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Stopped-flow kinetic studies of Ca(II) and Mg(II) dissociation in cod parvalbumin and bovine α-lactalbumin

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The dissociation kinetics of complexes of bovine α-lactalbumin and cod parvalbumin with Ca(II) and Mg(II) ions induced by mixing of a Ca(II)- or Mg(II)-loaded protein with a chelator of divalent cations (EDTA or EGTA) have been studied by means of the stopped-flow method with intrinsic protein fluorescence registration. Within the temperature interval from 10 to approx. 37 °C kinetic curves for Ca(II) removal from α-lactalbumin are monoexponential with a rate constant ranging from 0.006 to 1 s. Taking into account the rather low rate of fluorescence changes, one can assume that the limiting stage in this case is the dissociation of the single bound Ca(II) ion from the protein and not a conformational transition which occurs after Ca(II) dissociation. At temperatures above 37 °C the kinetic curves require at least two exponential terms for a satisfactory fit. The second exponential seems to be due to denaturation of the apo form of α-lactalbumin which takes place at these temperatures. The values of the dissociation rate constants for Mg(II) bound to α-lactalbumin practically coincide with those for Ca(II). Within the temperature interval 10–30 °C the kinetic curves for Ca(II) and Mg(II) removal from parvalbumin are best fitted by a sum of two exponential terms identified as arising from the dissociation of cations from the two binding sites. The values of the dissociation rate constants are within the range 0.03–0.8 and 0.18–5 s^{-1} for the Ca(II)-parvalbumin complex and 0.9–4.5 and 4–33 s^{-1} for the Mg(II)-parvalbumin complex. The equilibrium Ca(II)- and Mg(II)-binding constants for parvalbumin and α-lactalbumin have been measured over the same temperature intervals. This allows the evaluation of the association rate constants for complexes of these proteins with Ca(II) and Mg(II). A kinetic experiment in which Mg(II) was bound by α-lactalbumin provided a value for the association rate constant which is in good agreement with that evaluated.

1. Introduction

It is well known now that Ca(II) ions are second messengers that couple an extracellular stimulus to an intracellular response [1]. Reception of the intracellular messenger, Ca(II), is achieved by specific receptor proteins. The equilibrium Ca(II)-binding properties of Ca(II) receptor proteins are now well established, but the elucidation of the molecular mechanisms of Ca(II) regulation in biological systems requires knowledge of their kinetic properties. So far the kinetics of Ca(II) association and dissociation have mostly been studied for proteins such as troponin C and calmodulin [2–10] containing three to four Ca(II)-binding sites per molecule. A comparatively large number of binding sites in these proteins complicates the interpretation of kinetic data, since the details of the binding scheme for these proteins are still largely unknown. This is why it is of importance to study the kinetics of Ca(II) association and dissociation for proteins which possess one or at most two binding sites. Parvalbumin and α-lactalbumin are two such proteins.

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Parvalbumin is a small (molecular mass approx. 12 kDa), Ca(II)- and Mg(II)-binding (two binding sites per molecule with affinity 10^8–10^9 M^{-1} for Ca(II) and 10^3–10^5 M^{-1} for Mg(II)) protein found in large amounts in the sarcoplasm of the fast muscles of vertebrates [11]. Despite intensive physicochemical investigations of parvalbumin, its biological function remains obscure. It has been proposed that parvalbumin is a soluble relaxing factor that accelerates removal of Ca(II) from Ca(II)-specific regulatory sites of troponin to bring about relaxation [12]. According to this hypothesis, the binding sites of parvalbumin in resting muscle are occupied by Mg(II) ions which begin to be exchanged by Ca(II) ions during their release from the sarcoplasmic reticulum. The delay in Ca(II) binding due to Mg(II) dissociation permits Ca(II) ions to reach Ca(II)-specific regulatory sites of troponin C and hence to activate the contractile proteins. Later on during the relaxation stage the Ca(II)/Mg(II)-binding sites of parvalbumin compete successfully for Ca(II), removing it from troponin. It is obvious that examination of the validity of this hypothesis requires knowledge of the kinetic constants of association and dissociation of Ca(II) and Mg(II) for parvalbumin. Several investigators attempted to measure these kinetic constants [13–17], but their measurements being either indirect or direct were carried out at only one temperature which furthermore was not physiological.

