The Lipid Binding Activity of the Exchangeable Apolipoprotein Apolipophorin-III Correlates with the Formation of a Partially Folded Conformation†

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ABSTRACT: *Manduca sexta* apolipophorin-III, apoLp-III, is an exchangeable apolipoprotein of 17 kDa that contains no Trp, one Tyr, and eight Phe. The effect of pH on the kinetics of association of apoLp-III with dimyristoylphosphatidylcholine was studied. The pH dependence of the kinetics showed three distinct regions. Above pH 7, the reaction rate is slow and slightly affected by pH. A ∼40-fold increase in the rate constant is observed when the pH is decreased from 8 to 4, and a decrease in rate is observed below pH 4. Far-UV CD spectroscopy indicated that the secondary structure of the protein is not affected when decreasing the pH from 8 to 4.5. The pH dependence of the Tyr fluorescence showed three pH regions that resemble the regions observed in the kinetics. Comparison of the far-UV CD and fluorescence studies indicated the formation of a folding intermediate between pHs 4 and 7. This intermediate was also characterized by near-UV CD and fluorescence quenching. Fluorescence quenching studies with I^- and Cs^+ indicated a very low exposure of the Tyr residue in both native and intermediate conformations. The pH dependence of the near-UV CD spectra indicated that the native → intermediate transition is accompanied by a loss in the packing constrains of the Tyr residue. UV absorption spectroscopy of the Phe and Tyr residues indicated that the native → intermediate transition is also accompanied by the hydration of the Tyr residue and ∼4 Phe residues. This report shows, for the first time, the correlation between the increase in lipid binding activity of an exchangeable apolipoprotein and the formation of a compact but hydrated conformation near physiological conditions. These results suggest a direct correlation between the lipid binding activity and the internal hydration of the apolipoprotein. The similarity between the insect exchangeable apolipoprotein and the human counterparts, apoA-I, apoA-II, etc., and the recent demonstration of the presence of a molten globular like-state of human apoA-I near physiological conditions [Gursky, O., and Atkinson, D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2991−2995] suggest that this highly hydrated and compact state may play an important physiological role as the most active lipid binding state of the apolipoproteins in general.

The binding of exchangeable apolipoproteins to the lipoprotein surface responds to changes in physical−chemical properties of the lipoprotein lipid surface domain. The thermodynamics and kinetics of the binding process are dependent on the properties of both the lipid surface and the apolipoprotein. The association of an apolipoprotein with a lipid surface is a very complex process that involves several types of molecular interactions. The understanding of the driving forces for the interaction of water-soluble proteins with lipid membranes is a very difficult task. This is so because the insertion of a protein into a membrane involves several steps and in each of them electrostatic (attractive and repulsive) and hydrophobic interactions play different roles. In addition to the complexity of the system, we also have to consider the experimental limitations and the limited understanding that we have about the physical−chemical properties of the protein. The understanding of this biochemical problem is important because the homeostasis of the lipid metabolism is controlled to a great extent by the lipid binding properties of the exchangeable apolipoproteins.

Apolipophorin-III (apoLp-III)[1] 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 (*1, 2*). *Locusta migratoria* apoLp-III is the only full-length, exchangeable apolipoprotein whose structure has been determined by X-ray crystallography (*3*). A similar topology of the amphipathic α-helices was recently deduced for *Manduca sexta* apoLp-III by multidimensional NMR spectroscopy (*4*). The apoLp-III 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 insect apoLp-III molecules described so far share similar structural and functional features (*1, 5*). *Manduca sexta* and *Locusta migratoria* apoLp-III have been well characterized in terms of their physicochemical properties and functionality. They also share a large number of properties with human exchangeable apolipoproteins, such as apoA-I, apoA-II, and apoA-IV. Among the common properties are the presence of amphipathic α-helices characterized by typical distribu-

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[1] Abbreviations: PIF, partially folded intermediate; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; apoLp-III, apolipophorin-III.

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tions of polar and nonpolar residues (*6*), and the ability to form discoidal recombinant lipoproteins (*7*, *8*).

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 directly applicable to vertebrate apolipoproteins.

The role of the properties of the lipid surface in the interaction of apoLp-III with lipid has been studied in several model systems (*9*−*13*). On the other hand, the role of the properties of apoLp-III in binding to lipid was studied by site-directed mutagenesis (*14*) and by chemical modification of the apolipoprotein (*15*).

The initial goal of this study was to determine the role of protein charge in the kinetics of apoLp-III−dimyristoylphosphatidylcholine (DMPC) association. However, the pH-induced changes in the kinetics were surprisingly large, prompting a careful study of the structural changes of apoLp-III induced by pH. As a result of these studies, we observed that *Manduca sexta* apoLp-III adopts a partially folded conformation near physiological conditions. This conformational intermediate shares many properties with the conformational state identified as a molten globule. More relevant and not yet reported for any apolipoprotein, we observed a clear correlation between the kinetics of association of apoLp-III with liposomes of DMPC and the formation of the partially folded intermediate. Our study indicates that this intermediate represents the most active apolipoprotein conformation under physiological conditions.

METHODS

Purification of ApoLp-III. Manduca sexta apoLp-III was purified from the low-density lipoprotein of adult insects. The lipoprotein was isolated from the hemolymph by ultracentrifugacion in a KBr gradient as described before (*11*). ApoLp-III was released from the lipoprotein by heating it at 80 °C in a water bath for 20 min. The aggregated lipoprotein was then separated from the soluble apoLp-III by centrifugation at 45 000 rpm for 1 h. The supernatant containing pure apoLp-III was filtered through a 0.22 μm membrane, and Gdm·HCl was added to a final concentration of 2 M. The resulting solution was extensively dialyzed against 10 mM sodium phosphate buffer, pH 7.4. Afterward, the protein was loaded into a DE52 (Whatman, Maidstone England) column and eluted with increasing concentrations of NaCl. For all the experiments, the apoLp-III was previously desalted by gel filtration in a Sephadex 25G column (PD10, Pharmacia), and the quality of the preparation was checked by UV and CD spectroscopy.

