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# Amino acids and short peptides do not always stabilize globular proteins: A differential scanning calorimetric study on their interactions with bovine α-lactalbumin

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Amino acids and related compounds have been shown to confer stabilization to proteins; however, the generalization of such a statement is far from reality as there is a lack of experimental data on the interactions of amino acids and short peptides with different proteins. In particular, there are only a few reports[1–3] on direct differential calorimetric studies on these systems. Using DSC, we have studied the thermal denaturation of bovine α-lactalbumin in the presence of glycine, alanine, leucine, lysine, aspartic acid, glutamic acid, diglycine, triglycine, tetraglycine, pentaglycine, glycyl-leucine and glycyl-glycyl-leucine at neutral pH. It has been observed that these amino acids and peptides either do not provide thermal stabilization to α-lactalbumin or that the extent of stabilization is negligibly small. However, sucrose was able to confer thermal stability at neutral pH. All the thermal denaturations were found to be two-state (*i.e.* natured$\rightleftharpoons$denatured) and reversible and provide quantitative thermodynamic parameters associated with them. The results have been interpreted in terms of a fine balance between the preferential exclusion and binding which takes place during the course of the denaturation reaction. It follows from this study that the amino acids and peptides do not always stabilize globular proteins.

Stabilizing proteins and developing empirical formulations and protocols for retaining the activity of purified preparations of peptides and proteins are important issues in biochemical research. Efforts have been made to enhance protein stability by engineering disulfide bonds into proteins[4–8] or by adding cosolutes, so-called osmolytes.[1,2,9–12] It has been shown[2,10,12] in the past that certain amino acids provide thermal stability to proteins. It has also been stated[6,10] that glycine-based osmolytes appear to provide a general method of stabilizing proteins against thermal unfolding, even well beyond the physiological concentration range of osmolyte, and the degree of stabilization can be extraordinary. We have reported previously[1] that amino acids as well as short peptides provide thermal stability to lysozyme and that the peptides provided high thermal stability to lysozyme compared to their respective free amino acids.

Hen egg-white lysozyme and bovine α-lactalbumin are similar globular proteins with known X-ray structures[13] and with molecular weights of 14 600 and 14 200 u respectively. The primary sequences of these two proteins have been shown to be strikingly similar, 49 amino acid residues out of 129 and 123 being identical. The main polypeptide fold of the α-lactalbumin molecule is held together by four disulfide bridges maintaining a similar conformation to that of hen egg-white lysozyme, with significant changes in the loop region and at the C-terminus. Native forms of the proteins are roughly ellipsoidal in shape and contain two separate lobes.[13–14] In this study we show that amino acids and short peptides do not always stabilize globular proteins. The effects of glycine, alanine, leucine, lysine, aspartic acid, glutamic acid, diglycine, triglycine, tetraglycine, pentaglycine, glycyl-leucine and glycyl-glycyl-leucine on the thermal denaturation of α-lactalbumin have been studied. The results are compared with those obtained with hen egg-white lysozyme.

## Materials and Methods

Bovine α-lactalbumin (type III) was purchased from Sigma Chemical Co. and stock solutions were prepared by extensive dialysis of the protein at 4 °C in 20 mmol dm$^{-3}$ tris [tris(hydoxymethyl)aminomethane] buffer from Sigma at pH 7.0 with at least three changes. Glycine, alanine, leucine, lysine, aspartic acid, glutamic acid, diglycine, triglycine, tetraglycine, pentaglycine, glycyl-leucine, glycyl-glycyl-leucine and sucrose were purchased from Sigma Chemical Co. The compounds were of the best available purity grade and were used as supplied. Protein concentrations were determined spectrophotometrically on a Shimadzu double-beam spectrophotometer UV-160A using absorbance $E^{1\%}_{280} = 20.1$.[15]

