Insight into Lipid Surface Recognition and Reversible Conformational Adaptations of an Exchangeable Apolipoprotein by Multidimensional Heteronuclear NMR Techniques

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Apolipoporphin III (apoLp-III) from the insect Manduca sexta is a 166-residue (Mr 18,340) member of the exchangeable apolipoprotein class that functions to stabilize lipid-enriched plasma lipoproteins. In the present study, we present the secondary structure and global fold of recombinant apoLp-III derived from three-dimensional heteronuclear NMR spectroscopy experiments. Five discrete α-helical segments (21–30 residues in length) with well defined boundaries were characterized by four NMR parameters: medium range nuclear Overhauser enhancement contacts between proton pairs, chemical shift index, coupling constants, and amide proton exchange rates. An antiparallel arrangement of helical segments has been obtained based on the long range interhelical nuclear Overhauser enhancement contacts. The NMR solution structure reveals a globular, up and down helix bundle organization similar to that of Locusta migratoria apoLp-III (Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., and Holden, H. M. (1991) Biochemistry 30, 603–608). However, a short helix (comprised of 5 amino acids) has been identified in the region between helix 3 and helix 4. This helix is postulated to play a role in lipid surface recognition and/or initiation of binding. Our results also indicate the existence of buried polar and charged residues in the helix bundle, providing a structural basis for the relatively low stability of apoLp-III in its lipid-free state. It is suggested that the intrinsic low stability of lipid-free apoLp-III may be important in terms of its ability to undergo a reversible, lipid binding-induced, conformational change. This study underscores the striking resemblance in molecular architecture between insect apoLp-III and the N-terminal domain of human apolipoprotein E. The potential for application of NMR techniques to studies of the exchangeable apolipoproteins, possibly in their biologically active, lipid-associated state, has broad implications in terms of our understanding of the molecular basis of their physiological functions.

Plasma lipoproteins are stable noncovalent assemblies of lipids and specialized proteins termed apolipoproteins. The apolipoproteins can be broadly categorized as exchangeable or nonexchangeable. Whereas nonexchangeable apolipoproteins are integral, nontransferable components, the exchangeable apolipoproteins stabilize lipoproteins through reversible binding as a function of particle surface defects or exposed hydrophobic regions. Specific exchangeable apolipoproteins are known to regulate lipoprotein metabolism through modulation of lipid metabolic enzymes or by functioning as ligands for cell surface receptors. To date, approximately 11 distinct human plasma-exchangeable apolipoproteins have been isolated and characterized (1). The capacity to form a stable interaction with lipid surfaces represents a common function shared by all exchangeable apolipoproteins and is essential for the expression of their alternate functions. Plasma levels of certain exchangeable apolipoproteins are correlated with several human diseases including dyslipidemia and cardiovascular disease (2). For example, overexpression of apolipoprotein E (apoE) in transgenic mice results in a decrease in plasma cholesterol (3), whereas apoE knockout mice exhibit massive accumulation of remnant lipoproteins, leading to premature atherosclerosis (4, 5).

Structural studies on exchangeable apolipoproteins indicate that the amphipathic α helix provides an essential structural motif for lipoprotein binding (6). A major advance in our understanding of exchangeable apolipoprotein structure comes from studies using X-ray crystallography to determine the three-dimensional structures of the 22-kDa N-terminal domain of human apoE (7) and apolipoporphin III (apoLp-III) from the insect Locusta migratoria (8). These structures, which were determined in the lipid-free state, share a similar molecular architecture comprised of a bundle of elongated amphipathic α helices. Whereas the N-terminal domain of human apoE exists as a four-helix bundle, L. migratoria apoLp-III is organized as a five-helix bundle. The helices orient their hydrophobic faces toward the center of the bundle while their hydrophilic faces interact with the solvent. It has been postulated that association with a lipid surface triggers a dramatic conformational change wherein the loop regions between α-helical segments function as hinges about which the molecule opens to expose its hydrophobic interior, which then becomes available for lipid binding (8, 9). To date, no lipid-bound structure of an exchangeable apolipoprotein is available.

