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Thermodynamic analysis of the effect of calcium on bovine alpha-lactalbumin conformational stability

R. K. Owusu

Procter Department of Food Science, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, UK

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$\Delta G°$ Values for bovine alpha-lactalbumin (BAL) unfolding in a range of solvents, including simulated milk ultrafiltrate buffer, were determined. The denaturant used was guanidine hydrochloride. In Ca^{2+}-free solvents fluorescence and ultraviolet (UV) difference absorbance unfolding profiles were non-superimposable leading to $\Delta G°$ estimates of 15–17 kJ mol^{-1} and 21–28 kJ mol^{-1}, respectively. Therefore, the unfolding of BAL was consistent with a three-state reaction. The low and high $\Delta G°$ estimates may correspond to the loss of BAL 3° and 2° structure, respectively.

The presence of excess Ca^{2+}, two BAL unfolding transitions (I and II) were observed with both spectrophotometric techniques. Transition (II) was associated with a $\Delta G°$ value of 52 kJ mol^{-1} and is probably identical to a two-state BAL unfolding reaction described previously. Transition (I) appears to signify a novel feature of BAL structure.

INTRODUCTION

Alpha-lactalbumin (BAL) is of interest as the second most abundant whey protein (Fox, 1989) and because of the structural homology with hen egg-white lysozyme (Warme *et al.*, 1979). It is also a calcium metalloprotein (Hiraoka *et al.*, 1980; Stuart *et al.*, 1986) which functions as a subunit for lactose synthetase (Shukla, 1973).

Individual whey proteins and concentrates are increasingly used in food manufacture (Fox, 1989). This has stimulated research into the effects of heating on whey proteins, e.g. by differential scanning calorimetry (DSC) (Ruegg *et al.*, 1977; Pfeil, 1981; Bernal & Jelen, 1984; De Wit & Klarenbeek, 1984; Bernal & Jelen, 1985). However, DSC is insensitive to any structural change which does not result in heat being absorbed or evolved (Pfeil, 1981, 1988).

The reversible denaturation of BAL by heat, acids or inorganic denaturants has also been studied by ultraviolet (UV) difference spectroscopy (Sugai *et al.*, 1973; Kita *et al.*, 1976; Takese *et al.*, 1976; Nitta *et al.*, 1977; Kuwajima & Sugai, 1978), optical rotation (Sugai *et al.*, 1973), infrared spectroscopy (Takaseda *et al.*, 1973) and nuclear magnetic resonance spectroscopy (Kuwajima *et al.*, 1986). Circular dichroism and fluorescence measurements have also been important for the study of BAL structure (Ikeguchi *et al.*, 1986*a*; Kuwajima *et al.*, 1976, 1986; Kronman, 1989; Kuwajima *et al.*, 1989).

The study of BAL unfolding, using an optical method, in a solvent with a milk-like electrolyte composition is rare. Consequently, the denaturation of BAL by guanidine hydrochloride (GnHCl) was studied in simulated milk ultra filtrate (SMUF) buffers using UV difference or fluorescence spectrophotometry.

In SMUF buffer, without the complement of phosphate or citrate anions, the denaturation of BAL by GnHCl occurred in two phases. Similar multiple unfolding transitions in tris-HCl buffer (with 9 mM $CaCl_2$) were designated transition (I) and (II) (Owusu, 1992). This paper describes the determination of the standard Gibbs free energy ($\Delta G°$) for the unfolding of BAL in several SMUF buffers and other solvents (e.g. tris-HCl, K-phosphate and Na-cacodylate buffer) with or without 9 mM Ca^{2+}. Experimental $\Delta G°$ estimates for transitions (I) and (II) were compared with literature $\Delta G°$ values in an attempt to identify the former transitions. Based on current results, the possible effects of indigenous Ca^{2+} chelators on the stability of alpha-lactalbumin in milk are also briefly discussed.

