EFFECT OF UREA ON THE SOLUBILITY OF BOVINE $\beta$-LACTOglobulins

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Abstract

The effect of exposure of bovine $\beta$-lactoglobulin to 7M urea at 25°C on its solubility in 2M ammonium sulphate–0·5M sodium acetate–0·22M acetic acid (pH 5·2) is examined. The solubility of the B and C variants decreases with increasing reaction time at pH 5·2, the change for B being greater than that for C. There is appreciable loss of reversibility of the solubility change following prolonged reaction. After 30 min reaction at pH 3·5 there is almost complete loss of solubility of the B variant in “stopping” solution. The change is largely reversible in the early stages of reaction, but becomes increasingly irreversible on prolonged reaction. It is much more reversible in the presence of N-ethylmaleimide. Irrespective of reaction time at pH 8·9 the protein becomes almost insoluble in stopping solution. The solubility change is partly prevented by N-ethylmaleimide provided that the reaction mixture is diluted with water prior to adding it to the stopping solution. The solubility losses reflect unfolding of the protein and –SH/–SS– interchange reactions.

I. INTRODUCTION

$\beta$-Lactoglobulin is the dominant whey protein of ruminant milk, has an isoionic point near pH 5·2, and exhibits genetic polymorphism. The best known bovine variants (A, B, and C) have the following amino acid residue substitutions: A:B = Asp:Gly, Val:Ala; C:B = His:Gln. Each contains two cystine and one cysteine residues per 18,000 monomer unit (McKenzie et al. 1972), and their chemistry has been extensively investigated (for reviews see Timasheff and Townend 1962; McKenzie 1967, 1971).

It has been shown elsewhere that the bovine A, B, and C proteins undergo time-dependent optical rotation changes in urea > 4M at pH 5·2 (Ralston 1969; McKenzie and Ralston 1971, 1973a). In 7M urea at 25°C the reaction may be broken into a rapid primary change and a slow secondary stage. The primary denaturation change is not a simple two-state process. However, its half-time is only slightly dependent on protein concentration and its time dependence may be represented by two exponential terms. Thus it involves sequential or parallel reactions or both. During this period the rotation change is essentially reversible, but there is an appreciable decrease in reversibility with further increases in time. This loss in reversibility also occurs at other urea concentrations, and is eliminated in the presence

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of -SH reagents. The protein is more readily unfolded at pH 3·5 than at 5·2, and even more so at pH 8·9. However, the order of rate of loss of reversibility is pH 8·9 > 5·2 > 3·5. This work is part of a study on the chemistry of \( \beta \)-lactoglobulins and also on the mechanism of denaturation of a variety of proteins (McKenzie and Ralston 1971). The action of denaturant is followed by a variety of methods sensitive to different properties of the protein. The loss of solubility of a protein in salt solutions following denaturation has long been used as a criterion for denaturation (Neurath et al. 1944; Anson 1945; Putnam 1953; Joly 1965). The procedure is a simple one and consists of maintaining the protein in the relevant solvent under the conditions of denaturation and transferring a sample at measured time intervals into a "stopping" solution, so chosen that the native protein is soluble, but the denatured protein is insoluble (Levy and Warner 1954). In the present paper the changes in solubility of \( \beta \)-lactoglobulins A, B, and C are examined following exposure to urea, and compared with the optical rotation and other changes.

II. EXPERIMENTAL

(a) Reagents and Glassware

All reagents were analytical grade. Samples of urea were selected for low cyanate content and further purified by recrystallization from aqueous ethanol. Pyrex glassware of low heavy metal content was used and special precautions taken throughout to exclude trace metal contamination (McKenzie and Murphy 1970).

(b) Preparation of \( \beta \)-Lactoglobulin

Bovine \( \beta \)-lactoglobulins A, B, and C were prepared by method Ia of Armstrong et al. (1967). After crystallization the protein was redissolved, precipitated with ammonium sulphate, centrifuged, and stored as paste at 2°C. Samples were recrystallized as required.

(c) Protein and Urea Stock Solutions

\( \beta \)-Lactoglobulin crystals (c. 0·2 g) were dissolved in 0·25M NaCl (1 ml) and then filtered through 0·45-\( \mu \)m Millipore filters (Millipore Corp., Bedford, Mass.). Stock 10m urea solutions were made up freshly each day and at no stage was the temperature allowed to rise above 35°C during the dissolution of the urea.

(d) Reaction Mixtures

The appropriate volume of 10m urea was pipetted into a 5-ml Quickfit conical flask to give the required final urea concentration. This was followed by the relevant volumes of water, stock buffer solution (CH\(_3\)COOH–CH\(_3\)COONa for pH 5·2, HCl–NaCl for pH 3·5, HCl–Na\(_2\)B\(_2\)O\(_4\) for pH 8·9) and sufficient sodium chloride to give a final total ionic strength of 0·1. The stoppered flask was brought to temperature in a thermostatically controlled (±0·02°C) water-bath. When thermal equilibrium had been obtained protein stock solution was added with rapid mixing by swirling, with care being taken to avoid foaming.

