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# Apolipoprotein A-II: Chemical Synthesis and Biophysical Properties of Three Peptides Corresponding to Fragments in the Amino-Terminal Half†

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ABSTRACT: Three peptide fragments of apolipoprotein A-II corresponding to residues 17–31, 12–31, and 7–31 have been synthesized by solid-phase techniques and purified to apparent homogeneity. Each of these fragments contains residues 18–30, a region previously proposed to possess potential amphipathic helical properties. Secondary structural changes of these synthetic fragments accompanying their interaction with phospholipid have been studied by circular dichroism. The magnitude of this interaction has been evaluated from the yields and stoichiometry of lipid–protein complexes isolated by density gradient ultracentrifugation. Fragment 17–31, the smallest peptide containing the proposed amphipathic helix, did not interact with dimyristoylphosphatidylcholine (DMPC) single bilayer vesicles at 24 °C; upon addition of DMPC, no ellipticity change could be detected nor could a stable lipid–peptide complex be isolated. However, fragments 12–31 and 7–31 did interact with phospholipid; in the absence of lipid, both fragments had primarily disordered structures, but when isolated as DMPC–peptide complexes, both fragments possessed increased helical structure. The phospholipid:peptide molar ratio was 14:1 for fragment 12–31 and 27:1 for fragment 7–31. Studies of space-filling models of these fragments suggest that hydrophobicity and/or length are important properties of phospholipid binding apoproteins.

Apolipoprotein A-II (apoA-II)[1] is one of the major protein components of human serum high density lipoproteins (HDL). It possesses two identical polypeptide chains each containing 77 amino acids. These chains are cross-linked by a disulfide bond at cystine-6 (Brewer et al., 1972). The approximate distribution of its secondary structure is 35% $\alpha$ helix, 13% $\beta$ structure, and 52% disordered structure. Other physical properties of this apoprotein have been recently reviewed (Morrisett et al., 1977).

In this laboratory, considerable effort has been directed toward identifying the structural features of apoA-II which

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[1] Abbreviations used: HDL, high density lipoproteins; apoA-II, apolipoprotein A-II (mol wt 17 149), a major apoprotein of HDL; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; EDTA, ethylenediaminetetraacetate; Boc, *tert*-butyloxycarbonyl; TLC, thin-layer chromatography; DCC, *N,N'*-dicyclohexylcarbodiimide.

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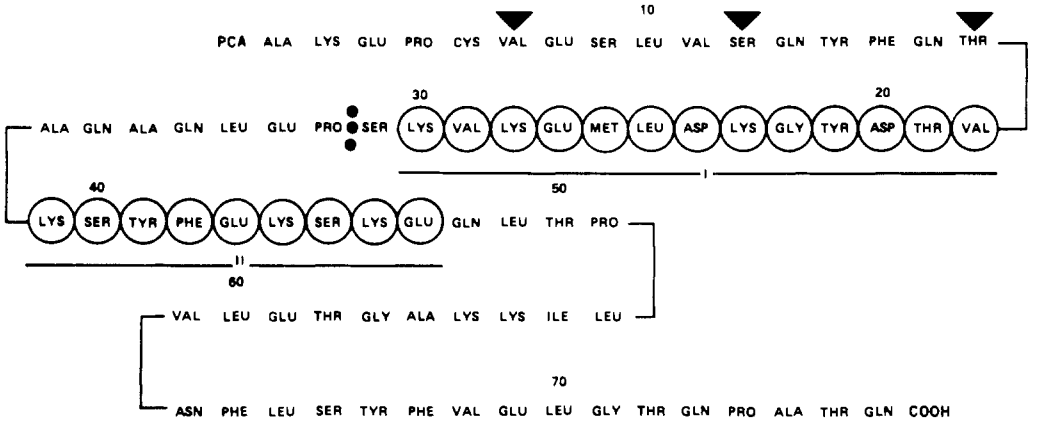


FIGURE 1: Amino acid sequence of human high density apolipoprotein A-II (apoA-II) as described by Brewer et al. (1972) and Lux et al. (1972b). The predicted amphipathic regions 18–30 and 39–47 previously described by Segrest et al. (1974) are indicated by circles. The beginning and ending of the fragments synthesized are indicated by triangles and dots, respectively. Each of the synthetic fragments contains the predicted amphipathic region 18–30.

