Structure and Dynamics of Human Apolipoprotein CIII*§

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Human apolipoprotein CIII (apoCIII) is a surface component of chylomicrons, very low density lipoproteins, and high density lipoproteins. ApoCIII inhibits lipoprotein lipase as well as binding of lipoproteins to cell surface heparan sulfate proteoglycans and receptors. High levels of apoCIII are often correlated with elevated levels of blood lipids (hypertriglyceridemia). Here, we report the three-dimensional NMR structure and dynamics of human apoCIII in complex with SDS micelles, mimicking its natural lipid-bound state. Thanks to residual dipolar coupling data, the first detailed view is obtained of the structure and dynamics of an intact apoCIII in its lipid-bound state. ApoCIII wraps around the micelle surface as a necklace of six 10-residue amphipathic helices, which are curved and connected via semiflexible hinges. Three positively charged (Lys) residues line the polar faces of helices 1 and 2. Interestingly, their three-dimensional conformation is similar to that of the low density lipoprotein receptor binding motifs of apoE/B and the receptor-associated protein. At the C-terminal side of apoCIII, an array of negatively charged residues lines the polar faces of helices 4 and 5 and the adjacent flexible loop. Sequence comparison shows that this asymmetric charge distribution along the solvent-exposed face of apoCIII as well as other structural features are conserved among mammals. This structure provides a template for exploration of molecular mechanisms by which human apoCIII inhibits lipoprotein lipase and receptor binding.

Apolipoproteins consist of five major classes, apo-A II through -E, and several subclasses. They are designed for lipid transport in blood and are attached to lipid droplets (lipoproteins) through amphipathic helices that intercalate into the single layer of phospholipids and cholesterol covering the droplet (1–3). The apolipoproteins regulate blood lipid levels by interaction with specialized endocytotic receptors and by modulating enzyme activities and lipid exchange reactions (4). They are therefore in focus with regard to disturbances in blood lipid metabolism (dyslipidemias), which in turn are associated with metabolic disorders like obesity, insulin resistance, diabetes type 2, and cardiovascular disease.

Apolipoprotein CIII (apoCIII) is the most abundant C-apolipoprotein in humans (2–4). It is found on very low density lipoproteins, chylomicrons, and high density lipoproteins (HDL). ApoCIII is predominantly expressed in liver and intestine from a gene cluster important for lipid regulation (ApoAI, AIV, -AV, and -CIII) (4, 5). ApoCIII inhibits lipoprotein lipase (LPL) and receptor-mediated endocytosis of lipoprotein particles by competing for space at the surface of the lipoproteins and by interfering with their binding to endothelial proteoglycans and to specific lipoprotein receptors (2, 4, 6). Overexpression of apoCIII in transgenic mice leads to severely increased plasma triglyceride levels due to accumulation of very low density lipoprotein-like lipoprotein remnants with increased apoCIII and decreased apoE content compared with controls (7). Overexpression of apoCIII in apoE−/− mice leads to similar results, demonstrating that the effects are not only connected to displacement of apoE (8). Knock-out of the apoCIII gene in mice leads to reduced levels of lipoproteins in blood (9). In human subjects, increased levels of apoCIII are often correlated to increased levels of triglycerides in blood and in turn to insulin resistance, cardiovascular disease, and diabetes type 2 (4).

Recent data suggest that apoCIII and apoAV are important opposing modulators of plasma triglyceride metabolism (5, 10). ApoCIII was found to directly affect insulin secretion by interfering with voltage-gated calcium channels in pancreatic β-cells (11). Interestingly, mutations in the apoCIII promoter are connected with longevity (12).

Lipid interaction is required for solubility as well as correct folding and function of most apolipoproteins (1–3). In addition, cumulative evidence suggests that apolipoproteins are dynamic and span multiple conformations required for their functions (13). Structural studies of apolipoproteins have therefore been as challenging as studies of membrane proteins, and high resolution structure information has remained relatively scarce. To date, apolipoprotein structures are available of the 22-kDa

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The atomic coordinates and structure factors (code 2jq3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† The 1H and 13C chemical shifts have been deposited in the BioMagResBank data base (http://www.bmrb.wisc.edu) under accession number 15268.

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and Figs. S1 and S2.

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2 The abbreviations used are: apo, apolipoprotein; LPL, lipoprotein lipase; LDL, low density lipoprotein; HDL, high density lipoprotein; RDC, residual dipolar coupling; NOE, nuclear Overhauser effect; r.m.s., root mean square; RAP, receptor-associated protein.
N-terminal domain of human/mouse apoE (14–16), Δ(1–43)apoAI (17) and of three intact apolipoproteins, apoAI (18), insect LpIII (19, 20), and apoAI (21), all in the lipid-free state. In the lipid-bound state, only recently the first low resolution (10 Å) x-ray model has been reported of an intact apolipoprotein, apoE (22). Also reported is the three-dimensional structure and dynamics of apoCII bound to micelles derived from NMR relaxation data (23). In addition, NMR has provided information on helix boundaries and local structure of lipid-bound intact apoCII (24), apoCl (25), and apoAI (26). No high resolution structural and dynamics information has yet been derived on intact apoCIII.

Human apoCIII consists of 79 amino acid residues. Structure predictions have indicated that two amphipathic helices are likely to be formed (1, 27). There is no consensus about which regions are most important for attachment to lipids (27–29). The structurally related apoCII is known to interact with LPL and activate this enzyme via the C-terminal helix (30), most likely in a dynamic fashion (23). ApoCIII inhibits LPL, but direct binding has not been shown (27). ApoCIII also inhibits binding of apoE/B-containing lipoproteins to members of the LDL receptor family (2, 6). Finally, apoCIII binds, like apoE, apoD, and apoAI, to the HDL receptor SR-BI (31, 32).

