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Occurrence and Characterization of Stable Intermediate State(s) in the Unfolding of Ovomucoid by Guanidine Hydrochloride

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Reversible unfolding of ovomucoid by guanidine hydrochloride, as followed by viscosity and difference-spectral measurements at 25°C, pH 6, occurred in two distinct steps involving at least three major conformational states, namely the native, intermediate and completely denatured states, occurring respectively in 60mM-sodium phosphate buffer, 3.5M-guanidine hydrochloride and 6M-guanidine hydrochloride. The overall native conformation of ovomucoid, as indicated by its intrinsic viscosity (5.24ml/g) and gel-filtration behaviour, differs significantly from that of a typical globular protein. Exposures of tyrosine residues in native ovomucoid measured by difference spectroscopy following perturbation with glycerol, ethylene glycol and dimethyl sulphoxide were, respectively, 0.42, 0.56 and 0.57. Of the exposed phenolic groups only one titrated normally ($pK_{int.}$, 9.91, electrostatic-interaction factor, w, 0.04). Results on difference spectra, solvent perturbation, phenolic titration and intrinsic viscosity (7.4ml/g) taken together showed that, although ovomucoid in 3.5M-guanidine hydrochloride was significantly unfolded, it retained a degree of native structure, removable with 6M-guanidine hydrochloride. In the latter, all the six tyrosine residues were available for titration, and the intrinsic viscosity of ovomucoid increased to 9.4ml/g. Furthermore, the characteristic fine structures in circular-dichroism spectra of ovomucoid, associated with the elements of native structure, were abolished in 6M-guanidine hydrochloride, suggesting that the completely denatured state is structureless and presumably behaves as a cross-linked random coil. The latter state has been shown by analysis of the results on guanidine hydrochloride-dependence of the transition, intermediate⇌denatured, to be less stable than the intermediate state under native conditions by about 46kJ/mol at 25°C. Attempts have been made to interpret the above results in the light of available information on the amino acid sequence of ovomucoid.

Equilibrium and kinetic studies have suggested that the reversible denaturation of proteins is generally a co-operative process involving essentially two major stable conformational states, i.e. the native and the denatured states (Tanford, 1968; Brandts, 1969). However, more sensitive kinetic probes show that the protein-unfolding and -refolding reactions rarely follow a two-state mechanism (Baldwin, 1975). In fact, marked deviation from a two-state model has been clearly demonstrated even by equilibrium studies in a few cases (Riddiford, 1966; Chignell *et al.*, 1972; Wong & Tanford, 1973), where it was shown that a stable intermediate state(s) accumulates in significant quantities on the pathway of folding and unfolding reactions. The characterization of stable intermediate state(s) would unquestionably be of great help for a proper understanding of the overall process of protein folding. So far, such characterization studies have rarely been undertaken. Here we report the results of our systematic studies on the conformational states of ovomucoid that are involved in its reversible unfolding by guanidine hydrochloride.

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Materials and Methods

Ovomucoid

By a method similar to that of Waheed & Salahuddin (1975*a*), ovomucoid was fractionated from egg-white first by trichloroacetic acid precipitation followed by $(NH_4)_2SO_4$ fractionation and sulphoethyl-Sephadex column chromatography in 0.014M-sodium acetate buffer, pH 4.6, by using a linear KCl gradient (0.014–0.3M). The least-acidic fraction, with 0.05% sialic acid, was pooled and used throughout these studies. The protein was homogene-

ous as indicated by a single symmetrical peak on gel filtration and a single protein band on polyacrylamide-gel electrophoresis. Other properties of ovomucoid as found in this study were: anti-tryptic activity, 98%; anti-chymotryptic activity, nil; hexose content, 8.3%; tryptophan content, nil; pI, 4.5; $A^{1\%}_{1cm}$, 4.16 at 278nm (and 3.9 at 280nm). The specific absorption coefficient near 278nm is in excellent agreement with that reported by Donovan (1967) and Davis *et al.* (1971), but differs significantly from that found for a similar ovomucoid preparation (Waheed & Salahuddin, 1975*a*), which showed one major and a minor protein band on polyacrylamide-gel electrophoresis. At 280nm the specific absorption coefficient measured for various ovomucoid preparations lies in the range 4.1–6.1 (see Melamed, 1966). The molecular weight of ovomucoid is taken as 27000 (Davis *et al.*, 1971). The protein concentration was determined by a dry-weight method (Ahmad & Salahuddin, 1974).

Sephadex G-200 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The marker proteins for gel-filtration experiments, *N*-acetyltyrosine ethyl ester and *N*-acetylphenylalanine ethyl ester were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guanidine hydrochloride was prepared from guanidinium carbonate by the method of Nozaki (1972); its 6M solution had an A_{225} of 0.11 and A_{275} of 0.002. Dimethyl sulphoxide, ethylene glycol and glycerol were of analytical grade. Other chemicals were of reagent grade.

