branes for the response of squalene epoxidase to SPF.

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Magnetic Resonance Studies of Apolipoprotein C-I Nitroxide Labeled or [^{13}C]Methyl Enriched at Methionine-38†

Tsung-Chang Chen, Roger D. Knapp, Michael F. Rohde, James R. Brainard, Antonio M. Gotto, Jr., James T. Sparrow, and Joel D. Morrisett*

ABSTRACT: One of the three proposed lipid-binding regions of the human apolipoprotein C-I (apoC-I) is an amphipathic helix which extends from residue 33 to residue 53 and includes a single methionine at sequence position 38. The involvement of the sequence around methionine-38 in phospholipid binding has been evaluated with paramagnetic and nuclear reporter groups attached to the thiomethyl moiety. This moiety has been spin-labeled with *N*-(2,2,6,6-tetramethylpiperidinyl-1-oxy)bromoacetamide or ^{13}C enriched with $^{13}CH_3I$. As determined from its EPR spectrum, the nitroxide at Met-38 of apoC-I had a rotational correlation time (τ_c) of 0.22 ns. When dimyristoylphosphatidylcholine (DMPC) was bound to the spin-labeled apoprotein, τ_c increased to 0.35 ns, indicating decreased motion for the methionyl side chain. The line width ($\nu_{1/2}$) and spin–lattice relaxation time (T_1) for the thiomethyl resonance of ^{13}C-enriched apoC-I in 10 mM phosphate buffer was 6.0 Hz and 320 ms, respectively. When the protein solution was made 1.6 M in Gdn·HCl, these values changed to 2.6 Hz and 970 ms, respectively. Upon addition of DMPC multilamellar liposomes to [^{13}C]apoC-I in 1.6 M Gdn·HCl, the line width increased to 4.7 Hz and the T_1 decreased to 380 ms. These results strongly suggest that methionine-38 of apoC-I resides in a region of the apoprotein which undergoes significant secondary and/or tertiary structural change upon disaggregation/unfolding in Gdn·HCl and upon interaction with phospholipid.

Apolipoprotein C-I (apoC-I)[1] is the smallest of the well-characterized human plasma apolipoproteins. It accounts for about 10 and 2% of the proteins in human plasma very low density lipoproteins and high density lipoproteins, respectively (Schaefer et al., 1978). The amino acid sequence of apoC-I has been determined by Jackson et al. (1974a) and Shulman et al. (1972, 1975) (Figure 1). The protein contains 57 residues which include a single methionine but exclude cystine, cysteine, tyrosine, and histidine. The total synthesis of apoC-I by the solid-phase method has been reported by Harding et al. (1976) and by Sigler et al. (1976). In aqueous solution at neutral pH, the apoprotein undergoes self-association which is attended by alteration in secondary structure. In acidic solution (e.g., pH 2.5), the apoprotein is monomeric and its secondary structure appears to be independent of protein concentration (Osborne et al., 1977a).

ApoC-I is involved in both the metabolism and structure of lipoproteins. It stimulates the activity of lecithin:cholesterol acyltransferase to the same extent with saturated and unsaturated phospholipid acyl donors (Soutar et al., 1975). Synthetic peptides containing residues 32–57, 24–57, and 17–57 stimulate enzymatic activity to the extent of 50, 60, and

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[1] Abbreviations used: Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; apoC-I, apolipoprotein C-I isolated from human very low density lipoproteins; DMPC, dimyristoyl-L-α-phosphatidylcholine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid; VLDL, very low density lipoproteins ultracentrifugally isolated at $d < 1.006$ g/mL; LDL, low density lipoproteins isolated at d 1.019–1.063 g/mL; HDL, high density lipoproteins isolated at d 1.063–1.210 g/mL.

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THR-PRO-ASP-VAL-SER-SER-ALA-LEU-ASP-LYS-LEU-LYS-GLU-PHE-GLY-ASN-THR-LEU-GLU-ASP-LYS-ALA-ARG-GLU-LEU-ILE-SER-ARG
10 20

ILE-LYS-GLN-SER-GLU-LEU-SER-ALA-LYS-MET-ARG-GLU-TRP-PHE-SER-GLU-THR-PHE-GLN-LYS-VAL-LYS-GLU-LYS-LEU-LYS-ILE-ASP-SER
30 40 50

FIGURE 1: Amino acid sequence of human plasma apoC-I as determined by Jackson et al. (1974a) and Shulman et al. (1975).

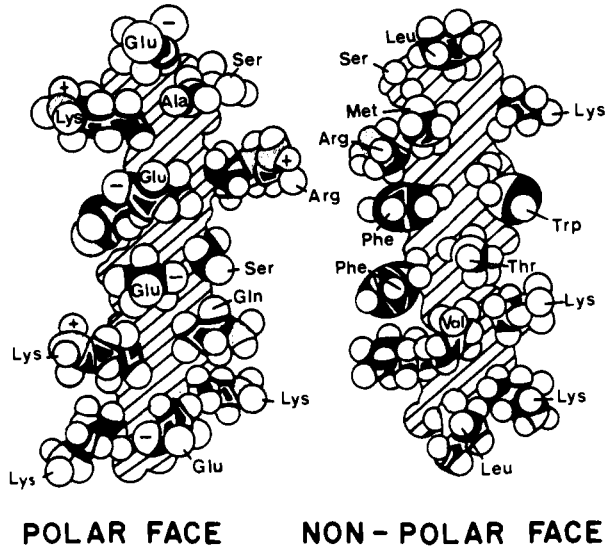

FIGURE 2: Space-filling model of a proposed amphipathic helical segment of apoC-I including amino acid residues 33–53.

