NMR Solution Structure and Dynamics of an Exchangeable Apolipoprotein, *Locusta migratoria* Apolipophorin III*

We report here the NMR structure and backbone dynamics of an exchangeable apolipoprotein, apoLp-III, from the insect *Locusta migratoria*. The NMR structure adopts an up-and-down elongated five-helix bundle, which is similar to the x-ray crystal structure of this protein. A short helix, helix 4′, is observed that is perpendicular to the bundle and fully solvent-exposed. NMR experimental parameters confirm the existence of this short helix, which is proposed to serve as a recognition helix for apoLp-III binding to lipoprotein surfaces. The *L. migratoria* apoLp-III helix bundle displays several characteristic structural features that regulate the reversible lipoprotein binding activity of apoLp-III. The buried hydrophilic residues and exposed hydrophobic residues readily adjust the marginal stability of apoLp-III, facilitating the helix bundle opening. Specifically, upon lipoprotein binding, the locations and orientations of the buried hydrophilic residues modulate the apoLp-III helix bundle to adapt a possible opening at the hinge that is opposite the recognition short helix, helix 4′. The backbone dynamics provide additional support to the recognition role of helix 4′ and this preferred conformational adaptation of apoLp-III upon lipid binding. In this case, the lipid-bound open conformation contains two lobes linked by hinge loops. One lobe contains helices 2 and 3, and the other lobe contains helices 1, 4, and 5. This preferred bundle opening is different from the original proposal on the basis of the x-ray crystal structure of this protein (Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, L. and Holden, H. M. (1991) Biochemistry 30, 603–608), but it efficiently uses helix 4′ as the recognition short helix. The buried interhelical H-bonds are found to be mainly located between the two lobes, potentially providing a specific driving force for the helix bundle recovery of apoLp-III from the lipid-bound open conformation. Finally, we compare the NMR structures of *Manduca sexta* apoLp-III and *L. migratoria* apoLp-III and present a united scheme for the structural basis of the reversible lipoprotein binding activity of apoLp-III.

Exchangeable apolipoproteins regulate the metabolism of lipids and plasma lipoproteins by their reversible lipoprotein binding activity (1). It is commonly believed that lipoprotein association induces a large conformational change in apolipoproteins (2). In the lipid-free state, apolipoproteins adopt an up-and-down helix bundle topology (3–5) in which the hydrophobic faces of the amphipathic helices are oriented toward each other to form a bundle interior and the hydrophilic faces are exposed to the solvent. This lipid-free helix bundle architecture is well demonstrated by the x-ray crystal structure of the N-terminal domain of human apoE (3), the x-ray crystal structure of apoLp-III from the insect, *Locusta migratoria* (4), and the NMR structure of apoLp-III from the insect *Manduca sexta* (5). On lipid binding, however, the apolipoprotein helix bundle is believed to open at putative hinge loops that connect helices, adopting an extended α-helical conformation that contains large flat hydrophobic surfaces for lipid binding (4, 6). Although recently published NMR structures of apoC-I (7), and a truncation mutant of apoA-I, apoA-I-(187–243) (8), in a lipid mimetic environment support this open conformation model, important questions are raised as to what triggers the lipid binding process and what structural features in the helix bundle regulate the bundle opening/recovery.

ApoLp-III is an exchangeable apolipoprotein found in the hemolymph of many insect species. It serves as a good model for structure-function studies of human exchangeable apolipoproteins (2). The x-ray crystal structure of *L. migratoria* apoLp-III provided the first high resolution structure of an exchangeable apolipoprotein and offered the possibility of correlating the structural knowledge of an apolipoprotein to its reversible lipid binding activity (4). We have reported an NMR structure of *M. sexta* apoLp-III (5, 9). Both apoLp-III adopt a similar up-and-down elongated five-helix bundle topology. We further described several important structural features that modulate the helix bundle opening/recovery of *M. sexta* apoLp-III on association/dissociation with the lipoprotein surface (9). In addition, a short helix was found between helices 3 and 4 in *M. sexta* apoLp-III (helix 3′), but was not reported in the original paper on the x-ray crystal structure of *L. migratoria* apoLp-III (4). This short helix, located at one end of the bundle, adopts an orientation that is nearly perpendicular to the long axis of the bundle and is fully exposed to the solvent. We hypothesized that this short helix played a critical role in initiating the lipid binding process of apoLp-III (5). Our experimental evidence using site-directed mutagenesis supported this concept (10). The fact that both apoLp-IIIAs share a similar...
helix bundle topology and an identical reversible lipoprotein binding activity leads us to speculate that *L. migratoria* apoLp-III may have a similar short helix that is responsible for lipid surface recognition. To test this hypothesis, we carefully studied the x-ray crystal structure of *L. migratoria* apoLp-III (Protein Data Bank code IAEF) and found a similar short helix that is located at the opposite end of the helix bundle from that of helix 3 of *M. sexta* apoLp-III. This short helix, helix 4, is located between helices 4 and 5 (Ala-132-Pro-132). Despite a different location from helix 3 of *M. sexta* apoLp-III, helix 4 in *L. migratoria* apoLp-III is also perpendicular to the long axis of the helix bundle and is fully solvent-exposed. This raises the question of whether the helix bundle of *L. migratoria* apoLp-III may open in a different manner if helix 4 serves as the recognition short helix. To confirm this short helix in solution, we carried out a NMR structural determination of *L. migratoria* apoLp-III.

