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Unfolding Behavior of Human α_1-Acid Glycoprotein Is Compatible with a Loosely Folded Region in Its Polypeptide Chain†

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Received March 6, 1990; Revised Manuscript Received May 18, 1990

ABSTRACT: The unfolding of human plasma α_1-acid glycoprotein (AGP) induced by heat or guanidine hydrochloride was studied under equilibrium conditions. In thermal unfolding, an intermediate state was detected by the appearance of unusual positive difference absorption bands in the 287-295-nm region, which occurred at lower temperatures than the common denaturation bands at 284 and 291 nm. The formation of this intermediate species apparently involves a local conformational change that perturbs the environment of tryptophyl residues, without affecting the secondary structure of the protein as judged from circular dichroism spectra. On the other hand, denaturation of the glycoprotein induced by guanidine hydrochloride seemed to follow a two-state model with no evidence of any intermediate species; however, the analysis of the transition curve indicated that the change in the accessibility to solvent of amino acid residues of AGP upon unfolding is significantly lower than those observed for other proteins. According to these results, it is proposed that part of the polypeptide chain in native AGP, namely, that from residue 122 to the C-terminus, may be "loosely" folded.

Many small proteins undergo unfolding transitions which closely follow a two-state mechanism under equilibrium conditions (Kim & Baldwin, 1982; Privalov, 1979). The thermodynamic study of these transitions has firmly established that the native state of proteins is only marginally stable; the Gibbs free energy change for the unfolding process, ΔG_U, is typically in the range of 20-60 kJ mol^{-1} under physiological conditions (Privalov, 1979; Pace 1975). This low value of ΔG_U results from a delicate balance between the changes in enthalpy, ΔH_U, and entropy, ΔS_U, that occur during protein denaturation. Both ΔH_U and ΔS_U are strongly temperature dependent due to the difference in heat capacity of denatured and native states, ΔC_{p_U}. It has been shown (Privalov, 1979; Privalov & Gill, 1988) that ΔH_U extrapolated to 110 °C is around 54 J g^{-1} for most globular proteins; however, some proteins suspected to be "loosely" folded display smaller ΔH values. Then, it seems that the unfolding thermodynamics of a protein reflects some features of the structural organization of the macromolecule.

In this work, we studied the unfolding of human α_1-acid glycoprotein (AGP)[1] induced by heat or guanidine hydrochloride (GuHCl). This plasma glycoprotein has a protein moiety with a molecular mass of 21 536 Da (Dente et al., 1985), and about 40% of its total mass is carbohydrate (Schmid, 1975). Although the physiological role of AGP is not clear at present, it has been found that this protein binds steroid hormones (Westphal, 1971) and a number of basic drugs (DeLeve & Piafsky, 1981). On the other hand, apart from an estimated content of secondary structures in the protein moiety (Aubert & Loucheux-Lefebvre, 1976), very little is known on the structure of this conjugated protein. The results presented in this paper indicate that human AGP is less stable than other common globular proteins; this lower stability seems to be attributable to a low degree of

† This work was supported in part by Secretaría de Educación Pública de México (PROIDES; Convenio C89-01-0221).

[1] Abbreviations: AGP, α_1-acid glycoprotein; GuHCl, guanidine hydrochloride; CD, circular dichroism.

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"compactness" in part of the polypeptide chain which may possibly be located between residue 122 and the carboxyl terminus.

Materials and Methods

Human AGP was isolated from the supernatant solution of Cohn fraction V (Cohn et al., 1946) obtained from pooled normal plasma according to the Beskorovainy and Winzler method (1961). The homogeneity of this preparation was established by agarose gel electrophoresis, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, gel filtration, and ion-exchange high-performance liquid chromatography. Protein concentration was determined spectrophotometrically using $A^{1\%}_{1cm,278} = 8.93$ (Schmid, 1975). Guanidine hydrochloride (GuHCl) grade I was obtained from Sigma Chemical Co. GuHCl concentration was calculated by measuring the refractive index of the solutions according to the method of Nozaki (1972). All other chemicals were of analytical grade.

Ultraviolet Difference Spectroscopy. Difference spectroscopy was performed on a VARIAN DMS-80 double-beam spectrophotometer equipped with an expanded-scale recorder. Thermal difference spectra were obtained using matched 1.0-cm cells with independent water thermostatic control. In each cell, the temperature was controlled with a precision of ±0.2 °C. The reference compartment was maintained at 15 °C while the sample cell was heated at a rate lower than 1 °C/min. Once the experiment temperature was reached, 10-min incubation was permitted, a lapse we found sufficient to attain thermal and conformational equilibria. Samples were disolved in 0.05 M glycylglycine buffer, pH 5.25, to obtain a concentration of 0.12 mg/mL.

