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ORIGINAL PAPER

An Equilibrium and a Kinetic Stopped-Flow Fluorescence Study of the Binding of Various Metal Ions to Goat Alpha-Lactalbumin

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Abstract Various metal ions bind to the protein α-lactalbumin prepared from goat milk. The stability of the protein after metal binding is compared with that of the apo-protein by monitoring the fluorescence of the tryptophan residues under equilibrium conditions. The kinetics of the metal binding is studied by stopped-flow fluorescence spectroscopy. By means of the Arrhenius plots, the activation energy with regard to the binding of the different ions is determined.

Keywords Metal binding · α-Lactalbumin · Binding kinetics · Fluorescence spectroscopy

Introduction

Goat α-lactalbumin (GLA) is a small globular protein consisting of 123 amino acids. Its three-dimensional structure was determined by X-ray spectroscopy [1] and is presented in Fig. 1. The protein consists of two structural domains: a large domain (residues 1–39 and 85–123) which comprises 4 α-helices and a shorter one (residues 40–84) with a three-stranded β-sheet. Like all other lactalbumins, GLA possesses a strong Ca^{2+}-binding site situated at the interface of the two domains. Calcium coordination involves the carboxyl oxygen of Asp residues 82, 87, and 88, the carbonyl group of the peptide backbone of Lys 79 and Asp 84, and two water molecules. Especially for α-lactalbumin originating from bovine (BLA), the binding of other metal ions has extensively been studied [2–5]. From these studies it results that a series of divalent metal ions including Cd^{2+}, Sr^{2+}, and Mn^{2+} bind to the Ca^{2+}-binding site but that Zn^{2+} binds to a distinct site which involves residues Glu 1, Glu 7, Glu 11, and Asp 37 in BLA [3]. It has also been shown that Co^{2+} and Mg^{2+} rather interact with the residues belonging to the Zn^{2+}-binding site [5, 6]. The binding of these ions to GLA, however, is not yet studied in detail. The sequence of GLA differs from that of BLA only by seven amino acids. Interestingly, inspection of the location of these amino acids within the spatial structure of LA reveals that in BLA, Glu 7 and Glu 11 are part of a large cluster of negative charges that also comprises Asp 37 and the Asp residues of the Ca^{2+}-binding site. In GLA the negativity of this region is reduced by the substitution of Glu 11 by a positively charged Lys. The larger stability of GLA compared to BLA and the smaller ΔC_p value for the binding of Ca^{2+} has recently been related to this difference in charge distribution [7].

In this contribution we present a systematic study of the effects of metal ion binding on the stability of GLA by observing the shift of λ_{max} during thermal denaturation of the protein. Furthermore the binding kinetics of Ca^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} to carefully purified apo goat α-lactalbumin is followed by stopped-flow fluorescence spectrocopy. These kinetic experiments performed at different temperatures allowed us to calculate the activation energies needed for the transition from the apo- to the metal-loaded state of the protein. Our equilibrium unfolding experiments reveal that the binding of the different metal ions stabilizes the protein, but the degree of stabilization varies for each ion. Furthermore, stopped-flow kinetic experiments show that the rate, at which the flouresence intensity decreases, strongly depends on the specific ion. The activation energy deduced from the Arrhenius plots

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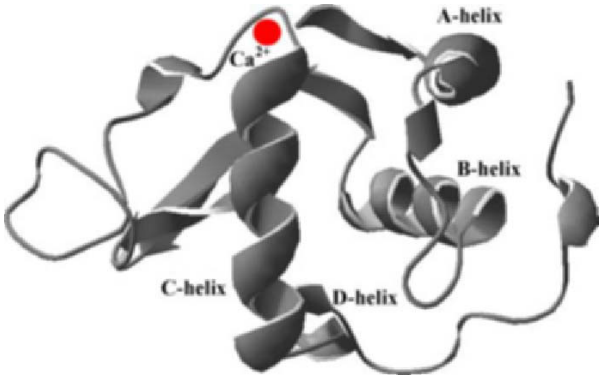

Fig. 1 Schematic representation of the three-dimensional structure of GLA with indication of the Ca^{2+}- and Zn^{2+}-binding site (1HFY). The picture was drawn using Molscript

makes clear that different sites of GLA are involved in metal binding.