Gel-filtration experiments

These were performed with native ovomucoid and marker proteins in 0.1M-sodium acetate buffer, pH5.0, as described by Ansari & Salahuddin (1973*a*). About 10mg of protein was applied to a Sephadex G-200 column (61cm×2.7cm) and the protein eluted with the sodium acetate buffer in 2ml fractions at a flow rate of 20ml/h. The column characteristics were as follows: total volume, 359ml; void volume, 130.5ml; internal volume, V_i, 250ml (Andrews, 1970). The marker proteins used in the calibration of the column, with their Stokes radii (Andrews, 1970) in parentheses, were bovine serum albumin dimer (4.30nm), bovine serum albumin monomer (3.55nm), ovalbumin (2.73nm), chymotrypsinogen A (2.24nm) and cytochrome *c* (1.64nm). A least-squares analysis of the gel-filtration data (Ackers, 1967) yielded the relation $\text{erfc}^{-1}\ K_d = 0.21\ a - 0.072$, where $\text{erfc}^{-1}\ K_d$ is the inverse error function complement of K_d for a given protein with Stokes radius a. Thus the Stokes radius of ovomucoid corresponding to its elution volume of 249ml was computed to be 2.75nm.

Viscosity measurements

The reduced viscosity, $\eta_{sp.}/c$, of ovomucoid was determined at 25±0.05°C with a Cannon–Fenske viscometer as described earlier (Ahmad & Salahuddin, 1974). The partial specific volume of the protein was taken to be 0.697ml/g (Davis *et al.*, 1971) in the calculation of $\eta_{sp.}/c$.

U.v.-absorption measurements

These were made on Beckman DK-2A ratio recording spectrophotometer equipped with a thermostatically controlled cell holder whose temperature was maintained at 25±0.2°C by circulating water from a thermostat type U 10. A few measurements were also made on Carl Zeiss spectrophotometer model VSU2-P.

Spectrophotometric titrations of ovomucoid

These were performed in the presence and absence of guanidine hydrochloride by the method of Crammer & Neuberger (1943) as described previously (Ansari & Salahuddin, 1973*b*; Qasim & Salahuddin, 1977).

U.v. solvent-perturbation difference spectroscopy

This was used as described by Laskowski (1966) to measure the degree of exposure of tyrosine residues of ovomucoid under native and denaturing conditions; 20% dimethyl sulphoxide, ethylene glycol and glycerol were used as perturbants. The composition of the model mixture solutions prepared with *N*-acetyltyrosine ethyl ester and *N*-acetylphenylalanine ethyl ester was based on six tyrosine and five phenylalanine residues per ovomucoid molecule (Beeley, 1976). Correction for light-scattering was routinely made by drawing a straight line under the spectrum from 360 to 270nm. Spectra obtained 5min and 1h after mixing the perturbant were identical. The results were expressed as $\Delta\varepsilon_{285-288}/\varepsilon_{275-278}$, where $\Delta\varepsilon_{285-288}$ is the increase in the molar absorption due to the solvent perturbation in the wavelength range 285–288nm and $\varepsilon_{275-278}$ is the molar extinction in the range 275–278nm, where the model mixture and the protein absorb maximally.

Circular-dichroic measurements

These were made with a JASCO J-20 automatic recording spectropolarimeter, maintained at 20°C by circulating water through a jacket covering the cell holder, by using cells of 0.1, 0.2, 0.5 and 1cm light-path. The observed ellipticity was converted into mean residue ellipticity, $[\theta]_{MRW}$, by a standard method (Ziegler & Bush, 1971).

Solutions for denaturation and renaturation were prepared as described by Ahmad & Salahuddin (1976). Although the unfolding and refolding

reactions attained equilibrium at all of the denaturant concentrations used in the present study within 10min, the equilibrium measurements were made after 12h; no difference was noticed between the observations made at 10min and those made after 12h. The pH values of the equilibrium solutions were close to pH6.