α-Lactalbumin is one of the two components of the lactose synthase system which catalyzes the final step in lactose biosynthesis in the lactating mammary gland [18]. One of the most important peculiarities of this small protein (molecular mass 14.2 kDa) is its high affinity for one Ca(II) ion per molecule with a binding constant of $(3–5) \times 10^8$ M^{-1} at 20 °C [20]. Moreover, it binds the cations Mg(II), Na(I), K(I) [21,22], Mn(II) and Zn(II) [23–25]. The physiological significance of binding of Ca(II) ions by α-lactalbumin is still unclear. So far only the equilibrium parameters for Ca(II) binding to α-lactalbumin have been studied, however, this protein could serve as an extremely convenient model for kinetic studies because it contains only one Ca(II)-binding site and this facilitates the interpretation of kinetic data. On the other hand, knowledge of the kinetic properties of α-lactalbumin could be rather useful in attempts to elucidate the physiological function of binding of metal ions to α-lactalbumin.

The binding of cations induces rather pronounced changes in the intrinsic tryptophan fluorescence of both parvalbumin and α-lactalbumin, which renders these proteins very suitable for stopped-flow kinetic studies.

Here we present some results of a stopped-flow kinetic study of Ca(II) and Mg(II) dissociation in cod parvalbumin and bovine α-lactalbumin at different temperatures. The results of this study in combination with equilibrium data have allowed us to obtain kinetic association constants and thermodynamic values of enthalpy and entropy changes for association and dissociation of complexes of parvalbumin and α-lactalbumin with Ca(II) and Mg(II) ions.

2. Materials and methods

α-Lactalbumin was isolated and purified from cow milk following a preparative sequence similar to that described by Kaplanas and Antanavichius [26]. Parvalbumin was prepared from white muscles of cod (*Gadus callarius* L.) as described by Haiech et al. [27]. The purity of the proteins was confirmed by SDS gel electrophoresis. Protein concentrations were evaluated spectrophotometrically using $\epsilon = 28\,542$ M^{-1} cm^{-1} at 280 nm for bovine α-lactalbumin [28] and $\epsilon = 7189$ M^{-1} cm^{-1} for cod parvalbumin [29].

All solutions were made using deionized water. Only plastic ware was used in this work.

Steady-state fluorescence measurements were performed with a laboratorymade spectrofluorimeter described earlier [30]. All fluorescence spectra were corrected for the instrumental spectral sensitivity.

Kinetic measurements were recorded with an SFL-25 stopped-flow apparatus (Hi-Tech Scientific, U.K.). Fluorescence was excited by light of the mercury line at 296.7 nm isolated by means of an M300 monochromator (Applied Photophysics, U.K.) and a broad-band ultraviolet filter. The mixing system and optical compartment of the

instrument were placed in a thermostat. The mixing volumes of the reagents were 0.3 ml. The instrumental dead time was approx. 10 ms. Fluorescence light passing through an H10 analyzing monochromator (Jobin Yvon, France) and/or a filter was registered with an EMI 9789 QA photomultiplier. The anode current was amplified by a UT-8105 high-gain electrometer (Tartu University, U.S.S.R.). Fluorescence traces were displayed on a V10 storage oscilloscope (Hihon Kohden, Japan) and/or a recorder.

The theoretical curves were calculated by fitting a single- or double-exponential function to the experimental kinetic data using an iterative non-linear least-squares procedure [31]. Fitting of the steady-state fluorescence data with theoretical curves was carried out using the same procedure on an M-4030 computer.