The abbreviations used are: apoE, apolipoprotein E; apoLp-III, apolipoporphin-III; HSQC, heteronuclear single-quantum correlation; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCOSY, total correlation spectroscopy; HNHA, amide proton to nitrogen to a proton correlation; CBCA(CO)NH, β proton to αβ carbon (via carbonyl carbon) to nitrogen to amide proton correlation; HCNACB, amide proton to nitrogen to αβ carbon correlation; HCCCH-TOCYS, proton-carbon-carbon-proton correlation using carbon total correlation spectroscopy; HMNC-J, heteronuclear multiple quantum coherence (NH-Hα scalar coupling); CSI, chemical shift index.
Given recent advances in the application of NMR spectroscopy to protein solution structure determination, this technique should be applicable to studies of exchangeable apolipoproteins. It has been shown by others that NMR techniques can be used to determine three-dimensional structures of proteins up to 30 kDa (10). Whereas most exchangeable apolipoproteins meet this size limitation, their tendency to self-aggregate in the absence of lipids hinders the application of NMR methods. Several investigators have used two-dimensional 1H NMR to study apolipoprotein peptide fragments in the range of 15–36 amino acids (11–14). To date, however, no NMR structure solutions of intact exchangeable apolipoproteins have been reported.

NMR structure determination is attractive because it can be used to study the conformations as well as the dynamic changes of exchangeable apolipoproteins in both lipid-free and lipid-bound states. In considering possible candidate apolipoproteins for solution structure determination by NMR, we found that apoLp-III (M, 18,300) meets several essential criteria. Although the x-ray crystal structure of L. migratoria apoLp-III has been solved (8), there are no reports of expression of this protein in bacteria, possibly due to the presence of complex carbohydrate moieties (15), which may be important for protein stability in the lipid-free state (16). On the other hand, the homologous apoLp-III from the sphinx moth, Manduca sexta, lacks covalent oligosaccharide moieties (17), is monomeric over a broad concentration range (18), and has been overexpressed in bacteria (19). Although the sequence identity between these two apoLp-III s is <30%, the two proteins are functionally indistinguishable (20).

The goal of our research is to understand the molecular basis of reversible conformational adaptations of the exchangeable apolipoproteins upon lipid binding. As the first step, we report here the solution secondary structure and global fold of lipid-free apoLp-III derived from three-dimensional heteronuclear NMR experiments. To our knowledge, this represents the first solution structure determination of an intact exchangeable apolipoprotein. Furthermore, it represents one of the largest α-helical proteins whose structure has been solved using NMR techniques (21). The NMR structure of M. sexta apoLp-III provides new insight into the mechanism of apoLp-III lipid surface recognition/binding and provides a structural rationale for the reversibility of its interactions with lipoprotein surfaces.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Isotope Labeling of Recombinant ApoLp-III—Bacterial expression of recombinant apoLp-III was carried out as described by Ryan et al. (19). For isotopic labeling experiments, a saturated overnight cell culture (grown in 2 x yeast tryptone medium at 37 °C) was diluted 1:100 (v/v) in M9 minimal medium containing 0.1% 2NH4Cl (Isotec, Miamisburg, OH) and/or 0.15% [6-13C]glucose (Isotec) as the sole nitrogen and/or carbon source with 0.1% 15NH4Cl (Isotec, Miamisburg, OH) and/or 0.15% D-[6-13C]glucose (Isotec). The culture was grown to an optical density of 0.60–0.90 at 590 nm, harvested by pelleting the cells and collecting the supernatant. Upon concentration to <50 ml, the culture supernatant was dialyzed against deionized H2O for 48 h and lyophilized. Protein was dissolved in water and subjected to high pressure liquid chromatography on a preparative Zorbax C8 reversed phase column. The purity of the sample was assessed by SDS-polyacrylamide gel electrophoresis, and the relative isotope enrichment was characterized by electrospray ionization mass spectrometry. Using the protocol described above, 70–100 mg of 13C, 15N double-labeled or 120–150 mg of 15N single-labeled apoLp-III was obtained per liter of cell culture with >99% purity and an isotope enrichment of >95%.