Results

Isothermal unfolding

The unfolding of ovomucoid by guanidine hydrochloride was followed at 25°C by difference-spectral and viscosity measurements and the results are graphically shown in Fig. 1. In both Figs. 1(*a*) and 1(*b*) the unfolding is seen to occur in two steps involving at least three major conformational states, namely the native (N), intermediate (X) and fully denatured (D) states. Both the transitions are reversible, as the points obtained in renaturation experiments lie on the curve drawn through the denaturation points (see Figs. 1*a* and 1*b*). The characteristic features of the two transitions, i.e. N⇌X and X⇌D, are summarized in Table 1. With regard to the first step of guanidine hydrochloride denaturation of ovomucoid, the transition followed by reduced viscosity (Fig. 1*a*) does not coincide with that measured by difference spectra (Fig. 1*b*). Thus the denaturant concentrations at and above which the first transition is complete are 2.5M from difference-spectral measurements and 3.25M from the viscosity method (see Table 1). In contrast, both $\eta_{sp.}/c$ and ΔA seem to measure, within experimental error, the same transition from the intermediate to the fully unfolded state. The coincidence of the two transitions as measured by the two properties, i.e. $\eta_{sp.}/c$ and ΔA, was more evident from the fact that, at a given guanidine hydrochloride concentration, the fraction denatured determined by difference-spectral measurements was identical, within experimental error, with that measured by $\eta_{sp.}/c$. This suggests that the transition X⇌D approximates to a two-state process. Admittedly, the observed coincidence of the transitions from state X to D is a necessary but not a sufficient criterion for the absence of a stable conformational state between states X and D. Despite the lack of unequivocal evidence for the two-state mechanism, it will be worth while to use the two-state approximation, as has been done for other protein denaturations (Pace, 1975), to obtain semi-quantitative information on the reversible transition X⇌D. Accordingly, the equilibrium constant, K, for the reversible transition, X⇌D, was calculated as a function of guanidine hydrochloride concentration by using the equilibrium data of Figs. 1(*a*) and 1(*b*) by the standard procedure (Tanford, 1968). The dependence of K on guanidine hydrochloride concentration was analysed with the relation (Aune & Tanford, 1969):

$$K = K^0(1 + ka_g)^{\Delta n} \qquad (1)$$

where K^0 is the equilibrium constant in absence of guanidine hydrochloride, a_g is the activity of guanidine hydrochloride, k is the binding constant, which is assumed to be the same in the X and D states, and Δn is the difference in the number of binding sites on D and X molecules. The implicit assumption in eqn. (1) is that the denaturing action of guanidine hydrochloride is due entirely to its binding to identical but non-interacting binding sites. The values of a_g were calculated from guanidine hydrochloride concentration according to Aune & Tanford (1969). A least-squares fit of the equilibrium data of Figs. 1(*a*) and 1(*b*) was obtained by a computer program. The following values of the constants of eqn. (1) were

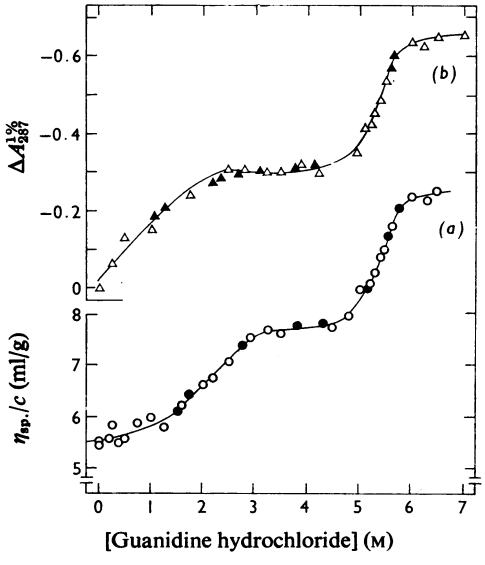

Fig. 1. *Effect of guanidine hydrochloride concentration on the reduced viscosity (a) and difference absorption at 287nm (b) of ovomucoid at 25±0.05°C under equilibrium conditions*

Open and filled symbols represent points obtained from denaturation and renaturation experiments respectively. For denaturation, ovomucoid was exposed at the indicated guanidine hydrochloride concentrations for 12h. For renaturation, the protein was first denatured with 7M-guanidine hydrochloride and then diluted with 60mM-sodium phosphate buffer, pH7.0. Light absorptions of protein solutions in the presence of guanidine hydrochloride were measured with appropriate guanidine hydrochloride solution as blank. Protein concentration was in the range 1–4mg/ml.

Table 1. *Characteristic features of guanidine hydrochloride-induced transition of ovomucoid at* 25°C, *pH*6
Experimental details are given in the legend to Fig. 1.

