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The Self-Association of the Reduced ApoA-II Apoprotein from the Human High Density Lipoprotein Complex†

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ABSTRACT: The molecular properties of the single linear chain form of human apoA-II, i.e., Cm apoA-II, have been evaluated by circular dichroism, polarization of fluorescence, difference absorption, and sedimentation equilibrium. The self-association of Cm apoA-II to a dimer resembles closely that of apoA-II though the free energy change is somewhat smaller. The dimerization of Cm apoA-II is accompanied by major changes in secondary and tertiary structure. The apoA-II molecule, therefore, represents a molecular association where the intramolecular structure is strongly dependent on the quaternary structure.

The two major protein components of the human high density lipoprotein complex (HDL)[1] are apoA-I and apoA-II, comprising 70 and 20%, respectively, of the proteins present. We have undertaken an investigation of the molecular properties of these proteins as a basis for understanding their behavior in HDL. We have reported that apoA-II self-associates to form a dimer (mol wt 34,760) at pH 7.4 with an association constant of $5 \times 10^4\ M^{-1}$ (Gwynne et al., 1975). The reaction was novel in that important changes in secondary and tertiary structure accompany the dimerization. Since apoA-II (mol wt 17,380) is a disulfide-linked dimer of a 77 residue chain (Brewer et al., 1972), it is possible to obtain the single chain by reduction of the disulfide bond. The liberated sulfhydryl group is subsequently protected by alkylation. Due to the structural changes involved in the self-association of apoA-II, it was of interest to compare the behavior of the single chain polypeptide with that of the double chain native molecule. The reduced molecule can also serve as a model for the apoA-II proteins of other species (rat and rhesus monkey) where apoA-II exists as a single chain which lacks cysteine residues (Herbert et al., 1974; Edelstein et al., 1973).

Methods

The preparation and purification procedures used for Cm apoA-II have been previously reported (Lux et al., 1972). It was shown by amino acid analysis that only the cysteine group is modified by reduction and alkylation with iodoacetic acid. Protein concentrations were determined by absorption at 280 nm employing a molar extinction coefficient of 5500 (calculated from amino acid analysis). A Radiometer pH meter was used for pH measurements. Glass redistilled water was used throughout and all chemicals employed were reagent grade with the exception of guanidine hydrochloride which was HEICO "Synthesized Extreme Purity".

Sedimentation equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a temperature control system and a photoelectric ultraviolet scanner. The double sector cells, containing epon filled aluminum centerpieces and quartz windows, were centrifuged in an ANG-Ti rotor. The layering fluid was omitted. Molecular weights were obtained by using the following equation:

$$M_W^{app} = \frac{2RT}{\omega^2(1 - \bar{\nu}\rho)} \frac{d \ln c}{dr^2} \quad (1)$$

where M_W^{app} is the apparent weight average molecular weight; R, the gas constant; T, the absolute temperature; ω, the angular velocity; $\bar{\nu}$, the partial specific volume; ρ, the solvent density; c, the protein concentration; and r, the distance from the center of rotation. For a monomer-dimer equilibrium eq 1 reduces to

$$C_r = C_{1,m}e^{AM_1(r^2-m^2)} + C_{2,m}e^{2AM_1(r^2-m^2)} \quad (2)$$

where C_r is the total concentration at distance r from the center of rotation; $C_{1,m}$ and $C_{2,m}$ are the concentrations of monomer and dimer at the meniscus; $(r^2 - m^2)$ corresponds to the radial distance from the meniscus (m); M_1 is the monomer molecular weight and $A = \omega^2(1 - \bar{\nu}\rho)/2RT$.

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[1] Abbreviations used are: HDL, high density lipoprotein; Cm apoA-II, reduced and carboxymethylated apoA-II; Gdn-Cl, guanidine hydrochloride.