Calorimetric measurements were performed on a micro DSC (SETARAM, France) at a scanning rate of 0.5 °C min$^{-1}$. Experimental details and the conversion of the traces obtained into excess heat capacity *vs.* temperature plots are the same as discussed earlier.[1,16] The resultant excess heat capacity *vs.* temperature scans were analysed by the EXAM program of Kirchhoff[17] and the thermodynamic parameters transition temperature ($t_{1/2}$), calorimetric enthalpy ($\Delta_{cal} H$), heat capacity of denaturation ($\Delta C_p$) and $N$, the number of moles of cooperative units per mole of protein in the calorimetric cell, were obtained by this curve deconvolution. The reversibility of the scans was checked by heating the sample to just above the transition maximum, cooling immediately and then reheating.

## Results and Discussion

Fig. 1 shows representative DSC endotherms, *i.e.*, the excess heat capacity *vs.* temperature plots, for holo α-lactalbumin at pH 7.0 in 20 mmol dm$^{-3}$ tris buffer, in the presence of 200 mmol dm$^{-3}$ diglycine and 1.5 mol dm$^{-3}$ sucrose. The transition temperature, $t_{1/2}$, for the unfolding of α-lactalbumin in 20 mmol dm$^{-3}$ tris buffer is 63.5 °C which is in excellent agreement with the literature.[18] The thermodynamic parameters obtained for α-lactalbumin from a series of DSC experiments in the presence of glycine, alanine, leucine, lysine, glutamic acid, aspartic acid, diglycine, triglycine, tetraglycine, pentaglycine, glycyl-leucine and glycyl-glycyl-leucine are summarized in Table 1. The $t_{1/2}$ values have an experimental error of ±0.1 K and $\Delta_{cal} H$ data have a deviation of 4% including errors in sample preparation, calibration constant and reproducibility. As shown in Fig. 2, the transition temperature,

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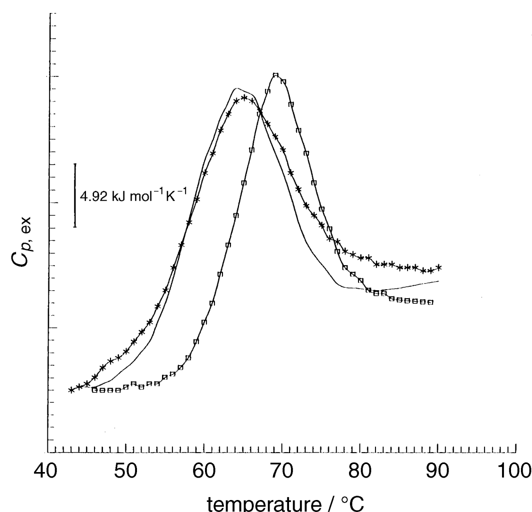


**Fig. 1** Representative DSC endotherms for α-lactalbumin at pH 7.0 in 20 mmol dm$^{-3}$ tris buffer (——), protein in the presence of 200 mmol dm$^{-3}$ diglycine (*) and in 1.5 mol dm$^{-3}$ sucrose (□)

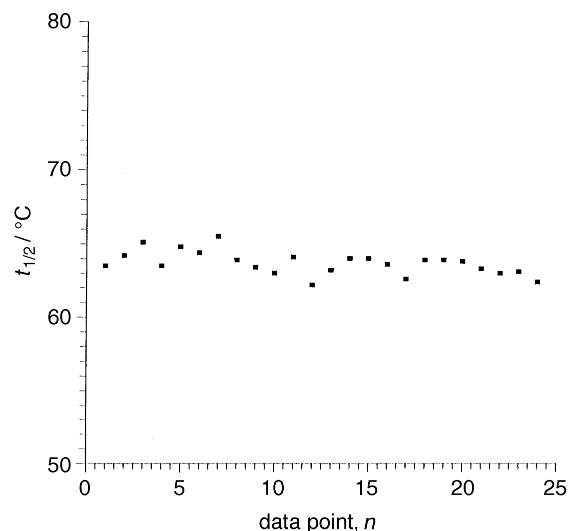


**Fig. 2** Plot of average $t_{1/2}$ values (■) *vs.* data points ($n$) for α-lactalbumin at pH 7.0 in 20 mmol dm$^{-3}$ tris buffer and in the presence of amino acids/peptides. Each point represents an average of three to four measurements.