The denaturation of AGP induced by GuHCl was studied at 20 ± 0.2 °C, using matched double-chamber cells of 1.0-cm light path per chamber. A mixture of protein and denaturant was placed in one chamber of the sample cell and solvent in the other chamber. On the other hand, protein and denaturant solutions with the same concentrations used for the sample cell were placed separately in the chambers of the reference cell. The samples were prepared by mixing an identical volume of an AGP stock solution, different volumes of 8.0 M GuHCl solution, and enough volume of 0.01 M sodium acetate buffer, pH 5.0, to reach the same final volume. The final AGP concentration was 0.18 mg/mL. In all cases, 8-h incubation at 4 °C was permitted.

Circular Dichroism. CD spectra were recorded on a JASCO J-500A spectropolarimeter calibrated with (+)-10-camphorsulfonic acid (Hennessey & Johnson, 1982). The scanning window was 0.7 nm, and the dynode voltage never exceeded 900 V. A water-jacketed cell holder was used to maintain the desired temperature with a precision of ±0.2 °C, and the actual temperature inside the cell was measured with a thermistor probe. Samples employed were prepared in the same way as those used in difference spectroscopy experiments. The mean residue ellipticity, $[\theta]_{mrw}$, was calculated with a mean residue molecular weight of 118 (Dente et al., 1985).

Thermodynamic Analysis. Under the assumption of a two-state reversible process, where the native (N) and unfolded (U) states of a protein are thought to be in equilibrium with each other, the fraction of the macromolecule in the unfolded state can be calculated by eq 1 where y is the observable

$$f_U = \frac{y - y_N}{y_U - y_N} \quad (1)$$

parameter and y_N and y_U are the values of y characteristic of the native and unfolded states, respectively. The equilibrium constant, K_U, and the free energy change for the process N $\rightleftarrows$ U can be calculated from f_U by using eq 2 and 3. Thermal

$$K_U = \frac{f_U}{1 - f_U} \quad (2)$$

$$\Delta G_U = -RT \ln K_U \quad (3)$$

unfolding data are generally analyzed by means of a van't Hoff plot ($\ln K_U$ vs $1/T$). The slope of such a plot provides values of the enthalpy change for the unfolding process, ΔH_U, since $[\partial(\ln K)/\partial(1/T)]_P = -\Delta H/R$. Moreover, the heat capacity change of the transition can be obtained from the variation of ΔH_U as a function of the temperature. However, the method outlined above requires the evaluation of the slope of the van't Hoff plot at different temperatures, a procedure that can introduce additional errors to the experimental data. Therefore, we decided instead to use an expression that directly relates f_U to the absolute temperature; this expression is shown in eq 4 and 5, where T_m is the temperature at which $K_U = 1$

$$f_U = K_U/(1 + K_U) \quad (4)$$

$$K_U = \exp[(\Delta H_m/RT_m - \Delta C_{p_U}/R)(1 - T_m/T)](T/T_m)^{\Delta C_{pU}/R} \quad (5)$$

and ΔH_m is the enthalpy change evaluated at T_m. Equation 4 follows immediately from eq 2, while eq 5 can be derived from eq 6 which gives the free energy change as a function

$$\Delta G_U = \Delta H_m(1 - T/T_m) - \Delta C_{p_U}[(T_m - T) + T \ln (T/T_m)] \quad (6)$$

of the temperature for a two-state transition of constant ΔC_{p_U} (Elwell & Schellman, 1977). Estimated values of T_m, ΔH_m, and ΔC_{p_U} were obtained by nonlinear least-squares fitting (Bevington, 1969).

Data obtained from the study of the GuHCl-induced unfolding of AGP were analyzed by eq 1–3 in order to calculate ΔG_U at different denaturant concentrations. The free energy change of the unfolding process in the abscence of denaturant, $\Delta G_U^{H_2O}$, was estimated by two extrapolation procedures. In the first method, it was assumed that ΔG_U varies linearly with GuHCl concentration (eq 7) and that this linear dependence

$$\Delta G_U = \Delta G_U^{H_2O} - m[\text{GuHCl}] \quad (7)$$

continues to zero concentration of denaturant (Pace, 1975). According to Pace (1975), the coefficient m in eq 7 is a relative measure of the transition steepness; i.e., it reflects the cooperativity of the unfolding process. The second procedure used was based on the transfer model of Tanford (Tanford, 1970; Pace, 1975); this model is represented by eq 8, where α is the

$$\Delta G_U = \Delta G_U^{H_2O} + \alpha \sum_i n_i \delta g_{tr,i} \quad (8)$$

average fractional change in the degree of exposure to solvent of amino acid side chains, n_i is the total number of side chains of type i present in the protein, and $\delta g_{tr,i}$ is the free energy of transfer of a group of type i from water to GuHCl solution.

Values of $\delta g_{tr,i}$ were calculated by parabolic interpolation from data reported by Pace (1975). Then ΔG_U was plotted as a function of $\sum_i n_i \delta g_{tr,i}$, and the values of α and $\Delta G_U^{H_2O}$ were estimated from a linear regression.