NMR Sample Preparation—Previous denaturation studies of lipid-free apoLp-III, monitored by circular dichroism spectroscopy, revealed a midpoint of guanidine HCl-induced denaturation of 0.35 M, corresponding to a ΔGm,25°C of 1.29 kcal/mol (22). Consistent with these results, a time course study of apoLp-III stability monitored by 1H/13C/15N HSQC experiments showed that apoLp-III in H2O at pH 7.0 was stable for >16 h. While peaks that corresponded to an unfolded state of the protein appeared at longer times (data not shown). Empirical stability optimization studies monitored by NMR resulted in the finding that apoLp-III was stable for >2 weeks in 250 mM potassium phosphate, 0.5 mM sodium azide, pH 6.4–6.5. In this buffer, the chemical shift dispersion in both the 1H and 15N dimensions was acceptable. Thus, for three-dimensional 1H/13C/15N-edited TOCSY, and HNHA experiments as well as two-dimensional 1H/15N HSQC and 15N/13C HMBC-J experiments, [15N]apoLp-III was dissolved in 250 mM potassium phosphate, pH 6.5, 0.5 mM NaN3 in 95% H2O, 5% D2O (1.2 mM final protein concentration). Likewise, this buffer and protein concentration of [13C, 15N]apoLp-III was employed for three-dimensional CBCA(CO)NNH, HNCA/HCCH-TOCSY, and simultaneous 13C- and 15N-edited NOESY experiments. All samples contained a small amount of 2,2-dimethyl-2-silapentanesulfonic acid as an internal standard for proton chemical shifts.

NMR Spectroscopy—All NMR experiments were carried out at 30 °C on a Varian Unity 600 NMR spectrometer equipped with three channels, a pulsed field gradient triple resonance probe with a z gradient, and a gradient amplifier unit. Carrier positions used in the various three-dimensional NMR experiments are as follows: 1H, 119.0 ppm; 13C, 43.0 ppm; 15N, 4.71 ppm. Two-dimensional 1H/15N HSQC spectra (23) were acquired with the following number of complex points and acquisition times: F1 (1H) 128, 91.5 ms; F2 (15N) 73, 8–32 transients. HMBC-J (24) spectra were recorded with the following number of complex points and acquisition times: F1 (1HN) 300, 214.5 ms; F2 (1H) 512, 73 ms, 8–32 transients. Pulse field gradient HCCCH-TOCSY (28) spectra were acquired on a double-labeled H2O sample with a mixing time of 16 ms (12 transients). The acquisition parameters for HCCCH-TOCSY were: F1 (1H) 512, 64 ms; F2 (1H–1H) 128, 35.6 ms; F3 (1C) 40, 128 ms; and 13C/15N-edited NOESY spectra were recorded with mixing times of 50 and 100 ms. The acquisition parameters for these experiments were: F1 (1H) 128, 35.6 ms; F3 (1H–1H) 512, 73 ms; and F2 (15N) 28, 20 ms. F3 (1H) 128, 20 ms; F2 (15N) 32, 22.9 ms. The same number of complex points and acquisition times was also used for 13C/15N-edited NOESY with a mixing time of 59 ms.

For backbone assignment of apoLp-III, 13N-edited NOESY and 15N-edited TOCSY spectra were used mainly due to the α-helical nature of the protein. HNCA/C and CBCA(CO)NNH spectra were used to confirm the backbone atom assignment obtained from 13N-edited NOESY and 13N-edited TOCSY spectra as well as to assign those residues that could not be assigned due to missing NH–NH connectivities in 13N-edited NOESY spectra. 1H/15N HSQC spectra of [15N]Leu, [15N]Lys, and [15N]Val specifically labeled apoLp-III facilitated assignment using the amide-amide walking strategy (34). Side-chain atoms were assigned using HCCCH-TOCSY, 13N-edited NOESY, 13N-edited TOCSY, and 15N- and 13C-edited NOESY spectra. All spectra were processed on SUN workstations using NMRPipe software from Delaglio et al. (35). The spectral assignment was achieved using Pipp software (36). Postacquisition solvent subtraction was employed in spectra where NH protons were detected in the acquisition time course study of apoLp-III stability monitored by 1H/15N HSQC experiments showed that apoLp-III in H2O at pH 7.0 was stable for >16 h. While peaks that corresponded to an unfolded state of the protein appeared at longer times (data not shown). Empirical stability optimization studies monitored by NMR resulted in the finding that apoLp-III was stable for >2 weeks in 250 mM potassium phosphate, 0.5 mM sodium azide, pH 6.4–6.5. In this buffer, the chemical shift dispersion in both the 1H and 15N dimensions was acceptable. Thus, for three-dimensional 1H/13C/15N-edited TOCSY, and HNHA experiments as well as two-dimensional 1H/15N HSQC and 15N/13C HMBC-J experiments, [15N]apoLp-III was dissolved in 250 mM potassium phosphate, pH 6.5, 0.5 mM NaN3 in 95% H2O, 5% D2O (1.2 mM final protein concentration). Likewise, this buffer and protein concentration of [13C, 15N]apoLp-III was employed for three-dimensional CBCA(CO)NNH, HNCA/HCCH-TOCSY, and simultaneous 13C- and 15N-edited NOESY experiments. All samples contained a small amount of 2,2-dimethyl-2-silapentanesulfonic acid as an internal standard for proton chemical shifts.
Assignment Strategy—For α-helical proteins in the 150-amino acid size range, the overall chemical shift dispersion is generally poor, especially in the $^1$H dimension. In $^1$H/$^15$N HSQC spectra of apoLp-III, the amide proton chemical shift dispersion is <2.5 ppm, with significant spectral overlap observed in the range of 7.5–8.5 ppm. By contrast, the chemical shift dispersion in the $^15$N dimension is relatively large, improving the overall cross-peak separation. Two independent methods were used to obtain the assignment of apoLp-III. One is through the amide-amide proton connectivities in the three-dimensional $^15$N-edited NOESY spectra. For residues with no water peak in the strip, slow exchange with solvent is concluded. In cases of weak intensity and medium or strong intensity water peaks in the strips, medium and fast proton exchange, respectively, is concluded.