Absorption Spectra. UV absorption spectra were recorded with a HP 8453 diode array spectrophotometer. The concentration of apoLp-III was calculated from the absorbance at 278 nm in the presence of 6 M Gdm·HCl (ϵ_{Tyr} = 1280 cm^{-1} M^{-1}) (*16*).

The second derivatives were calculated by the Savitzky−Golay differentiation technique using a filtering length of 9. The pH dependence of the difference between the second derivatives at 293 and 289 nm was studied at 25 °C. The pH of a solution of apoLp-III, in 10 mM sodium phosphate buffer, pH 7, was modified by successive additions of 0.1 or 1 M solutions of NaOH or HCl. The pH was determined with a calibrated Hamilton Biotrode electrode that was immersed into the cell of the spectrophotometer. Data were corrected for the dilution.

Quenching Measurements. Fluorescence spectra were collected in a Photon Technology International Model A1010 spectrofluorometer at 24 °C. Quenching experiments were carried out in 10 mm cells. The solutions of apoLp-III (∼15 μM) were excited at 285 nm, and the emission was collected between 290 and 360 nm every 0.25 nm. A fresh 5 M stock solution of KI (Mallinckrodt, Paris, KY) containing 50 μM thiosulfate was prepared in deoxygenated buffer and immediately used. Aliquots of the KI stock solution were added to 3 mL of the protein solution in 40 mM sodium phosphate buffer, pH 5.2 or 7.4, containing either 0 M NaCl or 500 mM NaCl. The total intensity was calculated by integration of the fluorescence spectra. Fluorescence data were corrected for the dilution and the Raman peak of the solvent. A similar protocol was used for CsCl (optical grade; BRL, Gaithersburg, MD).

Stern−Volmer quenching constants were obtained by nonlinear regression analysis of the data using the modified Stern−Volmer relation (*17*):

$$I_o/I = (1 + K[Q])e^{V[Q]}$$

Circular Dichroism. CD spectra were acquired in a 62A DS AVIV CD instrument (Lakewood, NJ) equipped with a Peltier temperature controller and interfaced to a personal computer. The ellipticity data were collected every 0.25 nm with a 1 s averaging time per point and a 1 nm band-pass. A 1−3 μM solution of apoLp-III was used in the far-UV region. In the near-UV, the concentration of the apoLp-III solutions was 40−80 μM. The CD spectra shown are the average of three background-corrected and smoothed scans. CD spectra were analyzed for secondary structure using the Contin program with the original (*18*) structure data set. Protein was determined spectrophotometrically (*16*).

Kinetics. The kinetics of apoLp-III−DMPC association was followed by the decrease in OD at 325 nm that accompanies the transformation of the multilamellar liposomes into discoidal lipoprotein particles (*19*). The kinetics was performed at 23.9 °C in a Hewlett-Packard diode array spectrophotometer equipped with a Peltier temperature-controlled cell holder. The spectrum was routinely acquired every 15 s using an averaging time of 0.5 s. Data were analyzed by nonlinear regression. The reaction was performed at a 4:1 lipid to protein molar ratio by adding 50 μL of a liposome suspension (2 mg/mL) to 1950 μL of the apoLp-III solution containing 520 μg of protein. At all pH values the reaction was complete after 24 h. Because the final OD at 325 nm was near zero, all decays in OD were normalized using $OD_\infty = 0$.

To obtain multilamellar liposomes, a thin film of DMPC was prepared on the bottom of a flat glass vial by drying 10 mg of DMPC in chloroform under N_2. The dried lipid was then incubated at 37 °C and mixed with 5 mL of the 20 mM buffer at the same temperature as the lipid. The following buffers were used: Tris base (pHs 7.5−11); sodium phosphate buffer (pHs 4.5−9); acetic acid/acetate (pHs 3.5−5); and HCl/KCl (below pH 3.5). The liposomes were obtained by vortexing the hydrated lipid film.

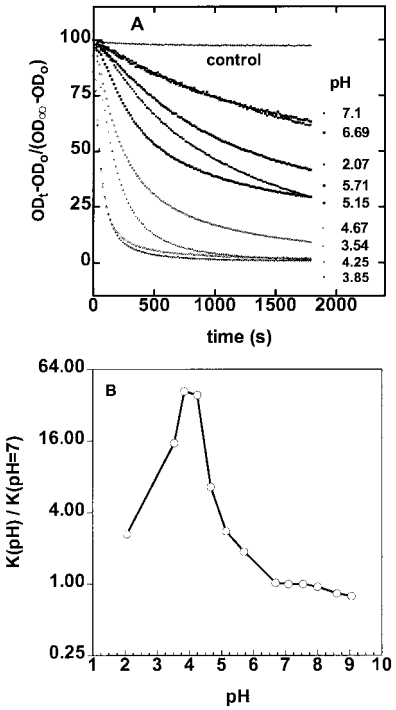

FIGURE 1: Kinetics of the association of *Manduca sexta* apoLp-III with multilamellar liposomes of DMPC. (A) Decay of optical density after adding 50 μL of liposomes (2 mg/mL) to 1.95 mL of an apoLp-III solution (520 μg of protein) equilibrated at 23.9 °C. Experiments were performed in 40 mM buffers of different pH. The pH was determined at the end of the experiment. (B) Effect of pH on the pseudo first-order rate constants. The control curve shows the typical decay in OD obtained in the absence of apoLp-III.

