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Thermodynamic stability of porcine β-lactoglobulin

A structural relevance

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The proposed biological function of β-lactoglobulins as transporting proteins assumes a binding ability for ligands and high stability under the acidic conditions of the stomach. This work shows that the conformational stability of non-ruminant porcine β-lactoglobulin (BLG) is not consistent with this hypothesis. Thermal denaturation of porcine BLG was studied by high-sensitivity differential scanning calorimetry within the pH range 2.0–10.0. Dependences of the denaturation temperature and enthalpy on pH were obtained, which reveal a substantial decrease in both parameters in acidic and basic media. The denaturation enthalpy follows a linear dependence on the denaturation temperature. The slope of this line is 9.4 ± 0.6 kJ·mol^{-1}·K^{-1}, which is close to the denaturation heat capacity increment $\Delta_d C_p = 9.6 \pm 0.5$ kJ·mol^{-1}·K^{-1}, determined directly from the thermograms. At pH 6.25 the denaturation temperatures of porcine and bovine BLG coincide, at 83.2 °C. At this pH the denaturation enthalpy of porcine BLG is 300 kJ·mol^{-1}. The denaturation transition of porcine BLG was shown to be reversible at pH 3.0 and pH 9.0. The transition profile at both pH values follows the two-state model of denaturation. Based on the pH-dependence of the transition temperature and the linear temperature dependence of the transition enthalpy, the excess free energy of denaturation, $\Delta_d G^E$, of porcine BLG was calculated as a function of pH and compared with that of bovine BLG derived from previously reported data. The pH-dependence of $\Delta_d G^E$ is analysed in terms of the contributions of side-chain H-bonds to the protein stability. Interactions stabilizing native folds of porcine and bovine BLG are discussed.

Keywords: β-lactoglobulin; porcine; stability; thermodynamics; DSC.

The protein β-lactoglobulin (BLG) has a long story of comprehensive studies of its physicochemical and biological aspects but it still remains a protein with undefined function. The most widespread hypothesis of its biological function refers to the role of BLG as a transport protein [1,2]. This view is supported by numerous data on binding of hydrophobic ligands to bovine BLG [3–6]. Structural data encourage this idea, providing an indication of possible binding sites for retinol and fatty acids in bovine BLG [1,6–8]. Bovine BLG stands out because of its high structural and proteolytic stability at low pH [1,6–16] but it readily loses its quaternary and tertiary structure at weakly basic pH [17–22]. These features are believed to play a protecting role for bound ligands under acidic conditions in the stomach and afford their release in the basic intestine [1]. In the light of these concepts, information on the conformational stability of BLG at different pH becomes of interest for functional considerations. Particularly, more light could be shed on this problem by involving nonruminant β-lactoglobulins that are known to differ from bovine BLG in their quaternary structure and binding properties [4,5,23–25].

Porcine BLG shows 66% sequence identity with bovine BLG [26]. It contains two disulfide bonds but does not have the free thiol group at Cys121. The latter property attracts an additional interest to porcine BLG as a naturally existing model for studying the role of the free thiol in postdenaturation processes of bovine BLG and, finally, in milk processing [27–29]. It is well known that thermal denaturation of bovine BLG at neutral and weakly basic pH is a complex, irreversible process. It involves the dissociation of bovine BLG dimer [20,30], unfolding and aggregation [21,31–35]. One of the most important consequences of these conformational changes is the exposure of the free thiol (Cys121) of bovine BLG, which is highly reactive and initiates the formation of intermolecular disulfide bonds [19,36–38]. Disulfide-mediated aggregation is considered to be the key factor of thermotropic gelation of bovine BLG. In accordance with this point, porcine BLG was reported to be unable to form thermally induced gels [39].

Porcine BLG remains up to now one of the poorly studied β-lactoglobulins. This is a small globular protein with a molecular mass of 18.5 kDa and pI of 4.6 [23,40,41]. Its amino acid sequence composed of 160 residues has been determined [42] and two main genetic variants designed as porcine BLG A and C were identified [40]. Recently the three-dimensional structure of this protein was reported [26]. Porcine BLG reveals a pH-dependent dimerization

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Abbreviations: BLG, β-lactoglobulin; HS-DSC, high-sensitivity differential scanning calorimetry

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behaviour reverse to that of bovine BLG: it exists in monomeric form at neutral and basic pH but tends to dimerization at acid pH [23,25,26]. No data on thermal denaturation of porcine BLG have been published up to now except some short remarks [39].

This paper presents a first study of the conformational stability of porcine BLG carried out by high-sensitivity differential scanning calorimetry (HS-DSC) at different pH. We report here evidence of the reversible character of thermal denaturation of this protein at acidic and basic pH. The dependence of the conformational stability of porcine BLG on pH is analysed and discussed in comparison with that of bovine BLG.

MATERIALS AND METHODS

Porcine BLG was purified from milk of Stamboek pigs (NIZO Food Research, the Netherlands) using a large-scale purification method [26]. Protein purity was checked by mass spectrometry and gel electrophoresis, revealing the presence of variants A (70%) and C (30%). The overall purity exceeded 95%. Bovine BLG (variants A and B) prepared from fresh milk was provided by NIZO (Netherlands Institute for Dairy Research).

Calorimetric measurements were carried out with a high-sensitivity differential scanning microcalorimeter DASM-4 (NPO BIOPRIBOR, Russia) within the temperature range of 10–110 °C at the heating rate of 1 K·min^{-1} and excess pressure of 0.2 MPa. The volume of the calorimetric cell was 0.5 mL and the protein concentration used was 3 mg·mL^{-1}. Primary data processing was carried out using the NAIRTA software (Institute of Biochemical Physics, Moscow). The denaturation enthalpy was determined as the area of the heat capacity peak over a baseline. The sigmoid baseline was simulated by spline interpolation of the thermogram segments before and after the transition [43]. Protein solutions for calorimetric measurements were prepared by dialysis against a corresponding buffer at 4 °C overnight. Protein concentration in solution after dialysis was determined spectrophotometrically assuming ε_{280} = 0.56 mL·mg^{-1}·cm^{-1} [23]. Buffer solutions used were: 40 mM glycine at pH 2–3 and pH 8–10, 25 mM acetate at pH 4.1 and 10 mM phosphate at pH 6.6. Renaturation tests were performed with solutions of porcine BLG heated for 10 min at the temperature corresponding to the completion of the denaturation transition according to the thermogram (90–93 °C) and then incubated at room temperature for 3.5 h.

Computer modelling for bovine and porcine BLG was carried out using the software SWISS PDP VIEWER 3.7 (b2). The following crystallographic structures taken from the SWISSPROT database were analysed: 1BEB, 1BSY, 2BLG and 3BLG for bovine BLG, and 1EXS for porcine BLG. The hydrogen bonding between two side-chain groups was assumed to be possible if the distance between proton donor and acceptor atoms of these groups did not exceed 3.2 Å. The PEAKFIT software was used for fitting of theoretical thermodynamic functions to experimental calorimetric data.

