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Thermodynamics of Mn^{2+}-binding to goat α-lactalbumin

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Abstract. By means of reaction calorimetry we measured the apparent enthalpy change, ΔH^{app}, of the binding of Mn^{2+}-ions to goat α-lactalbumin as a function of temperature. The observed ΔH^{app} can be written as the sum of contributions resulting from a conformational and a binding process. In combination with the thermal unfolding curve of goat α-lactalbumin, we succeeded in separating the complete set of thermodynamic parameters (ΔH, ΔG, ΔS, ΔC_p) into the binding and conformational contributions. By circular dichroism we showed that NH_4^+-ions, upon binding to bovine α-lactalbumin, induce the same conformational change as do Na^+ and K^+: the binding constant $K_{NH_4^+}^{app}$ equals $98 \pm 9\ M^{-1}$.

Key words: α-lactalbumin – Metal ion binding – Thermodynamics – Microcalorimetry

Introduction

All known α-lactalbumins of different species contain one high affinity Ca^{2+}-site and a second site which binds Zn^{2+}. The binding of different ion to bovine α-lactalbumin has been studied by spectroscopic and chromatographic techniques. A recent review article (Kronman 1989) summarizes this work and discusses still unresolved problems.

Above room temperature, the apo-form of α-lactalbumins is characterized in circular dichroism, by the absence of tertiary structure but conservation of secondary structure. However, upon cooling the apo-form to near 0 °C the band of the tertiary structure appears (Segawa and Sugai 1983). Binding of monovalent or divalent ions also restores the native structure with a drastic increase in the thermal stability which depends on the ion concentration (Hiraoka and Sugai 1984). By means of van't Hoff measurements, the enthalpy and heat capacity changes between the native and unfolded states were determined in the presence and absence of sodium (Hiraoka and Sugai 1984) and calcium (Kuwajima et al. 1986). It was demonstrated that the stability of the folded state is markedly enhanced by these ions and that this is almost entirely an entropic effect (Kuwajima et al. 1986).

Our laboratory approaches the binding of cations to apo-α-lactalbumin by measuring the enthalpy changes of the binding by batch microcalorimetry (Van Ceunebroeck et al. 1985; Desmet et al. 1987; Desmet and Van Cauwelaert 1988). As for the stability problem, we try to resolve the net thermodynamic contribution of the binding phenomenon to the overall stability. Although from the calorimetric measurements the enthalpy as well as the free energy change of the binding process can be measured, this technique suffers from the fact that binding (dehydration + electrostatic) and conformational contributions are measured together. Goat-α-lactalbumin turned out to be a convenient protein to resolve both contributions. Indeed, in the absence of metal ions, goat apo-α-lactalbumin refolds to the native-like state with a transition temperature of 25 °C (Segawa and Sugai 1983) so that practically the whole folding process takes place within the working range of our microcalorimeter (10–45 °C). Thus at low temperature, the apo-form is in the N-like state and one essentially measures the net heat of binding, while at high temperatures, both binding and conformational heats are observed. For the binding of Mn^{2+} to goat α-lactalbumin we have succeeded in separating the complete set of thermodynamic parameters (ΔH, ΔG, ΔS, ΔC_p) into binding and conformational contributions. Mn^{2+} has been chosen instead of Ca^{2+} for this work: indeed, although the ΔH-values for Ca^{2+} binding are more exothermic by 55 kJ mol^{-1} and therefore more precise, the breakpoint of the titration curve is so sharp that no accurate values of $K_{Ca^{2+}}^{app}$ can be calculated. Binding of Mn^{2+} also gives easily measurable values of ΔH but, as shown previously, $K_{Mn^{2+}}^{app}$ can be easily calculated from the titration curve.