Factor Analysis. With the aim to find out if our experimental data fitted a two-state transition, we analyzed them using the matrix eigenvector method known as factor analysis (Malinowski & Howery, 1980). In a first step, this technique is commonly used to determine the number of components or factors that can reproduce, by linear combination, a set of

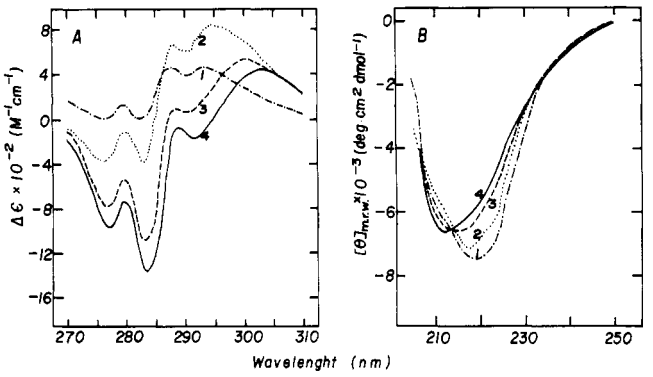

FIGURE 1: Thermal denaturation of 0.12 mg/mL AGP in 0.05 M glycylglycine buffer, pH 5.25. (A) Ultraviolet difference absorption spectra of AGP at different temperatures: (1) 29.9 °C; (2) 49.6 °C; (3) 58.2 °C; (4) 67.0 °C; reference cell at 15 °C. (B) Circular dichroism spectra recorded at (1) 16.8, (2) 44.6, (3) 49.5, and (4) 71.2 °C with a scanning window of 0.7 nm.

spectroscopic data (Hennessey & Johnson, 1981). Our spectroscopic data (either from difference absorption or from CD measurements) were grouped to form a data matrix, $[D]$, of m rows and n columns, where each column represented the spectrum observed at a given temperature. A symmetric square matrix was constructed by premultiplying the data matrix by its transpose, $[D]^T$. This square matrix was then diagonalized to find the $n \times n$ matrix of eigenvectors, $[Q]$, and the diagonal matrix of eigenvalues, $[\lambda]$, such that

$$[Q]^T[D]^T[D][Q] = [\lambda]$$

At this point, the original data matrix could be reproduced exactly by the product of two matrices:

$$[D] = [U][Q]^T$$

where

$$[U] = [D][Q]$$

However, the problem was to reproduce $[D]$ within experimental error with a minimum of eigenvectors. This restricted set of eigenvectors (principal factors, i.e., the factors with the higher eigenvalues) was found by computation of the residual standard deviation, RSD, according to

$$\mathrm{RSD} = \left[\frac{1}{m(n-f)}\sum_{i=f+1}^{n}\lambda_i\right]^{1/2}$$

where f is the number of factors in the restricted set and the λ_i values are the individual eigenvalues that form the diagonal entries in $[\lambda]$. The chosen value of f was the smallest one which gave an RSD lower or equal to the estimated experimental error.

Results

Thermal Unfolding. The thermal unfolding of AGP was studied by means of difference absorption spectroscopy and circular dichroism (CD). Figure 1A shows some of the spectra recorded at different temperatures (pH 5.25); the changes observed by both techniques were found to be more than 90% reversible by slow cooling of the AGP solution. The difference spectrum at high temperature (curve 4 in Figure 1A) displays three negative bands at 277, 284, and 291 nm which can be safely asigned to tyrosyl and tryptophyl residues that become exposed to the aqueous solvent when the protein is denatured (Donovan, 1969). It should be noted, however, that difference spectra at middle temperatures (curves 1 and 2 in Figure 1A) show two intense positive bands centered around 287 and 295 nm. According to Andrews and Forster (1972) and Anan-

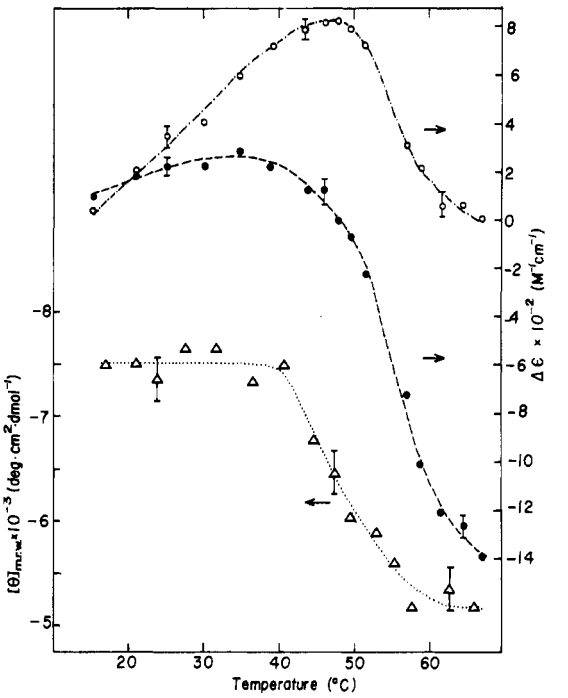

FIGURE 2: Effect of temperature on difference absorption at 295 nm (○) and 284 (●), and on ellipticity at 220 nm (△). Bars indicate experimental variation for each point.

