightly different for each sample.

within 2 min. NBS readily abolished 100% of the Trp fluorescence of all Trp mutants in reconstituted lipoprotein particles. In all cases, the reaction of NBS was equally efficient and fast, requiring less than 2 min for completion (data not shown). Moreover, the shape of the spectra obtained after the addition of NBS indicates that they reflect the contribution of scattering. The Trp residues located in any of the helices could not be distinguished by their reaction with NBS. These results indicate that the hydrophobic faces of the amphipathic α-helices of apoLp-III are readily exposed to NBS and suggest that none of the single Trp residues of the apoLp-III mutants resides in a transmembrane location.

*Spectral Corrections and Effect of the Quenchers on the Protein Structure.* One of the main quantitative limitations of fluorescence quenching studies in membrane preparations is given by the contribution of light scattering to the fluorescence spectrum. The small size of the discoidal lipoprotein particles, diameter < 20 nm, reduces considerably the impact of scattering on the fluorescence spectrum. However, even in this case, when the extent of quenching becomes significant, the effect of scattering must be taken into account in the calculation of the quenching efficiency. In doing this study we tested two approaches to remove the scattering. The first approach consisted of using, as a scattering correction factor, the spectrum obtained in lipoprotein particles prepared with a mutant containing no Trp residues (W113F/W128F). However, soon we realized that NBS was a highly efficient quencher for all the Trp mutants that could provide a fast and reliable method to correct each individual spectrum by use of its own background. Therefore, the data presented here were corrected by use of the spectra obtained after the addition of NBS.

Figure 2A shows the scattering-corrected fluorescence spectra of Trp-H1 in lipoprotein particles containing 100% POPC or 85% POPC and 15% either Tempo-PC, 5-doxyl-PC, or 16-doxyl-PC. A comparison with the spectra obtained after the addition of NBS, shown in Figure 2B, clearly shows that at high concentrations of quenchers the correction by scattering becomes significant.

The maximum concentration of nitroxide quencher used in our studies was 15 mol % (85% POPC). To study whether the presence of 15% of the nitroxide-labeled phospholipid could affect the structure of the proteins in the lipid-bound state, every sample was analyzed by circular dichroism to monitor changes in the secondary structure of the apolipoproteins. Figure 2C shows the original uncorrected CD spectra of the samples whose fluorescence spectra are shown in Figure 2A. It can be readily observed that the secondary structure of the proteins in lipoprotein samples containing 0% or 15% of any of the three nitroxide-labeled phospholipids was nearly undistinguishable. The small differences observed in the CD spectra are due to minor changes in protein concentration that take place during the dialysis. Similar results were obtained with all the mutants or at lower concentrations of nitroxide-labeled phospholipids (data not shown). In every case, and as previously reported for these mutants in reconstituted lipoprotein particles of DMPC (*20*), the proteins are highly α-helical in the lipid-bound state (~60% α-helical content).

*Trp Fluorescence Quenching by Nitroxide-Labeled Phospholipids.* The quenching efficiency of different quenchers was determined by comparing the fluorescence intensity (corrected for protein concentration and scattering) observed in lipoprotein particles made with no quencher (100% POPC) with the corrected fluorescence intensity of lipoproteins containing increasing concentrations of nitroxide-labeled phospholipid. To examine the location of the Trp residues relative to the lipoprotein bilayer surface, we compared the Trp fluorescence quenching efficiency of phospholipids carrying a nitroxide quencher at a shallow (Tempo-PC),