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MATERIALS AND METHODS

Reagents and methods were essentially as described previously (Owusu, 1992). Simulated milk ultrafiltrate and related buffers were prepared, with analytical grade chemicals from the British Drug Houses (BDH) Ltd (Poole, UK), according to the composition described by Ruegg and co-workers (Ruegg *et al.*, 1977). It was necessary to adjust the pH of the buffers to pH 6·6 using concentrated hydrochloric acid and therefore the level of chloride was in excess of 32·4 mM. Other SMUF buffer variants were also prepared by omitting Ca^{2+}/Mg^{2+} cations or citrate/phosphate anions from the standard recipe, i.e. SMUF (minus Ca^{2+} and Mg^{2+}) or SMUF (minus citrate and phosphate), respectively.

Determination of the unfolding free energy

$\Delta G°$ Values were determined from measurements of BAL Trp UV difference absorbance and fluorescence changes as a function of GnHCl concentration (Owusu, 1992). The fraction of unfolded BAL (F_u) is given by, $F_u = (y - yN) / (yU - yN)$, where yN is the UV difference absorbance change for the native (N) protein; yU is a similar change in property at very high GnHCl concentration for the unfolded (U) protein; y is the observed spectrophotometric change at intermediate concentration of GnHCl. The following relations enable the determination of the unfolding equilibrium constant (K_{eq}) and eventually $\Delta G°$; $K_{eq} = F_u/(1-F_u)$ and $\Delta G = -R\,T \ln K_{eq}$, where R is the gas constant and T is the temperature for experiments.

K_{eq} is a function of experimental conditions, e.g. pH, denaturant concentration ([GnHCl]) and temperature. That is, $K_{eq} = K°f(\text{pH, [GnHCl]}, T..)$ (Aune & Tanford, 1969; Tanford, 1970). Hence, in the absence of denaturant, at pH = 7·0 and at room temperature (T = 298 K), K_{eq} assumes the standard value, $K°$. An expression for the function f([GnHCl]), based on the ligand equilibrium binding (EB) model is $(1 + ka\pm)^{\Delta n}$; where k = denaturant binding constant, Δn = difference in the number of denaturant binding sites on the native and unfolded protein states and $a\pm$ = the mean ion activity of GnHCl, respectively. The value of k may be taken as 1·2 for the interaction of proteins with GnHCl or urea (Tanford, 1970). In logarithm form, $\Delta G = \Delta G° + \Delta n\,RT \ln (1 + ka\pm)$. Hence a plot of ΔG versus ln $(1 + ka\pm)$ will be linear with a slope of Δn and an $(x = 0)$ intercept value equal to $\Delta G°$ (kJ mol^{-1}).

An alternative expression for the function f([GnHCl]) is based on the empirical observation that ΔG shows a linear dependence upon denaturant concentration, i.e. $\Delta G = \Delta G° + m$ [GnHCl]. In this 'linear extrapolation' (LE) method, ΔG is plotted versus [GnHCl] to give a linear graph with slope equal to m (kJ mol^{-1} M^{-1}) and an $(x = 0)$ intercept value of $\Delta G°$ (kJ mol^{-1}). The slope (m) may be considered as a measure of the sharpness or cooperativity of the unfolding transition (Pace & Vanderburg, 1979). The above methods were reviewed recently (Pace, 1986; Schmidt, 1990).

RESULTS AND DISCUSSION

The $\Delta G°$ value of BAL unfolding was determined in the following buffers: (1), K-phosphate (0·1M, pH 7·0); (2), tris-HCl (0·1M, pH 7·0); and (3), tris-HCl (0·1M, pH 7·0; 9 mM $CaCl_2$) (Owusu, 1992). Other solvents used include: (4), SMUF (pH 6·6); (5), SMUF (minus Ca^{2+} and Mg^{2+}); (6), SMUF (minus citrate and phosphate); and (7), Na cacodylate buffer (0·05M, with 9 mM $CaCl_2$) (Tables 1 & 2).