On the basis of the x-ray crystal structure of *L. migratoria* apoLp-III, it was proposed that upon lipid binding the bundle opens in such a way that the helices spread on the lipid surface. Helices 1, 2, and 5 form a lobe, and helices 3 and 4 form the other lobe (4). The two lobes are linked by the putative hinge loops in which one loop contains helix 4. This hypothesis is based on an assumption that several conserved leucine residues located in the putative hinge serves as the recognition sites of apoLp-III for the lipoprotein surface. One recent report seems to support this hypothesis (11). An alternative model for the apoLp-III helix bundle opening is that it opens at hinges located at the opposite end to helix 4’, containing helices 1, 4, and 5 in one lobe and helices 2 and 3 in the other lobe. The helix bundle topology indeed allows for this alternative bundle opening. More importantly, this alternative model permits helix 4’ to serve as a recognition helix, similar to the role that helix 3 plays in *M. sexta* apoLp-III. The apparent difference between these two models is that the helix bundle opens at a different end of the bundle. An NMR solution structure and dynamics may provide critical information to identify the most likely conformation adaptation of *L. migratoria* apoLp-III on lipid binding.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Isotope Labeling of *L. migratoria* apoLp-III**—An efficient expression system of *L. migratoria* apoLp-III has been developed (12) that produces ~150 mg/liter protein. Briefly, saturated 2× YT overnight was diluted (1:100, v/v) in M9 minimal medium containing 0.1% 15NH4Cl and/or 0.2% D-13C6-glucose (Isotec, C. The protein production was carried out semiautomatically using a computer program to resolve ambiguous NOEs (19) in an iterative manner. Each iteration generated 50 NMR structures, which were analyzed for identification of distance restraint violations of >0.3 Å. NOEs were checked for ambiguity, followed by generation of a new distance restraint set for the next calculation. Thirteen iterations were carried out, and the final iteration generated 50 structures with no NOE violations of >0.3 Å or dihedral angle violations of >2.0°. The 20 lowest energy NMR structures within the final 50 structures were used for the structural statistics calculation.

**Structural Analysis**—The NMR and x-ray crystal structures of *L. migratoria* apoLp-III were analyzed using PROCHECK (20) and VADAR (21) programs. Both programs analyze the coordinates of proteins and give secondary structure locations. The VADAR program also provides the fractional solvent-accessible surface area (ASA) of the side chain of each residue as well as H-bonding information. From the fractional ASA of each side chain, we identified buried hydrophilic residues and exposed hydrophobic residues. The thresholds used were a side chain fractional ASA of <0.30 for the buried hydrophilic residues and a side chain fractional ASA of >0.30 for the exposed hydrophobic residues.

**15N Relaxation Measurements**—The 15N relaxation times *T*1 and *T*2, and 13C-15N NOEs were measured using inversely detected two-dimensional NMR methods (22, 23). Relaxation times *T*1 were determined by collecting seven points with delays of 10, 100, 200, 300, 400, 500, and 700 ms using a recycle delay of 1 s and 8 scans. Relaxation times *T*2 were measured by collecting seven points with delays of 10, 20, 30, 40, 50, 60, and 70 ms using a spin-lock power of 1.6 kHz, a 2.5-s recycle delay, and eight scans. To measure the heteronuclear NOEs, two spectra were acquired with and without proton saturation using 24 scans. Saturation was achieved by running a train of 120° HI pulses for 4 s after a 4-s recycle delay. All the data were recorded by using 256 and 512 complex points in *t*1 and *t*2 dimensions, respectively, and with spectral widths of 1316 Hz (15N) and 8000 Hz (1H). Relaxation times were fitted as single exponential decays to peak height data. Spin–spin relaxation time *T*2 was calculated from *T*1 and *T*2 according to Equation 1:

\[
\frac{1}{T_{2}} = \frac{1}{T_{1}} \sin^{2} \theta + \frac{1}{T_{1}} \cos^{2} \theta
\]  

(Eq. 1)

where θ = atan(Δω/Δω₀) and Δω and Δω₀ are the resonance offset and spin-lock field strength, respectively.

