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The Denaturation of Proteins: Two State? Reversible or Irreversible?

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One of the earliest workers on the heat coagulation of proteins, HARDY[1], considered that it occurs in two main stages: The first stage involves a preliminary change in the structure of the protein – the *denaturation* stage. The second and distinctly different stage is the process of *aggregation* and *coagulation* of the protein.

While the phenomenon of 'denaturation' is well known it has meant different things to different people. The technologist and biologist have often regarded it as only something to be avoided, e.g. the loss of solubility of a protein, the inactivation of an enzyme. The chemist has been more concerned with the chemical and physical aspects of changes in structure inherent in the primary phenomenon. Understanding of the phenomenon is still imperfect but it has reached the stage where it is realized that a study of denaturation can be of great value in gaining a better understanding of the biological function of proteins and the mechanism of enzyme action. An adequate theory of denaturation is in effect a theory of the structure of native and denatured proteins.

There has been a long controversy over the definition of *denaturation* as can be seen from a perusal of major reviews[2–8]. However, there is now some consensus of opinion that denaturation denotes a process in which the conformation of the polypeptide chain(s) within a protein molecule is changed in a major way from that typical of the native protein without the rupture of any primary covalent bonds linking one amino acid residue to another. Some authors, e.g. KAUZMANN[7], would include changes in conformation accompanying limited hydrolytic cleavage of peptide linkages in the term, denaturation. Others prefer to abandon the use of the term, and use another (e.g. transconformation[9]). Because the native protein itself can undergo subtle changes in conformation, exhibiting a motility[10], it becomes difficult to draw a clear distinction between such subtle changes and the more extensive conformational changes usually associated with denaturation. BRANDTS[11,12] and BILTONEN and LUMRY[13] attempt to distinguish between them in their excellent discussions, by restricting the term, denaturation, to those conformational changes involving a change in the molar heat capacity at constant pressure (C_p) of the protein. We shall consider this further below. The process of denaturation usually involves a change in the ordered structure of the native protein to a more disordered arrangement. Some proteins, e.g. various caseins[14], appear to occur in a disordered chain arrangement and hence cannot undergo denaturation in this sense, but may undergo other conformational changes.

The ordered structures of native proteins are 'co-operative' in nature, involving a number of amino acid residues. Major conformational changes are 'co-operative', the transition from the native state to the denatured state is usually a 'steep' transition, occurring within a narrow range of temperature or concentration of denaturant (Figure 1). Observations such as these have led some workers to consider denaturation in terms of a two state transition. Although this concept has been placed recently on a thermodynamic basis[8,12,13], it is not a new one. Some 25 years ago ANSON[4] stated the essential features of the 'all or none' denaturation reaction in non-thermodynamic language on p. 382 of his review.

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[6] W. KAUZMANN, in *The Mechanism of Enzyme Action* (Eds. W. D. McELROY and B. GLASS; Johns Hopkins Press, Baltimore 1954), p. 70.
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[12] J. F. BRANDTS, in *Structure and Stability of Biological Macromolecules* (Eds. S. N. TIMASHEFF and G. FASMAN; Marcel Dekker, New York 1969), p. 213.
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[14] D. C. POLAND and H. A. SCHERAGA, Biopolymers *3*, 401 (1965).

Let us consider the transition N⇄D, between the native macroscopic state (N) of a protein and the denatured macroscopic state (D). Each of these states is a distribution of microscopic states. BRANDTS[11,12] and other members of this school[13] consider that the transition is a two state one in which intermediate forms of the protein are negligible. POLAND and SCHERAGA[15] argue against this theory on statistical mechanical grounds, and consider that the mechanism is one of gradual unwinding of the protein molecule. There are well defined criteria for a two state transition; however, problems arise in their experimental application. The change in the experimental value of a given parameter ($\bar{\alpha}$) (such as optical rotation, light absorption, viscosity) for the transition N⇄D is shown in Figure 1. A major problem in determining the equilibrium constant of the reaction is to know what the values of the parameter for the pure native form ($\bar{\alpha}_N$) and for the pure denatured form ($\bar{\alpha}_D$) are in the transition region. They can only be obtained by extrapolation procedures. It is important to realise that a change in the perturbant (temperature, T, or pressure, P, or solvent composition, M) may not only shift the equilibrium between native and denatured forms but may also cause a change in the extent of unfolding of the denatured form (or of the native form). Such a change may be a gradual one or a cooperative one (Figure 1). This situation does not preclude a two state transition in a solution of constant composition and temperature. However, it does mean that the transition cannot be considered strictly as simple two state over a wide change in a given perturbant. Nevertheless providing the changes in $\bar{\alpha}_N$ and $\bar{\alpha}_D$ are small, and they are taken into account using a suitable extrapolation procedure, it is reasonable to treat the transition over the whole range of perturbant change by two state theory as BRANDTS[11,12] has done. It is also important to stress that the denatured form of the protein produced by one perturbant (e.g. temperature of an aqueous solution) is not necessarily the same as that produced by another perturbant (e.g. concentrated urea solution). In fact it is our experience that they are in general quite different.