Here, we present the three-dimensional structure and dynamics of intact human apoCIII in complex with SDS micelles, derived from a combination of NMR data: NOEs, 13C chemical shift-derived backbone dihedral angles, 3JHNH couplings, NMR multiple-field 15N-spin backbone relaxation, and 15N/1H residual dipolar couplings (RDCs). Thanks to the RDC data, the first detailed view is obtained of the structure and dynamics of an intact apoliprotein in a lipid-bound state. This structure provides a template for exploration of the molecular mechanisms by which human apoCIII inhibits LPL and receptor binding.

**EXPERIMENTAL PROCEDURES**

**Expression, Extraction, and Purification of 13C/15N-Labeled ApoCIII**

ApoCIII was expressed from a pET23b vector (a kind gift from Dr. Philippa Talmud (London, UK)), containing the full-length cDNA for human apoCIII, including the sequence for a His6 tag at the 3’-end (C-terminal His6 tag) preceded by two additional residues (Leu and Glu) (27). A detailed protocol is presented in the supplemental material. The 13C/15N-labeled M13 coat protein was expressed and purified as described elsewhere (33, 34).

**NMR Spectroscopy**

(a) Preparation of NMR Samples and Optimal Measurement Conditions

The NMR sample of apoCIII in complex with SDS micelles was prepared by lyophilized 15N/13C-labeled apoCIII as described (23), leading to the final ApoCIII-NMR sample: —0.5 mM apoCIII, 180 mM SDS-d25, 8% D2O, and 10 mM deuterated sodium acetate buffer (pH 5.0). In this final sample, the apoCIII/micelle ratio is 1:6, assuming 60 SDS molecules/micelle (23). For RDC measurements, we prepared a similar second sample (apoCIII-RDC), except for a slightly higher buffer concentration (50 mM). While keeping the apoCIII/SDS micelle ratio as low as possible (here 1:6) to prevent protein-protein interactions (23), relatively low SDS and buffer concentrations were used. This considerably reduced the overall tumbling time of the apoCIII-micelle complex and improved three-dimensional NMR spectra, as will be described elsewhere in more detail. All NMR experiments were carried out at 42.7 °C, determined to be optimal via 15N/1H HSQC experiments. Prior to each experiment, the temperature was calibrated using tetramethylammonium. The NMR sample for the 13C/15N-labeled M13 coat protein (gVIIIp) in complex with SDS micelles (M13-NMR) was similar to that for apoCIII. For RDC measurements, a similar second sample (M13-RDC) was prepared.

(b) NMR Measurements, Processing, and Resonance Assignment

The NMR experiments for assignment and derivation of experimental restraints are summarized in Table S4. Briefly, for backbone resonance assignments, 600-MHz CBCA(CO)NH, HNCACB, and HNCO were recorded (35) and a 600-MHz three-dimensional HNHA for 3JHNH coupling (36). To confirm the backbone assignments and derive NOE distance restraints, 800-MHz 1H/15N NOESY-HSQC and 1H/15N HSQC-NOESY-HSQC were recorded (35). All NMR spectra were processed using NMRPipe (37) and analyzed using XEASY (38) and NMRDraw (37). Further details on restraint extraction are described under “Structure Calculations.”

(c) Residual Dipolar Couplings

RDCs were measured in stretched polyacrylamide gels via a procedure modified from previously published ones (39) (i.e. practical procedures were developed to control alignment magnitude and buffer conditions (pH and salt)). Briefly, 4% (w/v) polyacrylamide gel (acylamide/ bisacrylamide, 37.5:1) was polymerized in the presence of apoCIII-SDS micelles (apoCIII-RDC sample) in a cylindrical Teflon tube with a 6-mm internal diameter. To induce alignment, the 6-mm diameter gel was then squeezed into an open-ended NMR tube (internal diameter 4.2 mm) using a laboratory-made funnel device (which will be described elsewhere).

The 1H-N RDCs were measured from five sets of 1H/15N 800-MHz IPAP-HSQCs (40). In addition, a decoupled 1H/15N HSQC was recorded, so that RDCs could be calculated as twice the difference of the peak positions of the (sharp) “down-component” in the IPAP and decoupled HSQC. The average r.m.s. deviation of the RDCs was 0.8 Hz. The RDC data on the M13 coat protein were measured in a similar fashion.

(d) NMR Relaxation Measurements and Analysis

15N T1 and T1p and 15N(1H) NOE measurements on the apoCIII-NMR sample were carried out at 600 and 800 MHz. Two and three sets of 15N T1 experiments were recorded with relaxation delays of 16, 256, 384, 512, 640, 768, 896, 1024, and 1280 ms at 600 and 800 MHz, respectively. Two sets of 15N T1p experiments (spin-lock field 3 kHz) were recorded using delays of 16, 32, 48, 64, 96, and 128 ms at 600 and 800 MHz each. The 15N(1H) NOE was acquired thrice at 600 MHz and twice at 800 MHz.
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MHz. The data points were recorded in an interleaved manner to minimize effects of spectrometer drift and temperature fluctuations. All of the data were processed with NMRPipe (37). \(^{15}\)N \(T_1\) and \(T_{1p}\), were calculated by nonlinear fittings of the delay-dependent peak intensities to an exponential decay function. \(^{15}\)N \(T_{1p}\) values were corrected for off-resonance effects. \(^{15}\)Ni(\(^{1}H\)) NOE values were calculated as the intensity ratio of cross-peaks from pairs of spectra acquired with and without \(^{1}H\) presaturation. The data obtained from the duplicate or triplicate measurements were averaged, and errors were derived. The \(T_1\), \(T_{1p}\), and NOE data were analyzed via PINATA (23, 42, 43).

Structure Calculations

(i) Overview of the Protocol

To derive a structure model of apocIII in complex with SDS micelles, we employed a protocol that is essentially as described previously for the apocII-SDS micelle complex (23). The main difference is that the restraints that define the relative helix orientations are now derived from RDCs, whereas previously they originated from the variation in the helix-specific correlation times as derived from \(^{15}\)N relaxation data. The protocol consists of two main parts. In this section (i), an overview is presented of the protocol, whereas in sections ii and iii, elements of the protocol are described in detail.