Method	Guanidine hydrochloride concentration (M)		
	Near onset of transition	At middle point of transition	At and above which the transition is complete
A. First transition, N⇌X			
(i) Reduced viscosity	1.25	2.1	3.25
(ii) Difference spectra	—	~1	2.5
B. Second transition, X⇌D			
(i) Reduced viscosity	4.5	5.4	6.0
(ii) Difference spectra	~4.5	5.4	6.0

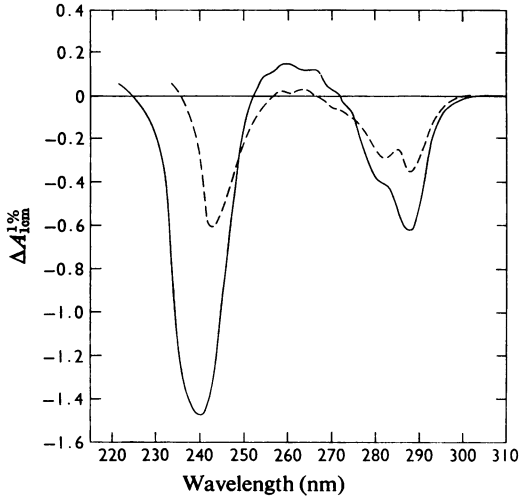

Fig. 2. *U.v. difference spectra of ovomucoid in* (----) 3.5M- *and* (——) 6M-*guanidine hydrochloride at* 25±1°C, *pH*6
Four well-matched silica cells of 1 cm light-path were chosen. The two cells in the sample compartment contained, respectively, ovomucoid in 3.5M- (or 6M-) guanidine hydrochloride and 60mM-sodium phosphate buffer, pH7.0. Likewise, the two cells in the reference compartment contained native ovomucoid in sodium phosphate buffer, pH7.0, and 6M-guanidine hydrochloride solution respectively. Extreme care was taken to ensure identical protein concentration in the sample and reference compartments. Protein concentrations in various experiments were in the range 2.2–2.9mg/ml.

computed from the experimental data: $\log K^0 = -8.3$, $\Delta n = 15$ and $k = 1.2$.

As shown above, three major conformational states of ovomucoid, i.e. the native, intermediate and fully denatured states, accumulate in significant amounts during its unfolding by guanidine hydrochloride. These conformational states, which exist respectively in 60mM-sodium phosphate buffer, pH7.0, in 3–4.5M-guanidine hydrochloride and at and above 6M-guanidine hydrochloride, were characterized by solvent perturbation, spectrophotometric titration and viscosity measurements. The fully denatured state was also characterized by circular dichroism.

Difference-spectral results

Native ovomucoid absorbs maximally at 277nm and its spectrum was blue-shifted by the addition of guanidine hydrochloride. This is shown by the spectral results, obtained at 3.5M- and 6M-guanidine hydrochloride, depicted in Fig. 2; the results at 3M-guanidine hydrochloride were identical with those found in 3.5M-guanidine hydrochloride. The 'wiggles' in the region 250–270nm represent perturbation of spectra of the phenylalanine residues, whereas the fine structures above 275nm (at 282 and 287nm) as well as the minimum near 240nm (Donovan, 1969) are indicative of the significant alteration of the environment of tyrosine residues of ovomucoid. These features in the difference spectra reveal that the protein chromophores are significantly exposed in 3.5M-guanidine hydrochloride and that the exposure was extensive in the presence of 6M-guanidine hydrochloride.

Solvent perturbation

Solvent-perturbation results are summarized in Table 2. Clearly, $\Delta\varepsilon/\varepsilon$ values for a model mixture are generally in excellent agreement with those reported earlier (Herskovits & Laskowski, 1968). In the native state, the exposure of tyrosine residues of ovomucoid as measured by perturbation with ethylene glycol and dimethyl sulphoxide is 0.56–0.57; the exposure from experiments with glycerol is 0.42.

Table 2. *Solvent-perturbation studies on ovomucoid under native and denaturing conditions*

The exposure of the tyrosine residues in ovomucoid is expressed relative to the model mixture and was obtained by dividing the $\Delta\varepsilon_{288}/\varepsilon_{278}$ value found for ovomucoid by $\Delta\varepsilon_{285}/\varepsilon_{275}$ value determined for the model mixture. The composition of the model mixture was based on six tyrosine and five phenylalanine residues per molecule of ovomucoid (Beeley, 1976). Protein concentration was in the range 3–4mg/ml.

	In 60mM-phosphate buffer, pH7.0			In 3.5M-guanidine hydrochloride		
	$\Delta\varepsilon_{285-288}/\varepsilon_{275-278}$			$\Delta\varepsilon_{285-288}/\varepsilon_{275-278}$		
Perturbant (20%, v/v)	Model mixture	Ovomucoid	Relative exposure	Model mixture	Ovomucoid	Relative exposure
Dimethyl sulphoxide	0.158	0.090	0.57	0.159	0.097	0.61
Ethylene glycol	0.073	0.041	0.56	0.074	0.046	0.62
Glycerol	0.057	0.024	0.42	0.059	0.033	0.56