The following experimental approaches are pertinent to the study of denaturation (see also[7,12]): 1. The determination of the change in several experimental parameters, sensitive to different properties of the protein (cf.[2,7]). The extent of change and rate of change of these properties should parallel one another exactly in a two state transition. Plots of equilibrium values of the property $\bar{\alpha}$ *vs* perturbation (T, or P, or M) should be of the form shown in Figure 1 and curves for different parameters should coincide for two state transitions. (This is a necessary, but not sufficient, condition.) If stable intermediate forms are present steps may occur in the plot. Only in the absence of stable intermediates may thermodynamic functions and the equilibrium constant (K_D) be calculated from equilibrium values of $\bar{\alpha}$. Note that experimental values of K_D can only be determined over a limited range ($K_D \approx 0.1$ to 10). 2. Determination of effect of temperature on the reaction. 3. Comparison of apparent heats of reaction (ΔH_{app}) and calorimetric heats of reaction (ΔH). Determination of the heat capacity change, ΔC_p [where, $\Delta C_p = \{\partial (\Delta H)/\partial T)\}_p$]. ΔH_{app} calculated from the effect of temperature on the reaction will, in general, only be equal to ΔH if there are no stable intermediates. 4. Examination of reversibility and determination of the rates of forward (k_1) and reverse reactions (k_{-1}). There are 2 questions of reversibility to be considered: a) can the native protein be recovered? b) is the reaction thermodynamically reversible? In a two state transition the forward and reverse reactions are first order and $k_1/k_{-1} = K_D$. 5. Examination of denaturation products by zone electrophoresis for heterogeneity, isolation and characterization (e.g. ORD, CD). 6. Determination of effect of pH on the reaction. 7. Comparison of behaviour of genetic variants of the protein. 8. Study of effect of microheterogeneity (*non* genetic in origin).

It is our experience that insufficient attention has been given to some of the above approaches, especially 5–8. While the original concept of HARDY in separating the processes of denaturation (conformational change) and aggregation is a valid one it is our opinion that some recent workers have failed to stress the importance of aggregation in the overall process

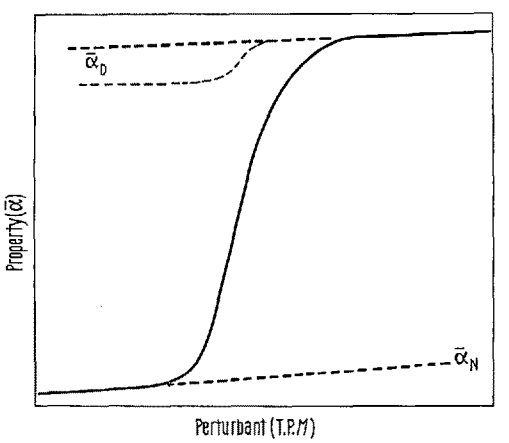

Fig. 1. Change in an experimental parameter ($\bar{\alpha}$) of a protein with change in perturbant for a two state transition. The perturbant may be temperature (T), or pressure (P), or solvent composition (M). —, represents experimental values of the property ($\bar{\alpha}$); --- $\bar{\alpha}_N$, represents extrapolated values of $\bar{\alpha}_N$ for the pure native form (N); --- $\bar{\alpha}_D$, represents extrapolated values of $\bar{\alpha}_D$ for the pure denatured form (D) assuming change in $\bar{\alpha}_D$ in a gradual way; ---, represents change in $\bar{\alpha}_D$ assuming change in a cooperative fashion (MCKENZIE, RALSTON: Denaturation).

[15] H. A. MCKENZIE, Adv. Protein Chem. *22*, 55 (1967).

for many proteins (e.g., p. 275 of ref.[8]). Since many proteins and enzymes contain –SH and –SS groups it is not surprising that irreversible inter- and intramolecular changes involving these groups may accompany denaturation and play an important role in the overall processes taking place.

Furthermore we believe that in many cases simple two state behaviour does not occur for the denaturation (unfolding) process itself. We shall now discuss in some detail an example in which two state behaviour is not observed, and the denaturation is accompanied (under certain conditions) by irreversible processes. Then we shall briefly discuss this example in relation to studies on other proteins and enzymes. The main discussion centres around our recent comparative study of the behaviour of genetic variants of bovine β-lactoglobulin in urea solution.

β-Lactoglobulin in urea solution. One of us has reviewed the chemistry of the β-lactoglobulins[14,16]. It suffices to point out here that each of the genetic variants is a dimer of molecular weight 36,000 daltons near the isionic point (ca. pH 5.2), consists of two identical chains, is a mixture of disordered chain, helical and β-conformations, and undergoes some dissociation to the monomer of molecular weight 18,000, and conformational transitions as the pH is varied from 5.2.

Recently a partial amino acid sequence for the A and B variants has been determined by FRANK and BRAUNITZER[17] and the positions of the single –SH group (per monomer) and two –SS groups have been determined by us[18]. A schematic representation of the monomer chain based on this work, is shown in Figure 2.