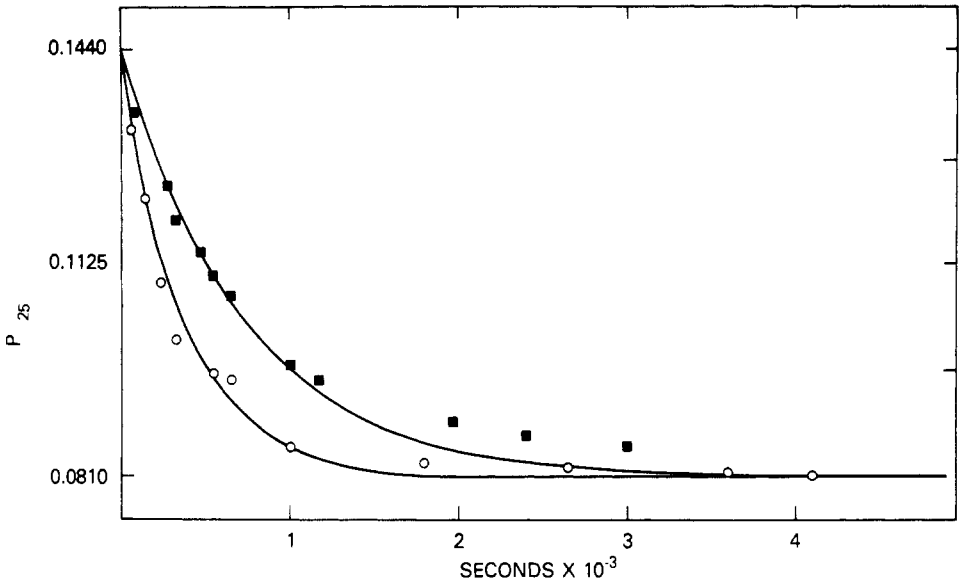

FIGURE 1: The rate of reduction under dry nitrogen of apoA-II (10.3 μM) in 0.01 M Tris, pH 8.2, 25° by dithiothreitol. (■) 0.1 M; (○) 0.2 M. The lines are theoretical first-order plots with rate constants of 1.4 and 2.8 × 10^{-3} $sec.^{-1}$. The points are experimental values. The ordinate is the polarization of tyrosyl fluorescence when excited with polarized light at 280 and observed at 305 nm.

Dimerization constants were obtained by a least-squares fit of the data to eq 2.

Fluorescent measurements were obtained by employing a Perkin-Elmer Model MPF III spectrofluorometer equipped with Hitachi 018-0054 polarizers and a thermostated cell holder. The excitation and emission wavelengths were 280 and 305 nm, respectively, since apoA-II contains tyrosyl but not tryptophanyl residues. Vertical polarized incident radiation was used for polarization measurements and the polarization of each solution was calculated by

$$P = (I_{VV} - GI_{VH})/I_{VV} + GI_{VH}) \quad (3)$$

where I is the fluorescent intensity and the first and second subscripts refer to the plane of polarization of the excitation and emission beams respectively (V, vertical; H, horizontal) and $G = I_{HV}/I_{HH}$. Excited lifetimes were measured with the TRW system (Chen et al., 1969).

The Perrin equation interrelates the polarization of fluorescence, the lifetime of the excited state, and the relaxation time of the molecule:

$$\left(\frac{1}{P} - \frac{1}{3}\right) \Big/ \left(\frac{1}{P_0} - \frac{1}{3}\right) = 1 + 3\tau/\rho \quad (4)$$

where P_0 is the limiting polarization; τ is the lifetime of the excited state, and ρ is the relaxation time.

Circular dichroic spectra were obtained with a Cary Model 60 spectropolarimeter (equipped with a temperature-controlled Pockels cell) and potassium dichromate was used to calibrate the instrument. Mean residue ellipticities were calculated by using the following equation:

$$[\theta]_\lambda = 113(\theta)_{obsd}/10lC \quad (5)$$

where $[\theta]_\lambda$ is the mean residue ellipticity at wavelength λ; $(\theta)_{obsd}$, the observed ellipticity; 113, the mean residue molecular weight of Cm apoA-II; l, the path length in centimeters; and C, the concentration in g/ml. Appropriate blanks were subtracted from the observed ellipticity and each value reported represents the average of duplicate determinations.