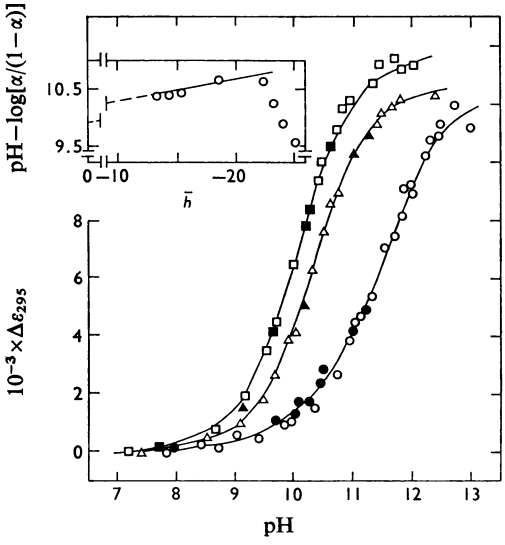

Fig. 3. *Spectrophotometric titration curves of ovomucoid in* 0.15M-*KCl* (○, ●), *in* 3.5M-*guanidine hydrochloride* (*plus* 0.15M-*KCl*) (△, ▲) *and in* 6M-*guanidine hydrochloride* (*plus* 0.15M-*KCl*) (□, ■)

For titration in 0.15M-KCl, protein solutions of different pH values were prepared by taking ovomucoid, KCl, water and KOH in calibrated volumetric flasks. The spectrum of each solution was recorded in the u.v. region against an appropriate blank solution. For titration studies in the presence of guanidine hydrochloride, ovomucoid was first exposed to the denaturant for 12h before adjustment of the pH with KOH. The pH of the solutions was measured immediately after the spectral measurements. $\Delta\varepsilon_{295}$ represents the increase in molar absorption of ovomucoid at a given pH over its molar absorption at pH7. The open and filled symbols denote the points obtained in the forward and reverse titrations. Inset: Linderstrøm-Lang plot for the titration data in 0.15M-KCl. Similar plots for the titration in 3.5M- and 6M-guanidine hydrochloride were obtained but are omitted for clarity. The protein concentration was in the range 0.7–1.1mg/ml.

These findings are in accord with those published previously (Herskovits & Laskowski, 1962; Donovan, 1967). In 3.5M-guanidine hydrochloride, the exposure of tyrosine residues of ovomucoid as measured by perturbation with dimethyl sulphoxide and ethylene glycol remains essentially the same as in the native state (see Table 2). However, the exposure as measured by glycerol perturbation increased from 0.42 in the native state to 0.56 in the intermediate state.

Spectrophotometric titration

Spectrophotometric titrations of the native, the intermediate and the fully denatured states of ovomucoid were performed at 30°C and the results are graphically shown in Fig. 3. The number of tyrosine residues ionized was computed by dividing $\Delta\varepsilon_{295}$ by 2300 (Crammer & Neuberger, 1943) for aqueous solutions and by 2450 (Nozaki & Tanford, 1967) for solutions containing 3.5M- (or 6M-) guanidine hydrochloride. In the native state, the titration was reversible only up to pH11.2, where 2.1 phenolic groups/molecule of ovomucoid were titrated. Above pH11.2 the titration became irreversible, owing to time-dependent alkaline denaturation. Of the six tyrosine residues in ovomucoid (Davis *et al.*, 1971; Beeley, 1976), five were titrated up to pH13.0. The titration results obtained in the pH range 7–11.2, where the titration was completely reversible, were thermodynamically analysed with the Linderstrøm-Lang (1924) equation:

$$\mathrm{pH} - \log[\alpha/(1-\alpha)] = \mathrm{p}K_{\mathrm{int.}} - 0.868\, w\bar{Z} \qquad (2)$$

where w is the electrostatic-interaction factor, $\bar{Z}$ is the net charge on the protein molecule, $\mathrm{p}K_{\mathrm{int.}}$ is the intrinsic dissociation constant of the two phenolic groups and α is the degree of dissociation of the groups at a given pH. Assuming that the protein does not bind salt ions, $\bar{Z}$ can be replaced by $\bar{h}$, the number of mol of H^+ ions bound to the protein; $\bar{h}$ can be obtained from the titration data (Waheed & Sala-

