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The Denaturation of Ovalbumin

CHANGES IN OPTICAL ROTATION, EXTINCTION AND VISCOSITY DURING SERIAL DENATURATION IN SOLUTIONS OF UREA

By F. S. STEVEN AND G. R. TRISTRAM

Departments of Biochemistry, Queen's College and St Salvator's College, The University, St Andrews, Scotland

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Anfinsen & Redfield (1956) suggested an A-shaped structure for the native ovalbumin molecule, one leg of the A terminating in the carbohydrate moiety and the other bearing the C-terminal group. This structure was supported by a study of the increasing chemical reactivity of the ϵ -amino groups of lysine in ovalbumin during serial ethanol denaturation (Steven & Tristram, 1958). The authors postulated an internal link, which ruptured when the concentration of ethanol reached 50-55% (v/v). In earlier work, Maurer & Heidelberger

(1951) observed a critical degree of deamination of ovalbumin above which the molecule appeared to uncoil, stressing the importance of amino groups in the maintenance of the native structure.

The present work is a study of the serial denaturation of ovalbumin in urea solutions complementary to the studies of Simpson & Kauzmann (1953), who observed that the rate of denaturation of a solution of ovalbumin at pH 7.6 increased rapidly in 7M-urea. It seemed probable that the extent of denaturation of ovalbumin as measured

by changes in physical properties, in urea solutions, would indicate a sudden increase at a critical urea concentration sufficient to rupture the postulated link. Schellman (1955) suggested there was a critical urea concentration for each protein at which the rate of denaturation was accelerated. If this is in fact true, the results presented here will not distinguish between the rupture of an internal link in ovalbumin and a non-specific action of urea on all proteins.

This critical urea concentration may be an indication of the energy required to rupture linkages within the protein essential for the maintenance of the native configuration. The concentration required might be expected to vary with the experimental conditions as well as with individual proteins.

METHODS

Materials. The (hen) ovalbumin was similar to that used by Steven & Tristram (1958). Urea (A.R.) as supplied by British Drug Houses Ltd. was used throughout.

Serial urea denaturation. The ovalbumin was dissolved and the solution brought to pH 11.6 with the addition of small quantities of 0.1N-NaOH. The pH was chosen in order to achieve a high protein concentration without danger of denaturation, which takes place above pH 12 (Crammer & Neuberger, 1943). The protein solution (10 ml.) was added to each of a series of flasks containing solid urea, each successive flask having 0.6 g. more urea than the previous one. In this way the initial urea strength was varied over the range 0–10M. The final strength, however, varies from 0 to 7.1M, owing to the increase in volume caused by the solution of urea. The initial urea strength will be used for convenience to describe the urea concentration, although both values are quoted in Fig. 1. The reaction mixtures were incubated at 20° for 18 hr., the pH in each adjusted to the initial value and the changes in physical properties were measured. These conditions will be referred to as the standard conditions in the following experiments.

RESULTS

Extinction. Crammer & Neuberger (1943) showed that the absorption maximum of ovalbumin in solution increased with pH both in wavelength and magnitude, the former increasing from 280 to 295 m μ during the conversion of the protein from the native into the denatured state. Glazer, McKenzie & Wake (1957) demonstrated a characteristic depression of the wavelength of maximum absorption during the urea denaturation of ovalbumin in acid solution from 280 to 277 m μ . The effect of pH on the absorption spectrum of denatured ovalbumin accounted for the differences observed by these two groups of authors.

In the present work the extinction was measured at 295 m μ ; the extinction due to the presence of the urea was determined in a protein-free control experiment and this value was deducted from the

observed extinction in the corresponding denaturation experiment. The changes in volume due to solution of urea were determined from the densities of the solutions, this necessarily introducing a correction factor for each concentration of urea to allow for the corresponding decrease in protein concentration.

The molar extinction coefficients (corrected for dilution and the absorption due to the urea) plotted against the initial urea molarity are presented in Fig. 1.

Iodination of tyrosine residues. Li (1942) studied the kinetics of the iodination of tyrosine and later (1945) employed the rates of iodination of these residues in pepsin and serum albumin to demonstrate increased chemical reactivity of tyrosine residues during urea denaturation.

In the present work samples of ovalbumin solution (1.5%, pH 11.6) were incubated under the standard conditions with urea. The final pH was adjusted to 5.7 by adding 1 ml. of M-acetate buffer of this pH containing 0.1M-KI. Each sample was treated with 2 ml. of aqueous 0.1N-I₂ solution and allowed to stand at room temperature for 30 min.