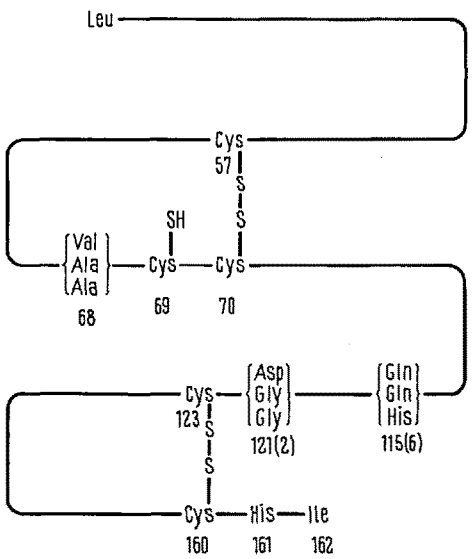

Fig. 2. Schematic diagram of the bovine β-lactoglobulin polypeptide chain (monomer) showing the position of the 2 disulphide bridges and the single sulphydryl group. The difference residues are indicated in the brackets in the order: A, B, C (A↔B, positions 68 and 121 or 122; B↔C, position 115 or 116). (There are also 2 rare variants: D has a Gln residue substituted for Glu at position 108 or 109; Droughtmaster has the amino acid composition of A, but has a carbohydrate moiety and a molecular weight of 20,000.) The diagram is based on refs. [17,18] (MCKENZIE, RALSTON: Protein denaturation).

The behaviour of β-lactoglobulin in urea solution is very dependent on pH. We shall first consider the behaviour at pH 3.5.

pH 3.5. Urea concentrations greater than 4 *M* caused a very rapid change in specific optical rotation at 578 nm, $[\alpha]_{578}$, for A, B and C variants. The rapid increase in laevorotation was followed by a small and very slow increase over the next 300 min, without further change over the next 48 h. The initial change was so rapid that a kinetic analysis could only be made in a few cases. The rapid change in $[\alpha]_{578}$ for the A variant in 6 *M* urea and the C variant in 5 *M* urea at 25 °C was first order in both cases at 25 °C. The kinetics for the entire rotation change for the A variant in 6 *M* urea were studied and the dramatic break between the rapid and slow reactions can be seen in the log plot of Figure 3a [where α is the optical rotation at a time t, and α_f is the final rotation]. Values of the final specific optical rotation at the end of the primary process, $[\alpha]_f$, for the A, B and C variants are given in Figure 4, showing that at pH 3.5 a given rotation change is brought about by a lower urea concentration for the C variant than for the A or B variants. The steep nature of each plot is strikingly similar to what one would expect for a two state process, but the existence of intermediates (shown below) belie this.

After only 10 min exposure to 7 *M* urea at 25 °C the change in $[\alpha]_{578}$ could be reversed completely to that of the native protein. However, the extent of reversibility decreased with increasing time of exposure to high urea concentration. This was confirmed by solubility measurements. We have shown (by methods similar to those used at pH 5.2 below) that a major part of the irreversibility at pH 3.5 is due to –SS–/–SH exchange reactions. This is contrary to expectation: it is generally assumed that –SS–/–SH exchange reactions are negligible at this low pH.

Evidence was obtained for the existence of intermediate states of β-lactoglobulin in urea solutions at low pH (ca. 3) and at reaction times when negligible irreversible products had formed. The final value of the optical rotation for the A, B and C variants in 7 and 8 *M* urea, at all wavelengths over the range 215–600 nm, was attained within 1 min of mixing at 25 °C. The optical rotatory dispersion (ORD) curves under these conditions were similar, a typical example being shown in Figure 3b. In 5.5 *M* urea the B variant

[16] H. A. MCKENZIE, in *Milk Proteins* (Ed. H. A. MCKENZIE; Academic Press, New York 1970), vol. II, chap. 14.

[17] G. FRANK and G. BRAUNITZER, Hoppe-Seyler's Z. physiol. Chem. *348*, 1691 (1967).

[18] G. B. RALSTON, Ph. D. Thesis, Australian National University (1969). – H. A. MCKENZIE, G. B. RALSTON and D. C. SHAW, to be published; see also discussion in ref. [16].

is 50% denatured at the end of the primary reaction step on the basis of $[\alpha]_{578}$ as experimental parameter. At wavelengths above 260 nm, the change in reduced rotation at any wavelength is almost exactly 50% of the corresponding change in $8M$ urea. However, a different picture emerges at wavelengths below 250 nm where an ORD curve, calculated from the curves for native and fully denatured protein, assuming 50% denaturation, deviates markedly from the experimental curve, as shown in Figure 3b. This is shown even more strikingly in the inset.

pH 5.2. The specific optical rotation at 578 nm, $[\alpha]_{578}$, for bovine β-lactoglobulin B and its rate of change with time (t), are strongly dependent on urea concentration (M) as shown in Figure 5a. In $6M$ urea the laevorotation at 578 nm increases slowly with time and does not reach a constant value even after 48 h. In $7M$ urea, the laevorotation increases rapidly over the first 30 min, and then changes slowly over 48 h. Thus the rates of change of rotation at these urea concentrations are much lower than the corresponding ones at pH 3.5. At urea concentrations of $8M$ and above there is rapid change in the first minute, and no further change.