Scheme I

tharayanan and Bigelow (1969a,b), these bands, which are rather unusual in protein difference spectra, could be originated from electrostatic effects on tryptophan chromophores; particularly, positive bands would be expected when the net charge around a tryptophyl residue becomes less positive or more negative. Nevertheless, a diminution in the polarity of the tryptophyl environment cannot be ruled out as a possible cause of the positive signals mentioned above (Andrews & Forster, 1972). Figure 2 shows the transition curves of AGP as followed by the difference absorption at 295 and 284 nm. It is evident that the onset of the positive band at the longest wavelength occurs at lower temperatures than the negative "denaturation" band. These results suggest that the thermal unfolding of AGP may be represented by Scheme I, where N and U are the native and unfolded forms of the protein, respectively, and X stands for at least one intermediate state, or an alternative native form, which is appreciably populated under equilibrium conditions. This proposal would be in agreement with the results of the calorimetric study of Halsall and Kirley (1982), who found that the ratio of calorimetric to van't Hoff enthalpies is around 1.22; such a value indicates that thermal unfolding of AGP departs from a two-state model.

According to the procedure outlined under Materials and Methods, we performed a factor analysis using 16 difference spectra corresponding to the range 15.3–67.0 °C and found that 2 abstract factors were necessary to reproduce the observed data within experimental error. This result clearly indicates the presence of an intermediate state in the thermal unfolding of AGP since for a two-state transition only one factor would be expected, inasmuch as the native-state difference spectrum is really a null vector. With the aim to identify physically significant factors, the spectrum of AGP at 67.0 °C (curve 4 in Figure 1A) was taken as representative

of the unfolded state and used to analyze all of the experimental spectra; this spectrum alone was insufficient to adequately reproduce the whole set of spectra, but when differences between reconstructed and experimental spectra were resubmitted to factor analysis, a second factor was obtained. Employing this second factor together with the unfolded-state factor, we were able to reproduce any of the spectra within experimental error. The spectral shape of the second factor, that may represent the intermediate state, was very similar to curve 2 in Figure 1A; i.e., it showed two maxima at 287 and 295 nm. As previously mentioned, these two positive bands are likely to be originated from charge or solvent perturbation of indole chromophores; hence, the interconversion of native and intermediate states seems to involve perturbations around the environment of tryptophyl residues in the AGP molecule, without altering significantly the hydrophobic interactions of buried amino acid residues.

The effect of temperature on the CD spectrum of AGP (pH 5.25) is illustrated in Figure 1B. There, the changes observed are mainly due to modifications in the secondary structure of the protein moiety since, as Aubert and Loucheux-Lefebvre (1976) have shown, the carbohydrate chains of this glycoprotein do not contribute significantly to the CD spectrum above 190 nm. The curves in Figure 1B clearly show an isodichroic point, indicating the presence of only two independent spectral forms. Indeed, when a set of 19 spectra covering the temperature range from 16.8 to 71.2 °C was factor-analyzed, 2 principal factors emerged. That is, the whole set can be explained in terms of only two spectra, i.e., those corresponding to native and unfolded forms of AGP (curves 1 and 4 in Figure 1B). Moreover, the transition profile constructed from ellipticity data at 220 nm approximately coincides with the curve corresponding to the negative difference absorption band at 284 nm (Figure 2). Thus, it seems that the putative intermediate in Scheme I possesses nativelike structure and that the conformational change leading to its formation is restricted to a local region of the molecule.

Although the presence of a stable intermediate state does not permit the use of direct van't Hoff analysis to calculate differences in enthalpy or heat capacity between the N and U states, it has been shown (Tanford, 1968; Cantor & Schimmel, 1980) that an apparent enthalpy change, ΔH_{vH}, can be obtained which is related in a rather complex way to the true ΔH_U. In general, it is expected that $\Delta H_{vH} < \Delta H_U$. In our case, considering one intermediate with the same circular dichroism spectrum as the native state, analysis of the transition monitored by ellipticity changes will provide apparent parameters which are given by the following expressions (see eq A3 and A4 in the Appendix):

$$\Delta H_{vH} = \Delta H_U - \Delta H_X\left(\frac{K_X}{1 + K_X}\right) \quad (9)$$

$$\Delta C_{p_{vH}} = \Delta C_{p_U} - \Delta C_{p_X}\left(\frac{K_X}{1 + K_X}\right) - \frac{\Delta H_X^2}{RT^2}\left[\frac{K_X}{(1 + K_X)^2}\right] \quad (10)$$

where subindex x refers to parameters for the conversion of N to X (N $\rightleftarrows$ X process). It should be noted that in eq 9 and 10 each of the terms in parentheses (or brackets) is greater than zero and less than unity; then, if we assume that ΔH_X and ΔC_{p_X} are positive, it is concluded that $\Delta H_{vH} < \Delta H_U$ and $\Delta C_{p_{vH}} < \Delta C_{p_U}$. However, since the formation of the X state seems to involve only a local conformational change in the AGP molecule, the associated enthalpy and heat capacity changes (ΔH_X, ΔC_{p_X}) may probably be small; therefore, ΔH_{vH} and $\Delta C_{p_{vH}}$ will likely approximate the true thermodynamic values, ΔH_U and ΔC_{p_U}.

