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The Thermodynamics of the Self-Association of the Reduced and Carboxymethylated Form of ApoA-II from the Human High Density Lipoprotein Complex†

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ABSTRACT: The thermodynamics of the self-association of the reduced carboxymethylated form of apoA-II protein (molecular weight 8690) from human serum high density lipoproteins has been obtained at neutral pH by ellipticity measurements. Changes in secondary and tertiary structure accompany the association. The association is endothermal at low and exothermal at high temperatures, and involves a decrease in heat capacity of 1250 cal/(mol deg) at 25°.

In order to characterize more fully the molecular organization of lipoprotein particles we have undertaken a study of the molecular properties of the isolated components of the high density lipoprotein complex. In two previous publications of this series the molecular properties of human apoA-II and reduced and carboxymethylated apoA-II, i.e., Cm apoA-II, were reported (Gwynne et al., 1975; Osborne, et al., 1975). Human apoA-II, a disulfide dimer of two identical chains of 77 residues (Brewer et al., 1972), was shown to self-associate to a dimer in aqueous solution (mol wt 17380 → 34760) (Gwynne et al., 1975). The single chain, Cm apoA-II (mol wt 8690), also self-associates in aqueous solution to form a dimer of molecular weight 17380 (Osborne et al., 1975). The dimer form of both molecules has appreciable secondary and tertiary structure, whereas the monomers possess little organized structure and resemble random coils.

The thermodynamics of these self-associations are quite unusual since increasing temperature first increases and then decreases the association constant of Cm apoA-II. The association of the unreduced, native molecule, apoA-II,

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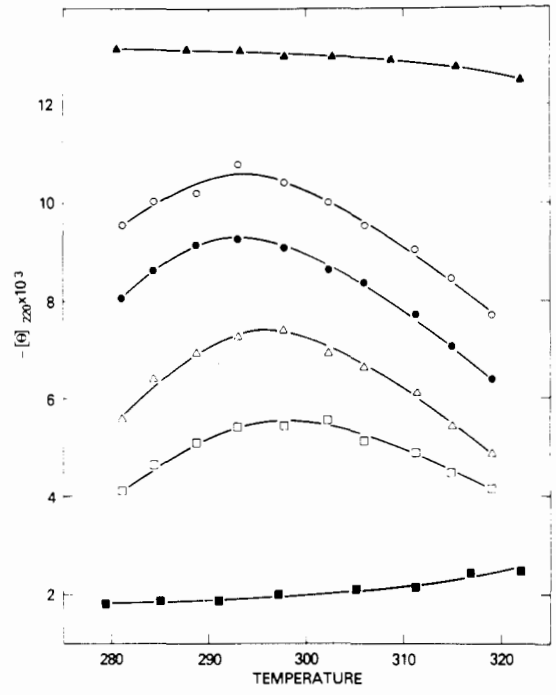

FIGURE 1: Effect of temperature on the mean residue ellipticity of Cm apoA-II at 220 nm. (○) 414.5 μM (0.01 M phosphate, pH 7.4); (●) 155.4 μM (0.01 M phosphate, pH 7.4); (△) 52.6 μM (0.01 M phosphate, pH 7.4); (□) 15.8 μM (0.01 M phosphate, pH 7.4); (▲) 339 μM (1.3 M phosphate, pH 7.4); (■) 42 μM (1.3 M Gdn·HCl, pH 7.4).

shows a similar dependence on temperature (Gwynne et al., 1975). The native molecule was not used for this study because the association constant is larger and solutions of lower concentrations would have to be measured. The ellipticities of such solutions are low and experimental errors would be greater.

Methods

The isolation and purification procedure used for human apoA-II has been reported (Lux et al., 1972). Reduction and carboxymethylation were carried out as previously described (Lux et al., 1972) and an amino acid analysis indicated that only the single cystine residue was reduced and alkylated with iodoacetic acid. A molar extinction coefficient of 5500 at 280 nm was used to determine protein concentrations (Osborne et al., 1975). 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."

A Cary Model 60 spectropolarimeter equipped with temperature controlled Pockels cell was used to measure the circular dichroic spectra. Mean residue ellipticities were calculated by using the following equation:

$$[\theta]_\lambda = (\theta)_{obsd}113/10lC \qquad (1)$$

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. Spectra were obtained between 260 and 215 nm for all solutions. We have used the mean residue ellipticity at the 220-nm trough to assess the equilibrium between species.