Experimental

Steady-state fluorescence was measured with a Perkin-Elmer LS55 Luminescence Spectrometer. The GLA concentration used was approximately 5 μM. The excitation wavelength was set at 280 nm, the bandpass for the excitation and emission slits was 5 and 3 nm, respectively. The cell holder was thermostated by circulating water from an external water bath. By means of a PID-control algorithm (Labview^{TM}) we imposed a stepwise heating program to the circulator thermostat. Adjustment of the imposed temperature was controlled by a Pt100 sensor dipped in the solution.

Kinetic binding experiments were performed on an SX.18MV sequential mixing stopped-flow fluorescence spectrometer from Applied Photophysics (Leatherhead, UK). The stopped-flow unit and the observation cell with a 2 mm path length were thermostated by circulating water from a temperature-controlled bath. A monochromator was used for excitation at 280 nm and the fluorescence emission was measured using a high-pass filter with a 320-nm cutoff. The dead-time of the instrument was estimated to be about 2 ms. Typically, kinetics were measured 10–12 times, averaged, and analyzed as a sum of exponential functions by using the manufacturer's software.

GLA was prepared from fresh milk whey and carefully purified and decalcified [8]. Ultrapure $SrCl_2$, $MnCl_2$ and $CoCl_2$ were purchased from Alfa Aesar. $MgCl_2$, $ZnCl_2$, $CaCl_2$ and $CdCl_2$ are products of Merck.

Binding was initiated by mixing ± 18 μM apo-BLA in 10 mM Tris(hydroxymethyl)-aminomethane (TRIS) at pH 7.5 with a solution of the different metal chlorides in the same buffer in a 1:1-mixing ratio. The metal ion concentration mentioned in the text always refers to values in the cell after mixing. All measured time traces consist of 1000 data points and were analysed using a double exponential function. As in all cases the contribution of the second phase appears to be small, only a single rate constant k was considered in the further analysis. The Arrhenius plots were fitted to the equation $\ln k = A - E_a/RT$ and the activation energy E_a was derived from the slope of the obtained linear curve.

Results

Thermal stability

GLA contains four tryptophan residues, the fluorescence of which is drastically dependent on their degree of exposition to the solvent. Ca^{2+}-binding at room temperature for example shifts the λ_{max} of the emission spectrum to lower values due to the more compact conformation in the Ca^{2+}-bound state. During thermal denaturation, on the contrary, λ_{max} shifts to longer wavelengths due to the transfer of Trp residues from the protein interior to the aqueous environment of the solvent. For apo-GLA, λ_{max} shifts from 320 nm in the native state at 10°C tot 343 nm in the unfolded state (Fig. 2A). The same figure shows the evolution of the wavelength maximum as a function of temperature in the presence of 0.20–0.25 mM metal ions. For all metal-loaded samples the λ_{max}-value at the lowest temperature lies in the range 317.5 ± 1.0 mm. In the unfolded state at higher temperatures this value amounts to 343 ± 0.5 nm with the exception for the Zn^{2+} sample where λ_{max} hardly reaches 340 nm. All the transition curves show the sigmoid form, characteristic for a simple two-state transition. The temperature at the transition midpoint, T_M, is a good measure for the relative stability of the various proteins. These values are presented in Table 1, showing that Ca^{2+} ions have a considerable effect on protein stability in contrast to Zn^{2+} that shifts the transition temperature only with 3.8°C.

Table 1 Transition midpoint of the unfolding of 0.25 mM metal-loaded GLA in 10 mM Tris at pH 7.5.

	Ca^{2+}	Sr^{2+}	Mg^{2+}	Mn^{2+}	Co^{2+}	Cd^{2+}	Zn^{2+}	Apo
T_M (°C)	66.71 ± 0.19	59.78 ± 0.17	55.68 ± 0.23	54.15 ± 0.15	47.34 ± 0.16	47.14 ± 0.30	35.15 ± 0.48	31.30 ± 0.33

Note. In the case of the zinc-loaded protein, 0.2 mM Zn^{2+} was used.