GUGGENHEIM[19] type plots for the rotation change in $7M$ urea, during the first 50 min of reaction, are curved, the rate decreasing with time (e.g. Figure 5b). Addition of denatured protein has no effect on the rate. Using the method of SIMPSON and KAUZMANN[20] the change in rotation may be broken down into a primary stage and a secondary stage and a value of the final rotation, α_f, for the primary process determined. The primary stage is not simple first order and can be described by 2 exponential terms. The overall primary and secondary processes cannot be described by 3 terms. The half time ($t_{1/2}$) for the primary process increases slightly with increasing protein concentration (2–18 g/l). Plots of log $(\alpha - \alpha_f)$ vs $t/t_{1/2}$ for different protein concentrations coincide.

The rate of change and the extent of change of $[\alpha]_{578}$ for the A, B and C variants are in the order A > B > C (i.e. C is the most stable).

In the early stages of the reaction the change in $[\alpha]_{578}$ is largely reversible, but the extent of reversibility decreases considerably with time. The kinetics of refolding are complex, and the complexity is greater the longer the reaction time and the higher the urea concentration (before dilution).

An estimate can be made of the final rotation values at the end of the rapid denaturation phase. These values for the A and B variants are plotted against urea concentration in Figure 4b. The steep nature of the plot is strikingly similar at first sight to that for a two state process, but again the complexity of the forward and reverse reactions belie this.

The rate of change of $[\alpha]_{578}$ does not parallel that of molar absorbancy difference ($\Delta\varepsilon$) with respect to native protein at 293 and at 286 nm, as shown in Figures 5c and 6.

In the temperature range 3–45 °C the rate of rotation change decreases with increasing temperature until 35 °C when the rate passes through a minimum. At 3.8 °C the changes in $[\alpha]_{578}$ are much more rapid and extensive than those at 25 °C. The rotation change in $6M$ urea followed first order kinetics ($t_{1/2} = 3.5$ min) and no slow reaction phase was observed. In $5M$ urea the kinetics were not simple first order.

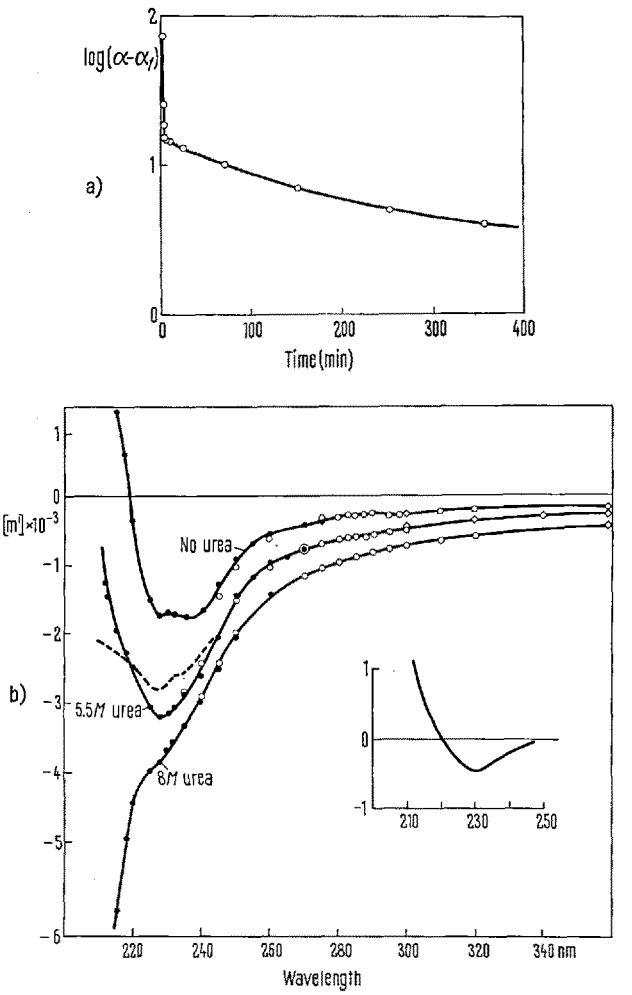

Fig. 3. a) Plot of log $(\alpha - \alpha_f)$ at 578 nm vs time for bovine β-lactoglobulin A in $6M$ urea solution at pH 3.5 (NaCl-HCl, I = 0.1) and 25 °C. b) Optical rotatory dispersion in the ultra violet region for bovine β-lactoglobulin B at pH 3.0 and 27 °C 1. in the absence of urea, 2. in the presence of $5.5M$ urea and 3. in $8M$ urea. The broken line represents the calculated value for 50% denatured protein, as described in the text. The triangles represent data for 1 cm path length and the circles represent data for 0.1 cm. The inset shows the difference between the experimental and calculated curves of the protein in $5.5M$ urea. The Cotton effects at 280–300 nm are too small to appear on the scale of the diagram. They will be discussed elsewhere (MCKENZIE, RALSTON: Protein denaturation).

[19] E. A. GUGGENHEIM, Phil. Mag. *2*, 538 (1926).
[20] N. C. PACE and C. TANFORD, Biochemistry *7*, 198 (1968).