Other Methods. The pH of the hemolymph was determined in individual insects with a calibrated Hamilton Biotrode electrode. The pH reading (6.5 ± 0.2) was done immediately after bleeding resting adult insects.

RESULTS

Effect of pH on the Lipid Binding Activity. The effect of pH on the kinetics of association of *Manduca sexta* apoLp-III with DMPC was followed by the clearance of liposomal turbidity at 23.9 °C (Figure 1). In the pH range covered (2−9), and at a protein to lipid ratio of 1:4, the apolipoproteins interact with DMPC, leading to a complete transformation of the liposomes into the much smaller discoidal apolipoprotein−lipid complexes. Figure 1A shows the turbidity decays obtained at some pH values during the first 30 min of reaction. The decrease in pH resulted in an increase in the reaction rate. However, the dependence of the kinetics on pH showed some interesting features, which can be seen from the plot of the apparent pseudo-first-order rate constants (Figure 1B). These rate constants were obtained by fitting the data to an exponential decay curve by nonlinear regression analysis. The dependence of the rate constants on pH can be divided into three separate regions. At alkaline pHs, the rate constants are small and slowly increase upon decreasing the pH to about 7. Below pH 7, a sharp rate increase was observed. This increase in rate reached a peak between pHs 3.5 and 4, where the reaction rate is up to ~40, times faster than at pH 7. Finally, a decrease in the association rate is observed below pH 4. This final decrease in rate could be related to the denaturation of the apolipoprotein, which according to a previous report

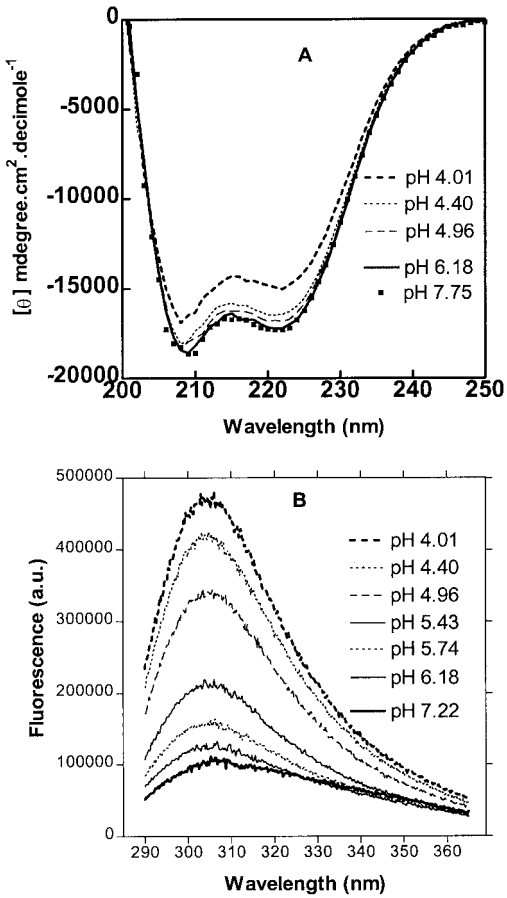

FIGURE 2: Effect of pH on the spectroscopic properties of *Manduca sexta* apoLp-III as observed by far-UV CD (A) and fluorescence (B). The figure shows the data obtained at 24 °C in one representative experiment.

occurs below pH 4 (*8*). It is important to remark that regarding size, the products of the reaction are similar at the various pH conditions. At all pHs, the OD^{325nm} reached near-zero values, indicating that in every case the interaction of apoLp-III with DMPC leads to the formation of small lipoprotein particles. Electron microscopy of negatively stained samples showed that at pH 5 and 7.5 the lipoprotein particles are indistinguishable and have a diameter of 17 nm (data not shown).

Because minor changes in the protein charge are expected to occur between pHs 4 and 7, the large increase in association rate observed in that pH range does not seem to be simply related to the change in net charge of the apolipoprotein. The membrane properties are not expected to change between pHs 4 and 7. Protonation of the phosphate group of the phosphatidylcholine takes place below pH 2 (*20*). Therefore, we considered the possibility that the breakpoint observed at pH 7 could be related to a new, pH-induced, conformational state of apoLp-III, and carried out a careful spectroscopic study of the effect of pH on the structure of apoLp-III.

pH Dependence of the Circular Dichroism and Fluorescence Spectra of apoLp-III. Figure 2 shows some of the CD and fluorescence spectra obtained at different pHs. It is readily observed that, although minor changes occur in the CD spectra, a major enhancement of Tyr fluorescence is observed between pHs 4 and 6. A better comparison of the results obtained by CD and fluorescence can be achieved

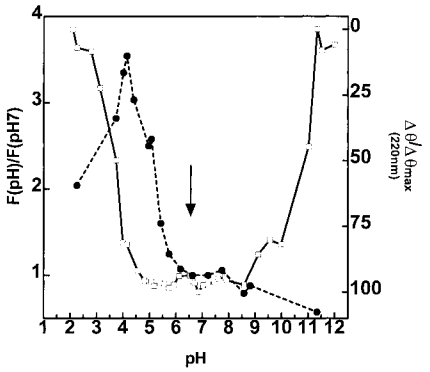

FIGURE 3: Comparison of the pH-induced changes in fluorescence intensity (●) and ellipticity at 220 nm (□) of apoLp-III. Measurements were done at 24 °C. The solutions of apoLp-III were prepared in 20 mM buffers. Data points indicate the average of at least three measurements ± SE. The arrow indicates the breakpoint in fluorescence intensity.