enable it to bind phospholipids. Initial studies established that reduction of the disulfide bond followed by alkylation did not significantly impair its lipid-binding properties (Jackson et al., 1973b). When phospholipid was bound to reduced apoA-II which had been tagged with a fluorescent or paramagnetic reporter group at the free sulfhydryl moiety, no changes in the spectroscopic properties of these groups were observed, suggesting that the segment of the apoprotein near the amino terminus did not contain structural features critical for phospholipid binding. In a subsequent study, it was observed that chemical cleavage of the protein with cyanogen bromide at the single methionine (sequence position 26) yielded two peptide fragments: the amino-terminal 1–26 fragment exhibited no lipid-binding capacity, while the carboxyl-terminal 27–77 fragment did bind phospholipid, but at a level lower than that of the uncleaved apoprotein (Jackson et al., 1973a). On the basis of sequence information (Brewer et al., 1972) and the amphipathic helix hypothesis (Segrest et al., 1974), it was predicted that apoA-II contained two amphipathic helical regions which comprise the principal lipid-binding determinants of this apoprotein. One of these regions, Lys-39-Glu-47, resides in the carboxyl-terminal half of the molecule. To evaluate the lipid-binding capacity of this segment, Mao et al. (1977) synthesized a series of four peptide fragments corresponding to residues 65–77, 56–77, 47–77, and 40–77 of intact apoA-II. As expected, the two shorter fragments which did not contain the predicted amphipathic helical segment did not form stable phospholipid–peptide complexes, and the longest fragment (40–77), which did contain the predicted amphipathic helix, did form a stable phospholipid–peptide complex. Quite unexpectedly, the fragment 47–77 which did not contain the predicted helix did form a stable complex. The purpose of the present study was to evaluate the contribution of the predicted amphipathic helical segment Val-18–Lys-30 to the lipid-binding properties of specific peptide segments within the amino-terminal half of apoA-II. Accordingly, three peptide fragments corresponding to residues 17–31, 12–31, and 7–31 have been synthesized. This paper describes the synthesis, purification, and lipid-binding properties of these peptides.

## Materials and Methods

Dimyristoyl-L-$\alpha$-phosphatidylcholine (DMPC) was obtained from Sigma. It was further purified on a Waters Prep LC/500 as described previously (Patel & Sparrow, 1978) and was judged homogeneous by thin-layer chromatography. Boc-amino acids were obtained from Bachem and Peninsula Labs. Urea and DCC were obtained from Schwarz/Mann. Solutions of 6 M urea were passed through a mixed-bed resin (Rexyn I-300, Fisher) shortly before use.

*Preparation and Purification of Synthetic Fragments of ApoA-II.* Three synthetic peptides were prepared in the present study; these corresponded to residues 17–31, 12–31, and 7–31 in apoA-II (Figure 1). Fragments were synthesized with a Schwarz Bio-Research peptide synthesizer using a modified polystyrene resin as described by Sparrow (1976). The modified resin was first reacted with Boc-*O*-benzylserine, corresponding to residue 31 of apoA-II, and was then subjected to a coupling program which was essentially the same as described previously (Mao et al., 1977). A 4:2:1 molar ratio of Boc-amino acid:DCC:reactive amine component was used, except for Val-29, Thr-19, Val-18, Gln-16, Phe-15, Gln-13, Ser-12, Val-11, and Ser-9, where an 8:4:1 ratio was employed. The coupling time was 60 min for 4:2:1 ratios and 120 min for 8:4:1 ratios. A portion of the solvated resin was removed from the synthesizer after the coupling of residues 17, 12, and 7. After cleavage with anhydrous HF, the fragments were initially fractionated by chromatography on a Bio-Gel P-10 column (2.6 × 90 cm) equilibrated with 0.1 M Tris-HCl, 6 M urea, pH 8.2. The appropriate fractions were pooled and desalted on a column of Bio-Gel P-2 (4.5 × 35 cm) equilibrated with 0.1 M ammonium bicarbonate and then lyophilized. The fragments were further purified by ion-exchange chromatography on sulfopropyl-Sephadex C-25 (1.6 × 30 cm) as described in the legend of Figure 2. The purity of the synthetic fragments was assessed by amino acid analysis and by polyacrylamide gel electrophoresis in 8 M urea at pH 8.2 (Figure 3).

