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The Effect of Calcium on Bovine α-Lactalbumin Conformational Transitions by Ultraviolet Difference and Fluorescence Spectrophotometry

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The unfolding of bovine α-lactalbumin (BAL) in guanidine hydrochloride (GnHCl) was studied by UV difference and fluorescence spectrophotometry. When BAL was dissolved in calcium-free solvents, nonsuperimposable unfolding profiles were observed. Thus, it may be concluded that the two spectrophotometric techniques are sensitive to different BAL chromophores or levels of structural organisation.

In a solvent containing calcium (0·1 M Tris–HCl buffer, pH 7·0; 9 mM calcium chloride) there was an increase in the molar UV difference absorbance change associated with GnHCl unfolding of BAL. Unfolding profiles were also biphasic, i.e. denaturation occurred in two stages at different GnHCl concentrations. This pattern of unfolding is different from the usual S-shaped profiles recorded for the three- or two-state BAL unfolding reaction. The results suggest that BAL possesses a more compact structure in the presence of calcium ions. The biphasic transition may arise from the loss of structure, which is unique to calcium-stabilised BAL.

INTRODUCTION

The measurement of conformation changes as a function or denaturant concentration is one way of determining protein stability. The free energy (ΔG°) of protein unfolding, determined in such a manner, is a thermodynamic function of state (Aune & Tanford, 1969*a*,*b*; Bolen & Santoro, 1988). In food science, reliable ΔG° estimates would help clarify relationships between protein stability and functional properties, such as film formation, emulsifier activity and gelation (Pour-El, 1979; Cherry, 1982; Fox, 1989). Thermodynamic parameters for protein unfolding are also increasingly important in the field of protein engineering (Cupo & Pace, 1983; Kellis *et al.*, 1989).

The methods of protein unfolding reaction thermodynamics involve a number of assumptions (Hermans, 1965; Lapanje, 1978; Privalov, 1979; Schellman & Hawkes, 1980). For example, it is commonly assumed that denaturation proceeds via a two-state mechanism, and that the ΔG° may be obtained from apparent values in the presence of denaturants by extrapolation to zero denaturant concentration (Schellman & Hawkes, 1980; Pace, 1986; Santoro & Bolen, 1988).

Further uncertainty arises because different analytical methods may be sensitive to different protein structural elements during the unfolding reaction. UV difference (at a wavelength of *c.* 280 nm), fluorescence and near-UV circular dichroism techniques may be sensitive to changes in amino acid side-chain exposure to solvent. By contrast, far-UV circular dichroism and NMR appear to be sensitive to changes in the pattern of peptide backbone folding (Saito & Wada, 1983*a*; Kuwajima *et al.*, 1986).

In this study, fluorescence and UV difference methods for monitoring protein unfolding were compared. Both techniques are frequently applied to studies of proteins in general (Wetlaufer, 1962; Udenfriend, 1962) and to studies of α-lactalbumin (Takase *et al.*, 1976; Permyakov *et al.*, 1981; Kronman, 1989). However, whether the two methods monitor equivalent structural elements and chromophores during the unfolding of BAL and other proteins is uncertain.

BAL was chosen as a model protein in this study since it apparently undergoes a two-state transition (with the loss of 3° structure) or a three-state transition (with the

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sequential loss of 3° and 2° structures) with varying types of denaturant (Kuwajima *et al.*, 1976; Kuwajima, 1977). In the presence of excess Ca^{2+} there may be a change from the three-state to a two-state unfolding reaction. In the latter case, both 3° and 2° structures are lost simultaneously (Ikeguchi *et al.*, 1986). Therefore, monitoring the unfolding of BAL in various solvents, with and without Ca^{2+}, should allow a thorough comparison of fluorescence and UV difference spectral changes as indices of protein unfolding.