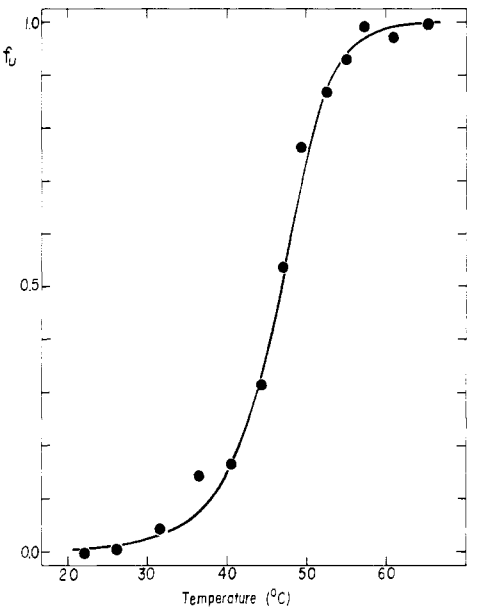

FIGURE 3: Transition curve for the thermal denaturation of AGP. The solid line represents the fitted function described under Materials and Methods, and dots represent values calculated from ellipticity data of Figure 2.

Table I: Thermodynamic Parameters of the Thermal Unfolding of α_1-Acid Glycoprotein at pH 5.25[a]

T_m (°C)	ΔH_{vH}[b] (kJ mol^{-1})	$\Delta C_{p_{vH}}$ (kJ mol^{-1} K^{-1})
46.6 ± 0.05	252 ± 25	8.8 ± 4.2

[a] Calculations were performed as described under Materials and Methods using ellipticity data of Figure 2. [b] Evaluated at T_m.

Ellipticity data in Figure 2 were used together with eq 1 to calculate fractions of unfolded AGP, f_U. In turn, these latter values (Figure 3) were fitted to the function described in eq 4 and 5 in order to estimate the melting temperature, T_m, and the apparent enthalpy and heat capacity changes. The results obtained by least-squares fitting are shown in Table I. The value of ΔH_{vH} (252 kJ mol^{-1}) should be extrapolated to 62 °C in order to be compared with the calorimetric parameters observed by Halsall and Kirley (1982) under conditions where polymerization did not affect significantly their results (pH 7.4, T_m = 62 °C; ΔH_{vH} = 401 ± 13 kJ mol^{-1}; and ΔH_{cal} = 493 ± 17 kJ mol^{-1}); under the assumption of constant ΔC_p, our ΔH_{vH} would be 387 kJ mol^{-1} at 62 °C, in good agreement with the corresponding value cited above.

In the case of ΔC_{p_U}, as far as we know no direct calorimetric determination has been done to date; however, Kirley et al. (1982) have estimated a ΔC_{p_U} of 6.7 ± 6.3 kJ mol^{-1} K^{-1} from a study of the binding of propranolol to AGP. Thus, in spite of the large uncertainity in its estimation, 8.8 kJ mol^{-1} K^{-1} seems to be a reasonable value for the heat capacity change that occurs during unfolding of this glycoprotein.

Unfolding Induced by Guanidine Hydrochloride. Figure 4A shows the difference absorption spectra of AGP corresponding to various GuHCl concentrations (pH 5.0). The changes observed were found to be 90% reversible by 3-fold dilution of denaturant. As in the case of thermal unfolding, two negative bands were present, centered at 292 and 285 nm, but in contrast, no significant positive bands were observed at any denaturant concentration. In addition, $\Delta\epsilon$ values for GuHCl unfolding were much larger than those corresponding to thermal unfolding (Figures 1A and 4A), indicating a greater

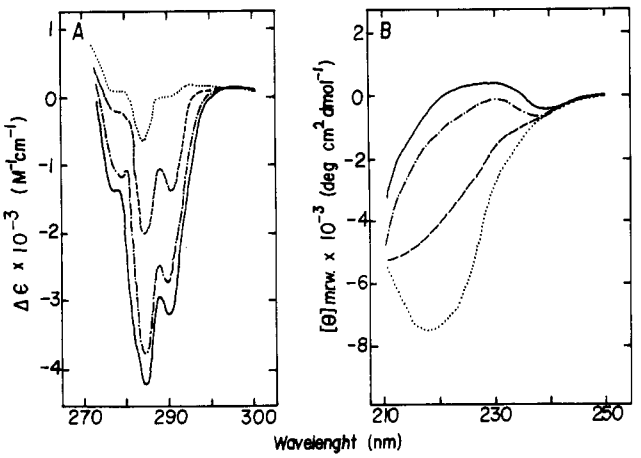

FIGURE 4: GuHCl denaturation of 0.18 mg/mL AGP in 0.01 M sodium acetate buffer, pH 5.0. (A) Ultraviolet difference spectra recorded at 1.9 (···), 2.2 (- - -), 2.8 (-·-), and 5.0 M (—) GuHCl concentration. (B) Circular dichroism spectra recorded at the following GuHCl concentrations: 0.0 (···), 2.0 (- - -), 4.0 (-·-), and 7.0 M (—).

