Thermodynamics of the binding of calcium and strontium to bovine α-lactalbumin

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Microcalorimetry and equilibrium gel filtration were used to determine the thermodynamic functions $\Delta H°$, $\Delta G°$ and $\Delta S°$ guiding the interaction of Ca^{2+} and Sr^{2+} with bovine α-lactalbumin. Two methods of nearly complete metal removal from the protein gave identical results. The single Ca- and Sr-binding site, which has moderate affinity for these ions ($K_{Ca} = 2.5 \times 10^6\ M^{-1}$ and $K_{Sr} = 5.1 \times 10^5\ M^{-1}$), displays unusually large enthalpy changes of $-118\ kJ \cdot mol^{-1}$ for Ca^{2+} and $-75\ kJ \cdot mol^{-1}$ for Sr^{2+}. The concomitant reaction entropies equal -273 and $-142\ J \cdot K^{-1} \cdot mol^{-1}$, respectively.

Microcalorimetry *α-Lactalbumin* *Ca^{2+} binding* *Sr^{2+} binding*

1. INTRODUCTION

Bovine α-lactalbumin (BLA), the non-catalytic subunit of the lactose synthetase enzyme complex, contains one calcium-binding site [1] with an affinity constant of $2.5 \times 10^6\ M^{-1}$ [2]. In contrast with intracellular Ca-binding proteins which serve as sensors of Ca^{2+}-transients and interact dynamically with the ion, calcium in extracellular proteins such as BLA merely serves a structural role and does not readily dissociate from the protein under physiological conditions. Removal of Ca^{2+} from BLA produces a conformational change closely resembling the one that occurs upon acid or heat denaturation [3], i.e. a two-state transition from the native-like state (N) to a partially destabilized intermediate (A or T form). This A or T form is characterized by a disorganized tertiary structure, as monitored by circular dichroism of the aromatic residues, but a compact shape and native-like secondary structure [1,4,5]. Complete unfolding (D state) occurs only in the presence of guanidine hydrochloride [1]. Based on the antagonistic effects of Ca^{2+} and heat on the N to A transition, Hiraoka and Sugai [6] suggested that the heat-induced transition involves dissociation of Ca^{2+} from the protein. From indirect measurements with guanidine HCl and heat, Pfeil [7] determined a standard enthalpy change of 122–130 $kJ \cdot mol^{-1}$ for the N to A transition in 40 mM imidazole HCl, pH 6.3, at 25°C. In this study, we used flow-microcalorimetry at 25°C to study the enthalpy change of binding of Ca^{2+} (ionic radius 0.99 Å) and the closely related Sr^{2+} (1.12 Å) to metal-free BLA. The decrease in enthalpy is unusually large for the two ions when compared to those occurring upon Ca-binding to the regulatory proteins calmodulin ([8]; unpublished), troponin C [9] and parvalbumin [10,11], which have comparable affinities for Ca^{2+}.

2. MATERIALS AND METHODS

BLA from Sigma contains 0.2–0.3 gion Ca^{2+} per mol protein, and was electrophoretically pure.

2.1. *Preparation of metal-free BLA*

Since treatment of BLA with Ca^{2+}-chelators may leave traces of the latter bound to the protein and disturb the binding parameters [12], two methods for metal removal were used in a comparative way.

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2.1.1. Ca-removal in the presence of EDTA

To a solution of BLA in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, solid ammonium sulfate was added to 95% final saturation and the pellet collected after centrifugation for 20 min at $50000 \times g$. The procedure was repeated once with EGTA. The pellet was dissolved in 20 mM Tris-HCl, pH 7.5, and equilibrated in the latter buffer by gel filtration on Sephadex G-25 (1×40 cm).

2.1.2. Ca-removal with HCl

A solution of BLA was adjusted to pH 1.7 with HCl suprapure and passed through a column (1×40 cm) of Sephadex G-25 equilibrated with 12 mM HCl. After lyophilisation, the sample was equilibrated in 20 mM Tris-HCl, pH 7.5, by gel filtration as described above. The protein obtained by the two methods contained 0.025 gion Ca^{2+} per mol protein.

2.2. *Metal and protein determination*

Protein concentration was determined by absorbance spectrophotometry at 280.4 nm, using a specific extinction coefficient of 2.01 $mg^{-1} \cdot ml \cdot cm^{-1}$ [13]. Ca^{2+} and Sr^{2+} concentrations were measured using a Perkin Elmer atomic absorption spectrophotometer model 2380 using titrisol standards.