MATERIALS AND METHODS

Bovine α-lactalbumin (BAL), containing 1 to 2 mol Ca^{2+} per mol protein, was used as supplied (Sigma Co. Ltd, Poole, UK, Type I grade). Guanidine hydrochloride (GnHCl) was puris grade (Fluka Chemical Ltd, Glossop, UK). Tris (hydroxymethylamino) methane (Tris), mono- and di-basic potassium phosphate salts, calcium chloride and all other chemicals were of AnalaR grade from BDH (Poole, UK).

UV difference spectra were measured, using a double beam spectrophotometer (Model SP1800), fitted with a thermostated cuvette block, in conjunction with a Model AR25 linear chart recorder (Pye Unicam Ltd, Cambridge, UK). GnHCl unfolding profiles were measured according to the semicontinuous method (Saito & Wada, 1983*a*) with modifications. Sample and reference cuvettes contained 2 ml of BAL (0·4–0·6 mg/ml); protein concentration was determined assuming that $E(285\text{ nm}) = 20{\cdot}1$ (for a 1% solution of BAL). At 10 min intervals, equal volumes (20–100 μl) of GnHCl (8 M) and buffer were added to the sample and reference cells, respectively, and a difference spectrum measured over the wavelength range 260–330 nm. The stepwise increase in GnHCl concentration ($C(i)$) can be described by, $C(i) = [S]V(t)/[V(o) + V(t)]$, where $[S]$, $V(o)$ and $V(t)$ are, respectively, the stock GnHCl concentration, initial volume of BAL and the cumulative volume of GnHCl added to the sample cuvette. The observed molar UV difference absorbance changes, i.e. E' (293) values, were then corrected for the continuous dilution of the BAL samples, e.g. $E(293) = E(293)'[1 + V(t)/V(o)]$. BAL unfolding profiles were then constructed by plotting $E(293)$ values against $C(i)$.

BAL intrinsic fluorescence was measured using a Perkin-Elmer 204 fluorescence spectrophotometer fitted with a digital output and a thermostated sample holder (Hitachi Instrument Co., Tokyo, Japan). Fluorescence measurements were made at excitation and emission wavelengths of 293 and 345 nm, respectively. Fluorescence unfolding profiles were measured in the same manner as described above except that a lower protein concentration (0·04–0·06 mg/ml) was used.

The fraction of unfolded protein (F_u) is given by, $F_u = (y - yN) / (yD - y)$ where y, yN and yD represent the $E(293)$ or fluorescence properties of BAL at intermediate, low or high GnHCl concentration, respectively. Values for yN and yD may show significant changes with GnHCl concentration owing to solvent perturbation of the native (N) and (D) denatured states (Wetlaufer, 1962). The standard corrections for solvent perturbation, involving the use of least square straight-line equation(s) to extrapolate to the expected values of yN and yD (cf. dashed lines, Fig. 2A–Fig. 3A), were applied until the fraction of solvent perturbed form was 0·5 or less (Wetlaufer, 1962; Pace, 1986).

RESULTS AND DISCUSSION

GnHCl unfolding of BAL resulted in a roughly 'W'-shaped UV difference spectrum. There were two negative maxima at wavelengths of 286 and 293 nm and a positive peak at 303 nm (Fig. 1).

The spectrophotometric properties of BAL showed pre- and post-denaturational changes as a function of GnHCl concentration (Fig. 2A and Fig 3A). Standard methods for calculating F_u under such circumstances were applied, as discussed above (Wetlaufer, 1962; Pace, 1986). The results of such analysis are shown as unfolding profiles consisting of a lot of F_u versus GnHCl concentration (Fig. 2B and Fig. 3B).