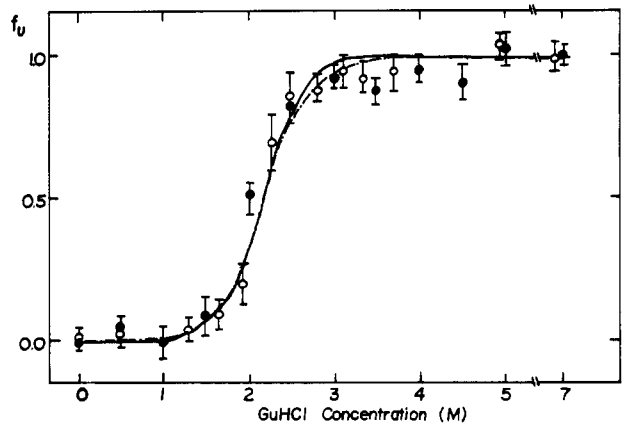

FIGURE 5: Transition curve of the GuHCl-induced denaturation of AGP. Solid and discontinuous lines represent the linear extrapolation method and Tanford's model, respectively. Data were obtained by difference spectroscopy (○) and circular dichroism (●); bars represent experimental variation.

exposure of aromatic residues in the unfolded state induced by GuHCl. The unfolding transition of AGP at pH 5.0, mediated by GuHCl, was also followed by changes in its CD spectrum (Figure 4B). Renaturation experiments demonstrated that the transition observed by CD changes was completely reversible. Comparison of Figures 1B and 4B shows that the CD spectrum of AGP at high GuHCl concentration is considerably less intense than the spectrum at high temperatures; similar observations have been reported for other globular proteins (Goto & Hamaguchi, 1987; Labhardt, 1982) and have been interpreted as indicative of residual structures in thermally unfolded proteins (Ptitsyn, 1987; Baldwin, 1986).

Figure 5 depicts the transition profiles calculated from difference absorption at 285 nm and ellipticity changes at 218 nm, in terms of the fraction of unfolded protein (eq 1). Since the two sets of experimental points fall on the same curve, the assumption of a two-state mechanism seems to be justified as a first approximation (Ghélis & Yon, 1982). Moreover, when data at other wavelengths (either from CD or from difference spectroscopy) were used, curves essentially identical with that in Figure 5 were obtained. Therefore, f_U values were used together with eq 2 and 3 to calculate free energy changes (Figure 6). The data of Figure 6 were fitted by least squares to either the linear (eq 7) or the Tanford (eq 8) model in order to obtain estimates of ΔG_U at zero denaturant concentration

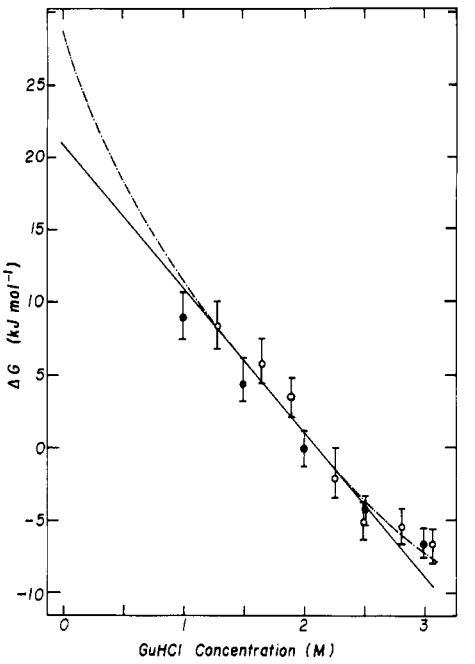

FIGURE 6: Extrapolation procedures for determining $\Delta G_U^{H_2O}$. Symbols are identical with those used in Figure 5.

Table II: Parameters That Characterize the Unfolding of α_1-Acid Glycoprotein by Guanidine Hydrochloride at pH 5.0 and 20 °C[a]

method	parameter	$[GuHCl]_{1/2}$[b] (M)	$\Delta G_U^{H_2O}$[c] (kJ mol^{-1})
linear extrapolation[d]	m = 10.2 kJ mol^{-1} M^{-1}	2.1	21.3
Tanford's model[e]	α = 0.12	2.1	28.9

[a] Calculations were performed as described under Materials and Methods using the data shown in Figure 5. [b] GuHCl concentration at the midpoint of the transition. [c] Free energy change in the absence of GuHCl. [d] From eq 7. [e] From eq 8.

