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Mechanism of Dissociation of Human Apolipoprotein A-I from Complexes with Dimyristoylphosphatidylcholine As Studied by Guanidine Hydrochloride Denaturation†

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ABSTRACT: The reversibility of the binding of human apolipoprotein A-I (apo A-I) to phospholipid has been monitored through the influence of guanidine hydrochloride (Gdn·HCl) on the isothermal denaturation and renaturation of apo A-I/dimyristoylphosphatidylcholine (DMPC) complexes at 24 °C. Denaturation was studied by incubating discoidal 1:100 and vesicular 1:500 mol/mol apo A-I/DMPC complexes with up to 7 M Gdn·HCl for up to 72 h. Unfolding of apo A-I molecules was observed from circular dichroism spectra while the distribution of protein between free and lipid-associated states was monitored by density gradient ultracentrifugation. The ability of apo A-I to combine with DMPC in the presence of Gdn·HCl at 24 °C was also investigated by similar procedures. In both the denaturation and renaturation of 1:100 and 1:500 complexes, the final values of the molar ellipticity and the ratio of free to bound apo A-I at various concentrations of Gdn·HCl are dependent on the initial state of the lipid and protein; apo A-I is more resistant to denaturation when Gdn·HCl is added to existing complexes than to a mixture of apo A-I and DMPC. There is an intermediate state in the denaturation pathway of apo A-I/DMPC complexes which is not present in the renaturation; the intermediate comprises partially unfolded apo A-I molecules still associated with the complex by some of their apolar residues. Complete unfolding of the α helix and subsequent desorption of the apo A-I molecules from the lipid/water interface involve cooperative exposure of these apolar residues to the aqueous phase. The energy barrier associated with this desorption step makes the binding of apo A-I to DMPC a thermodynamically irreversible process. Consequently, binding constants of apo A-I and PC cannot be calculated simply from equilibrium thermodynamic treatments of the partitioning of protein between free and bound states. Apo A-I molecules do not exchange freely between the lipid-free and lipid-bound states, and extra work is required to drive protein molecules off the surface. The required increase in surface pressure can be achieved by a net mass transfer of protein to the surface; in vivo, increases in the surface pressure of lipoproteins by lipolysis can cause protein desorption.

The binding of apolipoproteins to aggregates of amphipathic molecules, particularly phospholipids, has been studied extensively. The particular structural feature of apolipoproteins involved in binding is the amphipathic α helix [for a review, see Morrisett et al. (1977)]. This α helix is located in the lipid/water interface with polar amino acid residues mainly located on one side and exposed to the aqueous phase and with apolar residues on the opposite side embedded among the phospholipid hydrocarbon chains. Although the structural features of this interaction are relatively well understood, only a few reports on the thermodynamics of the lipid/protein interactions have been published.

By measuring the apparent equilibrium distribution of apolipoproteins between the free and lipid-associated states, Chung et al. (1979) obtained a value of −34.7 kJ/mol of apolipoprotein A-I (apo A-I)[1] for the standard free energy of association at 37 °C of apo A-I with egg phosphatidylcholine (PC) vesicles containing 20 mol % cholesterol and −36.9 kJ/mol of peptide of apo A-II for the binding of apo A-II. Hickson et al. (1981) and Pownall et al. (1981) calculated a free energy of binding for reduced and carboxymethylated apo A-II to dimyristoylphosphatidylcholine (DMPC) vesicles of −33.2 kJ/mol of carboxymethylated apo A-II, a value barely influenced by the aggregation state of DMPC, or the temperature of incubation between 5 and 37 °C. Calculation of the free energy of binding from the difference between the initial and final states is possible only if the reaction is reversible. In general, a reaction is considered to be reversible in the thermodynamic sense if, after allowing the reaction to occur in one direction, return to the initial state causes the variables characterizing the reaction to pass through the same values but in reversed order so that heats of reaction are of reversed sign (Prigogine & Defay, 1962). In the above reports on the calculation of the standard free energy of association of apolipoproteins with phospholipids, no systematic tests for reversibility were performed.

Until now, only the experiments of Tall et al. (1976, 1977) on the thermal denaturation of apo A-I in the absence and

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[1] Abbreviations: apo A-I, apolipoprotein A-I; CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; Gdn·HCl, guanidine hydrochloride; HDL, high-density lipoprotein; PC, phosphatidylcholine; VLDL, very low density lipoprotein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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presence of DMPC are such that the denaturation of apo A-I under both conditions can be tested against the above criterion for reversibility and equilibrium. Tall and co-workers (Tall et al., 1976) have shown that the thermal denaturation and subsequent renaturation of free apo A-I are reversible. However, the data of Tall et al. (1977) on the denaturation and renaturation of apo A-I/DMPC complexes clearly indicate that in this case the reaction exhibits hysteresis and is then thermodynamically irreversible. Thus, heating complexes of apo A-I and DMPC gives an endothermic transition centered around 90 °C which is associated with the denaturation of the complexes, but there is no corresponding exothermic transition upon cooling of the denatured complexes. On the contrary, a new exotherm associated with the renaturation of apo A-I appears at around 55 °C. After the system is held at 24 °C, during which time recombination of lipid and protein occurs, the original heating and cooling cycle could be repeated.

When a reaction is reversible, the Gibbs–Helmholtz equation describes the relationship between the enthalpy and the free energy as a function of the temperature (Prigogine & Defay, 1962). As mentioned above, Hickson et al. (1981) and Pownall et al. (1981) have reported that the free energy of binding for carboxymethylated apo A-II to DMPC does not change with temperature so it follows from the Gibbs–Helmholtz equation that the enthalpy of reaction is zero. This is inconsistent with the large heat of association observed upon recombination of apolipoproteins and DMPC (Massey et al., 1979, 1981a; Rosseneu et al., 1976a,b).