Sigmoidal or S-shaped BAL unfolding profiles were obtained in SMUF (Fig. 1), tris-HCl, K-phosphate

Table 1. Bovine alpha-lactalbumin conformational stability by Trp UV difference absorbance measurements

Buffer	$\Delta G°$ (kJ mol^{-1})		Slope[a]		$[GnHCl]_{1/2}$
	LE	EB	m	Δn	
1	15(1)	21(1)	6(0·3)	9(0·5)	2·2
2	21(2)	28(2)	9(1)	13(1)	2·3
3(I)[b]	14(1)	21(2)	12(1)	16	1·1
3(II)	43(3)	52(4)	14(2)	19	3·0
4	32	35(2)	10	12	3·2

Buffers were (1), K-phosphate (0·1M, pH 7·0); (2), tris-HCl (0·1M, pH 7·0); (3) tris-HCl (0·1M, pH 7·0; 9 mM CaCl); and (4), SMUF (pH 6·6).
[a]Slopes of the linear extrapolation (LE) and equilibrium binding (EB) plots, i.e. m (kJ mol^{-1} M^{-1}) and Δn (kJ mol^{-1}) respectively (see text).
[b]Biphasic transition observed in excess calcium.
Data are mean of triplicate experiments, SD of <0·2 not shown in parenthesis.
$[GnHCl]_{1/2}$ is the guanidine hydrochloride concentration at 50% BAL unfolding.

Table 2. Bovine alpha-lactalbumin conformational stability by Trp fluorescence measurements

Buffer	$\Delta G°$ (kJ mol^{-1})		Slope[a]		$[GnHCl]_{1/2}$
	LE	EB	m	Δn	
1	11(0·3)	15(0·4)	6(0·4)	9(0·2)	1·8
2	11(0·5)	17(0·6)	7	11	1·7
3(I)[b]	12(0·9)	18(2)	9(1)	13(1)	1·6
3(II)	41(4)	52(5)	16(3)	15(1)	3·2
4	11(2)	16(3)	6(2)	9(1)	2·4
5	13(1)	21(2)	7(2)	11(2)	1·8
6(I)[b]	17(2)	21(3)	16(2)	18(2)	1·2
6(II)	24(2)	29(4)	9(2)	3(2)	2·9
7(I)[b]	8(0·8)	13(0·7)	6(0·7)	9(0·5)	1·2
7(II)	35(3)	32(0·2)	9(0·8)	9	4·0

See Table 1 footnotes.
Additional buffers were: (5), SMUF (minus Ca^{2+} and Mg^{2+}); (6), SMUF (minus citrate and phosphate); and (7), Nacacodylate buffer (50 mM, with 9 mM $CaCl_2$).

buffers (Fig. 2, Owusu, 1992). However, with tris-HCl (9 mM $CaCl_2$) (cf. Fig. 3, Owusu, 1992) or SMUF (minus citrate and phosphate) buffer as solvent, biphasic denaturation profiles were obtained (Fig. 2). Figures 3 and 4 show the linear and non-linear EB plots for BAL unfolding of SMUF and SMUF (minus citrate and phosphate) buffers respectively.

$\Delta G°$ Values determined using the EB analysis were, from results reported in this paper and in the literature (Pace, 1986), on average 35% larger than values obtained using the LE analysis (Tables 1 & 2). Therefore comparisons between experimental and literature $\Delta G°$ values involved a consistent set of data determined by the same (EB) form of analysis.

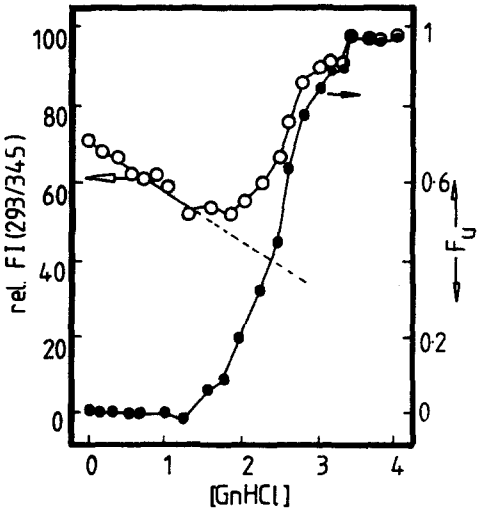

Fig. 1. Unfolding profile for alpha-lactalbumin in SMUF buffer: (○), relFI (293/345), relative fluorescence intensity (excitation/emission wavelength = 293/345 nm), plotted versus [GnHCl]; (●), fraction of unfolded protein (F_u) at different [GnHCl].