Fig. 1. Molar extinction coefficients of ovalbumin in urea solutions. O, Tyrosine: $\epsilon_{295 \text{ m}\mu}$, pH 11.6; ∇ , di-iodo-tyrosine: $\epsilon_{310 \text{ m}\mu}$, pH 12.0.

before the reaction was stopped by the addition of 2 ml. of 0.1N-Na₂S₂O₃. The precipitated protein was dissolved by adding 10 ml. of N-NaOH (bringing the final pH up to 12), the volume made up to 100 ml. and the extinction measured at 310 m μ . Crammer & Neuberger (1943) found the absorption maximum of di-iodotyrosine to be 315 m μ at pH 13. The absorption maximum of iodinated ovalbumin was 310 m μ at pH 12. The increase in the extinction, measured at 310 m μ , during urea denaturation should therefore be an indication of the degree of iodination of tyrosine residues. The increase in extinction cannot be used as a direct measure of iodination since the phenolic groups of tyrosine and di-iodotyrosine in the protein also absorb in the region 295–310 m μ at this pH.

A control experiment was set up in which the conditions were identical in all respects with those described above, except that the iodine and thio-sulphate were mixed before addition to the protein solution. In this way no iodination could take place; the absorption was measured at 310 m μ over the complete range of urea concentration and no increase was observed. The control indicated that no correction need be made for the absorption

caused by the reagents employed in the reaction measured at this wavelength.

The molar extinction coefficients at 310 m μ plotted against the initial urea molarity are presented in Fig. 1. The iodine reaction was carried out for 10, 20 and 30 min.; in each instance the increase in extinction for each solution remained constant.

Optical rotation. Rotations were measured at 20° with a Schmidt and Haensch (Berlin) polarimeter with the sodium D line. Serial urea denaturation of ovalbumin was carried out at 20° for 18 hr., under the following experimental conditions, before the rotations were observed: (a) standard conditions of 1.5% protein and pH 11.6; (b) standard conditions in the presence of 0.1M-sodium thioglycolate, which was added to the urea before mixing with the protein solution; (c) standard conditions but with the addition of 0.1M-Na₂SO₄ to the urea before mixing with the protein; (d) protein solutions made up in dilute HCl to pH 2.5.

The results obtained from these experiments are presented in Fig. 2 after allowing for the lowering of the protein concentration. The initial and final rotations, 30° and 100° respectively, are in good agreement with the values observed by Simpson & Kauzmann (1953). The slightly higher initial rotation observed in acid solution corresponds to a similar observation made by Schellman (1958b). Sulphate ions suppress the change in rotation over the whole range of urea concentration. The graphs indicate critical concentrations of urea at which the rotations become increasingly negative. The critical urea concentration is dependent on the experimental conditions, namely pH, the presence of a disulphide reducing agent or an inhibitor of denaturation such as sulphate ions.

Viscosity. Viscosities were measured at 20° with an Ostwald viscometer (79 sec. H₂O at 20°; Griffin and George Ltd., London). The small change in density between the control urea solutions and the urea solutions containing protein has been neglected. The small correction factor due to changes in refractive index in urea solution has also been ignored (Schellman, 1958a). The changes in viscosity observed during serial urea denaturation are presented in Fig. 3 as ($\Delta\eta/c$) plotted against the initial urea concentration, after correcting for the dilution of the protein concentration. The results are presented in this way for the following

reasons: If the reduced viscosity, $\left(\frac{[\eta_1 - \eta_0]}{\eta_0}\right)/c$, where c is the molar concentration, is employed at high concentrations of urea the change in viscosity, $\eta_1 - \eta_0$, is zero, while the value of η_0 continues to increase; consequently the graph of the reduced viscosity versus the urea concentration may show

Fig. 2. Optical rotation of ovalbumin in urea solutions. (O), Standard conditions (see text) of 1.5% protein, pH 11.6; (●), standard conditions in the presence of 0.1M-sodium thioglycolate; (Δ), standard conditions in the presence of 0.1M-Na₂SO₄; (□), protein solutions in dilute HCl at pH 2.5.

a maximum before a decline. When $\Delta\eta/c$ is employed under identical conditions, the graph levels off at high concentrations of urea, at which the ovalbumin molecule appears to be fully uncoiled. The graph of $\Delta\eta/c$ against molarity of urea actually records the change in viscosity of the protein independently of any changes in the viscosity of the solvent.

Serial urea denaturation of the protein solution (1.5%) was carried out under conditions identical with those employed for the rotation experiments

Fig. 3. Changes in viscosity (millipoises) of ovalbumin in urea solutions. The conditions are identical with those in Fig. 2, the symbols having the same meaning.

described above as (a), (b), (c) and (d). In each of these experiments the same type of graph was obtained as in the corresponding rotation experiment, the inflexion appearing at the same urea concentration. The increase in the final value of $\Delta\eta/c$ in alkaline pH as compared with the same conditions in the presence of thioglycollate or in acid pH is probably due to the cross-linking of the extended peptide chains through conversion of intramolecular disulphide bonds into intermolecular bonds (Frensdorff, Watson & Kauzmann, 1953). This type of cross-linking cannot take place at acid pH or in the presence of thioglycollate in alkaline solution.

