Reversible Unfolding of the Major Fraction of Ovalbumin by Guanidine Hydrochloride†

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ABSTRACT: The guanidine hydrochloride induced unfolding of the major fraction of ovalbumin (i.e., A_1 which contains two phosphate groups and constitutes about 77% of the total protein) was investigated systematically by difference spectral and viscosity measurements. As judged by the intrinsic viscosity (3.9 ml/g), the native protein conformation is compact and globular. Difference spectral results showed extensive disruption of the native structure by guanidine hydrochloride. The intrinsic viscosities of ovalbumin A_1 in 6 M guanidine hydrochloride with and without 0.1 M β-mercaptoethanol were 31.1 and 27.0 ml/g. These and optical rotation results indicated that the denatured protein existed in a cross-linked random coil conformation in 6 M guanidine hydrochloride alone. Strikingly, in contrast to whole ovalbumin, the denaturation of its A_1 fraction by guanidine hydrochloride was fully reversible and obeyed first-order kinetic law under different experimental conditions of pH, temperature, and the denaturant concentration. The monotonic variation of ΔH for the unfolding of ovalbumin A_1 by guanidine hydrochloride with temperature, the coincidence of the two transition curves obtained by measuring two independent properties (namely reduced viscosity and difference in light absorption at 288 nm (or 293 nm)) as a function of the denaturant concentration, and finally the adherence of the unfolding as well as refolding reactions to first-order kinetic law suggested that the transition of ovalbumin A_1 can reasonably be approximated by a two-state model. Analysis of the equilibrium data obtained at pH 7.0 and 25 °C according to Aune and Tanford (Aune, K. C., and Tanford, C. (1969), *Biochemistry 8,* 4586) showed that 12 additional binding sites for the denaturant with an association constant of 1.12 were freshly exposed by the unfolding process and that the native protein was marginally more stable (~6 kcal/mol) than its unfolded form even under native condition. The temperature dependence of the equilibrium constant for the unfolding of ovalbumin A_1 by guanidine hydrochloride which was studied in the range 10–60 °C at pH 7.0 can be described by assigning the following values of the thermodynamic parameters for the unfolding process: ΔH = 52 kcal/mol at 25 °C; ΔS = 153 cal deg^{-1} mol^{-1} at 25 °C; and ΔC_p = 2700 ± 400 cal deg^{-1} mol^{-1}.

Protein denaturation, in general, is a highly cooperative process which, for small globular proteins, may be approximated by a two-state model where the two states, namely the native and the denatured states, are *macroscopic* in nature such that within-state variations are possible (Lumry et al., 1966; Brandts, 1969; Tanford, 1970). Using the two-state model, results on reversible denaturation of a number of proteins have been quantitatively analyzed and useful conclusions have been drawn regarding the folding of a protein and its intrinsic thermodynamic stability in aqueous solution (Tanford, 1968, 1970; Brandts, 1969; Pace, 1975; Anfinsen and Scheraga, 1975). However, the scope of a denaturation study is severely limited in cases where the denaturation process exhibits little or no reversibility. One such unfolding reaction is the denaturation of ovalbumin by Gdn·HCl[1] (Holt and Creeth, 1972).

Available data on the molecular properties of ovalbumin (see Taborsky, 1974) suggest that the native protein exists in a compact and globular conformation containing 25–30% α helix and some β structure (Gorbunoff, 1969). Ovalbumin contains electrophoretically three distinguishable fractions, namely A_1, A_2, and A_3, which contain 2, 1, and 0 phosphate groups (Perlmann, 1955). The three fractions seem to possess the same overall native protein conformation (Ahmad, 1974; Ansari et al., 1975). The native structure of ovalbumin is altered among others by urea (Simpson and Kauzmann, 1953; Smith and Back, 1965) and Gdn·HCl (Schellman et al., 1953; Harrington, 1955; Gordon and Jencks, 1963; Castellino and Barker, 1968a; Warren and Gordon, 1971; Gordon, 1972; Holt and Creeth, 1972; Bull and Breese, 1975). Kauzmann and coworkers (Simpson and Kauzmann, 1953; Frensdroff et al., 1953) investigated systematically the urea denaturation of ovalbumin. They found that the process of unfolding is a complex one and did not follow the first-order kinetic law. Similar deviation from the first-order kinetic behavior was also noticed for the denaturation of ovalbumin by Gdn·HCl (Schellman et al., 1953). However, the latter unfolding process was not investigated in its essential details and the development of turbidity due to poor solubility of ovalbumin at lower Gdn·HCl concentration made it impossible to study the reaction quantitatively. In addition to the problem posed by the poor solubility of ovalbumin at lower Gdn·HCl concentrations, the unfolding reaction could not be fully reversed by trying different experimental conditions (Holt and Creeth, 1972). This has led Holt and Creeth (1972) to suggest that the folding of native ovalbumin is affected by "extra thermodynamic", presumably kinetic, factors. We found that the unfolding process was much simpler in the case of ovalbumin A_1, the major fraction of the protein which was soluble at all concentrations of Gdn·HCl (0–7 M), and the denaturation was completely reversible under a variety of experimental condi-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; SHEtOH, β-mercaptoethanol; 6 M Gdn·HCl–SHEtOH, 6 M guanidine hydrochloride containing 0.1 M β-mercaptoethanol.

tions, i.e., pH, temperature, Gdn·HCl concentration. Thus a thermodynamic study of the unfolding process appeared feasible. This paper describes for the first time a systematic study of the reversible denaturation of ovalbumin A_1 by Gdn·HCl. The results presented here not only invalidate the previous suggestion regarding folding of the native ovalbumin molecule (Holt and Creeth, 1972) but also provide much needed thermodynamic data on Gdn·HCl denaturation of a single-chain globular protein of a relatively large molecular weight. Such quantitative studies have been hitherto restricted to small globular proteins of molecular weight less than 30 000 (Tanford, 1968, 1970; Brandts, 1969; Pace, 1975).