100% of that exhibited by the naturally occurring apoprotein (Soutar et al., 1978). Binding of egg yolk phosphatidylcholine to the apoprotein has been demonstrated by several different biochemical and physical criteria (Jackson et al., 1974b). Three phospholipid-binding regions have been proposed for apoC-I (Segrest et al., 1974; Jackson et al., 1974b). The largest of these is a segment extending from Glu-33 to Leu-53 (Figure 2) and includes the single methionine at sequence position 38. Cyanogen bromide cleavage of apoC-I produces two fragments, 1–38 and 39–57. Fragment 39–57 does not interact with phospholipid, although the longer synthetic fragment, 32–57, does. Both of these experiments support the view but do not conclusively prove that Met-38 is at or near a segment of the apoprotein which is independently involved in phospholipid binding. This possibility has been pursued further through the study of apoC-I tagged specifically at Met-38 with a nitroxyl group or enriched with $^{13}CH_3$. A description of these studies is the subject of the present report.

Experimental Procedures

Materials

DMPC was obtained from Sigma Chemical Co. and purified to homogeneity by liquid chromatography (Patel et al., 1979). $^{13}CH_3I$ (90% enriched) was obtained from Koch Isotopes. Bromo[2-^{3}H]acetic acid was purchased from Amersham. 4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxy was purchased from Molecular Probes. The radioactive spin-label *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)bromo[2-^{3}H]acetamide was prepared according to the method of Price (1973). ApoC-I was isolated from very low density lipoproteins of patients with primary type IV or V hyperlipoproteinemia as described previously (Jackson et al., 1974a). The apoprotein was initially partially purified by DEAE-Sephadex chromatography, further purified by chromatography on SP-Sephadex C-25 in 20 mM sodium phosphate and 6 M urea, 5 °C, pH 3.7, and then deionized over Bio-Gel P-2 and eluted with NH_4HCO_3, pH 8.0. It exhibited one band on 7.5% polyacrylamide gels electrophoresed in 8 M urea at pH 4.3 and 8.2 and had an amino acid composition entirely devoid of cystine, tyrosine, and histidine.

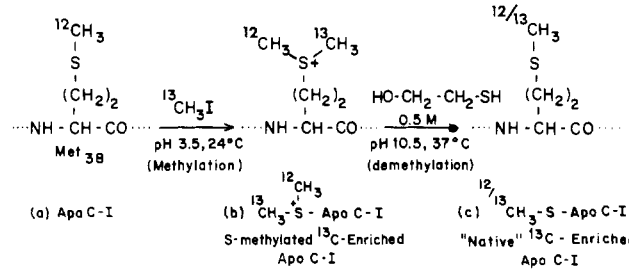

FIGURE 3: Reaction scheme for the $^{13}CH_3$ isotopic labeling of methionine-38 of apoC-I.

Methods

Preparation of SL-Met$_{38}$-apoC-I. The lyophilized apoC-I was dissolved in 6 mL of 0.1 M KCl and 6 M urea and the resulting solution adjusted to pH 3.5 with 1 N HCl. To this solution was added a 150 molar excess of Tempo-bromoacetamide. The reaction mixture was stirred at room temperature in the dark for 24 h and then extensively dialyzed against 0.05% acetic acid. Residual traces of unreacted spin-label were removed by gel-filtration chromatography over Bio-Gel P-2. The extent of labeling was determined from the specific activity of the apoprotein (uncertainty = ±1%) and from the loss of methionine from a 4 N methanesulfonic acid hydrolysate as determined by amino acid analysis (uncertainty = ±5%). On the basis of these criteria, apoC-I contained 0.80 ± 0.05 mol of spin-label/mol of apoprotein.

Preparation of [$^{12/13}$C-Met$_{38}$]apoC-I. Purified apoC-I was methylated with $^{13}CH_3I$ by using a slightly modified version of the Jones procedure (Jones et al., 1976; Figure 3). Lyophilized apoC-I (15 mg) was dissolved in 6 mL of 0.1 M KCl and 6 M urea and adjusted to pH 3.5 with 1 N HCl. To this solution was added a 150 M excess of $^{13}CH_3I$ (90% enriched). The solution was stirred at room temperature in the dark for 24 h. The reaction mixture was then dialyzed against 0.05% acetic acid and 0.02% NaN_3 and then against distilled water before finally lyophilizing. The resulting ^{13}C-labeled sulfonium salt was demethylated by using 0.5 M 2-mercaptoethanol at pH 9.5–10.0, 37 °C, for 18 h. The extent of methylation of apoC-I and demethylation of S-methylated apoC-I was determined by amino acid analysis of 4 M methanesulfonic acid hydrolysates (Liu & Chang, 1971).