by plotting the relative changes in fluorescence and ellipticity versus pH. Figure 3 shows the relative changes in fluorescence and ellipticity. The normalization was done by setting 100% as the maximum absolute value of ellipticity at 222 nm, which occurs at neutral pH, and 0% as the ellipticity determined in the presence of 2 M Gdm. Fluorescence data were expressed relative to the fluorescence observed at pH 7.4 and showed a pattern similar to that previously reported (*23*). The CD results are essentially identical to those previously reported (*8*). The results shown in Figure 3 clearly show that the onsets of the changes in fluorescence intensity and ellipticity are shifted by at least 1 pH unit. Whereas the ellipticity at 220 nm remained about constant between pHs 5 and 6, a 2.5-fold change in fluorescence intensity was observed in that pH range. Below pH 5, the fluorescence keeps on increasing, until pH 4 where it reaches a 3.6-fold increase. However, at pH 4, the presence of unfolded apoLp-III is already apparent as indicated by an 18% decrease in ellipticity at 222 nm. Below pH 4, the fluorescence decreases to a level that, at pH 2.0, is comparable to that observed in the Gdm-unfolded protein at neutral pH (data not shown). Concomitantly, and according to the far-UV CD data, a complete unfolding of the protein is achieved at pH 2.0.

These results indicate that a conformational change takes place below pH 6.5. According to the CD data, this conformational change appears to occur without any modification of the secondary structure. This low-pH conformational state becomes maximally populated at pH 4, or near pH 4, where the fluorescence intensity reaches a maximum. The small decrease in ellipticity observed at pH 4 and the complete unfolding observed at pH 2.5 indicate the presence of a small fraction of unfolded protein at pH 4. The fact that the fluorescence intensity at pH 4 or 3.5 was higher than that of the unfolded protein at pH 2, or at pH 7 in the presence of 2 M Gdm, indicates that the Tyr residue is in a medium of relatively low polarity.

Near-UV Circular Dichroism Spectroscopy and Second-Derivative Absorption Spectroscopy of ApoLp-III. The packing constraints of the Tyr residue within the protein core were studied by near-UV CD spectroscopy. The CD spectra of apoLp-III at pHs 5.2 and 7.4 are shown in Figure 4A. These spectra are characterized by the absorption bands of the Phe residues (apoLp-III contains eight), at 261 and 268

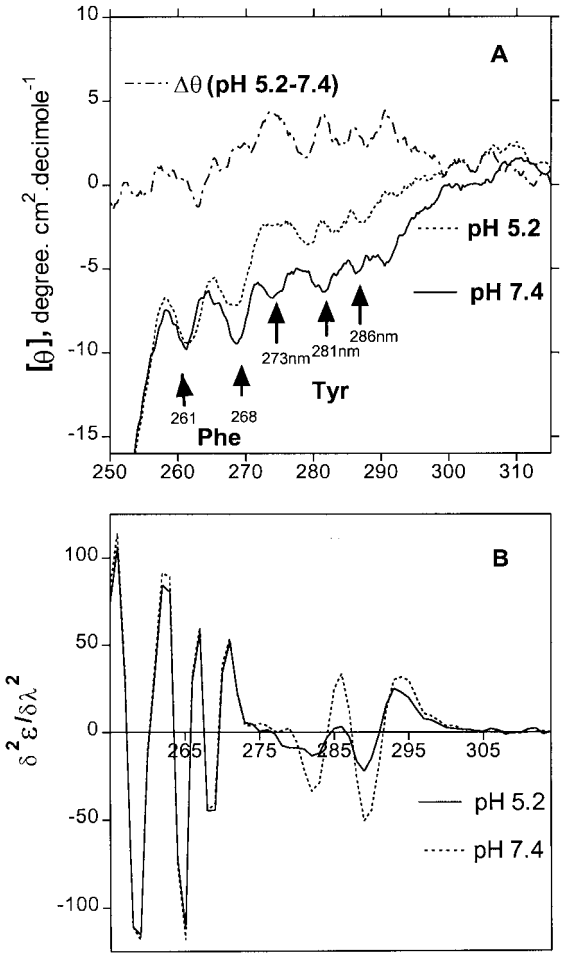

FIGURE 4: (A) Near-UV CD spectra of *Manduca sexta* apoLp-III in 40 mM sodium phosphate solutions of pH 5.2 (•••) or 7.4 (—). The difference spectrum is also shown (— • —); (B) UV absorption spectra (second derivative) of apoLp-III at pH 5.2 and 7.4.

nm, and the CD bands of the Tyr residue, at 273, 281, and 286 nm (*21*). Comparison of the spectra obtained at these two pHs indicates a considerable loss of intensity at pH 5.2 (see difference spectrum in Figure 4A). Most of the "hypochromic" effect observed is due to the reduction in the structural constraints of the Tyr residue. However, the hypochromic effect is also present in the absorption bands corresponding to Phe.

The second derivatives of the UV absorption spectra of apoLp-III also showed that, in the absorption region dominated by Tyr (above 270 nm), the decrease in pH from 7.4 to 5.2 is accompanied by significant spectral changes (Figure 4B). The changes in the second derivatives of the absorption spectrum of Tyr are related to the hydration of the residue (*22*). The pH-induced changes in the hydration of Tyr were monitored by means of the difference between the second derivatives at 293 and 289 nm (Figure 5). This extensive titration clearly showed two major sharp transitions. The first one is observed between pHs 4 and pH 7, resembling the transition observed by fluorescence. The second transition starts at pH 9 and ends at pH 11, and is due to the ionization of the Tyr (*23*) and concomitant protein unfolding. This transition is well accompanied by the change in ellipticity at 220 nm, whereas the first transition ($4 < pH < 7$) is shifted by at least 2 pH units with the changes in ellipticity at 220 nm. The fact that no change in the second