which is widely considered as a stability index of proteins, does not appreciably change even at very high concentrations of any of these cosolutes. Each data point in Fig. 2 represents an average of three to four experiments on thermal denaturation of α-lactalbumin in buffer or in the presence of a given concentration of cosolute. The first data point in Fig. 2 is an average of $t_{1/2}$ values for the thermal denaturation of α-lactalbumin in 20 mmol dm$^{-3}$ tris buffer, the second average data point is $t_{1/2}$ in the presence of 200 mmol dm$^{-3}$ glycine, the third data point is $t_{1/2}$ in the presence of 1000 mmol dm$^{-3}$ glycine and the remainder are in the order given in Table 1. Thermal denaturation in the presence of all amino acids and peptides was found to be two-state and reversible as reflected by the cooperativity ratio $\beta$ shown in Table 1 which was calculated by the procedure discussed earlier.[1,16]

Yancey *et al.*[9] have reported that amino acids are dominant stabilizing osmolytes in genetically diverse bioorganisms. Arakawa and Timasheff,[11] using high-precision densimetry, have studied the interaction of amino acids such as glycine, alanine and betaine with lysozyme and bovine serum albumin. Estimation of preferential hydration parameters for the above systems evidenced the 'preferential exclusion' of amino acids from the protein surface and the resultant hydration of the

**Table 1** Thermodynamic parameters associated with the thermal unfolding of α-lactalbumin (0.335295 mmol dm$^{-3}$) in the presence of amino acids, oligopeptides and sucrose at pH 7.0 and in 20 mmol dm$^{-3}$ tris buffer[a]

| | [amino acid/peptide/sucrose] /mmol dm$^{-3}$ | $t_{1/2}$/°C | $\Delta_{cal}H$/kJ mol$^{-1}$ | $\Delta C_p$/kJ mol$^{-1}$ K$^{-1}$ | $N$ | $\beta$ ($\Delta_{vH}H/\Delta_{cal}H$)[b] | data point ($n$) |
|---|---|---|---|---|---|---|---|
| | 0.00 | 63.5 | 274 | 4.68 | 0.97 | 1.02 | 1 |
| glycine | 200 | 64.2 | 255 | 3.93 | 0.99 | 1.01 | 2 |
| | 1000 | 65.1 | 271 | 3.28 | 0.96 | 1.02 | 3 |
| alanine | 200 | 63.5 | 266 | 5.90 | 0.96 | 1.01 | 4 |
| | 750 | 64.8 | 280 | 5.15 | 0.99 | 1.01 | 5 |
| lysine | 200 | 64.4 | 260 | 2.48 | 0.99 | 1.00 | 6 |
| | 750 | 65.5 | 280 | 3.02 | 0.97 | 1.01 | 7 |
| leucine | 5 | 63.9 | 273 | 3.20 | 1.02 | 1.01 | 8 |
| | 25 | 63.4 | 267 | 3.77 | 0.97 | 0.99 | 9 |
| glutamic acid | 200 | 63.0 | 261 | 3.65 | 0.98 | 1.01 | 10 |
| | 1000 | 64.1 | 291 | 7.56 | 0.97 | 1.04 | 11 |
| aspartic acid | 200 | 62.2 | 269 | 2.27 | 0.97 | 1.01 | 12 |
| | 50 | 63.2 | 273 | 3.14 | 0.97 | 1.01 | 13 |
| diglycine | 50 | 64.0 | 265 | 2.55 | 0.97 | 1.02 | 14 |
| | 200 | 64.0 | 252 | 3.80 | 1.04 | 1.03 | 15 |
| triglycine | 50 | 63.6 | 276 | 3.68 | 0.95 | 1.01 | 16 |
| | 150 | 62.6 | 249 | 2.12 | 1.03 | 0.96 | 17 |
| tetraglycine | 3 | 63.9 | 267 | 1.97 | 1.00 | 0.99 | 18 |
| | 8 | 63.9 | 285 | 3.95 | 0.97 | 0.98 | 19 |
| pentaglycine | 3 | 63.8 | 262 | 4.23 | 1.06 | 1.03 | 20 |
| glycyl-leucine | 50 | 63.3 | 269 | 5.56 | 0.95 | 1.03 | 21 |
| | 150 | 63.0 | 267 | 2.90 | 0.98 | 0.98 | 22 |
| glycyl-glycyl-leucine | 50 | 63.1 | 263 | 4.07 | 0.99 | 1.03 | 23 |
| | 150 | 62.4 | 261 | 3.02 | 0.98 | 1.00 | 24 |
| sucrose | 500 | 65.2 | 267 | 2.65 | 1.00 | 1.00 | |
| | 750 | 65.9 | 282 | 2.07 | 1.01 | 0.99 | |
| | 1000 | 66.1 | 289 | 2.28 | 1.01 | 0.99 | |
| | 1500 | 68.6 | 299 | 3.14 | 0.97 | 1.01 | |
| | 2000 | 70.1 | 295 | 3.44 | 0.96 | 1.02 | |