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 given wavelength, λ:

$$[\theta]_\lambda = f_M[\theta_M]_\lambda + f_D[\theta_D]_\lambda \qquad (2)$$

where f_M is the weight fraction and $[\theta_M]_\lambda$ is the mean residue ellipticity of monomer and f_D is the weight fraction and $[\theta_D]_\lambda$ is the mean residue ellipticity of dimer. Equation 2 reduces to:

$$[\theta]_\lambda = ([P]/C)[\theta_M]_\lambda + [(C - [P])/C][\theta_D]_\lambda \qquad (3)$$

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

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

The mean residue ellipticities of monomer and dimer were approximated by employing appropriate neutral salts as indicated in the Results section. The association constants were obtained by a least-squares fit of the data, including the values for monomer and dimer, to eq 3 and 4.

Results

The shape of the CD spectra of Cm apoA-II remains the same although the mean residue ellipticities change significantly with increasing concentration. The CD spectrum of Cm apoA-II is essentially composed of a mixture of the spectra for random and helical peptide chromophores (Osborne et al., 1975). The increase in magnitude, therefore, indicates the conversion of random peptide groups to an α-helical configuration. The effect of temperature on the mean residue ellipticities at 220 nm of four solutions of Cm apoA-II varying between 15.8 and 415 μM is shown in Figure 1. Each of the four curves shows a maximum in ellipticity near room temperature. A maximum has also been found in the difference absorption of Cm apoA-II near 28° (Osborne et al., 1975).

The mean residue ellipticities of the monomer and dimer species were obtained in the following manner. It has been shown previously that the ellipticity of a mixture of the two species is shifted to the monomer form by Gdn·HCl[1] and to the dimer form by phosphate. The ellipticities reached plateau values by ~1.4 M concentrations of each salt. The plateau values obtained with the two salts were slightly lower and higher than the ellipticity values found when the concentration was increased almost 100-fold, i.e., 5 and 450 μM (Osborne et al., 1975). We have determined the temperature dependence of the ellipticity values of the monomer and dimer form of Cm apoA-II in order to have these values for calculating the equilibrium constant as a function of temperature. Concentrations of salts and Cm apoA-II were used which should prevent the interconversion of the two species in the temperature range investigated. The phosphate solution (1.3 M) contained 339 μM Cm apoA-II while the Gdn·HCl (1.3 M) solution contained 42 μM of Cm apoA-II. The temperature dependence of the ellipticity of these two solutions is shown in Figure 1. Only at temperatures above ~35° were small changes observed in either solution.

Computation of Thermodynamic Parameters. Association constants were calculated employing the data in Figure

[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride.

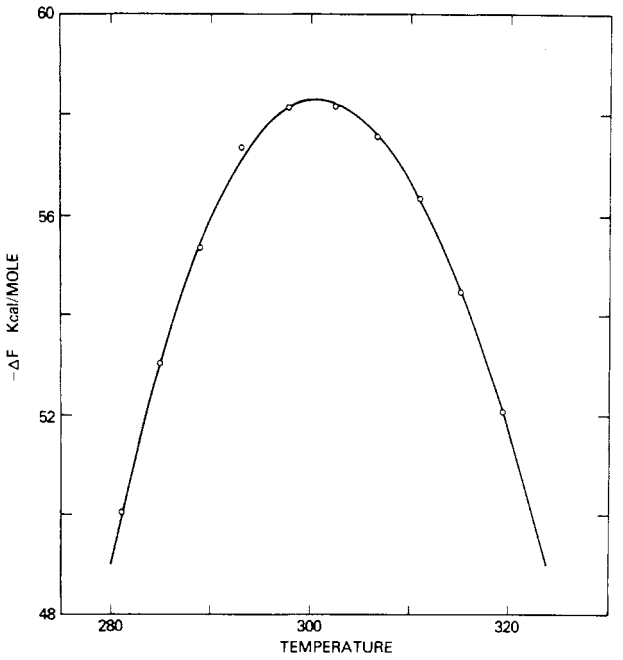

FIGURE 2: The temperature dependence of the free energy change for the dimerization of Cm apoA-II in 0.01 *M* phosphate at pH 7.4. The solid line represents the theoretical curve calculated from eq 6.