For a monomer–dimer association the mean residue ellipticity is related to the weight fraction of each species in solution by the following equation at a given wavelength, λ:

$$[\theta]_\lambda = f_M[\theta_M]_\lambda + f_D[\theta_D]_\lambda \quad (6)$$

where f_M is the weight fraction; $[\theta_M]$ is the mean residue ellipticity of monomer; f_D is the weight fraction; and $[\theta_D]$ is the mean residue ellipticity of dimer. Equation 6 reduces to:

$$[\theta]_\lambda = \frac{[P]}{C}[\theta_M]_\lambda + \frac{C - [P]}{C}[\theta_D]_\lambda \quad (7)$$

where [P] is the concentration of monomer and C is the total protein concentration. From the conservation of mass, the concentration of monomer is related to the equilibrium constant, K, and the total protein concentration by eq 8. The mean residue ellipticities of monomer, i.e. $[\theta_M]_\lambda$ and dimer $[\theta_D]_\lambda$, were estimated by employing appropriate neutral salts which shifted the equilibrium to one form or the other (see text). The association constants were obtained by a least-squares fit of the ellipticity data between 5 and 440 μM Cm apoA-II to eq 7 and 8.

$$2P \rightleftharpoons P_2$$
$$K = [P_2]/[P]^2$$
$$[P] = \frac{-1 \pm (1 + 8KC)^{1/2}}{4K} \quad (8)$$

Results

The disulfide bonds of many proteins are reduced rather slowly over many hours if denaturants are not added to unfold the protein (Cecil, 1963). The disulfide bond in apoA-II is apparently very accessible since reduction under nitrogen occurred in minutes when dithiothreitol was added to a solution of apoA-II at pH 8.2. The reduction could be conveniently followed by polarization measurements since a rather large fall accompanies the reaction. The polarization data approximately fit a first-order process (Figure 1) and the rate constants, 1.4 and 2.8 × 10^{-3} sec^{-1}, are proportional to the dithiothreitol concentration at the two levels studied.

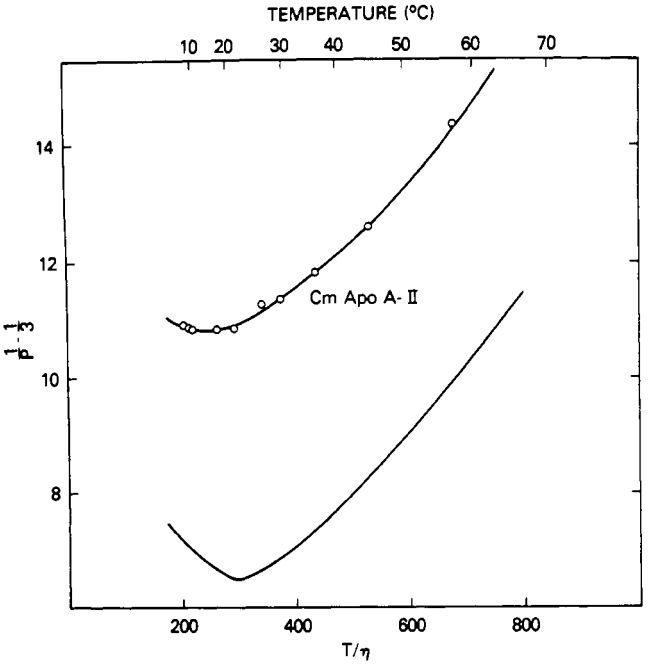

FIGURE 2: The Perrin plot of Cm apoA-II (35 μM) in 0.01 M phosphate (pH 7.4). The points are for Cm apoA-II, whereas the line represents unreduced apoA-II (Gwynne et al., 1975). The data show the effect of temperature on the polarization. The viscosity is in centipoise units.