3. Results and discussion

Cod parvalbumin contains one tryptophan residue per molecule [32], the fluorescence of which is changed drastically on binding of Ca(II) and Mg(II) ions. Fig. 1A shows the temperature dependences of the fluorescence parameters of cod parvalbumin in the apo, Mg(II)- and Ca(II)-loaded states. The shift of the fluorescence spectrum towards longer wavelengths is caused by transfer of the single tryptophan residue to an aqueous environment during the course of thermal denaturation of the protein. It is clearly seen that thermal denaturation of the Ca(II)-loaded protein occurs at temperatures above 60 °C whereas denaturation of the Mg(II)-loaded protein takes place above 30 °C. At all temperatures studied the tryptophan residue in the metal-free parvalbumin is located at the protein surface (long-wavelength position of the fluorescence spectrum); the thermal denaturation is reflected in a slight red shift of the spectrum which begins above 20 °C.

The corresponding data for bovine α-lactalbumin are presented in fig. 1B. In contrast to parvalbumin, thermal denaturation of α-lactalbumin results in an increase in the value of the fluorescence quantum yield.

Fig. 1 demonstrates rather significant differences between the fluorescence parameters of different states in the cases of parvalbumin and α-lactalbumin, which can be used to determine both steady-state and kinetic constants of association and dissociation for the complexes of these proteins with Ca(II) and Mg(II) ions.

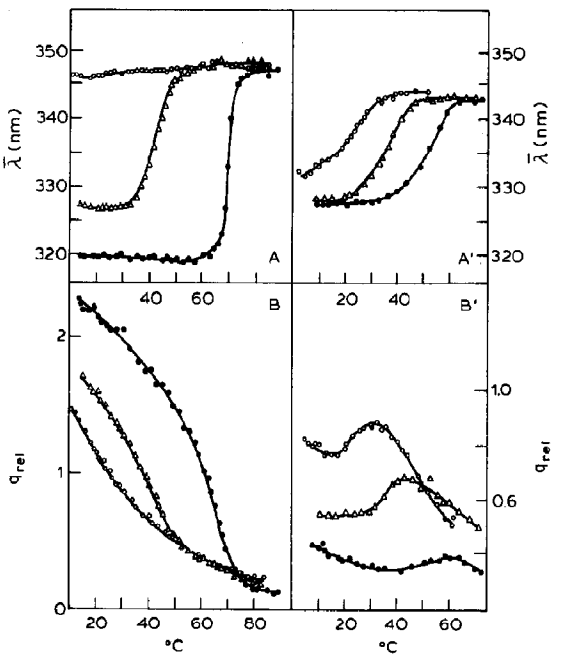

Fig. 1. Temperature dependence of fluorescence parameters for apo (○) (1 mM EGTA), Ca(II)-loaded (●) (1 mM $CaCl_2$) and Mg(II)-loaded (△) (1 mM EGTA, 6 mM $MgCl_2$) cod parvalbumin (A,B) and bovine α-lactalbumin (A′,B′) in 10 mM Hepes, pH 8. (A,A′) Fluorescence spectrum position; (B,B′) relative fluorescence quantum yield. Protein concentration 5×10^{-5} M. Excitation wavelength 280.4 nm.

Fig. 2 shows two kinetic curves plotted as the logarithm of the α-lactalbumin fluorescence intensity vs. time, obtained at two different temperatures. In these experiments Ca(II)-loaded protein was mixed with EDTA. The experimental data were fitted by a single- or double-exponential theoretical curve computed according to the equation:

$$F = f_1 \exp(-t/\tau_1) + f_2 \exp(-t/\tau_2). \quad (1)$$

In fig. 2 the continuous traces are theoretical curves fitted to the experimental points by varia-