In solvents without added calcium, UV difference and fluorescence unfolding profile were not superimposable. With Tris–HCl (0·1 M, pH 7·0) as solvent, the unfolding profile determined by fluorescence measurements occurred at a lower GnHCl concentration than that determined using $E(293)$ measurements (Fig. 2B). Similar results were obtained in studies where the solvent was K phosphate buffer (results not shown). This is confirmation that the unfolding of BAL, in a low calcium

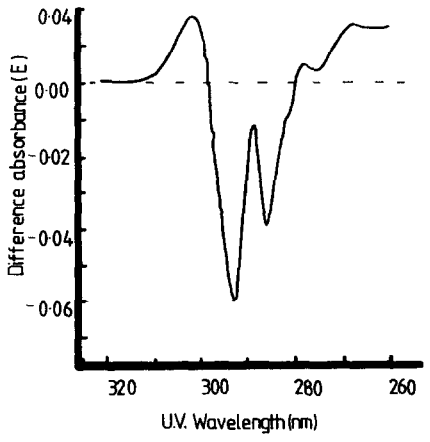

Fig. 1. A typical UV difference absorption spectrum for BAL. The sample and reference cuvettes contained respectively, BAL (0·2–0·3 mg/ml) in 4 M guanidine hydrochloride (GnHCl) and the same concentration of protein without GnHCl (see text for details).

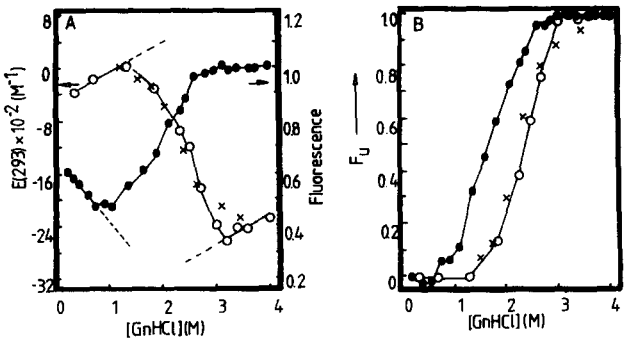

Fig. 2. (A) The effect of quanidine hydrochloride concentration ([GnHCl]) on the (A) BAL 293 nm molar UV difference absorption ($E(293)$, ○) or fluorescence (●). Conditions: Tris–HCl buffer 0·1 M, pH 7·0; 25°C; (-----) least-squares lines showing the spectrophotometric properties of the N and D states. (B) The effect of guanidine hydrochloride concentration ([GnHCl]) on the fraction of unfolded BAL (F_u); data and symbols as in Fig. 2A; X, test of reversibility, i.e. BAL initially dissolved in 4 M GnHCl was diluted and spectrophotometric measurements taken.

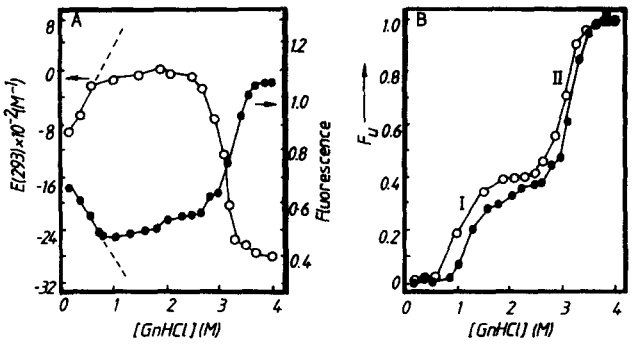

Fig. 3. (A) Legend is as shown for Fig. 2A. Conditions: Tris–HCl buffer (0·1 M, pH 7·0) with 9 mM calcium chloride. (B) Legend is as shown for Fig. 2B (from data in Fig. 3A); I and II represent two unfolding transitions.

solvent, involves more that two structural states (Tanford, 1968). At the wavelengths employed in this study, UV differences and fluorescence changes are both sensitive to changes in the environment or Trp residues and insensitive to other aromatic amino acids (Wetlaufer, 1962,; Undenfriend, 1969). Therefore, the two spectrophotometric methods were also probably sensitive to different classes of Trp residues.