huddin, 1975*b*). The inset of Fig. 3 shows that the curve is linear only up to pH 10.75, where $\Delta\varepsilon_{295}$ corresponds to the ionization of 1.2 phenolic groups; above pH 10.75 the curve deviated appreciably from linearity. This, as well as the fact that the curve of $\log[\alpha/(1-\alpha)]$ versus pH (not shown) was non-linear, indicates that the $pK_{int.}$ remains constant (Roxby & Tanford, 1971) only up to pH 10.75 and that a change in pH from 10.75 to 11.2 produced a reversible conformational change. The pH for the onset of alkaline denaturation of ovomucoid is slightly lower than pH 10.9 (Gorbunoff, 1968). From the analysis of the results up to pH 10.75 according to eqn. (2), the values of pK_{int} and w were computed to be 9.9 and 0.04 respectively. These results seem to suggest that an increase in pH of about 0.5 pH unit from pH 10.75 exposes an additional tyrosine residue of ovomucoid which becomes available for reversible titration. In 3.5M-guanidine hydrochloride, 5.1 tyrosine residues of ovomucoid titrated reversibly up to pH 12.4. The curve of $pH-\log[\alpha/(1-\alpha)]$ versus $\bar{h}$ was linear up to pH 12.4 and the values of $pK_{int.}$ and w were determined to be 10.2 ± 0.15 and 0.003 respectively; the average experimental error involved in the determination of $pK_{int.}$ was 15%. Likewise, in 6M-guanidine hydrochloride the titration was fully reversible up to pH 12.0, where $\Delta\varepsilon_{295}$ corresponds to the ionization of 5.6 tyrosine residues. The Linderstrøm-Lang curve was linear up to pH 12.0 and the computed values of $pK_{int.}$ and w were 9.90 and 0.005 respectively. Thus the value of w in 3.5M-guanidine hydrochloride was identical, within experimental error, with that found in 6M-guanidine hydrochloride.

Circular dichroism

The main objective of measuring circular-dichroism spectra of ovomucoid in the presence and absence of 6M-guanidine hydrochloride was to characterize the denatured state. As shown in Fig. 4 the u.v. circular-dichroism spectrum of native ovomucoid is characterized by a trough at 264nm, a small trough near 268nm, a band near 280nm and a trough near 292nm. These features are similar to those reported by Ikeda *et al.* (1968) and by Kay *et al.* (1974). According to the latter authors these spectral features arise from phenylalanine, tyrosine and cystine residues of the protein. The nature of the circular-dichroism curve can be explained in view of the reasonable suggestion that the narrow positive circular-dichroism bands of tyrosine residues at 278 and 284nm are superimposed on the broad negative band corresponding to the cystine residues of the protein (Kay *et al.*, 1974). Following Greenfield & Fasman (1969), the percentage of helix in native ovomucoid with $[\theta]_{MRW}$ at 208nm of -12777 degree·cm^2·$dmol^{-1}$ can be calculated to be 30%. It should be pointed out that the characteristic fine structures in the circular-dichroism spectra of the native ovomucoid disappear in 6M-guanidine hydrochloride. Interestingly, the spectrum obtained in 6M-guanidine hydrochloride is qualitatively similar to that of *NN'*-diacetyl-L-cystine bismethylamide in 6M-guanidine hydrochloride or to the computed circular-dichroism band of the disulphide groups of ribonuclease A in 6M-guanidine hydrochloride (Takagi & Izutsu, 1974); ribonuclease in 6M-guanidine hydrochloride behaves as a cross-linked random coil (Salahuddin & Tanford, 1970). This observation

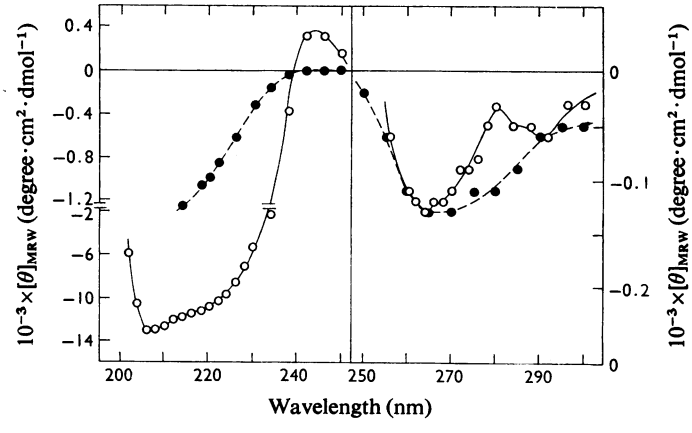

Fig. 4. *Circular-dichroism spectra of ovomucoid*

○, In sodium phosphate buffer, pH 7.0; ●, in 6M-guanidine hydrochloride. The data are expressed as mean residue ellipticities. The protein concentrations were in the range 0.5–0.7mg/ml.

suggests that in the denatured state the residue–residue interactions are abolished and ovomucoid probably exists as a cross-linked random coil.