[a] Each value represents an average of three to four experiments. [b] Subscript vH indicates van't Hoff enthalpy change.

protein at acidic and neutral pH values thereby thermally stabilizing the protein. From calorimetric studies, we have observed the stabilizing effect of some amino acids and oligopeptides on hen egg-white lysozyme at pH 2.0 in 40 mmol $dm^{-3}$ phosphate buffer.[1] The most obvious effect of all the amino acids and peptides was to raise the temperature of protein denaturation. It was also observed that peptides provide more thermal stabilization than the corresponding free amino acids. The calorimetric enthalpy in the presence of these amino acids and peptides was found to increase linearly in the concentration range studied. All the thermal transitions of lysozyme in presence of these cosolutes were found to be two-state and reversible. From the data given in Table 1, it is evident that all the amino acids and oligopeptides studied either do not stabilize α-lactalbumin or that the extent of stabilization is negligibly small at pH 7.0. The $\Delta_{cal} H$ values in the presence of these amino acids and peptides are nearly the same as those obtained in the absence of these cosolutes. These values in the absence and presence of amino acids and peptides are plotted *vs.* the data points ($n$) in Fig. 3. The deviation of individual values from the average is within the limits of experimental uncertainty. The average heat capacity of unfolding, $\Delta C_p$, in all cases is $3.41 \pm 1.28$ kJ $mol^{-1}$ $K^{-1}$. Recent heat capacity measurements of proteins, lysozyme and ribonuclease A using DSC in the presence of various cosolutes and at different pH conditions resulted in almost two-fold variation of the $\Delta C_p$ values for each protein.[19] At lower pH values (2.5 or 4.5 in 20 mmol $dm^{-3}$ sodium phosphate and acetate buffers respectively), α-lactalbumin did not give a DSC peak, hence we could not compare the results for α-lactalbumin and lysozyme at the same pH.

The stabilization/destabilization of proteins by addition of cosolute is currently best explained in terms of preferential interactions.[11,20,21] Preferential hydration, strengthening of the water layer around the macromolecule, by the addition of cosolute leads to the stabilization of proteins. Hydration[11,12,22] favours the compact native state and disfavours an increase in the surface area of the protein. In DSC experiments this situation is reflected by an increase in the transition temperature of the protein. On the other hand, preferential binding[21,23] of the cosolutes with the protein upon unfolding opposes the stabilization effect. Binding of a cosolute at a site depends on the difference between the intrinsic Gibbs free energies of interaction of the protein with the additive and with water. In the case of amino acids and peptides,