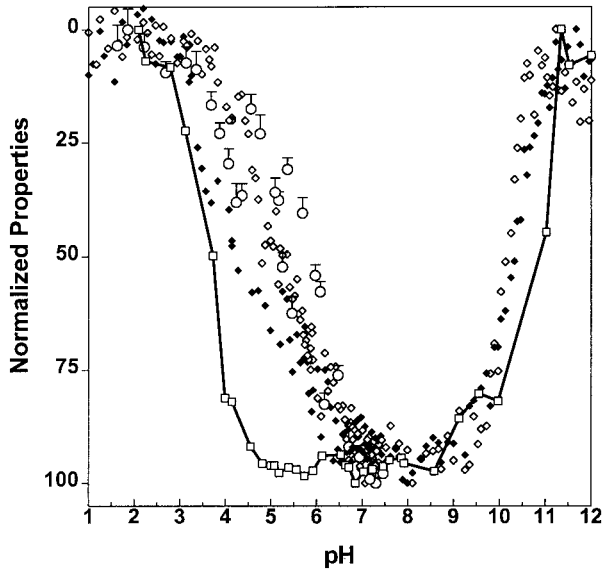

FIGURE 5: Effect of pH on the spectroscopic properties of apoLp-III. Near-UV CD data were collected at 273 nm (○); second-derivative difference absorption spectroscopy of Tyr, $\Delta(A''_{289} - A''_{293})$ (◇), and Phe, $\Delta(A''_{255} - A''_{252})$ (◆). Data were obtained at 25 °C. Other experimental details are given under Methods. For comparison purposes, the data obtained by CD at 220 nm (□) are also shown.

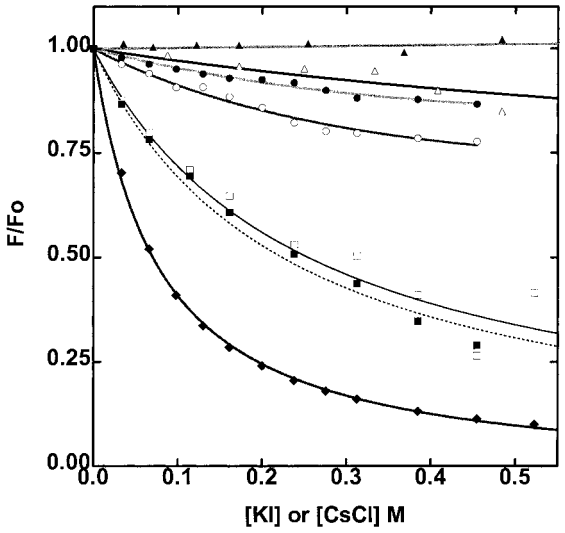

FIGURE 6: Quenching of Tyr fluorescence by iodide and CsCl. Data symbols: iodide quenching of apoLp-III at pHs 5.2 (▲) and 7.4 (△), and in the presence of 2 M Gdm at pHs 5.2 (■) and 7.4 (···); CsCl quenching of apoLp-III at pH 5.2 (○) and 7.4 (●). For comparison purposes, the quenching of free Tyr by iodide in the same buffer conditions and at pH 7.4 was also included (◆). The experiments were performed in 40 mM sodium phosphate buffer at 24 °C. Other experimental details are given under Methods.

derivative of the Tyr absorption bands is observed below pH 4, where the I→U transition takes place, indicates that in the intermediate the Tyr residue is as hydrated as in the unfolded protein at pH < 4. It is possible that the hydration of the Tyr in the intermediate be less extensive than the hydration in the unfolded state. However, the UV absorption features of the Tyr residue cannot distinguish between those two possibilities.

The changes induced by pH in the absorption region (250 nm > λ > 270 nm) monitor the changes in the environment and hydration of the Phe. These changes can be used to monitor the hydration of the Phe residues (*24*, *25*). We followed the pH-induced changes in the hydration of Phe by the difference between the second-derivative values at 255 and 252 nm (Figure 5). The profile observed indicates that the Phe also is affected by the transition that takes place below pH 7. However, the changes with pH are slower for Phe than those observed for Tyr, indicating that in the intermediate not all the Phe residues (seven total) become fully hydrated. A simple inspection of the curve suggests that roughly four or five Phe residues would become hydrated with the formation of the intermediate. The remaining two or tjree Phe become fully hydrated only when the protein unfolds.

Figure 5 also shows the pH dependence of the ellipticity at 273 nm. The notorious similarity between the pH dependence of the changes in the near-UV CD and in the second derivative of the Tyr absorption indicated that both techniques detect the same conformational transition. Overall the transition detected with these techniques is consistent with the results obtained by fluorescence. The major difference between the fluorescence and the near-UV absorption studies resides in the fact that the fluorescence intensity reaches a maximum between pHs 3.5 and 4.0 whereas below pH 4 either near-UV CD or second-derivative UV spectroscopy is insensitive to changes in the structure of apoLp-III. Fluorescence is the only isolated technique that can distinguish between the intermediate and the unfolded states of apoLp-III. Also, it is important to note that titration followed by the second derivative of the Phe absorption indicated that, as judged by the hydration of the Phe, only part of the protein core would become highly hydrated. This result suggests that in a significant part of the molecule, in the intermediate conformation, the tertiary interactions remain as tight as in the native state.