*Phospholipid-Binding Studies.* The secondary structure of the synthetic fragments of apoA-II in the absence and presence of phospholipid vesicles was studied by circular dichroism. Circular dichroic spectra of the peptide fragments and of isolated fragment–DMPC complexes were recorded on a Cary 61 spectropolarimeter as previously described (Morrisett et al., 1973). Single bilayer vesicles of DMPC (30 mg in 1 mL of buffer containing 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 1 mM $NaN_3$, pH 7.4) were prepared by sonication under UltraPure nitrogen for 45 min at 25 °C. After sonication, the DMPC solution was almost transparent. DMPC was stable to sonication as evidenced by retention of a single spot on thin-layer chromatography (silica gel; $CHCl_3$–$CH_3OH$–$H_2O$, 65:25:4). The peptide fragment and DMPC vesicles (1:3 w/w) were incubated for 12 h at 24 °C and then subjected to density gradient ultracentrifugation. Each

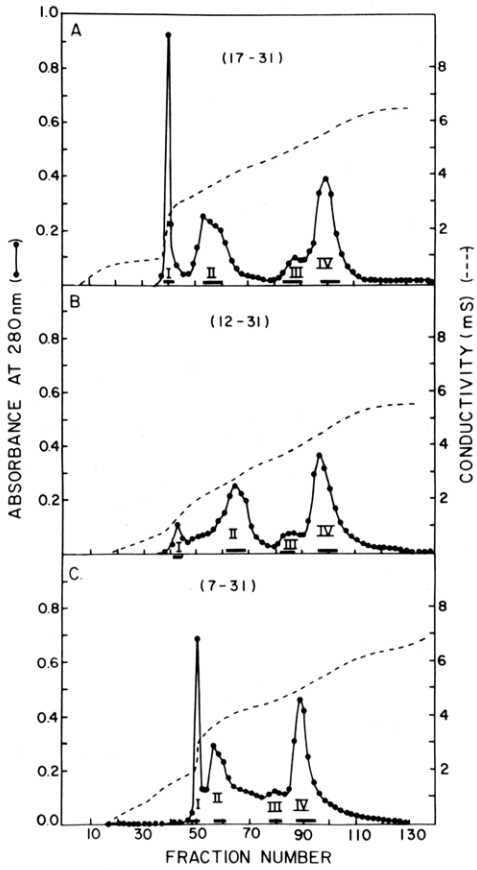


FIGURE 2: Purification of synthetic fragments of apoA-II by SP-Sephadex C-25. (A) Fragment 17–31; (B) fragment 12–31; and (C) fragment 7–31. The sample was applied to a column (1.6 × 30 cm) of SP-Sephadex C-25 at a concentration of 5 mg/mL and was eluted with the following gradient: (A) 300 mL of 0.02 M sodium acetate, 6 M urea, pH 4.2, and (B) 300 mL of 0.2 M sodium acetate, 6 M urea, pH 4.2. Fractions (3.5 mL) were collected and the flow rate was 20 mL/h. The fractions indicated by the bars were pooled, desalted, and lyophilized.

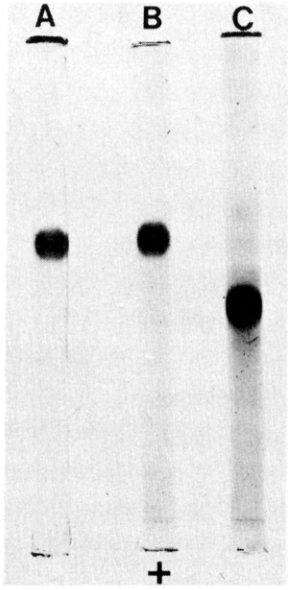


FIGURE 3: Polyacrylamide gel electrophoresis of synthetic apoA-II fragments. The fragment (approximately 40 μg) was run on a 7.5% polyacrylamide gel in 8 M urea at pH 8.2 for 3 h; the gels were stained with Coomassie blue. (A) Synthetic fragment 17–31; (B) synthetic fragment 12–31; and (C) synthetic fragment 7–31.

gradient (5 mL) was formed with a Buchler peristaltic pump and Densiflow gradient maker. The tubes were centrifuged in a Beckman SW 50.1 rotor at 45 000 rpm for 4 days at 24 °C. Centrifuged samples were fractionated by collecting 0.3-mL portions from the bottom of the pierced tubes. The density of each fraction was estimated by refractometry (Weast, 1974–1975) on a Bausch and Lomb refractometer. The presence of protein and/or phospholipid was determined by measuring absorbance at 280 nm. The actual protein concentration was determined by quantitative amino acid analysis. Phospholipid concentration was determined by phosphorus analysis (Bartlett, 1959) using a conversion factor of 21.9.