RESULTS

Denaturation thermograms of porcine BLG obtained at different pH are shown in Fig. 1. It is seen that a single endothermic heat capacity peak is observed within the whole pH range studied (a broad marginal peak of heat capacity at low temperatures seen at pH 10 was attributed to instability of the baseline and neglected). The peak position and height reveals a substantial dependence on pH. All thermograms were obtained in diluted buffer solutions with the ionic strength of 0.004–0.04 M depending on pH. Figure 1H shows thermograms of porcine BLG obtained at pH 6.6 in the same buffer with ionic strength of 0.004 M (solid line) and 0.04 M (points). They coincide completely indicating that variation in ionic strength within these limits does not affect the denaturation parameters of porcine BLG.

For most pH values a notable positive increment of heat capacity under denaturation, $\Delta_d C_p$, was detected (Fig. 1A–K). For a quantitative determination of $\Delta_d C_p$ the linear segments of baselines after and before denaturation were extrapolated to the transition temperature and subtracted. The slopes of postdenaturation segments of all thermo-

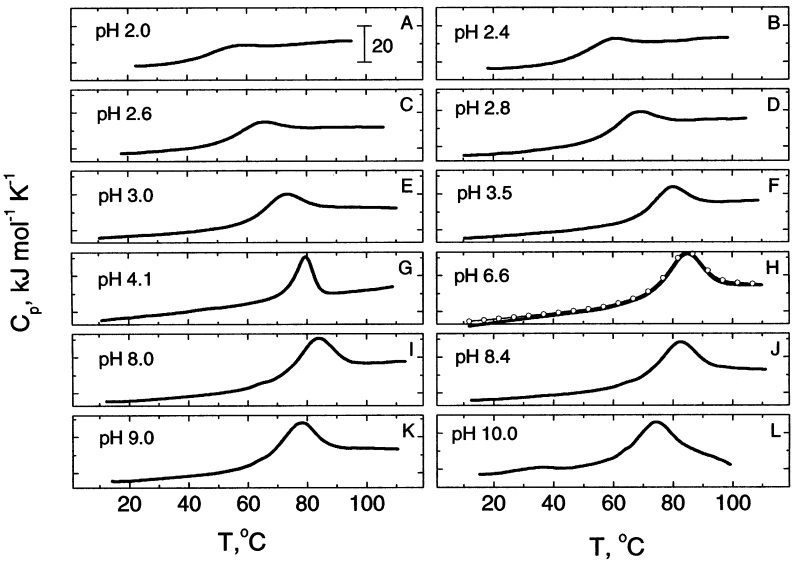


Fig. 1. Thermograms of porcine BLG at different pH. Solid lines – 40 mM glycine at pH 2–3.5 and pH 8–10; 25 mM acetate at pH 4.1 and 10 mM phosphate at pH 6.6. Points (H) – 10 mM phosphate, 0.036 M NaCl, pH 6.6. Scanning rate 1 K·min^{-1}; protein concentration 3 mg·mL^{-1}.

grams (except panels G and L) are close to zero (slightly positive or negative) within the limits of baseline stability. The only remarkable exception is seen for panels G and L. At pH 10.0 a negative slope of the postdenaturation baseline arises (Fig. 1L). This feature is known to result from an aggregation of denatured protein and/or from secondary exothermic chemical reactions caused by extreme pH and temperatures such as hydrolysis of S–S bonds with subsequent aggregation via thiol–disulfide exchange, deamidation of asparagine and glutamine residues, etc. [44]. The contribution of secondary processes can affect the estimation of the denaturation heat capacity increment, $\Delta_d C_p$ from thermograms. For this reason the thermogram at pH 10.0 could not be used for calculation of this parameter. The thermogram at pH 4.1 has a positive baseline slope after denaturation but the heat capacity peak is asymmetric and abnormally narrow. The protein solution taken from the calorimetric cell after the scan showed a significant turbidity indicating aggregation and precipitation of the denatured protein. For this reason, the data at pH 4.1 were also excluded from consideration in the determination of the denaturation heat capacity increment.

Thus, taking into account the thermograms at all pH except pH 10.0 and pH 4.1, we have determined the value of the denaturation heat capacity increment $\Delta_d C_p = 9.6 \pm 0.5$ kJ·mol^{-1}·K^{-1}.

The transition temperature, T_d, and enthalpy, $\Delta_d H$, of porcine BLG are plotted as a function of pH in Fig. 2A,B. The enthalpy at pH 4.1 was not taken into account because of probable exothermic contribution of aggregation of the denatured protein. Both denaturation temperature and enthalpy are maximal at neutral pH (pH 6–7) and decrease markedly to the left from this region (Fig. 2A,B). In basic medium the function T_d(pH) changes only slightly (Fig. 2A), while a decrease in $\Delta_d H$ is more pronounced (Fig. 2B). It is noteworthy that the dependences T_d(pH) and $\Delta_d H$(pH) are not symmetrical and their maximum does not correspond to the isoelectric point of porcine BLG (pI 4.6).

Correlation between $\Delta_d H$ and T_d obtained at different pH is given in Fig. 3. A linear dependence $\Delta_d H(T_d)$ is observed with the slope 9.4 ± 0.6 kJ·mol^{-1}·K^{-1}. This value is close to the denaturation heat capacity increment $\Delta_d C_p = 9.6 \pm 0.5$ kJ·mol^{-1}·K^{-1} obtained directly from the calorimetric curves. This result indicates that the transition enthalpy is a function of temperature but not of pH and that the value of $\Delta_d H$ derived from thermograms at 10.0 can be included in the analysis. In other words, the secondary postdenaturation processes at this pH affect $\Delta_d C_p$ but do not contribute significantly to the heat effect of the denaturation. In fact, slow irreversible processes such as protein aggregation do not perturb markedly the relatively fast denaturation transitions at the heating rates used normally in HS-DSC studies (about of 1 K·min^{-1}) [45,46].

To test the reversibility of thermal denaturation of porcine BLG, renaturation experiments were carried out at acidic and weakly basic pH, where no signs of secondary processes were observed. Figure 4 shows the calorimetric curves for the native and renatured samples of porcine BLG at pH 3.0 and pH 9.0. It is seen that the renatured samples recover the position and profile of the denaturation peak characteristic of the native protein. The degree of the enthalpy recovery is 90% at pH 3.0 and 84% at pH 9.0.