Materials and Methods

Ovalbumin A_1. Ovalbumin was isolated from egg white as described by Kekwick and Cannan (1936) and was recrystallized five times. The protein was homogeneous on a Sephadex column and in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, but it gave three bands on polyacrylamide gel electrophoresis corresponding to its three fractions A_1, A_2, and A_3 which respectively contained 2, 1, and 0 phosphate groups (S. A. Kidwai, unpublished results) as shown earlier by Perlmann (1955). Ovalbumin A_1 was isolated from ovalbumin by carboxymethylcellulose column chromatography as described by Rhodes et al. (1958). The fraction A_1 contained 2 phosphate groups and repeatedly gave a single band in polyacrylamide gel electrophoresis (S. A. Kidwai, unpublished results). The molecular weight of ovalbumin A_1 will be taken to be the same as found for ovalbumin, i.e., 43 000 (Castellino and Barker, 1968b). The protein concentration was determined by heating a known weight of the isoionic preparation of ovalbumin A_1 obtained by passing an extensively dialyzed protein solution through a mixed-bed, ion-exchange column prepared by the method of Dintzis (1952).

Guanidine hydrochloride (Gdn·HCl) was prepared from guanidine carbonate (BDH, England) and purified according to the method of Nozaki and Tanford (1967). A 6 M Gdn·HCl solution had an absorbance at 225 nm which was less than 0.15. β-Mercaptoethanol was purchased from Sigma Chemical Co. Other chemicals were of analytical grade.

Light absorption measurements were made on a Beckman Model DK-2A ratio recording spectrophotometer equipped with a thermostatically controlled cell holder whose temperature was maintained within ±0.05 °C by circulating water from an ultrathermostat type NBE (East Germany). In some experiments which did not require automatic recording, absorbance was measured with a Carl Zeiss Jena spectrophotometer, Model VSU2-P.

Viscosity Measurements. The details of the procedure for viscosity measurements have already been described (Ahmad and Salahuddin, 1974). Cannon-Fenske viscometers with a flow time of about 400 s for water at 25 °C were generally used. However, in some kinetic experiments an Ostwald type viscometer with a flow time of 70 s for water at 25 °C was used. The reduced viscosity, η_R, was determined as described by Tanford (1955) using the time of fall for the protein solution and the solvent in the viscometer, the partial specific volume of ovalbumin A_1 (Dayhoff et al., 1952), and the density of the solvent. The intrinsic viscosity, $[\eta]$, and Huggins constant (Huggins, 1942) were obtained from a least-squares analysis of the results on η_R as a function of the protein concentration.

Preparation of Protein Solutions. Protein solutions for the equilibrium and kinetic studies of the denaturation of ovalbumin A_1 were prepared as follows. For denaturation experiments, a known weight of the stock protein solution was taken in a 5-ml volumetric flask. To the latter were added 0.025 M sodium phosphate buffer (pH 7.0) and a requisite weight of solid Gdn·HCl. When required, the pH was adjusted by the addition of HCl or NaOH. The protein solution in Gdn·HCl was incubated for a period that was found to be sufficient by kinetic experiments for the completion of the reaction. A similar procedure was employed in preparing the protein solutions for renaturation experiments with the only exception that a concentrated solution of ovalbumin A_1 was first denatured with 7 M Gdn·HCl and then diluted with 0.025 M sodium phosphate buffer, pH 7.0. The latter buffer was used throughout this study unless stated otherwise. The molar extinction of the protein in the phosphate buffer was determined to be 30.1×10^4 M^{-1} cm^{-1}.

The kinetics of Gdn·HCl denaturation was studied at pH 7.0 and at different temperatures and Gdn·HCl concentrations by viscosity measurements. For denaturation, a known volume of Gdn·HCl solution of the desired concentration in the phosphate buffer, pH 7.0, was taken in the viscometer. A small volume of a concentrated solution of ovalbumin A_1 was added. The rate of the denaturation process was determined by measuring the time of fall immediately after mixing the two solutions in the viscometer. The rate of renaturation was measured similarly except that a concentrated solution of ovalbumin A_1 in Gdn·HCl of desired concentration was the last reagent to be added to the viscometer. Thus it was possible to record the progress of the denaturation reaction, both in the forward and reverse directions, beginning from 1 min after adding the last reagent.

Results

The Native State. The intrinsic viscosity of ovalbumin A_1 in 0.025 M sodium phosphate buffer, pH 7.0, and at 25 °C was found to be 3.9 ml/g which is the same as that reported for whole ovalbumin (see Frensdroff et al., 1953). The viscosity increment was calculated from $[\eta]$ as described elsewhere (Ansari et al., 1975) and was found to be 4.2 which corresponds to an axial ratio, a/b, of 3.5 and a frictional ratio, f/f_0, of 1.15. Thus the hydrodynamic behavior of native ovalbumin A_1 as measured by intrinsic viscosity is consistent with that of a compact and globular conformation.

The Denatured State. The ultraviolet spectra of ovalbumin A_1 in 0.025 M phosphate buffer, pH 7.0, showed a peak at 279 nm, a shoulder at 286 nm, and "wiggles" in the range 250–270 nm. The spectra showed hypochromism and the usual blue shift (from 279 to 277 nm) upon denaturation by 6 M Gdn·HCl. In addition, the difference spectra of the native protein measured against the protein solution of equal concentration in 6 M Gdn·HCl showed fine structures (two troughs near 293 and 288 nm, shoulder at 281 nm, and "wiggles" in the range 250–270 nm) that indicated extensive protein unfolding and subsequent exposure of tryptophan, tyrosine, and phenylalanine residues of ovalbumin A_1 in 6 M Gdn·HCl.