**RESULTS**

**Quality of the NMR Structure**—Fig. 1 shows an ensemble of 20 NMR structures and a ribbon representation of the energy-minimized average structure of lipid-free *L. migratoria* apoLp-III. The coordinates of these 20 NMR structures and an energy-minimized average structure have been deposited into the Protein Data Bank (code 1LS4). Table I lists the structural statistics for the 20 deposited NMR structures and the energy-minimized average structure. The root mean square deviation of the well defined secondary structure regions of the 20 NMR structures to the energy-minimized average structure is 0.62 ± 0.07 Å for the backbone and 1.05 ± 0.08 Å for the heavy atoms. A PROCHECK analysis of the energy-minimized average NMR structure indicated that 83% of the residues lie in the most
favored region, 14.6% of the residues lie in additionally allowed regions, and 2.4% of the residues lie in generously allowed regions of the Ramachandran plot. There are no residues located in the disallowed regions.

**Description of ApoLp-III Structure**—The NMR structure of *L. migratoria* apoLp-III consists of five long amphipathic α-helices that form an up-and-down antiparallel elongated helix bundle with short loops of 3–9 residues connecting the helices. A short helix, helix 4’, is observed between helices 4 and 5. The location of each helix is: helix 1, residues 10–34; helix 2, residues 37–68; helix 3, residues 72–87; helix 4, residues 96–125; short helix 4’, residues 129–132; and helix 5, residues 134–161. Such a helix location is nearly identical to that of the x-ray crystal structure (helix 1, residues 10–32; helix 2, residues 37–68; helix 3, residues 72–87; helix 4, residues 96–125; short helix 4’, residues 129–132; and helix 5, residues 134–156), except for helix 5. Although helix 5 in the x-ray crystal structure is located between residues 134 and 156, helix 5 in the NMR structure extends to residue 161. The helix bundle topology of the NMR structure is also nearly identical to the x-ray crystal structure with a root mean square deviation (backbone superposition) of 2.06 Å between the two structures. A careful comparison between the NMR and the x-ray crystal structures indicates that the five helix segments are superimposed very well with a smaller root mean square deviation, whereas the loops and two terminals are superimposed less well. This is

| Structural statistics | *<SA>* | *<SA>*<sup>a</sup> |
|-----------------------|-------|---------------|
| r.m.s.d. from distance restraints (Å) | 0.016 ± 0.001 | 0.015 |
| Intraresidue (540) | 0.007 ± 0.002 | 0.005 |
| Sequential (772) | 0.013 ± 0.002 | 0.012 |
| Medium range (1203) | 0.012 ± 0.001 | 0.009 |
| Long range (235) | 0.010 ± 0.001 | 0.010 |
| r.m.s.d. from torsion angle restraints<sup>c</sup> | 0.42 ± 0.10 | 0.43 |
| φ Angles (133) | 0.50 ± 0.08 | 0.49 |
| X-PLOR potential energies (kcal mol<sup>–1</sup>) | 180.4 ± 8.7 | 178 |
| E (total) | 6.9 ± 0.5 | 7 |
| E (bond) | 144.4 ± 4.6 | 142 |
| E (ang) | 14.2 ± 0.7 | 15 |
| E (imp) | 8.2 ± 2.0 | 6 |
| E (vdw) | 2.5 ± 1.2 | 2 |
| E (NOE) | 4.1 ± 0.9 | 6 |
| Structural r.m.s.d. from idealized geometry | 0.00 ± 0.00 | 0.00 |
| Bond (Å) | 0.46 ± 0.01 | 0.40 |
| Angle (°) | 0.27 ± 0.01 | 0.30 |
| Improper (°) | 0.62 ± 0.07 | 1.05 ± 0.08 |

<sup>a</sup> *<SA>* is the energy-minimized average structure of 20 NMR structures.

<sup>b</sup> r.m.s.d., root mean square deviation.

<sup>c</sup> NOE force constant = 50 kcal/mole Å<sup>2</sup>.

<sup>d</sup> Torsion angle force constant = 200 kcal/mole Å<sup>2</sup>. The torsion angle restraints are generated using TALOS calculation based on chemical shift information.

<sup>e</sup> Improper angle restraints are generated using TALOS calculation based on chemical shift information.