Effect of External Quenchers on Tyrosine Fluorescence. The hydration of the Tyr residue detected by second-derivative UV spectroscopy could also be due to a localized melting of the helical segment containing the only Tyr residue of the protein. If this hypothetical melting involved only few amino acid residues, or was compensated by the folding of a previously unfolded domain, it could be undetectable by far-UV CD. To investigate the possibility of a localized unfolding involving the only Tyr residue, the accessibility of the Tyr residue was investigated by fluorescence quenching. These studies were limited to protein solutions at pH values of 5.2 and 7.4, where the secondary structure is not affected and the presence of unfolded protein can be discarded. Figure 6 shows the apoLp-III fluorescence quenching plots obtained with Cs^+ and I^-, at pHs 5.2 and 7.42. This figure also shows the quenching curves obtained with free Tyr and apoLp-III in the presence of Gdm. These sets of data served the purpose of checking the experimental conditions and providing a reference for a fully exposed Tyr residue. Because the fluorescence lifetime of apoLp-III at different pHs is about constant and similar to that observed in free Tyr (*23*), the Stern−Volmer constants obtained from the data analysis are directly related to the exposure of Tyr residues. In the absence of NaCl, a slightly higher accessibility of the Tyr residue was observed with I^- quenching at pH 7.4 ($K_{s,v} = 0.83 \pm 0.03$ M^{-1}) than at pH 5.2 ($K_{s,v} = 0.41 \pm 0.02$ M^{-1}). Comparison of the $K_{s,v}$ values obtained

for apoLp-III at pH 5.2 and 7.4 with those constants obtained for free Tyr ($K_{s,v}$ = 13.0 M^{-1}) and for apoLp-III in the presence of denaturing concentrations of Gdm [$K_{s,v}(I^-$, pH 5.2) = 3.83 ± 0.43 M^{-1}; $K_{s,v}(I^-$, pH 7.4) = 3.96 ± 0.96 M^{-1}] also indicates that at both pHs the Tyr residue is marginally accessible to the solvent. In the presence of 500 mM NaCl (data not shown), which reduces the charge effects, the quenching ability of I^- was about the same as in the absence of NaCl, indicating the absence of large charge effects on the quenching. This is consistent with the low degree of Tyr exposure inferred from the quenching data. Moreover, the Stern−Volmer constants obtained with Cs^+ at pH 7.4 [$K_{s,v}$(CsCl) = 0.64 ± 0.26] and at pH 5.2 [$K_{s,v}$-(CsCl) = 0.00 ± 0.32 M^{-1}] indicate both a low exposure of the Tyr residue in native apoLp-III at both pH values and a slightly higher Tyr accessibility at pH 7.4 than at pH 5.2.

Quenching studies with uncharged quenchers, such as acrylamide, avoid charge effects and have the potential advantage of partitioning into the protein core. There is one report showing the use of acrylamide to quench Tyr fluorescence in proteins (*26*). We tried to study the accessibility of the Tyr residue in apoLp-III at different pHs using acrylamide. However, the large absorption of acrylamide at the excitation and emission wavelengths prevented us from obtaining quality data.

DISCUSSION

Identification and Properties of the Partially Folded State of ApoLp-III. The first evidence about the presence of an unfolding intermediate was provided by the fluorescence studies. The presence of a fluorescence maximum indicated the existence of at least one intermediate in the pH-induced unfolding of apoLp-III. The identification of intermediates is usually difficult because their properties fall between the properties of the native and unfolded states. The great sensitivity of fluorescence to monitor the formation of an intermediate in apoLp-III was given by the efficient quenching of the Tyr fluorescence observed in the native state. This quenching was previously described and investigated (*23*), but its origin is not clear as of yet.

The differences between the breakpoints in the pH dependence of the ellipticity at 220 nm (onset at ∼pH 4.3) and the fluorescence intensity (onset ∼pH 6.5) indicated that this intermediate state has about the same secondary structure as the native state and becomes highly populated below pH 6.5. The fact that the fluorescence intensity reached a peak at pH 3.5, and at lower pHs decreased to a value close to that observed in the guanidine unfolded protein at neutral pH, suggested that the Tyr residue was not fully exposed in the "intermediate state" observed between pHs 3.5 and 6.5. The lower quantum yield of apoLp-III at pH 2 (unfolded protein) than at pH 3.5−4 suggested that the intermediate has a compact tertiary structure. The compactness of the intermediate was also studied by fluorescence quenching with external collisional quenchers. The very low accessibility of the Tyr residue at both pH values, 5.2 and 7.4, and for both quenchers, Cs^+ and I^-, also indicates that the intermediate state has a compact structure. These fluorescence studies also indicated that the possible localized melting in the helix containing the Tyr residue was not taking place. At this point, we had two possibilities: (1) the protein adopted a second nativelike structure; (2) the protein adopted a partially folded conformation. To distinguish between these two possibilities, we studied the effect of pH on the ellipticity at 273 nm, as determined by CD, and on the UV absorption of the Tyr and Phe residues. The results obtained showed a sharp decrease in ellipticity (apparent p*K* 5.6), which accompanied the changes in fluorescence intensity. The decrease in ellipticity at 273 nm indicated a random distribution of packing arrangements around the Tyr residue. Interestingly, in the absorption region of Tyr, the pH titration curve obtained by second-derivative UV absorption spectroscopy matched the behavior of the near-UV CD titration. This similarity indicated that the formation of the intermediate is accompanied by an increase in the hydration of the protein, or at least of the protein domain containing the Tyr residue (middle region of helix 5). To obtain more information about the hydration of the protein, we studied the pH-induced changes in the spectral region of Phe. *Manduca sexta* apoLp-III contains seven Phe residues distributed among four out of the five α-helices (*17*). Therefore, the spectral changes in the UV absorption region of Phe provide a better indication about the global changes in the hydration of the protein. As described under Results, the titration showed that the hydration of the Phe residues starts around pH 6.5, well before the protein unfolds. However, the titration also indicated that, conversely to the case of the Tyr residue, the complete hydration of the Phe residues is observed only when the protein is fully unfolded. The titration curve suggested that between four and five Phe residues become hydrated in the intermediate conformation.