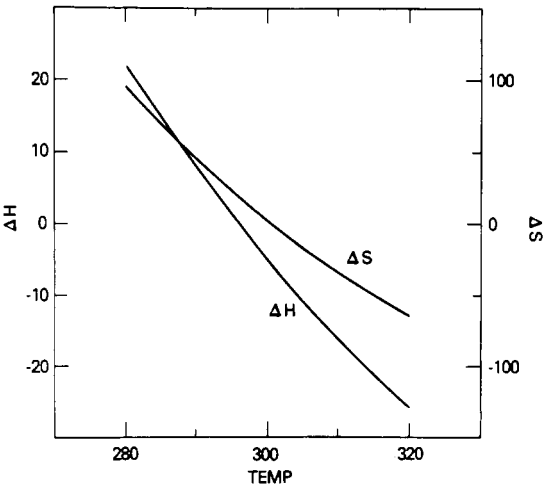

FIGURE 3: Thermodynamic parameters obtained from the free energy curve in Figure 2 for the dimerization of Cm apoA-II in 0.01 *M* phosphate (pH 7.4). The values were calculated from eq 7 and 8, respectively. The enthalpy is in units of kcal/mol. The entropy is in cal/(mol deg).

1 with the procedure indicated in the Methods section. The free energy change goes through a maximum near 28°. A graphical determination of the enthalpy from the slope of the van't Hoff plot at various temperatures would give inexact values. Therefore, the association constants were plotted as the free energy change against temperature (Figure 2). A regression analysis of the curve in Figure 2 was performed by using eq 5-8.

$$\Delta F = -RT \ln K = A + BT + CT^2 + DT^3 \quad (5)$$

$$\Delta F = 468650 - 4141.4T + 11.801T^2 - 0.10894T^3 \quad (6)$$

$$\Delta H = \delta(\delta F/T)/\delta(1/T) = 468650 - 11.801T^2 + 0.21788T^3 \quad (7)$$

$$\Delta S = \frac{\delta \Delta F}{\delta T} = 4141.4 - 23.602T + 0.32682T^2 \quad (8)$$

The result of the best fit analysis of the experimental data is represented by the solid line in Figure 2 and the coefficients are shown in eq 6.

The enthalpy and entropy changes were computed by eq 7 and 8. It is evident from Figure 3 that the changes in both functions vary continuously with temperature. The change in heat capacity is negative, indicating that the heat capacity for the monomer state is greater than that for the dimer. This result is consistent with other studies on the denaturation of proteins where the denatured state was found to have a larger heat capacity than the native state.

Discussion

The noncovalent interactions responsible for the stabilization of the native form of globular proteins have received considerable attention both from an experimental and theoretical point of view (Kauzmann, 1959; Hagler et al., 1973). A measure of the magnitude of these interactions can be obtained from the thermodynamic constants of protein reactions where there is a large change in organization, as in a denaturation reaction. There are thermodynamic reasons, as well as x-ray evidence, indicating that the principal interactions which stabilize the native conformations of proteins are the so-called hydrophobic effects (Tanford 1970; Dickerson and Geis, 1969). The latter are largely determined by the energetics of the interaction of the nonpolar side chains with the aqueous solvent (Kauzmann, 1959; Némethy and Scheraga, 1962).

Earlier investigations of protein denaturation usually showed enthalpy changes which were independent of temperature. More recent studies, over a broader temperature range, reveal nonlinear van't Hoff plots. The curvature indicates a dependence of the enthalpy on temperature and consequently a difference in heat capacity between the native and denatured states.

The denaturation of globular proteins usually involves highly cooperative transitions of many or most of the noncovalent interactions. In the present case, the association of Cm apoA-II involves a structural change comparable in magnitude to those observed in the thermal transitions of small globular proteins.

The free energy change of Cm apoA-II (and apoA-II) association is strongly dependent on temperature with a maximum occurring at 28°. All the measurements used to evaluate the equilibrium, i.e., difference absorption, ellipticity, and polarization, show maxima near 28° (Osborne, et al., 1975). The equilibrium between the monomer and dimer forms of Cm apoA-II is dependent on the same variables, i.e., salts, pH, denaturants, etc., as is found in most denaturation reactions. The major difference between the Cm apoA-II and other protein denaturations in the neutral pH region is that Cm apoA-II unfolds (and dissociates) with either increasing or decreasing temperature from 28°. Although many proteins dissociate at low temperatures, few have been shown to unfold (Lauffer, 1975).