<sup>f</sup> The atoms of well defined secondary structure regions: residues 11-34, 41-65, 71-88, 100-124, and 136-160.
especially true in the loop between helices 2 and 3, and the loop between helices 3 and 4. Interestingly, the short helix, helix 4, and the loop residues before and after this short helix are better superimposed between the two structures as compared with the other loops. The fact that helix 4 (highlighted in red in Fig. 1) is better superimposed indicates that this short helix is conserved in both structures, suggesting a potentially important role in the function of apoLp-III.

Existence of Helix 4—To confirm the existence of helix 4, we carefully examined the NMR parameters, such as NOEs, coupling constants, amide exchange rates, and chemical shift index, of the corresponding region, Gln127, Glu128, Ala129, Trp130, Ala131, Pro132. Fig. 2 shows these NMR parameters, providing strong NMR evidence that the helix 4 exists in the region of residues 128–132. Structure calculation generated the 20 lowest energy structures that all contained this short helix between residues 128 and 132. VADAR calculation of the energy-minimized averaged structure indicates that whereas Glu-127 and Glu-128 are nearly completely exposed (Gln-127, fractional ASA of 0.77; Glu-128, fractional ASA of 0.65), Ala-129 is completely buried (ASA, 0.06), and Trp-130, Ala-131, and Pro-132 are partially buried (Trp-130, fractional ASA of 0.47; Ala-131, fractional ASA of 0.42; and Pro-132, fractional ASA of 0.37). This is different from the x-ray crystal structure in which Trp-130 is completely buried (Gln-127, fractional ASA of 0.84; Glu-128, fractional ASA of 0.79; Ala-129, fractional ASA of 0.39; Trp-130, fractional ASA of 0.04; Ala-131, fractional ASA of 0.56; Pro-132, fractional ASA of 0.53), but consistent with the recent results by Weers et al. (24). We suggest that this difference is mainly because helix 4 starts at residue Glu-128 in solution but at residue Ala-129 in the x-ray structure.

Buried Hydrophobic Residues, Exposed Hydrophobic Residues, and Buried H-bonds—VADAR calculation indicated several hydrophobic residues that are buried in the hydrophobic interior of the protein and several exposed hydrophobic residues that are located on the bundle surfaces. A total of 22 buried/partially buried hydrophobic residues and a total of 14 exposed/partially exposed hydrophobic residues were observed. Table II indicates that both buried/partially buried hydrophilic residues and exposed/partially exposed hydrophobic residues are evenly distributed in each helix of L. migratoria apoLp-III.

Fig. 3 shows a helix wheel diagram of L. migratoria apoLp-III on the basis of its NMR structure, providing both the location and orientation of each residue. Strikingly, Fig. 3 demonstrates that buried/partially buried hydrophilic residues are not evenly located/oriented between helix-helix interfaces. Although only a few buried/partially buried hydrophilic residues are located in the interfaces between helices 1 and 4 (Asn-18), between helices 1 and 5 (Thr-20, Glu-27, Thr-31, and Thr-144), and between helices 2 and 3 (Lys-52, Thr-59, Asn-78), most buried/partially buried hydrophilic residues are located in the interfaces between helices 2 and 5 (Gln-47, Glu-65, Ser-61, Lys-68, Glu-139, and Ser-154) and between helices 3 and 4 (Ser-87, Gln-100, Ser-103, Asn-110, Gln-114, and Lys-121). A similar observation has also been obtained for the exposed/partially exposed hydrophobic residues. VADAR calculation further indicates that several buried hydrophobic residues form either intrahelical H-bonds or buried interhelical H-bonds. Table III lists 11 interhelical hydrogen bonds that are either completely buried or partially buried. The fractional ASA data listed in Table III provide quantitative data on the extent of burial of the buried H-bonds. For example, the H-bond between Thr-59 and Ala-81 is completely buried with the fractional ASA of both residues less than 0.69, whereas the rest are partially buried, with the ASA values of either one residue between 0.30 and 0.38 or both residues between 0.10 and 0.20. Fig. 4 demonstrates that buried H-bonds are mostly located in the interfaces between helices 2 and 5 (5 interhelical H-bonds) and between helices 3 and 4 (4 interhelical H-bonds). One buried interhelical H-bond was found between helices 2 and 4, and another one between helices 2 and 3. No buried H-bond was observed either between helices 1 and 4 or between helices 1 and 5.