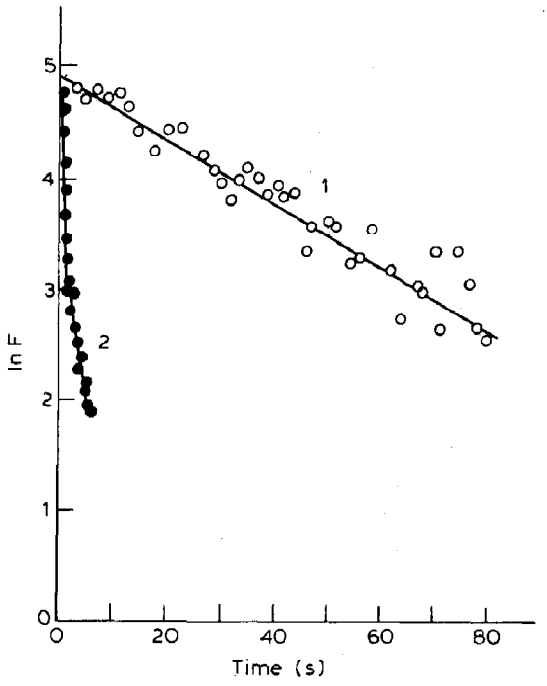

Fig. 2. Stopped-flow traces of Ca(II) release from bovine α-lactalbumin (logarithm of fluorescence intensity vs. time). Ca(II)-loaded α-lactalbumin (5×10^{-5} M) was mixed with 3.5 mM EDTA, 10 mM Hepes, pH 8. Curve 1, 24°C; curve 2, 40°C. Curves are theoretical traces computed according to eq. 1.

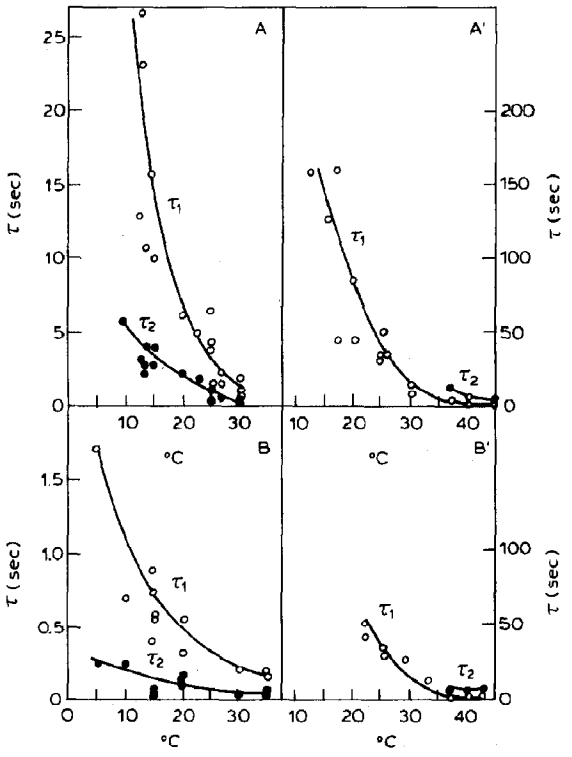

Fig. 3. Temperature dependences of τ obtained from the stopped-flow traces of Ca(II) (A,A') and Mg(II) (B,B') release from cod parvalbumin (A,B) and bovine α-lactalbumin (A',B').

tion of the f_i and τ_i parameters. The kinetic curves for α-lactalbumin obtained at temperatures below approx. 37°C are monoexponential, while those obtained at higher temperatures require a double-exponential function for a satisfactory fit. As concerns parvalbumin all kinetic data obtained in the temperature range 10–30°C were found to be best fitted by a sum of two exponential terms.

Fig. 3 represents τ_1 and τ_2 as functions of temperature for cod parvalbumin and bovine α-lactalbumin. This figure contains the data for both Ca(II) (A, A') and Mg(II) (B, B') release. In Mg(II) dissociation experiments the Mg(II)-loaded protein (1 mM EGTA, 3 mM $MgCl_2$, 10 mM Hepes, pH 8) was mixed with 3.5 mM EDTA (10 mM Hepes, pH 8).

It is significant that the processes monitored by the intrinsic fluorescence are rather slow: the values of τ_1 and τ_2 for Ca(II) release are within the range of several seconds to several tens of seconds (or even greater than 100 s as in the case of α-lactalbumin). This fact, along with the difference in rates of Ca(II) and Mg(II) release from cod parvalbumin, suggests that the limiting stage in the kinetic process, as monitored by the changes in the intrinsic fluorescence parameters, is not the conformational rearrangement after cation release (which should be much faster in small proteins like parvalbumin and α-lactalbumin), but rather the dissociation of Ca(II) and Mg(II) ions.