2.3. *Proton release*

Mixing an unbuffered solution of $CaCl_2$ and of Ca-free BLA (0.1 mM), both carefully adjusted to pH 7.5 and kept under nitrogen atmosphere, displays a decrease in pH of 0.03 units ± 0.01, corresponding to a release of 0.003 gion H^+ per mol BLA. In a similar experiment with Sr^{2+} the decrease in pH was 0.01 units ± 0.01. This experiment, and the fact that the equilibrium constant is independent of the proton concentration in the pH range 5.5–8.5 [2], indicate that no protons are released upon Ca^{2+}- or Sr^{2+}-binding.

2.4. *Equilibrium filtration experiments*

Although the equilibrium binding constant of Ca^{2+} to BLA was carefully determined at room temperature [2], it was thought necessary to perform some control equilibrium Ca-binding studies on BLA, depleted of metals by either of the procedures described above. For this purpose, 1 ml of 8.5 μM metal-free BLA, prepared either way, was passed through a column (1×40 cm) of Sephadex G-25 equilibrated with 20 mM Tris-HCl, pH 7.5, 1.2 μM $CaCl_2$ in a temperature-controlled room (25.0 ± 0.2°C) and eluted under experimental conditions where equilibrium is reached [13]. Ca^{2+} and protein concentrations in the eluted fractions (0.6 ml each) were determined as described above. K_{Sr} was determined by a similar procedure using 1 ml of 24 μM BLA and 2 and 3 μM free Sr^{2+}, respectively.

2.5. *Microcalorimetry*

Enthalpy changes were determined in an LKB 10700 flow microcalorimeter [15,16] (LKB, Stockholm, Sweden) at 25.0 ± 0.1°C. A 50–100 μM stock solution of metal-free BLA in 20 mM Tris-HCl, pH 7.5, and varying concentrations of $CaCl_2$ or $SrCl_2$ in the same buffer were introduced at equal flow rates in the mixing chamber of the microcalorimeter by means of a 2-channel peristaltic pump at total rate of 9 $ml \cdot h^{-1}$. The observed thermal effects in the measuring cell were calibrated by electric simulation immediately after each experiment. For each concentration studied, the heat of dilution of Ca^{2+} and Sr^{2+} was determined in an independent experiment without protein, but under otherwise identical conditions. Assuming a simple interaction of BLA with Sr^{2+} and a 1:1 stoichiometry, K_{Sr} was estimated iteratively from the calorimetric titration curve: the minimum of $\Sigma(\Delta H_{calc.} - \Delta H_{exper.})$ upon varying $K_{Sr} = f([BLA \cdot Sr^{2+}])$ yields the values of K_{Sr} and ΔH.

3. RESULTS AND DISCUSSION

3.1. *Equilibrium gel filtration*

Assuming a 1:1 complex, the equilibrium constant K_{Ca} of BLA for Ca^{2+} determined in our study equals 2.5×10^6 M^{-1} for the protein treated with the metal chelator, and 1.2×10^6 M^{-1} for the acid-treated BLA. These are single point determinations, and they compare very well with the value obtained by Bratcher and Kronman [2], i.e. 2.5×10^6 M^{-1}. Hence the two methods of preparing metal-free BLA are equally good (see also below). The mean affinity constant of BLA for Sr^{2+} (K_{Sr}) at 2 and 3 μM free Sr^{2+} amounted to 5.2×10^5 M^{-1}. The Ca^{2+}/Sr^{2+} selectivity ratio of 5 is distinctly lower than the one in intracellular regulatory

Ca-binding proteins, where the ratio is about 30 [17].

3.2. *Microcalorimetry*

Fig.1 depicts the enthalpy changes observed upon titration of BLA with Ca^{2+} and with Sr^{2+}. Curves pass through the origin and reach plateau values at 1 gion per mol protein indicating that saturation is reached and that only one high affinity binding site is present in the protein for both Ca^{2+} and Sr^{2+}. No significant difference in the titration was observed when the protein was metal-depleted by either the chelator- or acid-procedure. Punctual measurements of Sr^{2+} binding at 10 and 50 mM Tris-HCl, also shown on fig.1, indicate that enthalpy changes do not strongly depend on ionic strength, which is also reflected by the fact that the affinity constant is independent of ionic strength [2]. The affinity constant for Sr^{2+} estimated from the titration curve of fig.1, amounts to $5.1 \times 10^5\ M^{-1}$, in close agreement with the above-mentioned direct binding studies. The thermodynamic parameters of the ion interactions of BLA are summarized in table 1. The overall enthalpy change upon titration of BLA with Ca^{2+} in the absence and presence of 250 μM Sr^{2+} (not shown) displays a difference which is identical to the enthalpy change occurring when metal-free BLA binds Sr^{2+}, indicating straight competition at one high affinity site. Ca^{2+}- and Sr^{2+}-binding exhibit strong negative reaction enthalpies and entropies, indicating that binding is essentially enthalpy-driven with an overall gain in order. As enthalpy changes are directly related to changes in the bond strength, the latter are much stronger in BAL than in the intracellular Ca-binding proteins, where they vary from 0 to -35 kJ per mol of site [8–11]. One explanation, favored by studies on the secondary structure of these proteins, is that, in BLA, the donor oxygens have a prearranged orientation, ideally suited for complex formation [18], whereas this would not be the case for the flexible Ca-binding sites in intracellular calcium binding proteins. It should be noted that our enthalpy value for Ca^{2+} binding is very close to the one determined by Pfeil [7], indicating that the same A⇄N transition is monitored in both cases.

