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Apoprotein stability and lipid–protein interactions in human plasma high density lipoproteins*

(protein denaturation/two-state transition/differential scanning calorimetry/atherosclerosis/hydrophobic binding sites)

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ABSTRACT **Temperature-dependent conformational changes of the principal apoprotein of human plasma high density lipoprotein (HDL), apoA-I, have been studied in the isolated apoprotein, in complexes of apoprotein with phospholipid, and in intact HDL. Differential scanning calorimetry shows that in solution apoA-I undergoes a reversible, two-state thermal denaturation (midpoint temperature 54°). The enthalpy (2.4 cal/g) (10.0 J/g) and specific heat change (0.08 cal/°C per g) (0.33 J/°C per g) associated with the denaturation were used to calculate the free energy difference (ΔG) between native and unfolded apoA-I at 37°. ΔG (2.4 kcal/mol) (10.0 kJ/mol) is less than that of other globular proteins (typically 8–14 kcal/mol) (33–59 kJ/mol), indicating that at 37° native apoA-I has a loosely folded conformation. Turbidity studies show that apoA-I is able to solubilize phospholipid in its native but not in its denatured form. Mixtures of apo-HDL (the total apoprotein of HDL) or apoA-I with dimyristoyl lecithin show a thermal transition at about 85° not present in the lecithin or the apoprotein alone, which indicates that the native conformation of the apoprotein is stabilized by phospholipid. Scanning calorimetry of intact HDL shows a high-temperature endotherm associated with disruption of the HDL particle, suggesting that in HDL the conformation of apoA-I is also stabilized by interaction with lipid. The loosely folded conformation of native, uncomplexed apoA-I may be specially adapted to the binding of lipid, since this process may involve both hydrophobic sites on the surface of the protein and concealed apolar amino acid residues that are exposed by a cooperative, low energy unfolding process.**

A knowledge of the structure and thermodynamic stability of serum lipoproteins is important to an understanding of their metabolism and role in diseases such as atherosclerosis. The human plasma high density lipoproteins (HDL) are spherical particles (1, 2) consisting of about 50% protein, 22% phospholipids, 3% free cholesterol, 14% cholesterol esters, and 8% triglycerides (3). The proteins (apo-HDL) include approximately 60–65% apoA-I [molecular weight (M_r) 28,331], 30% apoA-2 (M_r 17,380) and 5–10% low M_r C-peptides (4, 5). The proteins and polar head groups of phospholipids occupy the surface of the lipoprotein particle while the apolar lipid moieties form a hydrophobic core (1, 2). Further, the conformations of the apoproteins of HDL appear to be stabilized by interaction with lipids (3, 4). In the present study, we show that the principal apoprotein of human HDL, apoA-I, undergoes a reversible temperature-dependent conformational change with unusual thermodynamic properties. A comparison with other water-soluble proteins suggests a loosely folded structure of the native apoprotein, which we relate to its special function of solubilizing lipid.

Abbreviations: M_r, molecular weight; HDL, plasma high density lipoproteins; DSC, differential scanning calorimetry; DML, dimyristoyl lecithin; apo-HDL, the total apoproteins of HDL.

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METHODS

Human HDL was isolated from the plasma of normal fasting male donors by ultracentrifugal flotation between 1.090 and 1.19 g/ml. The purity of HDL preparations was confirmed by immunodiffusion and immunoelectrophoresis (6). HDL was delipidated by chloroform–methanol extraction (7), and apoA-I and apoA-2 were prepared by Sephadex G-200 column chromatography (8). ApoA-I and apoA-2 migrated as single bands on polyacrylamide disc gel electrophoresis. Dimyristoyl lecithin (DML) was purchased from Sigma Chemical Co. and was chromatographically pure.

Differential scanning calorimetry (DSC) experiments were performed on a Perkin-Elmer DSC-2B, calibrated with cyclohexane and indium. The temperature (T_d), enthalpy (ΔH), and heat capacity change ($\Delta C_p{}^d$) associated with protein denaturation were determined as shown in Fig. 1b according to Privalov (9). Ribonuclease was used as a reference protein and gave values of T_d, ΔH_d, and $\Delta C_p{}^d$ very similar to those reported by Privalov (9). Experiments were performed at various protein concentrations in 20 or 50 μl sealed pans at heating rates of 1.25, 2.5, and 5°/min and cooling rates of 10°/min. The values obtained for apoA-I denaturation were not dependent on heating rate.

Mixtures of apo-HDL or apoA-I and unsonicated DML were equilibrated at 37° for up to 96 hr. Equilibrium, as judged by clearing of turbidity of the mixture, was reached within 12 hr with apo-HDL and within 96 hr with apoA-I. Protein concentrations in all samples were estimated by the method of Lowry *et al.* (10).