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Abbreviations: BLA, bovine α-lactalbumin; GLA, goat α-lactalbumin; HLA, human α-lactalbumin; CD, circular dichroism

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Materials and methods

GLA was prepared from fresh milk. After elimination of the fat by centrifugation and the caseins by Na_2SO_4 precipitation, the whey was dialyzed exhaustively and lyophilized. The proteins were dissolved in *Tris* 50 mM pH 7.5, EDTA 10 mM and applied to a phenyl-Sepharose column (Lindahl and Vogel 1984). Apo-GLA binds to the column in these circumstances while the whey proteins are eluted with *Tris* 50 mM, 1 mM EDTA pH 7.5. To elute the GLA fraction, the eluent is changed to *Tris* 50 mM, 1 mM Ca^{2+}, pH 7.5. BLA was purchased from Sigma. The apo-form of BLA and GLA was prepared as previously (Desmet and Van Cauwelaert 1988) according to the method of Hiraoka and Sugai (1984), except that we omitted the guanidinium hydrochloride. Since the eluting buffer contains 5 mM NH_4HCO_3, the lyophilization time has to be strictly controlled. If the time is too short, NH_4^+-ions remain in the dry sample (they behave like an alkali metal and therefore influence the conformational change). If the time is too long, some of the protein may denature. Therefore, an alternative method was used. α-Lactalbumin was purified on the Sephadex G-100 column in its Ca^{2+}-form in NH_4HCO_3 solution. Lyophylization has no influence on the Ca^{2+}-form which can be stored for a long time. Before use, the protein was demetallized as above on a Sephadex G-100 column in *Tris* buffer 10 mM or any other buffer and stored in solution.

$MnCl_2$ was a gold label product from Aldrich. Batch micro-calorimetric titrations were carried out as described previously in detail (Desmet and Van Cauwelaert 1988). In a typical experiment, 5 ml apo-α-lactalbumin, 2 mg · ml^{-1}, were titrated with 152 µl, 6 mM, of the cation, both in the appropriate buffer. All the experimental data have been fitted to the equations quoted in the text using the procedure of Bevington (1969) as described earlier (Desmet et al. 1987).

Results

A. Binding of NH_4^+-ions to α-lactalbumin

It has been stressed earlier (Kronman 1989; Desmet et al. 1987) that incomplete removal of monovalent cations during the preparation of a protein sample may lead to erroneous data. For instance, since the enthalpy change of the binding of a cation to α-lactalbumin is largely due to the conformational change from the unfolded apo-form (A) to the native (N)-state, its value is very sensitive to the fraction of the protein in the apo-form at the start of a calorimetric titration and, hence, also to the presence of cations. Samples prepared in an NH_4HCO_3-buffer and lyophylised for too short a period (less than one day), showed an appreciably decreased exothermicity in their heat of binding to cations (results not shown).

Therefore, we determined $K_{NH_4^+}^{app}$, the apparent binding constant of NH_4^+-ions to apo-BLA at 25 °C by means of CD at 270 nm. The residual ellipticity as a function of the NH_4^+-concentration is shown in Fig. 1. From this curve,

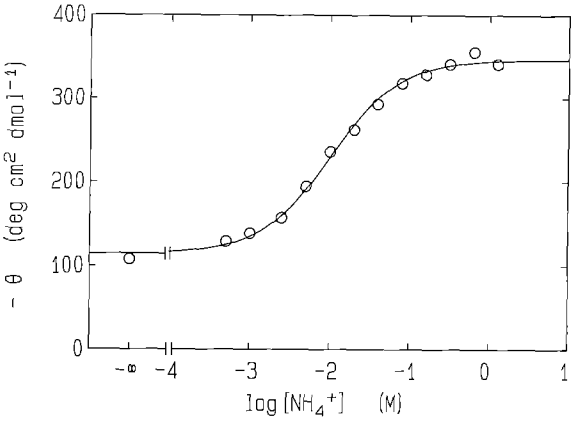

Fig. 1. The residual ellipticity of bovine α-lactalbumin at 270 nm as a function of the NH_4^+-concentration. The protein concentration was 26 µM and the temperature was 25 °C. The data points were fitted to the expression for metal binding to one single site and this yielded an apparent binding constant, $K_{NH_4^+}^{app}$ of 98 ± 9 M^{-1}. Note that the signal at saturating concentrations of NH_4^+ is characteristic of BLA in its native state