($\Delta G_U^{H_2O}$) and the corresponding parameters of each model. Table II summarizes the results obtained.

Discussion

Our results showed that under equilibrium the thermal unfolding of AGP departs from a two-state model; i.e., at least one species X different from the native or unfolded protein can be detected in appreciable amounts as the temperature is increased. A similar conclusion was reached by Halsall and Kirley (1982) from the calorimetric studies of AGP thermal transition. These authors have mentioned that the variability in amino acid sequence within a given sample of this glycoprotein (Schmid et al., 1973) may be a cause for this apparent multistate transition; however, they have alternatively proposed that the AGP molecule may contain a region which is readily perturbed by ethanol and that undergoes a conformational change which only slightly affects the main transition endotherm. This later proposal would be consistent with our results since the X state in Scheme I apparently differs from the native state in the conformation around some tryptophyl residues, but otherwise maintains the secondary structure typical of native AGP.

As mentioned under Results, the formation of X state is accompanied by a perturbation of the tryptophyl environment that seems to involve either a decrease in polarity or a charge effect. Human α_1-acid glycoprotein has three tryptophyl residues in its polypeptide chain at positions 25, 122, and 160 (Schmid et al., 1973; Dente et al., 1985); of these residues, tryptophyl-160 is located in a highly charged region which

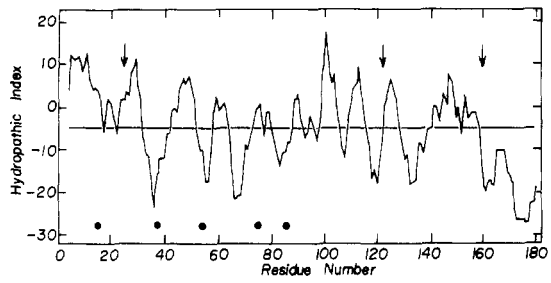

FIGURE 7: Hydrophobicity profile of AGP determined by using the hydropathy scale of Kyte and Doolittle (1982), with a span setting of seven residues. The maxima represent regions of relative hydrophobicity and the minima regions of relative hydrophilicity. The arrows show the positions of tryptophyl residues, and the dots indicate asparaginyl residues where carbohydrate chains are linked.

includes one aspartyl and two lysyls at positions 159, 161, and 162. Thus, a conformational change occurring in this region would likely be reflected in an electrostatic perturbation of residue 160. On the other hand, if the perturbation is due to a decrease in polarity of the medium, then the tryptophyl involved will be expected to be one which is exposed to aqueous solvent in the native state. According to Schmid et al. (1976), tryptophyl-122 is partially reactive with 2-hydroxy-5-nitrobenzyl bromide, while the other two are unreactive and, therefore, assumed to be buried. Moreover, the hydropathy profile of AGP (Figure 7) constructed following the procedure of Kyte and Doolittle (1982) shows that residues 122 and 160 are located in regions whose hydrophilicity is greater than the average while tryptophan-25 lies in a markedly hydrophobic zone. Therefore, whichever the perturbation mechanism being operative, it may be thought that the conversion of N to X produces a conformational change involving residues 122 or 160, or both.

Since thermal unfolding of AGP departs from a two-state model, thermodynamic parameters derived from standard van't Hoff analysis are expected to be smaller than true calorimetric values (Cantor & Schimmell, 1980; Tanford, 1968). However, eq 9 and 10 indicate that if the enthalpy and heat capacity changes associated with the N to X transition are small, as seems to be the case for AGP in view of the limited conformational change taking place, the van't Hoff parameters (ΔH_{vH}, $\Delta C_{p_{vH}}$) will closely approximate the true unfolding values (ΔH_U, ΔC_{p_U}). Thus, with all due reserve, the value of $\Delta C_{p_{vH}}$ estimated in this work (8.8 ± 4.2 kJ mol^{-1} K^{-1}) can be used to extrapolate the ΔH_U of unfolding (493 kJ mol^{-1} at 62 °C) determined by Halsall and Kirley (1982) to other temperatures. At 110 °C, ΔH_U would amount to 915 ± 200 kJ mol^{-1}; when only the polypeptide moiety of AGP (21.5 kDa) is considered, a specific enthalpy change of 38 ± 8 J g^{-1} is obtained. This value is lower than 54 J g^{-1} which is characteristic for globular proteins with a compact structure, while it compares well with values corresponding to proteins whose structures are thought to be "loosened" throughout or in part (Privalov, 1979).

In contrast to thermal unfolding, GuHCl-induced denaturation of AGP seems to follow a two-state model. Whether the X state mentioned above is unstable in the presence of GuHCl or simply is not detected under these conditions is a question that cannot be solved on the basis of our data. In any case, the transition of AGP seems to be as cooperative as those of other proteins, if judged from the value of the slope of the linear extrapolation procedure, m (Table II). This parameter, which is a measure of the transition steepness (Pace, 1975), lies in the range 7.9–16.2 kJ mol^{-1} M^{-1} for the proteins studied by Green and Pace (1974). On the other hand, $\Delta G_U^{H_2O}$ for the unfolding of AGP at 25 °C was estimated to be only 25.1 kJ mol^{-1} (average of the two values of $\Delta G_U^{H_2O}$ in Table II) which is certainly close to the lower limit of stability for globular proteins under physiological conditions (Privalov, 1979; Pace, 1975).