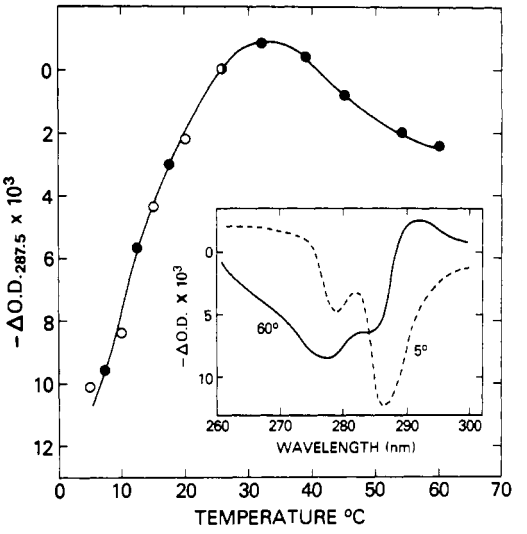

FIGURE 3: The effect of temperature on the difference absorption at 287.5 nm of Cm apoA-II (56 μM) in 0.01 M phosphate (pH 7.4). The reference solution at the same concentration was maintained at 26°. The open circles were obtained while decreasing the temperature and the closed circles while increasing the temperature. Inset: Difference absorption spectra of a 56 μM solution of Cm apoA-II at 5 and 60°. The reference solution was maintained at 26°.

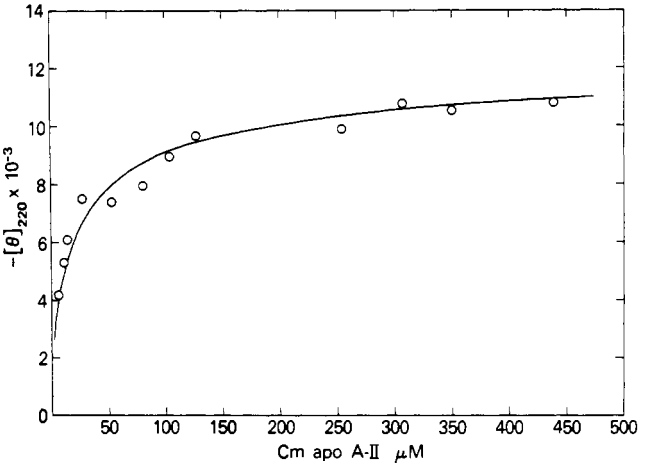

FIGURE 4: The concentration dependence of the mean residue ellipticity of Cm apoA-II at 220 nm in 0.01 M phosphate, pH 7.4, 24°. The line represents a theoretical curve for a monomer-dimer equilibrium with an association constant of $2.3 \times 10^4\ M^{-1}$.

Effect of Temperature

(*1*) *Polarization of Tyrosyl Fluorescence.* The polarization of unreduced apoA-II does not follow the Perrin equation when the viscosity is changed by altering the temperature of the solution (Gwynne et al., 1975). Instead of observing increasing polarization with decreasing temperature an inversion is found near 25° and the polarization decreases with decreasing temperature (Figure 2). This effect results from the molecular dissociation that occurs at temperatures below 25°. The polarization of Cm apoA-II shows almost no temperature dependence below ~30° and presumably originates from structural changes similar to those observed with apoA-II (Figure 2). Though the polarization values are very different for the two molecules, the effects of temperature resemble each other.

(*2*) *Difference Absorption.* The thermal dependence of the absorption at 287.5 nm of the difference spectra of a 56 μM Cm apoA-II solution in 0.1 M phosphate (pH 7.4) is illustrated in Figure 3. The reference solution was maintained at 26°. A reversible decrease in absorption, i.e., "blue-shift", was observed with increasing or decreasing temperature from 30° and indicates the exposure of tyrosyl chromophores to the aqueous environment (Yanari and Bovey, 1960). The difference spectra observed at high and low temperatures were different from each other (Figure 3, inset). The difference between the spectra would be smaller if account were taken of the effect of temperature on the absorption of the exposed residues, i.e., as is observed with acetyltyrosinamide. A similar maximum in absorption was observed with unreduced apoA-II near 25° (Gwynne et al., 1975).