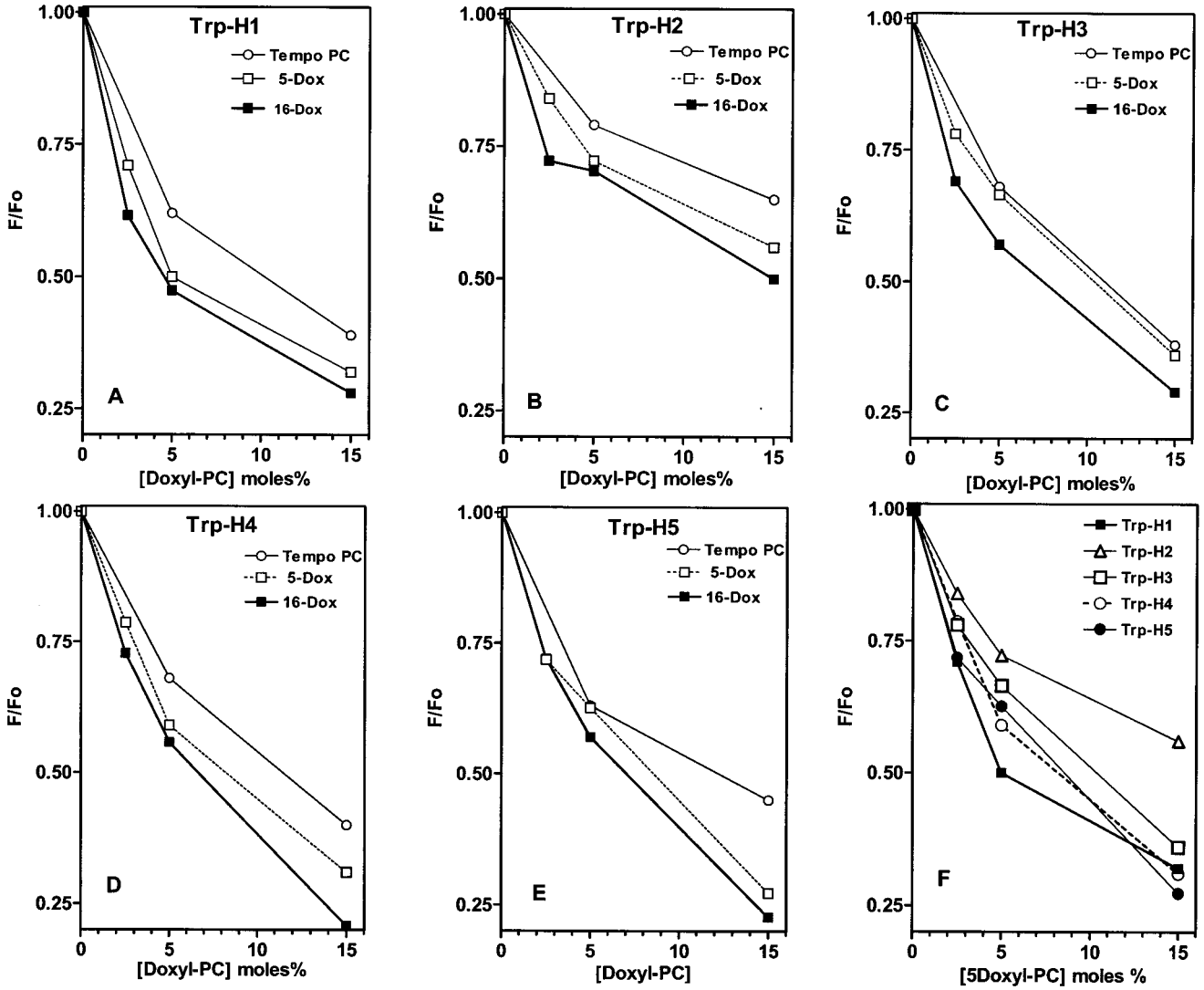


FIGURE 3: Nitroxide-PC quenching of single-Trp mutants of apoLp-III in discoidal lipoprotein particles. Panels A−E show the concentration dependence of the quenching of Trp fluorescence by three different nitroxide-labeled phosphatidylcholines. The quenchers with their respective symbols are indicated in the figure, and each panel (A−E) shows the data for a different single-Trp mutant. To allow a visual comparison of the quenching data obtained with different single-Trp mutants, panel F shows the quenching curves obtained for each of the five mutants with 5-doxyl-PC. Data represent the average of two independent samples simultaneously prepared.

medium (5-doxyl-PC), or deep (16-doxyl-PC) depth in the membrane.

Figure 3 (panels A−E) shows the quenching profiles obtained with the three nitroxide quenchers for each of the five single-Trp mutants. The first observation of this study shows that there is a clear pattern of quenching efficiency; 16-doxyl-PC showed the highest quenching efficiency of the nitroxide PCs for all the mutants, whereas the lowest quenching efficiency was observed for Tempo-PC. Although the difference in quenching efficiency between Tempo-PC and 16-doxyl-PC is large, only a small difference is observed between 5-doxyl-PC and 16-doxyl-PC. This pattern strongly suggests that the Trp residues, and therefore the helices, reside closer to the acyl chains than to the polar headgroups of the phospholipid molecules. Figure 3F shows the concentration dependence of the quenching efficiency of 5-doxyl-PC for all the mutants. The comparison of these data shows a significantly lower quenching efficiency for the Trp residue in helix 2 than for the Trp residues located in helices 1, 3, 4, and 5 (Figure 3F and Table 1). Similar conclusions are obtained when the data obtained with Tempo-PC or 16-doxyl PC are compared. On the other hand, only minor differences are observed between the quenching profiles of the mutants containing Trp residues in helices 1, 3, 4, and 5.

*Comparison with Previously Reported Data.* The nitroxide phospholipids used in this study have been amply used to study the depth of membrane insertion of Trp residues in proteins and several model systems (*14*−*16*, *27*−*29*). Table 1 shows some of the quenching data reported in other studies for shallow and deeply inserted Trp residues. We have obtained the nitroxide phospholipid from the commercial source used in most reported studies. However, to check the quality of the quenchers and provide a reference to which other studies could be related, we have also studied the efficiency of TOE fluorescence quenching by different nitroxide phospholipids. These data are included in Table 1. It can be seen that the quenching observed with apoLp-III mutants is higher than that of shallow Trp residues, such as TOE and cytochrome $b_5$. Also, whereas the quenching of shallow Trp residues is greater for 5-doxyl-PC than for 12- or 16-doxyl-PC, the opposite pattern is observed for all Trp mutants; a higher quenching efficiency is observed when the depth of

Table 1: Fluorescence Quenching by Nitroxide-Labeled Phospholipid of Apolipophorin III Mutants in Discoidal Lipoproteins of POPC: Comparison with Other Studies and Model Systems

| sample | $\lambda_{max}$[a] (nm) | Tempo-PC[b] ($F/F_0$, %) | 5-doxyl-PC ($F/F_0$, %) | 12-doxyl-PC ($F/F_0$, %) | 16-doxyl-PC ($F/F_0$, %) | $F_{Tempo}/F_{16Dox}$[c] |
|---|---|---|---|---|---|---|
| Trp-H1 | 332 | 39 | 32 | ND | 28 | 1.39 |
| Trp-H2 | 341 | 65 | 56 | ND | 50 | 1.30 |
| Trp-H3 | 339 | 38 | 36 | ND | 28 | 1.36 |
| Trp-H4 | 325 | 40 | 31 | ND | 21 | 1.90 |
| Trp-H5 | 327 | 45 | 26 | ND | 22 | 2.04 |
| TOE[d] | 336 | 18 | 34 (40) | (59) | 63 | 0.28 |
| Cyt b5[e] | 350 | ND | 64 | 72 | ND | |
| DT W206[f] | 338 | 70 | 58 | 44 | ND | |
| DT W281[f] | 338 | 80 | 70 | 55 | ND | |
| DT W364[f] | 334 | 49 | 41 | 25 | ND | |
| pLeu11[f] | 336 | 53 | 40 | 21 | ND | |
| pLeu 19[f] | 317 | 67 | 59 | 8.5 | ND | |
| pLeu23[f] | 328 | 68 | 67 | 15 | ND | |
| apoA-I[g] | 324-334 | ND | 54-80 | 40-67 | ND | |