Collective consideration of these studies raises the question of whether the binding of apolipoproteins to phospholipids is a reversible process. We have addressed the question of the reversibility of the binding of apo A-I to phospholipids by investigating the reaction paths of the denaturation and renaturation of apo A-I/DMPC complexes under the influence of Gdn·HCl, which is known to dissociate apo A-I from high-density lipoprotein (HDL_3) (Gwynne et al., 1975; Nichols et al., 1976; Forte et al., 1979) and to denature apo A-I complexed with DMPC (Verdery & Nichols, 1974; Swaney & Braithwaite, 1980; Massey et al., 1981b). The average structure of apo A-I and the distribution of protein between free and bound states on addition of Gdn·HCl to apo A-I/DMPC complexes have been monitored. The denaturation of such complexes follows a reaction path quite different from that of the renaturation process; the desorption of apo A-I from the lipid/water interface is the irreversible step involved. Consequently, the binding of apo A-I to phospholipids in such a system is a thermodynamically irreversible process so that the affinity of apo A-I for phospholipids cannot be calculated by using the standard approaches of equilibrium thermodynamics to analyze the data.

Experimental Procedures

Materials

Apolipoprotein A-I was isolated from the total HDL fraction ($1.063 < d < 1.21$ g/mL) of human serum by the urea–Sephacryl column chromatographic method of Scanu et al. (1969) as modified by D.-J. Reijngoud (unpublished results). [^{125}I]Apo A-I with a specific activity of 80 dpm/ng was prepared according to McFarlane (1958). Chromatographically pure L-α-phosphatidylcholine, β,γ-dimyristoyl, was purchased from Calbiochem (LaJolla, CA), Na^{125}I from New England Nuclear (Boston, MA), [^{14}C]DMPC from Applied Science Laboratories, Inc. (State College, PA), Scintiverse from Fisher Scientific Co. (Pittsburgh, PA), RbCl from Chemical Dynamics (South Plainfield, NJ), and Gdn·HCl from Bethesda Research Laboratories (Bethesda, MD). Other chemicals were analytical grade.

Methods

DMPC Vesicles. Weighed amounts of DMPC and [^{14}C]-DMPC were mixed in redistilled chloroform/methanol (2:1 v/v), and the solvent was evaporated under N_2; any remaining solvent was removed under vacuum for 2 h at 40 °C. Dispersions of DMPC (10 mg/mL) in buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, and 0.02% w/v NaN_3) were sonicated with a Branson Model 350W sonifier equipped with a tapered microtip by using a power setting of 4 for 4 min at a temperature between 25 and 50 °C.

Association of Apo A-I/DMPC. Apo A-I, freshly desalted from 4 M Gdn·HCl by passage over a short (5 × 1 cm) Bio-Rad P2 column (Pownall et al., 1978a), and [^{14}C]DMPC vesicles were incubated at the indicated apo A-I:DMPC ratio in buffer supplemented with various concentrations of Gdn·HCl for 24 h at 24 °C. The order of addition was Gdn·HCl as an 8 M solution, apo A-I, DMPC, and buffer. Before addition of DMPC, the concentration of Gdn·HCl was always >2 M. For circular dichroism spectroscopy and density gradient ultracentrifugation, the protein concentrations in the incubation medium were 0.06–0.10 and 0.25–0.30 mg/mL, respectively. After incubation, the samples were cooled on ice to 4 °C and dialyzed overnight against buffer to remove Gdn·HCl, since hardly any reaction between apo A-I and DMPC occurs at this temperature (Pownall et al., 1978a).

Dissociation of Apo A-I/DMPC. Freshly desalted apo A-I and [^{14}C]DMPC vesicles were incubated at the indicated molar ratios of apo A-I and DMPC in buffer for 24 h at 24 °C at a protein concentration of about 1 mg/mL. After incubation, NaBr was added to increase the density to 1.21 g/mL, and the solution was centrifuged for 24 h at $226000g_{max}$ at 4 °C. After centrifugation, the top 2 mL from the 10-mL solution was collected and dialyzed overnight against buffer at 4 °C. Gdn·HCl was added to samples of these lipid/protein complexes which were then incubated at various Gdn·HCl concentrations in buffer at 24 °C. Aliquots were withdrawn at appropriate intervals and the circular dichroism spectra recorded. After incubation, samples were prepared for density gradient ultracentrifugation by cooling to 4 °C and dialyzing overnight against buffer.

Circular Dichroism (CD). CD spectra were recorded at 24 °C with a JASCO J-41A spectropolarimeter calibrated with a 0.1% (w/v) *d*-10-camphorsulfonic acid solution. The molar ellipticity at 220 nm was calculated according to $[\theta]_{220} = (MRW)\theta_{220}/(10lc)$, where θ_{220} is the observed ellipicity at 220 nm in degrees, MRW the mean residue molecular weight (=114) (Scanu et al., 1975), l the path length in centimeters (0.1 cm), and c the protein concentration in grams per milliliter.

Density Gradient Ultracentrifugation. At 4 °C, 2 mL of sample containing lipid/protein complexes was placed in a centrifuge tube, and another 1.85 mL to which 0.75 g of RbCl had been added was layered beneath the first layer. Centrifugation was performed in a Beckman SW 60 rotor for 24 h at 55 000 rpm at 10 °C. Longer runs led to a progressive release of apo A-I from 1:100 mol/mol apo A-I/DMPC complexes. The combined influence of high salt concentrations and the centrifugal fields led to a release of 2% of total protein in 24 h. Shorter spins did not give a complete separation of free and DMPC-associated apo A-I. After centrifugation, the gradients were separated into 25–30 fractions in preweighed tubes (7 drops/fraction). After fractionation, the tubes were weighed, and the refractive index (n) was measured. If ^{125}I

label was present, the fractions were counted in a Beckman Gamma 300 γ counter prior to the measurement of the refractive index. Aliquots were taken from the fractions for the determination of [^{14}C]DMPC by liquid scintillation in a Beckman LS230 liquid scintillation counter and for the determination of protein by fluorescence in a Turner Model 430 spectrofluorometer equipped with a broad-band film polarization filter to suppress interference by scattering of multilamellar vesicles of DMPC (excitation wavelength 285 nm; emission wavelength 360 nm). A solution of tryptophan in buffer was used for calibration; this was added to the cuvette to correct for quenching by RbCl ([RbCl] $\leq$ 0.08 M). The densities (d) of the fractions were calculated according to $d = 8.6154n - 10.4901$; this relationship was obtained by linear interpolation of the relevant data for RbCl (Washburn, 1928, 1930). If ^{125}I radioactivity was measured, the counting efficiency was corrected for the variation in density of the fractions. Gradient profiles of DMPC and apo A-I as a function of the distance of the midpoint of the fraction from the axis of rotation were subsequently calculated. The ranges of the recoveries were volume = 100 ± 1%, DMPC = 100 ± 10%, protein = 100 ± 30%, and ^{125}I = 100 ± 5%. "Free apo A-I" was considered to be the fraction of the total apo A-I fluorescence present in the fractions devoid of DMPC (less than 1 mol of DMPC/mol of apo A-I).