Intrinsic Viscosity. The intrinsic viscosities of ovalbumin A_1 in 6 M Gdn·HCl and in 6 M Gdn·HCl containing 0.1 M SHEtOH were determined at pH 7.0 and 25 °C, and the respective values were 27.0 and 31.1 ml/g. The latter is lower than the 34.6 ml/g measured for ovalbumin in 6 M Gdn·HCl–SHEtOH by Castellino and Barker (1968b) who determined the protein concentration using a value of 7.35 for $E_{1\,cm}^{1\%}$ at 280 nm which is higher than that (7.0) found in this study. However, when we used $E_{1\,cm}^{1\%}$ of 7.35 at 280 nm for the determination of protein concentration, the value of $[\eta]$ went up from 31.1 to 32.6 ml/g. Moreover, the Gdn·HCl de-

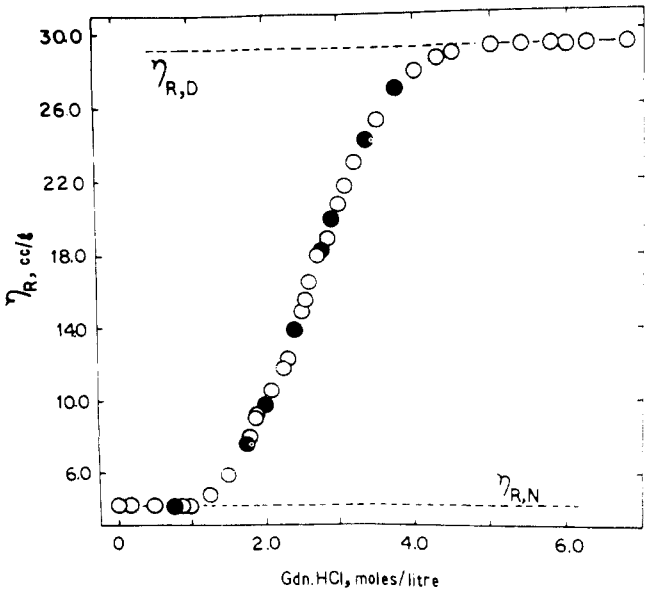

FIGURE 1: The effect of Gdn·HCl concentration on the reduced viscosity of ovalbumin A_1. Open and filled circles represent denaturation and renaturation experiments, respectively. All measurements were made at pH 7.0 and 25 °C. Protein concentration was 3.8 mg/ml.

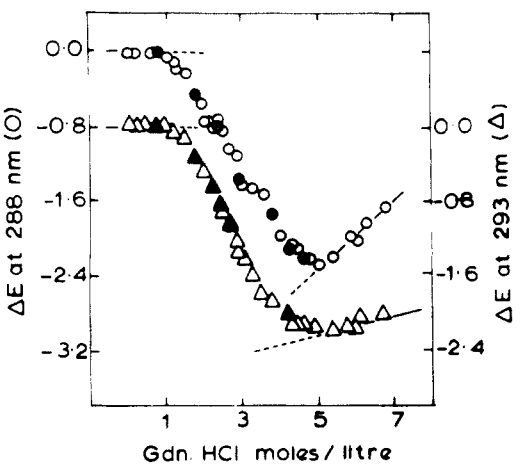

FIGURE 2: The effect of Gdn·HCl concentration on ultraviolet light absorption by ovalbumin A_1 at 288 and 293 nm and at pH 7.0, 25 °C. Open circles and triangles represent results from denaturation experiments, while the corresponding filled ones represent results from renaturation experiments. Protein concentration was in the range 1–2 mg/ml. For calculation of ΔE see text.

natured ovalbumin, especially its A_3 fraction, may have undergone some aggregation, raising the intrinsic viscosity somewhat. This can be checked by the value of the Huggins constant which has not been reported (Castellino and Barker, 1968b). The Huggins constant for ovalbumin A_1 in 6 M Gdn·HCl–SHEtOH was determined to be 0.31 which is normal for a linear randomly coiled protein in 6 M Gdn·HCl–SHEtOH (Tanford et al., 1967a). For the randomly coiled ovalbumin A_1 with the disulfide bond reduced, $[\eta]$ was calculated by Tanford's equation (1968) to be 34 ml/g which is 9.6% higher than the experimental value. Although this difference is experimentally significant, it is not beyond the limit of the uncertainty of Tanford's equation especially when the latter is applied to conjugated proteins. Ovalbumin A_1 contains as many as 2 glucosamine and 6 mannose units linked to its asparagine residue (Kornfeld and Kornfeld, 1976). The net effect of the single carbohydrate branching would be to reduce the radius of gyration and, hence, the intrinsic viscosity (Graessley, 1968) of ovalbumin A_1 in a linear random coil conformation.

The intrinsic viscosity of ovalbumin A_1 in 6 M Gdn·HCl alone was 13% lower than that in 6 M Gdn·HCl–SHEtOH. This indicated that the protein behaves as a cross-linked random coil in 6 M Gdn·HCl. If ovalbumin A_1 behaves as a random coil, free from any noncovalent structure, its optical rotation in 6 M Gdn·HCl, according to Tanford et al. (1967b), should be equal to the sum of the rotations of its amino acid residues. The mean residue rotation of ovalbumin A_1 at 589 nm was calculated from its amino acid composition (Fothergill and Fothergill, 1970) by the procedure described by Tanford (1968) and was about −84° which compares well with the value of −81° measured in 6 M Gdn·HCl–SHEtOH (A. salahuddin, unpublished data). In addition, the levorotation in 6 M Gdn·HCl decreased slightly on reduction of the disulfide bond. The disruption of the ordered structure around the disulfide bond would have produced an increase in the levorotation.

Isothermal Denaturation. The Gdn·HCl induced transition of ovalbumin A_1 from the native to the denatured states was followed at pH 7.0, 25 °C, by measuring η_R and ΔE as a function of the increasing concentrations of the denaturant. The results are depicted in Figures 1 and 2; ΔE represents the decrease in absorbance due to the denaturation of a 1% solution of ovalbumin A_1 in a cell of 1-cm light path. The transition starts above 1 M Gdn·HCl and is seen to be completed at 4.5 M Gdn·HCl where intrinsic viscosity of the protein was measured to be the same as in 6 M Gdn·HCl. The experimental points obtained from the denaturation and the renaturation experiments lie on the same curve, suggesting that the Gdn·HCl denaturation of ovalbumin A_1 is reversible. This view is strongly supported by our kinetic results. It is to be noted that the changes in η_R and ΔE with the denaturant concentration were independent of the protein concentration. The Gdn·HCl induced transition of whole ovalbumin also starts above 1 M Gdn·HCl and is completed near 4 M Gdn·HCl (Schellman et al., 1953; Gordon, 1972; Holt and Creeth, 1972; Bull and Breese, 1975). However, unlike its A_1 fraction, ovalbumin is not soluble at lower Gdn·HCl concentrations (Schellman et al., 1953; Gordon and Jencks, 1963; Holt and Creeth, 1972; Ahmad, 1974) and the denaturation process is not reversible (Holt and Creeth, 1972).