In the presence of N-ethyl maleimide (2 moles NEM: 1 mole –SH) the rate of change of $[\alpha]_{578}$ was much greater than in its absence. The kinetics in $6M$ urea were apparent first order. The effect of dilution from $7M$ urea to $6M$ urea was immediate change of $[\alpha]_{578}$ to the final value attained in $6M$ urea. This is in contrast to the behaviour in the absence of NEM, where the effect of dilution depends on the time of reaction in $7M$ urea prior to dilution to $6M$.

The increasing irreversibility with increasing time of exposure to $7M$ urea solution was also demonstrated by studies of the solubility in $2M$ ammonium sulphate of the products of the denaturation. An examination by starch gel electrophoresis of the reaction products after 24 h in $7M$ urea at 25 °C, revealed a number of slow moving bands. The products represented by these bands were not reversed on the removal of urea. These bands were eliminated when the denatured protein was treated with 2-mercaptoethanol prior to application of the samples to the gel.

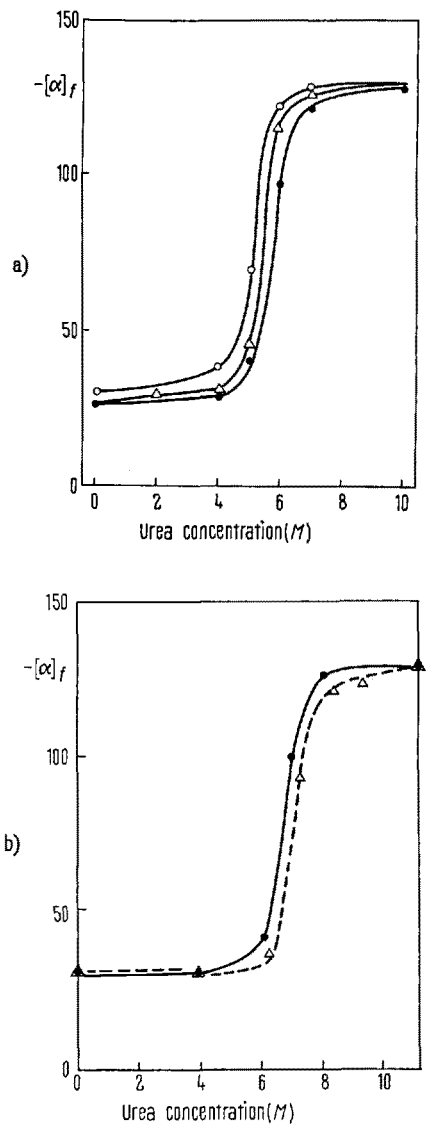

Fig. 4. Values of the optical rotation at 578 nm at the end of the primary step of the denaturation reaction at 25 °C for the bovine β-lactoglobulin variants and urea concentrations shown. a) pH 3.5; b) pH 5.2. –●–, signifies the A variant; –△–, signifies the B variant; –○–, signifies the C variant (McKenzie, Ralston: Protein denaturation).

pH 6.1, 6.9. Complex kinetics were found in most cases and results for pH 6.1 and 6.9 were very similar. There was a very rapid reaction phase followed by a slower one. It was found from reversibility experiments that $[\alpha]_f$ for the rapid phase was that of an equilibrium position and the subsequent change was largely irreversible.

An examination was made of solutions of the B variant that had been exposed to $7M$ urea at pH 7.5 for various times by starch gel electrophoresis and Sephadex G 100 chromatography. There were increasing amounts of high molecular weight material ($> 36{,}000 \leq 100{,}000$) with increasing time of exposure to urea and material of molecular weight 54,000 (i.e. a trimer of the monomer of molecular weight 18,000) was an important product. Protein of molecular weight of ca. 36,000 daltons was isolated by chromatography, after exposure to $7M$ urea for 1 h and after 24 h. The former had an ORD curve, identical with the native protein, the latter had one that was similar to, but not identical with, the native protein. It was shown that the irreversible products involve extensive –SH/–SS exchange.

Conclusions for β-lactoglobulin. We shall describe the above and other results, for the pH range 3–9, in detail elsewhere (including a discussion by G. B. Ralston of the origin of pH effects for the C variant). The general picture that has emerged is that the behaviour of β-lactoglobulin in urea is complex. This complexity is partly due to irreversible aggregation reactions accompanying the denaturation process. However, aggregation is not the only cause of complexity: the primary unfolding reaction itself cannot be described adequately in terms of a two state process. The kinetics of the denaturation and renaturation reactions are in general not simple first order, except for a limited range of denaturation conditions. In most cases two exponential terms are needed to describe the primary stage of denaturation. At the end of this stage, under pH conditions where the onset of aggregation is negligible, the ORD of β-lactoglobulin does not correspond to a simple mixture of native and fully denatured protein. Different parts of the native protein (e.g. α-helical and β-structure segments) would appear to unfold at different rates. The kinetics of the unfolding reactions are consistent with parallel or sequential unfolding of these segments (although a general mechanism may involve varying contributions from 'branching' reactions according to denaturation conditions. The relative importance of parallel and sequential reactions will be discussed elsewhere).