(e) Determination of Solubility

Magnesium sulphate was used by Christensen (1952) to precipitate denatured protein. Its use was avoided in the present work because the presence of divalent cations might cause co-precipitation of native \( \beta \)-lactoglobulin with the denatured protein. Ammonium sulphate was chosen for this reason and for those discussed by Green (1931) and Dixon and Webb (1961). The optimum composition of the stopping solution was determined in the following way. Concentrated stopping solution [\( \text{(NH}_4\text{)}_2\text{SO}_4–\text{CH}_3\text{COOH–CH}_3\text{COONa} \)] (c. 4·5 ml) was added rapidly by means of a wide
bore pipette to 0·25 ml of β-lactoglobulin in urea after various reaction times, and the volume then adjusted to 5·0 ml with concentrated stopping solution. The mixture was held at room temperature for 20 min, and the precipitate then removed by centrifugation at 17,000 r.p.m. for 30 min at 22±2°C using a Sorvall RC 2B centrifuge with an SS-34 rotor. The supernatant was carefully removed and its absorbance measured at 278 nm against solvent of identical salt concentration. The concentration of (NH₄)₂SO₄ in the stopping solution was varied so that the final concentration was 0·2M, but the final concentration of acetate buffer was kept constant at 0·5M CH₃COONa–0·22M CH₃COOH. A final concentration of 2M (NH₄)₂SO₄ was chosen because native β-lactoglobulin is soluble at this concentration, but maximal precipitation of protein was obtained in a solution of β-lactoglobulin B that had been held in 7M urea at pH 5·2 and 25°C for 24 hr (Fig. 1). The apparent pH of this stopping solution was 5·2; it was established in separate experiments that variation of pH of the stopping solution over the range 5·0–5·4 did not appreciably alter the amount of protein precipitated.

When it was desired to determine the amount of protein that could revert to a soluble form in the salt solution on the dilution of urea, the following method was used. Water (or pH 5·2 buffer in some experiments) was added to 0·25 ml protein solution to give a volume of 1·5 ml. After standing for 20 min at room temperature a solution 20/7M in ammonium sulphate, 5/7M in sodium acetate, and (2·2)/7M in acetic acid was added to give a final volume of 5·0 ml, and an ammonium sulphate concentration of 2M. After a further 20 min the precipitate was removed by centrifugation and the absorbance of the supernatant measured at 278 nm.

(f) pH Measurements

Calibration of the electrode assembly was made by the procedure of Bates (1964). No attempt was made to set up pH scales for concentrated urea or for concentrated ammonium sulphate solutions. Thus the pH measurements in these solutions are apparent pH values and no theoretical interpretation is made of these values.

III. RESULTS

(a) Solubility after Reaction at pH 5·2

The change in solubility of bovine β-lactoglobulins B and C after various times in 7M urea solution at pH 5·2 and 25°C was studied using the ammonium sulphate stopping solutions and methods described in Section II. The effect of rapid addition of stopping solution is shown in the upper curves of Figure 2. After 30 min reaction about 60% of the protein was precipitated by 2M ammonium sulphate at pH 5·2. This may be compared with the corresponding optical rotation change which is about
65% of the maximum change in 7M urea (McKenzie and Ralston 1971, 1973a). Increasing amounts of protein were precipitated by the ammonium sulphate solution with increasing reaction times until virtually all the protein was precipitable after 24 hr. The order of change in solubility for the B and C variants was qualitatively similar to that shown for the optical rotation change (i.e. B > C).

The effect of diluting the reaction mixture with water and then allowing it to stand for 20 min prior to the addition of the ammonium sulphate–buffer solution was examined (Fig. 2). At the end of 30 min reaction only 24 ± 4% of the protein was insoluble in the stopping solution under these conditions. Longer exposure to 7M urea resulted in increasing insolubility, until 48 hr when 93 ± 3% of the protein was precipitated by the ammonium sulphate. However, the prior dilution of the urea with water at this reaction time resulted in the immediate development of turbidity and at the end of the standing period 76% of the protein was precipitated.

When a solution of β-lactoglobulin A (9·0 g/l) containing 5M urea was allowed to react for 48 hr at 3·8°C, 95% of the protein was precipitated by immediate addition of stopping solution at 3°C. However, warming of the 48-hr reaction mixture to 25°C prior to addition of stopping solution (c. 20°C) resulted in only 48% precipitation of the protein. When the reaction mixture was diluted with water at 25°C, prior to the addition of the ammonium sulphate–buffer solution, only 17% of the protein was precipitated.

(b) Solubility after Reaction at pH 3·5

Direct addition of stopping solution to β-lactoglobulin B (9·1 g/l) in 7M urea at pH 3·6, at times in excess of 30 min, resulted in complete precipitation of protein (Fig. 3). Dilution with either water or 0·5M CH₃COONa–0·22M CH₃COOH buffer, pH 5·2, before addition of the stopping solution resulted in a considerable reduction in the amount of protein precipitated by 2M ammonium sulphate–buffer solution. However, the amount precipitated in this procedure increased slowly with time of reaction in 7M urea until, after 48 hr, 60% could be precipitated (Fig. 3).