It is known that, for native BAL, fluorescence emissions are associated with intramolecular energy transfer from Trp 26 and 104 to Trp 60 (Sommers & Kronman, 1980; Kronman, 1989). The numbers refer to the position of the amino acids in the BAL 1° structure. Fluorescence from Trp 60 is also quenched by the proximity of two disulphide bonds (Cowgill, 1967; Sommers & Kronman, 1980). Increases in the distances between the disulphide bonds and Trp 60, e.g. as a result of a protein unfolding, reduce the quenching effect. This explains the BAL fluorescence increase at a high GnHCl concentration (Fig. 2A and Fig. 3A). In the absence of a Trp 60, α-lactalbumin unfolding is not accompanied by a fluorescence increase at 345 to 350 nm (Sommers & Kronman, 1980). That such fluorescence increases occur at a relatively low GnHCl concentration implies that Trp 60 and/or the disulphide groups are within a relatively exposed or unstable part of the native BAL structure. Alternatively, it could be that one or both of these groups is particularly sensitive to the slightest alteration of the native BAL structure.

The chromophore(s) responsible for the UV difference change, when BAL is denatured with GnHCl, can be partially identified from $E(293)$ values for α-lactalbumins from different species (Takase *et al.*, 1978). The proteins differ in the total numbers and positions of Trp residues. There are four Trp residues in BAL, i.e. Trp 26, Trp 60, Trp 104 and TRP 118. Within human α-lactalbumin (HAL), Trp 26 is replaced by a leucine (Leu), while for guinea pig α-lactalbumin (GPAL), Trp 60 is replaced by a phenylalanine (Phe) residue (Kronman, 1989). In the following discussion the three α-lactalbumin variants are designated, BAL(–), HAL(Trp 26/Leu) and GPAL(Trp 60/Phe), respectively.

The $E(293)$ value when BAL(–), GPAL(Trp 60/Phe) or HAL(Trp 26/Leu) is unfolded in GnHCl was estimated (from Fig. 3 in the paper of Takase *et al.*, 1978) as –3200/M, –3800/M and 2400/M, respectively. Partial $E(293)$ values due to changes in the environment of specific Trp residues were then calculated by assuming that the contribution of Trp residues to the total $E(293)$ value for each protein is additive (Wetlaufer, 1962; Takase *et al.*, 1978; Sommers & Kronman, 1980).

With GnHCl unfolding of BAL, the partial $E(293)$ change due to Trp 26 was –800/M. This figure arises from the subtraction of the $E(293)$ value of HAL(Trp26/Phe) from the value for BAL (–), i.e. –3200 – (–2400). The combined partial $E(293)$ value of Trp 104 and 118, estimated in a similar fashion, was –3000/M. By comparison the partial $E(293)$ value for Trp 60 was positive (+600/M). Therefore, according to the UV difference approach, the greatest change in environment probably occurred in the vicinity or Trp 104 and/or 118 during the GnHCl unfolding of BAL. Indeed, as Trp 118 is believed to be exposed to solvent in the native BAL structure (Warme *et al.*, 1979), the important residue must be Trp 104.

From the above discussion, the nonsuperimposable unfolding profiles (Fig. 2B) may arise because $E(293)$ results mainly reflect changes in the environment of Trp 104. In contrast, the important chromophore for fluorescence measurements, at the emission/excitation wavelengths used in this study, seems to be Trp 60 and its interaction with two disulphide bonds in BAL. Evidently, in a low calcium solvent, BAL conformation changes near these groups occur with varying degrees of ease.

The above results (Fig. 2B) can also be restated in terms of the classical three-state denaturation reaction of BAL dissolved in low calcium buffers (Kuwajima *et al.*, 1976; Kuwajima, 1977). That is, fluorescence and *E*(293) unfolding profiles may correspond to the loss of BAL 3° structure and 2° structure, at low and high GnHCl concentrations, respectively.