Backbone Dynamics—To determine the potential contributions of protein dynamics to the structure-function of apoLp-III, we measured backbone 15N longitudinal (T1) and transverse (T2) relaxation times, as well as 15N-1H heteronuclear NOEs. Fig. 5 shows the results of these measurements, indicating that five long helical segments display less motion, whereas the loop regions are much more flexible. These can be seen clearly in R1 (1/T1), R2 (1/T2), and 15N-1H heteronuclear NOEs data in which the helical segments display plateaus, and the loops show either sharp increases in R1 or dramatic decreases in R2 and NOEs. This is an expected dynamic behavior for a helix bundle protein. However, different loops display different flexibility. Although loop 1 between helices 1 and 2 displays a similar motion as the helix segments, loop 3 between helices 3 and 4 displays the most flexibility in all loops. This is reasonable, because loop 3 with 9 residues is the longest loop, and loop 1 only contains 4 residues. Interestingly, loop 2 contains 3 residues but displays a much higher flexibility compared with loop 1. For loop 4, which contains the short helix 4, it also displays large motion in residues 125–128. In addition, residues 122–124 located at helix 4, which is adjacent to the N-terminal end of loop 4, show more mobility than the rest of the residues in this helix. Helix 4, on the other hand, only shows moderate motion and maintains certain rigidity. Nevertheless, helix 4 is a relatively flexible short helix. Loop 1 is located at the opposite end from helix 4 and loop 2. This side of the bundle also contains loop 3 (see Fig. 4, B and D). However, loop 3 does not reach the very end of the bundle (~14 Å away between loops 1 and 3 (Fig. 4, B and D) because helix 3 is approximately 6–8 residues shorter than the other four long helices. Thus, only loop 1 forms one end of the bundle that is opposite to the helix 4 end. Taken together, 15N backbone relaxation data suggest that the two ends of the apoLp-III helix bundle display significantly different dynamics. Although the end containing loop 2 and helix 4 seems to have greater mobility, the loop 1 end shows much less motion and is as rigid as...
the long helices that form the bundle. Overall, model-free analysis of the $^{15}$N relaxation data also indicates that the apoLp-III helix bundle displays mostly fast motions at nano- and picosecond time scales, whereas slow motions at milli- and microsecond time scales are not detectable. This is because all the relaxation data can be fitted very well to an axially symmetric rotational top in the absence of slow conformational exchange (i.e. $R_\alpha = 0$), except for residue 36 located at loop 1.

**DISCUSSION**

**Lipid-free Helix Bundle and Lipid-bound $\alpha$-Helical Open Conformation—** *L. migratoria* apoLp-III shares a remarkably similar topology with the x-ray crystal structure of the apoE LDL receptor binding domain (3) and the NMR structure of *M. sexta* apoLp-III (5). These three structures represent a typical structural topology of lipid-free apolipoproteins, which is an up-and-down elongated helix bundle in which hydrophobic residues are oriented toward each other to form a hydrophobic core and hydrophilic residues are pointed toward the solvent. The helix segments of the bundle adopt an antiparallel fashion, which are connected by short loops. The only exception is the third loop in *L. migratoria* apoLp-III, which contains 9 residues. Therefore, helix 3 contains 20 residues, at least 5 residues less than the other helices. In addition, this loop also displays much greater mobility than other loops. The helix bundle architecture of lipid-free apoLp-III clearly explains the water-soluble and monomeric properties of this protein; however, the exposed hydrophilic surfaces of the helix bundle is thought to be incapable of binding to the lipoprotein surface.

In contrast to the lipid bundle of lipid-free apoLp-III, the structures of apoC-I and an apoAI truncation mutant, apoAÎΔ (187–243), which is generated in a lipid-mimetic environment, display an extended $\alpha$-helical open structure (7, 8). While no interhelical interactions have been observed in either structure, this open structure is analogous to the proposed lipid-associated open conformation of apoLp-III (4). Interestingly, an x-ray crystal structure of an apoAI truncation mutant, apoAÎΔ (1–43), displays a similar molecular architecture as the extended open $\alpha$-helical conformation even in the absence of lipids. This structure is tetrameric, curved into a horseshoe shape, in which four monomers in the asymmetric unit associate via their hydrophobic faces, whereas their hydrophilic faces point to the solvent (25). Biophysical and limited proteolytic digestion studies suggested that apoAÎΔ(1–43) retained the lipid-bound conformation even in the absence of lipid (26). In the case of apoLp-III, although currently we do not have a lipid-bound structure available, experimental evidence indicates that the apoLp-III helix bundle opens at hinge loops on lipid association to adopt an extended $\alpha$-helical open conformation (27, 28). The helical segments form two lobes linked by flexible loops and pointing in opposite directions (4). This open conformation provides a large exposed hydrophobic surface area that is available for lipid binding, whereas the hydrophilic surfaces point to the solvent. Physiologically, apoLp-III exists in either a lipid-free or lipid-bound state that readily converts from one to the other depending on the metabolic setting of the insect life stages. During this conversion, hydrophobic helix-helix interactions in the lipid-free helix bundle structure are replaced by helix-lipid interactions in the lipid-bound open conformation. Experimental data have indicated that the lipid-bound structure is more stable, suggesting that the lipid-bound...
conformation is thermodynamically favored (29). However, structural features that regulate the conformational interconversion of apoLp-III remain to be determined.