The presence of the –SH reagent, N-ethylmaleimide (1·12 × 10⁻⁴M), in the urea solution prevented some loss of solubility in 2M ammonium sulphate–buffer, but 25% was still precipitated after 48 hr (Fig. 3).
There was no difference in solubility between the products of the reaction in 10M and 7M urea on dilution with stopping solution after reaction times from 30 min to 48 hr.

Fig. 3.—Fraction of protein insoluble in the 2M ammonium sulphate–buffer solution, pH 5·2, after various times of reaction for bovine β-lactoglobulin B (c. 10 g/l) in 7M urea at pH 3·5, 25°C.

- Fraction insoluble following rapid addition of salt solution. ○ Fraction insoluble on prior dilution of the urea with water before addition of salt solution.
- Fraction insoluble on prior dilution, but with N-ethylmaleimide present throughout.

(c) Solubility after Reaction at pH 8·9

Rapid addition of 2M ammonium sulphate–acetate buffer mixture to a solution of the B variant (9·7 g/l) in 7M urea at pH 8·9, 25°C, resulted in almost complete precipitation of the protein at all times. The amount of material precipitated was not altered appreciably by dilution with water or acetate buffer prior to addition of the ammonium sulphate solution. The presence of N-ethylmaleimide (1·12 × 10⁻³m) in the 7M urea reaction did not reduce the amount of protein precipitated by rapid addition of the stopping solution. However, the amount was reduced when the urea was diluted prior to the addition of stopping solution.

The high loss of solubility of the denatured protein in 2M ammonium sulphate that occurred when N-ethylmaleimide was present could have been due to either (1) complete reaction of the –SH group to produce a protein derivative with a lower solubility than the native protein, or (2) incomplete reaction of the –SH group resulting in some aggregation and heterogeneity. If only one kind of protein molecule were present, as in (1), the curve for loss of solubility versus ammonium sulphate concentration should show a sharp single-step rise, similar to that observed for the native protein in Figure 4, but occurring at a lower salt concentration. Real heterogeneity, as in (2), should result in a less steep curve or even a series of discrete steps. The effect of ammonium sulphate concentration on the fraction of protein precipitated, with the salt solution added subsequently to the dilution of the urea with acetate buffer, was examined and the results are shown in Figure 4. The precipitation curve for products of the reaction in urea with N-ethylmaleimide present exhibits a low profile indicating some degree of heterogeneity.

IV. DISCUSSION

The changes in solubility of bovine β-lactoglobulin in ammonium sulphate solution, following exposure to concentrated urea, broadly parallel the changes in optical rotation at 578 nm; e.g. there is a time-dependent change in 7M urea at pH 5·2 and 25°C, a more rapid change at pH 3·5, and a very rapid one at pH 8·9. It has been shown elsewhere that changes in several properties (e.g. optical rotation,
difference spectra) of β-lactoglobulin in urea do not exhibit synchrony even during the primary unfolding change (McKenzie and Ralston 1971). Thus it was not expected that the optical rotation and solubility changes would be precisely synchronous, especially on prolonged reaction where there are irreversible changes in optical rotation.

The solubility criterion is a very simple one to apply experimentally but adequate care must be taken in the choice of stopping solution. However, the solubility changes cannot be easily interpreted in terms of structural changes in the protein because the solubility is affected, not only by conformational changes, but also by intra- and intermolecular –SH/–SS– exchange reactions and non-covalent aggregation reactions that may accompany the denaturation. Nevertheless, it is possible by performing the salting-out experiments under a variety of conditions, including tests for reversal, to gain some insight into the structural changes involved.

In the present work solubility tests have been carried out under conditions in which the stopping solution is rapidly mixed with the denatured protein, and under those in which the protein–urea is diluted with water then allowed to stand for a short period prior to mixing with the stopping solution. The latter measurements reveal that there is increasing loss of reversibility of the solubility change with increasing reaction time. The irreversible changes are slow at pH 3·5, somewhat faster at pH 5·2, and much more rapid at pH 8·9. Again there is similarity with the optical rotation change, which is reversible during the primary unfolding change, but becomes irreversible on prolonged reaction. The solubility measurements made after reaction at 3·8°C for 48 hr indicate that the irreversible changes are slower at 3·8 than at 25°C.

The considerable decrease in loss of reversibility of the solubility and optical rotation changes when –SH reagents are present suggests the involvement of –SH/–SS– interchange reactions in causing the irreversible changes. It was expected originally that such changes would occur to some extent at pH 5·2 and appreciably at pH 8·9, but be negligible at pH 3·5. However, it is evident from the effects reported here and elsewhere (McKenzie et al. 1972) that interchange is appreciable at pH 3·5. In a study of the effects of urea on the solubility of bovine serum albumin, Kauzmann and Douglas (1956) concluded that both inter- and intramolecular –SH/–SS–
interchange occurs at neutral and higher pH, but is negligible near pH 3.5. A re-examination of their results suggests to us that some intramolecular interchange may also have occurred at pH 3.5. In the following paper (McKenzie and Ralston 1973b) a detailed examination of the products of the reaction of \( \beta \)-lactoglobulin in urea is reported.

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VI. REFERENCES

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