Part I. Structure Calculations Performed Entirely within X-PLOR-NIH 2.1 with Its Standard Simulated Annealing Protocol in Torsion Angle Space and NMR Restraints Corresponding to NOE, CDIH, JCOUP, and SANI (RDC) Terms in the Available Potential—Starting from an \(\alpha\)-helical conformation (section iiia) a set of 100 apocIII structures were calculated using X-PLOR-NIH 2.1 with its standard SA protocol in torsion angle space (section iib) (44). In these calculations, restraints obtained from the following NMR experiments/data were used: NOEs (section iib), \(^{13}\)C chemical shift-derived (via TALOS) backbone dihedral angles (CDIH) (section iic), \(^{3}\)J\(^{HNH}\) couplings (JCOUP) (section iic), and \(^{15}\)N/\(^{1}H\) RDCs (SANI) (section iid). The resulting set of structures shows good structure characteristics (Table S2a). The structures show well defined secondary structures of the helices (data not shown). However, the relative orientations of the helices are not fully defined by these restraints. This is due in part to the local nature of the “classical” NMR restraints and the lack of long range NOEs (as evident from separate calculations without RDCs). That inclusion of the RDCs neither fully defines the relative helix orientations is due to the intrinsic symmetry relations in the geometric equations of the RDCs. Hence, to fully define the three-dimensional structure of the apocIII-micelle complex, the micelle has to be taken into account. This is done in Part II. To proceed, the 10 lowest energy structures were submitted to part II.

Part II. Structure Derivation of the Protein-Micelle Complex—To define the relative helix orientation and to position the protein on the surface of the micelle, additional “global” restraints were employed. They were (a) \(H_N^2-N\) RDCs and (b) two further additional restraints, namely (b1) the hydrophobic moments of the amphipathic helices should point toward the center of the micelle, and (b2) the structure should fit to the geometrical size of the micelle. All calculations in Part II, except in Step 1 (see below), were carried out in X-PLOR and Protein Constructor, a program written by a few of us, which employs calls on XPLOR and aims to smooth structure calculations based on “global” constraints (for a more detailed description, see section iiib).

In Step 1, a Monte Carlo simulation is performed to fit for each helix the calculated to the experimental RDCs using the dipolar wave formalism (45, 46). These Monte Carlo fittings were performed via Matlab scripts using the dipolar wave formulations by Mesle et al. (45) and/or by Mascioni et al. (46) and via a procedure incorporated into Protein Constructor, which employs the dipolar wave formulation by Mascioni et al. (46). This resulted in a set of angles (three per helix rigid body: \(\theta\), \(\varphi\), and \(\rho\)) that defines the helix orientations in three-dimensional space, albeit with some unavoidable degeneracy intrinsic to derivation of angles from RDCs. This degeneracy is resolved by the geometric requirements resulting from “global” restraints mentioned above.

In Step 2, the 10 “best” helix structures of Part I were then supplied to Protein Constructor. Each of the structures was logically divided into fragments detected by the program as helices or joints. Based on the results from Part I, the apocIII molecule was segmented into six helical regions in the following order: helix 1 (residues 8–18), helix 2 (residues 20–29), helix 3 (residues 33–43), helix 4 (residues 46–54), helix 5 (residues 55–65) and helix 6 (residues 74–79). The rest of the molecule was treated as joints and enumerated in appropriate consecutive order. The average length of each joint between helices was estimated based on the 100 structures calculated in Part I of the structure determination. Those values together with the three angles (\(\varphi\), \(\theta\), and \(\rho\)) orienting helices axes in three-dimensional space were submitted to the Protein Constructor as parameters. Having this information and being equipped with the algorithms able to calculate helical axes and hydrophobic moments from the structure, the Protein Constructor appropriately positioned each helix one after another according to the supplied information on the surface of the micelle. The orientations of the short junctions between helices were determined in the first approximation as averages of the orientations of the adjacent helices. Putting the length of the hydrophobic moments equal to the size of the radius of the micelle and requiring the hydrophobic moments to point to the same place in space, we ensured that all helices stayed on the surface of the micelle.

In Step 3, the unavoidable disruptions at helical junctions were then “healed” in Protein Constructor via a loop closure algorithm in 200 iterations (47).

In Step 4, the resulting structures were then minimized as rigid bodies in Protein Constructor via a call to XPLOR-NIH using positional constraints on C\(_\alpha\) atoms (i.e. the C\(_\alpha\) atoms of residues 8, 24, 29, 38, 50, 60, and 76 were constrained in space, corresponding to about one C\(_\alpha\) atom per helix rigid body).

In Step 5, the five lowest energy structures were then refined by XPLOR-NIH via a gentle Cartesian space SA protocol (500 to 25 K, step 25 K) followed by 3000 steps of Powell energy

\(^3 J\) Zdunek, M. Lindgren, M. Karlsson, P. O. Westlund, and S. Wijmenga, manuscript in preparation.
minimization employing all NMR restraints. Each of the five structures generated 10 new final structures. During this refinement, the seven Cα backbone atoms of Step 4 (in the middle or near the middle of each helix) were kept in space to ensure the helices stayed on the micelle. Additionally, the NOE constraints were loosened somewhat by uniformly putting the lower bound to 2 Å for all distances (upper bound as calculated before).

In Step 6, the top 10 of the 50 calculated structures were then accepted and analyzed with respect to their spatial features, energy, and constraints violations. Structures (final and some intermediate) were displayed via the VMD program (48).