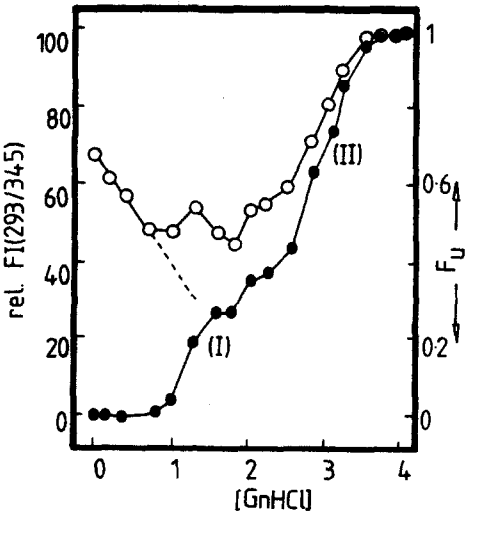

Fig. 2. Unfolding profile for alpha-lactalbumin in SMUF (minus citrate and phosphate) buffer. A bi-phasic transition is seen as I and II. Symbols are as in Fig. 1.

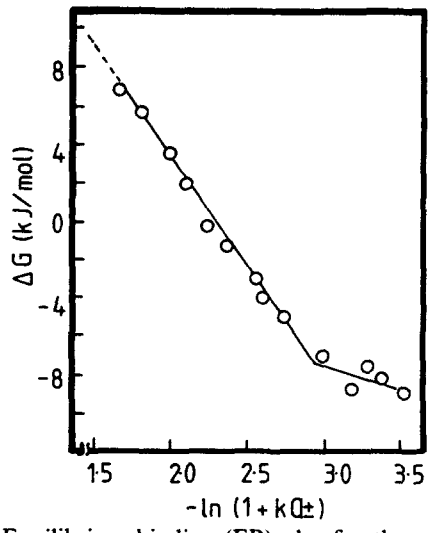

Fig. 3. Equilibrium binding (EB) plot for the estimation of $\Delta G°$ for alpha-lactalbumin in SMUF buffer. Data from Fig. 1 (see text).

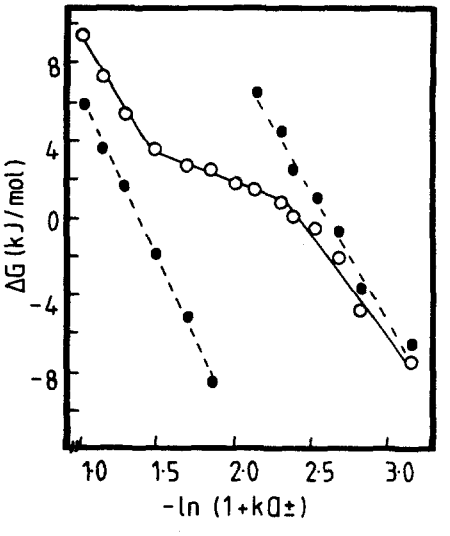

Fig. 4. Equilibrium binding (EB) plot for the estimation of $\Delta G°$ for alpha-lactalbumin in SMUF (minus citrate and phosphate) buffer: (○), data from Fig. 2; – – ● – –, unfolding treated as two transitions (see text).

BAL conformational stability in calcium-free buffers

The GnHCl unfolding of BAL in a Ca^{2+}-free buffer apparently occurs via a three-state reaction, i.e. $N \rightleftharpoons A \rightleftharpoons U$; where N is the native protein, A is a thermodynamically stable intermediate and U is the unfolded BAL. The unfolding process may involve the successive loss of BAL 3° structure ($N \rightleftharpoons A$) and 2° structure ($A \rightleftharpoons U$) at a low and high GnHCl concentration. The $N \rightleftharpoons A$ transition represents the full extent of BAL denaturation by heat or acid (Kuwajima *et al.*, 1976; Kuwajima & Sugai, 1978; Ikeguchi *et al.*, 1986*b*). In the discussion to follow, literature $\Delta G°$ values for these reactions are designated $\Delta G°$ (N,A) and $\Delta G°$ (N,U), respectively.