In view of the molecular sizes of the products of the action of urea, denaturation appears to proceed via the monomer of molecular weight 18,000. Also bovine β-lactoglobulins A and B (and to a less extent C) are probably largely dissociated to the monomer in urea solution $> 3M$ at pH < 3.5 (see, e.g.[20]) and at pH > 7.5. At pH 5.2 where dissociation is less extensive, the rate of denaturation increases with decreasing protein concentration (i.e. increasing dissociation to monomer). The first unfolding step of the primary reaction is very rapid at low and high pH, but relatively slow at pH 5.2. The second step of the primary phase appears to be comparatively slow, although it becomes fast for high urea concentrations at high pH. When N-ethyl maleimide (NEM) is present the whole primary reaction at pH 5.2 is faster and becomes simple first order. It is of interest that NEM is known to dissociate the protein completely to monomer at pH 8 even in the absence of urea[21] (see also[22]).

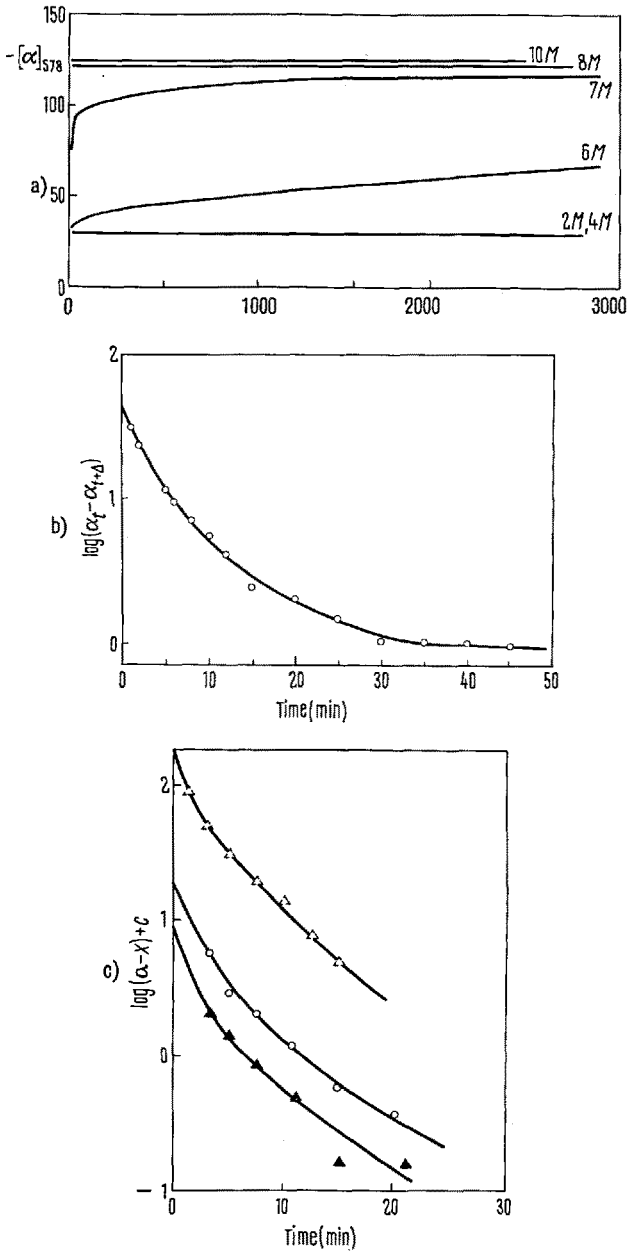

Fig. 5. Effect of urea on bovine β-lactoglobulin B at pH 5.2 (CH_3COOH–CH_3OONa-NaCl, I = 0.1) and 25 °C. a) Effect of time on $[\alpha]_{578}$ for various urea concentrations. b) GUGGENHEIM plot for the change in $[\alpha]_{578}$ for the protein (8.4 g/l) in 7 M urea. Δ = 10 min. c) Plots of log $(a - x) + c$ vs time for a property with value, a, for the native protein, and, x, at a time t. An arbitrary constant, c, has been included to enable all data to be plotted conveniently. △, signifies data for $[\alpha]_{578}$; ○, signifies molar absorbancy index difference at 293 nm ($\Delta\varepsilon_{293}$); ▲, signifies $\Delta\varepsilon_{286}$ (MCKENZIE, RALSTON: Protein denaturation).

The unfolded material from the second step undergoes –SH/–SS exchange reactions (and possibly –SH oxidation) to yield irreversible products. Those formed at moderate urea concentrations may be partially refolded on removal of urea. The irreversible reactions also take place, albeit slowly, at low pH. The latter observation and the complexity of the denaturation reaction lead us to consider it inadvisable to calculate thermodynamic functions from the data. On the other hand PACE and TANFORD[20] concluded that at pH 2.5 to 3.5 the urea denaturation is a simple two state process, and no irreversible reactions occur. Their conclusions appear to have been modified subsequently[8].

Other proteins. To what extent are our conclusions for β-lactoglobulin applicable to the denaturation of other proteins? It might be considered at first sight that β-lactoglobulin is an isolated complex case, and is not a model example to use since it contains –SH and –SS groups. However it is well to stress that many other proteins and enzymes contain these groups. Furthermore many examples of denaturation are not simple two state-processes even when the additional processes of aggregation are not occurring.