(ii) Part I

(a) Generation of ApoCIII Starting Structures—The starting model of apoCIII was generated in α-helix conformation along the whole sequence from residue 1 to 79 using Protein Constructor. The coordinates of the apoCIII model were then submitted to the program X-PLOR, where the hydrogen atoms were built in, and the model of the molecule was energy-minimized using 1000 step of the Powell minimization algorithm.

(b) Distance Constraints—1H/15N-edited NOESY-HSQC and HSQC-NOESY-HSQC spectra were used to extract the experimental intensities of cross-peaks between protons. NOE peak volumes, obtained using peakint in XEASY, were converted into distances by calibrating average NOE intensities in helical regions against corresponding standard helical distances. To ensure the consistency of the derived distance values and to take into account the effects of spin diffusion, the distance constraints were additionally evaluated using the experimental intensities and the complete relaxation matrix algorithm within the program MARDIGRAS (49). In total, 185 distances were obtained in this way. The ranges of the distances proposed by MARDIGRAS were tighter than seemed realistic. Therefore, new lower and upper distance bounds were calculated for every distance according to Folmer et al. (50), so that MD calculations could be performed smoothly. The average values of the upper and lower bounds were 0.745 Å. In total, we have used 185 distance constraints. The distances were operationally divided into six groups: HA(i)-HN(i + 1), 57 distances; HA(i)-HN(i + 2), 14 distances; HA(i)-HN(i + 3), 21 distances; HA(i)-HN(i + 4), 10 distances, and HA(i)-HN(i + 5), 1 distance; HN(i)-HN(i + 1), 72 distances; and HN(i)-HN(i + 2), 7 distances, HN(i)-HN(i + 3), 1 distance, and HN(i)-HN(i + 4), 2 distances.

(c) Torsion Angle Constraints—Backbone torsion angles were derived using TALOS (51). For those residues for which the chemical shift and the output from TALOS indicated an α-helical conformation, the φ and ψ torsion angles were kept at values calculated by TALOS and with the ranges ±30°. For the residues for which the calculated values were close to but not evidently indicating helical conformation, the ranges were increased to ±45°. These residues were primarily located on the junctions between sequences with strict helical conformations (i.e., residues 19–22, 30–33, 44–46, 55, and 65–69). No torsion angle constraints were used for residues 1 and 79. Additionally, 74 JHNHA-coupling constants (no couplings for residues 1, 26, 54, 69, and 73) obtained from NMR measurements were used directly in the X-PLOR force field for constraining ϕ torsion angles of the backbone.

(d) Residual Dipolar Coupling Constraints—In all calculations, we used 70 available N-HN RDC constraints obtained from NMR experiments.

(e) Simulated Annealing Protocol—From the apoCIII starting structure in α-helical conformation and using all of the NMR constraints (see above), the simulated annealing (SA) algorithm of the X-PLOR-NIH 2.1 (44) was used to generate 100 structures of the molecule. The SA calculations were carried out using the IVM (52) implementation in torsion angle space. The protein was decomposed for torsion angle dynamics using the default algorithm included in the program. The rings of aromatic residues were treated as rigid bodies. The energy function consisted of the covalent terms (bonds, angles, improper) and the van der Waals nonbonded potential, and three terms of experimental contributions: NOE, CDIHE, J couplings, and RDC restraints. Before entering into dynamics, the structure of apoCIII was energy-minimized using 1000 steps of the Powell minimization algorithm. The SA protocol consisted of three distinct MD periods: a high temperature period at 3500 K with all energy terms included except the atomic repulsion, a period at the temperature as before but with atomic repulsion added incrementally in 10 cycles of 100 steps each, and a cooling period of 10000 steps in which the temperature was reduced (in 25-step cycles) from 3500 to 100 K. The integration step of the molecular dynamics run was set to an initial value of 10 fs and gradually decreased to 3 fs at the end of each run. The resulting 100 structures of apoCIII were energy-minimized using 3000 steps of Powell minimization algorithm. When the N-HN RDC restraints were included, the SANI force field in XPLOR-NIH 2.1 was employed with a floating alignment PAS and the axial component set of alignment Dα set to −7 Hz and the rhombicity R set to 0.26. The latter values followed from the Monte Carlo fit of calculated to experimental RDC values using the dipolar wave formulation as described in Part II.

(iii) Part II

(a) Protein Constructor—Protein Constructor can generate protein structures for a given sequence and conformation (α-helix, β-strand, random) or from a list of specified consecutive backbone torsion angles (φ and ψ) or from existing helical fragments of refined structures according to the specified directions of the helical axes. It can perform small rotations and/or translations of selected fragments and has the ability to create smooth junctions between adjacent portions of the molecule. Other features are back-calculation of RDCs from a given three-dimensional structure, comparison of structures using r.m.s. deviation, generation of torsion angles, and replacement of amino acid residues for given positions in a three-dimensional protein structure. We used Protein Constructor for generation of the global structure, calculation of hydrophobic moments (and their directions) of the helices of apoCIII, finding the helical axes, orientation of the helices in three-dimensional space in agreement with RDC experimental constraints, and analysis of intermediate results using the theoretical analysis described by Mascioni et al. (46) and the SVD (singular value decomposition) approach by Losonczi et al. (53).
(b) Generation of the SDS-micelle Structure—The all atom model of individual SDS molecules was created within X-PLOR as described previously (23).

c) Determination of Helical Axes—To perform rotations and translations of small domains (here helices) in a uniform way in three-dimensional space, an algorithm for calculating the helical axis from Cartesian coordinates was applied as described previously (23). The algorithm is implemented both in X-PLOR command language and as a feature within Protein Constructor.

d) Hydrophobic Moment Calculations—The hydrophobic moments were calculated largely as described previously (23).

RESULTS

NMR Spectra and Resonance Assignment

Good quality NMR spectra were obtained of $^{13}$C/$^{15}$N apoCIII in complex with SDS micelles as demonstrated by $^1$H/$^{15}$N HSQC (Fig. 1). Complete sequential resonance assignments were obtained of $^{13}$C/$^{15}$N triple resonance experiments, whereas NOEs were assigned via three-dimensional $^1$H/$^{15}$N NOESY-type experiments.