Overall, the spectroscopic characterization of the intermediate indicates the following: (A) The intermediate has the same secondary structure as the native state. The domain containing the Tyr residue is hydrated, and allows the existence of different packing arrangements around the Tyr residue. (C) The structural changes that accompany the native to intermediate transition affect a large extent of the protein structure. Spectroscopy of the Phe residues suggested that roughly 60% of the protein core becomes hydrated upon the formation of the intermediate conformation. Tight tertiary interactions would be maintained in the remaining 40% of the protein core. (D) Compared to the native state, the intermediate constitutes a partially folded, but still compact conformation of apoLp-III.

Role of the Partially Folded Conformation in the Kinetics of Association of ApoLp-III with DMPC. In this study, we attempted to investigate the role of electrostatic interactions in the kinetics of association of apoLp-III with DMPC by changing the pH of the reaction media and relating the reaction rates to the net charge of the protein. However, the sharp increase in rate of apoLp-III insertion observed below pH 7, and the presence of a maximum between pHs 3.5 and 4, indicated that the kinetics of this lipid−protein interaction were being affected by a change in the structure of the protein. In other words, the changes in the kinetics did not respond to a simple change in the net charge of the protein but rather to the pH-dependent changes in the dynamics and/or structure of the protein. As discussed above, the spectroscopic characterization of apoLp-III confirmed that between pHs 4 and 7 the protein goes through a structural transition, which involves the equilibrium between the native conformation and a partially folded conformation.

The strong correlation found between the kinetics of apoLp-III−DMPC association and the concentration of the unfolding intermediate indicates that this conformational state is responsible for the increase in association rate observed when the pH is decreased from 7 to 4. Furthermore, the second structural transition, which is observed below pH 4 and corresponds to the formation of the unfolded state, is also accompanied by a change in the kinetics. However, contrary to the formation of the intermediate, the formation of the unfolded state correlates with a decrease in the association rate. Overall, the spectroscopic and the kinetic studies identify three regions of pH that show a strong and distinct correlation between the kinetics of the reaction and the concentrations of the native protein, the partially folded intermediate, and the unfolded protein.

The association of apoLp-III with DMPC liposomes involves the insertion of the protein into the membrane core. The insertion of a globular protein into a membrane is a complex process that involves several steps. In each of these steps, the energy barriers are of different nature and have different contributions from electrostatic and hydrophobic interactions. Because of the complexity of the insertion process, elucidation of the limiting steps and energy barriers is not a simple task. The insertion rate of a globular protein into a bilayer is determined by the properties of both the lipid surface and the protein. We can assume that in our experiments there are no significant changes in the properties of the DMPC membrane surface. Therefore, the increase in reaction rate that correlates with the formation of the partially folded intermediate can be attributed to changes in the structure and/or dynamics of the protein.

Because the hydrophobic faces of the amphipathic α-helices are buried in the globular protein, large conformational changes are required to allow the interaction between the hydrophobic residues of the protein and the acyl chains of the phospholipid. Some of these conformational changes are expected to occur once the protein is adsorbed or bound to the membrane, but it is also likely that a conformational change leading to an activated protein state could occur in the water-soluble state of the protein. This activated conformation would allow the binding of the protein in a configuration competent with the posterior insertion of the protein into the membrane. The correlation between the kinetics of apoLp-III−DMPC association and the concentration of the partially folded intermediate indicates that this conformation facilitates the insertion of the protein. Because of that, the partially folded intermediate can be seen as an activated state of the protein in solution. Our results indicate that the conformation and/or conformational flexibility of the native state of apoLp-III in the aqueous phase constitutes an important energy barrier for the insertion of the protein into the membrane. The formation of the partially folded intermediate produces a significant decrease of this energy barrier and a concomitant activation of the apolipoprotein. A possible contribution to the decrease in the energy barrier could be represented by the hydration of the protein core that takes place with the formation of the intermediate.

Even though the native state is more abundant than the partially folded conformation at pH 6.5 (physiological pH), the higher lipid binding activity of the partially folded conformation suggests that it represents the most active form of apoLp-III under physiological conditions. This is supported by two facts. First, the rate constant for the interaction of the partially folded conformation with multillamelar liposomes is 50-fold higher than that of the native state. Second, because the breakpoints for the changes in hydration and packing of the protein core occur near pH 7 and are maximum near pH 4, we estimated that at pH 6.5 there is a low but significant concentration of partially folded intermediate (10−15 mol %). Thus, at pH 6.5, the relative contribution of the partially folded intermediate to the reaction rate would be 5−10 times higher than that of the native state.

Partially folded conformations have been described for human apoA-I and apoA-II under near-physiological conditions (*27, 28*). The relationship between the lipid binding activity and the abundance of the partially folded forms of apoA-I and apoA-II has not been studied. However, the fact that a partially folded conformation is the predominant form of these apolipoproteins suggests that this conformation could represent the most active and metabolically relevant conformation of apolipoproteins in general.

Relationship between the Partially Folded Intermediate of apoLp-III and a Molten Globular Conformation. The molten globular conformation refers to a partially folded conformation characterized by the absence of tight packing of side chains groups, a high compactness, a nativelike secondary structure (*29*−*31*), and an increased hydration of the protein core (*32*). The structural properties of the partially folded intermediate of apoLp-III are consistent with most of the properties that define the so-called molten globular state. A thermodynamic study of the unfolding of apoLp-III will allow a better definition of the conformational properties of the intermediate. However, the comparison with the molten globular state is relevant because the molten globular conformation has been linked to the lipid binding activity of several proteins (*32*). Particularly interesting is the fact that human apoA-I adopts a molten globular like-state at 37 °C and pH 7 (*27*).