Helix 4 / H11032 may initiate apoLp-III-lipoprotein interaction. Helix 4 / H11032 in L. migratoria apoLp-III, similar to helix 3 / H11032 in M. sexta apoLp-III, is located at one end of the bundle and is solvent-exposed. Experimental evidence suggested that apoLp-III initiated its binding to the lipid surface via one end of the bundle (28, 30). It has been proposed previously that conserved leucine residues located in the hinge loops play an important role in lipid surface recognition (4, 11) on the basis of the assumption that the lipoprotein surface is hydrophobic. In fact, the lipopro-

| TABLE III | Buried/partially buried H-bonds in L. migratoria apoLp-III NMR structure |
|-----------|---------------------------------------------------------------|
|           | Helix 1 | Helix 2 | Helix 3 | Helix 4 | Helix 5 |
| Helix 1   |         |         |         |         |         |
| Helix 2   | Thr-59s-Ala-81b | Thr-45s-Gln-100s | Gln-47s-Ser-154s | 0.05–0.09 | 0.37–0.90 |
| Helix 3   | Lys-121s-Gln-76s | Ser-87s-Asn-110s | Ala-91b-Ser-103s | 0.30–0.69 | 0.29–0.26 |
| Loop 3    | Asp-90b-Ser-103s | 0.04–0.20 | 0.62–0.20 |
| Helix 4   |         |         |         |         |         |
| Helix 5   |         |         |         |         |         |

*a* "s" stands for side chain atom and "b" stands for backbone atom that forms the H-bonds.

The number represents the fractional solvent-accessible surface area of the side chain atoms of a residue that forms the buried/partially buried H-bond.

Fig. 4. Buried H-bonds in L. migratoria apoLp-III. Helices 2 and 3 are shown in light blue and helices 1, 4, and 5 are shown in green. The short helix, helix 4', is highlighted in red. The side chain heavy atoms are shown in stick model for those residues that form buried H-bonds. The H-bonds are shown in yellow dotted lines. The H-bonds formed by the residues in the front helices are highlighted in pink, whereas the H-bond-forming residues in the back helices are shown in yellow. A, buried H-bonds between helices 2 and 5; B, buried H-bonds between helices 2 and 3; C, buried H-bonds between helices 3 and 4; D, no buried H-bonds are observed between helices 1 and 5.

Fig. 5. 15N relaxation rates $R_1$ and $R_2$ and heteronuclear 15N-{1H} NOE data of L. migratoria apoLp-III. The data were recorded at 500 MHz. Error bars are shown in the figure; some of the data points display a larger error, whereas the error bars for most of the data points cannot be clearly seen because of the error being smaller than the size of the symbols.

Helix 4' May Initiate apoLp-III-Lipoprotein Interaction—Helix 4’ in L. migratoria apoLp-III, similar to helix 3' in M. sexta apoLp-III, is located at one end of the bundle and is solvent-exposed. Experimental evidence suggested that apoLp-III initiated its binding to the lipid surface via one end of the bundle (28, 30). It has been proposed previously that conserved leucine residues located in the hinge loops play an important role in lipid surface recognition (4, 11) on the basis of the assumption that the lipoprotein surface is hydrophobic. In fact, the lipopro-
tein surface contains a phospholipid monolayer that is not hydrophobic. Instead, it is the surface-located diacylglycerol that provides the binding sites for apoLp-III (31, 32). In addition, an amphipathic α-helix serves as a secondary structural motif for lipid binding (1). On the basis of this concept, we propose that helix 4′ is the first structural motif that initiates *L. migratoria* apoLp-III interaction with the lipoprotein surface. This is supported by the ^15^N relaxation measurements shown in Fig. 5, which indicate that the helix 4′-containing end of the apoLp-III helix bundle displays greater mobility than the other end that contains loop 1. In fact, the loop 1 end is quite rigid, with the relaxation rates similar to the five long helices in the bundle. It is commonly accepted that binding between two proteins requires a certain flexibility of the binding motifs that facilitates the induced fit binding process. The rigidity of the loop 1 end likely disfavors such an induced fit binding process. On the other hand, the helix 4′ end not only provides an amphipathic helix motif for lipid surface recognition, but also possesses the required flexibility for binding. In addition, this hypothesis is analogous to our previous proposal for the helix 3′ in *M. sexta* apoLp-III (5, 9). We demonstrated that replacing helix 3′ by a 4-residue β-turn significantly decreased the lipid binding activity. This is because the mutant fails in competing for the binding sites with wild-type apoLp-III (10).