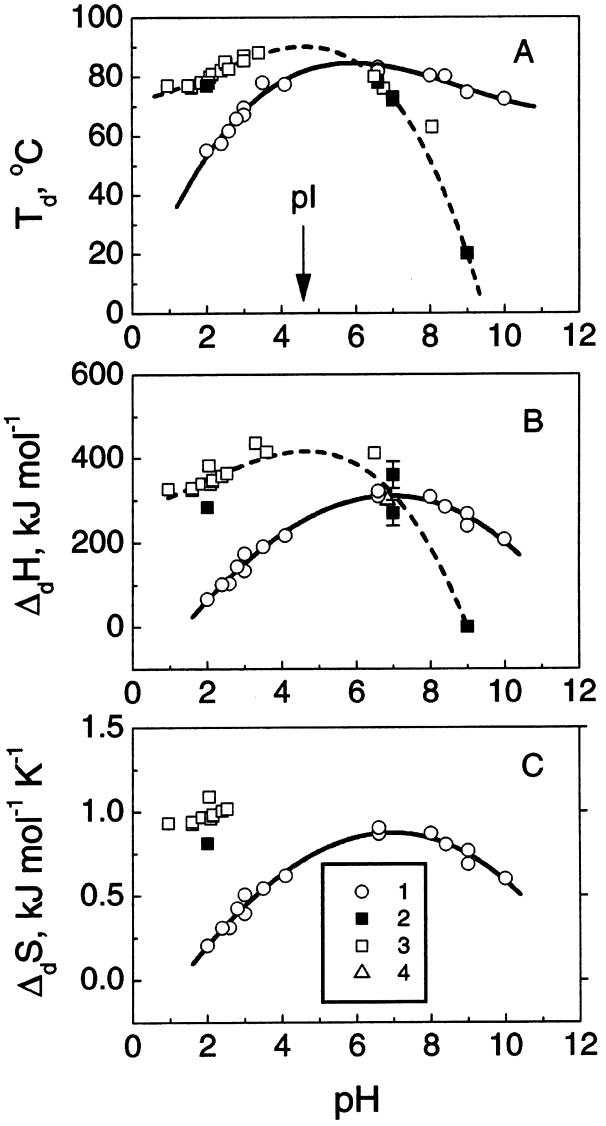


Fig. 2. pH-dependences of the denaturation temperature, T_d (A); enthalpy, $\Delta_d H$ (B) and entropy, $\Delta_d S$ (C) for porcine and bovine BLG. 1, porcine BLG; 2, bovine BLG (both this work); 3, bovine BLG (DSC data reported previously at pH 2.6–2.8 [49], at pH 2.0 [14], at pH 1.5–3.0 [12], at pH 6.75 and pH 8.05 [21], at pH 6.5 [67], at pH 0.9–2.5 [13], at pH 2.05 [15]); 4, bovine BLG, isothermal calorimetry data at pH 6.85 [32]. The pI of porcine BLG is marked by an arrow. The solid and dashed lines represent polynomial approximations of the experimental dependences for porcine and bovine BLG, respectively. The diameter of circles corresponds to the experimental errors.

These results provide unambiguous evidence for the reversibility of thermal denaturation of porcine BLG in both acidic and basic media.

Porcine and bovine BLG show not only different refolding ability after thermal denaturation but also remarkable differences in their stability. Denaturation thermograms for these proteins are compared at acidic and basic pH in Fig. 5. At pH 9.0 no co-operative endothermic transitions are observed for bovine BLG, suggesting its tertiary structure to be destroyed already at room temperature (Fig. 5A). Porcine BLG shows a

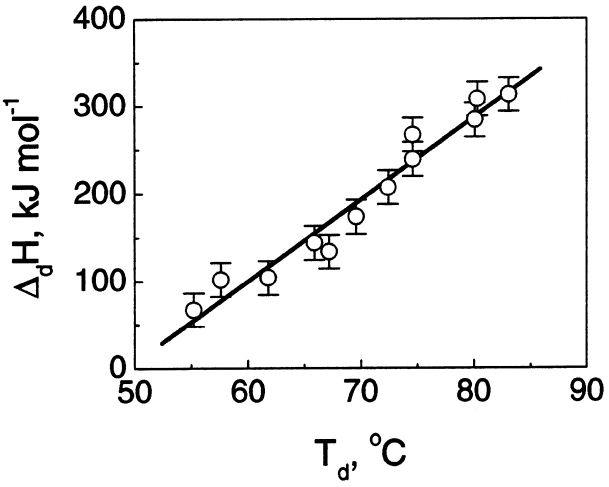


Fig. 3. Correlation between the denaturation enthalpy and temperature of porcine BLG according to Kirchhoff's law. The slope of the straight line is 9.4 ± 0.6 kJ·mol^{-1}·K^{-1}.

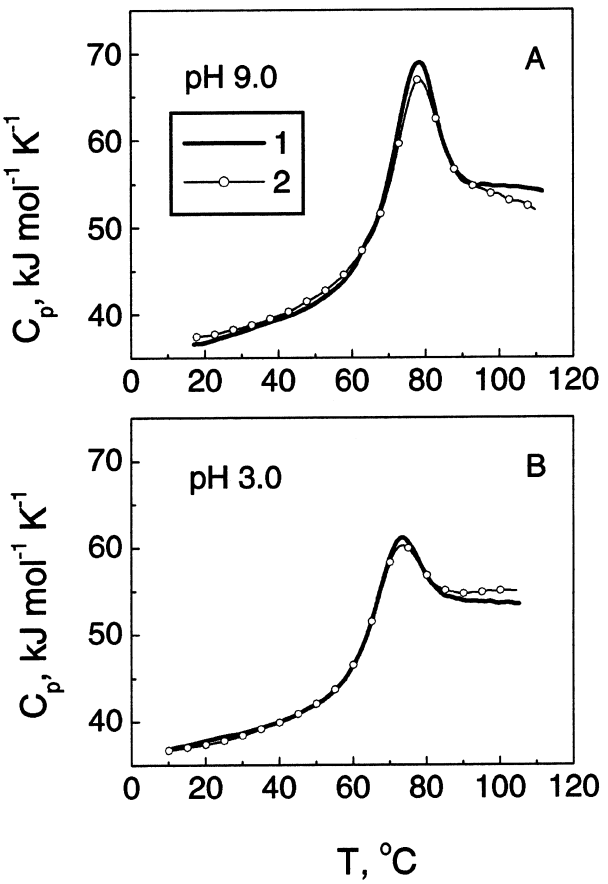


Fig. 4. Thermograms of native (1) and renatured (2) porcine BLG at pH 9.0 (A) and pH 3.0 (B).

well-defined co-operative denaturation transition at 76 °C under these conditions. An opposite situation is observed at pH 2.0: bovine BLG reveals the highly co-operative denaturation transition at about 80 °C whereas porcine BLG undergoes a diffuse transition with the marginal denaturation enthalpy (Fig. 5B). Thus, the tertiary structure of two β-lactoglobulins differs as a result of interactions which are sensitive to pH changes. It can be suggested that these interactions are unlikely to be of pure electrostatic origin: the two BLGs do not differ substantially in their average charge since they have similar pIs.