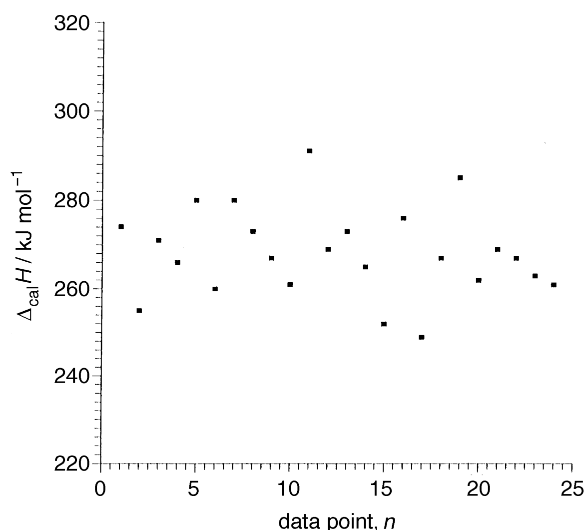


**Fig. 3** Plot of average $\Delta_{cal} H$ values (■) *vs.* data points ($n$) for α-lactalbumin at pH 7.0 in 20 mmol $dm^{-3}$ tris buffer and in the presence of amino acids/peptides. Each point represents an average of three to four measurements.

preferential binding results due to interactions of the hydrogen-bonding and hydrophobic groups of these cosolutes with those exposed on the protein when it unfolds. Thus protein stability depends on the fine balance between patterns of interactions of the native and unfolded protein with cosolutes, which involves a combination of preferential exclusion and binding that changes during the course of the reaction. In amino acids and peptides, the hydrogen-bonding sites are the terminal amino and carboxy groups and the peptide linkages in oligopeptides. The non-polar side chains of hydrophobic amino acids result in hydrophobic interactions with the non-polar groups of the protein in the denatured state. The binding is dependent on the chemical nature of the surface of the protein in contact with the solvent and it increases upon protein unfolding due to the exposure of additional hydrophobic sites and peptide bonds.

The DSC results obtained for α-lactalbumin in the presence of amino acids and peptides studied at neutral pH indicate that the influence of preferential interactions is nearly zero in these systems. The balance of preferential exclusion and binding of the peptides is retained throughout the denaturation process. It appears that the balancing of the preferential hydration of α-lactalbumin results due to an increase in the preferential binding of these cosolutes with the protein in the denatured state. The reason for this may be the lower electrostatic repulsion between the charged cosolute and the protein at neutral pH than at low pH, thus resulting in more effective binding, so much so that it balances the stabilizing effect which could result due to preferential hydration.

In view of the absence or negligibly small effect on thermal stabilization of α-lactalbumin by the amino acids and peptides studied, it seemed of interest to examine the effect of sucrose which has been shown[24] to induce strong preferential hydration in some globular proteins leading to an increase in their thermal stability. Table 1 also summarizes the thermodynamic parameters associated with the thermal unfolding of α-lactalbumin in the presence of sucrose. Both $t_{1/2}$ and $\Delta_{cal} H$ values of denaturation in the presence of sucrose were found to increase with an increase in the concentration of the saccharide, although the increase is not dramatic. For example, 2 mol $dm^{-3}$ sucrose increased $t_{1/2}$ and $\Delta_{cal} H$ by 6.6 °C and 21 kJ $mol^{-1}$ respectively. The results show that the preferential hydration of α-lactalbumin induced by sucrose at pH 7.0 outweighs the attenuation of this effect by preferential binding of the hydroxy groups of the saccharide with the hydrogen-bonding groups of the amino acid residues and peptide groups exposed on protein unfolding. The factors that have been shown to affect protein stability in sugar solutions is their relative water-structure-promoting tendencies. Positive values of the Gibbs free energy of transfer have been obtained in the literature for amino acids and peptides in sugar solutions relative to water. Oakenful and Fenwick[25] and Bull and Breese[26] have also suggested that sugars which strongly promote the water structure are the more effective stabilizers. Consistent with these explanations, sucrose does lead to stabilization of α-lactalbumin.