[a] $\lambda_{max}$ is the wavelength of the fluorescence emission maximum. [b] $F/F_0$ is the ratio of fluorescence (percent) in lipoprotein particles containing 15% of the indicated nitroxide quencher to that in samples prepared without quencher (100% POPC). The data represent the average of two samples simultaneously prepared and analyzed. [c] $F_{Tempo}/F_{16dox}$ represents the ratio of fluorescence intensities obtained in lipoproteins containing 15% Tempo and lipoproteins containing 15% 16-doxyl-PC. The ratios were calculated from the data shown in the third and sixth columns of the table, respectively. [d] Shallow Trp residues: TOE data from this study and, between parentheses, from Abrams and London (*16*). [e] Data for cytochrome $b_5$ are from Abrams and London (*16*). [f] Deeply membrane-inserted Trp residues: Data for the Trp residues from diphtheria toxin (DT W206, DT W281, and DT W364) were reported by Malenbaum et al. (*29*); pLeu peptides were studied by Ren et al. (*28*). pLeu 19 has a Trp residue at the bilayer center, whereas pLeu 23 and pLeu 11 are off-center by ~2 and 5 Å, respectively. [g] The values indicate the range of $\lambda_{max}$ and $F/F_0$ values reported by Maiorano and Davidson (*18*) for two different single-Trp mutants in discoidal particles of different size.

the quencher is increased. Moreover, with any of the three quenchers, the quenching efficiency observed against the Trp mutants of apoLp-III is of the same order, or higher, than that reported for deeply embedded Trp residues. This fact suggests that the Trp residues of apoLp-III are very close to the acyl chains.

A comparison of the ratios of fluorescence quenching achieved with Tempo-PC and 16-doxyl-PC may also serve as a qualitative indicator of the penetration depth of the Trp residues (*10*, *11*). A comparison of the ratios obtained in this study for TOE and for the Trp mutants of apoLp-III (last column in Table 1) indicates that the Trp residues of all mutants are deeper into the bilayer structure than the Trp residue in TOE. Further distinctions can be seen if the ratios of quenching (Tempo-PC to 16-doxyl-PC) obtained with different mutants are compared. According to these ratios the Trp residues in helices 4 and 5 would represent a group of deeply embedded Trp residues, whereas the Trp residues in helices 1 and 3 would represent a group of intermediate penetration depths, followed by the Trp in helix 2 that would correspond to the shallowest Trp residue.

*Trp Fluorescence Quenching by Nitroxide-Labeled Stearic Acids.* Titration of the lipoproteins with doxyl fatty acids allows construction of quenching curves with a large number of data points from just one lipoprotein sample. This fact constitutes a great advantage as compared to the quenching curves obtained with nitroxide-labeled phospholipids, where, for practical reasons, only a small number of data points can be obtained, and each data point comes from a different sample. However, given the differences in the partition coefficients of different doxyl fatty acids, their use as depth-dependent quenchers is not straightforward and would require the independent determination of their membrane-binding constants. This problem has limited the use of doxyl fatty acids to explore the topology of membrane-bound proteins, and consequently, nitroxide-labeled phospholipids are used more often than doxyl fatty acids. Despite this problem, doxyl fatty acids can still be used to obtain a qualitative comparison of the distances of approach of different Trp residues to a membrane. In other words, we cannot use doxyl fatty acids to determine the depths of insertion of a Trp residue, but we can use any given doxylstearic acid to compare the relative distances of approach to the membrane of different Trp residues (different single-Trp mutants).

We extended our studies on the topology of the α-helices of apoLp-III in the lipid-bound state by determining the quenching curves of the five single-Trp mutants using 5-doxyl- and 16-doxylstearic acid. As expected from the study with nitroxide phospholipids, these two fatty acids quenched the fluorescence of the Trp mutants in the lipid-bound state. The upper panels of Figure 4 show the spectra of two of the mutants obtained at increasing concentration of 5-doxyl-SA, and the lower panels show the quenching curves obtained for each of the five mutants of apoLp-III with 5-doxyl-SA and 16-doxyl-SA. Comparison of the quenching constants obtained for each of the five single-Trp mutants with either 5-doxyl-SA or 16-doxyl-SA (Table 2) suggests that there are differences in the exposure to lipid of the Trp residues of different mutants. The rates of quenching suggest that the Trp residues in helices 1, 4, and 5 reside closer to the acyl chains of the bilayer disk than the Trp residues in helices 2 and 3.