Equilibrium results of Figures 1 and 2 will be analyzed with the assumption that Gdn·HCl denaturation of ovalbumin A_1 involves only the native (N) and the denatured (D) states. The equilibrium constant (K) for the denaturation process was calculated at different Gdn·HCl concentrations from the measured values of η_R and ΔE. In Figure 1, $\eta_{R,N}$ and $\eta_{R,D}$ are seen to be independent of the denaturant concentration. Similarly ΔE for the native state showed little or no dependence on Gdn·HCl concentration. However, ΔE for the denatured state does exhibit marked dependence on Gdn·HCl concentration which at two wavelengths is described by the straight lines of Figure 2 obtained by the method of least-squares.

Evidence for the Two-State Transition. The assumption that Gdn·HCl denaturation of ovalbumin A_1 approximates to a two-state process seems reasonable in view of the following considerations. (i) The enthalpy change (ΔH) for Gdn·HCl denaturation of ovalbumin A_1 was found to be a monotomic function of temperature in the transition region. No maximum was found in the curve between ΔH and temperature. This is what one should expect for a two-state transition (Lumry et

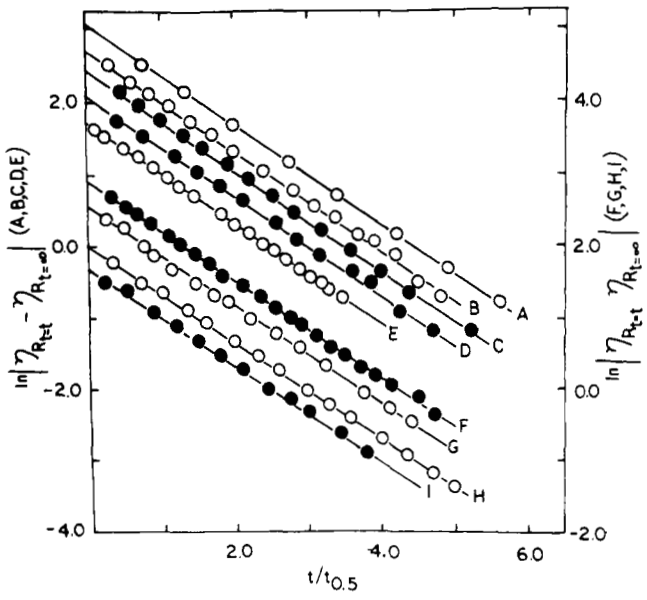

FIGURE 3: Kinetic data for denaturation and renaturation of ovalbumin A_1 under different experimental conditions. Open circles represent results from denaturation experiments at 2.0 M Gdn·HCl, pH 7.0, 45 °C (A); 1.72 M Gdn·HCl, pH 3.0, 25 °C (B); 2.0 M Gdn·HCl, pH 7.0, 25 °C (E); 2.20 M Gdn·HCl, pH 5.2, 25 °C (G); 2.30 M Gdn·HCl, pH 7.0, 15 °C (H) Filled circles represent results from renaturation studies at 1.25 M Gdn HCl, pH 2.2, 25 °C (C); 1.90 M Gdn·HCl, pH 3.8, 25 °C (D); 2.30 M Gdn·HCl, pH 6.6, 25 °C (F); 2.24 Gdn·HCl, pH 4.8, 25 °C (I). Protein concentration was generally near 3.8 mg/ml.

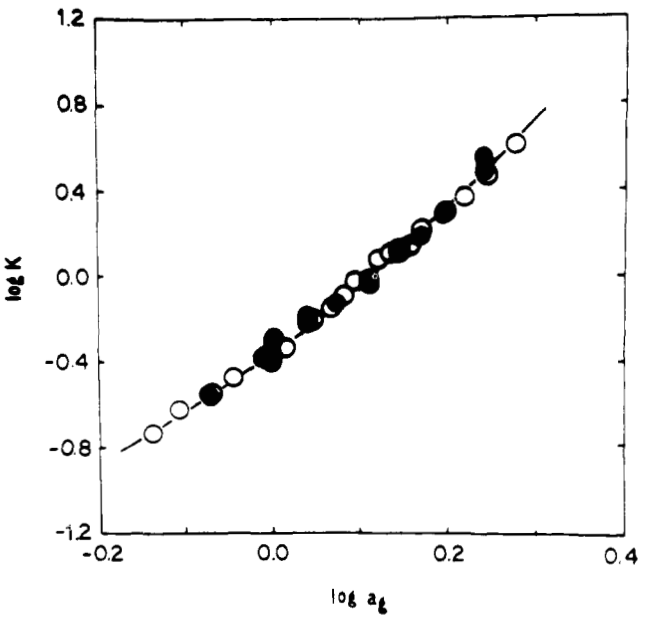

FIGURE 4: The effect of Gdn·HCl concentration on log K calculated from the data of Figures 1 (○) and 2 (●). Solid line was drawn according to eq 1.

al., 1966). (ii) At a given Gdn·HCl concentration, the value of K calculated from viscosity results (Figure 1) was the same as that determined from ΔE (Figure 2), suggesting that the same transition was measured by the two properties. (iii) Kinetic criterion is also important. More convincing evidence for the two-state transition of ovalbumin A_1 induced by Gdn·HCl is provided by the results on the kinetics of unfolding and refolding. Some representative data in the form of a first-order plot of ln $(\eta_{R,t=t} - \eta_{R,t=\infty})$ vs. $t/t_{0.5}$ are shown in Figure 3. Here $\eta_{R,t=t}$ and $\eta_{R,t=\infty}$ are respectively reduced viscosities at time t and that after the attainment of equilibrium by the denaturation of ovalbumin A_1, and $t_{0.5}$ is the half-period for the reaction. The curves in Figure 3 are seen to be linear at different Gdn·HCl concentrations, pH's, and temperatures. This is also true for the refolding reaction (D → N). Additionally, a change in protein concentration from 2 to 10 mg/ml had no effect on the kinetics of denaturation and renaturation of ovalbumin A_1. These results showed that Gdn·HCl induced unfolding (a) is reversible and that (b) the denaturation reaction followed first-order kinetics in both directions. This is also evident from the fact that a linear extrapolation of $\eta_{R\ t=t}$ to zero time gave reduced viscosity which was identical, within experimental error, with $\eta_{R,N}$ (for N → D) or $\eta_{R,D}$ (for D → N). These results suggest the absence of stable intermediates between the native and the denatured states of ovalbumin A_1.