Effect of Concentration

(*1*) *Ellipticity.* In order to evaluate whether the structural changes observed by polarization and difference absorption involved molecular dissociation, the effect of concentration was investigated over almost a 100-fold range as seen in Figure 4. It was found that the negative mean residue ellipticity at 220 nm of Cm apoA-II increased with concentration as was found for apoA-II. It is evident, therefore, that the self-association of Cm apoA-II involves the acquisition of appreciable secondary structure.

The ellipticity values of the dissociated and associated species could not be obtained directly by covering a wider range in concentration. Instead, the effects of several salts were evaluated on their ability to shift the equilibrium to the limiting forms involved in the Cm apoA-II equilibrium. It has been shown in numerous studies that certain salts, which usually follow the Hofmeister series, stabilize while others destabilize the native conformation of a variety of proteins (von Hipple and Schleich, 1969). Sulfate and phosphate, for example, usually enhance the stability while Gdn·Cl and thiocynate tend to lower the transition temperatures of proteins. The influence of these salts on associat-

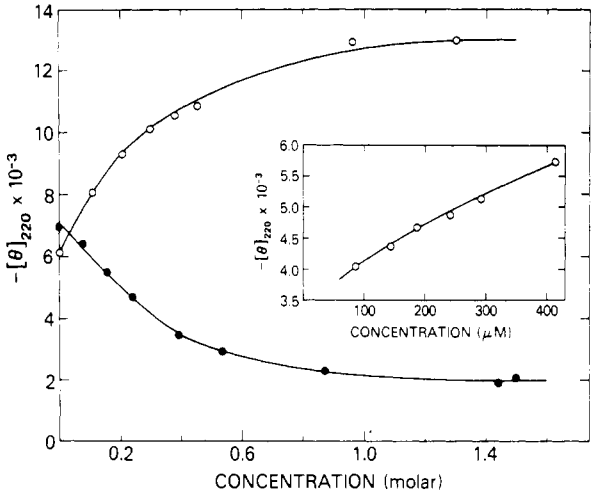

FIGURE 5: The effect of sodium phosphate (○) and Gdn·Cl (●) on the mean residue ellipticity at 220 nm of a 44 and 47 μM, respectively, solution of Cm apoA-II at 25°. Inset: The concentration dependence of the ellipticity of Cm apoA-II in 0.62 M Gdn·Cl.

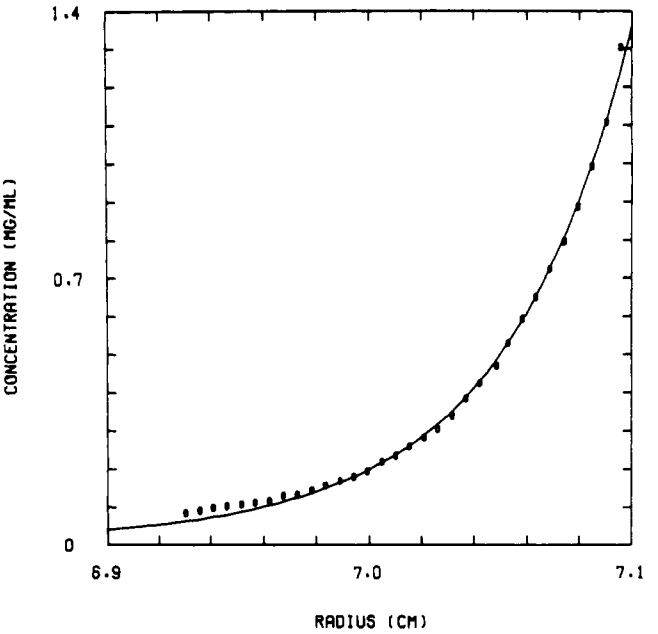

FIGURE 6: Plot of the concentration of Cm apoA-II against radial distance from the center of rotation in 0.01 M phosphate–0.1 M KCl (pH 7.4) at 25°. The rotor speed was 40,000 rpm. The line, calculated using a monomer molecular weight of 8690 and a partial specific volume of 0.74, represents a theoretical curve for a monomer–dimer equilibrium with an association constant of 6×10^4 M^{-1}.

ing systems has received less attention but one would expect them to work in the same way since the interactions involved in protein association are of the same kind as those involved in stabilizing the conformation of single chain proteins (Kawahara et al., 1965; Perutz et al., 1968; Reeke et al., 1974).