Table I: Amino Acid Composition of Synthetic Fragments of ApoA-II[a]

| amino acid | synthetic A-II (17–31) | synthetic A-II (12–31) | synthetic A-II (7–31) |
|---|---|---|---|
| Asp | 2.00 (2) | 1.99 (2) | 2.02 (2) |
| Thr | 1.59 (2) | 1.83 (2) | 1.77 (2) |
| Ser | 1.01 (1) | 1.67 (2) | 2.55 (3) |
| Glu | 1.08 (1) | 3.07 (3) | 3.97 (4) |
| Gly | 1.17 (1) | 0.97 (1) | 1.01 (1) |
| Val | 1.95 (2) | 2.04 (2) | 4.03 (4) |
| Met | 0.91 (1) | 1.00 (1) | 1.13 (1) |
| Leu | 1.03 (1) | 1.04 (1) | 2.02 (2) |
| Tyr | 1.07 (1) | 1.82 (2) | 1.87 (2) |
| Phe | 0 (0) | 1.01 (1) | 0.96 (1) |
| Lys | 2.73 (3) | 2.99 (3) | 2.90 (3) |
| total | 15 | 20 | 25 |

[a] The results of duplicate analyses from 6 N HCl hydrolysates (22 h, 110 °C) are shown. Analyses were performed on a Beckman Model 119 analyzer equipped with an Autolab integrator. No corrections for destruction during hydrolysis have been made. The theoretical number of residues is shown in parentheses after each experimental value.

## Results

*Synthesis and Purification of Synthetic Fragments.* The general strategy used for the synthesis of fragments of apoA-II has been described previously (Mao et al., 1977). At the appropriate residue, a portion of the peptide resin was removed from the synthesizer reaction vessel and treated with anhydrous HF to remove all protecting groups and to release the peptide from the resin. After a partial purification on Bio-Gel P-10 to remove truncated peptides, the fragments were further purified by ion-exchange chromatography on sulfopropyl-Sephadex C-25 (Figure 2). The fractions of peak IV, indicated by the bars, were pooled, desalted, and lyophilized.[2] The amino acid composition of each fragment so purified is given in Table I. The compositions were in good agreement with the theoretically expected values. Each fragment migrated as a single band on a 7.5% polyacrylamide gel in 8 M urea at pH 8.2 (Figure 3). The overall yield from the Ser resin to purified peptide was 17.5% for fragment 7–31 and even higher for the two shorter fragments.

*Phospholipid-Binding Properties of Synthetic Fragments.* Fragment 17–31, the smallest proposed amphipathic helix-containing fragment among the three synthesized, did not bind to DMPC vesicles, as evidenced by the absence of any change in the circular dichroic spectrum (Figure 4A) and by the failure to form a lipid–peptide complex that could be isolated by density gradient ultracentrifugation (Figure 5C). Amino acid analysis of the phospholipid-containing fractions at the top of the gradient indicated that there was no protein associated with the lipid. After incubation with DMPC vesicles,

[2] Peak I contained a nonproteinaceous, ninhydrin-negative material which is probably a degradation product of the polystyrene beads. Peaks II and III contained peptides whose amino acid analyses were inconsistent with their expected composition.

Table II: Hydrophobicity of Peptide Fragments and Composition of Peptide Fragment-DMPC Complexes

| synthetic fragment | no. of amino acids | hydrophobicity[a] index | $[\theta]_{222}$ (deg cm$^2$ dmol$^{-1}$) fragment | $[\theta]_{222}$ (deg cm$^2$ dmol$^{-1}$) fragment-DMPC complex | composition of complex[b] lipid/protein (w/w) | composition of complex[b] lipid/protein (molar ratio) |
|---|---|---|---|---|---|---|
| 17-31 | 15 | −831 | 1630 | *c* | *c* | *c* |
| 12-31 | 20 | −854 | 1170 | 5150 | 4.0 | 14 |
| 7-31 | 25 | −935 | 1170 | 8240 | 6.5 | 27 |

[a] The average hydrophobicity per residue was calculated using the hydrophobicity assignments of Bull & Breese (1974). [b] The compositions were determined on the isolated complexes. [c] No significant amount of complex was isolated.