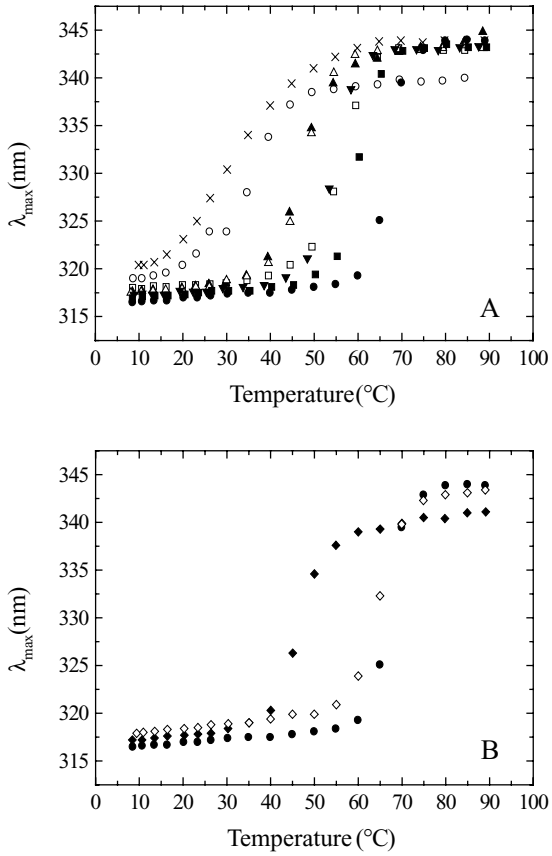

Fig. 2 (A) Thermal unfolding curves for apo-GLA (×) in 10 mM Tris, pH 7.5 and for GLA with 0.25 mM of Ca^{2+} (●), Sr^{2+} (■), Mn^{2+} (▼), Cd^{2+} (▲) Mg^{2+} (□), Co^{2+} (△) and Zn^{2+} (○). (B) The effect of 0.25 mM of Co^{2+} (◇) or 0.2 mM Zn^{2+} (◆) on 0.25 mM Ca^{2+}-GLA (●)

Addition of metal ions to Ca^{2+}-GLA does not alter its stability except for Co^{2+} and Zn^{2+} (Fig. 2B). The addition of 0.2 mM Zn^{2+} to GLA with 0.25 mM Ca^{2+} shifts the thermal transition temperature from 66.7 to 46.6°C.

Stopped-flow fluorescence spectroscopy

The binding of Ca^{2+} to apo-GLA causes pronounced conformational changes in tertiary structure, observed by a blue shift and a decrease in quantum yield in the fluorescence spectrum. The binding kinetics thus can be determined from stopped-flow fluorescence experiments in which apo-GLA in 10 mM Tris, pH 7.5 and 25°C is mixed with the same buffer containing 0.5 mM Ca^{2+} in a 1:1 ratio. The evolution of the total fluorescence intensity as a function of time is depicted in Fig. 3 and can be fitted with a double exponential function. In this specific case the rate constants k equal 14.3 and 1.52 s^{-1}. The amplitude of the two phases is 1.57 and 0.27, respectively.

The first point of each trace corresponds with the fluorescence intensity characteristic for apo-GLA and the last point with that of Ca^{2+}-GLA. These initial and final values of the time traces were plotted as a function of temperature for the binding of 0.1 mM Ca^{2+} (Fig. 4). The final points, corresponding to Ca^{2+}-GLA show a gradual decrease in fluorescence intensity due to thermal quenching. The temperature dependence of the initial values reflects the unfolding of apo-GLA. These results show that from 10°C on, the effect of thermal quenching is counteracted by the unfolding of apo-GLA resulting in an increase of the fluorescence signal. Once the protein is completely unfolded, thermal quenching becomes again the dominant factor.