Amino Acid Analysis. For the determination of *S*-methylmethionine, aliquots of the methylated reaction products were hydrolyzed in 4 N methanesulfonic acid at 110 °C for 24 h and analyzed on a Beckman 121 amino acid analyzer fitted with a 0.9 × 16 cm column of PA-35 resin which was eluted at 65 °C with citrate buffer (pH 5.28) containing 0.3 N Na^+. *S*-Methylmethionine eluted 22.1 min after lysine, 14.3 min after histidine, and 9.4 min before ammonia (1.5-h total analysis time). For the simultaneous determination of me-

thionine and *S*-methylmethionine, Beckman resin AA-15 (0.9 × 56 cm) was used. With this system, the elution time of *S*-methylmethionine was 5.7 min after lysine and 12.8 min before ammonia. The specificity of the alkylation reaction used in the present study was further evaluated by reacting the Beckman amino acid standard mixture with Tempo-bromoacetamide or $^{13}CH_3I$ under conditions identical with those used for protein modification. The quantities of amino acids measured in these control reaction mixtures were not significantly different from those in the starting mixtures, with the exception of methionine.

Magnetic Resonance Measurements. EPR spectra were recorded at 24 °C on a Varian E-12 spectrometer operated at 9.5 GHz. Samples were contained in 100-μL Corning micropipets sealed at one end. Nitroxide rotational correlation times (τ_c) were calculated as described by Stone et al. (1965). ^{13}C NMR spectra were recorded on a Varian XL-100-15 spectrometer operated at 25.2 MHz in the pulsed Fourier transform mode. Acquisition conditions are given in the figure legends. Spin–lattice relaxation times (T_1) were determined by the inversion-recovery method utilizing a ($180°$–t–$90°$–nT_1) sequence (Freeman & Hill, 1971). T_1 values were usually obtained from 7 to 11 points per experiment, with values of t ranging from 0.05 to 1.60 s. The ambient probe temperature was 37 °C. Line widths and relaxation times were calculated by the algorithm NTCFT 1004 supplied by Nicolet Technology, Inc.

Circular Dichroism Measurements. Duplicate spectra (250 → 210 nm) were obtained for each sample on a Jasco J-500C spectropolarimeter at 25 °C. The spectra were processed by using a Jasco CD data processor. The mean residue ellipticities were calculated from

$$[\theta]_\lambda = \frac{\theta_{obsd} \times 116}{10lc}$$

where θ_{obsd} is the observed ellipticity, 116 is the mean residue weight for apoC-I, l is the path length in centimeters, and c is the protein concentration in grams per milliliter. The percent helicity of the protein was estimated from the empirical relationship derived from the CD spectra of completely random and completely helical poly(L-lysine) (Greenfield & Fasman, 1969; Morrisett et al., 1973).

$$\%\ \alpha\ \text{helix} = \frac{-[\theta]_{222} + 3000}{36000 + 3000} \times 100$$

Analytical Ultracentrifugal Measurements. Sedimentation equilibrium experiments were performed on a Spinco Model E analytical ultracentrifuge. Double-sector cells with charcoal-filled epon center pieces and quartz windows were used in an AN-D rotor at 31 410 and 39 460 rpm for 18, 30, and 60 h. The temperature was maintained at 25.9 °C. The layering fluid was omitted, and each cell contained 150 μL of buffer and 100 μL of sample. Data collection, reduction, and analysis were performed by the direct search curve fitting methods described by Aune et al. (1977).

Quasi-elastic Light-Scattering Measurements. The size of the DMPC–apoC-I complex was determined from a population of LDL eluted from a Sepharose CL-4B at the same volume. These LDL were measured by quasi-elastic light scattering, using methods described previously (Morrisett et al., 1974; Aune et al., 1977).

Calculations of Spin–Lattice Relaxation Times and Natural Line Widths. Spin–lattice relaxation times and natural line widths [$\nu_{1/2} = 1/(\pi T_2)$] were calculated for varying effective rotational correlation times according to equations given for ^{13}C–^1H dipolar relaxation (Lyerla & Grant, 1972) by using the treatment of Doddrell et al. (1972) for isotropic rotation.