Secondary Structure of ApoCIII

The secondary structure of apoCIII was determined from a combination of NOEs; $\Delta C^\beta$, $\Delta C^\alpha$, $\Delta C^\gamma$, and $\Delta H^\alpha$ chemical shifts (Fig. 2a); and TALOS-derived $\varphi$ and $\psi$ angles. The TALOS angles and $\Delta C^\alpha$ shifts (Fig. 2b) indicate the region from residue 1 to 7 to be random coil. From 8 to 64, the backbone takes on an $\alpha$-helix conformation except for two regions where clear deviations are seen (30/31–33 and 44–45) and one region where they are less evident (19–22). Here, $\psi$ of 20 and 21 deviate slightly, whereas $\varphi$ is somewhat increased for 20–22. For 19 and 21, the $\Delta C^\alpha$ values are below the average of the $\alpha$-helical regions, whereas the $\Delta C^\beta$ values of 19–22 approach zero. In the long helical region 47–64, $\Delta C^\alpha$ shows two lobes separated with a minimum at 54, for which in addition $\Delta C^\beta$ approaches zero. We therefore consider 54 as a break in the long 47–64 $\alpha$-helix. From 65–73, the backbone is unstructured (see below). From 74–79, $\varphi$, $\psi$, and $\Delta C^\beta$ indicate the presence of $\alpha$-helix. The recombinant apoCIII ends with an unrelated insert of 2 residues (Leu80 and Glu81), followed by a C-terminal 6-residue His tag. Leu80 and Glu81 show helical conformation based on chemical shifts, whereas the last 6 residues do not display a defined secondary structure. Leu80 and Glu81 may stabilize the last helix. In summary, we can define the following helices: h1 (positions 8–18), h2 (positions 20/23–29), h3 (positions 34–43), h4

FIGURE 1. $^1$H/$^{15}$N HSQC spectrum of $^{13}$C/$^{15}$N-labeled apoCIII in complex with SDS micelles. Peaks are labeled as assigned. The unlabeled cross-peak, at 7.65/117.8 ppm, appears after a few weeks.

ApoCIII Structure

that points from the nucleus of the $\alpha$-carbon of the $n$th residue toward the geometric center of its side chain. The two most widely used hydrophobicity scales are by Kyte and Doolittle (56) and Eisenberg (57). Both hydrophobic scales above were incorporated as alternatives in Protein Constructor (and previously also used in scripts to X-PLOR).
ApoCIII Structure

Relaxation Data at Two Magnetic Fields Show Pico- and Nanosecond Time Scale Internal Motions

To establish internal dynamics, $T_1$, $T_{1\text{rho}}$ and $^{13}$N(1H) NOE data were measured at 600 and 800 MHz (Fig. S1). The unstructured terminal regions (positions 1–7 and 82–87) and large loop (positions 65–73) are clearly visible in the $T_1$, $T_{1\text{rho}}$ and $^{13}$N(1H) NOE data (Fig. S1). Analysis of the relaxation data shows that the backbone undergoes both pico- and nanosecond time scale motions, similar to what we observed for apoCII (23). The mean helix order parameters for the pico- and nanosecond time scale motions (Table S1) indicate, as for apoCII, well formed helices ($S^2$ values are high, $\sim 0.8$) that undergo domain motion of the helices (presence of nanosecond time scale motion, $S^2 \sim 0.7$). A more detailed analysis of the dynamics will be presented elsewhere.

Dipolar Wave Analysis of the Dipolar Couplings Shows That Helices Are Curved and Their Three-dimensional Orientations Are Averaged

Thanks to careful optimization, accurate $H^N$-N RDCs could be measured in stretched polyacrylamide gels (Fig. 3). A convenient route to extract qualitative and quantitative information on both global (three-dimensional) structure and dynamics of a protein from RDCs is formed by the analytical expressions of the dipolar wave formulation for RDCs (45, 46). They express RDCs in terms of a number of parameters that define the orientation of the dipolar vectors (e.g., $H^N$-N) in the alignment tensor frame and in the context of the three-dimensional protein structure. Briefly, in a helix, the $H^N$-N vectors lie around the helix axis on a cone with opening angle $\delta$ (15.8° in standard $\alpha$-helices). The position on the cone or phase $\rho$ of the $H^N$-N vector is defined relative to the helix phase $\rho_0$, which in turn is defined with respect to the phase of $H^N$-N vector of the middle residue in the helix $i_{\alpha}$. $\rho_0$ also depends on the residue number $i$, $\rho = \rho_0 + (i - i_{\alpha})360°/n$, where $n$ represents the number of residues per turn (3.6 in standard $\alpha$-helices). The helix orientation is described by the polar angles $\theta$ and $\phi$ and by the phase $\rho_0$; these define the helix axis orientation and helix phase in the principle axis frame of the alignment tensor. From the set of $(\theta, \phi, \rho_0)$ for each helix, the three-dimensional conformation of the protein can be derived. Here we applied this formalism to interpret the RDCs of apoCIII in its micelle-bound state, first qualitatively and then quantitatively.