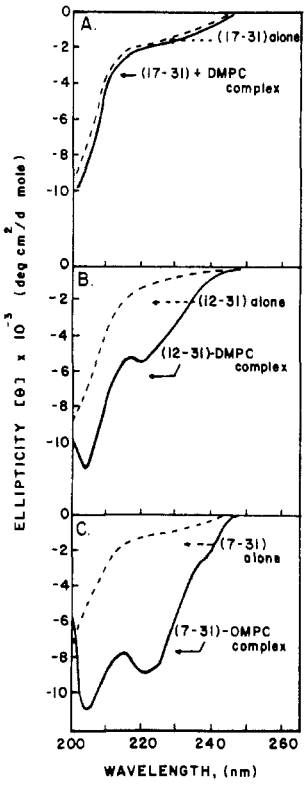


FIGURE 4: Circular dichroic spectra of fragments and fragment–DMPC complexes. The spectra shown in panel A are for fragment 17–31 alone and an incubated mixture of fragment with DMPC (1:3 w/w). The spectra in panels B and C are for DMPC–fragment complexes reisolated by density gradient centrifugation.

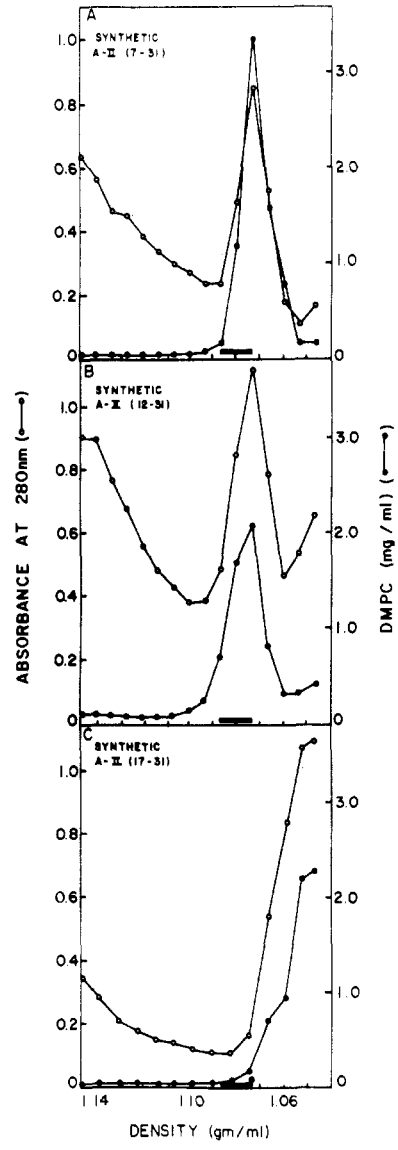


FIGURE 5: Ultracentrifugal behavior of complexes formed by synthetic fragments of apoA-II and DMPC. Sonicated DMPC vesicles were added to 1 mL of synthetic fragments in buffer containing 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 1 mM $NaN_3$, pH 7.4. Concentration of fragments was 1.03, 0.93, and 0.70 mg/mL for fragment 7–31, 12–31, and 17–31, respectively. The peptide fragment and DMPC vesicle (1:3, w/w) mixtures were incubated for 12 h at 24 °C and then subjected to density gradient ultracentrifugation (see Materials and Methods). The lower optical density for unbound peptide (17–31) in the high density range of panel C is due to the lower extinction coefficient for this peptide (contains one tyrosine) relative to the other two peptides (contain two tyrosines).

fragments 12–31 and 7–31 formed complexes with DMPC which were isolated ultracentrifugally (Figure 5A,B). The purity and stability of these complexes were evaluated by ultracentrifugation using a narrower density gradient ($d$ = 1.05–1.10) in KBr for 2 days at 45 000 rpm. Both complexes banded symmetrically at $d$ = 1.08 and were found to be free of unbound protein and lipid (not shown). The molar ratios of phospholipid to peptide were 14:1 and 27:1 for fragments 12–31 and 7–31, respectively (Table II). The CD spectra of the complexed peptides show significant increases in ellipticity at 222 nm when compared with the unbound peptides (Figure 4, Table II). Approximately 29% helical content was calculated for fragment 7–31 in the isolated complex.