In order to establish the validity of this suggestion we carried out a stopped-flow experiment in which Mg(II), bound to parvalbumin, was substituted by Ca(II). Polar cod parvalbumin was used, since its fluorescence parameters change significantly during this process, in contrast to cod parvalbumin. In this experiment the Mg(II)-loaded polar cod parvalbumin (0.07 mM protein in 1 mM

EGTA, 1.5 mM $MgCl_2$) was mixed with 10 mM $CaCl_2$, 1 mM EGTA (10 mM Hepes, pH 8) at 7°C. We found that the Mg(II)-to-Ca(II) exchange kinetic curve coincides with the curve for Mg(II) dissociation in cod parvalbumin at the same temperature.

As the association rate constant of Mg(II) by α-lactalbumin is sufficiently low to monitor this process by our stopped-flow apparatus, we performed an experiment in which α-lactalbumin in the apo state (0.05 mM protein, 1 mM EGTA) was mixed with Mg(II) (3 mM $MgCl_2$, 1 mM EGTA; both in 10 mM Hepes, pH 8) at 10°C. The association rate constant estimated from the experimental curve was 56 ± 15 M^{-1} s^{-1}. This is in good agreement with the calculated value (96 ± 10 M^{-1} s^{-1}). Thus, both the above experiments demonstrate validity of the suggestion that in the dissociation experiments the limiting stage is really Mg(II) and Ca(II) dissociation.

It is appropriate to mention here that we carried out the dissociation stopped-flow experiments at several EGTA and EDTA concentrations (from 5×10^{-3} to 5×10^{-1} M) and the results obtained were the same within the limits of experimental error. In conjunction with the Mg(II)-to-Ca(II) exchange and Mg(II)-binding experiments, this demonstrates that EGTA and EDTA at the concentrations used here, do not affect the dissociation rate.

The experimental data obtained using various methods [13,33–35] show that binding of Ca(II) and Mg(II) to parvalbumin is a successive process:

$$P \overset{K_1}{\rightleftharpoons} PMe \overset{K_2}{\rightleftharpoons} MePMe, \tag{2}$$

where P denotes protein, Me signifies metal cation and K_1 and K_2 are equilibrium constants for the first and second sites of parvalbumin, respectively:

$$K_1 = \frac{k_1^+}{k_1^-}, \; K_2 = \frac{k_2^+}{k_2^-} \tag{3}$$

(k_1^+ and k_2^+, association rate constant; k_1^- and k_2^-, dissociation rate constants for the two binding sites).

Fig. 1A demonstrates that within the temperature range 10–30°C in a stopped-flow experiment, we monitor the transition of Ca(II)- or Mg(II)-loaded parvalbumin to the native apo-protein. One cannot observe transition of the apo-protein from the native to the thermally denatured state because these states have almost the same fluorescence parameters. Therefore, it is reasonable to identify the two exponential terms in the experimental kinetic curves for parvalbumin as arising from the dissociation of Ca(II) (or Mg(II)) from the two binding sites of the protein, i.e., from the stopped-flow data we can determine

$$k_1^- = 1/\tau_1 \text{ and } k_2^- = 1/\tau_2. \tag{4}$$

It is well known from activated-state theory [36] that

$$k^+ = \frac{kT}{h} \exp\left(- \frac{\Delta H^+ - T\Delta S^+}{RT} \right), \tag{5}$$

$$k^- = \frac{kT}{h} \exp\left(- \frac{\Delta H^- - T\Delta S^-}{RT} \right) \tag{6}$$

(where k is Boltzmann's constant, R the gas constant, h Planck's constant, and ΔH and ΔS the enthalpy and entropy of formation of the activated complex).

$$K = \exp\left(- \frac{\Delta H - T\Delta S}{RT} \right), \tag{7}$$

$$\Delta H = \Delta H^+ - \Delta H^-, \quad \Delta S = \Delta S^+ - \Delta S^-. \tag{8}$$