In terms of Tanford's model (eq 8), the unfolding of a protein is characterized by a fractional change in the degree of exposure of amino acid side chains, α. For typical globular proteins, it has been found (Pace, 1975; Tanford, 1970) that α varies between 0.17 and 0.35. In comparison, the estimated value for α was 0.12 (Table II) in the case of AGP. This low α value might be explained by an appreciable deviation from the two-state behavior, as has been shown to be the case for an immunoglobulin κ-type light chain (Rowe & Tanford, 1973). However, if the unfolding transition does not deviate too much from the two-state model, the low α would be indicative of relatively small changes in the solvent accessibilities of amino acid residues upon unfolding of the macromolecule. This would imply that in comparison with other proteins, either the native state of AGP has a larger fraction of solvent-exposed groups or the unfolded form has a smaller solvent accessibility. Considering that the transfer of a tyrosyl residue from the interior of a protein to the aqueous solvent would cause a difference molar absorption, $\Delta\epsilon$, of −700 and −130 M^{-1} cm^{-1} at 285 and 292 nm, respectively, while corresponding values for the transfer of a tryptophyl residue would be −1190 (285 nm) and −1620 M^{-1} cm^{-1} (292 nm) (Donovan, 1969; Herskovits & Sorensen, 1968), the difference spectrum of AGP in 5 M GuHCl (Figure 4A) appears to be consistent with the exposure of approximately two tryptophyls and three tyrosyls. These figures indicate that at least the aromatic residues being considered show a high degree of solvent accessibility in 5 M GuHCl since in native AGP two or three tryptophyls and four to five tyrosyls are assumed to be buried (Schmid et al., 1976) and expected to become exposed upon unfolding. On the other hand, the possibility that native AGP may have a fraction of exposed residues larger than other proteins seems to be supported by the hydrophilic properties of its carboxyl-terminal cyanogen bromide fragment (Ikenaka et al., 1972); as seen in Figure 7, the polypeptide region comprising residues 132–183 shows a highly hydrophilic character, and, therefore, it is reasonable to assume that several residues in this region may be exposed to the solvent.

In summary, the results of this study seem to be consistent with a picture of the AGP molecule in which part of the polypeptide chain is "loosely" folded, or at least less compactly structured than the rest of the chain. This proposal would explain the conformational change observed at low temperature during thermal unfolding, as well as the apparent high solvent accessibility of the native glycoprotein. Furthermore, according to our results, it is probable that the "low compactness" region be located between residues 122 and 183, a region where no carbohydrate chains are linked to the protein moiety (Figure 7). Therefore, carbohydrate residues probably do not play a significant role on the structural characteristics of this part of the polypeptide chain. It would be interesting to further study the influence of ligand binding on the unfolding behavior of AGP in order to elucidate whether the apparent low degree of compactness is related to the binding properties of this conjugated protein.

ACKNOWLEDGMENTS

We are very grateful to Centro Nacional de Transfusión Sanguínea and Cutter Laboratories de México for the generous supply of the supernatant of Cohn fraction V from which α_1-acid glycoprotein was isolated.

Appendix

If in a transition involving three states (N, X, U) the property being measured is the same for two of them (e.g., N and X), the apparent equilibrium constant calculated under the assumption of a two-state process, K_{vH}, will be

$$K_{vH} = f_U/(f_N + f_X) \quad \text{(A1)}$$

where f_U, f_N, and f_X are the fractions of each of the states. Dividing by f_N in the numerator and denominator of A1, and introducing the equilibrium constants $K_U = f_U/f_N$ and $K_X = f_X/f_N$, we obtain

$$K_{vH} = K_U/(1 + K_X) \quad \text{(A2)}$$

From this equation and the general relationship $\partial (\ln K_i)/\partial T = \Delta H_i/RT^2$, the apparent enthalpy change, ΔH_{vH}, can be expressed by the equation:

$$\Delta H_{vH} = \Delta H_U - \Delta H_X[K_X/(1 + K_X)] \quad \text{(A3)}$$

where ΔH_U and ΔH_X are the enthalpy changes for the true unfolding process (N to U) and for the conversion of N to X, respectively.

Since in general $\Delta C_{p_i} = \partial \Delta H_i/\partial T$, straightforward differentiation of eq A3 followed by rearrangement leads to

$$\Delta C_{p_{vH}} = \Delta C_{p_U} - \Delta C_{p_X}[K_X/(1 + K_X)] - \Delta H_X^2/RT^2[K_X/(1 + K_X)^2] \quad \text{(A4)}$$

where the meaning of subindexes is the same as above.

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