Most of the molten globules extensively characterized were obtained under acidic conditions at pH 4 or lower (*30*). In this regard, the partially folded conformation adopted by apoLp-III near physiological pH (the physiological pH of the insect blood is 6.5) indicates a similarity with the human apoA-I and a difference with most of the previously described molten globules. It also suggests a physiological role for partially folded conformations in the biological activity of exchangeable apolipoproteins.

The molten globular state has been proposed to play a physiological role in translocation of proteins across membranes (*33*). Several reports have shown the apparent involvement of the molten globular state in the binding of proteins to lipid membranes (*32*). However, most of the studies linking the molten globular state to the membrane binding activity were done under quite extreme experimental conditions and did not show a link between the formation of the molten globule and the physiological role of the protein. An exception to this was given by a study showing that the binding of diphtheria toxin to negatively charged membranes at pH 5 correlates with the formation of a molten globule (*34*). This example was physiologically relevant because the toxin would be activated at the low pH found in lysosomes. Regarding the lipid binding activity, a major difference between our study and those previously reported

resides in the fact that we used zwitterionic membranes, whereas the lipid binding studies reported for diphtheria toxin, α-lactalbumin, and colicin A were carried out with negatively charged lipid membranes.

REFERENCES

1. Blacklock, B. J., and Ryan, R. O. (1994) *Insect Biochem. Mol. Biol. 24*, 855−873.
2. Soulages, J. L., and Wells, M. A. (1994) *Adv. Protein Chem. 45*, 371−415.
3. Breiter, D. R., Kanost, M. R., Benning, M. M., Wesemberg, G., Law, J. H., Wells, M. A., Rayment, I., and Holden, M. (1991) *Biochemistry 30*, 603−608.
4. Wang, J., Gagné, S. M., Sykes, B. D., and Ryan, R. O. (1997) *J. Biol. Chem. 272*, 17912−17920.
5. Smith, A. F., Owen, L. M., Strobel, L. M., Chen, H. D. R., Kanost, M. R., Hanneman, E., and Wells, M. A. (1994) *J. Lipid Res. 35*, 1976−1984.
6. 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.
7. Wientzek, M., Kay, C. M., Oikawa, K., and Ryan, R. O. (1994) *J. Biol. Chem. 269*, 4605−4612.
8. Ryan, R. O., Oikawa, K., and Kay, C. M. (1993) *J. Biol. Chem. 268*, 1525−1530.
9. Zhang, Y., Lewis, R. N. A., McElhaney, R. N., and Ryan, R. O. (1993) *Biochemistry 32*, 3942−3952.
10. Kawooya, J. K., Meredith, S. C., Wells, M. A., Kezdy, F. J., and Law, J. H. (1986) *J. Biol. Chem. 261*, 13588−13591.
11. Soulages, J. L., and Wells, M. A. (1994) *Biochemistry 33*, 3245−3251.
12. Soulages, J. L., Salamon, Z., Wells, M. A., and Tollin, G. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5650−5654.
13. Soulages, J. L., van Antwerpen, R., and Wells, M. A. (1996) *Biochemistry 35*, 5191−5198.
14. Narayanaswami, V., Wang, J., Kay, C. M., Scraba, D. G., and Ryan, R. O. (1996) *J. Biol. Chem. 271*, 26855−26862.
15. Upadhayaya, M., Oikawa, K., Kay, C. M., Scraba, D. G., Bradley, R., and Ryan, R. O. (1995) *Arch. Insect Biochem. Physiol. 30*, 211−223.
16. Elwell, M. L., and Schellman, J. A. (1977) *Biochim. Biophys. Acta 494*, 367−383.
17. Eftkin, M. R., and Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199−227.
18. Provencher, S. W., and Glockner, J. (1981) *Biochemistry 20*, 33−37.
19. Pownall, H. J., Massey, J. B., Kusserow, S. K., and Gotto, A. M., Jr. (1978) *Biochemistry 17*, 1183−1188.
20. Eibl, H., and Wooley (1979) *Biophys. Chem. 10*, 261−271.
21. Woody, R. W., and Dunker, A. K. (1996) in *Circular Dichroism and the Conformational Analysis of Biomolecules* (Fasman, G. D., Ed.). pp 109−157, Plenum Press, New York.
22. Ragone, R., Colonna, G., Balestrieri, C., Servillo, and L. Irace, G. (1984) *Biochemistry 23*, 1871−1875.
23. Narayanaswami, V., Frolov, A., Schroeder, F., Oikawa, K., Kay, C. M., and Ryan, R. O. (1996) *Arch. Biochem. Biophys. 334*, 143−150.
24. Mach, H., Thomson, J. A., Middaugh, C. R., and Lewis, R. V. (1991) *Arch. Biochem. Biophys. 287*, 33−40.
25. Mach, H., and Middaugh, C. R. (1994) *Anal. Biochem. 222*, 323−331.
26. Follenius, A., and Gerard, D. (1983) *Photochem. Photobiol. 38*, 373−376.
27. Gursky, O., and Atkinson, D. (1996a) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2991−2995.
28. Gursky, O., and Atkinson, D. (1996b) *Protein Sci. 5*, 1874−1882.
29. Ptitsyn, O. B. (1992) *Protein Folding* (Creighton, T. E., Ed.) pp 243−300, Freeman, New York.
30. Fink, A. (1995) *Methods Mol. Biol. 40*, 343−360.
31. Kawajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87−103.
32. Ptitsyn, O. B. (1995) *Adv. Protein Chem. 47*, 83−229.
33. Bychkova, V. E., Pain, R. H., and Ptitsyn, O. B. (1988) *FEBS Lett. 238*, 231−234.
34. Van der Goot, F. G., Gonzalez-Maas, J. M., Lakey, J. H., and Patus, F. (1991) *Nature 354*, 408−410.

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