RESULTS AND DISCUSSION

DSC of solutions of apoA-I and apo-HDL shows a reversible transition between 43 and 71° with the midpoint T_d at 54° (Fig. 1a and b). From the DSC thermogram, it is possible to measure both the calorimetric enthalpy of the transition in cal/g (ΔH_{cal}) (1 cal = 4.184 J) from the area under the curve, and the effective enthalpy in cal/mol (ΔH_{vH}) using an expression derived from the van't Hoff equation for a two-state transition (9):

$$\Delta H_{vH} = 2R^{1/2} T_d M_r^{1/2} \Delta C_d^{1/2} \quad [1]$$

where R is the gas constant, ΔC_d in cal/°C per g is the excess heat capacity at T_d (see Fig. 1b), and M_r is the molecular weight of the protein. If $\Delta H_{cal} \times M_r = \Delta H_{vH}$, then the protein transition represents a two-state denaturation, i.e., a cooperative unfolding of the protein molecule with no significant population of intermediate states (9). The thermal

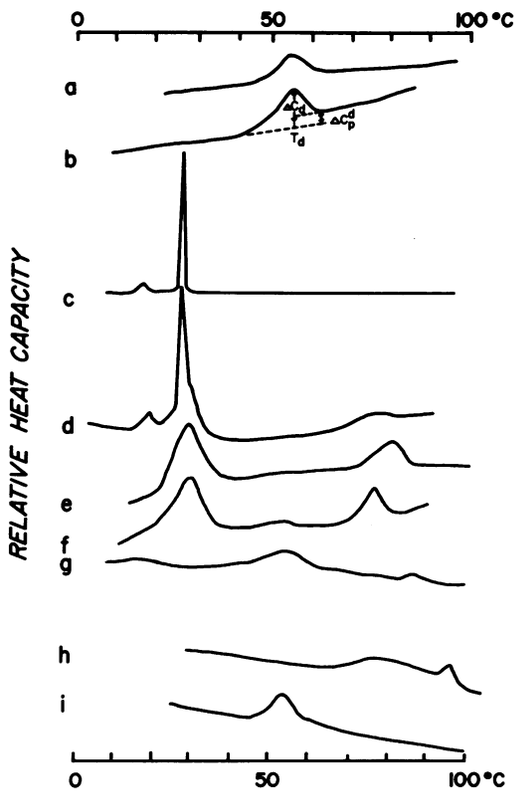

FIG. 1. DSC heating curves of (a) 2% apo-HDL, (b) 2.2% apoA-I, (c) DML in excess water, (d–g) DML–apo-HDL mixtures in excess water (about 2% solids) in ratios (w/w), (d) 83:17, (e) 45:55, (f) 30:70, (g), 21:79, and (h) intact HDL and (i) denatured HDL. All experiments were performed in Na_2CO_3–HCO_3 buffer, pH 9.2.

denaturation of several globular proteins has been shown to conform to a two-state model (9, 11). For apoA-I, ΔH_{cal} = 2.4 ± 0.2 cal/g and ΔH_{vH} = 64 ± 7.8 kcal/mol. The mean value from six experimental thermograms of $\Delta H_{cal} \times M_r / \Delta H_{vH}$ = 1.03 ± 0.02, and thus the thermal transition of apoA-I can be described to a first approximation as a two-state denaturation. Solutions of apoA-2 show no transition at 54°. The transition observed at 54° in solutions of apo-HDL gives ΔH_{cal} = 1.6 ± 0.04 cal/g apo-HDL, which is equivalent to 2.5 cal/g of apoA-I, confirming that the transition in apo-HDL is due to denaturation of its major component, apoA-I.

The interpretation that the thermal transition of apoA-I is due to a cooperative unfolding process is supported by the circular dichroism and ultra-violet spectroscopic studies of solutions of apoA-I (12) and apo-HDL (3) which indicate a major conformational change associated with a helix-coil transition between 45–70°. Given that the denaturation of apoA-I is a two-state process, it is possible to estimate a ΔH value from the published spectroscopic data (12), using the relationship:

$$\Delta H = -Rd(\ln K)/d(1/T) \quad [2]$$

where K is a thermodynamic equilibrium constant for the native-denatured transition (13). In dilute solution the UV difference spectra of apoA-I (12) give a ΔH value of 40–80 kcal/mol, which is consistent with our value (64 ± 7.8 kcal/mol). Furthermore, the parameters obtained for apoA-I denaturation by DSC were independent of concentration in the range 0.58–3.4% (g/100 ml). Thus, over a wide range of concentrations, possible changes in the state of aggregation of apoA-I did not appear to have a major effect on the temperature or ΔH of denaturation.