Using a linear anamorphosis of eq. 5:

$$\ln\left(\frac{k^-}{T} \frac{h}{k} \right) = - \frac{\Delta H^-}{RT} + \frac{\Delta S^-}{R}, \tag{9}$$

one can obtain the values of ΔH^- and ΔS^-. Fig. 4 illustrates the dependence of $\ln\{(k^-/T)(h/k)\}$ vs. $1/T$ for Ca(II) dissociation from cod parvalbumin. Similar plots were also constructed for Mg(II) removal. The values of ΔH_1^-, ΔH_2^- and ΔS_1^-, ΔS_2^- obtained from these plots are collected in table 1.

Table 1 also lists the values of the enthalpy and entropy of equilibrium Ca(II) and Mg(II) binding for the two sites of cod parvalbumin, ΔH and ΔS, as determined from the temperature dependence of the equilibrium binding constants for both kinds of metal ions according to eq. 7 (fig. 5). The equilibrium constants, K_1 and K_2, for Ca(II) and Mg(II) binding were measured over the tempera-

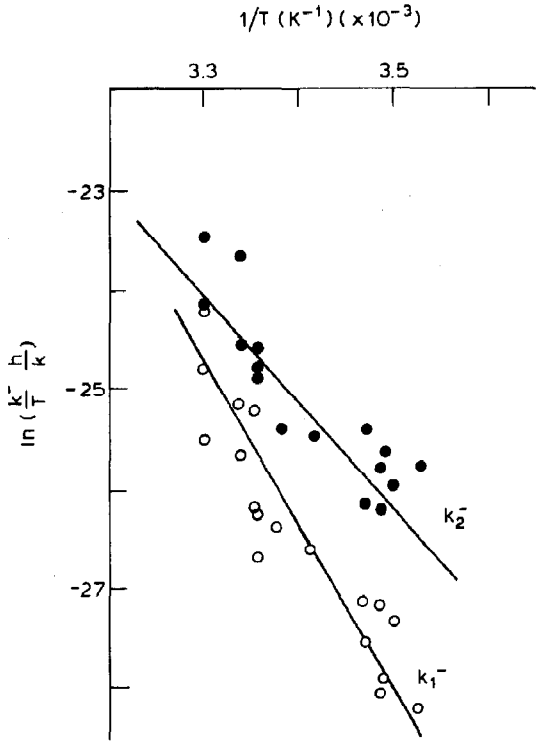

Fig. 4. Dependence of $\ln\{(k^-/T)(h/k)\}$ vs. $1/T$ for Ca(II) removal from cod parvalbumin (eq. 9).

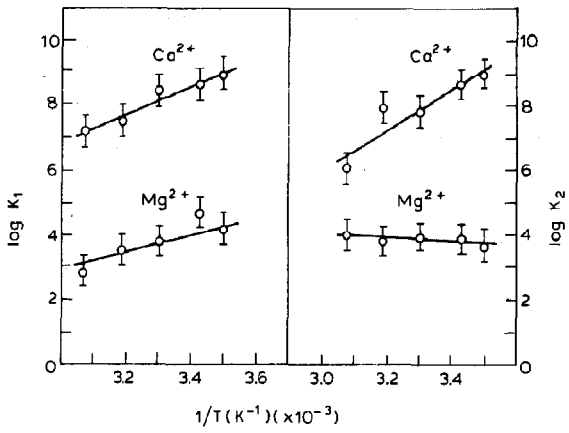

Fig. 5. Dependence of logarithms of equilibrium constants of Ca(II) and Mg(II) for two sites of parvalbumin vs. $1/T$.

ture interval 10–50 °C by means of methods described previously [20,22, 33, 37].