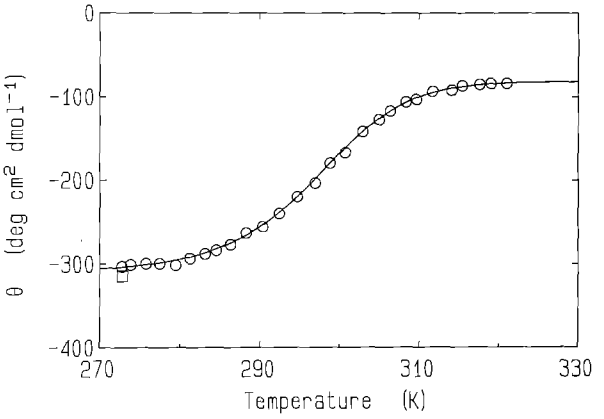

Fig. 2. Thermal unfolding of goat α-lactalbumin monitored by the CD-signal at 272 nm. [GLA] = 35 µM, 10 mM *Tris* pH 7.5. o: apo-GLA, □: Ca^{2+}-GLA ([Ca^{2+}] = 0.5 mM). The curve was fitted as explained in the text

$K_{NH_4^+}^{app} = 98 \pm 9\ M^{-1}$ is calculated. This value is of the same order of magnitude as $K_{Na^+}^{app}$ and $K_{K^+}^{app}$ (Hiraoka and Sugai 1985). Since NH_4^+ is known in inorganic chemistry as a pseudo-alkali metal ion, it is logical to assume that NH_4^+ also binds in a competitive way to the Ca^{2+}-site. The suggestion by Kronman (1989) that one should work in an NH_4^+-buffer to avoid the presence of other monovalent cations should therefore not be followed.

B. Thermal unfolding of goat α-lactalbumin

The thermal unfolding curve of GLA has been obtained using the CD signal at 272 nm, which is indicative of the protein tertiary structure (Fig. 2). Therefore, the ellipticity values can be fitted to the expression:

$$\theta(T) = \theta_U + (\theta_N - \theta_U) f_N(T) \quad (1)$$

It is assumed that θ_U and θ_N, the values for the unfolded apo-form and folded protein respectively, are constant over the entire unfolding region. $f_N(T)$ denotes the fraction of protein in the folded, native state at a temperature T, and its temperature dependence can be written as follows:

$$f_N(T) = 1/(1+K_U(T)) \tag{2}$$

with

$$K_U(T) = \exp(-\Delta G_U(T)/RT) \tag{3}$$

and

$$\Delta G_U(T) = \Delta H_U(T^\circ) - T\,\Delta S_U(T^\circ) + \Delta C_{p,U}\left(T - T^\circ - T\ln\frac{T}{T^\circ}\right) \tag{4}$$

$\Delta H_U(T^\circ)$ and $\Delta S_U(T^\circ)$ are the van't Hoff standard enthalpy and entropy changes at a reference temperature T°. T° has been chosen as 298.15 K. $\Delta C_{p,U}$ is the difference in molar heat capacity between the folded and unfolded state. During the fit, θ_N has been kept fixed at -314 deg cm^2 dmol^{-1}, the value which was experimentally obtained for Ca^{2+}-GLA (Fig. 2).

The four-parameter fit yielded the following values: $\theta_U = -81 \pm 2$ deg cm^2 dmol^{-1}, $\Delta H_U(T^\circ) = 127 \pm 3$ kJ mol^{-1}, $\Delta S_U(T^\circ) = 0.43 \pm 0.01$ kJ mol^{-1} K^{-1}, $\Delta C_{p,U} = 3.1 \pm 0.3$ kJ mol^{-1} K^{-1}. The curve itself coincided nearly perfectly with the one published by Segawa and Sugai (1983), although the latter was obtained in slightly different conditions. The values that we found after digitizing and fitting their data were about 10–15% larger than ours. These thermodynamic unfolding data obtained above are collected in Table 1, but for comparison with calorimetric experiments, they are written for the reverse process, so that the sign becomes negative.