RESULTS

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atoms on the basis of sequential NH–NH connectivities in $^{15}$N-edited NOESY spectra.

Fig. 2 shows C\textsubscript{b}(i-1)-C\textsubscript{a}(i-2)-N(i)-NH(i) connectivities from a CBCA(CO)NNH spectrum together with the corresponding C\textsubscript{b}(i)-C\textsubscript{a}(i)-N(i)-NH(i) and C\textsubscript{b}(i-1)-C\textsubscript{a}(i-1)-N(i)-NH(i) (in different sign) connectivities identified by an HNCACB experiment for residues Lys73–Thr86 of apoLp-III. An independent assignment, obtained on the basis of these two spectra, was used to confirm the assignment derived from $^{15}$N-edited NOESY and $^{15}$N-edited TOCSY spectra. For residues Ala\textsuperscript{4}-Gly\textsuperscript{5}-Gly\textsuperscript{6}-Asn\textsuperscript{7}-Ala\textsuperscript{8}, where no dNN connectivities were found, and residues Ser\textsuperscript{33}-Lys\textsuperscript{34}-Asn\textsuperscript{35}-Thr\textsuperscript{36}-Gln\textsuperscript{37}-Asp\textsuperscript{38}-Phe\textsuperscript{39}, where significant overlap was observed for NH protons, assignment could not be made on the basis of the $^{15}$N-edited NOESY spectrum alone. In this case, HNCACB and CBCA(CO)NNH spectra were used to obtain the assignment. Side-chain assignment was obtained by analysis of HCCH-TOCSY, $^{15}$N-edited TOCSY, $^{15}$N-edited NOESY, and $^{13}$C-edited NOESY spectra. The $^{15}$N-edited TOCSY spectrum was not very useful in this regard, however, since it generally showed the connectivities only up to a protons. Three-dimensional HCCH-TOCSY provided a powerful spectrum for side-chain assignment, although, in some cases, chemical shift overlap was observed in some planes, creating ambiguity for some side-chain assignments. In these cases, three-dimensional $^{15}$N-edited NOESY and $^{13}$C-edited NOESY spectra were used to confirm the assignment.

Fig. 3 shows a complete assignment of the $^{1}H/^{15}$N HSQC spectrum of apoLp-III in 50 mM potassium phosphate, pH 7.0. The assignments are shown by the residue name (one-letter code) and number. Cross-peaks connected by dotted lines in the upper right corner are derived from the side-chain amine groups of Asn and Gln residues. Cross-peaks connected by dotted lines in the upper right corner of Fig. 3 were derived from the side-chain amine groups of Asn and Gln residues. Interestingly, several cross-peaks derived from backbone N–H groups (such as Ser\textsuperscript{24}, Ser\textsuperscript{128}, Ser\textsuperscript{66}, and Ser\textsuperscript{29}) were located in this region of the spectrum. We have recently reported a strategy that results in specific labeling of peptide backbone nitrogen atoms without side-chain nitrogen atom labeling (34). The $^{1}H/^{15}$N HSQC spectrum of the backbone nitrogen specifically labeled apoLp-III sample confirmed the assignment of these four Ser residues (data not shown).