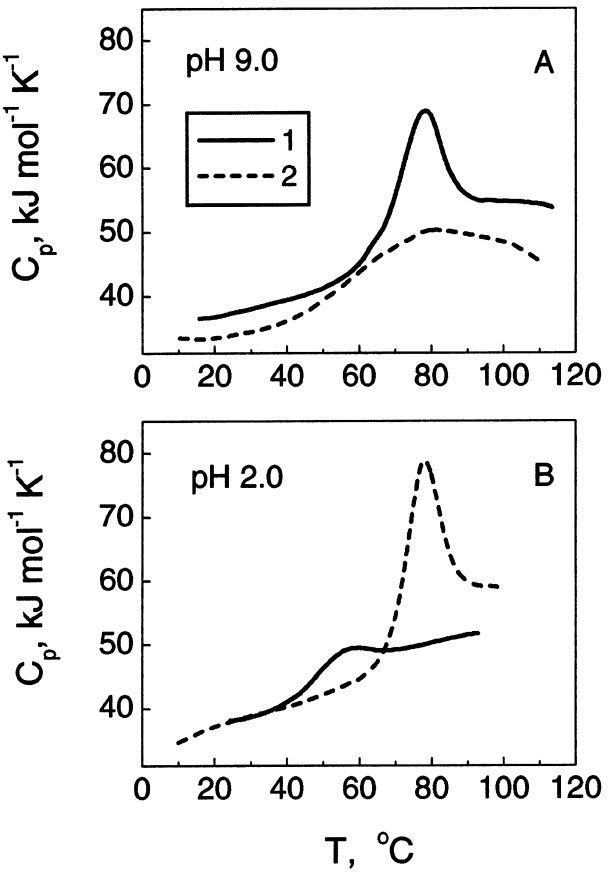


Fig. 5. Thermograms of porcine (1) and bovine (2) BLG at pH 9.0 (A) and pH 2.0 (B).

For the reversible transitions one can calculate the transition entropy as $\Delta_d S = \Delta_d H/T_d$. Figure 2C presents the transition entropy for porcine BLG as a function of pH.

DISCUSSION

Reversibility and two-state mechanism of thermal denaturation of porcine BLG

Despite extensive experimental data on thermal and solvent denaturation of β-lactoglobulins there is no clear understanding of the key factors that stabilize the tertiary structure of these proteins. Particularly, most work in this field has been carried out with bovine BLG as the most accessible and practically significant protein. Several studies of bovine BLG have been performed using differential scanning calorimetry [12–15,21,32,47–49]. A variety of data on T_d and $\Delta_d H$ were obtained that were dependent on the protein concentration and heating rates. However, a

considerable body of such data (in particular, obtained at neutral and basic pH) is nonequilibrium and therefore cannot be used for quantitative estimates of the conformational stability. The main problem is that the denaturation (both thermal and solvent) of bovine BLG at neutral and basic pH is a complex irreversible process that is kinetically controlled [21,37,47,50–52] and involves dissociation of BLG dimer, unfolding and aggregation [21,31–33,53].

A principal reason for the irreversibility of bovine BLG denaturation is known to be intramolecular and intermolecular thiol–disulfide exchange reactions leading to an incorrect refolding and/or disulfide-mediated aggregation of the unfolded protein. The key role of the highly reactive free thiol group of Cys121 in these events has been highlighted [15,19,31,50]. The reactivity of this thiol is high enough to initiate the thiol-disulfide aggregation at acid pH, making the denaturation of bovine BLG under these conditions only partially reversible [15]. All these problems seem to be overcome by elimination of the free thiol from the structure of BLG. This could be achieved either by chemical or by genetic modification of Cys121 in bovine BLG [15,19,50,54]. A particular possibility is provided by nonruminant β-lactoglobulins lacking free thiols, such as porcine BLG.

The data on the renatured porcine BLG presented in Fig. 4 testify that thermal denaturation of this protein is a reversible process both at acidic and basic pH. This result points to the evident difference between the unfolding behaviour of porcine and bovine BLG. This difference is particularly pronounced at basic pH, where bovine BLG undergoes the multistage irreversible thermal transition [21,32]. Thus, the key role of the free thiol of bovine BLG in the hindering of its refolding is unambiguously confirmed.

At room temperature porcine BLG is presumably a monomer at neutral pH and tends to dimerize in acid medium [25]. However, a single symmetric denaturation peak of heat capacity is observed on the thermograms in the whole pH range studied (Fig. 1). The values of the denaturation enthalpy measured at different pH from 2 to 10 follow the same linear temperature dependence (Fig. 3). It means that the enthalpy of dimer dissociation of porcine BLG does not contribute to the calorimetric enthalpy. For comparison, the enthalpy of dimer dissociation of bovine BLG estimated from sedimentation equilibrium at pH 3 and 20 °C is of about 50 kJ per mol of dimers [55]. This value is no more than 6% of the denaturation enthalpy assuming the dimer fraction to be 100%. Note that this level of heat effects cannot be measured by modern DSC instruments. Thus, we can conclude that calorimetric parameters determined in this work at all pH values refer to the unfolding transition and characterize the stability of tertiary structure of porcine BLG.

Reversibility of the denaturation transition of porcine BLG allows one to analyse its profile in terms of thermodynamics. The simplest model of conformational transitions in proteins assumes a transition between two thermodynamically stable conformations; native and denatured. In Fig. 6 the experimental calorimetric curves for porcine BLG at pH 3.0 and pH 9.0 are shown together with their best fits according to the two-state model. The fact that the transition profile is well approximated by the two-state model implies that no broadening of the peak is detected because of different stability of variants A and C present in the preparation. Thus, we can conclude that porcine BLG

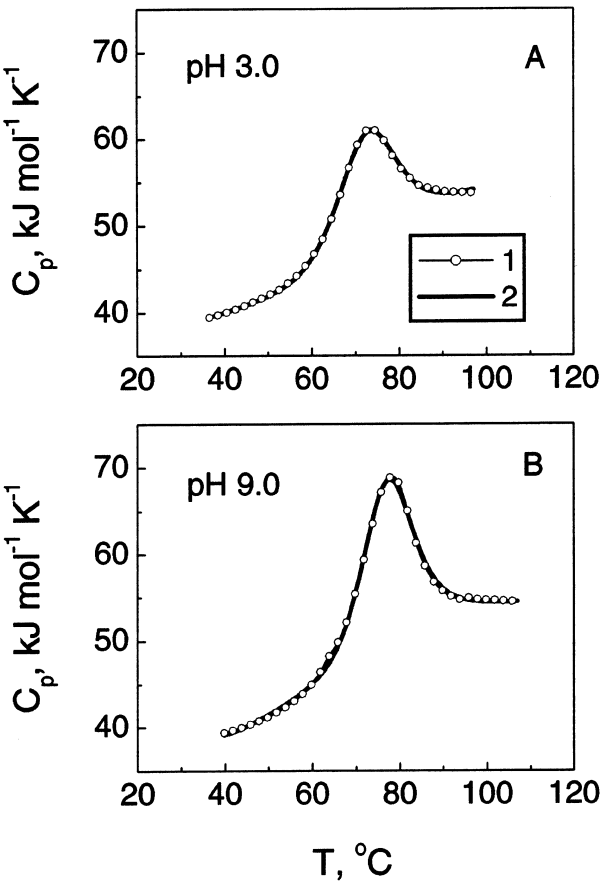


Fig. 6. Two-state approximation of the denaturation profile of porcine BLG at pH 3.0 (A) and pH 9.0 (B). 1, experimental; 2, the best-fit curve. The standard fit error is 1.4% and 1.9% of the maximal excess heat capacity for pH 3.0 and 9.0, respectively. A part of experimental points is omitted for more clarity of presentation.