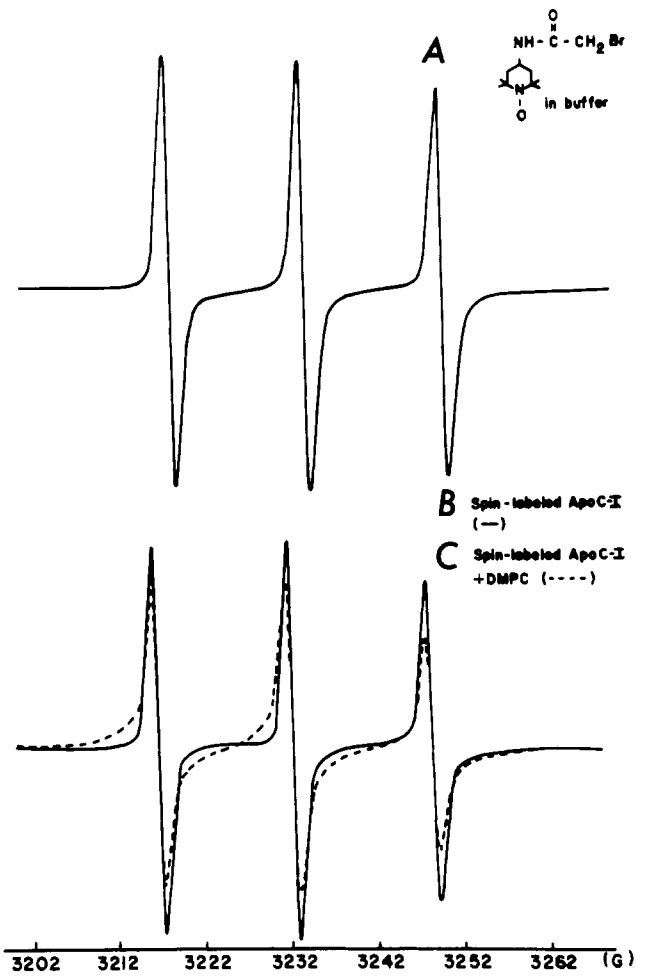

FIGURE 4: EPR spectra of (A) the alkylating spin-label Tempo-bromoacetamide (0.1 mM), (B) (—) apoC-I spin-labeled with Tempo-bromoacetamide at methionine-38, 0.1 M spin-labeled apoprotein alone, and (C) (---) spin-labeled apoprotein to which multilamellar DMPC liposomes have been added (4 g of lipid/g of protein). The buffer used for all three spectra was 10 mM phosphate, 100 mM NaCl, and 1 mM NaN_3, pH 7.4. Temperature = 25 °C.

Protein and Phospholipid Quantitation. Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951) with bovine serum albumin as a standard. Its concentration was determined spectrophotometrically ($E^{278}_{1\%} = 6.67$). Phospholipid concentration was determined by phosphorus analysis (Bartlett, 1959) with anhydrous Na_2HPO_4 as a gravimetric standard and 21.9 as a conversion factor for DMPC.

Results

The covalent attachment of the Tempo-acetamido group to the Met-38 thioether moiety of apoC-I caused only a modest change in its EPR spectrum. The persistence of the three narrow lines indicated that the motion of the attached label was fast and almost isotropic. From this type of narrow line spectrum it is possible to estimate a rotational correlation time[2] (τ_c) (Stone, 1965) from the peak height ratios. For the apoprotein alone, a value of 2.2×10^{-10} s was obtained. When multilamellar DMPC liposomes were added to the spin-labeled apoprotein, the shape and amplitude of the three hyperfine

[2] This calculation assumes the motion is isotropic, a condition which is not entirely fulfilled despite the high degree of mobility (Stone et al., 1965).

Table I: Line Widths ($\nu_{1/2}$) and Spin-Lattice Relaxation Times (T_1) for [$^{13}CH_3$-S-Met$_{38}$]apoC-I

	$\nu_{1/2}$ (Hz)	T_1 (s)	τ_c determined from $\nu_{1/2}/3$	τ_c determined from $3T_1$	τ_c of particle
[^{13}C] apoC-I (~0.5 mM) in 10 mM phosphate buffer with 1.6 M Gdn·HCl, pH 7.4	2.6 ± 0.8	0.971 ± 0.097	1.2×10^{-10}	1.6×10^{-11}	8.4×10^{-9} [a]
[^{13}C] apoC-I (~0.5 mM) in 10 mM phosphate buffer with 1.6 M Gdn·HCl, pH 7.4					
+10 mM DMPC	4.7 ± 0.5	0.381 ± 0.068	2.5×10^{-10}	4.0×10^{-11}	
+20 mM DMPC	15.0 ± 5.0	[c]	8.9×10^{-10}		4.6×10^{-6} [b]
[^{13}C] apoC-I (~0.5 mM) in 10 mM phosphate buffer with 2-chloroethanol (50%), pH 7.4	28.4 ± 5.4	0.819 ± 0.164	3.2×10^{-9}	1.9×10^{-11}	8.4×10^{-9} [a]
[^{13}C] apoC-I (~0.5 mM) in 10 mM phosphate buffer containing 0.1 M NaCl, 1 mM NaN_3, pH 7.4	6.0 ± 0.5	0.320 ± 0.048	3.1×10^{-10}	5.0×10^{-11}	8.4×10^{-9} [a]

[a] For isotropic tumbling of globular, monomeric apoC-I. [b] For isotropic tumbling of the DMPC–apoC-I complex indirectly determined by quasi-elastic light scattering to have a diameter of 309 Å. [c] Insufficient signal/noise to obtain reliable T_1 values.

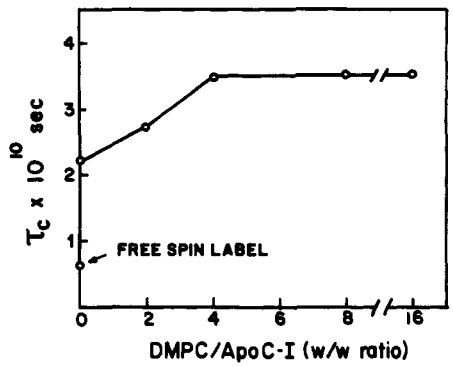

FIGURE 5: Effect of DMPC multilamellar liposomes on the rotational correlation time of the nitroxyl group on spin-labeled apoC-I.

lines changed significantly (Figure 4C). When the phospholipid was added in a titration fashion, a stepwise diminution of the high-field/center-field line height ratio was observed, and the corresponding correlation time was increased until a DMPC/apoC-I ratio of 4 g/g was reached, at which point a limiting estimated correlation time of 3.5×10^{-10} s was attained (Figure 5).