The Secondary Structure of ApoLp-III—Fig. 4 summarizes short and medium range NOE connectivities ($|i-j| \leq 4$), amide exchange rates, $^{3}J_{NN}$ coupling constants, and chemical shift index data for apoLp-III. The medium range (2 $\leq |i-j| < 4$) NOEs, especially $d_{NN}(i, i+3)$ NOEs, together with strong $d_{NN}(i, i+1)$ and weak $d_{NN}(i, i+3)$ NOEs, indicate that the peptide bonds adopt a folded structure (i.e. a helix), due to the 3.6-residue repeats in the helical structure. The observed slow amide proton exchange rates for specific residues suggest these protons are hydrogen-bonded, which prevents proton exchange with the solvent, again consistent with an $\alpha$-helical structure. The chemical shift index is a consensus of $\alpha$ proton and $\alpha$ and $\beta$ carbon atom chemical shift deviation from random coil values, which correlates with specific secondary structure elements (41). In the case of apoLp-III, the observed negative CSI for most residues in the protein is consistent with $\alpha$-helical
secondary structure. Likewise, the observed lack of consensus positive CSI for any residue indicates an absence of \( \beta \) structure in apoLp-III. Finally, the presence of 3\( \tilde{J}_{\alpha N} \) coupling constants, 6.0 Hz (shown by filled circles in Fig. 4) provides further evidence of a helix structure for given residues. Whereas reliance on a single parameter may cause ambiguity, the use of four independent NMR measurements permits conclusive identification of secondary structure elements within the protein sequence. The consensus NMR parameters for apoLp-III shown in Fig. 4 clearly identify five discrete \( \alpha \)-helical regions (21–30 residues in length) with well defined boundaries. These data are in accord with structural information on apoLp-III from another insect, \( L. \) migratoria (8), despite the fact that these proteins share \( 30\% \) sequence identity. In \( M. \) sexta apoLp-III, the NMR data also reveal a short helix, termed helix 3\( _{9} \), located between helix 3 and helix 4 just beyond Pro\( ^{95} \). This helix includes residues Asp\( ^{96} \)–Glu\( ^{100} \). The existence of this short helix is confirmed not only by medium range NOEs \( d_{\alpha N}(\text{Glu}^{99}, \text{Asp}^{96}), d_{\alpha N}(\text{Glu}^{100}, \text{Val}^{97}), d_{\alpha N}(\text{Lys}^{99}, \text{Val}^{97}), \) and \( d_{\alpha NN}(\text{Glu}^{100}, \text{Glu}^{98}) \), but also by slower amide proton exchange rates, smaller 3\( \tilde{J}_{\alpha N} \) coupling constants, and chemical shift index criteria.

The five major helices in apoLp-III range between 21 and 30 residues and, other than helix 3\( _{9} \), are connected to one another by short 5–7-residue flexible loop segments. For example, helix 4 and helix 5 are connected by a seven-residue loop comprising amino acids 129–135. Two \( d_{\alpha N}(i,i+2) \) NOEs were found between residue Glu\( ^{131} \) and Lys\( ^{135} \), indicating turn-type structure in the loop. Helix 5 contains a proline at position 139 which, interestingly, does not break the helix. The C terminus

### Table I

| HELIX | PRIMARY SEQUENCE |
|-------|------------------|
| 0     | DAPAGGINAEEEMHKAEKFOQTKFSQNGSLSYNSKNTQDFNKALDKGSDSV |
| 1     | LOQLSASSGSSGIRGAKAELRQARQNYEKTAEELRKAHPDVEK |
| 2     | ANAKFDKQLGAHAVGTTGQESQKLKEVASMNEETNKKLAPKKQAADDVFK |
| 3     | HAEEVQK(HEAATQO |
| 4     | H4F5 |
| 5     | H5F6 |

**Comparison of the secondary structures of apoLp-III**

| HELIX | \( M. \) sexta \( ^{a} \) | \( L. \) migratoria \( ^{b} \) |
|-------|------------------|------------------|
| Helix 1 | 10–31 | 7–32 |
| Helix 2 | 38–68 | 35–66 |
| Helix 3 | 72–93 | 70–86 |
| Helix 3\( _{9} \) | 96–100 |  |
| Helix 4 | 104–128 | 95–121 |
| Helix 5 | 137–164 | 129–156 |

\( ^{a} \) The secondary structure of apoLp-III from \( M. \) sexta was obtained by NMR techniques in this study.