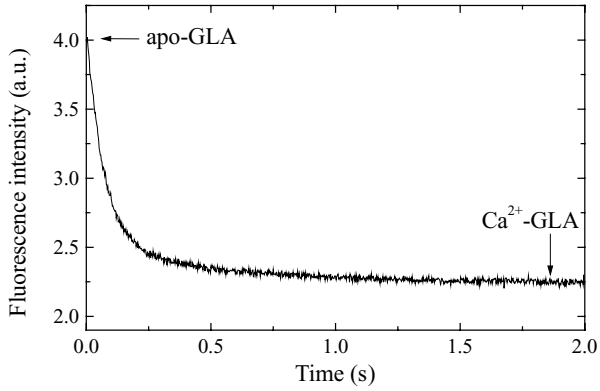

Fig. 3 Evolution of the fluorescence intensity as a function of time upon binding of Ca^{2+} to apo-GLA. In this SF-experiment, 18 μM apo-GLA is used in 10 mM TRIS, pH 7.5 and 25°C and is mixed with the same buffer containing 0.5 mM Ca^{2+} in a 1:1 ratio

Arrhenius plots

The evolution of the rate constant as a function of the inverse of the temperature is shown in Fig. 5. This Arrhenius plot is linear in the temperature range between 5 and 40°C and can be fitted to the equation $\ln k = A - E_a/RT$ (solid lines). The slope of this curve is a measure for the activation energy of the

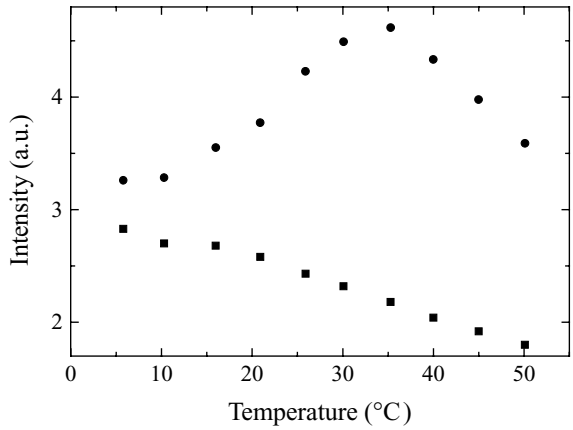

Fig. 4 Kinetic parameters for the binding of 0.1 mM Ca^{2+} to apo-GLA at different temperatures. The initial (●) and final (■) intensities of each of the individual traces are shown between 5 and 50°C

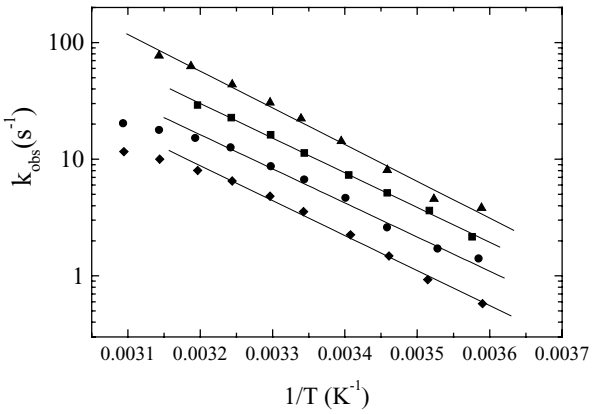

Fig. 5 Arrhenius plot for the binding of 2.5 mM (▲), 0.5 mM (■), 0.1 mM (●), and 0.05 mM (◆) Ca^{2+} to apo-GLA. The solid lines represent linear fits through the data points in the temperature region between 5 and 41°C

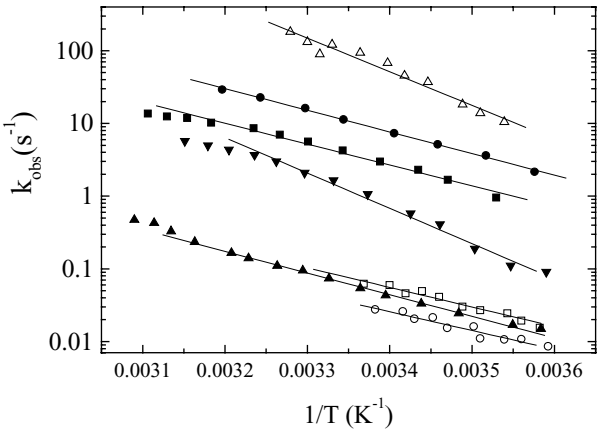

Fig. 6 Arrhenius plot for the binding of Ca^{2+} (●), Sr^{2+} (■), Mn^{2+} (▼), Cd^{2+} (▲), Mg^{2+} (□), Co^{2+} (△) and Zn^{2+} (○) to apo-GLA. In all cases the final concentration of the metal ion equals 0.25 mM except for Zn^{2+} (0.20 mM). The solid lines represent the linear fit through the data points