The Dependence of K on Gdn·HCl Concentration. The equilibrium constant (K) for the denaturation of ovalbumin A_1 depends on Gdn·HCl concentration. The slope of the linear plot between log K and log (Gdn·HCl) was determined to be 5.5. When ΔG, the free energy change for the denaturation reaction, was plotted against the denaturant concentration as recommended by Pace (1975), a linear curve with a slope of 1.25 kcal mol^{-1} M^{-1} was obtained.

The variation of K with the denaturant concentration can better be described in terms of binding of the denaturant molecule to the protein. Assuming that the denaturing action of Gdn·HCl is due entirely to its binding to identical but noninteracting binding sites that are available in a greater number in the denatured state than in the native state of the protein, the dependence of K on the activity of Gdn·HCl (a_g) can be described by the help of the following equation (Aune and Tanford, 1969):

$$K = K_0(1 + k'a_g)^{\Delta n} \quad (1)$$

where K_0 is the equilibrium constant in the absence of Gdn·HCl; k' is the binding constant which is assumed to be the same in the native and the denatured states; and Δn is the difference between the number of binding sites on the denatured and the native molecules of the protein. A least-squares fit of the experimental data of Figure 1, which gave K in the range $0.2 \leq K \leq 5$, to eq 1 was obtained by a computer program. The values of K_0, k', and Δn were computed to be 8.71×10^{-3}, 0.81, and 8.75, respectively. With these values of the constants, a curve was drawn between log K and log a_g according to eq 1 and is shown in Figure 4. The actual equilibrium data obtained from viscosity and difference spectral measurements (Figures 1 and 2) are shown by open and filled circles. The activity of Gdn·HCl was calculated from its concentration as described by Aune and Tanford (1969).

If the denaturing action of Gdn·HCl is primarily due to its cation, i.e., $GdnH^+$ (Aune and Tanford, 1969), only the binding of $GdnH^+$ should be considered in accounting for the variation of K with the denaturant concentration. Since the activity of $GdnH^+$, i.e., a_{GdnH^+}, is not available, it will be assumed that $a_{GdnH^+} = a\pm = (a_g)^{1/2}$, so that eq 1 would become

$$K = K_0(1 + ka_\pm)^{\Delta n} \quad (2)$$

where $a_\pm$ is the mean ion activity of Gdn·HCl. The parameters of eq 2 were computed as described above from the best fit of the experimental data of Figures 1 and 2 to eq 2. The data giving K in the range $0.2 \leq K \leq 5$ were included in the analysis.

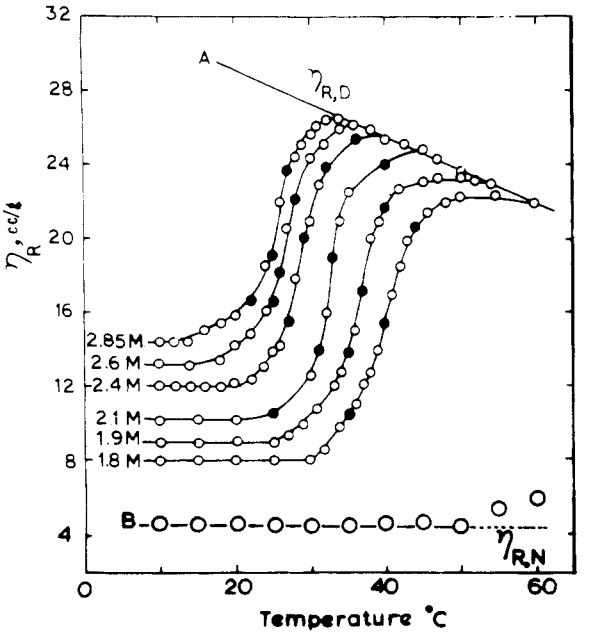

FIGURE 5: The effect of temperature on the Gdn·HCl denaturation of ovalbumin A_1 at pH 7.0. The concentration of the denaturant for each melting curve is shown on the left. The curve A for $\eta_{R,D}$ followed the equation: $\eta_{R,D}$ = 0.209(temperature) + 34.21. Open and filled circles represent denaturation and renaturation points, respectively. Protein concentration was 3.8 mg/ml.

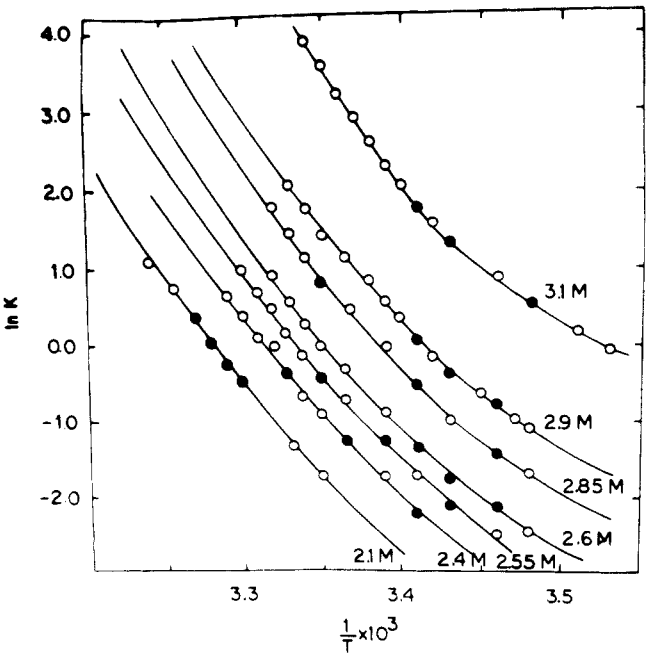

FIGURE 6: Temperature dependence of K for Gdn·HCl denaturation of ovalbumin A_1. Experimental conditions were the same as in Figure 5. Solid curves were drawn according to eq 3.