## Discussion

An amphipathic helix possessing sharply defined topography with one side polar and the other side nonpolar has been proposed as a basic structural element of lipid-binding apolipoproteins (Segrest et al., 1974). This hypothesis holds that the polar side of the helix interacts with the polar head groups of phospholipid, while the hydrophobic side of the helix interacts with methylene carbons near the fatty acyl carboxyl termini. Such interactions necessarily require that the long axis of the protein helix be oriented orthogonal to the long axis of the fatty acyl chains. Thermodynamic and geometric considerations for the amphipathic helix hypothesis have been described by Segrest (1977). In the original description of the model, several amphipathic regions for apoA-II, apoC-I, and

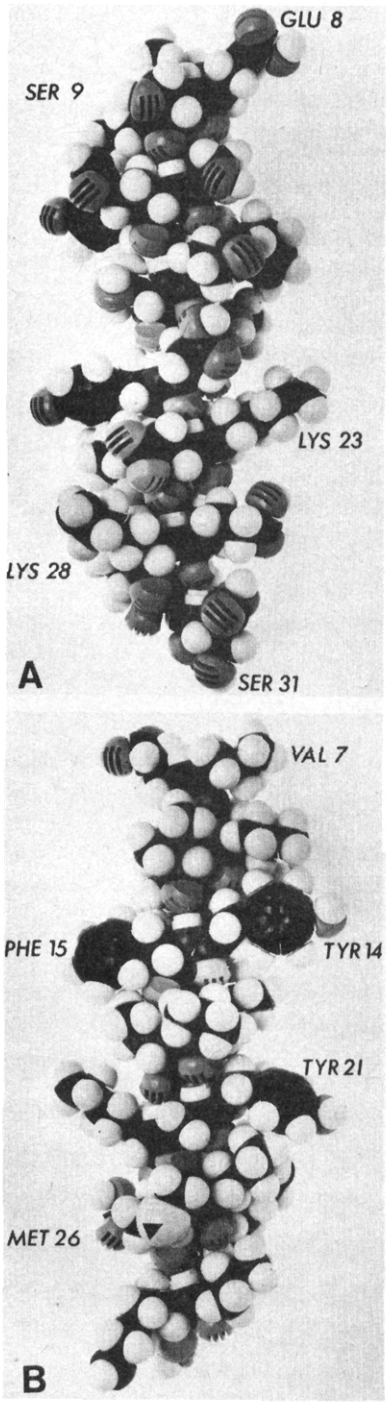


FIGURE 6: Corey–Pauling–Koltun space-filling model of the synthetic fragment 7–31 of apoA-II. Both the polar (A) and nonpolar (B) faces are shown. On the polar face are seen a number of hydrophilic amino acids such as Glu-8, Ser-9, Ser-12, Thr-17, Asp-20, Lys-23, Glu-27, Lys-28, and Ser-31 whose side chains favor preferential hydration of this side of the helix and/or interaction with the polar head groups of phospholipids. On the nonpolar face are observed numerous hydrophobic residues such as Val-7, Leu-10, Val-11, Tyr-14, Phe-15, Val-18, Tyr-21, Leu-25, Met-26, and Val-29 whose side chains resist hydration and enhance interaction of this side of the helix with hydrophobic surfaces such as those of phospholipid fatty acyl chains.

apoC-III were predicted. ApoA-II was proposed to contain only two such regions, residues 18–30 and 39–47. It was reported that the amino-terminal cyanogen bromide fragment which spans 1–26 did not interact with egg phosphatidylcholine significantly, whether the disulfide linkage at residue 6 was intact or not (Jackson et al., 1973a; Lux et al., 1972a). This result was attributed to the disruption of the predicted amphipathic region 18–30 by chemical cleavage at Met-26. In an effort to directly test this aspect of the amphipathic helix hypothesis, we have synthesized by solid-phase methods three peptide fragments, all of which contain the predicted amphipathic region 18–30. The fragment 17–31, which contains two amino acids more than the predicted amphipathic region, did not bind DMPC. The calculated average hydrophobicity (Bull & Breese, 1974) for fragment 17–31 was −831 cal/residue (Table II). The hydrophobicity of this region might be below that required for binding phospholipids as suggested previously in model peptide studies (Sparrow et al., 1975, 1977a). Alternatively, this 15-residue peptide may simply be too short to develop sufficient helical structure for binding. Based on these considerations of peptide length and average hydrophobicity, as well as our observation that fragment 17–31 does not bind to DMPC vesicles, it is not surprising that Kroon et al. (1978) observed no binding of fragment 22–31 to egg phosphatidylcholine vesicles since this fragment of apoA-II contains only 10 residues and possesses an average hydrophobicity of −743 cal/residue.