C. Calorimetric experiments

We have measured, by means of reaction calorimetry, the "apparent" enthalpy change $\Delta H^{app}_{Mn^{2+}}$ for the addition of Mn^{2+}-ions to GLA as a function of temperature (Fig. 3). "Apparent" means that the values for $\Delta H^{app}_{Mn^{2+}}(T)$ must not be interpreted as the heat of binding of Mn^{2+} to fully folded or unfolded apo-GLA. Indeed, for apo-GLA, an equilibrium exists between the unfolded (U) and the folded N-like state:

$$\text{apo-}U\text{-GLA} \overset{K_c}{\rightleftharpoons} \text{apo-}N\text{-GLA} \tag{5}$$

K_c is the equilibrium constant for the conformational change from the U- to the N-state. Furthermore, evidence exists that metal ions only bind to the N-state (Segawa and Sugai 1983), so that one can write:

$$Mn^{2+} + \text{apo-}N\text{-GLA} \overset{K_{b,Mn^{2+}}}{\rightleftharpoons} Mn^{2+} \cdot N\text{-GLA} \tag{6}$$

where $K_{b,Mn^{2+}}$ is the "true" binding constant of Mn^{2+} to folded N-GLA. Both equilibria are characterized by their own thermodynamic parameters: ΔG_c, ΔH_c, ΔS_c, $\Delta C_{p,c}$ and ΔG_b, ΔH_b, ΔS_b and $\Delta C_{p,b}$ for which we could derive values from the data presented in Figs. 3 and 4.

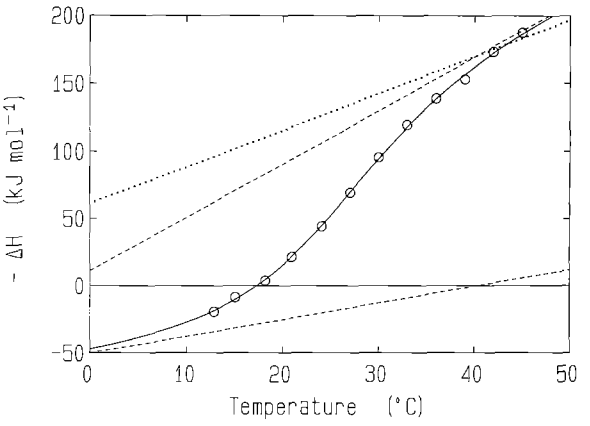

Fig. 3. The apparent enthalpy change for Mn^{2+}-binding to GLA, $\Delta H^{app}_{Mn^{2+}}$, obtained by batch microcalorimetry, as a function of temperature, *Tris* 10 mM pH 7.5. The dashed line touching the $\Delta H^{app}_{Mn^{2+}}$-curve at low temperatures is $\Delta H_{b,Mn^{2+}}$. The tangent at high temperatures is $\Delta H_{b,Mn^{2+}} + \Delta H_c$. The dotted line is ΔH_c. The error bars on the data points (± 5 kJ mol^{-1}) are smaller than the size of the symbols