The following best values of the parameters were obtained: k = 1.12 and log K_0 = −4.34.

Intrinsic Stability of Ovalbumin A_1. Extrapolation of K to zero Gdn·HCl concentration would formally represent the equilibrium constant, K_0, for the reaction N ⇌ D, in absence of Gdn·HCl. The value of K_0 would, therefore, provide an estimate of the free energy of stabilization of the native ovalbumin A_1 over its denatured form at pH 7.0 and 25 °C. The free energy, ΔG_{H_2O}, corresponding to the value of K_0 determined from eq 2 above, comes out to be 5.92 kcal/mol at pH 7.0, 25 °C.

The Dependence of K on Temperature. The effect of temperature on Gdn·HCl induced transition of ovalbumin A_1 was studied at pH 7.0 in the temperature range 10–60 °C. The transition curves at six different concentrations of Gdn·HCl are shown in Figure 5; the curves obtained at 2.5, 2.7, 2.9, and 3.1 M Gdn·HCl were omitted for clarity. The reduced viscosity of ovalbumin A_1 in 0.025 M phosphate buffer, pH 7.0, i.e., $\eta_{R,N}$, which represents the viscosity value in the pretransition region, was measured to be 4.2 ml/g at a protein concentration of 3.8 mg/ml. As evident from curve B of Figure 5, $\eta_{R,N}$ is independent of temperature up to 50 °C. Above 50 °C, the reduced viscosity increased with heating due to thermal denaturation. The reduced viscosity in the posttransition region, i.e., $\eta_{R,D}$, decreased on heating at all the ten concentrations of the denaturant. The temperature dependence of $\eta_{R,D}$ was found to be similar at all of the ten Gdn·HCl concentrations and can be described by curve A of Figure 5. At each of the ten Gdn·HCl concentrations used in this study, a linear extrapolation of $\eta_{R,D}$ from higher (or lower) temperature to 25 °C gave an $\eta_{R,D}$ of 29 ml/g at a protein concentration of 3.8 mg/ml. This is identical, within experimental error, with the reduced viscosity of ovalbumin A_1 in 6 M Gdn·HCl at 25 °C. This suggests that, regardless of the denaturant concentration, the product of heat and Gdn·HCl denaturation of ovalbumin A_1, in the Gdn·HCl concentration range 1.8–3.1 M, is as extensively unfolded as the denatured protein in 6 M Gdn·HCl, 25 °C. At each Gdn·HCl concentration, the experimental points obtained by cooling are seen to fall on the same curve as that found by heating (see Figure 5). This showed that the effect of temperature on Gdn·HCl denaturation of ovalbumin A_1 is reversible at each Gdn·HCl concentration in the range, 1.8–3.1 M. At 2 M Gdn·HCl the denaturation of ovalbumin A_1 by Gdn·HCl was found to obey the first-order kinetic law both at 25 and 45 °C. Also the reaction followed first-order kinetics at 2.3 M Gdn·HCl and at 15 °C. It, therefore, seems reasonable to assume that Gdn·HCl denaturation of ovalbumin A_1 approximates to a two-state model at temperatures other than 25 °C in the range 15–45 °C. Thus the results of Figure 5 can be used in the calculation of equilibrium constant, K, at different temperatures. The values of $\eta_{R,D}$ and $\eta_{R,N}$ required in such calculations were computed respectively from curves A and B of Figure 5. The results are shown in Figure 6 in the form of a van't Hoff plot between ln K and $1/T$.

The temperature dependence of K can be described by an equation (Brandts and Hunt, 1967) of the form

$$\ln K = A + (B/T) + C'T + DT^2 \qquad (3)$$

A best fit of the experimental data on ln K as a function of temperature given in Figure 6 to eq 3 was obtained by the method of least-squares using a computer program. The values of the constants A, B, C', and D thus determined were 1124, −60 850, −5.784, and 9.046×10^{-3}, respectively. With these values of A, B, C', and D, solid lines of Figure 6 were drawn according to eq 3 at different Gdn·HCl concentrations. The enthalpy change (ΔH), entropy change (ΔS), and heat capacity change (ΔC_p) for the denaturation of ovalbumin A_1 were determined by the help of appropriate equations (Tanford, 1968).

The heat capacity change (ΔC_p) for the denaturation of ovalbumin A_1 was found to be somewhat dependent on temperature. Such a temperature dependence of ΔC_p has also been noted for other protein denaturations (Brandts and Hunt,

1967; Pohl, 1968; Brandts, 1969). It should, however, be emphasized that a small uncertainty in the determination of K will be greatly magnified in the evaluation of ΔC_p. In fact, the changes in ΔC_p with temperature in the range 15–35 °C were found to be small, about 15%, and the experimental error in the actual determination of ΔC_p was as high as 18%. The average value of ΔC_p in the temperature range 15–35 °C comes out to be +2700 cal mol^{-1} deg^{-1} which is expectedly higher than the value (1400 cal mol^{-1} deg^{-1} below 40 °C) for the process, native → activated state, calculated from the kinetic data on the denaturation of ovalbumin in absence of any denaturant (see Waissbluth and Grieger, 1974). It is noteworthy that recently Privalov and Khechinashvili (1974) on the basis of their studies on the thermal denaturation of several proteins have concluded that the temperature dependence of ΔC_p is much smaller than found previously.

TABLE I: Parameters Characterizing the Gdn·HCl Denaturation of Ovalbumin A_1 at 25 °C and pH 7.0.

$C_{1/2}$[a] (mol/l.)	2.63
Log K_0	−4.34
ΔG_{H_2O} (kcal/mol)	5.92
Δn	12.3
k	1.12
ΔH (kcal/mol)	51.6
ΔS (cal deg^{-1} mol^{-1})	153
ΔC_p (cal deg^{-1} mol^{-1})	2700 ± 400[b]

[a] The concentration of Gdn·HCl corresponding to the middle point of the isothermal transition illustrated in Figure 1. [b] The value represents the average value of ΔC_p computed in the temperature range 15–35 °C and the range represents experimental uncertainty of about 18% in the determination of ΔC_p.