Interestingly, such a short helix is also found in the x-ray crystal structure of the apoE N-terminal domain, which is located between helices 1 and 2. The short helix, helix 1′, in the apoE N-terminal domain is located in the most conserved region of apoE with unknown biological functions. Fig. 6 shows a striking similarity of this short helix in all three lipid-free apolipoproteins in terms of its location, orientation, and solvent-exposed nature. The fact that all three lipid-free apolipoproteins contain a short helix that is located in the most conserved region of the sequences makes it conceivable that this short helix may play an important role in triggering the apolipoprotein lipid binding activity. We are currently carrying out a mutagenesis study of helix 4′ to test this hypothesis.

**Buried Hydrophilic Residues May Adjust the Helix Bundle Stability of apoLp-III**—Lipid-free apolipoproteins are marginally stable proteins with a free energy of unfolding (ΔG) of <4.5 kcal/mol (33). Indeed, the ΔG of *L. migratoria* apoLp-III is 2.48 kcal/mol (11). In the lipid-free helix bundle, hydrophobic residues are oriented toward the protein interior, escaping from the solvent, and the hydrophilic interhelical interaction stabilizes the bundle. Although the hydrophobic interaction between helices contributes to the helix bundle stability, we suggest that the buried/partially buried hydrophilic residues in the otherwise hydrophobic interior significantly destabilize *L. migratoria* apoLp-III. In a similar manner, the exposed/partially exposed hydrophobic residues in the otherwise aqueous environment further destabilize this protein. We further suggest that an intrinsic marginal stability of apoLp-III may facilitate the helix bundle opening, promoting the reversible lipoprotein binding activity of this protein. Although helix-helix interactions determine the lipid-free helix bundle stability, this hydrophobic interaction will be replaced by helix-lipid interactions once apoLp-III binds to the lipoprotein surface. A competition between the helix-helix interaction and helix-lipid interaction determines the reversible lipoprotein binding activity of apoLp-III. A stable apoLp-III may prevent its bundle from opening, prohibiting apoLp-III from binding to the lipoprotein surface, whereas a stability that is too low makes apoLp-III unable to recover its helix bundle structure from a lipid-bound open α-helical conformation. Thus, a marginal stability of apoLp-III is well suited for the reversible helix bundle opening/recovery and lipoprotein binding activity.

**Buried Hydrophilic Residues May Dictate the Helix Bundle Opening upon Lipid Binding**—We noticed that helix 4′ in *L. migratoria* apoLp-III is located at the opposite end from helix 3′ in *M. sexta* apoLp-III. This fact implies that the helix bundle may open in a different manner from the helix bundle opening in *M. sexta* apoLp-III (9), if helix 4′ indeed serves as a recognition helix. Fig. 3 shows that the stability of the helix-helix interfaces can be readily adjusted by placing buried/partially buried residues in a different location and orientation. Such a mechanism allows apoLp-III to accurately refine this adjustment for a bundle opening upon binding to lipoprotein. For example, Fig. 3 demonstrates that more buried/partially buried hydrophilic residues are observed in the interfaces be-
tween helices 2 and 5 as well as helices 3 and 4 than the other interfaces. These buried/partially buried hydrophilic residues interrupt the hydrophobic interhelix interaction, resulting in a less stable interface than those between helices 1 and 4, helices 1 and 5, and helices 2 and 3. As a consequence, one way for the helix bundle to open will involve repositioning the helices in such a way that helices 2 and 3 are moved away from the bundle center to one side, and helices 1, 4, and 5 are moved to the opposite side. In this case, apoLp-III uses its loops at the opposite end from helix 4’ as the hinges for its helix bundle opening (Fig. 7B). Such a helix bundle opening effectively allows apoLp-III to use helix 4’ as the recognition helix. It is worth noting that this possible opening is indeed different from the opening in *M. sexta* apoLp-III, which is similar to Fig. 7A (9), supporting our previous speculation that helix 4’ may initiate the apoLp-III-lipid interaction.