An effect of calcium on BAL structure can be discerned from *E*(293) values in various solvents. In this study, the *E*(293) value for GnHCl unfolding of BAL was −1968 (±22)/M, −2342 (±130)/M and 2840 (±54)/M with K phosphate, Tris–HCl or Tris–HCl (9 nM calcium chloride) as buffer, respectively. That is, *E*(293) values were significantly greater for BAL unfolding in the presence of Ca^{2+}. The magnitude of molar UV difference values is a function of the magnitude of net changes in the environment of chromophores during the unfolding of proteins in various solvents (Wetlaufer, 1962). Therefore, the greater *E*(293) value for the GnHCl unfolding of BAL in the presence of 9mM calcium chloride is consistent with a more folded or solvent-inaccessible native structure. BAL presumably exists in a less compact native conformation in Tris–HCl or K phosphate buffers (without Ca^{2+}). Finally, the high *E*(293) estimate for BAL denaturation in Na phosphate buffer (i.e. −3200/M, above) suggests that, like Ca^{2+}, Na+ can also increase the structure of native BAL (Hiraoka & Sufai, 1985; Kuwajima *et al.*, 1986).

In the presence of excess Ca^{2+} (Tris–HCl buffer with 9mM $CaCl_2$), the unfolding profile for BAL showed a trough at a GnHCl concentration of 1 to 2M. After corrections for native and denatured state solvent perturbation effects, the resulting profiles revealed the presence of two unfolding transitions (I and II, Fig. 3B).

The biphasic transition (Fig. 3B) can be attributed to the presence of calcium chloride; e.g. compare results in Fig. 2B with those in Fig. 3B. BAL is a calcium metalloprotein (Hiroaka *et al.*, 1980) with a high affinity for a single Ca^{2+}. The value of the association constant is 10^6–10^9/M (Permyakov *et al.*, 1981; Segawa & Sugai, 1983; Hamano *et al.*, 1986; Ikeguchi *et al.*, 1986; Kronman, 1989). However, the binding affinity for calcium is reduced in the presence of competing cations (Hamano *et al.*, 1986; Kuwajima *et al.*, 1986). Consequently, a concentration of 1 to 10 mM Ca^{2+} is necessary to saturate the BAL Ca^{2+} binding site in non-deionised solvents.

In the only other study of BAL unfolding in a solvent containing a high (>5 mM) calcium chloride concentration, the unfolding reaction apparently changed from a three-state to a two-state transition in the presence of 12 mM calcium chloride (Ikeguchi *et al.*, 1986). There is evidence also from this study to support such a view. Thus, it can be seen that, for BAL unfolding in 9 mM calcium chloride (0·1 M Tris–HCl, pH 7·0; Fig. 3B), UV difference and fluorescence profiles were, within experimental error, superimposable. Indeed, quantitative data presented elsewhere (Owusu, 1991) identify transition II (Fig. 3B) as the same two-state transition reported previously (Ikeguchi *et al.*, 1986).

It can be seen in Fig. 3B that there is a second unfolding (transition I) for BAL in the presence of a high concentration of calcium chloride. No such transition was observed previously (Ikeguchi *et al.*, 1986). However, the later study differed from this one, in using sodium cacodylate and not Tris–HCl buffer as solvent. As both Na^+ and Tris cations can interact with BAL (Rawitch & Gleason, 1971; Hiroaka & Sugai, 1985), it is possible that the differences in results may be related to choice of solvents in the two studies. The possible role of solvent choice on the effects of Ca^{2+} on BAL structure and stability is currently under study.

A multiphasic unfolding transition (Fig. 3B) is generally thought to be characteristic of a protein with multiple domains with different stability. On the other hand, structural domains with equal stability could unfold synchronously to give a simple S-shaped denaturation profile. For example, lysozyme, a structural analogue of α-lactalbumin (Warme *et al.*, 1979), possesses two domains, but does not show a multiphasic unfolding profile (Aune & Tanford, 1969*b*). However, a single domain protein could conceivably exhibit a multiphasic unfolding profile, if it had regions with large differences in stability (Saito & Wada, 1983*a*,*b*; Jaenicke, 1987).