Discussion

Results on intrinsic viscosity showed that the overall conformation of ovalbumin A_1 under native condition is globular. The native protein structure was extensively unfolded in 6 M Gdn·HCl as shown by the exposure of aromatic amino acid residues, including nine tyrosine residues. The product of unfolding of ovalbumin A_1 by 6 M Gdn·HCl is, in fact, structureless and behaves as a random coil as revealed by the results on intrinsic viscosity and optical rotation of the protein in 6 M Gdn·HCl with and without 0.1 M SHEtOH. Ovalbumin A_1 contains one disulfide bond (Fothergill and Fothergill, 1970) which imposed a physical constraint on the otherwise randomly coiled ovalbumin A_1 and consequently the intrinsic viscosity was 13% lower in 6 M Gdn·HCl than in 6 M Gdn·HCl–SHEtOH.

The equilibrium and kinetic results on the Gdn·HCl denaturation of ovalbumin A_1 clearly showed that the transition from the native to the denatured state is fully reversible at different denaturant concentrations (1–7 M), temperature (10–60 °C), and at pH values between pH 1 and 7 (Ahmad, 1974). This finding is significant, for a previous attempt to achieve a complete refolding of whole ovalbumin denatured by Gdn·HCl was not successful (Holt and Creeth, 1972). This has led the latter authors to suggest that "extra-thermodynamic" factors affect the folding of native ovalbumin. The main practical difficulty in demonstrating the reversibility of the Gdn·HCl denaturation of whole ovalbumin was the fact that the protein was insoluble at lower denaturant concentrations (Schellman et al., 1953; Gordon and Jencks, 1963; Ahmad, 1974). Preliminary studies carried out in this laboratory indicated that, of the three fractions of ovalbumin, the fraction A_3 devoid of any phosphate groups is least soluble in water and in dilute solutions of Gdn·HCl; the major fraction A_1 with two phosphate groups showed maximum solubility in water and in Gdn·HCl solutions of concentration less than 2 M. It seems surprising that such a minor alteration at the posttranslational level in the primary structure of ovalbumin A_3 as is made by the phosphorylation of its two serine residues should have such a striking effect on the solubility of the protein. The presence of charged phosphate groups presumably enables the protein molecule to interact with the aqueous solvent with significant gain in free energy.

Results on (a) the coincidence of the two transition curves obtained by the two independent properties, i.e., η_R and ΔE, (b) the variation of ΔH with temperature, and (c) on the kinetics of folding and refolding of ovalbumin A_1 in presence of Gdn·HCl suggested that the denaturation of ovalbumin A_1 by Gdn·HCl can reasonably be approximated by a two-state model. This would mean that the denaturation process essentially involves only two *macrostates* which we refer to as the *native* and the *denatured* states and that the intermediate state(s), if any, is either too unstable or accumulates in too low quantity to be detectable by the experimental techniques used in this study. These results, however, do not preclude the possibility of the existence of more than one *microstates* within one (or both) of the *macrostates,* for it is conceivable that the different *microstates* within a given *macrostate* of ovalbumin A_1 that are indistinguishable by η_R and ΔE may be detectable by a sensitive probe such as nuclear magnetic resonance. One such within-state variation which is not a part of the actual protein unfolding reaction has been recently shown by Brandts et al. (1975) to arise from the cis–trans isomerism of the proline residues in a denatured protein. These within-state variations are likely to involve very small local conformational changes which would not be related to the "major" conformational change that accompanies protein denaturations (Tanford, 1968), such as the denaturation of ovalbumin A_1 by Gdn·HCl. In this connection it will be of interest to refer to the extensive studies of Kauzmann and his co-workers (Simpson and Kauzmann, 1953; Frensdroff et al., 1953) on the denaturation of whole ovalbumin by urea. Equilibrium studies of the denaturation process were not made and the kinetics were studied only in one direction, i.e., denaturation. The first-order kinetic plots were independent of the protein concentration but showed some curvature which was interpreted to mean that the denaturation reaction involved several stable intermediates. The denaturation of whole ovalbumin by Gdn·HCl was found to be kinetically similar (Schellmann et al., 1953). However, the curvature in the kinetic plot was slight, which, in view of the present findings, seems to be probably due to the presence of the three different fractions of the whole ovalbumin. The fact that Gdn·HCl denaturation of a mixture of the three fractions of ovalbumin did not fully follow first-order kinetic law, which was obeyed by the denaturation of its fraction A_1 alone, suggests that the rates of denaturation of any two or all of the three fractions of the protein are different.

A summary of the results characterizing the reversible denaturation of ovalbumin A_1 by Gdn·HCl is given in Table I. The value of Δn suggests that 12 binding sites with an association constant of 1.12 were newly exposed in the denaturation of ovalbumin A_1. This value of Δn is strikingly very low and is incompatible with the proposal that the peptide groups (Robinson and Jencks, 1965) offer principal binding sites

since all of the 368 peptide groups of ovalbumin A_1 are available for binding in the denatured state. The aromatic side chains with largest free energy of transfer from water to Gdn·HCl solution (Nozaki and Tanford, 1970) are also expected to represent binding sites. There are 31 aromatic amino acid residues in ovalbumin A_1 that are mostly buried in the native state and exposed in the denatured state. Some of these aromatic side chains are conceivably involved in the binding of the denaturant.

The value of ΔG_{H_2O} in Table I would formally represent the intrinsic thermodynamic stability of the native conformation of ovalbumin A_1 in aqueous solution. The free energy of stabilization of the native structure of ovalbumin A_1 turns out to be 6 kcal/mol at neutral pH and 25 °C. This would mean that the native state is only marginally more stable than the completely unfolded state even under native condition. This seems to be the general conclusion which emerges from the studies of protein denaturation (Tanford, 1970; Rowe and Tanford 1973; Pace, 1975; Kita et al., 1976), where the native globular conformation appears to be stabilized by 4–15 kcal/mol. A possible implication of the inherently low stability of a native protein conformation is that a protein may not be able to retain its unique native structure even after a few amino acid replacements in its primary structure. This point of view is supported by the well-known structural and functional differences between normal human hemoglobin and its several mutant forms.