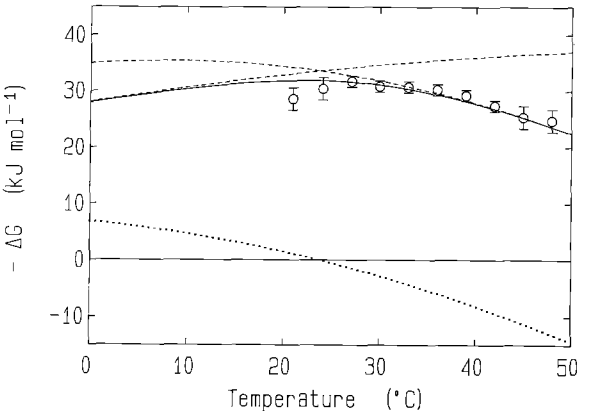

Fig. 4. The apparent free energy change for Mn^{2+}-binding to GLA, $\Delta G^{app}_{Mn^{2+}}$ obtained from the calorimetric titration curve at each temperature. Data below 20 °C could not be reliably calculated because of the small calorimetric signal. The line starting as a tangent to the $\Delta G^{app}_{Mn^{2+}}$-curve at low temperatures is $\Delta G_{b,Mn^{2+}}$. The line starting tangentially at high temperatures is $\Delta G_{b,Mn^{2+}} + \Delta G_c$. The dotted line is ΔG_c as obtained from the thermal unfolding curve of GLA (Fig. 2)

The observed, apparent enthalpy change for the saturation of apo-GLA with Mn^{2+} at the temperature T can be written as the sum of a conformational and a binding process. Using the constant ΔC_p-model, both are a linear function of temperature:

$$\Delta H^{app}_{Mn^{2+}}(T) = \Delta H_b(T^\circ) + (T - T^\circ)\,\Delta C_{p,b} + [1 - f_N(T)]\,[\Delta H_c(T^\circ) + (T - T^\circ)\,\Delta C_{p,c}] \tag{7}$$

The data in Fig. 3 were fitted to this equation, where for $f_N(T)$, the results of the thermal unfolding experiment were used. In essence, it all adds up to the search for the two tangents to the curve at low and high temperatures respectively. The former one represents the enthalpy change for the binding of Mn^{2+} to N-GLA, $\Delta H_{b,Mn^{2+}}(T)$ (reaction 6), while the latter one includes both the binding

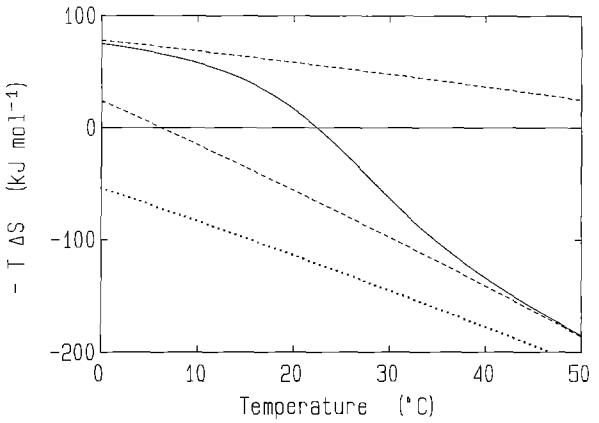

Fig. 5. Entropy changes for Mn^{2+}-binding to GLA as a function of temperature. The full line is $\Delta S^{app}_{Mn^{2+}}$. The tangent to this curve at low temperatures is $\Delta S_{b,Mn^{2+}}$. The tangent at high temperatures is $\Delta S_{b,Mn^{2+}} + \Delta S_c$. The dotted line is ΔS_c

and conformational heat, $\Delta H_{b,Mn^{2+}}(T) + \Delta H_c(T)$ (reactions 5+6). At $T° = 25°C$, we found $\Delta H_{b,Mn^{2+}}(T°) = +19 \pm 7$ kJ mol^{-1} (endothermic!) and $\Delta H_c(T°) = -129 \pm 12$ kJ mol^{-1}. Note that the latter calorimetric value agrees very well with the value found earlier for the van't Hoff enthalpy for the opposite, unfolding process, $\Delta H_U(T°) = 127 \pm 3$ kJ mol^{-1}. From the slopes of these two curves, we calculated the following values: $\Delta C_{p,b,Mn^{2+}} = -1.2 \pm 0.5$ kJ mol^{-1} and $\Delta C_{p,c} = -2.7 \pm 0.3$ kJ mol^{-1} K^{-1}. Again within the limits of error, $\Delta C_{p,c}$ is in good agreement with the value from the unfolding experiment $\Delta C_{p,U} = 3.1 \pm 0.3$ kJ mol^{-1} K^{-1}.