It is clear from fig. 1 that in the temperature interval 10–45 °C, α-lactalbumin first undergoes a transition from the Ca(II)- or Mg(II)-loaded state to the native form and then to the thermally denatured apo state. The transition from the native to thermally denatured apo state is more pronounced at sufficiently high temperatures. Therefore, it is reasonable to identify the second exponential component in kinetic data which appears at temperatures above 37 °C as arising from this process. Hence, the single-exponential process observed below 37 °C appears to correspond to the transition of α-lactalbumin from the metal-loaded to native apo state. The thermodynamic parameters of the dissociation and association kinetics of α-lactalbumin complexes with Ca(II)

Table 1

Values of the enthalpy and entropy of equilibrium binding of Ca(II) and Mg(II) to parvalbumin and α-lactalbumin (ΔH, ΔS) and of the enthalpy and entropy of formation of an activated complex during the course of dissociation (H^-, ΔS^-) and association (ΔH^+, ΔS^+) of complexes of cod parvalbumin and α-lactalbumin with Ca(II) and Mg(II)

Cation	Binding site	ΔH (kcal/mol) (±2)	ΔS (cal/mol per K)) (±5)	ΔH^- (kcal/mol) (±2)	ΔS^- (cal/mol per K) (±5)	ΔH^+ (kcal/mol) (±5)	ΔS^+ (cal/mol per K) (±5)
Parvalbumin							
Ca(II)	1	−19	−26	33	66	14	41
	2	−28	−57	21	26	−7	−31
Mg(II)	1	−12	−23	13	+3	1	−21
	2	3	27	10	−5	13	23
α-Lactalbumin							
Ca(II)	1	−18	−20	30	48	12	28
Mg(II)	1	−11	−24	22	21	10	−2

and Mg(II) ions, determined in the same way as for parvalbumin, are collected in table 1. The values of the enthalpy and entropy of equilibrium binding, ΔH and ΔS, for α-lactalbumin were obtained earlier [22].

We used the data collected in table 1 to compute the constants K, k^+ and k^- for cod parvalbumin and bovine α-lactalbumin as functions of temperature. The curves are presented on a semilogarithmic scale in figs. 6 and 7. It is evident that the values of the association rate constants for the complexes of parvalbumin with Ca(II) ions fall within the range 10^7–10^9 M^{-1} s^{-1}, i.e., they approach the diffusion-controlled limit. The association rate constants for complexes of both parvalbumin and α-lactalbumin with Mg(II) ions are much lower than those for the complexes with Ca(II). The Mg(II) and Ca(II) dissociation rate constants are rather close to each other in the case of both proteins. Thus, the difference between the values of the equilibrium binding constants for Ca(II) and Mg(II) ions is due to the difference in values of the association rate constants.

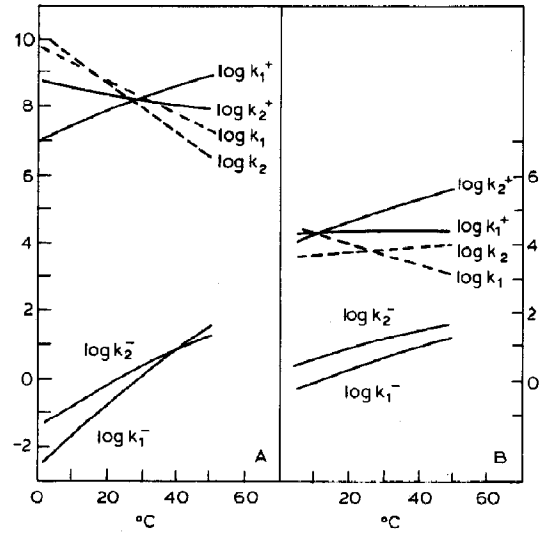

Fig. 6. Temperature dependences of logarithms of rate constants of association and dissociation and equilibrium constants of association for complexes of cod parvalbumin with Ca(II) (A) and Mg(II) (B) ions computed using the data listed in table 1.