binding process. As a result, E_a equals 56 ± 2.6 kJ/mol for the binding of 0.1 mM Ca^{2+} to GLA. At higher temperatures, the Arrhenius plot deviates from linearity.

The same experiment was also performed using various Ca^{2+} concentrations. As expected, the apparent rate constant increases with increasing Ca^{2+} concentration but the slope of the Arrhenius plot remains the same, leading to comparable E_a values (Fig. 5 and Table 2). Besides the ability to bind Ca^{2+}, GLA also binds other divalent metal ions. In this work we have measured the kinetics of the binding of Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, and Zn^{2+} to apo-GLA. In all cases, a lot of metal concentrations were applied except for Zn^{2+}, due to precipitation of the solution at high metal concentration [9]. The difference in concentration has only an effect on the rate constant and does not alter the value of the activation energy (Fig. 6 and Table 2).

For Cd^{2+} and Sr^{2+}, which are known to bind to the Ca^{2+}-site in bovine α-lactalbumin, the activation energy corresponds, within error margins, to the value determined for the Ca^{2+}-binding to apo-GLA (Table 2). This result suggests that the activation energy is primarily dependent on the nature of the binding site and is not markedly influenced by the specific properties of the binding ion.

Table 2 Rate constant and activation energy for the binding of Ca^{2+}, Sr^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+} to apo-GLA.

Metal ion	k at 20°C (s^{-1})	E_a (kJ/mol)
Ca^{2+} (0.5 mM)	6.95	60 ± 1.5
Ca^{2+} (0.1 mM)	2.05	56 ± 2.6
Sr^{2+} (2.5 mM)	2.55	54 ± 2.4
Cd^{2+} (0.25 mM)	0.041	57 ± 2.0
Mn^{2+} (2.5 mM)	0.59	93 ± 4
Co^{2+} (0.25 mM)	7.17	88 ± 3
Mg^{2+} (0.25 mM)	0.053	51 ± 3
Zn^{2+} (0.20 mM)	0.024	48 ± 3

It has been shown previously that Mg^{2+} binds to the Zn^{2+}-binding site in BLA, a protein that differs only by seven amino acids from GLA [5]. The activation energy for the binding of Mg^{2+} to apo-GLA coincides with that of Zn^{2+} (Fig. 6 and Table 2), confirming that both ions bind to the same site.

Co^{2+} is a metal ion which is believed to bind to the Zn^{2+}-binding site in BLA [10]. However, the activation energy for the binding of Co^{2+} to GLA is remarkably higher than the value obtained for Zn^{2+} suggesting that in this case, Co^{2+} binds to another distinct site.

The activation energy of Mn^{2+} is different from the value obtained for the Ca^{2+}-binding to apo-GLA (Fig. 6 and Table 2), but is rather close to the value obtained for the Co^{2+}-binding site.

Conclusions

In this work it has been shown that the thermal stability of goat α-lactalbumin in its apo- and in its metal-bound form can be determined by monitoring the wavelength at which the Trp fluorescence intensity reaches a maximum. Furthermore, stopped-flow fluorescence spectroscopy can give valuable information on the binding kinetics of metal ions to the protein and on their specific binding site.

From the equilibrium experiments it clearly results that the different divalent metal ions stabilize GLA in a different way with unfolding temperatures ranging from 35 to 67°C.

Our kinetic binding experiments suggest that Co^{2+} that was thought to bind to the Zn^{2+}-binding site, binds to another distinct site. On the other hand Mn^{2+} ions show comparable

activation energy as Co^{2+} ions upon binding to GLA. The binding characteristics of Ca^{2+}, Sr^{2+} and Cd^{2+} to GLA are analogous with the binding to BLA.

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