The accuracy of the "free apo A-I" measurement depends on there being no reassociation of free apo A-I with DMPC during an 18-h dialysis at 4 °C against buffer. Control experiments were performed to correct for the combined effect of reassociation and copurification of apo A-I and DMPC. When apo A-I and DMPC were mixed at a 1:100 molar ratio and dialyzed at 4 °C for 18 h after density gradient centrifugation, 9% of the free apo A-I was found to be associated with DMPC. The equivalent figure for 1:500 complexes was 32%. These factors were used to correct for the maximal releasable apo A-I; "corrected free apo A-I" (F_c) for the 1:100 complex was free apo A-I/0.91 and the 1:500 complex F_c = free apo A-I/0.68. The corresponding values for "bound apo A-I" were $1 - F_c$. In Figures 3 and 4B, ln free apo A-I/bound apo A-I was calculated from $\ln F_c/(1 - F_c)$.

Electron Microscopy. Lipid-containing dispersions were examined by negative staining procedures as described previously (Goldfine et al., 1981).

Analytical Procedures. Protein was determined by the $NaDodSO_4$–Lowry procedure (Markwell et al., 1978) and phosphorus according to Sokoloff & Rothblat (1974). The molar Gdn·HCl concentrations (C) in the incubation media were determined according to $C = 60.87n - 81.16$. This relationship was obtained by linear interpolation of the relevant data on Gdn·HCl solutions reported by Kielley & Harrington (1960).

Results

Free Apo A-I. Reynolds (1976) has shown that the denaturation of free apo A-I by Gdn·HCl yields the same relationship between the molar ellipticity and the Gdn·HCl concentration in either a forward or a reversed titration of the protein with Gdn·HCl. Another test for reversibility is shown in Figure 1 where the effects of Gdn·HCl on the molar ellipticity of apo A-I in solution (Figure 1A) and on the midpoint of the thermally induced denaturation (Figure 1B) are depicted. The midpoint of Gdn·HCl-induced denaturation is similar, whether obtained from an isothermal titration at 25 °C of apo A-I with Gdn·HCl (Figure 1A, C_m = 1.0 M) or from the extrapolation of the midpoints of thermally induced denaturation as a function of the concentration of Gdn·HCl

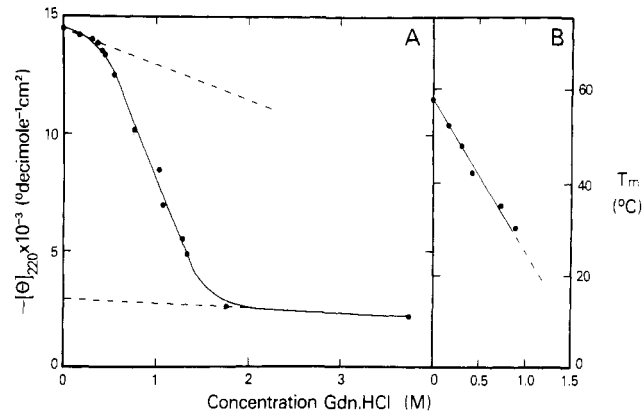


FIGURE 1: Denaturation of free, human apo A-I by guanidine hydrochloride. (A) Variation of the molar ellipticity at 220 nm, $[\theta]_{220}$, with Gdn·HCl concentration. (B) Influence of Gdn·HCl concentration on the midpoint (T_m) of the thermal unfolding, as detected by $[\theta]_{220}$.

(Figure 1B; T_m = 25 °C at C = 1.0 M). This confirms that the denaturation of free apo A-I is a reversible process [cf. Tall et al. (1976) and Edelstein & Scanu (1980)].

Free DMPC. No leakage of carboxyfluorescein from the interior of egg PC vesicles was observed when unilamellar vesicles containing 200 mM carboxyfluorescein were incubated with up to 3 M Gdn·HCl for 24 h [cf. Weinstein et al. (1977)]. At higher concentrations of Gdn·HCl, quenching of the carboxyfluorescein fluorescence became predominant. In agreement with this, Chen et al. (1980) showed that 1.6 M Gdn·HCl had no influence on the natural-abundance ^{13}C NMR spectrum of DMPC multilamellar liposomes. Furthermore, DSC studies on the gel–liquid-crystalline transition of hydrated DMPC in the absence and presence of 6 M Gdn·HCl showed that Gdn·HCl did not alter markedly the essential features of the endothermic transitions. In the presence of 6 M Gdn·HCl, the peak maximum temperatures of the reversible, pretransition, and gel to liquid-crystalline transitions are decreased by 3–5 °C compared to their values in water (Phillips, 1972). The transitions are also broadened so that the end of the gel to liquid-crystalline transition occurs 5 °C higher; this occurs without any detectable alteration in the enthalpy of transition. These results indicate that the bilayer structure of DMPC remains intact in the presence of these levels of Gdn·HCl (D.-J. Reijngoud and M. C. Phillips, unpublished results).