Two interesting qualitative features are directly apparent from Fig. 3. First, within each helix, the dipolar waves of apoCIII (Fig. 3, top) do not run parallel to the horizontal axis as for a straight helix but curve up or down, indicating curvature of the helix axes, probably to accommodate the micelle surface. Second, in the apoCIII helices, the amplitudes of the dipolar wave

structure calculations, $\alpha_2$ was chosen to run from 20 to 29. b, secondary structure of apoCIII and absolute value of the backbone angles ($\phi$ and $\psi$) as derived from TALOS. Helices derived here are indicated as black horizontal bars, and uncertainty in the start of the second helix is indicated as a gray bar. Previously predicted helices are shown as gray horizontal bars (top) (1).
ApoCIII Structure

Quantitative structure and dynamics information can be obtained using the dipolar wave formulation by fitting the calculated RDCs onto the measured RDCs. This was done for apoCIII using Matlab scripts (see “Experimental Procedures”), so that apoCIII structure parameters as well as information on its dynamics are derived (Table 1 and Fig. 4). First, optimal and good quality fits are only obtained when the opening angles for the H\textsubscript{N}-N vectors in the helices are small, much smaller than expected for rigidly attached standard α-helices. Compare for instance the amplitude of the dipolar waves of the apoCIII helices (Fig. 3, top) with the wave amplitude of the second (micelle-inserted) helix of M13 (Fig. 3, bottom). As discussed below, the small amplitude can effectively be taken into account by employing smaller than standard opening angles δ for the H\textsubscript{N}-N vectors in the helices.

Quantitative structure and dynamics information can be obtained using the dipolar wave formulation by fitting the calculated RDCs onto the measured RDCs. This was done for apoCIII using Matlab scripts (see “Experimental Procedures”), so that apoCIII structure parameters as well as information on its dynamics are derived (Table 1 and Fig. 4). First, optimal and good quality fits are only obtained when the opening angles for the H\textsubscript{N}-N vectors in the helices are reduced from the standard 15.8° (Fig. 4, top versus bottom, and Table 1). Reducing the axial component while leaving δ at 15.8° improves the fit (Fig. 4, top correlation plot, left and right) but does not lead to as optimal a fit as to when δ is reduced (Fig. 4, bottom). Thus, indeed the amplitudes of the waves are reduced compared with standard helices (Table 1). This reduced wave amplitude suggests the presence of conformational averaging of helix orientations on the micelle surface. Second, optimal fits also require that curvature of varying degree be introduced into the helices (Table 1). Third, the best fit number of residues per turn equals the standard value of 3.6 for the six helices (1). Finally, a set of θ, φ, and ρ values for each helix is obtained, which define the helix orientations and thus the three-dimensional conformation of apoCIII, albeit with some degeneracy due to intrinsic symmetries in the RDC equations. This degeneracy is resolved by geometric constraints employed in the final structure calculations described under “Experimental Procedures.” Due to the dynamics, the angles, θ, φ, and ρ, that define the helix orientations must be viewed as averages. Below, the structure of apoCIII in the complex is described.

Three-dimensional Structure of ApoCIII in Complex with SDS Micelles

Structure calculations of apoCIII in complex with SDS micelles were performed (see “Experimental Procedures”). The final structures show good structure characteristics (Table S2) and are displayed on the SDS micelle surface in three views (Fig. 5). The three-dimensional structure consists of six ~10-residue helices. ApoCIII was previously predicted to consist, like apoCI/II (23, 25), of two long ~22-residue helices (positions 8–29 and 40–67) connected by an unstructured loop (1, 27). We now find that the two long helices in apoCIII are each broken up into two shorter ones (h1/2 and h4/5) and that the unstructured region (residues 30–39) is largely helical (h3; residues 33–43). At the C-terminal end, helix 6 is formed. All helices are connected via semiflexible hinges and are curved (Figs. 5 and 6A), so that they wrap tightly around the micelle, thereby optimizing interaction with the curved micelle surface. The semiflexible hinges allow apoCIII to easily adapt to the different diameters of its natural binding partners, which range in diameter from 75–1200 nm (chylomicrons) to 25–75 nm (very low density lipoproteins), 18–25 nm (LDL), and 5–12 nm (HDL), with the last coming closest to the 4.4-nm diameter of SDS micelles. The helix curvature is expected to be less, in particular for the larger particles.

We finally note that within each helix, the side chain orientations are well defined in both the structures derived from the experimental NMR data alone (Part I) and the final structures (Part II). To confirm this, we have recalculated for the 10 best structures the variation of the orientation of the hydrophobic moment vectors within each helix, which represent the varia-

### Table 1

Structure parameters of apoCIII as defined from dipolar waves fits

| Helix | θ\textsuperscript{a} | φ\textsuperscript{a} | ρ\textsuperscript{a} | δ | C\textsubscript{Cu}\textsuperscript{a} |
|-------|-----------------|-----------------|-----------------|---|------------------|
| 1     | 62 (1)          | 150 (25)        | 3               | 1.5| 0.26             |
| 2     | 72 (2)          | 15              | 3               | 1.5| 0.26             |
| 3     | 55 (1)          | 272 (38)        | 7               | 0.5|                  |
| 4     | 73 (2)          | 270 (50)        | 6               | 0.0|                  |
| 5     | 79 (3)          | 280 (60)        | 11              | 0.0|                  |
| 6     | 50 (3)          | -117            | 3               | 1.0|                  |

\textsuperscript{a}Helices 1–6 are defined as 8–18, 20–29, 33–43, 46–53, 55–64, and 74–79.

Dipolar wave formulation, angle definitions: θ, opening angle of the cone around the helix axis on which the H\textsubscript{N}-N vectors reside (δ = 15.8° for standard helix); ρ, phase of the H\textsubscript{N}-N vector on the cone, ρ = ρ\textsubscript{0} + (i\textsuperscript{im}ΔΩ)/136°, where i is the number of residues per helix turn (n = 3.6 residues per turn for standard helices), i is the residue number, and ρ\textsubscript{0} is the phase in the middle of the helix at residue i\textsuperscript{im} |[θ\textsubscript{0}, φ\textsubscript{0}|, polar angles that define the helix axis orientation; all parameters are expressed in the principal axis frame of the alignment tensor.