According to the current 'molten globule' concept, intermediates of BAL infolding are compact, though possessing a greater intrinsic flexibility than the native state (Dolgikh *et al.*, 1981, 1985; Pfeil, 1988). This concept has yet to be reconciled with the alternative view that BAL unfolding intermediates have little or no 3° structure (Kuwajima *et al.*, 1976). A possible explanation for the biphasic GnHCl unfolding profile, which accommodates both of these views, is that BAL assumes a more rigid and/or compact native structure in the presence of a high concentration of Ca^{2+}. Evidence for such a structural change, based on the magnitude of the molar UV difference change for BAL denaturation in different solvents, was presented above. Therefore, the initial unfolding of BAL in GnHCl (Transition I; Fig. 3B) may be due to the loss of structure which is unique to BAL in the presence of a high concentration of Ca^{2+}.

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REFERENCES

Aune, K. C. & Tanford, C. (1969*a*) Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. I. Dependence on pH at 25°C. *Biochemistry*, **8**, 4579–85.

Aune, K. C. & Tanford, C. (1969*b*). Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. II. Dependence of denaturant concentration at 25°C. *Biochemistry*, **8**, 4586–90.

Bolen, D. W. & Santoro, M. M. (1988). Unfolding free energy change determined by the linear extrapolation method. 2. Incorporation of $G(N–D)$ values in a thermodynamic cycle. *Biochemistry*, **27**, 8069–74.

Cherry, J. P. (ed.) (1982). *Food Protein Deterioration: Mechanism and Functionality* (ACS Symp. Ser. 206), American Chemical Society, Washington, DC.

Cowgill, R. W. (1967). Fluorescence and protein structure: 11. Fluorescence quenching by disulphide and sulfhydryl groups. *Biochim. Biophys. Acta.*, **140**, 37.

Cupo, J. F. & Pace, C. N. (1983). Conformational stability of mixed disulfide derivatives of beta-lactoglobin B. *Biochemistry*, **22**, 2654–58.

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. G., Bychkova, V. E., Semisotnov, G. V., Venyuaminov, S. Yu. & Ptitsyn, O. B. (1981). Alpha-lactalbumin: Compact state with fluctuating tertiary structure. *FRBS Lett.*, **136**, 311– 15.

Dolgikh, D. A., Abuturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Gilmanshin, R. I., Lebedev, Yu. O., Semisotnov, G. V., Tiktopulo, E. I. & Ptitsyn, O. B. (1985). Compact state of a protein molecule with pronounced small-scale mobility: Bovine alpha-lactalbumin. *Eur. Biophys. J.*, **13**, 109–21.

Fox, P. F. (ed.) *Development in Dairy Chemistry 4: Functional Milk Proteins.*, Elsevier Applied Science, London, 1989.

Hamano, M., Nitta, K., Kuwajima, K. Sugai, S. (1986). Binding strength of calcium-ion to bovine alpha-lactalbumin. *J. Biochem.*, **100**, 1617–22.

Hermans, J. (1965). Methods for the study of reversible denaturation of proteins and interpretation of data. *Meth. Biochem. Anal.*, **13**, 81–111.

Hiroaka, Y., Segawa, T. Kuwajima, K., Sugai, S. & Murai, N. (1980). Alpha-lactalbumin: A calcium metalloprotein. *Biochem Biophys. Res. Comm.*, **95**, 1098–104.

Hiraoka, Y. & Sugai, S. (1985). Equilibrium and kinetic study of sodium- and potassium-induced conformational changes of apo-alpha-lactalbumin. *Int. J. Peptide Protein Res.*, **26**, 252–61.

Ikeguchi, M., Kuwajima, K. & Sugai, S. (1986). Ca^{2+} induced alternation in the unfolding of alpha-lactalbumin. *J. Biochem.*, **99**, 1191–201.

Jaenicke, R. (1987). Folding and association of proteins. *Prog. Biophys.*, **49**, 117–237.

Kellis, J. T., Nyberg, K. & Fershst, A. R. (1989). Energetics of complementary packing in a protein hydrophobic core. *Biochemistry*, **28**, 4914–22.

Kronman, M. J. (1989). Metal-ion binding and the molecular conformational properties of alpha-lactalbumin. *CRC Crit. Rev. Biochem.*, **24**, 565–667.