Intrinsic viscosity

The intrinsic viscosity of ovomucoid in 60mM-sodium phosphate buffer, pH7.0, at 25°C was measured to be 5.24ml/g. The axial ratio (a/b), calculated from the intrinsic viscosity by the procedure of Ansari *et al.* (1975), is 6.2 (and frictional ratio, $f/f_0 = 1.33$) for the unhydrated ovomucoid molecule. Assuming that the hydration of ovomucoid is similar to that of ovalbumin, which binds about 0.2g of water/g of protein (Tanford, 1961), the respective values of a/b and f/f_0 would be 4.9 and 1.24. The Stokes radius of native ovomucoid as determined by gel filtration was 2.75nm, which according to Andrews (1970) would correspond to a frictional ratio (f/f_0) of 1.4. Thus both intrinsic viscosity as well as gel-filtration data indicated marked asymmetry in the overall native conformation of ovomucoid. In 3.5M-guanidine hydrochloride, the intrinsic viscosity of the protein increased to 7.4 ± 0.2ml/g, which represents an increase of 41% in the effective hydrodynamic volume of the protein due to its unfolding by guanidine hydrochloride. However, this value of intrinsic viscosity is substantially lower than the value (9.4 ± 0.4ml/g) measured in 6M-guanidine hydrochloride. Evidently ovomucoid in 3.5M-guanidine hydrochloride contained considerable residual native structure, which was partly or wholly removed by 6M-guanidine hydrochloride. The value of Huggins constant computed from the plot of reduced viscosity versus protein concentration (Huggins, 1942) was 0.7 in 6M-guanidine hydrochloride, which suggests that ovomucoid in 6M-guanidine hydrochloride behaves as a flexible polypeptide chain. It should be pointed out that, in 6M-guanidine hydrochloride, the intrinsic viscosity of commercial ovomucoid preparation, which contains a significant quantity of lysozyme (Donovan, 1967), was earlier reported to be 8.1ml/g (Ahmad & Salahuddin, 1975), which is significantly lower than the value reported in the present study. Previous underestimation of the intrinsic viscosity may be attributed to the presence of lysozyme, whose intrinsic viscosity in 6M-guanidine hydrochloride is 6.5ml/g (Aune, 1968).

Discussion

The overall native conformation of ovomucoid, as shown by the hydrodynamic results presented here and elsewhere (see Melamed, 1966; Davis *et al.*, 1971; Waheed & Salahuddin, 1975*a*), is substantially different from that of a typical globular protein. Thus marked asymmetry in the native protein molecule is clearly indicated from its measured intrinsic viscosity (5.24ml/g) and its related parameters (axial ratio = 6.2; frictional ratio = 1.33); the intrinsic viscosity of a typical globular protein lies in the range 3–4ml/g (Tanford, 1961). Consistent with this view is the observed gel-filtration behaviour of native ovomucoid. The Stokes radius (2.75nm) and frictional ratio (1.40), calculated from the gel-filtration data, are much higher than those expected for a globular protein of mol.wt. 27000. Despite this asymmetry, there is a high degree of structural organization in the native protein molecule. Circular dichroism, for example, suggests the presence of a sizable secondary structure, and spectral results implicate extensive protein folding and consequent 'burial' of several aromatic amino acid residues in the native structure of ovomucoid. As judged from the solvent-perturbation results, 43–44% of the tyrosine residues are 'buried' in the native state, and the remaining 'exposed' chromophores are not equally exposed, since the exposure measured by dimethyl sulphoxide or ethylene glycol is not the same as that found by glycerol perturbation. Of the 'exposed' tyrosine residues, only one could be titrated up to pH10.75 without any change in the native protein conformation; above pH10.75 alkaline denaturation took place. We emphasize that the exposure of tyrosine residues as shown by solvent perturbation may not necessarily be the same as that measured by phenolic titration. Short-range electrostatic interactions might have been responsible for the failure of even 'exposed' phenolic groups to titrate up to pH10.75. A tentative identification of such tyrosine residues may be attempted in the light of the amino acid sequence being currently studied extensively in the laboratory of M. Laskowski, Jr. From the available sequence data (I. Kato & M. Laskowski, Jr., personal communication) it seems that the six tyrosine residues 37, 46, 73, 102, 141 and 161 are equally distributed among the three separate structural domains of hen ovomucoid and that only tyrosine-102 in the second domain and tyrosine-141 of the third domain have adjacent negative charges because of aspartic acid-103 and glutamic acid-140. The net effect of the presence of proximal negative charge would be to raise the $pK_{int.}$ of the tyrosine residue. It is possible that one or both of these 'exposed' tyrosine residues were not available for titration up to pH10.75 in the native ovomucoid. Not even a tentative conclusion can be drawn from the sequence data with regard to the nature of accessibility of the remaining tyrosine residues, although, from the proximity of the two hydrophilic carbohydrate moieties that are presumably fully exposed, one may suspect that tyrosine-73 is least shielded from the solvent and is consequently able to titrate normally without any conformational change.

The intermediate conformational state of ovomucoid that occurs at 3.5M-guanidine hydrochloride is appreciably but not completely unfolded.