isoforms do not differ substantially in the stability of their tertiary structure. In contrast, some differences of stability of bovine BLG variants A and B have been reported although the data are somewhat contradictory [12–15,48].

pH-dependence of the denaturation parameters of porcine BLG; comparison with bovine BLG

Figure 2 summarizes the thermodynamic denaturation parameters of porcine BLG, T_d, $\Delta_d H$ and $\Delta_d S$, at different pH. Corresponding parameters for bovine BLG, obtained in this work and extracted from the literature, are also plotted in Fig. 2 for comparison with those of porcine BLG.

We should note that the denaturation parameters for bovine BLG refer mostly to acidic pH, where its denaturation is partially reversible and not affected by dimer dissociation. At neutral and basic pH, the literature data for bovine BLG are represented mainly by values of the transition temperature obtained by HS-DSC at protein concentrations of 3–4 mg·mL^{-1} and heating rate of 1 K·min^{-1} (the same calorimetric conditions as we applied for porcine BLG).

As seen from Fig. 2A, the dependences T_d(pH) are notably different for porcine and bovine BLG. In the acidic

pH range, porcine BLG reveals a drastic decrease in the denaturation temperature while that of bovine BLG is less dependent on pH and remains relatively high even at extremely low pH. In the basic medium, the dependence T_d(pH) for porcine BLG is more gradual but that of bovine BLG shows a sharp drop.

Figure 2B,C depicts pH-dependences of the denaturation enthalpy and entropy of porcine and bovine BLG. At neutral and basic pH, the calorimetrically measured denaturation heat effect for bovine BLG includes (in addition to the heat of unfolding) an endothermic contribution of dimer dissociation and exothermic contribution of postdenaturation aggregation. Therefore, in this pH range the enthalpies for bovine BLG estimated by HS-DSC cannot be compared precisely with those for porcine BLG. Roughly, the unfolding enthalpy of bovine BLG is ≈ 300 kJ·mol^{-1} at pH 6.75–7.0 as derived from deconvolution of isothermal calorimetric curves [32] and about zero at pH 9.0 where no co-operative transition was observed for bovine BLG (Fig. 5A). It is seen from Fig. 2B that values of $\Delta_d H$ and $\Delta_d S$ for porcine BLG in the acid pH range are markedly lower than those for bovine BLG. Moreover, they are lower than those for other globular proteins so far investigated [56].

Porcine BLG is characterized by notably low values of the denaturation entropy, particularly at acid pH. One could assign this low entropy either to a relatively disordered folding of the protein or to some restrictions for configurational freedom in the denatured state. Our estimations for the denaturation heat capacity increment of porcine BLG give $\Delta_d C_p = 9.4 \pm 0.6$ kJ·mol^{-1}·K^{-1} as derived from the dependence $\Delta_d H(T_d)$ (Fig. 3). This value coincides with the literature data for bovine BLG B [12,15] but is somewhat higher then that reported for bovine BLG A [13,14]. A relatively high value of $\Delta_d C_p$ for porcine BLG indicates a high denaturation change in hydration of the protein groups, predominantly non-polar ones [56–58]. This result shows that the fold of porcine BLG is packed rather tightly. Consequently, the low denaturation entropy relates most likely to configurational restrictions in the denatured state of this protein. This point is of particular interest and needs additional experimental support.

In accordance with the thermodynamic data presented in Fig. 3 one could expect a cold denaturation transition of porcine BLG in aqueous solution at temperatures about 23 °C and higher depending on pH. However, calorimetric curves obtained within temperature range 10–110 °C show only one co-operative transition related to heat denaturation (Fig. 1). The question arises why the thermodynamic data derived from heat denaturation of porcine BLG are not supported by direct calorimetric observation of its cold denaturation? A definitive answer to this question is not possible without additional search of the experimental conditions needed for detection of low temperature transitions in porcine BLG. The observation of cold denaturation may be complicated due to existence of a high activation barrier of unfolding at low temperatures. Data available to date indicate rather high time constants of cold denaturation (up to several tens of minutes). It should be noted that direct calorimetric detection of protein cold denaturation was in most cases possible in the presence of denaturants [12,14,59,60]. The denaturants not only increase the temperature of cold denaturation but also lower the activation barrier. An experimental correlation between heat and cold denaturation in the absence of denaturants, as predicted by extrapolation of thermodynamic functions of heat denaturation, remains questionable [61]. Indeed, the prediction of cold transition at low temperatures based on the extrapolation of free energy functions determined at high temperatures is rather poor because of large errors, particularly for proteins with denaturation temperatures as high as 80 °C (such as porcine BLG). The extrapolation can be hindered also by temperature-sensitive rearrangements of protein quaternary structure. The cold denaturation of porcine BLG could be expected in acid medium where this protein is maximally destabilized. However, porcine BLG forms dimers at low temperatures in acid medium stabilized by additional hydrogen bonds [25,26]. For this reason the conditions of cold denaturation predicted for the monomeric form of porcine BLG can deviate from those for its dimer.

Thermodynamic model for pH-dependent stability of proteins; side-chain H-bonds

A general measure of pH-induced changes in the conformational stability of a protein can be the excess Gibbs free energy of denaturation, $\Delta_d G^E$:

$$\Delta_d G^E = \Delta_d G(\mathrm{pH}) - \Delta_d G(\mathrm{pH}_0) \quad (1)$$

where $\Delta_d G = \Delta_d H - T\Delta_d S$ and pH_0 is a reference pH.

As shown in Fig. 3, values of the denaturation enthalpy obtained for porcine BLG at different pH follow the same temperature dependence approximated by a straight line. Thus, one can consider that within the temperature range studied, $\Delta_d H$ is a linear function of temperature:

$$\Delta_d H = \Delta_d H(T_d) + \Delta_d C_p(T - T_d) \quad (2)$$

In this case, the temperature dependence of the denaturation free energy can be presented in the form:

$$\Delta_d G(T) = \Delta_d H(T_d)(1 - T/T_d) + \Delta_d C_p(T - T_d) - \Delta_d C_p T \ln(T/T_d) \quad (3)$$

where T_d is a function of pH.