The $\Delta G°$ value for BAL unfolding in tris-HCl was 28 (±1) kJ mol^{-1}. For BAL unfolding in K-phosphate buffer $\Delta G°$ was 21(±2) kJ mol^{-1}. Both studies involved UV difference measurement (Table 1). Such $\Delta G°$ values compare with $\Delta G°$ (N,U) values of 28·5 kJ mol^{-1} (Sugai *et al.*, 1973). 23·8 kJ mol^{-1} (Ahmad & Bigelow, 1982) and 23–26 kJ mol^{-1} (Takase *et al.*, 1974). The agreement between $\Delta G°$ and $\Delta G°$ (N,U) values suggests that, in the absence of added Ca^{2+} (e.g. with tris-HCl or K-phosphate buffer as solvent), the unfolding of BAL is consistent with a three-state transition (Kuwajima *et al.*, 1976). Furthermore, Trp UV difference absorbance changes at 293 nm appear to be sensitive only to the loss of BAL 2° structure. Otherwise, a bi-phasic, rather than a simple S-shape, unfolding profile would have been recorded.

Fluorescence monitoring of the unfolding of BAL in tris-HCl or K-phosphate buffer led to $\Delta G°$ estimates of 15–17 kJ mol^{-1} (Table 2; solvents 1 and 2). This is in good agreement with ΔG (N,A) values of 16–17 kJ mol^{-1} measured for the heat or acid denaturation of BAL (Kuwajima *et al.*, 1976; Ikeguchi *et al.*, 1986*b*). Therefore, for BAL in Ca^{2+}-free solvents, Trp fluorescence changes at 345 nm are apparently sensitive only to the loss of 3° structure.

BAL conformational stability in excess Ca^{2+}

In 0·1M tris-HCl (with 9 mM $CaCl_2$) or SMUF (minus citrate and phosphate) buffer, the unfolding of BAL was shifted to higher GnHCl concentration (cf. $[GnHCl]_{1/2}$ values, Tables 1 & 2). Denaturation profiles determined using both Trp UV difference absorbance and fluorescence measurements were also bi-phasic (above). If the unfolding of BAL was treated as a monophasic process then non-linear EB graphs were obtained. However, straight-line EB (Fig. 4) and LE graphs (not shown) were produced when transitions (I) and (II) were treated separately. The boundary between the two transitions was found by inspection. $\Delta G°$ Estimates for transition (I) and (II) are given in Tables 1 and 2.

In the presence of Ca^{2+} (9 mM $CaCl_2$) $\Delta G°$ for transitions (I) and (II) were 18–21 kJ mol^{-1} and 52 kJ mol^{-1}, respectively (Tables 1 & 2, solvent 3). Importantly, there was no difference in $\Delta G°$ values obtained from UV difference or fluorescence profiles. Hence the nature of the unfolding reaction for BAL may be altered in the presence of Ca^{2+} (in contrast with Ca^{2+}-free solvents) such that UV difference and fluorescence monitoring are sensitive to the same BAL conformational transition(s).

There seems one other study to which the present may be compared in terms of the high concentration of Ca^{2+} used. In Nacacodylate buffer (50 mM with 50 mM NaCl and 12 mM $CaCl_2$, pH 7·0), the unfolding of BAL apparently changed from a three-state to a two-state reaction. The conformational stability of BAL, determined by near or far UV circular dichroism, increased from 21 kJ mol^{-1} to 51 kJ mol^{-1} (Ikeguchi *et al.*, 1986*b*); the original data were obtained using the LE form of analysis and have been increased by 35% to allow for the in-built underestimation of $\Delta G°$. From a comparison of $\Delta G°$ values, transition (II) from this study seems identical to the two-state transition reported previously (Ikeguchi *et al.*, 1986*b*).