Accordingly, in 3.5M-guanidine hydrochloride the blue shift in the protein spectra, the characteristic fine structures in the difference spectrum (see Fig. 2), the increased spectral perturbation by the three different perturbing solvents, and availability of five titratable tyrosine residues as against one group in the native state, all showed considerable disruption of the native protein structure in 3.5M-guanidine hydrochloride. If the above interpretation of the results of phenolic titration of native ovomucoid is correct, it appears that, in addition to tyrosine residues 73, 102 and 141, two additional tyrosine residues were made available for titration as a result of unfolding by 3.5M-guanidine hydrochloride; tyrosine-102 and -141 were presumably 'normalized' by the high ionic strength provided by 3.5M-guanidine hydrochloride. That ovomucoid was unfolded by 3.5M-guanidine hydrochloride was further confirmed by the viscosity data, which showed 41% increase in the effective hydrodynamic volume of the protein produced by 3.5M-guanidine hydrochloride. However, as shown below, the intermediate state still retains significant residual native structure that is removable by 6M-guanidine hydrochloride.

The fully denatured state of ovomucoid that occurs at and above 6M-guanidine hydrochloride is much more unfolded than the intermediate state at 3.5M-guanidine hydrochloride. This is evident from the increased exposure of aromatic chromophores, including phenylalanine and tyrosine residues of the protein, revealed by the difference spectrum, availability of all the six tyrosine residues for titration with normal $pK_{int.}$, and from a marked (27%) increase in the intrinsic viscosity (7.4ml/g) of the protein in the intermediate state. The presence of residual native structure in the intermediate state was clearly indicated by the fact that addition of guanidine hydrochloride beyond 3.5M produced a second transition (see Fig. 1). However, both viscosity and phenolic-titration results did not show that ovomucoid was structureless in 6M-guanidine hydrochloride. On the other hand, circular-dichroism data obtained in 6M-guanidine hydrochloride suggested that the completely denatured ovomucoid was free from residue–residue interaction characteristic of the native protein structure, and behaved as a cross-linked random coil.

The conformational fluctuations in guanidine hydrochloride-denatured ovomucoid, which appears to be free from residue–residue interaction, would be severely limited owing to the physical constraint imposed by the presence of eight disulphide cross-links (Beeley, 1976). This in turn will minimize or even preclude the possibility of the existence of stable intermediate state(s) on the pathway of refolding. Under the circumstances the intermediate state, if any, is expected to occur only transiently. However, the results on the guanidine hydrochloride unfolding of ovomucoid have conclusively shown that the unfolding process involves more than one step. For the first step, the transition measured by viscosity is not the same as that followed by difference-spectral measurements. The results of Fig. 1 and Table 1 are consistent with the process $N \rightleftharpoons X_1 \rightleftharpoons X \rightleftharpoons D$, so that the intermediate states X_1 and X are indistinguishable by difference-spectral measurements, but seem to have significantly different effective hydrodynamic volumes. On the other hand, the two properties measured the same transition from X to D. The free energy change, ΔG^0, for the unfolding process $X \rightleftharpoons D$ in the absence of guanidine hydrochloride would formally represent the free energy of stabilization of state X over state D. The value of ΔG^0 computed from the best fit of the equilibrium results on the dependence of K on denaturant concentration was about 46kJ/mol. This would imply that state X of ovomucoid is thermodynamically more stable than state D by 46kJ/mol.

As stated above, ovomucoid consists of three separate structural domains, I, II and III, consisting of respectively 68, 65 and 50 amino acid residues. The hydrophobicities of the three domains computed from their amino acid compositions (I. Kato & M. Laskowski, Jr., personal communication) and the free energies of transfer of amino acid side chains (Nozaki & Tanford, 1971) are respectively 155, 134 and 96kJ/mol at 25°C. Therefore it would not be unreasonable to suspect that the guanidine hydrochloride unfolding of the first domain will require relatively higher denaturant concentrations than those of the domains II and III. From the position and number of the disulphide cross-links in the three separate domains of ovomucoid it would appear that the denaturation should cause greater expansion of domain III than of domain I. It is noteworthy that the percentage increase in intrinsic viscosity in going from state N to X is 1.5 times that involved in the transition $X \rightleftharpoons D$. This would suggest that the unfolding of the III domain is perhaps a part of the $N \rightleftharpoons X$ transition. Whether the unfolding of the II domain contributes to this transition or whether it represents a separate transition that could not be separated by the experimental probes used in this study remains to be answered. Available evidence appears to suggest that the $X \rightleftharpoons D$ transition may represent the unfolding of the I domain. Unequivocal identification of the two separate transitions observed during the guanidine hydrochloride denaturation of ovomucoid in terms of unfolding of the individual domains requires more extensive data than are presently available.

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