1:500 mol/mol Complexes of Apo A-I and DMPC. Incubation of apo A-I and sonicated dispersions of DMPC at a molar ratio of 1:500 (protein:lipid) leads to the formation of mainly vesicular complexes in which apo A-I adsorbs to DMPC vesicles without disruption of the vesicles [cf. Jonas et al. (1981)]. Electron micrographs of our preparations confirmed this, and the effects of increasing concentrations of Gdn·HCl on $[\theta]_{220}$ of such complexes are summarized in Figure 2. Figure 2A presents $[\theta]_{220}$ at 0 h (i.e., measured <3 min after the addition of Gdn·HCl) and after 72-h incubation while Figure 2B shows how $[\theta]_{220}$ varies with time of incubation. In contrast to the situation for free apo A-I in solution, the denaturation of lipid-bound apo A-I by Gdn·HCl is a multistage process (cf. Figures 1 and 2). Thus, at 0 h of incubation, two stages in the denaturation can be discerned, one centered around 2.7 M and another around 5.7 M Gdn·HCl (Figure 2A). Furthermore, the effect of Gdn·HCl concentration on the structure of apo A-I occurs by two distinguishable reactions (Figure 2B). Between 0 and 3 M Gdn·HCl, the decrease in $[\theta]_{220}$ is so rapid that it is complete before the first measurement. Increasing the concentration

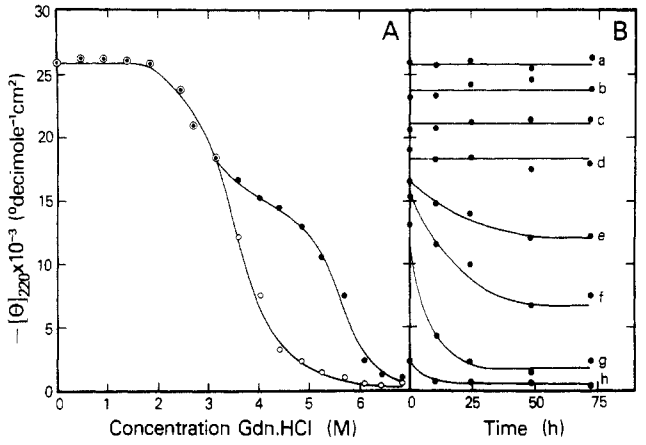


FIGURE 2: (A) Influence of Gdn·HCl concentrations on the molar ellipticity at 220 nm, $[\theta]_{220}$, of apo A-I in 1:500 mol/mol complexes with DMPC after 0- (●) and 72-h (○) incubations. (B) Effects of different Gdn·HCl concentrations are shown as a function of the time of incubation: (a) 0.00, (b) 2.44, (c) 2.69, (d) 3.15, (e) 3.62, (f) 4.01, (g) 4.84, and (h) 6.11 M.

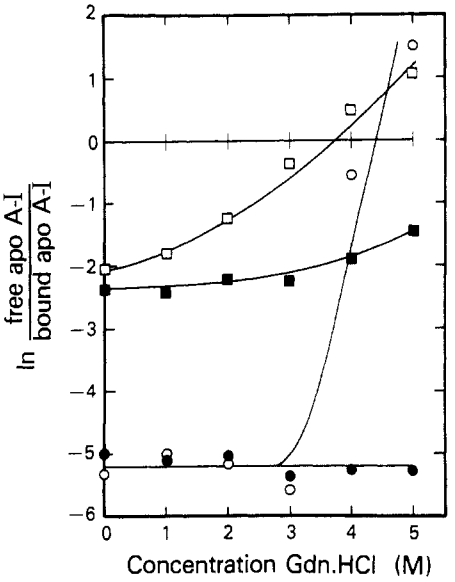


FIGURE 3: Effect of Gdn·HCl concentration on the ratio of free/bound apo A-I after 0 h (closed symbols) and 72 h (open symbols) of incubation with 1:100 (□, ■) and 1:500 (○, ●) mol/mol apo A-I/DMPC complexes, as measured by density gradient centrifugation.

of Gdn·HCl above 4 M gives rise to the appearance of a second reaction whose rate increases with increasing Gdn·HCl concentration and which is only complete after 48 h of incubation. At >6 M Gdn·HCl, both reactions become so rapid that the decrease in $[\theta]_{220}$ is complete before the first measurement. As a consequence of this time dependence, after a 72-h incubation the biphasic decrease in $[\theta]_{220}$ observed at 0 h disappears and $[\theta]_{220}$ decreases monotonically with increasing concentrations of Gdn·HCl (Figure 2A). Accordingly, the concentration of Gdn·HCl required for half-maximal denaturation decreases from 4.8 to 3.6 M.

The effect of Gdn·HCl concentration on the ratio of apo A-I free in solution to that associated with DMPC vesicles after 0 and 72 h of incubation, as determined by density gradient centrifugation, is summarized in Figure 3. The reported ratios for the 0-h incubation of free/bound apo A-I are upper limits because the fraction of free apo A-I ($F_c \simeq 0.006$; 5–10 ng/mL) was at the detection limits of our fluorescence measurements. It is apparent from Figure 3 that immediately after addition of Gdn·HCl (0-h incubation) there is no release of apo A-I from the complexes at all concentrations applied. In contrast, a 72-h incubation with 4–5 M Gdn·HCl induces the release of 0.37 and 0.72 of the total apo A-I initially associated with DMPC. Comparison of the data in Figures 2 and 3 indicates that the changes in $[\theta]_{220}$ between 0 and 72 h are concomitant with the appearance of free apo A-I in solution.

The effect of Gdn·HCl concentration on the renaturation of 1:500 mol/mol apo A-I/DMPC complexes is shown in Figure 4; the variations in $[\theta]_{220}$ and the distribution of apo A-I between solution and DMPC vesicles obtained from centrifugal data are shown in Figure 4, panels A and B, respectively. The incubations were carried out for 24 h after which time renaturation is essentially complete. Decreasing the concentration of Gdn·HCl from 6.5 to 0 M produces a continuous increase in the $[\theta]_{220}$ of apo A-I so that the intermediate plateau observed with the denaturation is absent (cf. Figures 2A and 4A). Comparison of the final values of $[\theta]_{220}$ at the various concentrations of Gdn·HCl shows that there is a difference of 0.8 M in the Gdn·HCl concentration required for a 50% change in $[\theta]_{220}$ during the renaturation and denaturation of apo A-I/DMPC complexes.