ApoC-I treated with an excess of $^{13}CH_3I$ under appropriate conditions (Figure 3) resulted in the formation of an S-methylated sulfonium salt derivative. The ^{13}C NMR spectrum of this derivative contained a dominant resonance at 25.2 ppm from Me_4Si, with a line width of 7.0 Hz. Demethylation of *S*-methylmethionine derivatives by using dithiothreitol has been reported by Jones et al. (1976). In the present study, mercaptoethanol was found to be a better demethylating agent. At equal concentrations (0.5 M) of dithiothreitol and mercaptoethanol, demethylation of *S*-methylmethionine sulfonium iodide proceeded to the extent of 87 and 98%, respectively, at 37 °C in 18 h. When the *S*-methyl derivative of apoC-I was treated with mercaptoethanol, it reverted rapidly to "native" apoC-I which was enriched about 45% in ^{13}C at the thiomethyl group. The ^{13}C NMR spectrum of this material contained a dominant resonance at 15.5 ppm downfield from Me_4Si, with a line width of 6.0 Hz.

Although isotopic enrichment permitted running the ^{13}C NMR experiments at about 0.5 mM apoC-I (about 3.3 mg/mL), this concentration is high enough to cause self-association (Osborne & Brewer, 1977b) which could have significant effects on the line width and T_1. In order to minimize the complicating effects of self-association, the lipid-binding studies of ^{13}C-enriched apoC-I were performed in 1.6 M Gdn·HCl. Osborne et al. (1977a) have reported that in guanidine solutions of this concentration or higher, apoC-I does not self-associate as determined by circular dichroism. We have corroborated this observation by using sedimentation equilibrium analysis in the analytical ultracentrifuge. A plot of logarithm of the fringe displacement vs. the square of radical distance ($\ln f$ vs. r^2) for the apoprotein at an initial concentration of 1.0 mg/mL in 1.6 M Gdn·HCl was linear (data not shown). The curve-fitting analysis indicated that apoC-I in 1.6 M Gdn·HCl is greater than 88% monomeric. The average molecular weight obtained from the sedimentation equilibrium experiment was 6810 while the molecular weight determined from the amino acid composition was 6634. Since the estimated concentration of apoprotein in the bottom of the analytical ultracentrifuge cell exceeded 4.5 mg/mL, the apoC-I is expected to be largely (>88%) monomeric at the concentrations (3.3 mg/mL) used in the NMR experiments.

After demonstrating the low level or absence of apoC-I self-association in 1.6 M Gdn·HCl, it became essential to determine what, if any, effect this concentration of guanidine had on the spectrum of DMPC liposomes. No significant difference in the natural abundance ^{13}C NMR spectrum of DMPC liposomes in phosphate buffer from that of liposomes in phosphate buffer containing 1.6 M Gdn·HCl was observed (Figure 6A,B). Multilamellar liposomes were used in preference to single bilayer vesicles because the former gave spectra of significantly lower intensity, thereby facilitating observation of the thiomethyl carbon resonance at 15.5 ppm (Figure 6C). The line width and spin–lattice relaxation time of apoC-I enriched with ^{13}C at Met-38 in Gdn·HCl, in 2-chloroethanol, and in phosphate buffer are presented in Table I. These data and the corresponding effective correlation times will be considered in detail under Discussion.

To facilitate interpretation of the NMR data obtained for apoC-I in the presence of the various solutes and ligands described in Table I, the effects of these substances on the secondary structure of the apoprotein were independently assessed by circular dichroism. Increasing concentrations of Gdn·HCl up to 1.6 M produced marked decreases in the helicity of the protein (Table II). This loss of helicity was more than reversed by the addition of DMPC which caused an increase in θ_{222} until a protein/lipid weight ratio of about 1:4 was reached. This is the same ratio at which no further change in the correlation time of spin-labeled apoC-I was observed (Figure 5). The effects of 2-chloroethanol on the secondary structure of apoC-I were also evaluated. In neat 2-chloroethanol, a very high ellipticity at 222 nm of about 32 000 deg cm^2 dmol^{-1} was observed. The dilution of this solution to 50% produced virtually no change in the CD spectrum. However, further dilution to 25% caused a significant drop to about 22 000 deg cm^2 dmol^{-1}. ApoC-I in a solution containing no 2-chloroethanol but at the same concentration as that of the 25% 2-chloroethanol (0.094 mg/mL) measurement had an ellipticity of about 14 000 deg cm^2 dmol^{-1}.