The effects of phosphate, a structure former, and Gdn·Cl, a structure breaker, on the mean residue ellipticity at 220 nm of Cm apoA-II are seen in Figure 5. The two salts have about equal and opposite effects on the ellipticity values with no further changes observable above ~1 M of each salt. The limiting mean residue ellipticity values in phosphate and Gdn·Cl are only slightly different from that reached by changing the concentration of Cm apoA-II. In order to show that Gdn·Cl simply displaced the equilibrium to the dissociated form, the concentration dependence of the ellipticity of Cm apoA-II was evaluated in 0.62 M Gdn·Cl. As seen in the inset in Figure 5, the mean residue ellipticity was dependent on the concentration of Cm apoA-II.

The data in Figure 4 were analyzed as indicated in the Methods section using the limiting mean residue ellipticities in Gdn·Cl and phosphate as the values of monomer and dimer, respectively. The solid line in Figure 4 represents the theoretical curve for an association constant of 2.3×10^4 M^{-1} (see next section).

(2) *Sedimentation Equilibrium.* In order to formulate the limits of the self-association it was necessary to evaluate the molecular weight of the associated species. Since the previous measurements do not give the molecular sizes of the interacting species, sedimentation equilibrium studies were performed to obtain this information. The ionic strength of the Cm apoA-II was increased by adding 0.10 M KCl to the 0.01 M phosphate solution to eliminate second virial effects. The sedimentation equilibrium data were analyzed by a computer program to give the best fit (see Methods). The data, using a monomer molecular weight of 8690 and a partial specific volume of 0.74, could be fitted by a monomer–dimer equilibrium with an association constant of $6 \pm 1 \times 10^4$ M^{-1} (Figure 6).

Effect of pH

The ellipticity of Cm apoA-II (39 μM) was constant between pH 6.5 and 9.0 but fell rapidly between pH 9.0 and

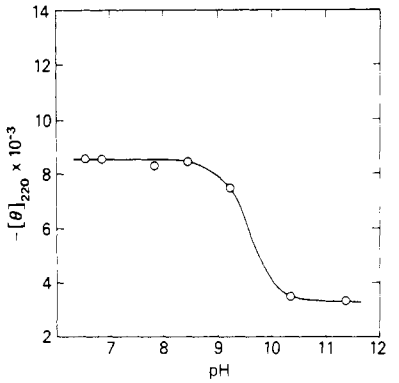

FIGURE 7: The effect of alkaline pH on the mean residue ellipticity of Cm apoA-II (39 μM) at 25°. The buffer was 25 mM in phosphate–25 mM D,L-lysine (pH 7.4).

10 at 25° (Figure 7). The fall in ellipticity appears to reflect the pH dependence of the equilibrium since the mean residue ellipticity increased between 50 and 415 μM of Cm apoA-II at pH 9.6. An association constant of 1×10^4 M^{-1} was calculated using the same values for the mean residue ellipticities of the monomer and dimer as at pH 7.4.

The mean residue ellipticity of Cm apoA-II changed from −8500 to −11,800 when the pH was reduced from 6.0 to 3.5. It is not clear whether this increase in negative ellipticity results from an increase in the association constant of the monomer–dimer reaction or from larger aggregates since Cm apoA-II is less soluble in this pH region.

Discussion

ApoA-II, extracted from HDL, occurs as a disulfide linked dimer of a single polypeptide chain of 77 residues in some species and as a single chain in other species which

lack a cystine residue. Since apoA-II is the second most abundant protein in HDL in many species, its interactions with the lipid and protein components of HDL could indicate its structural role in the lipoprotein molecule. Its interaction with the phospholipid, lysolecithin, will be presented elsewhere.[2]

A comparison of the properties of the native and reduced molecules is of interest in understanding the effect of molecular size on the properties of apoA-II. Of particular interest is the state of association since rather drastic changes in secondary and tertiary structure accompany the self-association of apoA-II. There are few high molecular weight polypeptides whose secondary and tertiary structures change significantly with molecular association. Only the behavior of glucagon has been well-documented in this respect (Gratzer and Beaven, 1969; Swann and Hammes, 1969).