Another particular feature of the interaction of

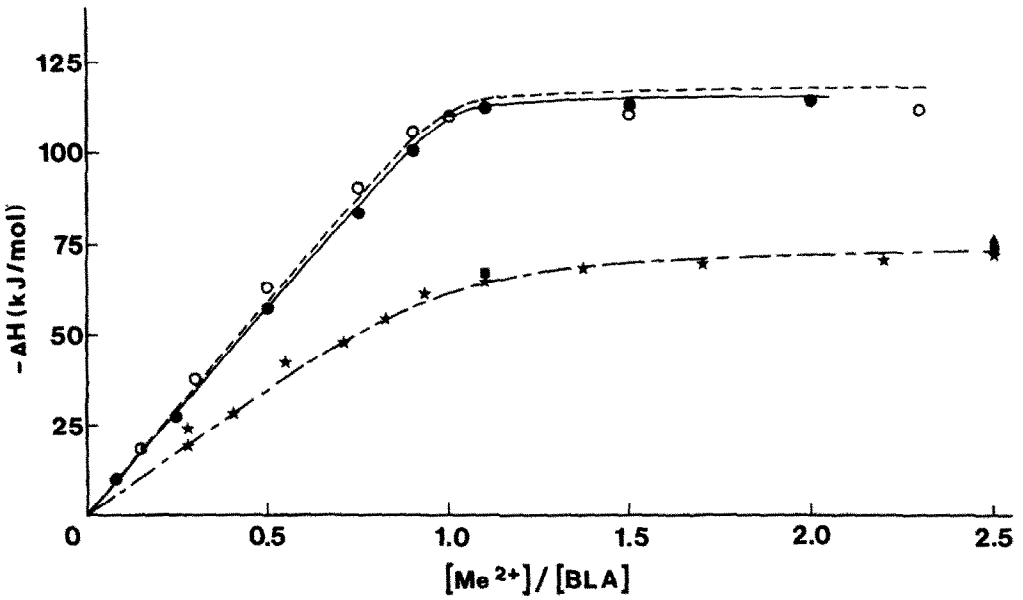

Fig.1. Enthalpy changes at 25.0°C for Ca^{2+} binding to BLA, which was depleted of metal ions with the procedure of HCl (○) or EDTA (●), and for Sr^{2+} binding to BLA in 10 (■), 20 (*) and 50 (▲) mM Tris-HCl buffer. All the curves were calculated from the Ca^{2+} and Sr^{2+} binding parameters of table 1. If proton release is significant, the observed enthalpy titration curves, calculated on a molar basis of BLA, would have to be corrected for -0.14 kJ and -0.05 kJ in the case of Ca^{2+} and Sr^{2+}, respectively, for protonation of the Tris buffer.

Table 1

Thermodynamic parameters for the interaction of Ca^{2+} and Sr^{2+} with BLA

	K_{eq} (M^{-1})	$\Delta G°$ ($kJ \cdot mol^{-1}$)	$\Delta H°^{b}$ ($kJ \cdot mol^{-1}$)	$\Delta S°$ ($J \cdot K^{-1} \cdot mol^{-1}$)
Ca^{2+}	2.5×10^{6} [a]	−36.5	−118	−273
Sr^{2+}	5.1×10^{5}	−32.6	− 75	−142

$\Delta G° = -RT \ln K$; $\Delta S° = (\Delta H° - \Delta G°)/T$

[a] Taken from [2] and confirmed in this study
[b] The accuracy of calorimetric measurements is ± 2.5%

Ca^{2+} and Sr^{2+} with BLA is the large negative reaction entropies which, in contrast to intracellular regulatory proteins [8–11] or organic chelators [19], are positive, i.e. in the latter cases there is a balance between gain of conformational order in the protein and loss of order upon dehydration of the ion. In BLA, the gain in conformational order is such that it outweighs the dehydration entropy and occurs even at the expense of an overall enthalpy decrease. Thus Ca^{2+} plays a crucial role in the overall structure of BLA, much more than in regulatory proteins.

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