The data on the distribution of apo A-I between the aqueous and lipid phases show that initially the fraction of free apo

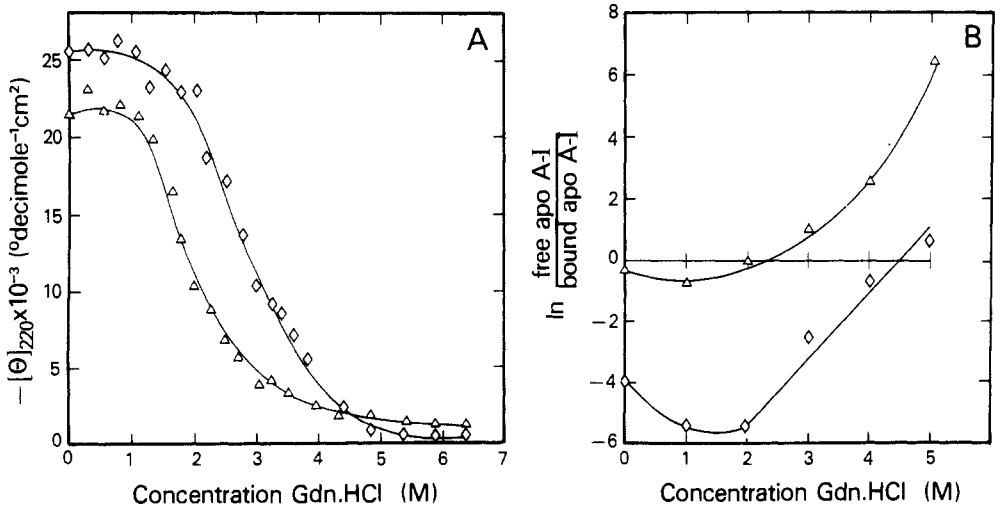


FIGURE 4: Effect of Gdn·HCl concentration on (A) the molar ellipticity at 220 nm, $[\theta]_{220}$, and (B) the ratio of free/bound apo A-I in a recombination of apo A-I and DMPC after a 24-h incubation at 24 °C for 1:100 (△) and 1:500 (◇) mol/mol apo A-I/DMPC, as measured by density gradient centrifugation.

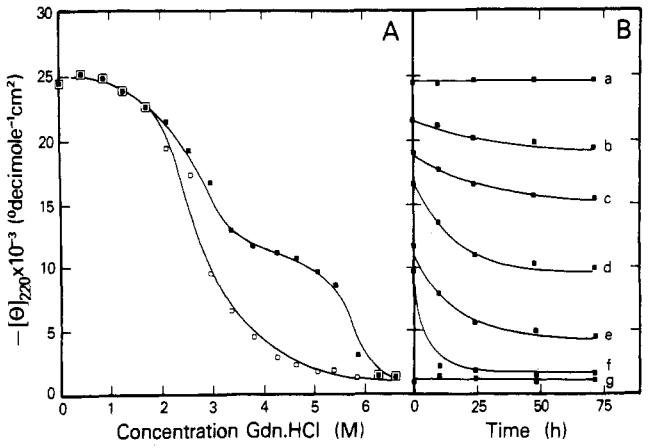


FIGURE 5: (A) Influence of Gdn·HCl concentration on the molar ellipticity at 220 nm, $[\theta]_{220}$, of apo A-I in 1:100 mol/mol complexes with DMPC after 0-h (■) and 72-h (□) incubations. (B) Effects of different Gdn·HCl concentrations are shown as a function of the time of incubation: (a) 0.00, (b) 2.15, (c) 2.57, (d) 2.98, (e) 3.79, (f) 5.08, and (g) 6.28 M.

A-I in the aqueous phase decreases with increasing concentration of Gdn·HCl to 1 M (Figure 4B). This presumably arises because addition of 1 M Gdn·HCl dissociates oligomers of apo A-I (Swaney & O'Brien, 1978; Edelstein & Scanu, 1980) which are incapable of adsorbing to DMPC vesicles. Between 1 and 2 M distribution of apo A-I between solution and complex is similar to that observed for apo A-I/DMPC complexes in the denaturation study (cf. Figures 3 and 4B). At [Gdn·HCl] >2 M, binding of apo A-I to DMPC decreases so that only ~40% of the total protein is bound at 5 M Gdn·HCl.

1:100 mol/mol Complexes of Apo A-I and DMPC. In view of the data of Nichols et al. (1976) and Forte et al. (1979) demonstrating that the size of the particle onto which apo A-I is bound is an important determinant in the response of apo A-I to Gdn·HCl, the effect of this denaturant has been studied on 1:100 mol/mol apo A-I/DMPC complexes and mixtures. Electron microscopy showed 1:100 mol/mol apo A-I/DMPC complexes to be discoidal in shape [cf. Andrews et al. (1976) and Morrisett et al. (1977)]. As with the 1:500 complexes, two stages centered around 2.5 and 5.5 M Gdn·HCl can be observed in the decrease of $[\theta]_{220}$ during denaturation of the 1:100 complex (Figure 5A). The second stage is not observed after 72-h incubation, and half-maximal denaturation occurs at 2.8 M Gdn·HCl (Figure 5B). The disappearance of this stage upon prolonged incubation is due to the presence of a slow reaction which is not completed before 48 h of incubation (Figure 5B). In contrast to the 1:500 complex, during denaturation of the 1:100 complex the second reaction can be detected at concentrations of Gdn·HCl as low as 2.1 M; the comparable concentration for the 1:500 complex is 3.6 M (cf. Figures 5B and 2B). It should be noted that our values of $[\theta]_{220}$ are not affected by spectral distortions due to absorbance (A) changes since addition of ≤3 M Gdn·HCl to 1:100 complexes had no effect on A_{220}, while between 3 and 6 M Gdn·HCl A_{220} values were maintained to within ±20%.