Table II: Summary of Circular Dichroic Studies of ApoC-I in Guanidine Hydrochloride, in Guanidine Hydrochloride Plus DMPC, and in 2-Chloroethanol

Gdn·HCl (M)	apoC-I/DMPC (g/g)	$-$ deg cm^2 dmol^{-1}	% helicity	2-chloro-ethanol (%)	$-$ deg cm^2 dmol^{-1}	% helicity
0	1/0	21900	64[a]	100	31700	89
0.07		17600	53	75	31700	89
0.22		15700	48	50	30100	85
0.45		11000	36	25	21900	64
0.68		10200	34	0	14100	44[a]
0.98		7500	27			
1.29		6300	24			
1.60		4800	20			
1.60	1/0.3	6300	24			
1.59	1/0.7	7900	28			
1.57	1/1.1	9800	33			
1.56	1/1.4	12600	40			
1.53	1/2.0	15300	47			
1.50	1/2.5	19200	57			
1.47	1/3.1	23500	68			
1.44	1/3.8	27000	77			

[a] The difference in helicities of these two samples is attributable to differences in protein concentration (Osborne et al., 1977a). In the guanidine experiment, [apoC-I] = 1.0 mg/mL; in the chloroethanol experiment, [apoC-I] = 0.094 mg/mL.

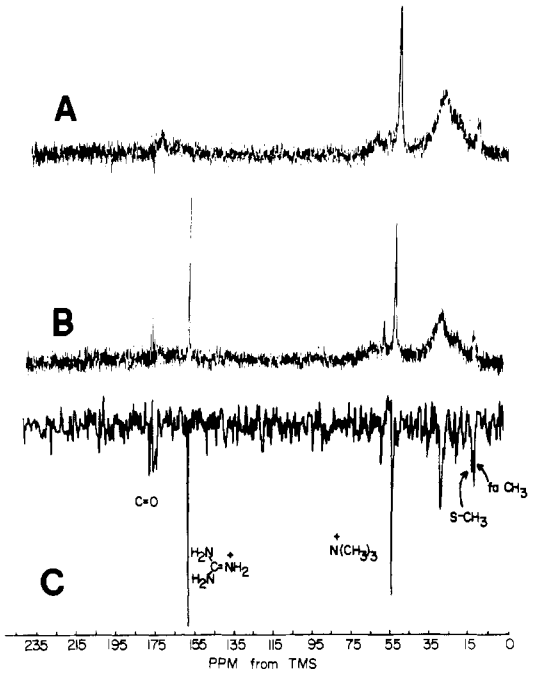

FIGURE 6: ^{13}C NMR spectra (25.2 MHz): (A) fully relaxed (t = 1.60 s) spectrum of DMPC mutlilamellar liposomes (20 mg/mL) in 10 mM phosphate buffer at pH 7.4; (B) fully relaxed spectrum of DMPC multilamellar liposomes in 10 mM phosphate buffer containing 1.6 M Gdn·HCl; (C) partially relaxed (t = 0.05 s) spectrum of DMPC multilamellar liposomes in 10 mM phosphate and 1.6 M Gdn·HCl, pH 7.4, to which [^{13}C]apoC-I has been added. Conditions: 16384 accumulations, 1.36-s acquisition time, 6024-Hz spectral width, 25 °C.

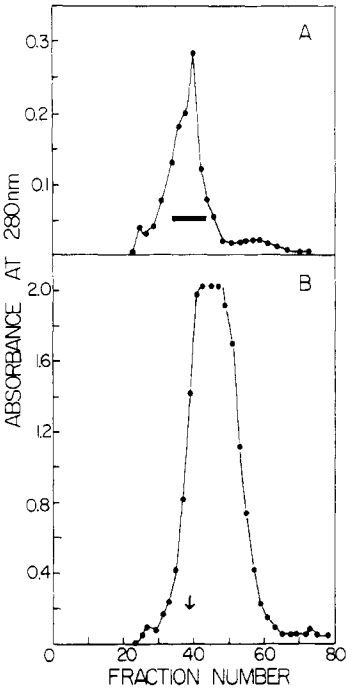

FIGURE 7: Isolation of apoC-I–DMPC complex by Sepharose CL-4B chromatography. (A) Elution profile of a mixture of apoC-I (1.4 mg) and DMPC multilamellar liposomes (6 mg). The lipid–protein mixture was incubated at 25 °C for 24 h before chromatography. (B) Elution profile of LDL (55 mg). Fraction 39 contained LDL with an average hydrated diameter of 309 ± 11 Å as determined by quasi-elastic light scattering. Conditions: 10 mM phosphate, 1.6 M Gdn·HCl, 100 mM NaCl, 1 mM NaN_3, pH 7.4; 1 mL/fraction; 10 mL/h flow rate; 1.6 × 40 cm column; 1-mL sample load.

These data are compared in terms of percent helicity in Table II.

The complex resulting from the interaction of apoC-I with DMPC under conditions similar to that used for the NMR experiment (i.e., 1.6 M Gdn·HCl) was isolated by gel-filtration chromatography on Sepharose CL-4B (Figure 7). ApoC-I produced a significant reduction in the size of the DMPC aggregates from multilamellar liposomes which elute in the void volume (fraction 25) to particles which elute somewhat more slowly (fraction 39) and are substantially smaller. The size of this complex was determined indirectly from a population of LDL particles eluting at the same volume under identical conditions (Figure 7B). These LDL had diameters of 309 ± 4 Å as determined by quasi-elastic light scattering. The stoichiometry of the complex eluting at the volume indicated by the solid bar (Figure 7A) was 3.2 g of DMPC/g of apoprotein.