The wide range of temperature in which the free energy of association of Cm apoA-II can be measured without modifying the solvent offers a distinct advantage in obtaining thermodynamic parameters. It has usually been necessary to change the pressure, pH, salt, or solvent composition with more stable proteins to obtain data over a large enough temperature range to observe the difference in heat capacities between the native and denatured molecules (Brandts, 1964a; Brandts and Hunt, 1967; Hermans and

Acampora, 1967; Biltonen and Lumry, 1969, 1971; Brandts et al., 1970; Shiao et al., 1971; Hawley, 1971; Zipp and Kauzmann, 1973). These methods of expanding the temperature scale are always questionable since the treatment of intramolecular conformational transitions usually assumes a two-state theory (Lumry et al., 1966; Tanford, 1968). Unfortunately, it is frequently difficult to be sure that the structures of the initial and final states remain the same when solution conditions are altered significantly.

The thermodynamic constants have been evaluated from the coefficients of the dependence of the free energy change on temperature. It should be emphasized that if the degree of curvature in the ΔF vs. T curve is not large, the coefficients C and D in eq 6 will be small and the error in their evaluation will be significant. It is important, therefore, to cover a temperature range in which there is adequate curvature in the dependence of the free energy on temperature.

The thermodynamic parameters computed from eq 7 and 8 show that the enthalpy and entropy of association decrease continuously with increasing temperature and that the equilibrium is controlled by the entropy change at low temperature and the enthalpy change at high temperature. The heat capacity change is about -1250 cal/(mol deg) at 25°. We have not reported the heat capacity as a function of temperature since the heat capacity is determined from the second derivative of the free energy and extremely precise values of K are needed to depict the dependence of ΔC_p on temperature.

The large change in heat capacity indicates that the unfolded monomer has a higher heat capacity than the folded dimer. This change in heat capacity is in accord with other studies of protein denaturation where the denatured molecule was found to have a higher heat capacity than the native form. Brandts has developed a theory of protein denaturation based on experiments with chymotrypsinogen and ribonuclease in which there is an important change in heat capacity (Brandts, 1964a,b; Brandts and Hunt, 1967). The studies on protein denaturation in which the effects of pH were combined with thermal curves have been reevaluated with similar results (Shiao et al., 1971). Only in the case of solutions of β-lactoglobulin in 4.5–5.5 M urea or 3.4 M guanidine hydrochloride was extensive denaturation observed in a single solvent at both high and low temperatures. The heat capacity change was independent of temperature between 10 and 55° and equal to 2100 cal/(mol deg) (Pace and Tanford, 1968).

There are numerous examples of proteins which dissociate with decreasing temperature but few in which the free energy change has been measured over a large enough temperature range to observe a maximum. Two self-associations where this has been achieved are those of glutamic dehydrogenase and α-chymotrypsin. Analysis of the free energy change of association of glutamic dehydrogenase between 10 and 40° showed a linear dependence of the enthalpy on temperature with positive values below 28° and negative values above 28°, and a heat capacity change of -600 cal/(mol deg) (Reisler and Eisenberg, 1971). In the dimerization of α-chymotrypsin the enthalpy changed from positive to negative at 21° and the heat capacity change between 0 and 31° was -700 cal/(mol deg) (Aune et al., 1971).

All the associations and denaturations that have been discussed are accompanied by changes in heat capacity. If we can exclude changes in ionization constants in the thermal denaturations and associations, we have only to consider two important sources of the heat capacity changes. These are the rupture of intramolecular hydrogen bonds, principally peptide, which form α-helical and β-structures, and the exposure of nonpolar groups to the solvent. The transfer of the nonpolar side chains of alanine, valine, and norleucine from ethyl alcohol to water has been shown by Brandts (1964) to result in heat capacity changes in the same direction as those observed when proteins are denatured. There is no evidence available that the thermal rupture of hydrogen bonds involves a change in heat capacity. An extensive analysis of the significance of the heat capacity changes in protein denaturation will be presented elsewhere (H. Edelhoch and J. C. Osborne, manuscript in preparation).

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