*Emission Wavelength and Heterogeneity*. The position of the fluorescence maximum is sensitive to the polarity of the Trp residue environment, and for globular proteins there is a good correlation between the position of the maximum and the exposure of the indole group to the solvent (*30*). Ren et al. (*27*) also found a correlation between the position of the fluorescence maximum and the apparent depth of insertion of the Trp residues in bilayers, and they reported that the position of the maximum is shifted to the red as the Trp residue moves from the bilayer center toward the polar headgroups. We have recently reported the emission wavelength of the five single-Trp mutants in discoidal particles of DMPC and shown that the Trp residues in helices 2 and 3 emit at longer wavelengths than the residues in helices 1,

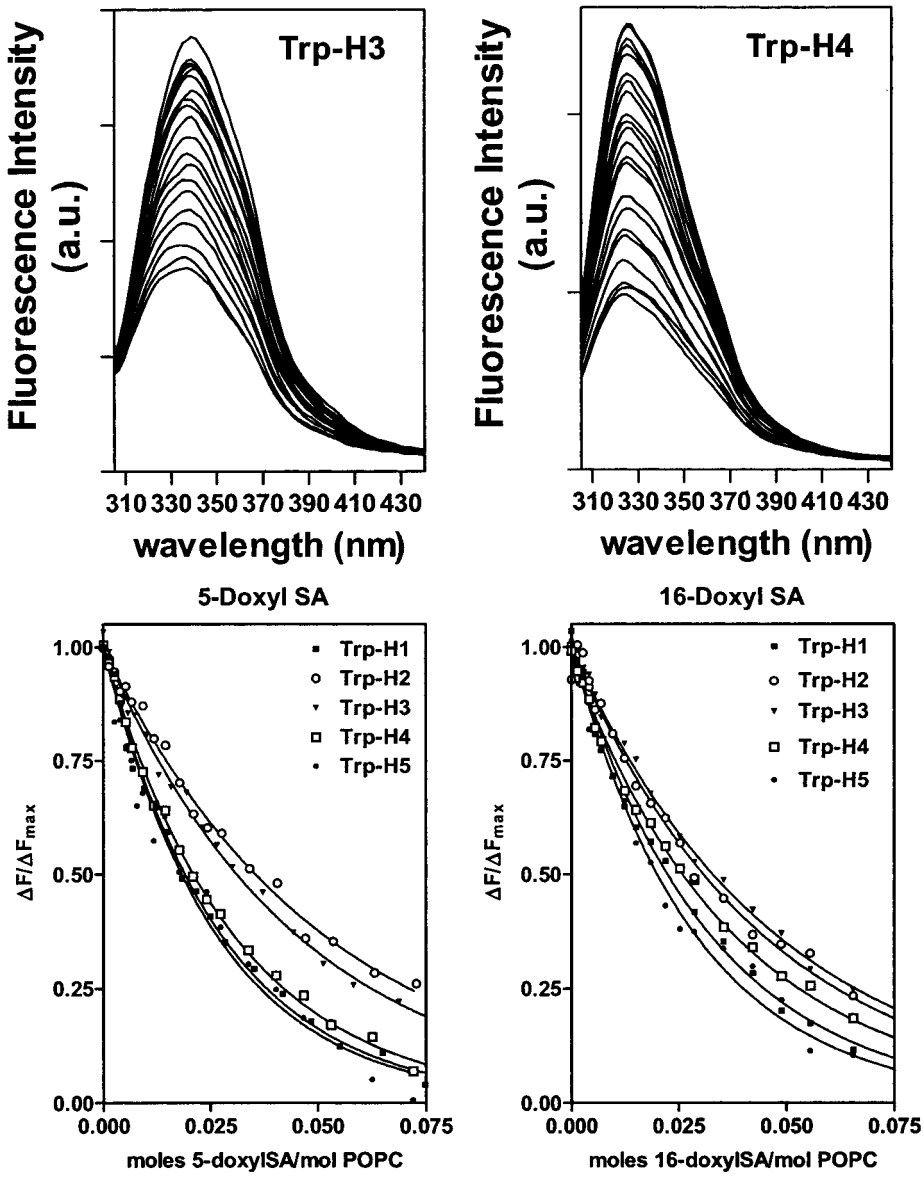


FIGURE 4: Trp fluorescence quenching by doxylstearic acids in discoidal lipoproteins of apoLp-III and POPC. The upper panels show the Trp emission spectra of Trp-H3 and Trp-H4 mutants in discoidal particles of POPC at increasing concentrations of 5-doxyl-SA. The quenching data were obtained in samples of nearly identical concentration of phospholipid (0.24 mg of POPC/mL). The samples were titrated with a stock solution of doxyl-SA in ethanol. The lower panels show the quenching curves obtained for each of the five mutants of apoLp-III with 5-doxyl-SA and 16-doxyl-SA, respectively. The curves represent the best fit of the data to the equation indicated under Experimental Procedures.

Table 2: Trp Fluorescence Quenching by Doxylstearic Acids in Discoidal Lipoproteins of ApoLp-III and POPC[a]

| | quenching constants | |
|---|---|---|
| protein | 5-doxylstearic acid | 16-doxylstearic acid |
| Trp-H1 | 36.3 ± 2.3 | 30.9 ± 2.6 |
| Trp-H2 | 19.4 ± 2.4 | 22.5 ± 2.9 |
| Trp-H3 | 22.1 ± 1.9 | 21.0 ± 2.1 |
| Trp-H4 | 32.9 ± 1.4 | 26.1 ± 1.5 |
| Trp-H5 | 37.8 ± 6.2 | 34.6 ± 3.3 |

[a] The quenching constants (±SE) are dimensionless because the concentration units of the quencher were expressed in moles of quencher per mole of phospholipid. The constants were obtained by nonlinear fitting of the data to a monoexponential decay as indicated under Experimental Procedures.

4, and 5. This pattern is also observed in discoidal particles of POPC (Table 1). A simple inspection of the data shown in Figure 5 clearly shows that the emission spectra of the Trp residues in helices 2 and 3 are significantly red-shifted as compared to the Trp residues in helices 1, 4, and 5. When the correlation reported by Ren et al. (*27*) is applied, this result indicates that the Trp residues in helices 2 and 3 would reside closer to the region of the polar headgroups of the phospholipid bilayer. On the same basis, the Trp residues in helices 4 and 5 would reside in a highly nonpolar environment, perhaps closer to the bilayer center.