SIMPSON and KAUZMANN[23] made a thorough study for ovalbumin in urea near pH 7 and concluded that the optical rotation change observed consisted of a primary and secondary stage and that the former could be described in terms of 2 exponential terms. It was also shown that irreversible exchange and aggregation reactions also occurred and these involved both –SH/–SS exchange and non covalent interactions[23,24]. Their results were confirmed by our group[25,26] and it was also shown[26] that the rate of optical rotation change at 589 nm does not parallel that of the change in

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[23] R. B. SIMPSON and W. KAUZMANN, J. Am. chem. Soc. *75*, 5139 (1953).
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[26] A. N. GLAZER, H. A. MCKENZIE and R. G. WAKE, Nature, Lond. *180*, 1286 (1957); Biochim. biophys. Acta *69*, 240 (1963).

difference spectra at 287 nm. McKENZIE and SMITH[27] found that only limited unfolding of protein occurs in heat denaturation of ovalbumin at low pH (compared with urea denaturation), but that aggregation is very extensive in the presence of electrolytes and dominated the overall process. SMITH and BACK[28] provided an example of how heterogeneity of non-genetic origin can affect the experimentally observed denaturation. They showed that a form of ovalbumin highly resistant to denaturation may be present in some ovalbumin samples. Recently HO CHO and KAUZMANN[29] have shown that even when the presence of this form is allowed for, the urea denaturation of ovalbumin is still not a two state process even at low pH. They have also demonstrated the presence of intermediates in the denaturation by circular dichroism measurements. At high urea concentrations, ovalbumin, like β-lactoglobulin, would appear to approach a random chain conformation.

A simple two state reaction is also not shown in the urea denaturation of conalbumin[30], in the guanidine hydrochloride denaturation of para-myosin[31] and of carbonic anhydrase[32]. KAUZMANN and KHALIFAH[33] have made a thorough study of the urea denaturation of the principal component of sperm whale metmyoglobin. They found that the rate of change of absorption at 230 nm paralleled that of the Soret band, each being approximately first order. The half time went through a maximum at 7.3 *M* urea as the urea concentration was varied. It was concluded that the reversible rate constant has an inverse dependence on urea concentration. This was confirmed by a direct experimental study of the reversal kinetics. However, the refolding half time was dependent on protein concentration, evidence for aggregation of denatured protein and for some degree of irreversibility being obtained. Even when allowance was made for dissociation of haem at low protein concentration it was found difficult to apply thermodynamic theories of denaturation.

On the other hand LUMRY, BILTONEN and BRANDTS[12,13,34] consider that thermally induced reversible transitions for ribonuclease, α-chymotrypsin, and chymotrypsinogen are two state processes. Their results for ribonuclease may be contrasted with those of SCOTT and SCHERAGA[35], and SIMONS et al.[36] who have found the denaturation to be more complex. SALAHUDDIN and TANFORD[37] have recently concluded from their measurements that the denaturation of ribonuclease by guanidine hydrochloride involves only two states. BARNARD[38] reached different conclusions. TANFORD et al.[39] found that below ca. 35 °C the guanidine hydrochloride denaturation of lysozyme involves only 2 states (native and cross linked random coil) but that above 35 °C a third state ('heat denatured state') contributes to the equilibrium properties. However, KING and BRADBURY[40] have suggested that the