The pH-dependence of T_d can be derived from Fig. 2A by a polynomial approximation of the experimental data (solid line for porcine BLG and dashed line for bovine BLG). This dependence in combination with Eqns (1–3) permits one to calculate the free energy of denaturation as a function of pH at some reference temperature. While calculating this function it was assumed $\Delta_d C_p = 9.4$ kJ·mol^{-1}·K^{-1} for porcine BLG (Fig. 3) and $\Delta_d C_p = 8.4$ kJ·mol^{-1}·K^{-1} for bovine BLG (the average value estimated from the literature data [12–15,21,32]). The reference pH was taken as pH 6.25, where both proteins have approximately the same denaturation temperature of 83.2 °C (Fig. 2A). The dependences $\Delta_d G^E(\mathrm{pH})/RT$ at this temperature calculated from experimental data for porcine and bovine BLG are shown in Fig. 7. They show that bovine BLG is more stable than porcine BLG at acid pH while porcine BLG is much more stable than bovine BLG at basic pH. This means that according to the stability data this nonruminant BLG does not agree with the proposed

function of β-lactoglobulins as transport proteins intended for protection of ligands under acid conditions in the stomach [1]. This is also supported by the absence of binding ability of porcine BLG to fatty acids [23,25].

Let us consider the dependences $\Delta_d G(\mathrm{pH})$ in more detail. One of the existing concepts assumes such dependences to be related to a pH-induced perturbation of intramolecular side-chain H-bonds, which contribute to the overall conformational stability of a protein [62]:

$$\Delta_d G(\mathrm{pH}) = \Delta_d G_{res} + \Delta_d G_H(\mathrm{pH}) \quad (4)$$

where $\Delta_d G_H(\mathrm{pH})$ is the pH-dependent free energy of denaturation and $\Delta_d G_{res}$ takes into account all other contributions to the denaturation free energy. The function $\Delta_d G_H(\mathrm{pH})$ is determined by parameters of H-bonds between side-chain groups capable of ionization:

$$\Delta_d G_H = -RT\sum_i \ln\left[1 - \frac{\exp(-\Delta_{HB}G_i/RT)}{1+\exp(-\Delta_{HB}G_i/RT) + 10^{(\mathrm{pH}-pK_{D,i})} + 10^{(-\mathrm{pH}+pK_{A,i})}}\right] \quad (5)$$

where $\Delta_{HB}G_i$ is the free energy of formation of the i-th H-bond, and $pK_{A,i}$ and $pK_{D,i}$ are the ionization parameters of hydrogen acceptor and donor for this bond. The summation is taken over all H-bonds formed by ionogenic side chains. Then one gets

$$\Delta_d G^E(\mathrm{pH}) = \Delta_d G_H(\mathrm{pH}) - \Delta_d G_H(\mathrm{pH}_0) \quad (6)$$

For applying Eqns (5) and (6) to the analysis of experimental dependences $\Delta_d G^E(\mathrm{pH})$ for porcine and bovine BLG an assumption should be made on the possible types and parameters of side-chain H-bonds in these proteins. In the case of bovine BLG it is possible to use data of crystallography [61], potentiometric titration [19] and computer modelling as a starting point for such analysis.

Thermodynamic analysis of side-chain H-bonding in bovine BLG

One of the key side-chain H-bonds in bovine BLG was identified by crystallography [7,63]. This is the H-bond between the carboxyl group of the residue Glu89 (proton donor) and the carbonyl oxygen of Ser116 (proton acceptor). This group is suggested to be an anomalous carboxyl group with $pK \approx 7.3$ as was found by Tanford *et al.* [17]. According to crystallographic data [63] the carboxyl group at residue Glu89 is buried at pH 6.2 but becomes more accessible at pH 8.2. These data are consistent with deprotonation of this group and breaking of the H-bond in this pH region. Thus, the crystallographic data provide an explanation for the so-called 'Tanford transition'; drastic structural changes occurring at pH 7.5–8.0 [16–18,63,64]. According to computer modelling, one more carboxyl group at residue Glu158 is presumed to be hydrogen bonded with carbonyl oxygen at residue Thr154. Most likely, this carboxyl group is the 'normal' one with $pK \approx 4.8$, as only one carboxyl group of bovine BLG was found to be titrated at pH 7.3, whereas all other carboxyl groups have pK 4.8 [17]. The crystallographic structure of this protein shows that one more carboxyl group at Glu108 has an accessibility as low as that at Glu89 [63]. The carboxylate ion of Glu108 appears to be hydrogen bonded with the α-amino group of the N-terminal residue Leu1. The accessibility of Glu108 does not change when pH increases from pH 6.2–8.2 [63]. This implies that this group does not contribute to the conformational changes related to the Tanford transition; however, it may be of importance for the overall stability of bovine BLG. Finally, the computer modelling identified two tyrosines, Tyr20 and Tyr102, located within ≈3.0 Å from carboxylate oxygens at residues Glu157 and Asp96, respectively. These residues could form side-chain H-bonds in which tyrosines serve as proton donors and carboxylate ions as proton acceptors.

Thus, guided by the above structural analysis, we can calculate a theoretical function $\Delta_d G^E(\mathrm{pH})$ for bovine BLG by Eqns (5) and (6) and fit it to the experimental dependence $\Delta_d G^E(\mathrm{pH})$ shown in Fig. 7. In doing so the following types of side-chain H-bonds have been taken into account: 'anomalous' COOH···O = C < (Type 1) and 'normal' COOH···O = C < (Type 2), where O is the carbonyl oxygen of a residue; $COO^-\cdots H_3N^+-$, where H_3N^+- is the N-terminal α-amino group (Type 3); and $COO^-\cdots H_3N^+-$ or $COO^-\cdots HO^-$, where H_3N^+- is the ε-amino group of Lys and HO^- is the hydroxyl group of Tyr (Type 4). The values of pK_A and pK_D for 'normal' groups were taken from potentiometric titration data for bovine BLG at 25 °C [17] and extrapolated to 83.2 °C by the van't Hoff equation using the known values of the ionization enthalpy [65]. The value of pK_{D1} at 83.2 °C for the H-bond involving the Tanford 'anomalous' carboxyl group was used as an adjustable parameter since the ionization enthalpy for this group may differ from that of the 'normal' carboxyl group. Another adjustable parameter was the average free energy of the H-bond formation, $\Delta_{HB}G$, that was assumed to be the same for all types of H-bonds.