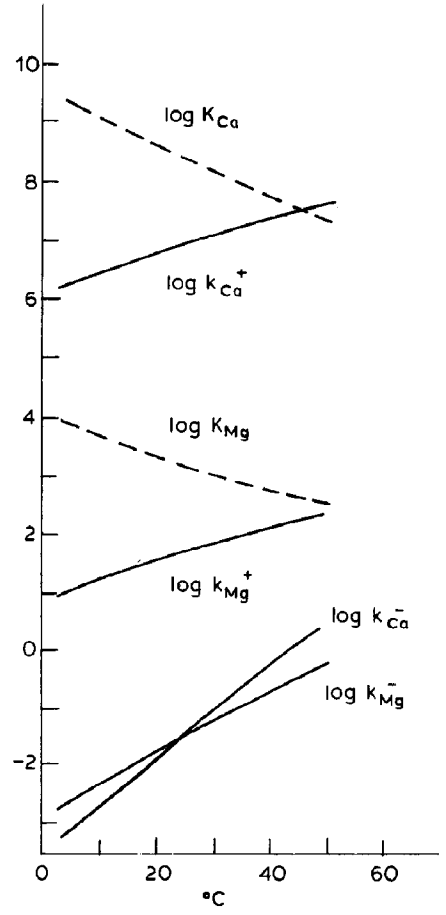

Fig. 7. Temperature dependence of logarithms of rate constants of association and dissociation and equilibrium constants of association for complexes of bovine α-lactalbumin with Ca(II) and Mg(II) ions computed using the data presented in table 1.

It should be noted that the equilibrium Ca(II)-binding constants for parvalbumin and α-lactalbumin decrease as the temperature is raised from 0 to 50 °C while the equilibrium Ca(II)-binding constants for calmodulin practically do not depend upon temperature within the same temperature range [38]. This shows that despite similarities in structure of the binding sites of parvalbumin and calmodulin there exist some fine differences in their arrangement.

It is of interest to compare our data with the results obtained by other authors. Breen et al. [16] have studied the kinetics of dissociation of com-

plexes of cod parvalbumin with Ca(II) ions at 24.8° C and pH 5.8. They have found that their kinetic curves are best fitted by a sum of two exponential terms with relaxation times of 0.93 and 0.17 s. These values are lower than those obtained in the present study, however, the difference is not significant, taking into account the difference between the pH values in our experiments and in those of Breen et al. In the work by Ogawa and Tanokura [17] the kinetic curves of dissociation of complexes of bull-frog parvalbumin with Ca(II) ions, measured at 20° C and pH 6.8, were fitted by a single exponent with a relaxation time of 0.67 s which is also somewhat lower than the values obtained in the present work. At the same time the value of the relaxation time determined by Ogawa and Tanokura for Mg(II) (0.2 s) is in a good agreement with our data.

It is of importance to compare the relaxation times obtained here for the dissociation of Mg(II)-parvalbumin complexes with the contraction and relaxation times of skeletal muscle. At physiological temperatures (10–15° C for cod-fish) τ_1 and τ_2 amount to 900 and 100 ms, respectively, while the duration of the contraction and relaxation stages for fish skeletal muscle is several tens of milliseconds [39]. This suggests that during a single twitch of a skeletal muscle Mg(II) ions in parvalbumin will not have enough time to be exchanged for Ca(II) if this exchange follows the common competition scheme. This means that parvalbumin cannot serve as a soluble relaxing factor that accelerates Ca(II) removal from troponin C during a single twitch. One can assume that the Mg(II)-Ca(II) exchange in parvalbumin can be achieved only after several successive contraction-relaxation cycles. Another possibility for parvalbumin to be the relaxing factor could be its direct interaction with some contractile proteins, for example, troponin.

Knowledge of the Ca(II) association rate constant for α-lactalbumin and Ca(II) concentration during activation of a cell ($\sim 10^{-6}$ M) allows one to evaluate the time of activation of α-lactalbumin by Ca(II) and hence of the lactose synthase system at physiological temperature (37° C) to be about 10 s. The time of dissociation of Ca(II) for α-lactalbumin is also several seconds. The involvement of Mg(II) ions slows down these processes. This suggests that the Ca(II)-dependent physiological process, in which α-lactalbumin is involved, is rather slow.

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