Fragment 12–31 with an average hydrophobicity of −854 cal/residue did bind DMPC vesicles, though not to the extent of fragment 7–31 which has a hydrophobicity of −935 cal/residue. Both fragments showed significant conformational changes upon interaction with phospholipids. While length and hydrophobicity of nonpolar residues are suggested as two important requisites for an amphipathic helical region that binds phospholipid (Table II), careful evaluation of the importance of these properties requires a systematic study in which the length of the peptide is kept constant and the hydrophobicity of the apolar residues is changed, and vice versa. This study is currently underway in our laboratories.

Analyses of the circular dichroic data of this and previous studies indicate that the entire predicted amphipathic regions cannot be involved in the formation of $\alpha$-helical structure. For instance, if the 18–30 region of fragment 7–31 did form an amphipathic helix upon binding phospholipid (Figure 6), the $\alpha$-helical content of this region in the fragment–DMPC complex should be more than 50%. However, the experimentally observed value is only 29%. The same problem arises with apoC-III in which the region 40–67 was originally predicted as an amphipathic helix. The predicted $\alpha$-helical content of fragment 41–79 of apoC-III of the isolated fragment–DMPC complex should be greater than 70%; however, only 38% was obtained (Sparrow et al., 1977b). In principal, this problem could be resolved by determination of bond angles $\psi$ and $\phi$ at individual amino acid residues. Recent peptide conformational studies have demonstrated the utility and power of the nuclear Overhauser effect (Urry et al., 1978) and vicinal coupling constants (Bystrov et al., 1975; Bystrov, 1976) in measuring these bond angles which permit direct determination of local secondary structure. Experiments of this type involving apolipoproteins isotopically substituted at specific nuclei are in progress.

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# High-Resolution Proton Magnetic Resonance Study of Porcine Colipase and Its Interactions with Taurodeoxycholate†

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ABSTRACT: A high-resolution 270-MHz proton NMR study of procine colipase I has been performed, and the resonances in the aromatic region of the spectrum have been assigned to amino acid residues by pH titration and decoupling experiments. The apparent p$K_a$ values of the three tyrosines were calculated to be 10.2, 10.3, and 11.8 with one of the tyrosines having properties of a "buried" residue. A tentative assignment to the amino acid residues in the primary sequence of colipase will be discussed. The effects of taurodeoxycholate (TDC) and a positively charged deoxycholate derivative on the aromatic region of the colipase NMR spectrum indicate that all tyrosines and one histidine are affected by the bile-salt binding, suggesting that the TDC molecules bind near these residues to a hydrophobic region on colipase. Measurements and calculations on the line width of the C(18) methyl group resonance suggest that the line-width increase of this resonance upon interaction of TDC with colipase to a large extent can be explained as due to the slower tumbling of the TDC molecules bound to colipase.

The hydrolysis of the dietary triglycerides in the intestinal lumen is mainly carried out by the enzyme pancreatic lipase with its cofactor colipase. The cofactor function of colipase is to bring the lipase molecule to the substrate surface in the presence of bile salts (Borgström & Erlanson, 1973). The colipase molecule has been purified (Mayliē et al., 1971; Erlanson & Borgström, 1972) and its primary and secondary structure characterized (Charles et al., 1974; Erlanson et al., 1974). The colipase molecule studied in the present investigation is composed of 100 amino acid residues (Figure 1). Among these, three are tyrosines, two are phenylalanines, and two are histidines. NMR[1] has proved to be a useful tool in studies of changes in the microenvironment of macromolecules upon ligand binding and in studies of the dynamics of molecular interactions (e.g., Dwek, 1973; Wütrich, 1977). In order to gain information on structural features of the colipase molecule and to interpret the interaction between colipase and TDC on the molecular level, a high-resolution proton NMR study has been performed with special reference to effects observed on the tyrosines, phenylalanines, and histidines of

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[1] Abbreviations used: CD, circular dichroism; cmc, critical micellar concentration; NMR, nuclear magnetic resonance; TDC, taurodeoxycholate; UV, ultraviolet; DSS, 2,2-dimethylsilapentane-5-sulfonate.

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