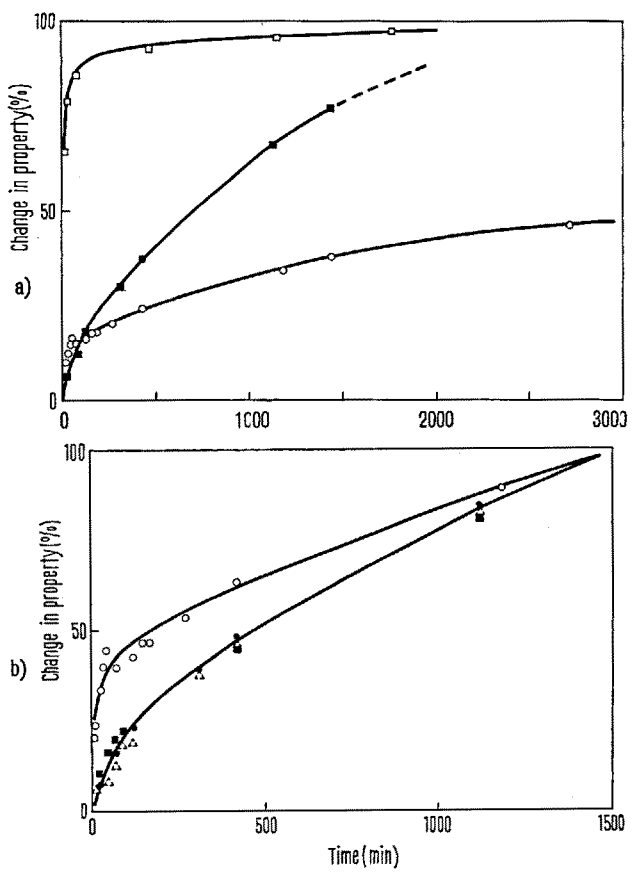

Fig. 6. Comparison of the change with time of $\Delta\varepsilon$ and $[\alpha]_{578}$ for the B variant (0.8 g/l) in 6 *M* urea at pH 5.2 and 25 °C. a) The change expressed as a percentage of the total change produced in 9 *M* urea. ○, signifies $[\alpha]_{578}$; ■, signifies $\Delta\varepsilon_{293}$. The change for $\Delta\varepsilon_{293}$ in 7 *M* urea is presented for comparison and is signified by □. b) The change expressed as a percentage of the total change occurring in 24 h. ○, signifies $[\alpha]_{578}$; ■, signifies $\Delta\varepsilon_{293}$; △, signifies $\Delta\varepsilon_{286}$; ●, signifies $\Delta\varepsilon_{237}$ (McKENZIE, RALSTON: Protein denaturation).

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hydrophilic exterior of lysozyme unfolds more readily than the hydrophobic core in urea at pH 2.8. The action of ethanol-water on trypsin has been interpreted by POHL[41] as a two state process (cf.[42]).

Following the classic paper of MIRSKY and PAULING[43] the denaturation of proteins was interpreted largely in terms of rupture of peptide hydrogen bonds. However, more recently it has become fashionable to lay great stress on the importance of the hydrophobic bond and the large heat capacity changes for denaturation reactions[12,13]. It is difficult to reconcile the hydrophobic interpretation with the temperature dependence of 'heat' denaturation in general, and with the effect of pressure on the model compound[44] 4-octanone, and on ribonuclease[45]. KAUZMANN and KLIMAN[44] found that the solubility of 4-octanone in water, at constant temperature, increases with increasing pressure up to 1500 to 2500 kg/cm² when it is ca. 1.5 times the value at 1 atm. Thereafter it decreases becoming equal to the value at 1 atm when the pressure is 4200, 5300 and 6500 kg/cm² at 15, 25 and 35 °C respectively. They found the solubility at 15 °C is greater than that at 35 °C for pressures up to 4500 kg/cm². The volume change on solution is ca. −28 cm³/mole at 1 atm (15–35 °C), 0 cm³/mole at 2000 kg/cm², and +3 to +5 cm³/mole at pressures above 2000 kg/cm². It was concluded that 4-octanone is a good compound to use as a model for hydrophobic interactions. On the basis of this model it would be expected that proteins would have a greater tendency to denature at pressures up to 4500 kg/cm² at 15 °C and up to 6000 kg/cm² at 35 °C, than at 1 atm. Assuming the solubility trends for 4-octanone continue at higher pressures native protein conformations should be stabilized at such pressures. On the other hand, as KAUZMANN and KLIMAN stress, pressures below 3000–4000 kg/cm² do not denature many proteins. Indeed the native conformations, except that for ribonuclease, appear to be stabilized by these pressures. When the pressure is increased above 4000–6000 kg/cm² at 10–70 °C all proteins and enzymes studied to date are denatured. The volume change of −45 cm³/mole observed by BRANDTS et al.[45] for the conformational change for ribonuclease in water at 25 °C is much smaller than one would expect if hydrophobic bonding is a major structural force in ribonuclease. The contribution of the hydrophobic bond in protein denaturation will be reviewed elsewhere by H. McK., who concludes that in many cases side chain hydrogen bonds are the more decisive factor[46].

Since this manuscript was prepared a review on microcalorimetry by I. WADSÖ has appeared in Q. Rev. Biophys. *3*, 383 (1970). WADSÖ stresses that heat capacity determinations on protein solutions are quite difficult to perform and that, although the required precision can be achieved with the best isoperibol type calorimeters, large quantities of protein (ca. 100 ml, 1% solution) are required.

Zusammenfassung. Untersuchungen der Denaturierungsvorgänge sind für ein Verständnis der Proteinstruktur und -funktion von grosser Bedeutung. Es werden Methoden besprochen, die am ehesten geeignet sind, die Fragen zu beleuchten, ob die Denaturierung einen Zwei-Formen-Prozess darstellt und ob sie reversibel oder irreversibel ist. Sie werden an Beispielen der Harnstoffdenaturierung genetischer Varianten des Rinder-β-Lactoglobulins erläutert. Es wird ausserdem erörtert, in wieweit sich die Schlussfolgerungen auf die Denaturierung anderer Proteine übertragen lassen.

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SPECIALIA

Les auteurs sont seuls responsables des opinions exprimées dans ces brèves communications. – Für die Kurzmitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed in the authors' brief reports. – Ответственность за короткие сообщения несёт исключительно автор. – El responsable de los informes reducidos, está el autor.

A New Solution of the Equilibrium Equation for an Isothermal Gas Sphere

In our recent paper[1] we have extended the region of validity of the Fowler's solution for the Lane-Emden equation of index 3. In this paper we have obtained a new solution of the equilibrium equation in the (ξ, ψ) plane for an isothermal gas sphere, satisfying the required boundary conditions and asymptotically approaching the singular solution at infinity. The solution governs the density distribution at and around the centre.