The increase in polarization with association of Cm apoA-II was much smaller than was found with apoA-II and was therefore not used to evaluate the association constant (Gwynne et al., 1975). The smaller change in polarization is presumably due to the smaller relaxation times of the monomer and dimer forms of Cm apoA-II. The association could also be measured by difference absorption since the magnitude of this change with association should be quite large judging from the effects of temperature as seen in Figure 3. It was not used, however, since much smaller amounts of Cm apoA-II were required for ellipticity measurements. We have, therefore, taken advantage of the large change in ellipticity of Cm apoA-II with self-association to assess the equilibrium. Since none of these methods gives information on the size of the molecular species involved in the equilibrium, the molecular weights were determined by sedimentation equilibrium. The distribution of Cm apoA-II as a function of radial distance in the centrifuge cell was fitted by a monomer–dimer (mol wt 8690–17,380) equilibrium. The ellipticity data were analyzed, therefore, by a monomer–dimer equilibrium using the limiting ellipticity values obtained in phosphate and Gdn·Cl for the monomer and dimer, respectively. An association constant of $2.3 \times 10^4\ M^{-1}$ was computed by a least-squares best fit analysis of the data and the theoretical curve is shown in Figure 4. The association constants calculated from the best fits of the ellipticity and sedimentation data were somewhat different but this difference is considered to be within the range of experimental errors of the two methods. Moreover, the two solutions also differed by 0.10 M KCl, since this was added to the centrifuged solution to reduce the effects of nonideal solution behavior. The ellipticity of Cm apoA-II increases with increasing KCl concentration (unpublished observations) and therefore the association constant would be higher in the presence of added salt.

The formation of the dimer of Cm apoA-II results in important changes in mean residue ellipticity and in tyrosyl absorption which reflect alterations in the secondary and tertiary folding of the polypeptide chains. Since the CD spectra of the monomer and dimer show the same extrema at 208 and 220 nm, the increase in magnitude should result from the formation of α helices from random peptide groups. The difference in mean residue ellipticity between the monomer and dimer is $-11{,}000$ and represents an increase in helical content of 35% when analyzed by the method of Chen et al. (1974). In the experiment shown in Figure 3, a decrease in absorption at 287.5 nm of 3.2% (relative to 280-nm absorption) occurs between 25 and 5°. Investigation of the temperature dependence of the monomer–dimer equilibrium on Cm apoA-II indicates that there is approximately 50 and 30% dimer present in the solution at 25 and 5°, respectively.[2] Consequently, the relative decrease in absorption ($\Delta A_{287.5}/A_{280}$) resulting from the dissociation of dimer to monomer should be about 16%. The magnitude of the blue shift that occurs by dissociation is therefore comparable to that observed by high temperature or guanidine hydrochloride denaturation of many tyrosyl containing proteins (Wetlaufer, 1962). The temperature dependent dissociation and unfolding afford an explanation of the unusual temperature effects observed by polarization below 25°.

The reduced form of apoA-II associates to form a dimer (mol wt 17,380) with only a slightly smaller free energy change than that found for the unreduced form (Gwynne et al., 1975). The structural changes produced in each molecule with association resemble each other quite closely. It is evident, therefore, that the disulfide bond in apoA-II imposes very little restraint on the folding of its two component chains other than to inhibit their interaction. The properties of the monomer molecules of reduced and native apoA-II more closely resemble those of a denatured protein whereas the structure of the dimer molecules of both forms is closer to those of a native protein. The association reaction is markedly dependent on experimental variables, such as temperature, pH, and salt ions. These control not only the association but also the structure, since the two are interdependent.