Fig. 4 shows a comparison of the temperature dependence of the standard Gibbs free energies of denaturation for α-lactalbumin and lysozyme in buffer and in the presence of triglycine and sucrose. This comparison is a representation of the stabilizing capacity of the amino acids and peptides towards these two structurally homologous proteins. It is obvious from the figure that lysozyme is conferred a high degree of stability by 40 mmol $dm^{-3}$ triglycine in the entire temperature range studied. However the curves of α-lactalbumin in the buffer and in the presence of 150 mmol $dm^{-3}$ triglycine overlap indicating that the peptide is unable to confer thermal stability to the protein. Thermal denaturation of α-lactalbumin is less spontaneous in the presence of sucrose than in buffer alone, but the extent of stabilization of

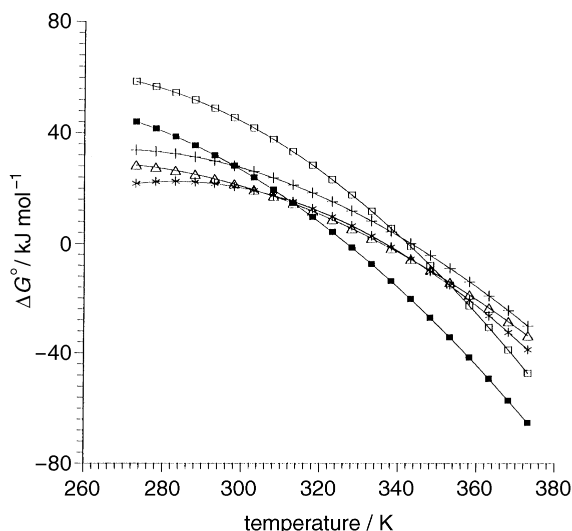


**Fig. 4** Temperature dependence of the standard Gibbs free energies of denaturation for α-lactalbumin and lysozyme in buffer and in the presence of triglycine and sucrose. (*) α-Lactalbumin in 20 mmol $dm^{-3}$ tris buffer, pH 7.0; (△) α-lactalbumin in 150 mmol $dm^{-3}$ triglycine; (+) α-lactalbumin in 2 mol $dm^{-3}$ sucrose; (■) lysozyme in 40 mmol $dm^{-3}$ phosphate buffer, pH 2.0; (□) lysozyme in 40 mmol $dm^{-3}$ triglycine.

this protein is not high even at a concentration of 2 mol $dm^{-3}$ sucrose as compared to just 40 mmol $dm^{-3}$ triglycine to lysozyme.

## Conclusions

Differential scanning calorimetric results on the thermal denaturation of α-lactalbumin in the presence of glycine, alanine, leucine, lysine, aspartic acid, glutamic acid, diglycine, triglycine, tetraglycine, pentaglycine, glycyl-leucine and glycyl-glycyl-leucine indicate that these cosolutes either do not enhance thermal stabilization of the protein or that the extent of stabilization is negligibly small within experimental deviation. Thermal denaturation in the presence of all these cosolutes has been found to be two-state and reversible and provide quantitative thermodynamic parameters which are nearly the same as those obtained in their absence. By contrast, in our earlier studies[1] on the interaction of amino acids and oligopeptides with hen egg-white lysozyme at pH 2.0, it was observed that 250 mmol $dm^{-3}$ glycine and alanine increase $t_{1/2}$ by nearly 17 °C, and that peptides provide more thermal stabilization than the corresponding free amino acids. The results clearly indicate stronger preferential hydration of lysozyme in the presence of amino acids and peptides. The results of the present study show that the fine balance of preferential exclusion and preferential binding is maintained during the denaturation reaction in the presence of these cosolutes for α-lactalbumin at pH 7.0. This comparison also suggests that the amino acids and oligopeptides do not always stabilize globular proteins.

This work was supported by a research grant from the Department of Science and Technology, New Delhi, India.

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*Paper* 6/05798E; *Received* 20*th August*, 1996