\( ^{b} \) The secondary structure of apoLp-III from \( L. \) migratoria is from the x-ray crystal structure by Breiter et al. (8).
of helix 5 extends to residue 164 of the protein. Although residues 165 and 166 display $d_{NN}(i, i+3)$ and $d_{NN}(i, i+2)$ NOEs, other NMR parameters indicate these residues do not adopt a stable helical secondary structure. These residues appear to be more flexible, as judged by fast or medium amide proton exchange rates and the larger $J_{aa}$ coupling constants for these residues. A summary of the secondary structure of M. sexta apoLp-III and comparison with L. migratoria apoLp-III is shown in Table I.

**Interhelical Contacts in the Helix Bundle**—Whereas the consensus NMR parameters shown in Fig. 4 identify secondary structure elements, long range NOEs were used to characterize helix topology and the tertiary structure of apoLp-III. These NOEs identify proton pairs <5.0 Å apart in space, despite the fact that they may reside on amino acids from distant regions of the primary sequence. Fig. 5 summarizes >300 interhelical long range NOEs derived from $^{15}$N-edited NOESY spectra at 150-ms mixing time. The helical wheel diagrams shown are based on the secondary structure of apoLp-III identified by the consensus NMR parameters described above. This depiction clearly demonstrates that each helix is amphipathic, with well segregated hydrophobic and hydrophilic surfaces. Analysis of the pattern of long range NOEs indicates that the helices in apoLp-III adopt an up and down, antiparallel orientation. For example, helix 1 contacts with both helix 4 and helix 5. We determined 46 interhelical NOEs between helix 1 and helix 5 and another 46 between helix 1 and helix 4. Long range NOEs were observed between residues 10 and 127, 20 and 153, and 29 and 159. These NOEs indicate a parallel orientation between helix 1 and helix 5 and an antiparallel orientation between helix 1 and helix 4. Interestingly, many more NOEs were found between the N terminus of helix 1 and the C terminus of helix 4 than those between the C terminus of helix 1 and the N terminus of helix 4, suggesting stronger interhelical contacts in one end of the two helices. Helix 2 interacts with three helices, helix 3 (70 interhelical NOEs), helix 4 (26 interhelical NOEs), and helix 5 (42 interhelical NOEs). Helix 2 contacts both helix 3 and helix 5 in an antiparallel manner, with contacts through the entire length of these three helices. Helix 2 contacts helix 4 weakly in a parallel orientation since interhelical NOEs were identified only between the two N termini and a few residues in the center region of the two helices. Other than contacting helix 2, helix 3 interacts with helix 4 in an antiparallel arrangement. A total of 37 interhelical NOEs were found between helix 3 and helix 4, with NOEs observed along the entire length of the two helices.

The interhelical contacts among helices in apoLp-III are mainly from hydrophobic residues such as leucine, valine, isoleucine, phenylalanine, and alanine. These hydrophobic contacts likely exert a stabilizing force in terms of the globular fold of apoLp-III. However, Fig. 5 also indicates that charged residues, such as Lys$^{105}$ and Glu$^{113}$, and several polar residues such as Gln$^{29}$, Asn$^{40}$, Ser$^{47}$, Gln$^{53}$, Ser$^{58}$, Gln$^{109}$, Gln$^{113}$, Ser$^{119}$, Gln$^{120}$, and Gln$^{156}$ are located within the hydrophobic core of the helix bundle. These buried charged and polar residues, which are available for contact with the other residues, may contribute to the low intrinsic stability of this protein in the lipid-free state. Long range NOEs were identified between all residues in helix 3' and several residues in the N terminus of helix 2, indicating the existence of interactions between helix 2 and helix 3'. These NOEs demonstrate that helix 3' is in close proximity to the N terminus of helix 2, suggesting the possible topology of helix 3'. Long range NOEs were also identified in the loops that connect different helices. For example, NOE contacts were found between residues in loop 1 and residues located in the N termini of helix 2 and helix 4; from loop 2 to
loop 4, the N terminus of helix 5 and the C terminus of helix 3; and from loop 4 to loop 2. These long range NOEs allow us to identify the global fold and tertiary structure of apoLp-III.