The reason for the above earlier onset of the second reaction in the decrease in $[\theta]_{220}$ can be deduced from the observations of the release of apo A-I at various concentrations of Gdn·HCl after 0 and 72 h of incubation (Figure 3). Immediately after addition of >1 M Gdn·HCl (0-h incubation), apo A-I is released from 1:100 complexes whereas no protein is released from 1:500 complexes (Figure 3). Incubation with any concentration of Gdn·HCl for 72 h induces release of apo A-I with 50% of the total apo A-I being released at 3.7 M rather than the 4.4 M observed with 1:500 complexes (Figure 3). These Gdn·HCl concentrations are not a function of the density gradient ultracentrifugation method used to separate free and bound apo A-I because gel filtration of 1:100 complexes incubated in 4 M Gdn·HCl for 24 h on a Sepharose 4B-CL column showed that the apo A-I was distributed equally between the free and bound states (J. Gryn, unpublished results). These above data demonstrate that the discoidal complexes are more susceptible than the vesicular complexes to denaturation by Gdn·HCl; our results probably give an underestimate of the difference in Gdn·HCl concentration because the 1:500 complexes contain some discoidal particles (Jonas et al., 1981).

Renaturation of the 1:100 apo A-I/DMPC complexes gives qualitatively similar results to those obtained with the renaturation of 1:500 complexes (Figure 4). $[\theta]_{220}$ of apo A-I in the presence of DMPC increases monotonically with decreasing concentrations of Gdn·HCl. The midpoint of renaturation occurs at 2.0 M Gdn·HCl so that the difference between the concentration of Gdn·HCl at which half-maximal renaturation and denaturation occurs is 0.8 M. This difference is the same as that observed with the 1:500 complex (cf. Figure 2A, 72 h, and Figure 4A). The distribution of apo A-I between the aqueous and lipid phases as a function of Gdn·HCl concentration is shown in Figure 4B. Qualitatively, apo A-I partitions between the two phases in a similar way for both 1:100 and 1:500 complexes, although with the 1:100 complex the proportion of apo A-I which is free is higher at all Gdn·HCl concentrations.

Exchange of Apo A-I between Solution and 1:100 mol/mol Apo A-I/DMPC Complexes. From the comparison of the ratio of free/bound apo A-I for the denaturation and renaturation of 1:100 complexes, it is clear that apo A-I does not reach a true equilibrium distribution between the aqueous and lipid phases. For further investigation of this mechanism, either 1:100 complexes of [^{125}I]apo A-I/DMPC were incubated with added unlabeled apo A-I or [^{125}I]apo A-I was added to a mixture of unlabeled apo A-I and DMPC at a 1:100 mol/mol ratio which had already been allowed to recombine for 24 h at 24 °C. The added apo A-I was incubated for 24 h at 24 °C to allow any exchange to occur; previous work (Shepherd et al., 1977; Pownall et al., 1978b; Lagocki & Scanu, 1980; Rosseneu et al., 1981; Van Tornout et al., 1981) has shown that displacement of apo A-I from a lipid/water interface by apolipoproteins is essentially complete within a few minutes.

Incubation of 1:100 complexes of [^{125}I]apo A-I/DMPC in the absence of added apo A-I yields the same ratio of free/bound protein for [^{125}I]apo A-I and unlabeled apo A-I, indicating that iodination does not modify the affinity of the protein for DMPC. Addition of an approximately equal amount of unlabeled apo A-I to 1:100 complexes of [^{125}I]apo A-I/DMPC causes desorption of [^{125}I]apo A-I with a concomitant incorporation of unlabeled apo A-I into the complexes, as indicated by an increase in the ratio of free/bound [^{125}I]apo A-I from 0.12 to 0.23 and a decrease in the ratio of free/bound unlabeled apo A-I. However, the specific activities of apo A-I in the aqueous and lipid phases do not equilibrate during a 24-h incubation (specific activity of free/bound apo A-I = 3.78). Consistent with this, [^{125}I]apo A-I added to recombinants of unlabeled apo A-I and DMPC also does not equilibrate (specific activity of free/bound apo A-I = 0.17).

Discussion

The binding reaction of apo A-I and DMPC has to be reversible in order for the standard free energy of transfer of apo A-I from the aqueous to the lipid phase to be calculated

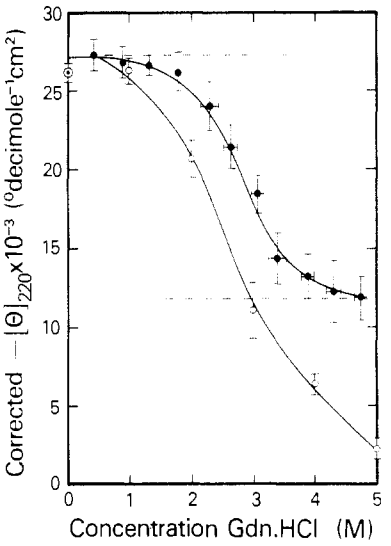


FIGURE 6: Effect of Gdn·HCl concentration on the molar ellipticity at 220 nm, $[\theta]_{220}$, of apo A-I bound at the surface of DMPC particles: (●) denaturation of 1:100 and 1:500 mol/mol apo A-I/DMPC complexes after 0- and 72-h incubations; (○) renaturation of 1:100 and 1:500 mol/mol apo A-I/DMPC complexes after 24 h of incubation. The error bars represent ±SD (n = 4).

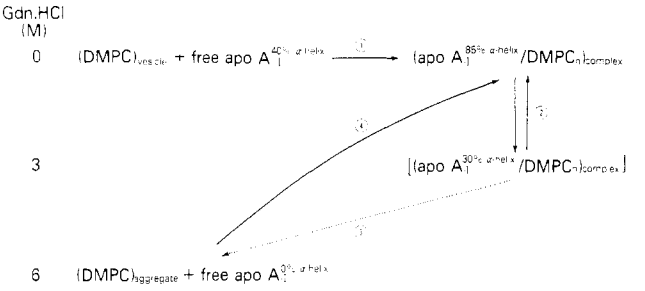
from the distribution of apo A-I between these two phases. However, the present investigation of the isothermal denaturation and renaturation of 1:100 and 1:500 mol/mol apo A-I/DMPC complexes shows clearly that the final values of both $[\theta]_{220}$ and the ratio of free/bound apo A-I at various concentrations of Gdn·HCl are dependent on the initial state of apo A-I and DMPC. At both molar ratios of apo A-I and DMPC, apo A-I is more resistant to denaturation by Gdn·HCl when the denaturant is added to the complex than when it is added to the mixture of apo A-I and DMPC.