The calorimetric data may be used to derive thermodynamic parameters describing the stabilization of conformation of macromolecules at physiological temperatures (9, 14). Application of the Gibbs-Helmholtz equation permits calculation of the free energy difference (ΔG) between native and denatured apoA-I as a function of temperature, assuming that at T_d, $\Delta G = 0$ (see refs. 9 and 14). Thus,

$$\Delta G(T_0) = \Delta H_{T_d}(1 - T_0/T_d) + \Delta C_p{}^d[T_0 - T_d + T_0 \ln (T_d/T_0)] \quad [3]$$

where $\Delta G(T_0)$ is the free energy difference between the native and denatured protein at T_0, a temperature remote from the transition, and $\Delta C_p{}^d$ is the difference in the heat capacities of the native and denatured proteins (see Fig. 1b). Since for apoA-I, $\Delta C_p{}^d$ = 0.07–0.09 cal/°C per g, the term in equation (3) containing $\Delta C_p{}^d$ is negative and small and the first term containing ΔH_{T_d} is dominant. The enthalpy of denaturation of apoA-I (2.4 cal/g) is small in comparison with other globular proteins (9, 11, 13) and results in ΔG (37°) = 2.4 kcal/mol. This ΔG is strikingly less than that obtained for other globular proteins by calorimetry (8–14 kcal/mol) (9) or other methods (9–16 kcal/mol) (15–17) at similar temperatures. This means that the free energy of stabilization of the native structure of apoA-I at 37° is much lower than that of other water-soluble proteins, such as ribonuclease or lysozyme. Although apoA-I is globular (18) there must be special features in the tertiary structure of the molecule which minimize the stabilizing effects of long-range interactions. A similar interpretation is suggested by the previous finding that at 25° apoA-I is completely denatured in 1.7 M guanidine·HCl (19), whereas concentrations of 6–8 M are required to denature most proteins (20). A relatively loosely folded but helix-containing conformation of apoA-I with a low free energy of stabilization may be important in its function of lipid binding.

The effect of phospholipid, the major lipid of HDL, on the conformational stability of apoA-I was investigated in mixtures of apo-HDL, or apoA-I, with dimyristoyl lecithin (DML). The unsonicated aqueous suspensions of DML were turbid both above and below the gel-liquid crystalline transition (23°). Addition of apo-HDL readily caused clearing of the turbidity above 23° but not below. Furthermore, if apo-HDL and DML were mixed above the denaturation temperature of apoA-I, for instance at 55°, the dispersion remained turbid and cleared only when cooled below the renaturation temperature. These experiments indicate that interaction of phospholipid and apo-HDL requires both a liquid crystalline state of the phospholipid and an intact tertiary structure of apoA-I. ApoA-I alone also caused spontaneous clearing of a turbid suspension of DML. However, the time required to achieve equilibration was much longer. Thus, as previously suggested (21), apoA-2 may facilitate interaction of apoA-I with phospholipid.

In mixtures of apo-HDL or apoA-I with DML equilibrated at 37°, major transitions may be observed at three temperatures (Fig. 1c–g): (*1*) at 23°, the gel-liquid crystalline transition of DML; (*2*) at the denaturation temperature of apoA-I (T_d = 54°); and (*3*) at high temperature (T about 85°). Since this last transition is not present in aqueous preparations of apo-HDL, or DML alone, it must represent denaturation of a lipoprotein complex. The enthalpies of these

three transitions depend on the stoichiometry of the mixture. With increasing amounts of apo-HDL, (*a*) the phospholipid transition diminishes, broadens, and finally disappears at 75% apo-HDL, (*b*) the enthalpy of the high-temperature transition (T about 85°) reaches a maximum between 30 and 45% apo-HDL, suggesting that the optimum stoichiometry of the complex lies within this range, and (*c*) with greater than 60% apo-HDL, a transition at 54°, representing uncomplexed apoA-I, appears and increases in size, to become the only transition at 100% protein.

To determine if similar interactions of phospholipid and apoprotein occur in plasma lipoproteins, we have studied intact human HDL. When heated to 70°, solutions of HDL become turbid. Polarizing microscopy of HDL shows that above 70° oil droplets appear, which on cooling to 20° form focal conics that have melting behavior typical of cholesterol ester droplets (22). Thus, above 70°, the HDL particle is disrupted with liberation of cholesterol esters.

DSC of intact HDL shows no thermal transition at 54° but on further heating an irreversible, double-peaked endotherm is observed at 70–95° (Fig. 1h). On cooling from 95°, an exotherm is present with a maximum at 54° and on subsequent heating and cooling there is a reversible, repeatable transition at 54° with characteristics identical to those of the apoA-I denaturation. Since no transition is present at 54° in the lipids extracted from HDL or in apoA-2, the transition is presumed to be due to liberated apoA-I. When expressed as cal/g of apoA-I the enthalpy is about 2.4, indicating that most, or all, of the apoA-I dissociates from the heat-disrupted HDL particle. The complex, high-temperature endotherm associated with disruption of HDL probably represents several processes: unfolding of apoA-I which, in intact HDL, has adopted a more stable conformation (as in apoHDL–DML mixtures); possible conformational changes of apoA-2 and C-peptides; and liberation of cholesterol esters.

The binding of phospholipid to the water-soluble apoproteins is primarily hydrophobic (23–27) and is thought to involve apolar surfaces of protein α-helices (27). Our results indicate a unique looseness of folding of the apoA-I molecule which would be consistent with readily accessible sites for lipid binding. The extensive binding of phospholipid to apoA-I may involve both apolar sites on the surface of the protein and apolar parts of amphipathic helices (28) exposed by unfolding of the tertiary structure. The ease of unfolding of the apoA-I native structure and the relative stability of apoA-I in phospholipid–apoprotein complexes and in HDL make this a thermodynamically favorable process.

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