Tertiary Structure and Global Fold—Fig. 6 shows a cylinder diagram of the global fold and tertiary structure of apoLp-III. This diagram clearly demonstrates a five-helix bundle structure for lipid-free *M. sexta* apoLp-III that is similar to the x-ray crystal structure of *L. migratoria* apoLp-III (8) as well as the four-helix bundle architecture of the N-terminal domain of human apoE (7). The short helix, helix 3*, which connects helix 3 and helix 4, orients almost perpendicular to helix 3 and helix 4 and is positioned at the end of the elongated helix bundle. In terms of proposed helical repositioning upon lipid association (8), helix 3* may function in initiating conformational opening of the protein. Interestingly, a similar short helix is present in the N-terminal domain of apoE (7).

**DISCUSSION**

Previous NMR studies on exchangeable apolipoproteins have been limited to small fragments ranging from 15 to 36 residues using two-dimensional NMR techniques. For example, Rozek et al. (42) and Buchko et al. (12) reported NMR structures of two synthetic fragments of apolipoprotein C-I, whereas Lycksell et al. (11) investigated the structural properties of a 30-residue synthetic fragment of human apolipoprotein C-II (43). In a recent study, Wang et al. (13) determined the structure of a peptide fragment (residues 166–185) of apolipoprotein A-I and two fragments of apoE (14), which range from 14 to 18 residues, in complex with sodium dodecyl sulfate and dodecyl phosphocholine micelles. Using a similar approach, we have solved the structure of a 36-residue C-terminal fragment of *M. sexta* apoLp-III in the presence of SDS. The present study is unique in that we report the first NMR structure of an intact exchangeable apolipoprotein. We suggest that extension of this approach to the lipid-associated state of apolipoproteins employing model lipid surfaces such as dodecyl phosphocholine micelles, points to a potentially fruitful research direction.

Following an extensive empirical search for optimal conditions for NMR experiments, we found that apoLp-III is stable for >2 weeks in 250 mM phosphate buffer at pH 6.4–6.5. This is a critical step toward the practice of three-dimensional NMR studies of apoLp-III. It was known from previous denaturation studies that apoLp-III is relatively labile and susceptible to environmental perturbation (22). Indeed, the low stability of apoLp-III is a common property of most exchangeable apolipoproteins. For example, the midpoint of guanidine HCl-induced denaturation of the 10-kDa C-terminal domain of human apoE was found to be 0.7 M guanidine HCl (44); that for human apolipoprotein A-II was 0.6 M (45), and that for human apolipoprotein A-IV was found at 0.4 M (46). A potentially important factor contributing to the low stability of apoLp-III has been identified in the NMR structure of *M. sexta* apoLp-III. Although hydrophobic interactions are the major force contributing to stabilization of the helix bundle structure, Fig. 5 indicates that there are several charged residues, such as Lys*<sup>105</sup>, Glu*<sup>13</sup>, as well as several polar residues (e.g. Gln*<sup>20</sup>, Asn*<sup>40</sup>, Ser*<sup>17</sup>, Gln*<sup>53</sup>, Ser*<sup>56</sup>, Gln*<sup>109</sup>, Gln*<sup>113</sup>, Ser*<sup>119</sup>, Glu*<sup>120</sup>, and Gln*<sup>156</sup>) located in the interior of the helix bundle. We suggest that charged and polar residues localized in otherwise nonpolar regions of the molecule contribute to the low stability of apoLp-III. This hypothesis can be tested by site-specific mutagenesis experiments coupled with denaturation studies.