It is possible to derive, directly from the calorimetric titration curves, values for the apparent binding constant of Mn^{2+} to apo-GLA, $K^{app}_{Mn^{2+}}(T)$. Using an expression analogous to Eq. (3), one can thus obtain the apparent changes in Gibbs free energy for the binding of Mn^{2+} to apo-GLA as a function of temperature $\Delta G^{app}_{Mn^{2+}}(T)$. Its relationship to the binding and conformational contribution can be found as follows:

$$K^{app}_{Mn^{2+}} = K_c K_{b,Mn^{2+}}/(1 + K_c) \quad (8)$$

$$\Delta G^{app}_{Mn^{2+}} = \Delta G_c + \Delta G_{b,Mn^{2+}} + RT \ln (1 + \exp(-\Delta G_c/RT)) \quad (9)$$

All variables are temperature dependent. Both $f_N(T)$ and $\Delta G_c(T)$ are known from the thermal unfolding experiment. $\Delta G_{b,Mn^{2+}}(T)$ can be written as a function of enthalpy and entropy changes:

$$\Delta G_{b,Mn^{2+}}(T) = \Delta H_{b,Mn^{2+}}(T°) + \Delta C_{p,b,Mn^{2+}}(T - T°) - T[\Delta S_{b,Mn^{2+}}(T°) + \Delta C_{p,b,Mn^{2+}} \ln (T/T°)] \quad (10)$$

The experimental data, presented in Fig. 4 were fitted to the combined expressions 9 and 10. The only adjustable parameter was $\Delta S_{b,Mn^{2+}}(T°)$ and its value was determined as $\Delta S_{b,Mn^{2+}}(T°) = 0.18 \pm 0.03$ kJ mol^{-1} K^{-1}. Filling in the value for $\Delta S_{b,Mn^{2+}}(T°)$ in (10) gave $\Delta G_{b,Mn^{2+}}(T°) = -34 \pm 2$ kJ mol^{-1}.

Finally, to complete the set of data, we derived $\Delta S_c(T°)$ from the known values for $\Delta G_c(T°)$ and $\Delta H_c(T°)$: $\Delta S_c(T°) = -0.43 \pm 0.04$ kJ mol^{-1} K^{-1}, not unexpectedly, a rather large, negative value, remarkably well compensated by the large, exothermic conformational enthalpy change.

Table 1. Thermodynamic data for the folding reaction of goat α-lactalbumin and for the binding of Mn^{2+} to the folded protein at the reference temperature, $T° = 25°C$. The data were obtained by applying the fitting procedures as explained in the text

	$\Delta G(T°)$ (kJ mol^{-1})	$\Delta H(T°)$ (kJ mol^{-1})	$\Delta S(T°)$ (kJ mol^{-1} K^{-1})	ΔC_p (kJ mol^{-1} K^{-1})
Conform.[a]	0.5 ± 0.1	-127 ± 3	-0.43 ± 0.01	-3.1 ± 0.3
Conform.[b]	0.5 ± 0.1[a]	-129 ± 12	-0.43 ± 0.04	-2.7 ± 0.3
Binding[b]	-34 ± 2	19 ± 7	0.18 ± 0.03	-1.3 ± 0.5

[a] Data are derived from thermal unfolding experiment with apo-GLA in the absence of metal ions
[b] Data are derived from calorimetric experiments

All results at the reference temperature $T° = 25°C$ are summarized in Table 1 and the temperature dependence of all thermodynamic parameters is drawn in Figs. 3, 4 and 5.