To investigate the presence of multiple protein-binding configurations, we compared the quenched component spectra (spectrum in lipoproteins made with 100% POPC minus spectrum in the presence of 15% nitroxide PC) for each of the three quenchers. The rationale behind this comparison is based on the assumption that, in the presence of a heterogeneous population of quencher-accessible Trp residues, one would expect a red shift in the quenched spectra of the Trp residues as the position of the quenchers becomes closer to

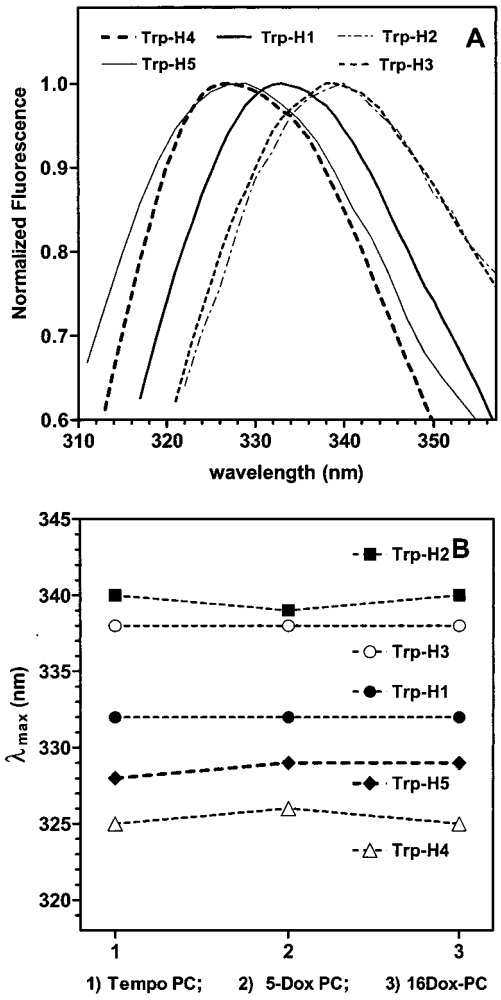


FIGURE 5: Emission wavelength and heterogeneity. Panel A shows the emission spectra of the single-Trp mutants in discoidal lipoproteins particles of POPC. Panel B shows the variation of $\lambda_{max}$ of the quenched fluorescence spectra with the position of the nitroxide group in the quencher. $\lambda_{max}$ values were obtained from the spectra of discoidal lipoproteins containing 15 mol % nitroxide PC. The symbols corresponding to each single-Trp mutant are indicated in the figure.

the membrane surface. A plot of the $\lambda_{max}$ values obtained for each of the Trp mutants with each of the three quenchers is shown in Figure 5. Interestingly, the $\lambda_{max}$ of the spectra of the quenched Trp residues does not seem to be dependent on the depth of the quencher. Thus, approximately the same $\lambda_{max}$ is obtained for any of the three quenchers, suggesting that the quenched Trp residues represent a considerably homogeneous population of fluorophores.

## DISCUSSION

One of the main features of exchangeable apolipoproteins resides in their ability to bind reversibly to the surface of lipoprotein particles. The transference of an apolipoprotein from the water-soluble, globular state to the lipid-bound state is believed to involve a large conformational change, where the hydrophobic sides of the amphipathic helices, initially buried in the protein core (lipid-free state), become exposed to the hydrophobic lipid domains in the lipid-bound state. Depending on the structure of the lipoprotein, two models are used to describe the interaction of the α-helices of exchangeable apolipoproteins with lipid surfaces. For spherical lipoproteins, it has been proposed that the amphipathic α-helices have an orientation parallel to the phospholipid monolayer surface (*31*). In this case, the hydrophobic faces of the helices are embedded in the phospholipid monolayer, whereas the hydrophilic faces remain at the lipid surface and strengthen the lipid−protein interaction by means of ionic interactions with the charged groups of the phospholipid. On the other hand, in discoidal lipoprotein particles the apolipoprotein is assumed to stabilize the lipoprotein particle by interacting with the acyl chains along the periphery of the lipid bilayer disk (*32*, *33*). The model of interaction for discoidal lipoproteins is supported by the size, stoichiometry, and geometry of the recombinant discoidal lipoprotein particles, and it has been proposed that both vertebrate apolipoproteins (*31*−*34*) and insect apolipoproteins (*4*, *9*, *35*) adopt an extended conformation interacting with the nonpolar acyl chains of the phospholipid bilayer. However, consistent with the properties of the apolipoproteins and the stability of the lipoprotein particles, the topological models adopted for discoidal lipoproteins have not been supported by data reporting on the interaction of specific regions of the protein with specific domains of the lipid bilayer. The studies presented here were directed to explore whether all, or some, of the amphipathic α-helices of apoLp-III interact with the lipid membrane in discoidal lipoprotein particles. To this purpose we have compared the quenching data of each of the five single-Trp mutants with quenching data previously reported for proteins or model peptides containing shallow and deeply embedded Trp residues.