Discussion

The value of any extrinsic reporter group used to probe a biological system depends on the sensitivity of that group to various environmental perturbations in the system. In the present study, both the Tempo-acetamido and the $^{13}CH_3$ groups were sensitive to such perturbations. The nitroxyl

radical covalently attached to Met-38 of the apoprotein had an estimated rotational correlation time calculated from its EPR spectrum of 0.22 ns. This value is substantially shorter than the 8.43-ns correlation time estimated from the Stokes–Einstein relationship for monomeric apoC-I by assuming a molecular weight of 6634, a hydrated partial specific volume of 0.87, a spherical shape, and a solution viscosity of 0.012 poise at 25 °C. This comparison suggests that the Tempo-acetamido group attached to Met-38 is free to undergo considerable internal motion, since the correlation time estimated from the molecular weight assuming a spherical shape represents a lower limit for the correlation time of the entire apoprotein. Addition of lipid caused the correlation time for the Tempo-acetamido group to increase, suggesting that lipid binding results in restriction of the spin-label motion. These constraints may be caused either by direct motional restrictions resulting from the presence of lipid near the spin-label or by indirect restrictions resulting from conformational changes in the protein induced by lipid binding. The sensitivity of the EPR spectrum to the addition of lipid ceased when a weight ratio of 4.0 g of lipid/g of protein was reached (Figure 5). This value for the stoichiometry of the lipid–apoprotein complex is in reasonable agreement with that obtained independently from circular dichroic measurements (Table II). These spectroscopically obtained stoichiometries are slightly above the value of 3.2 g of lipid/g of protein obtained by chemical analysis of the complex isolated by gel-filtration chromatography (Figure 7A).

The sensitivity of EPR permitted execution of the spin-label experiment at a protein concentration on the order of 0.1 mM, a concentration below that at which the complicating effects of protein self-association would be expected (Osborne et al., 1977a). However, for the ^{13}C experiments, even with 45% enrichment of the methionyl group with ^{13}C, it was necessary to use about 0.5 mM apoprotein, a concentration at which significant self-association in phosphate buffer was quite possible. Consequently, we elected to perform the ^{13}C NMR lipid-binding experiments in 1.6 M Gdn·HCl. Although the extent of self-association and the amount of secondary structure of apoC-I is markedly reduced by Gdn·HCl, the apoprotein and phospholipid interact strongly in its presence. Addition of DMPC to the apoprotein in Gdn·HCl results in changes in the circular dichroic spectrum (Table II) characteristic of phospholipid binding to apoproteins in nondenaturing buffer solutions (Jackson et al., 1974b). Moreover, a stable lipid–apoprotein complex could be isolated by using gel-filtration chromatography (Figure 7).

The addition of phospholipid to apoC-I in Gdn·HCl also was associated with significant changes in the ^{13}C spectrum of the ^{13}C-labeled methionine. Addition of a 20-fold molar excess of DMPC increased the line width of the thiomethyl resonance from 2.6 to 4.7 Hz and decreased its spin–lattice relaxation time from 0.971 to 0.381 s. Although the motion of the thiomethyl group is expected to be complex, including contributions from various types of internal rotations in addition to overall rotation of the entire apoprotein, we have chosen, for comparative purposes, to estimate an effective rotational correlation time from the experimentally observed line widths and spin–lattice relaxation times, assuming isotropic rotational reorientation of the thiomethyl group. It should be stressed that the derived effective correlation times may not be related in a precise way to the diffusion rates of internal motions and overall rotation or to the extents of the internal angular motions. However, for comparative purposes, longer effective correlation times may be related to decreased rates of motion and/or decreased angular fluctuations in motions of the thiomethyl group. It should be noted that the effective correlation time estimated from the line width should be regarded as an upper limit, since the observed line width may have significant contributions from sources other than spin–spin relaxation. However, for the ^{13}C NMR spectra of macromolecules, the natural line width often makes the dominant contribution to the experimental line width; consequently, effective correlation times estimated from line widths are frequently useful in a comparative way. Effective correlation times for the thiomethyl group estimated from the line width and spin–lattice relaxation times are given in Table I. The effective correlation times estimated from both line widths and T_1's increase when phospholipid is added to the system, suggesting that the motion of this group is restricted in the complex relative to the free apoprotein in Gdn·HCl. The molecular details of this restriction are difficult to define. Possible contributions include (1) a longer rotational correlation time for the peptide backbone due to its association with a much larger particle, (2) decrease in the local motion of the methionine side chain induced by the presence of surrounding phospholipid, and (3) decrease in the local motion of the methionine side chain induced by increased secondary structure of the apoprotein. These changes may be envisioned with a space-filling model of the amphipathic helical segment which contains Met-38 (Figure 2). Further addition of DMPC (to a 40:1 molar ratio) resulted in significant loss of signal to noise ratio due to sample dilution and increased resonance line widths. However, the line width of the ^{13}C Met-38 resonance was estimated graphically to be approximately 15 Hz, suggesting that addition of phospholipid up to a 4:1 weight ratio resulted in further restriction in the motion of the thiomethyl group.