The following discussion of these observations will be presented in three parts: (1) molecular mechanisms involved; (2) feasibility of deriving thermodynamic parameters describing the lipid/protein interaction; and (3) the physiological significance of the phenomena.

Molecular Mechanisms. Inspection of the denaturation of apo A-I/DMPC complexes shows that this process involves two consecutive reactions. Separation of the reactions is possible when the effects of Gdn·HCl on surface-bound and free apo A-I are considered; this is achieved by correcting the observed average $[\theta]_{220}$ for the amount of apo A-I free in solution as determined from ultracentrifugation. Figure 6 shows these data for the denaturation and renaturation of both the 1:100 and 1:500 apo A-I/DMPC complexes. It is apparent that for the denaturation of these complexes, irrespective of the molar ratio, surface-bound apo A-I can be only partially unfolded by Gdn·HCl essentially regardless of the time of incubation with Gdn·HCl. The slow step in the overall denaturation is the desorption of this partly unfolded apo A-I from the lipid/water interface (see Scheme I). In the renaturation, no such intermediate structure is observed.

It is likely that the partly unfolded protein molecules are attached to the lipid surface by means of their most apolar residues. This is illustrated by the observations of Swaney & Braithwaite (1980) and by Massey et al. (1981b) on the effect of Gdn·HCl on the emission maximum of Trp fluorescence when apo A-I/DMPC complexes are exposed to Gdn·HCl. A major red shift is observed indicative for the exposure of Trp to the aqueous phase at around 5 M Gdn·HCl; this coincides with the desorption reaction observed by our centrifugation data (step 3 in Scheme I). Thus, the need for a cooperative

Scheme I



displacement of such apolar residues from the lipid/water interface stabilizes the intermediate state during the denaturation process. Furthermore, it makes the desorption reaction essentially an irreversible process.

(A) Denaturation of Apo A-I on the Surface of Complexes with DMPC. The denaturation of apo A-I while attached to the surface of the complex involves a helix to coil transition (see step 2 in Scheme I). Since these types of transitions are normally reversible, it can be assumed that the denaturation of surface-bound apo A-I is reversible and accordingly can be evaluated quantitatively as a reversible, two-state, process. In general, there are two possible explanations for the DMPC-induced increase in stability of apo A-I to denaturation by Gdn·HCl (Elwell & Schellman, 1979): either the native, lipid-associated apo A-I increases in stability or Gdn·HCl becomes less effective in bringing about the denaturation when lipid is present. These two possibilities can be distinguished by an analysis of the surface denaturation of apo A-I according to a model in which the unfolding of the protein is driven by the enhancement of the binding of Gdn·HCl to the protein due to the exposure of binding sites upon unfolding (Tanford, 1970; Aune & Tanford, 1969). These binding sites are the peptide bonds and aromatic residues in proteins (Robinson & Jencks, 1965; Lee & Timasheff, 1974). This model of binding then leads to the following relation between the free energy of denaturation (ΔG_D) and the Gdn·HCl activity (a) (Aune & Tanford, 1969):

$$\Delta G_D = \Delta G_D^\circ - \Delta nRT \ln (1 + ka) \quad (1)$$

where ΔG_D° is the standard free energy of denaturation and a measure of the stability of the native structure, Δn the moles of Gdn·HCl bound per mole of protein needed to bring about the transition and as such a measure of the effectiveness of Gdn·HCl in inducing denaturation, and k the average association constant of Gdn·HCl with the binding sites on the protein [according to Pace & Vanderburg (1979), k = 0.6 M^{-1}). The ΔG_D is calculated according to

$$\Delta G_D = -RT \ln K_D \quad (2)$$

in which K_D is obtained from the relationship

$$K_D = \frac{[\theta]_N - [\theta]}{[\theta] - [\theta]_D} \quad (3)$$

where $[\theta]_N$ and $[\theta]_D$, respectively, are the extrapolated molar ellipticities for the native and the denatured forms of apo A-I at the particular activity of Gdn·HCl and $[\theta]$ is the observed molar ellipticity at that activity of Gdn·HCl. In Figures 1 and 6, the extrapolations are shown for both native and denatured apo A-I. Pace & Vanderburg (1979) in their discussion of eq 1 showed that the evaluation of ΔG_D° is most accurately achieved by using the mean ionic activity of Gdn·HCl ($a_\pm$) as the concentration scale. When Δn is evaluated by using

the ionic activity ($a_+ = [a_\pm]^{1/2}$), it becomes almost equal to the change in absolute solvation of the protein over the range of Gdn·HCl concentrations required for denaturation.

When the mean ionic activities for Gdn·HCl are calculated according to Pace & Vanderburg (1979), the analysis of the denaturation of free apo A-I (Figure 1) according to eq 1 yields a value for $\Delta G_D^\circ = 4.2 \pm 0.5$ kcal/mol of apo A-I. Similar analysis of the data of Edelstein & Scanu (1980) for free apo A-I gives $\Delta G_D^\circ = 4.1$ kcal/mol of apo A-I, in close agreement with our value. Using ionic activities for Gdn·HCl in eq 1, Δn associated with the observed denaturation of free apo A-I is 36 ± 1 mol of Gdn·HCl/mol of apo A-I. Our analysis of the data of Edelstein & Scanu (1980) gives $\Delta n = 34$ mol of Gdn·HCl/mol of apo A-I. Absolute solvation data for free apo A-I (Edelstein & Scanu, 1980) can be used to calculate the increase in bound Gdn·HCl over the transition range for the denaturation of free apo A-I [0.4–2 M Gdn·HCl; cf. Figure 1 and Edelstein & Scanu (1980)]. At 0.4 M Gdn·HCl, 27 mol of Gdn·HCl/mol of apo A-I is bound, which increases to 63 mol of Gdn·HCl/mol of apo A-I at 2 M Gdn·HCl. This increment of 36 mol of Gdn·HCl/mol of apo A-I is identical with that calculated by applying eq 1 to the denaturation of free apo A-I.