For membrane-embedded fluorophores, depth-dependent quenching data are often analyzed by the parallax method developed by Chattopadhyay and London (*10*) or the distribution analysis developed by Ladokhin (*11*). We have not applied these methods to our data analysis because at least one of the assumptions on which they are based is not likely to be met by our system. These two models provide estimates of the depth of penetration of a fluorophore into a membrane under the assumption that the minimum distance of lateral separation between fluorophore and quencher is small and constant for fluorophores located at different depths. Because the amphipathic α-helices of the apolipoproteins are likely to surround the bilayer disk periphery, the concentration of the quencher seen by a Trp residue located in any α-helix will be highly dependent on the lateral separation between the lipid surface and the helix. Thus, for polypeptide chains bound to a lipid surface the quenching efficiency of a given quencher could be highly affected not only by the depth of the fluorophore but also by the lateral separation as well as by the orientation of the Trp residue at the membrane protein interface. Because of these considerations, we have not applied the parallax method or the distribution analysis commonly used to determine the penetration depth of Trp residues in a membrane bilayer. Despite these quantitative limitations, the quenching efficiency of nitroxide-labeled lipids is dependent on the average distance of separation between quencher and fluorophores. Therefore, we have used the quenching data to compare the relative distances of separation from the acyl chains of the bilayer of Trp residues located at different positions along the apoLp-III polypeptide chain. Also, in an attempt to determine which of the amphipathic α-helices of apoLp-III make a direct interaction with the lipid surface,

we have compared our quenching data with data previously reported for other membrane-bound proteins and model peptides.

*How Close to the Lipid Bilayer Are the Trp Residues?* The data in Table 1 show that the quenching efficiency of nitroxide PC observed with apoLp-III mutants is higher than that reported for shallow Trp residues (*16*) and of the same order, or higher, than that reported for deeply embedded Trp residues (*28*, *29*). The fact that we observed high efficiencies of quenching for all the quenchers and with each of the five single-Trp mutants of apoLp-III would suggest that the Trp residues of apoLp-III are very close to the acyl chains. Thus, the quenching efficiencies of nitroxide-labeled PC (15 mol %) observed with the single-Trp mutants of apoLp-III are higher than those reported for several single-Trp mutants of the transmembrane domain of diphtheria toxin (*29*) and of the same order as the quenching efficiencies of model peptides containing deeply embedded Trp residues (*28*). This comparison adds strong support to a model where apoLp-III would adopt an extended conformation in the lipid-bound state, in which all α-helices interact with the lipid surface. Further support for this model comes from the fact that because the α-helices are likely to surround the disk periphery, the effective concentration of quencher would be much lower for the Trp residues of apoLp-III than that seen by a Trp residue embedded in the membrane bilayer. Thus, for a Trp residue located near the disk periphery, close to the acyl chains but not inserted into the bilayer, we would expect an effective concentration of quencher at least 50% lower than that observed for Trp residues with transmembrane locations. If this were the case of the Trp residues of apoLp-III, we should have observed a significantly lower quenching efficiency as compared to Trp residues embedded in a bilayer. Therefore, the high quenching efficiency observed appears to be indicating a close interaction of the nonpolar side of the amphipathic α-helices with the acyl chains of the phospholipid. This type of interaction would make the effective concentration of quencher almost as high as that seen by transmembrane Trp residues. According to this rationale, it would appear that all helices of apoLp-III are in close contact with the hydrophobic periphery of the discoidal bilayer.

On the other hand, the comparison among the quenching constants deduced from the study with 5- and 16-doxylstearic acids suggests that there are differences between the accessibility of the quenchers to the Trp residues located in different α-helices. Thus, the study with both fatty acid quenchers indicates that the Trp residues in helices 2 and 3 have a lower exposure to the nitroxide groups than the Trp residues in helices 1, 4, and 5. This result is consistent with a previous report from our lab where it was shown that the Trp residues in helices 2 and 3 are more accessible to water-soluble quenchers than the Trp residues in helices 1, 4, and 5 (*20*). Also, the differences between the quenching constants are correlated to some extent with the red shift of the fluorescence maximum of the Trp residues in helices 2 and 3. These results suggest that there is a difference between the interactions of helices 2 and 3 with the lipid and the interaction of helices 1, 4, and 5. One likely explanation would be that helices 2 and 3 reside at a longer average distance from the acyl chains of the bilayer than the remaining helices. On the other hand, the large efficiency of quenching observed against the Trp residues located in helices 2 and 3, as compared to the quenching efficiencies reported for other membrane-embedded Trp residues, suggests a close interaction of these Trp residues with the acyl chains. Thus, we cannot conclude that helices 2 and 3 do not interact with the lipid surface. It is difficult to identify the reasons for the lower quenching constants or quenching efficiencies found with these two Trp mutants. Perhaps, the differences observed are only due to the degree of penetration into the lipid matrix of the Trp residues located in helices 2 and 3. A partial embedding of these Trp residues could be the cause of their sensitivity to acrylamide quenching previously reported (*20*), the position of the fluorescence maximum, and the lower quenching rate observed with doxylstearic acids (this study). Interestingly, and supporting the possibility of a partial embedding, there is a correlation between the position of the Trp residues relative to the hydrophobic face of the amphipathic α-helices and the accessibility of the Trp residues to lipid quenchers. Figure 6 shows the helical wheel diagrams of the five helices of apoLp-III. This diagram shows that the residues in helices 1, 4, and 5 are located near the center of the hydrophobic face of the amphipathic α-helices, whereas the Trp residue in helix 3 is at the interface between the polar and hydrophobic faces. The location of the Trp in helix 3 could explain its longer wavelength of emission as compared to the emission of the Trp residues in helices 1, 4, and 5. On the other hand, a simple inspection of the helical wheel of helix 2 shows that this helix has a small hydrophobic face and the Trp residue is leaning toward the edge of the hydrophobic face. Therefore, this feature of helix 2 and the location of the Trp residue in the helical wheel could also explain why, when compared to the Trp in helices 1, 4, and 5, the Trp residue in helix 2 is characterized by a red-shifted emission spectrum. The location of the Trp relative to the polar and hydrophobic faces of the helices could explain the lower quenching efficiency of the nitroxide phospholipids against the Trp residues located in helices 2 and 3. The lower accessibility of the Trp residues in helices 2 and 3 compared to the lipid quenchers could also be explained by assuming that these two helices constitute a helical hairpin that is closely but loosely bound to the discoidal lipid bilayer. This consideration is based on a recent study on the structure of apoA-I in discoidal lipoproteins. Córsico et al. (*37*) showed that a two-helix central domain of human apoA-I is loosely bound in discoidal lipoproteins of POPC (*37*).