Discussion

Before starting the discussion and interpretation of the thermodynamic data, two points must be underlined. First, we have demonstrated experimentally that the use of NH_4^+ ions in buffer solutions must be avoided since a CD-titration of apo-α-lactalbumin with NH_4^+-ions showed that NH_4^+-ions, just like Na^+ and K^+, also induce a conformational change from the *U*-state to the *N*-state. This is further supported by the fact that a calorimetric titration of apo-BLA with Ca^{2+} produced a smaller exothermic effect when the protein sample, which was prepared by lyophilization from a NH_4HCO_3 buffered solution, was not lyophilized for long enough.

Secondly, we are convinced that Mn^{2+}-ions bind to the Ca^{2+}-site on apo-bovine or apo-goat α-lactalbumin. In the literature on α-lactalbumin, there is not full agreement on this problem. The pro and contra arguments are extensively reviewed by Kronman (1989). On the basis of his data and those of Gerken (1984), Kronman proposes that the potentially coordinating atoms are the carboxylate oxygens of Glu 1, 7 and 11, the peptide carbonyls of Gln 2, Thr 4 and Asp 83 and the hydroxyl of Thr 4. We observed by batch microcalorimetric titrations, that competitive binding occurs on BLA between Ca^{2+} and Mn^{2+} (Desmet and Van Cauwelaert 1988). Furthermore, we showed (Desmet et al. 1989) that Mn^{2+} also binds to equine lysozyme, which contains an identical Ca^{2+}-binding site to that on BLA, but lacks the Glu-coordinating groups, proposed for the Mn^{2+}-site.

By CD, it can be demonstrated that titration of apo-GLA and apo-HLA with Mn^{2+} induces spectroscopically the same conformational transition as Ca^{2+} does (data not shown). This is remarkable since in GLA Glu 11 is replaced by Lys 11 and in HLA Glu 1 and Glu 7 are replaced by Lys 1 and Leu 7. If the Mn^{2+}-site indeed includes the negatively charged carboxylate oxygens, then

these replacements would eliminate two strong binding ligands from the site. A further argument for a same binding site for Mn^{2+} and Ca^{2+} is the fact that the difference between $\Delta H_{Ca^{2+}}$ and $\Delta H_{Mn^{2+}}$, as measured by batch microcalorimetry, is -55 kJ mol^{-1} for the binding of both ions to BLA, GLA, HLA and equine lysozyme, which strongly suggests that the same ligands are involved.

In conclusion, we believe, as was also stressed by Murakami et al. (1982), but on less firm grounds, that Mn^{2+} binds to the Ca^{2+}-elbow, defined by X-ray cristallography by Stuart et al. (1986).

Thermodynamic data are often hard to interpret on a submolecular basis. This is mainly due to the fact that the values obtained include contributions from numerous altering interactions, especially in the case of macromolecules changing their conformation. Then, comparison with model systems (such as EDTA for metal binding sites, hydrocarbons for hydrophobic interactions, site directed mutagenesis, etc.) sometimes makes it possible to resolve the data into contributions from different types of interactions.

In the present study, the choice of Mn^{2+}-binding to goat α-lactalbumin has enabled us to separate the measured, apparent thermodynamic parameters into binding and conformational contributions. Since our main goal is to try to understand the mechanism by which metal binding can increase a protein's thermal and proteolytic stability, we thought it was of the utmost importance to know the exact share of the binding in the overall thermodynamic data.

Earlier, we predicted the heat of binding of Ca^{2+} to bovine α-lactalbumin, $\Delta H_{b,Ca^{2+},BLA}$, to lie around -30 kJ mol^{-1}, at 25 °C (Desmet et al. 1987). Since, for GLA, the difference between ΔH_b for Ca^{2+} and Mn^{2+} is also about -55 kJ mol^{-1} (data not shown) as in the case of the bovine protein (Desmet et al. 1989), then, we find a value for the heat of binding of Ca^{2+} to GLA, $\Delta H_{b,Ca^{2+},GLA}$, of about -35 kJ mol^{-1}, very close to what we had expected earlier for BLA (Desmet et al. 1987).