Because of the absence of bi-phasic transition in the above study, GnHCl denaturation of BAL was re-examined with Nacacodylate (50 mM, pH 7·0 with 9 mM $CaCl_2$) as solvent. Unfolding was monitored by fluorimetry as described above. Transitions (I) and (II) were observed with associated $\Delta G°$ values of 13 kJ mol^{-1} and 32 kJ mol^{-1} respectively (Table 2). The studies of solvent effects on BAL denaturation are in progress and a fuller account of results will be given at a later date.

Interestingly, the $\Delta G°$ value for Ca^{2+}-free BAL, i.e. 22·8 kJ mol^{-1} (Hiraoka & Sugai, 1984) is similar to the $\Delta G°$ value for Ca^{2+}-bound BAL dissolved in Ca^{2+}-free solvents (Table 1, solvents 1 and 2). The effect of a high ionic strength and/or competitive binding of Na^+ or tris cations (Rawitch & Gleason, 1971) to BAL could facilitate the dissociation of Ca^{2+} from Ca^{2+}-bound BAL and equilibration with the surrounding solvent. Holo-BAL, in a Ca^{2+}-free solvent, may possess a conformation somewhat akin to the conformation of the Ca^{2+}-free protein (Hiroaka & Sugai, 1984).

Apo-BAL possesses an expanded 3° structure with a greater intrinsic flexibility compared to the N-state (Dolgikh *et al.*, 1985; Pfeil, 1988; Kronman, 1989). The implication is that an increase in conformational stability of BAL (Tables 1 & 2, solvent 3) as a result of Ca^{2+} binding may be interpreted in terms of a decrease in intrinsic flexibility.

In this context, it is noteworthy that the slopes of both the LE and EB graphs increased when BAL was denatured in the presence of excess Ca^{2+} (Tables 1 & 2). m (kJ mol^{-1} M^{-1}) is an index of unfolding co-operativity whilst Δn (kJ mol^{-1}) is the apparent difference in the number of GnHCl binding sites in the N- and U-states (Pace, 1986). The increases in these parameters suggest that BAL, in the presence of excess Ca^{2+}, has a more compact, inflexible conformation with less accessible GnHCl binding sites. Transition (I) may be due to the loss of a feature of 3° structure which is unique to the compact, Ca^+-bound, BAL structure.

The degree of Ca^{2+} stabilisation of BAL can be seen from the increase in $\Delta G°$ by about 26 kJ mol^{-1} (Ikeguchi *et al.*, 1986*b*; Table 1). This corresponds to a $3·6 \times 10^4$–fold increase in the conformational stability of BAL at 25°C. Considering transition (I) as an integral part of the effect of Ca^{2+}, the stabilisation effect amounts to 50 kJ mol^{-1} (Table 1) or about a further 5000-fold. Thus, the stabilisation of BAL by Ca^{2+} may be three orders of magnitude greater than suggested by previous results.

In SMUF buffer (solvent 4, Table 1) the stabilisation of BAL by Ca^{2+} was more moderate, i.e. 14 kJ mol^{-1} or 280-fold. Hence the degree of BAL stabilisation in SMUF buffer is about 6×10^5-fold lower than the stabilising effect observed with an equivalent concentration (9 mM) of Ca^{2+} in tris-HCl buffer. The activity of Ca^{2+} would be lower in SMUF buffer owing to Ca^{2+} binding by chelators (see below).

The $\Delta G°$ value for BAL dissolved in SMUF or SMUF (minus Ca^{2+} and Mg^{2+}) buffer was lower than the $\Delta G°$ value in SMUF (minus citrate and phosphate) buffer (Table 2, solvents 4 to 6). Also a bi-phasic unfolding transition was only seen with BAL dissolved in the latter solvent which did not contain chelating species. Based on such results, indigenous chelators in milk, e.g. citrate, phosphate and the caseins, would reduce the Ca^{2+} stabilisation of BAL by competing with the protein for Ca^{2+} binding.

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