The data presented in Figure 6 for surface-bound apo A-I were subjected to a similar analysis, yielding $\Delta G_D^\circ = 5.5 \pm 1.2$ kcal/mol of apo A-I and $\Delta n = 35 \pm 3$ mol of Gdn·HCl/mol of apo A-I. It is apparent that the stabilities of surface-bound and free apo A-I against denaturation of Gdn·HCl and the moles of Gdn·HCl required to induce denaturation are essentially the same. However, the denaturation of surface-bound apo A-I does not occur until higher concentrations of Gdn·HCl are present. Since the increase in Gdn·HCl needed for denaturation is not due to an increased stability of the native form of apo A-I in the presence of DMPC, the effectiveness of Gdn·HCl must be diminished when lipid is present. The reason for this decrease is the occupation of binding sites for Gdn·HCl on apo A-I by DMPC. A lower estimate of the number of binding sites occupied by DMPC can be obtained if Δn for surface-bound apo A-I is compared to the change in absolute solvation of free apo A-I over the range of 2–5 M Gdn·HCl, the transition range for denaturation of complexed apo A-I (see Figure 6). From the data of Edelstein & Scanu (1980), it can be calculated by linear interpolation between 2 and 6 M Gdn·HCl that this change amounts to +77 mol of Gdn·HCl/mol of apo A-I. Since $\Delta n = 35$ for the denaturation of the apo A-I/DMPC complex, it can be concluded that more than half of the Gdn·HCl binding sites are masked by DMPC. This is consistent with our present understanding of the localization of apo A-I in lipid/protein complexes [for a review, see Morrisett et al. (1977)]. Apo A-I molecules in these complexes must be intercalated between phospholipid molecules so that about half of the peptide bonds and most of the aromatic side chains are masked from the aqueous phase [cf. the fluorescence data of Swaney & Braithwaite (1980) and Massey et al. (1981b)].

(*B*) *Desorption.* Step 3 in Scheme I represents the irreversible step in the unfolding path of surface-bound apo A-I. This irreversibility is presumably due to the requirement for cooperative exposure of apolar residues to the aqueous phase. The energy barrier of this cooperative hydration step is such that appreciable desorption rates can only be observed if an additional source of energy such as an increase in the surface pressure in the complex becomes available. A possible cause of such an effect could be the partial unfolding of surface-bound apo A-I by Gdn·HCl.

Since the protein is distributed over the entire external surface of the vesicular 1:500 complexes, it is possible to make a reasonable assessment of the influence of apo A-I on the surface pressure in the PC bilayer. For vesicles (radius $\simeq$100 Å) containing about 2500 PC molecules, of which two-thirds are in the outer shell of the bilayer, geometrical considerations lead to an average surface area of 76 Å^2/PC molecule [cf. Huang & Mason (1978) and Watts et al. (1978)]. This surface area is equivalent to a surface pressure of about 12 mN m^{-1} for a liquid-expanded monolayer of PC molecules spread on an air/water interface [for a review, see Phillips (1972)]. Insertion of an average of five molecules of apo A-I into the surface of a vesicle (to give an apo A-I:PC molar ratio = 1:500) with an average surface area of 15 Å^2/residue (Phillips & Sparks, 1980; Shen & Scanu, 1980) leads to a decrease in the average surface area from 76 to 65 Å^2/PC molecule; for a liquid-expanded PC monolayer, this is equivalent to an increase in surface pressure from 12 to 23 mN m^{-1} (Phillips, 1972). This final surface pressure is comparable to the collapse pressure of absorbed apo A-I at an air/water interface (Phillips & Sparks, 1980) at which point protein molecules tend to desorb from interfaces (Graham & Phillips, 1979; MacRitchie, 1978). Consequently, unfolding of the protein in the surface of the complex will tend to increase the surface pressure above its own collapse pressure and accordingly lead to desorption of the protein.

Thermodynamics. A quantitative description of the binding of apo A-I to DMPC from the partitioning of the protein between the aqueous and lipid phases is possible only if the binding reaction is thermodynamically reversible. Our data showing that the denaturation of apo A-I/DMPC complexes by Gdn·HCl after 72 h of incubation does not coincide with the renaturation of apo A-I with DMPC prove that for both 1:100 and 1:500 complexes the overall denaturation is irreversible. Thus, equilibrium thermodynamic parameters cannot be calculated simply from the effects of Gdn·HCl, or from the ratio of free/bound apo A-I in such systems. Since hysteresis is observed in the denaturation and renaturation of apo A-I/DMPC complexes upon repetitive heating and cooling (Tall et al., 1977), similar considerations apply to the use of heats of denaturation to derive free energies of stabilization.

Physiological Significance. In vivo, apolipoproteins redistribute between the different classes of lipoproteins [for a review, see Eisenberg & Levy (1975)], and our data on the nature of the association and dissociation of apo A-I and DMPC suggest that these processes do not involve thermodynamic equilibria. In our experiments on the replacement of bound apo A-I by free apo A-I in the discoidal 1:100 complexes, a net mass transfer of protein from the water to the lipid/protein complex was observed. Other data on the replacement of bound apolipoproteins by free apolipoproteins in discoidal complexes by Rosseneu et al. (1981) also indicate that a net mass transfer is observed when bound apo A-I is replaced by free apo A-II. In their experiments, the replacement ratio was 1 bound apo A-I replaced by 2 free apo A-II. In the above displacements of apolipoproteins, the driving force is derived from the adsorption free energy of the excess protein mass moving to the interface, whereas in the metabolic interconversion of lipoproteins the additional source of energy that drives the redistribution of apolipoproteins comes from enzymatic reactions. For example, upon hydrolysis of the core lipids of VLDL or chylomicrons by lipoprotein lipase, the surface pressure of the lipoprotein can be increased by the movement of the polar lipids produced to the surface, thereby promoting the desorption of apolipoproteins from the surface

[cf. Eisenberg et al. (1972) and Eisenberg & Olivecrona (1979)].

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