C\textsubscript{Cu}, curvature of the helix axis (° per residue); when curvature is present, the angles [θ, φ] depend on residue number, e.g. when the helix axis is parallel to the x-axis of the alignment tensor, θ = θ\textsubscript{0} + n C\textsubscript{Cu}i, i.e. the value of θ at the position of C\textsubscript{Cu}i residue in the helix. D\textsubscript{Ω} = 0.26 Hz as derived from dipolar wave fit and histogram method. In parentheses is given the r.m.s. deviation of the MC fit values from 10 variations assuming 0.8 Hz experimental RDC error; r.m.s. deviation of the MC fit is ~ 1.4 Hz for standard helix δ of 15.8° and ~ 0.5 Hz when δ is as given (see also Fig. 4).

FIGURE 3. H\textsubscript{N}-N RDCs of apoCIII (top) and M13 (bottom) both in complex with SDS micelles and measured in 4% acrylamide gel, showing their dipolar waves. Helical regions are indicated as horizontal bars. M13 consists of a surface helix (h1, residues 8–18) connected to a trans-membrane helix (h2, residues 25–45), which restricts motion of both the trans-membrane helix and the surface helix so that their RDCs display full-amplitude dipolar waves, in contrast to the surface helices of apoCIII.
ApoCIII Structure

FIGURE 5. Three-dimensional structure of apoCIII-SDS micelles as derived by NMR. In the left and in the middle, two views are shown that illustrate helix curvature and side chain orientations. The axis system corresponds to that of the alignment tensor frame. N and C represent the positions of the N terminus and C terminus, respectively; in the middle, side chains are shown in white. The right panel shows a front view of the overlay of the 10 best structures in the complex together with the hydrophobic moments of the helices displayed as arrows; note that the arrows point to the interior of the micelle and show relatively little variation, indicative of the relatively minor variation in the side chain orientation within each helix. We find that the values fall within 20° for all helices (ranges: h1, 11°; h2, 12°; h3, 17°; h4, 11°; h5, 9°; h6, 13°). Consequently, functional roles of the side chain orientation can be reliably inferred from the structures.

(a) Lipid Interaction—The lipid interaction in human apoCIII extends over all six helices and originates mainly from hydrophobic residues that point with their side chains to the interior of the micelle (Fig. 6B). The hydrophobic residues of human apoCIII are functionally conserved among other mammals (Table 2). Additional contributions to the lipid interaction stem from charge interactions, between “anchoring” charge residues in the protein and charges in the lipid head groups as discussed by Segrest et al. (1). One can recognize two different helix classes in human apoCIII (Fig. 6B). In the two class A helices (h4/5), the positive residues face the negative lipid head groups, whereas the negative residues line the polar face (Fig. 6B). In fact, two or more anchoring residues, operationally defined here as (a) positive residues located at the polar to non-polar interface or (b) as negative residues located at the polar face, are present in these helices (Table 2, columns 4 and 5, human apoCIII). In the G* helices (h1/2/3), we find in each, one or two anchoring residues (Table 2, columns 1–3, human apoCIII). Helix 6 (also class G*) plays a minor role. The anchoring residues are, like the hydrophobic residues, functionally conserved in apoCIII among mammals (Table 2, columns 1–5).

The importance of one of the anchoring residues is well illustrated by the human mutation K58E (58). This resulted in decreased plasma concentrations of apoCIII and increased levels of apoE on HDL, indicating lower lipid affinity of the mutant apoCIII.

The hydrophobic moments of the class A and G* helices (Table S1, H_M) are relatively similar, as predicted by Segrest (1). Thus, the number of charge interactions from anchoring residues of each helix indeed makes the distinction between class A and G* helices (Tables 2 and S1, C_p). The relative lipid affinity can then be read off from the total number of anchoring residues. The total number of anchoring residues is relatively constant among the mammals (varies from 7 to 10; Table 2), indicating that the degree of lipid affinity is evolutionarily conserved at a more or less fixed level.

It is also interesting to compare the lipid interaction of apoCIII with that of apoCI and -CII. ApoCI and -CII consist of four and three class A helices, respectively; the class A helices each have 4 – 6 anchoring residues (Table 2 and Fig. S2); apoCII contains in addition a highly mobile non-class A helix, which may be disregarded for the present discussion (Table 2). The total number of anchoring residues in human apoCI, -CII, and -CIII is 21, 12, and 9, respectively. Hence, it appears that the lipid affinity of the three apoS follows the trend, apoCI >> apoCII ≈ apoCIII.
solvency-exposed Lys residues (Lys17, Lys21, Lys24) line the apex—The most salient feature of human apoCIII is that three Face Arg residue at this position (Table 2). For the Lys17 and Lys24 mals, except for pig apoCIII, which has the functionally similar This similarity suggests the interesting possibility that lipid- interacting interactions, Lys17 and Lys24 in human apoCIII, are and is necessary for sufficient binding (60). Such a remote and is necessary for sufficient binding (60). Such a remote stabilizing interaction is absent in all apoCIIIs. Local stabilizing interactions, Lys17 and Lys24 in human apoCIII, are also absent in some mammals. Thus, the apoCIII receptor binding is expected to be weak. Interestingly, apoCI, which

Thus, the solvent-exposed face of mammalian apoCIII displays positive charges in the N-terminal and negative charges in the C-terminal region. Conservation of the lipid interaction as well as of the charge distribution on the polar face suggests that these aspects are of functional importance. ApoCI and -CII display purely negative polar faces (Table 2). Thus, in contrast to apoCIII, they do not have solvent-exposed positive residues.

(c) Remarkable Similarity between the Conserved Conformation around Residue Lys21 on ApoCIII and the Lys-binding Motif of the LDL Receptor Family—The structure of the multidomain LDL receptor (59) and the complex of two of its domains with the receptor-associated protein (RAP) (60) demonstrate the molecular details of the binding of RAP/apoE/apoB with the LDL receptor family (Table S3). Effective binding appears to derive from multiple weak charge interactions on more than one receptor domain (60). The central interaction involves a Lys residue, Lys256, in RAP and Lys146 in apoE, which binds into a Lys-binding pocket on a receptor domain, the Lys-binding motif (60) (Fig. 6D). The motif itself interacts weakly, and receptor interaction is stabilized by additional local (RAP, Lys253 and His259, apoE, Lys143 and Arg150) and remote (RAP, Arg296; apoE, Arg172) charge interactions.