It is conceivable that the low stability of apoLp-III is required for its known reversible lipoprotein binding activity. Based on the crystal structure of *L. migratoria* apoLp-III, it was proposed that the helix bundle opens about putative hinge regions, exposing its hydrophobic interior, which interacts di-
rectly with lipid surfaces (8). This open conformation model is supported by the NMR structure of apoLP-III presented in this study. Whereas conformational opening of apoLP-III may disrupt certain interhelical contacts within the protein, this is more than compensated by subsequent replacement of helix-helix contacts with helix-lipid interactions. It is recognized that association with lipoprotein surfaces and consequent conformational opening of apoLP-III is induced by the content of surface monolayer-localized diacylglycerol. On the other hand, dissociation from lipoprotein surfaces and return to the helix bundle conformation results from depletion of lipoprotein diacylglycerol content (47–50). Thus, in essence, apoLP-III association/dissociation with lipoproteins is dictated by the lipid composition of the particle. The intrinsic reversibility of apoLP-III conformational changes permits the protein to function in multiple association/dissociation events during its lifetime in plasma.

Compared with the crystal structure of L. migratoria apoLP-III, the NMR structure of M. sexta apoLP-III reveals an overall similar molecular architecture. One apparent difference, however, is the presence of a short helix, helix 3, in M. sexta apoLP-III. Interestingly, a similar short helix was also found in the x-ray crystal structure of human apoE N-terminal domain (7). In human apoE, this short helix, which involves nine residues, connects helix 1 and helix 2 and orients perpendicular to helix 1 and helix 2. The short helix found in the NMR structure of M. sexta apoLP-III is in a similar orientation. It is worth noting that helix 3 is a flexible helix displaying amide exchange rates that are faster than those from the other helices.

The helix-short helix-helix organization in this molecule may comprise an important structural element in apoLP-III in terms of initiating interactions with lipoprotein surfaces. It is noteworthy that helix 3 is positioned where, in the open conformation model, it could initiate contact with lipid surfaces. We have proposed a structural model to describe the interactions between apoLP-III and the phospholipid monolayer of insect lipophorins based on 31P NMR studies designed to evaluate the mobility of phospholipids in different lipophorin sub-species (48). In this model, we suggested that diacylglycerol partitioning into the particle surface monolayer creates a binding site for apoLP-III interaction. It is recognized that a strong correlation exists between lipophorin diacylglycerol content and apoLP-III binding, and recently we have provided direct experimental evidence for surface localization of diacylglycerol in lipophorin particles known to bind apoLP-III (49). We speculate that surface diacylglycerol intercalates between phospholipids in the monolayer surface, creating a gap between phospholipid head groups. This defect is effectively repaired by apoLP-III binding.

An important question, however, is whether hydrophobic interactions are responsible for initiation of binding or whether ionic interactions localize apoLP-III at the particle surface, positioning the protein to "respond" to surface defects created by diacylglycerol partitioning into the surface monolayer. Souлагes and Wells (51) have presented a "hydroporphic sensor" hypothesis to describe the initiation of interactions between exchangeable apolipoproteins and lipoproteins. According to this hypothesis, a two-step sequential mechanism for binding of apoLP-III to lipoprotein surfaces has been proposed. The first step involves a recognition process (through exposed hydrophobic amino acids) consisting of the adsorption of apoLP-III to a nascent hydrophobic defect in the phospholipid bilayer caused by the presence of diacylglycerol. This is followed by a conformational opening to expose the protein interior. Souлагes and Wells suggest (51) that in L. migratoria apoLP-III, conserved leucines located in the loops between helices are responsible for the initial association process. An alternative proposal is that helix 3 may function in initiation of stable lipid binding. This concept is supported by disulfide bond engineering studies, which indicate that apoLP-III interactions with lipoproteins is oriented at the end of the molecule containing helix 3 (52). Helix 3 contains five residues (Asp, Val, Glu, Lys, and Glu), four of which possess charged hydrophilic side chains. The suggestion that charge-charge interactions between the phospholipid head groups and helix 3 provide a relatively long range attraction, which localizes apoLP-III in close proximity to the lipoprotein surface, is supported by studies with model phospholipids (53). Once the recognition process is complete, surface-exposed diacylglycerol could trigger opening of the helix bundle, resulting in formation of a stable binding interaction. Judging by the fact that the N-terminal domain of apoE contains a similar short helix, an analogous binding mechanism may be suggested for human exchangeable apolipoprotein-lipoprotein interactions. Although further experiments are required to determine the precise mode of lipid association of apoLP-III, it is likely that concepts developed with this model protein may have important implications for amphipathic-exchangeable apolipoproteins in general.

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