The results are compared in Table 1; the optical absorption studies on tyrosine and di-iodotyrosine are not included, since only conditions (a) were examined, although the inflexion occurred at an initial concentration of 5-6M-urea, as was observed in the corresponding rotation and viscosity changes.

DISCUSSION

The serial urea denaturation of ovalbumin, as measured by changes in certain physical properties, occurs in such a way as to suggest a critical urea concentration above which the configuration of the protein molecule is greatly altered. It is suggested that this critical urea concentration causes the rupture of some linkage which is essential for the maintenance of the native structure. Schellman (1955) suggested that the high electrostatic charge associated with a protein molecule at extremes of pH would produce repulsive forces within the chain and thus enhance the action of hydrogen-bond breakers. This would account for the difference observed in the critical urea strength of 7M at pH 7.6 (Simpson & Kauzmann, 1953) and 5-6M at pH 11.6 in the present work. The critical urea strength is lowered in acid and thioglycollate solutions indicating respectively the acid lability and possible structural importance of disulphide bonds in ovalbumin. The disulphide bonds in ovalbumin are not attacked by thioglycollate in the absence of urea, in contrast with the uncoiling

Table 1. Variation of the critical urea concentration with experimental conditions

In all experiments denaturation was continued for 18 hr. at 20°.

Experimental conditions	pH	Added reagent	Critical urea molarity (initial concn.)	
			$[\alpha]_D^{20}$	$(\Delta\eta/c)$
(a)	11.6	—	5-6	5-6
(b)	11.6	0.1 M-Thioglycollate	1-2	2-3
(c)	11.6	0.1 M- Na_2SO_4	6-7	6-7
(d)	2.5	—	2-3	2-3

action of thioglycollate on insulin (Schellman, 1958c).

The results suggest that a rapid uncoiling of the ovalbumin molecule occurs after a certain degree of denaturation has been exceeded, which is in agreement with the serial ethanol-denaturation study previously described.

SUMMARY

1. The degree of denaturation produced in ovalbumin by urea solutions under various conditions has been followed by physical means.

2. During denaturation optical rotation and viscosity show parallel changes and indicate that there is a critical urea concentration. This urea concentration is thought to be associated with the rupture of a linkage important for maintaining the configuration of the native protein.

3. Changes in extinction coefficient at 295 $m\mu$ and, after iodination, at 310 $m\mu$ also exhibit a similar dependence upon urea concentration.

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Kinetics and Equilibria in Systems Containing Haem, Carbon Monoxide and Pyridine

By M. H. SMITH*

Department of Biochemistry, University of Sheffield

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The object of the work described here was to compare the kinetic behaviour of haem compounds of varying degrees of complexity. A single reaction, combination with carbon monoxide, was studied to determine if the rate of combination was variable and the structural factors upon which any variation depended. Three groups of haem compounds were chosen: free haem, the haemochromogens and the haemoproteins. Myoglobin was taken as representative of the last-named group, being formally similar to the simpler haem compounds in having only one haem group per molecule. It also combines reversibly with carbon monoxide, and is very similar in many of its properties to haemoglobin. For the haemochromogen group the compounds with pyridine were chosen, since pyridine has been very widely used and more information was available than for other possible ligands. The effect of changing the side chains on the porphyrin nucleus has also been examined, and mesohaem and deuterohaem and their derivatives

have been prepared in addition to protohaem. The main problem therefore was to determine the rates of combination of carbon monoxide with three closely related haems, three pyridine haemochromogens and three myoglobins (nine reactions in all), the whole representing a gradual increase in the complexity of the haem-conjugated molecule.

The results with myoglobin conveniently stand alone and are dealt with in the next paper (Smith & Gibson, 1959). The present paper is divided into two parts: the first deals with experiments designed to establish the nature of the materials being studied, and the conclusions drawn about their structure; the second gives the results of the kinetic experiments.

It has been generally accepted that the iron atom in a haem molecule is able to combine with only two other groups, one on each side of the haem ring (cf. Lemberg & Legge, 1949). Although the concept of facultative hepta-co-ordination could resolve certain experimental and theoretical difficulties, its use seems to lead, in the end, to even greater perplexities than exist at present. In this work it

* Present address: John Harrison Laboratory of Chemistry, University of Pennsylvania, Philadelphia, 4.