The initial fitting of the function $\Delta_d G^E(\mathrm{pH})$ for bovine BLG according to Eqns (5) and (6) was performed taking the number and types of side-chain H-bonds from structural data for this protein. These are: $n_1 = 1$ (Type 1); $n_2 = 1$ (Type 2); $n_3 = 1$ (Type 3) and $n_4 = 2$ (Type 4). The result of the fitting is shown in Fig. 7A by a dashed line. It is seen that this approximation is poor: the stability of bovine BLG is highly underestimated at acid pH. As shown previously [66] the slope of the dependence $\Delta_d G^E(\mathrm{pH})$ in acid medium is proportional to the number of protons bound by a protein during its unfolding or, in other words, to the number of side-chain H-bonds involving carboxylate ions (Type 4) and disrupted during protein unfolding. For this reason we have performed the second fitting with $n_4 = 1$ (dotted line in Fig. 7A). It is seen that in this approximation the function $\Delta_d G^E$ at acid pH has increased but remains too low as compared with the experimental one. In the final fitting the parameter n_4 was adjusted. Additionally, the number of H-bonds of Type 2, n_2, was used as an adjustable parameter for better

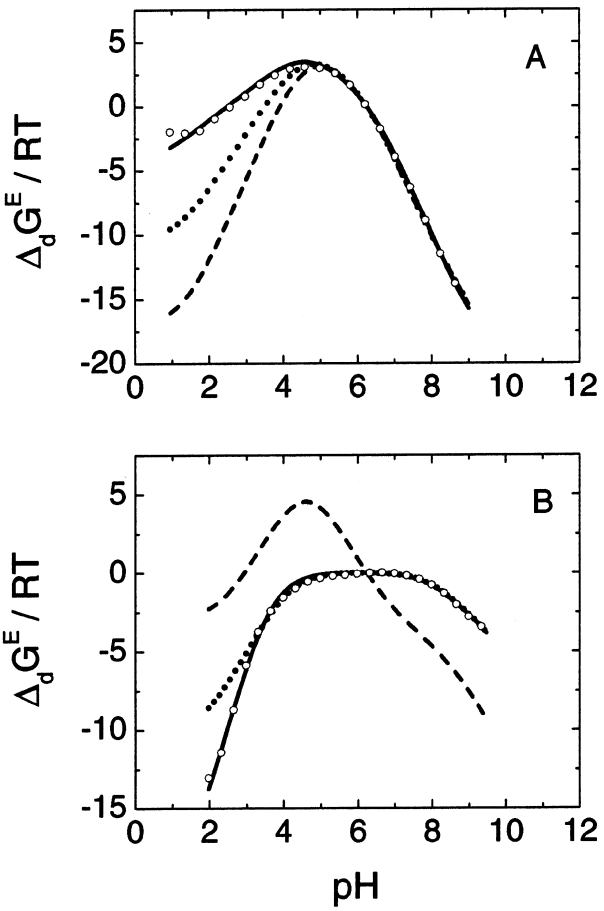


Fig. 7. pH-dependences of the excess free energy of denaturation for bovine (A) and porcine (B) BLG. Points: experimental; lines: obtained by fitting of Eqns (5) and (6) to the experimental data using the following parameters of the model: A: dashed line: $n_1 = 1$, $pK_{D1} = 6.1$, $pK_{A1} = 0$; $n_2 = 1$, $pK_{D2} = 4.6$, $pK_{A2} = 0$; $n_3 = 1$, $pK_{D3} = 6.2$, $pK_{A3} = 4.6$; $n_4 = 2$, $pK_{D4} = 9.2$, $pK_{A1} = 4.6$; dotted line: $n_1 = 1$, $pK_{D1} = 6.25$, $pK_{A1} = 0$; $n_2 = 1$, $pK_{D2} = 4.6$, $pK_{A2} = 0$; $n_3 = 1$, $pK_{D3} = 6.2$, $pK_{A3} = 4.6$; $n_4 = 1$, $pK_{D4} = 9.2$, $pK_{A1} = 4.6$; solid line: $n_1 = 1$, $pK_{D1} = 6.3$, $pK_{A1} = 0$; $n_2 = 0.9$, $pK_{D2} = 4.6$, $pK_{A2} = 0$; $n_3 = 1$, $pK_{D3} = 6.2$, $pK_{A3} = 4.6$; $n_4 = 0$, $pK_{D4} = 9.2$, $pK_{A1} = 4.6$ (see Table 1). B: dashed line: $n_1 = 1$, $pK_{D1} = 7.3$, $pK_{A1} = 0$; $n_2 = 2$, $pK_{D2} = 4.6$, $pK_{A2} = 0$; $n_3 = 0$, $pK_{D3} = 6.2$, $pK_{A3} = 4.6$; $n_4 = 2$, $pK_{D4} = 9.2$, $pK_{A1} = 4.6$; dotted line: $n_1 = 1$, $pK_{D1} = 7.9$, $pK_{D1} = 0$; $n_2 = 0$, $pK_{D2} = 4.6$, $pK_{D2} = 0$; $n_3 = 0$, $pK_{D3} = 6.2$, $pK_{A3} = 4.6$; $n_4 = 2$, $pK_{D4} = 10.1$, $pK_{A1} = 4.1$; solid line. $n_1 = 1$, $pK_{D1} = 7.9$, $pK_{A1} = 0$; $n_2 = 0$, $pK_{D2} = 4.6$, $pK_{A2} = 0$; $n_3 = 0$; $n_4 = 4.0$, $pK_{D4} = 10.3$, $pK_{A1} = 3.6$ (See Table 1). T = 83.2 °C.

verification of the predictions made by computer modelling. The result of the final fitting is shown in Fig. 7A by a solid line. The agreement between experimental dependence $\Delta_d G^E$(pH) and that calculated by Eqns (5) and (6) can be considered as satisfactory. The best fit parameters representing the characteristics of side-chain H-bonds for bovine BLG are summarized in Table 1.

As deduced from Table 1, three side-chain H-bonds are responsible for the pH-dependence of the stability of bovine BLG. Two of them (Type 1 and Type 2) involve carboxylate groups as proton donors. These bonds are stable at acid pH and dissociate at pH > pK_D, and provide a high stability of bovine BLG in acid medium. In this respect, our result is in agreement with the tertiary structure of bovine BLG as determined by crystallography and NMR [7,16,63]. The Type 4 H-bonding does not contribute to the stability of this protein ($n_4 = 0$). In fact, it is not surprising for the H-bond Glu157–Tyr20 because the residues are located at the surface of the protein molecule and thus are most likely hydrated [63]. The H-bond Asp96-Tyr102 is partially buried [63], but it could have a minor effect on the stability of bovine BLG. Thus, the increased stability of bovine BLG in acid medium is mainly related to the hydrogen bonding with participation of the carboxyl groups.

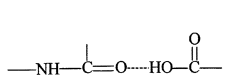
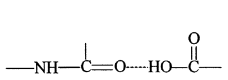
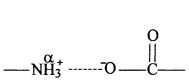
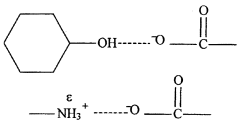
Thermodynamic analysis of side-chain H-bonding in porcine BLG

Let us now examine side-chain H-bonds contributing to the stability of porcine BLG. According to the crystallographic structure [26], the carboxyl group at Glu89 is buried and located within 2.43 Å from the carbonyl oxygen at Ser116, that points to a high probability of an H-bond. It means that the 'anomalous' character of this carboxyl group is most likely conserved in porcine BLG. Two more H-bonds of Type 2 involving carboxyl groups could be detected: Asp33–Asp88 and Asp96–Asp100 (the distances 2.66 Å and 2.44 Å). Then, carboxylate ions at Glu156 and Glu69 are candidates for H-bonding with Tyr42 and Lys60, respectively (2.69 Å and 2.57 Å). The N-terminal part of porcine BLG is substantially modified as compared with that of bovine BLG, thus providing no possibility for hydrogen bonding of Type 3 between the carboxylate ion and α-amino group like in bovine BLG.