Kuwajima, K. (1977). A folding model of alpha-lactalbumin deduced from the three-state denaturation mechanism. *J. Mol. Biol.*, **114**, 241–58.

Kuwajima, K. Nitta, K., Yoneyama, M. & Sugai, S. (1976). Three-state denaturation of alpha-lactalbumin by guanidine hydrochloride. *J. Mol. Biol.*, **106**, 359–73.

Kuwajima, K., Harushima, Y. & Sugai, S. (1986). Influence of Ca^{2+} binding on the structure and stability of alpha-lactalbumin studied by circular dichroism and nuclear magnetic resonance spectra. *Int. J. Pept. Protein Res.*, **27**, 18–27.

Lapanje, S. (1978). *Physicochemical Aspects of Protein Denaturation.* Wiley Interscience, Chichester, UK, pp. 180–225.

Owusu, R. K. (1992). Thermodynamic analysis of the effect of calcium on bovine alpha-lactalbumin conformation stability. *Food Chem.* (in press).

Pace, C. N. (1986). Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Meth. Enzymol.*, **131**, 266–80.

Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A. & Burstein, E. A. (1981). Calcium binding to alpha-lactalbumin: Structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. *Biochem. Biophys. Res. Comm.*, **100**, 191–7.

Pfeil, W. (1988). *Biochemical Thermodynamics*, ed. M. M. Jones. Elsevier, Oxford, pp. 53–99.

Pour-El, A. (ed.) (1979). *Functionality and Protein Structure* (ACS Symp. Ser. 92). American Chemical Society, Washington, DC.

Privalov, P. L. (1979). Stability of proteins: Small globular proteins. *Adv, Protein Chem.*, **33**, 167–241.

Rawitch, A. B. & Gleason, M. (1971). the interaction of alpha-lactalbumin with tris cation and other cationic hydroxamines. *Biochem. Biophys. Res. Comm.*, **45**, 590–20.

Saito, Y. & Wada, A. (1983*a*). Comparative study of GnHCl denaturation of globular proteins. I. Spectroscopic and chromatographic analysis of the denaturation curves of ribonuclease A, cytochrome C and pepsinogen. *Biopolymers*, **22**, 2105–22.

Saito, Y. & Wada, A. (1983*b*). Comparative study of GnHCl denaturation of globular proteins. II. A phenomenological classification of denuration profiles of 17 proteins. *Biopolymers*, **22**, 2123–32.

Santoro, M. M. & Bolen, D. W. (1988). Unfolding free energy changes determined by the linear extrapolation method. I. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. *Biochemistry*, **27**, 8063–8.

Schellman, J. A. & Hawkes, R. B. (1980). *Protein Folding: Proc. 28th German Biochemical Society, Regensburg*, ed. R. Jaenicke. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 331–41.

Sommers, P. B. & Kronman, M. J. (1980). Comparative fluorescence properties of bovine, goat, human and guinea pig alpha-lactalbumin. Characterization of the environments of individual tryptophan residues on partially folded conformers. *Biophys. Chem.*, **11**, 217–32.

Takase, K., Niki, R. & Arima, S. (1976). Thermodynamics of the temperature dependent denaturation of alpha-lactalbumin. *Agric. Biol. Chem.*, **40**, 1273–7.

Takase, K., Niki, R. & Arima, S. (1978). Environment of tryptophan residues in alpha-lactalbumin. *J. Biochem.*, **83**, 371–8.

Tanford, C. (1968). Protein denaturation. *Adv. Protein Chem.*, **23**, 121–282.

Udenfriend, S. (1962). *Fluorescence Assay in Biology and Medicine.* Academic Press, London, pp. 191–229.

Warme, P. K., Monany, F. A., Rumball, S. V., Tuttle, R. W. & Scheraga, H. A. (1979). Computational structures of homologous proteins. Alpha-lactalbumin for lysozyme. *Biochemistry*, **13**, 768–82.

Wetlaufer, D. B. (1962). Ultraviolet spectra of protein and amino acids. *Adv. Protein Chem.*, **17**, 303–90.