An alternative bundle opening (shown in Fig. 7A) cannot be excluded. This alternative bundle opening, originally proposed by Breiter et al. (4) on the basis of the x-ray crystal structure, uses the opposite end from the helix 4’ for recognition. In this case, helix 4’ and the loop between helices 2 and 3 serve as the hinges. As a consequence, helices 3 and 4 will be repositioned to one side and helices 1, 2, and 5 to the opposite side. Although the helix bundle topology of apoLp-III allows for this alternative opening, such an opening is unlikely to be a preferred one, because the interfaces between helices 2 and 3 and helices 1 and 4 are more stable than those between helices 3 and 4 and helices 2 and 5. Furthermore, this alternative opening will also eliminate the potential role of helix 4’ in the lipoprotein surface recognition. In addition to the above models, other conformational adaptation models may also be possible. For example, a recent report suggested that the conformational flexibility of helices 1 and 5 is a key property of *L. migratoria* apoLp-III for lipoprotein binding activity (34).

**Buried H-bonds May Guide a Specific Helix Bundle Recovery**—The helix bundle recovery from the lipoprotein-bound open conformation is equally important to the reversible lipoprotein binding activity of apoLp-III as is its helix bundle opening. In the lipoprotein-bound state, the helix-lipid interaction stabilizes the open conformation that otherwise exposes a large hydrophobic surface in aqueous solution. Once the apoLp-III binding sites are eliminated from the lipoprotein surfaces because of a different metabolic setting during different insect life stages, such a helix-lipid interaction will be removed, and the open conformation will be unstable. Recovery of the helix bundle will reestablish the hydrophobic helix-helix interactions, avoiding this situation. The hydrophobic interaction likely initiates the helix bundle recovery process, resulting in both lobes repositioning around the hinge loops in the lipid-bound open conformation. In other words, helices 2 and 5 and helices 1, 4, and 5 move toward each other. Hydrophobic interactions are unlikely to accomplish recovery of the unique apoLp-III helix bundle, because it is less specific. We suggest that buried interhelical H-bonds may provide a specific driving force that guides the unique apoLp-III helix bundle recovery. Fig. 4 shows that these buried interhelical H-bonds are predominantly located at the interfaces between the two lobes of the open conformation (10 H-bonds), whereas only one buried H-bond is found within one lobe (between helices 2 and 3). Such a spatial arrangement strongly suggests that the interlobe H-bonds are critical in guiding the two lobes to specifically fold into the lipid-free apoLp-III helix bundle. We realize that NMR data do not provide a direct detection of the interhelical H-bonds from side chain atoms of both residues; however, several interhelical H-bonds listed in Table III are from side chain to backbone atoms. For these buried H-bonds, NOEs between side chain and backbone atoms of the H-bonding residues are observed. For example, for the H-bond between backbone –HN of Ala-91 and side chain –OH of Ser-103, a NOE between HN of Ala-91 and Hβ of Ser-103 was observed. In addition, the HN of Ala-91 is a slow exchange amide proton, potentially because of the H-binding. These data provide indirect NMR experimental evidence for the H-bonds listed in Table III.

**Comparison of *M. sexta* ApoLp-III and *L. migratoria* ApoLp-III NMR Structures**—Although two apoLp-III s only share 29% sequence identity, they adopt a similar five-helix bundle structure, shared with several common structural features such as the short recognition helix, buried hydrophilic residues, and buried H-bonds. In addition, they also share a reversible lipoprotein binding activity and many biophysical properties.
such as marginal stability. Our previous results on *M. sexta* apoLp-III (9) and this present study on *L. migratoria* apoLp-III indicate that the helix bundle topology of apoLp-III is well suited for the reversible helix bundle opening/recovery between the lipid-free and lipid-bound conformations, thus promoting its lipoprotein binding activity. The short helix, however, is located at the opposite end between two apoLp-IIIIs, suggesting that the two apoLp-IIIIs may use a different end for recognition. In support of this suggestion, we show that the possible helix bundle opening of two apoLp-IIIIs uses the hinges at an opposite end of the bundle. Although *L. migratoria* apoLp-III possibly opens its helix bundle using a model shown in Fig. 7B, *M. sexta* apoLp-III preferably opens its helix bundle using a model similar to Fig. 7A, except that the recognition short helix is helix 3′ (9). Thus, the lipid-free apoLp-III NMR structures provide a structural rationale of their reversible lipoprotein binding activity. In addition, such a structural rationale includes several new hypotheses/concepts for the structural features that regulate the reversible lipid binding activity of apoLp-III, and these new hypotheses/concepts require further experiments to verify. We are currently carrying out experiments to test these hypotheses.

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