The analysis of the experimental dependence $\Delta_d G^E$(pH) for porcine BLG was carried out using Eqns (5) and (6) in the same manner as for bovine BLG. In the first approximation we have used the number and types of H-bonds predicted by structural consideration, i.e. $n_1 = 1$, $n_2 = 2$, $n_3 = 0$, and $n_4 = 2$. The result is shown in Fig. 7B by a dashed line. It is seen that in this approximation the stability of the protein is overestimated at acid pH and underestimated at basic pH. As it was shown above for bovine BLG, the increased stability in acid medium arises from contribution of H-bonds involving carboxyl groups. In the second approximation we have reduced the number of H-bonds of Type 2 to zero, i.e. $n_2 = 0$ (dotted line in Fig. 7B). This allowed us to get a good approximation of the stability function in basic medium. However, the slope of the function $\Delta_d G^E$(pH) at acid pH is too low suggesting an underestimated number of H-bonds of Type 4. Finally, we have used n_2 and n_4 as adjusted parameters of fitting. Because no data of potentiometric titration are available for porcine BLG, the pK values of the proton donors and acceptors participating in H-bonding of Type 4 were also varied. The best-fit curve $\Delta_d G^E$(pH) obtained for porcine BLG is shown in Fig. 7B by a solid line and the corresponding parameters of side-chain H-bonds derived from the fitting are listed in Table 1.

First of all, the contribution of 'normal' carboxyl groups to H-bonding in porcine BLG is apparently negligible ($n_2 = 0$). On the other hand, the value $n_4 = 4$ is obtained for H-bonding with participation of Tyr and/or

Table 1. Parameters of the side-chain H-bonds estimated by approximation of the experimental dependences $\Delta_d G^E$(pH)/RT at T = 83.2 °C for bovine and porcine BLG according to Eqns (5) and (6).

	Type 1			Type 2			Type 3			Type 4			
													
BLG	n_1	pK_{D1}	pK_{A1}	n_2	pK_{D2}	pK_{A2}	n_3	pK_{D3}	pK_{A3}	n_4	pK_{D4}	pK_{A4}	$\Delta_{HB}G$, kJ mol^{-1}
Bovine	1[a]	6.3±0.1[b]	≈0	0.9±0.1[b]	4.6[c]	≈0	1	6.2[c]	4.6[c]	0.00±0.01[b]	9.2[c]	4.6[c]	−27.5±0.5[b]
Porcine	1[a]	7.9±0.1[b]	≈0	0.00±0.01[b]	4.6[c]	≈0	–	–	–	4.0±0.1[b]	10.3±0.1[b]	3.6±0.1[b]	−13.7±0.6[b]

[a] Data from crystallography [26,63] and computer modelling. [b] Obtained as the best-fit parameter set (see text). [c] Calculated according to the van't Hoff equation for $T = 83.2$ °C using data of potentiometric titration at 25 °C [17] and experimental values of the enthalpies of ionisation of the ionogenic groups [65].

Lys residues, which is higher than expected from the crystallographic data. The reason for this discrepancy is probably related to the conditions of crystallization of porcine BLG corresponding to pH 3 [26]. Calorimetric data show that at pH 3 the tertiary structure of this protein is substantially destabilized. As deduced from Table 1, the H-bonds of Type 4 in porcine BLG will be unstable at pH < 3.6 at 83.2 °C. Clearly, it cannot be excluded that at pH 3 some H-bonds of this type can dissociate also at low temperature and thus could not be detected by crystallography. The side-chain H-bonds with participation of Tyr and Lys residues may be the key interactions providing the increased stability of monomeric porcine BLG at basic pH.

It should be noted that our result is consistent with the existence in porcine BLG of an 'anomalous' carboxyl group similar to that found in bovine BLG [17]. According to our prediction, this group has pK 7.9 at 83.2 °C (Table 1). This value is higher than pK for the 'anomalous' carboxyl group in bovine BLG (pK 6.3). At present it is not possible to give a quantitative interpretation of these values, as they are affected both by temperature and hydrogen bonding. We can only predict the existence of an 'anomalous' carboxyl group in porcine BLG that is homologous to that in bovine BLG. If so, then the question arises: will deprotonation of this 'anomalous' carboxyl group lead to a structural transition in porcine BLG similar to the Tanford transition in bovine BLG? Our data on the stability of porcine BLG allow one to suggest that titration of the 'anomalous' carboxyl group in basic medium will have little effect on the overall structure of this protein. The structure of porcine BLG in basic medium is maintained by several side-chain H-bonds involving Tyr and Lys residues, which are stable up to pH 10.3 (Table 1). In bovine BLG the H-bonding of this type is absent (Table 1) and the most 'basic' proton donors at 25 °C are the 'anomalous' carboxyl group at Glu89 (pK 7.3) and the α-amino group of Leu1 (pK 7.4). This is why the titration of these groups is crucial for the tertiary structure of bovine BLG.

Recently it was shown that a dimeric form of porcine BLG is stabilized in acid medium [26]. According to crystallographic data, the side-chain H-bonding on the dimer interface of porcine BLG involves the carboxyl group of Glu9 of one monomer as proton donor and the carbonyl oxygen of Thr142 of another monomer as proton acceptor. We have shown that this type of H-bonding (Type 1 by the classification given in this work) is stable at acid pH. Two intramolecular H-bonds of this type are thought to be the key interactions providing an increased stability to the monomeric form of bovine BLG in acid medium (see Table 1). It seems that the same type of interaction could give a main contribution into stabilization of the porcine BLG dimers.

CONCLUSIONS

In contrast to bovine BLG, the thermal denaturation of porcine BLG is reversible in both acidic and basic pH. Apparently, it is related to the absence in this protein of free thiol groups, which can provoke a postdenaturation aggregation via thiol–disulfide exchange.

The existence in porcine BLG of an 'anomalous' carboxyl group similar to that found in bovine BLG can be predicted. However, deprotonation of this group will not lead to a conformational transition in porcine BLG similar to the Tanford transition in bovine BLG. The structure of porcine BLG in basic medium is maintained by several co-operative side-chain H-bonds involving Tyr and Lys residues, which are stable up to pH 10.0.

According to the calorimetric data, porcine BLG does not show high stability of tertiary structure at acid pH or drastic destabilization at weakly basic pH as bovine BLG does. This suggests that a low proteolytic stability of porcine BLG under acidic conditions is most likely, which is not consistent with the concept of a transporting function for this protein. This function dictates that a protein must first be capable of ligand binding, and second must retain the ligand-adapted native fold under the acidic conditions of the stomach. Neither of these properties is evident for porcine BLG.

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