*Doxyl-PC Data Supports a Model Where the Apolipoprotein Surrounds the Lipid Bilayer of the Discoidal Lipoprotein.* The comparison of the Trp-quenching efficiency of phospholipids carrying a nitroxide-labeled PC at a shallow, intermediate, and deep depth in the membrane allows inferring the location of a Trp residue relative to the membrane surface (*16*, *27*, *28*). Tryptophan octyl ester (TOE) has been used as model compound for a Trp residue located near the membrane surface. Confirming previous studies, we observed that the fluorescence of TOE was quenched more efficiently by nitroxide phospholipids when the quencher was closer to the membrane polar headgroups (Table 1). Similarly, for cytochrome $b_5$ (*16*), which has a Trp residue inserted near the membrane surface, a decreasing efficiency was observed as the depth of the quencher was increased. On the other hand, studies of model systems containing Trp

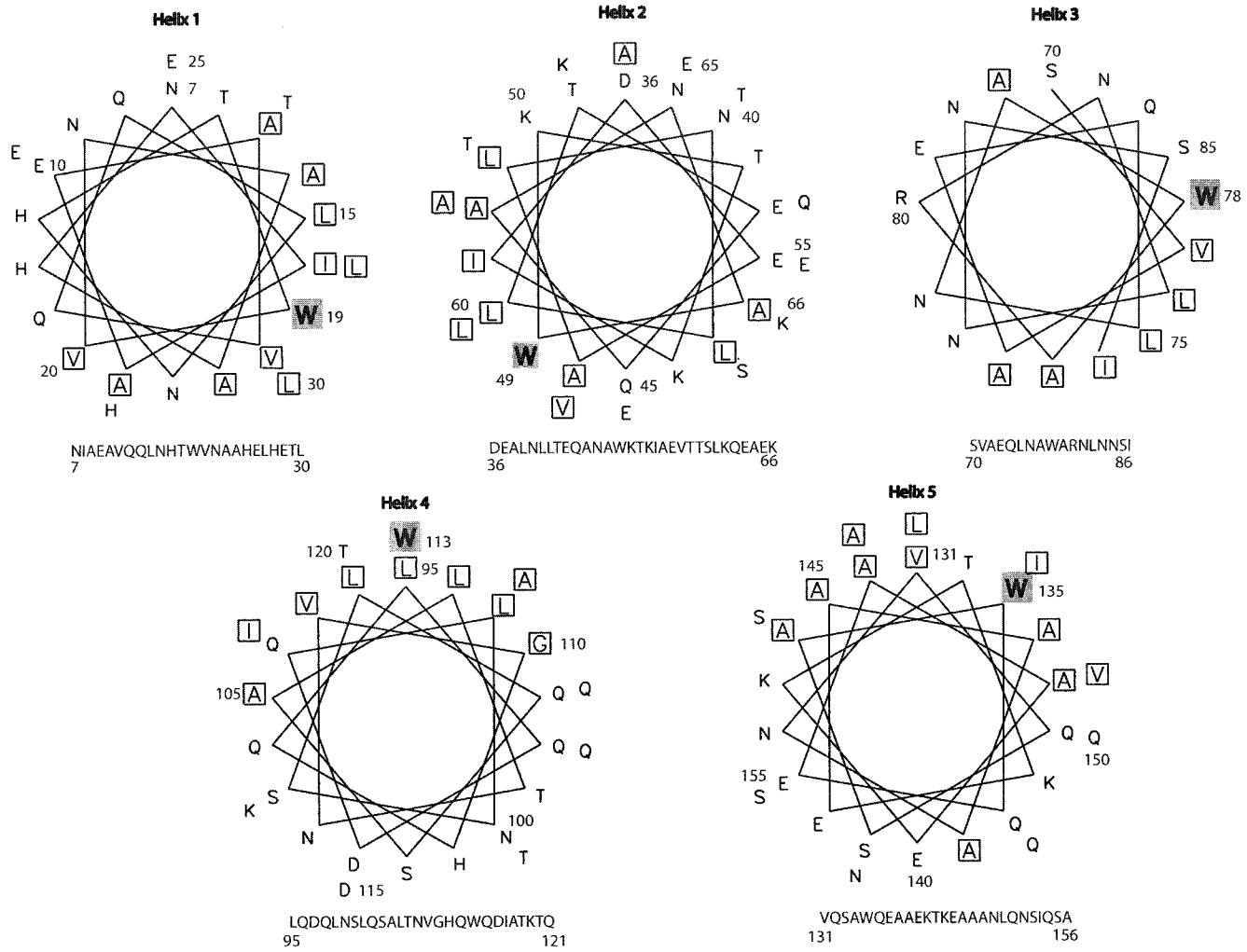


FIGURE 6: Helical wheel representation of the α-helices of apoLp-III. The Trp-containing α-helices of the single-Trp mutants of apoLp-III are represented as helical wheel diagrams. The sequence of each helix is indicated below the corresponding helical wheel. The hydrophobic residues are enclosed in squares, and the Trp residues are highlighted.

residues embedded in the membrane core show that the quenching efficiency increases as the depth of the probe is increased (*27*, *28*).