The rather "bad", endothermic binding of Mn^{2+}-ions to the Ca^{2+}-site of α-lactalbumins has been explained in terms of a rigid site, unable to collapse around ions, smaller than which it is "designed" for (Desmet et al. 1988). In this case, one would expect for the entropy of binding of Mn^{2+} to GLA, $\Delta S_{b,Mn^{2+},GLA}$, a value comparable to the entropy change for Mn^{2+}-binding to EDTA, i.e. 0.20 kJ mol^{-1} K^{-1} (Martell and Smith 1974). Our value for $\Delta S_{b,Mn^{2+},GLA}$ of 0.18 kJ mol^{-1} K^{-1} is indeed only slightly lower. It richly compensates for the unfavourable endothermicity of the binding and it illustrates the strong entropic driving force of metal ion binding.

In (5), we assumed that metal ions, binding to folded, native α-lactalbumin, do not induce further conformational changes. The evidence for this came from the indistinguishable spectroscopic properties of metal-free and metal-bound N-GLA (Fig. 2). However, for the change in heat capacity upon Mn^{2+}-binding to GLA, $\Delta C_{p,b,Mn^{2+}}$, the obtained, negative value of -1.3 kJ mol^{-1} K^{-1} is surprisingly large compared to the conformational part of the change in heat capacity, $\Delta C_{p,c} = -2.7$ kJ mol^{-1} K^{-1} (calorimetric) to -3.1 kJ mol^{-1} K^{-1} (van't Hoff value). This suggests that the bound metal ion further chains up the protein structure, reducing the number of internal degrees of freedom in the vicinity of the metal site.

For the conformational part of the enthalpy change upon unfolding of GLA, $\Delta H_{U,GLA}$, we have obtained both a van't Hoff and a calorimetric value (see Table 1), which agree well with each other. These values can be compared with the data in the literature for unfolding of BLA (Pfeil 1981: $\Delta H_{U,BLA}$ (25 °C) = 130 ± 17 kJ mol^{-1}; Kuwajima et al. 1986: $\Delta H_{U,BLA}$ (25 °C) = 159 kJ mol^{-1}; Hiraoka and Sugai 1984: $\Delta H_{U,BLA}$ (25 °C) = 187 ± 18 kJ mol^{-1}). However, an attempt to correlate the observed differences in unfolding enthalpy between BLA and GLA with differences in primary sequence, would be complicated by the fact that the data in the literature are not accurate enough for this purpose and always contain contributions from the binding of the metal ion (either Na^+ or Ca^{2+}).

The thermal unfolding curves of BLA and GLA (Segawa and Sugai 1983, Fig. 2) clearly demonstrate the highest thermal stability for the latter protein. In the apo-form, their transition temperatures are 15 °C and 25 °C for BLA and GLA respectively and the differences in free energy change for unfolding, $\Delta\Delta G_U$, amounts to 7 kJ mol^{-1}.

Engineering a protein molecule in order to stabilize its tertiary structure is in most cases accomplished by improving intramolecular interactions (BLA and GLA could be regarded as a natural example of this). Still, the above results suggest that strategic introduction of one or more well-designed metal binding sites could be a lucrative alternative. The design and creation of a Ca^{2+}-site in lysozyme is a nice example of this (Kuroki et al. 1989). Our results show that metal binding sites have to be carefully designed for one particular metal ion because of the stringent radius effect. Yet, in this case, the binding may be expected to be enthalpically favourable (slightly exothermic), while the entropic driving force is bound to improve the strength of binding (to an extent, proportional to the hydration of the free metal ion). Finally, an important advantage of stabilization by creation of a metal binding site, is the fact that protein stability becomes strongly dependent on the free concentration of the metal ion (see for example Hiraoka and Sugai 1984). Increasing the metal concentration increases the protein's transition temperature and this way, one can in fact make a protein "as stable as desired".

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