To investigate the effect of apoprotein secondary structure on the NMR spectrum of ^{13}C-labeled apoC-I, we recorded the ^{13}C spectrum of the apoprotein in the helix-inducing solvent, 2-chloroethanol.[3] In 50% 2-chloroethanol, the line width of the ^{13}C Met-38 resonance increased markedly to 28 Hz. In contrast, the spin–lattice relaxation time remained within experimental error of that observed for the apoprotein in Gdn·HCl. These observations suggest that a simple isotropic rotational model is not adequate to describe the motion of the thiomethyl group in 2-chloroethanol. The spin–lattice relaxation and spin–spin relaxation rates are sensitive to the spectral density functions at different frequencies. In particular, the spin–spin relaxation rate and its contribution to the line width are very sensitive to the spectral density at zero frequency. Consequently, the observed line width is frequently more sensitive to the degree of anisotropy of the motion than is the spin–lattice relaxation time. A possible but rather tentative explanation for the observed behavior in 2-chloroethanol is that the threefold symmetry axis of the thiomethyl group becomes quite restricted in its angular fluctuations but that rotation about the symmetry axis remains quite rapid. Additional relaxation measurements at different magnetic field strengths are required to adequately test this possibility. The available data do suggest that the formation of α helix does not alter the spin–lattice relaxation time significantly and that the decrease in the T_1 observed in the presence of phospholipid is a result of the phospholipid and not solely due to α-helx formation.

The relaxation rate of [^{13}C-Met]apoC-I under self-associating conditions (10 mM phosphate, 0.1 M NaCl) is similar to those measured for apoC-I in the presence of phospholipid,

[3] In 2-chloroethanol, apoC-I at 0.5 mg/mL is ~70% monomeric and 30% dimeric as determined by analytical ultracentrifugation.

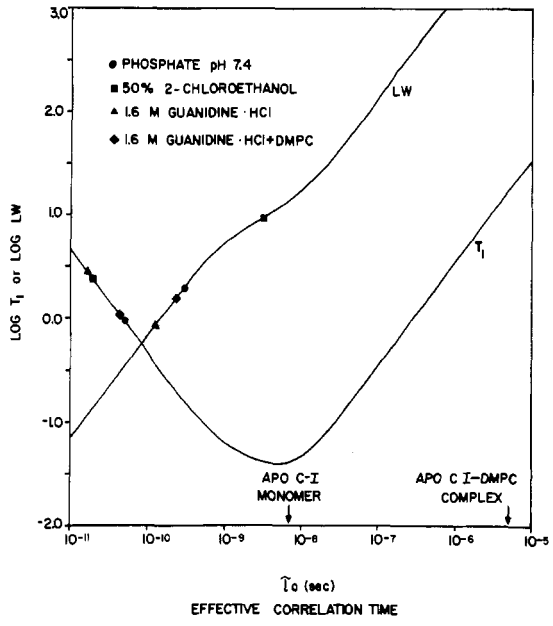

FIGURE 8: Log–log plot of T_1 (s) and natural line width (Hz) vs. effective correlation time assuming isotropic rotational reorientation. Arrows on abscissa indicate rotational correlation times for the entire apoC-I monomer and the apoC-I–DMPC complex estimated from the Stokes–Einstein relationship. The solid lines relating T_1 and line width to correlation time were calculated from equations for ^{13}C–^{1}H dipolar relaxation (Lyerla & Grant, 1972) by using the treatment of Doddrell et al. (1972) for isotropic rotation.

suggesting that the sites and mechanism for self-association may be quite similar to those for interaction with phospholipid, at least with respect to Met-38.

Comparison of the effective correlation times for the thiomethyl group estimated from the NMR relaxation rates with the rotational correlation times calculated for the entire apoprotein or complex reveals that under all the conditions examined, the thiomethyl group undergoes considerable internal motion, since its effective correlation time is considerably less than that estimated for isotropic rotation of the entire apoprotein[4] or complex (Figure 8). Jones et al. (1976) have reached similar conclusions regarding Met-55 and Met-131 of sperm whale myoglobin.

Results from these studies with Tempo-acetamide labeled and ^{13}C-enriched Met-38 as probes of the dynamic changes in apoC-I induced by phospholipid binding have shown that formation of a phospholipid–apoprotein complex is associated with changes in the EPR and NMR spectra of these probes. These data suggest that the region surrounding Met-38 is involved in phospholipid binding.

In a previous study (Jackson et al., 1974b), it was shown that cyanogen bromide cleavage of apoC-I at Met-38 afforded two fragments in which the ability to bind phospholipid and activate lecithin:cholesterol acyltransferase was either reduced or absent. In a separate study (Soutar et al., 1975), it was shown that a synthetic peptide fragment of apoC-I containing residues 32–57 in which Met-38 is intact binds phospholipid and activates the enzyme. The present study has provided two additional pieces of evidence that implicate the region containing Met-38 in phospholipid binding. This implication provides further support for the view that this region is an important integral part of the amphipathic helix spanning residues 33–53 which has been proposed to be one of the three lipid-associating regions of apoC-I.

[4] Self-associated apoC-I would be expected to have an even larger rotational correlation time, and, consequently, the correlation time estimated for the monomer represents a lower limit on the rate of rotational reorientation of the apoprotein species of the solution.

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