The data obtained with nitroxide-labeled PC clearly show that for all Trp mutants the pattern of fluorescence quenching observed in recombinant lipoproteins resembles that of deeply embedded Trp residues. In other words, the quenching efficiency increases with the depth of the nitroxide group. The fact that we observed a lower quenching efficiency with Tempo-PC than with any other nitroxide PC is consistent with a location of the protein along the disc's periphery. Nitroxide groups are quenchers characterized by a critical radius of about 11 Å (*16*), and quenching by these groups does not require contact with the fluorophore. This implies that, in the context of a bilayer thickness, nitroxide groups have a moderately long range of action, and therefore, quenching is expected to occur from the quenchers located in both membrane monolayers. Consequently, and as initially described by Chattopadhyay and London (*10*), a fluorophore located near the acyl chains will be subjected to a higher effective concentration of quenchers when the nitroxide groups are located near the bilayer center. Thus, a Trp residue located near position 5 of the acyl chain will be partially quenched only by the Tempo-PC located in one of the monolayers, whereas it will be quenched by the nitroxide groups of both monolayers for either 5-doxyl- or 16-doxyl-PC. These considerations support the interpretation of the quenching data as due to the location of the protein around the periphery of the discoidal particle, with a predominant interaction of the α-helices with the phospholipid acyl chains.

*Arrangement of the α-Helices and the Apolipoprotein Molecules in a Discoidal Lipoprotein.* The arrangement of the helices of an apolipoprotein in a discoidal lipoprotein and the relative orientation of different apolipoprotein molecules in a lipoprotein complex remain as a matter of speculation. The most studied discoidal lipoproteins are those made with apoA-I, for which at least five different models have been reported to explain the organization of α-helices and apolipoprotein molecules in discoidal lipoproteins (*34*, *38*−*41*). The alternative models have been divided into two groups. One group assumes that the α-helices are parallel to the acyl chains of the phospholipid (picket-fence models), whereas the second group of models assumes that the α-helices orient perpendicular to the phospholipid acyl chains (belt and hairpin models). The challenge represented in the elucidation of the organization of the apolipoproteins in discoidal lipoproteins is illustrated by the fact that even though the reported models have been based on experimental results, none of them can fully explain the results obtained with different experimental approaches. On the other hand,

the organization of apoLp-III in discoidal lipoproteins has not been as extensively studied as the organization of apoA-I. On the basis of the dimensions of the disks and their molecular weight and chemical composition, a model was proposed wherein apoLp-III adopts an open conformation, interacting with the acyl chains around the perimeter of the bilayer disk (*9*). According to this model the α-helices are oriented perpendicularly to the acyl chains. This orientation of the α-helices was subsequently supported by a total internal reflection IR spectroscopy study (*36*), which indicated that the average orientation of the helices' axis of apoLp-III is perpendicular to the acyl chains of the phospholipid. The present study provides two additional pieces of information relevant to the structure of the discoidal lipoprotein: it provides the first experimental evidence indicating that the five α-helices of apoLp-III interact with the lipid, and as well it provides experimental support to a structural model where the α-helices surround the periphery of the lipid bilayer interacting with the phospholipid acyl chains. It must be noted that our study cannot distinguish whether the orientation of the helices is perpendicular or parallel to the acyl chains. On the other hand, the study by Raussens et al. (*36*) cannot distinguish whether the α-helices interact with polar headgroups or the acyl chains. Taken together, the study by Raussens et al. and the present study support a model in which the α-helices are arranged perpendicularly to the phospholipid acyl chains surrounding the periphery of the discoidal bilayer. Also, because five α-helices that are perpendicular to the acyl chains cannot be accommodated in the thickness of a bilayer, it is deduced that the apolipoprotein molecule probably adopts a highly extended conformation. This type of lipid-bound conformation would require a large conformational change of the protein including a major opening of the helix bundle. Supporting the existence of a major conformational change, it has been recently reported that tethering of helices 1 and 5 abolishes the ability of apoLp-III to form discoidal lipoproteins (*42*). Although a picket fence model appears to be unlikely, we still need to learn what is the precise arrangement of the α-helices around the disk periphery. For instance, it is important to distinguish whether the configuration of the helices correspond to a beltlike model or an end-to-end model. Studies directed to investigate this subject are underway in our laboratory.

*Concluding Remarks.* The ability of NBS to react with all Trp residues suggested an open conformation of apoLp-III and indicated the absence of transmembrane helical domains. Perhaps more important, NBS provided us with a means to obtain accurate estimates of the quenching efficiency by removing the unavoidable contribution of scattering. This approach is likely to be useful to the study of many other membrane-interacting proteins by fluorescence quenching.

This study is unique in the sense that we have systematically scanned the interaction of the amphipathic α-helices of apoLp-III with the lipid surface using several lipid quenchers. Similar studies have not been carried out with any of the known exchangeable apolipoproteins.

The pattern of quenching observed with different nitroxide lipids is consistent with a model in which the apolipoprotein surrounds the periphery of the disk bilayer.

Comparison of the extent of quenching observed with the Trp mutants with the extent of quenching reported for other membrane-embedded Trp suggests that all the helices of apoLp-III interact with the acyl chains of the phospholipid. The differences in exposure to the lipid quenchers observed between the Trp residues in helices 2 and 3 and the Trp residues in helices 1, 4, and 5 could be due to small changes in the lateral insertion depth of the Trp residues arising from the relative location of the Trp residues in the hydrophobic face of the helix. Additional studies with other single-Trp mutants or model amphipathic peptides could provide the clues underlying the observed differences between the Trp residues located in different helices.

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BI010949D