Considering the size of ovalbumin A_1, the dependence of K on Gdn·HCl concentration is unusually small. It is unlikely, although possible, that, in the sequence of reactions $N \rightleftharpoons N_1 \rightleftharpoons D$, only the latter step could be monitored by η_R and ΔE as N and N_1 were indistinguishable with respect to these two properties and that the $N \rightleftharpoons N_1$ transition involved exposure of a small number of unusual amino acid residues making a disproportionately large contribution to the dependence of K on Gdn·HCl concentration.

The change in enthalpy (ΔH) for the Gdn·HCl denaturation of ovalbumin A_1 is 52 kcal/mol at 25 °C and exhibits a rather strong dependence on temperature which gives rise to a large and positive ΔC_p for the denaturation process. The conclusion is valid regardless of the fact whether or not ΔC_p is temperature independent. This significant feature of the denaturation of ovalbumin A_1 is invariably shared by all the protein denaturations known to date (Tanford, 1968, 1970; Pace, 1975) and has been attributed to the exposure of hydrophobic residues due to protein unfolding. The solvent molecules are ordered about the exposed hydrophobic groups. It is the melting of the ordered solvent structure which gives rise to a large and positive ΔC_p. Furthermore, such an ordering of the solvent molecules about the freshly exposed groups, which occurs in the denatured state, would make a negative contribution to the change in entropy (ΔS) for the denaturation of ovalbumin A_1. In the absence of a negative contribution by the solvent ordering, the value of ΔS would have been much larger than 153 cal deg^{-1} mol^{-1} (see Table I). The value of ΔS is obviously low especially for a process in which the protein goes from an ordered (the native) to a fully disordered (the denatured) state.

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Ultracentrifuge Studies of the Binding of IgG of Different Subclasses to the Clq Subunit of the First Component of Complement†

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ABSTRACT: Normal IgG and myeloma proteins of the IgG1, 2, 3, and 4 subclasses were mixed with human Clq and studied in the analytical ultracentrifuge for complex formation. Binding of IgG to Clq is apparent both from the enlargement of area and from the increase in sedimentation rate of the well-separated schlieren peak of Clq. The accurate determination of binding parameters requires that sedimentation rates be corrected for hydrodynamic interaction, and area measurements corrected for the Johnston–Ogston effect. At the highest immunoglobulin concentrations employed in these studies, more than ten IgG molecules are bound to each Clq. If we assume that the number of binding sites must be an integral multiple of 6, then the data best support a 12 binding site model, although an 18 site model cannot be ruled out. Myeloma IgG proteins of all subclasses bind to Clq, with affinities decreasing in the order G3 > G1 > G2 > G4. No binding of IgA to Clq could be detected.

The first component of complement (Cl) is a protein–calcium complex composed of at least three subunits, Clq, Clr and Cls (Lepow et al., 1963; Naff et al., 1964). A fourth subunit, Clt, has been reported (Assimeh and Painter, 1975). Clq has a number of sites specific for the binding of IgM and IgG. The location of the binding site on IgG has been reported to be within the C_H2 domain of the F_c subunit (Kehoe and Fougereau, 1970; Yasmeen et al., 1976), but the C_H3 domain may also be involved in the binding of Clq (Allan and Isliker, 1974; Ovary et al., 1976). The binding of antibody to Clq at multiple sites appears to trigger the activation of Clr (Naff and Ratnoff, 1968; Valet and Cooper, 1974b; Ziccardi and Cooper, 1976) which in turn results in proteolytic cleavage and activation of Cls (Valet and Cooper, 1974a; Sakai and Stroud, 1974). This initiates the classical pathway since Cls possesses proteolytic and esteratic activity for C4, C2, and synthetic substrates. Some recent reviews are by Müller-Eberhard (1975), Cooper (1973), Lepow (1972), Kinsky (1972), and Ruddy et al. (1972).

Isolation and purification of Clq has been performed by several different procedures (Müller-Eberhard and Kunkel, 1961; Agnello et al., 1970; Calcott and Müller-Eberhard, 1972; Volanakis and Stroud, 1972; Sledge and Bing, 1973b), and the molecule has been examined with the electron microscope (Svehag et al., 1972; Shelton et al., 1972; Knobel et al., 1975). It is a remarkable object, composed of a central body from which radiate six fibrillar arms ending in six peripheral subunits. The molecule can be dissociated to yield noncovalently bonded subunits (Calcott and Müller-Eberhard, 1972; Volanakis and Stroud, 1972; Reid et al., 1972; Yonemasu and Stroud, 1972; Heusser et al., 1975). Upon reduction and alkylation, three polypeptide chains are produced (Reid et al., 1972; Yonemasu and Stroud, 1972; Heusser et al., 1975; Bhattacharyya et al., 1974; Lowe and Reid, 1974), all of which appear to have a collagen-like region (Calcott and Müller-Eberhard, 1972; Reid et al., 1972; Bhattacharyya et al., 1974; Lowe and Reid, 1974).

The binding of immunoglobulins to Clq has been studied by ultracentrifugation (Müller-Eberhard and Calcott, 1966), affinity chromatography (Sledge and Bing, 1973a), and by a "$C\bar{1}$ binding assay" (Augener et al., 1971). Digestion studies indicate that the peripheral subunits are sites of attachment of the Clq to IgG (Knobel et al., 1974).

In this communication we report the results of binding studies between Clq and myeloma proteins of the IgG1, 2, 3, and 4 heavy chain subclasses, as well as normal IgG. These studies have all been performed with the ultracentrifuge. Since high concentrations of immunoglobulins must be employed to saturate the binding sites on Clq, some sedimentation anomalies occur and must be corrected to allow quantitative interpretation of binding data. These anomalies cause an apparent reduction of the amount of immunoglobulin bound to Clq. Therefore, a minimum number of binding sites can be estimated from the uncorrected sedimentation data. It ranges from

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