A remarkable similarity exists between the three-dimensional conformation of Lys17, Lys21, and Lys24 of human apoCIII (Fig. 6C) and that of the corresponding Lys/His residues on RAP (Lys253, Lys256, and His259) (Fig. 6, C and D). Thus, the solvent-exposed Lys21 of human apoCIII could, like Lys256 in RAP domain 3 (D3), interact with the Lys-binding pocket on the negatively charged receptor domain LA4, whereas Lys24 can, like Lys256 in RAP, aid in stabilizing the interaction with LA4 and similarly Lys21, like His259 in RAP. This similarity suggests the interesting possibility that lipid-bound apoCIII, via these residues, could interact with the LDL receptor. Note, however, that in RAP, the remote residue Arg296 (Arg172 in apoE) further stabilizes the interaction and is necessary for sufficient binding (60). Such a remote stabilizing interaction is absent in all apoCIIIs. Local stabilizing interactions, Lys17 and Lys24 in human apoCIII, are also absent in some mammals. Thus, the apoCIII receptor binding is expected to be weak. Interestingly, apoCI, which

In contrast to the N-terminal helices, seven negative residues line the polar face of the C-terminal class A helices 4/5 and adjacent flexible loops (Fig. 6B and Table 2). Also, this feature is largely conserved among other mammals (Table 2, column I).
TABLE 2
Sequence comparison of mammalian ApoCIII and comparison with ApoC-I and -II

| ApoC-I | ApoC-II | ApoC-III |
|--------|---------|----------|
| H      | Ma      | P        |
| NPKQWKR | NPKQWKR | NPKQWKR |
| K      | K       | K        |
| NNKQWKR | NNKQWKR | NNKQWKR |
| K      | K       | K        |
| NNKQWKR | NNKQWKR | NNKQWKR |
| K      | K       | K        |
| NNKQWKR | NNKQWKR | NNKQWKR |

DISCUSSION

Biological Functions of ApoCIII

Inhibition of Lipolysis—Lipolysis of lipoproteins occurs by the enzyme LPL, which resides on the cell surface and needs to associate to lipoproteins in order to carry out the reaction. ApoCIII can inhibit lipolysis in part by preventing lipoproteins from approaching the negatively charged cell surface (8) by displacing apoE, the apolipoprotein with the highest affinity for negatively charged surfaces, from the particle or by causing conformational changes to apoE. Repulsion between the solvent-exposed negative charges on apoCIII and the cell surface could further reduce the interaction. ApoCIII might also displace some apoCIII from the lipoprotein surface, thus lowering the direct activation of LPL (61). Finally, yet unproven direct protein-protein interactions between apoCIII and LPL could play a role (62).

ApoCIII Interaction with SR-BI, the HDL Receptor—The HDL receptor SR-BI (31) binds a variety of apolipoproteins, including apoCIII, -E, -D, and -AI (32), most likely via their class A amphipathic helices (63). The strong binding of human apoCIII to SR-BI (31) can be understood from the presence of seven negative residues on the polar face of region 41–71, which includes the class A helices 4 and 5 (Table 2).

Inhibition by ApoCs of ApoE/B Binding to the LDL Receptor Family—The main previously proposed mechanism for the inhibition by apoCs (I, II, and III) of apoE-mediated binding of lipoproteins to the LDL receptor is that apoCs displace apoE from the lipoprotein particles (2). This displacement can either be complete or involve only the N-terminal domain of apoE (2). ApoE interacts with the LDL receptor via positive charges on helix 4 (K-motif) of its N-terminal domain with one of the negative domains of the LDL receptor. Complete displacement of apoE from the lipoprotein or displacement of only the N-terminal domain (which then folds into a receptor-inactive helix bundle) diminishes or prevents the receptor interaction.

The apoCIII structure presented here is compatible with such a mechanism, and comparison with the apoC-I and apoCIII structures provides insight into the differences in inhibitory strength between the apoCs. First, the higher lipid affinity of apoC-I versus apoCIII (see above) explains the observed higher apoE displacement and higher inhibitory strength of apoC-I versus apoCIII. With apoE displaced from the lipoprotein particle, the apoCs prevent interaction with the LDL receptor by repulsion between the negative receptor domains and the negative polar faces of their class A helices. This repulsion is strong and similarly large in apoC-I, -CII, and -CIII, since the number of negative charges is large and similar (Table 2). Thus, this explains well why apoC-I inhibits better than apoCIII. Second, the similar lipid affinity of apoC-I versus apoCIII (see above) is in accord with the similar capacities of apoC-I and apoCIII to displace apoE (64). Factors other than lipid affinity are expected also to play a role (e.g. a balance between repulsive and attractive charge interactions with receptor domains). In this respect, it is interesting to note the K-motif on apoCIII, which is absent on apoC-I (and apoCII). The weak attractive interaction with the receptor exerted by the K-motif may at least in part explain why apoC-I is found to inhibit better than apoCIII (64). In conclusion, from the presented structure and the above analysis, one...
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would predict the observed weak inhibition by apoCIII relative to both apoCII and apoCI (apoCII \(\gg\) apoCII \(\gg\) apoCIII). The relative importance of these different mechanisms could be experimentally tested with mutant forms of the apoCs.

In summary, this structure may provide a productive template for further exploration of the molecular mechanisms for the action of apoCIII. We suggest that a subtle interplay of repulsive and attractive receptor interactions with class A helices and K-motifs, respectively, together with specific properties of the lipid affinity, may explain the effects of apoCIII on apoE/B-mediated receptor binding, proteoglycan binding, and lipolysis.

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