ApoA-II and its reduced form appear to represent polypeptides which lack enough nonpolar residues to provide the hydrophobic environment to fold the molecule into a globular structure. This is accomplished, however, by molecular association. The association is dependent on minor changes in solution properties of pH, temperature, etc., in much the same way that a native structure becomes dependent once its threshold of stability is reached by adding an organic denaturant. The interactions in apoA-II are clearly weaker, and result in a much less stable molecule than is found with most proteins. This flexibility in structure could, of course, be related to its function in its parent molecule HDL.

[2] Manuscript in preparation.

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Proton Magnetic Resonance Relaxation Studies on the Structure of Mixed Micelles of Triton X-100 and Dimyristoylphosphatidylcholine†

Anthony A. Ribeiro and Edward A. Dennis*

ABSTRACT: Proton magnetic resonance and gel chromatographic studies on mixtures of phospholipid and the nonionic surfactant Triton X-100 have shown that at temperatures above the thermotropic phase transition of the phospholipid and below the cloud point of Triton, mixed micelles are present at molar ratios above about 2:1 Triton/phospholipid. Proton T_1 and T_2* (from line widths) relaxation times are reported for protons in Triton micelles and in mixed micelles of Triton and dimyristoylphosphatidylcholine at a molar ratio of 3:1 Triton/phospholipid. The T_1 values and their temperature dependence and the activation energies of the various Triton proton groups appear to reflect internal motions of the Triton molecules in the micelle. Measurements of the T_1/T_2* ratio and frequency dependence (55–220 MHz) suggest that the hydrophobic *tert*-butyl group in Triton is observed under extreme narrowing conditions. The T_1 and T_2* values of Triton are unchanged in the presence of phosphatidylcholine. The T_1 values of various protons of dimyristoylphosphatidylcholine in mixed micelles are similar to those reported for the phospholipid in sonicated vesicles, which are used as membrane models, and presumably the same coupled trans–gauche motions dominate. The T_2* values for the terminal methyl and choline methyl protons in the phospholipid are longer than those reported for these groups in vesicles. Hence, the motion of the phospholipid in the mixed micelles appears to be less restricted than in vesicles. T_1 measurements in H_2O/D_2O mixtures are consistent with the idea that water does not penetrate the hydrophobic core of the mixed micelles, while water does solvate the polar oxyethylene and choline methyl groups. Titration with Mn^{2+} confirms that the oxyethylene and choline methyl groups are on the exterior of the mixed micelle while the hydrophobic groups are located in the micellar interior.

Previous ^{1}H nuclear magnetic resonance (NMR) (Dennis and Owens, 1973; Ribeiro and Dennis, 1974a) and gel chromatographic studies (Dennis, 1974a) have led to the suggestion that phosphatidylcholine and the nonionic surfactant Triton X-100 form mixed micellar structures at high molar ratios of Triton/phospholipid. These mixed micelles provide one form of the phospholipid which lipolytic enzymes such as phospholipase A_2 can utilize as substrate (Dennis, 1973) and the phospholipase A_2–dipalmitoylphosphatidylcholine–Triton X-100 system provides an artificial, characterizable system for studying the effect of thermotropic phase transitions and lipid phase separations on biological activity (Dennis, 1974b). Mixed micelles of phospholipid and surfactant are also important in membrane studies since they are formed when Triton X-100 and other surfactants are employed in the solubilization of membrane-bound proteins (Helenius and Söderlund, 1973; Makino et al., 1973).

Using continuous wave ^{1}H NMR techniques, we have shown that the phospholipid molecules in mixed micelles give rise to full or nearly full intensities and narrow line widths (Dennis and Owens, 1973; Ribeiro and Dennis, 1974a), whereas unsonicated dispersions of phospholipid which have a multibilayer structure do not (Penkett et al., 1968; Chan et al., 1973). Furthermore, the Triton molecules in Triton micelles and mixed micelles have similar intensities and line widths (Dennis and Owens, 1973; Ribeiro and Dennis, 1974a). Preliminary T_1[1] measurements have suggested that the T_1 relaxation times are also similar in Triton micelles and mixed micelles (Ribeiro and Dennis,

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[1] Abbreviations used are: diacylphosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; TMS, tetramethylsilane; cmc, critical micelle concentration; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time.