A calorimetric study of Ca^{2+} binding to two major isotypes of bullfrog parvalbumin

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Microcalorimetric titrations of the two major isotypes of parvalbumin (PA1 and PA2) from bullfrog skeletal muscle with Ca^{2+} in the presence and absence of Mg^{2+} have been carried out at 25°C and pH 7.0. The observed enthalpy titration curves were analyzed by the least-squares method. The measured enthalpy changes (ΔH) of Ca^{2+} binding are – 33.2 (PA1) and – 16.3 kJ/mol site (PA2), and the entropy changes (ΔS) are 28 (PA1) and 76 J/mol per K (PA2) in the absence of Mg^{2+}. When 5 mM Mg^{2+} is present, the enthalpy change of PA2 (– 26.7 kJ/mol) is about twice as large as that in the absence of Mg^{2+}, whereas that of PA1 (– 34.6 kJ/mol) is about the same. The entropy changes are 8 (PA1) and 29 J/mol per K (PA2). Both enthalpy and entropy changes are favorable for the Ca^{2+}-binding reactions of PA1 and PA2 irrespective of the presence of Mg^{2+}.

Microcalorimetry *Parvalbumin* *Ca^{2+} binding* *Mg^{2+} binding* *Enthalpy titration*

1. INTRODUCTION

Parvalbumins are water-soluble, acidic, low-M_r Ca^{2+}-binding proteins, which are present in large quantities mostly in white muscles of lower vertebrates [1,2]. Though their definite physiological function remains unknown [3], the physicochemical properties and structures of parvalbumins have been extensively studied because their primary structures are homologous to those of troponin C and calmodulin [4]. They contain two Ca^{2+}-binding sites per molecule, which also bind Mg^{2+} competitively (Ca^{2+}–Mg^{2+} sites) [2].

Parvalbumins are ubiquitously present in muscles from various sources and are classified into two genetically different categories, i.e., α- and β-types [5,6]. Some muscles contain only α-type parvalbumin(s) and others only β-parvalbumin(s). Frog skeletal muscle is especially interesting in that it contains both α- and β-parvalbumins [7–10]. So far there have been no systematic studies on the thermodynamic properties of α- and β-type parvalbumins.

Here, enthalpy titrations of the two isotypes of parvalbumin from bullfrog, *Rana catesbeiana,* with Ca^{2+} (or Mg^{2+}) were carried out to elucidate the difference in the thermodynamic properties between α- and β-parvalbumins. The results may also be relevant to the amount of 'labile' heat production which occurs in intact frog muscles early in tetanic contractions [11].

2. MATERIALS AND METHODS

The two isotypes of parvalbumin, PA1 and PA2, were extracted from skeletal muscles of bullfrogs, *R. catesbeiana,* according to Yagi et al. [12] and purified by column chromatography as described by Haiech et al. [13] with a slight modification. Each of the isolated isotypes of bullfrog parvalbumin was dialyzed against 1 mM $NaHCO_3$ and 0.2 mM dithiothreitol. The purity of each isotype was confirmed by polyacrylamide gel electrophoresis with and without SDS [14–16], and by isoelectric focusing [17]. The amount of Ca contaminated in protein solutions was determined with an atomic absorption spectrometer (Seiko SAS 727) to be less than 0.2 mol/mol protein. The protein concentrations were determined by the biuret method as described [18].

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Calorimetric titrations were carried out at 25°C in an LKB batch microcalorimeter equipped with twin gold cells. Titrations with Ca^{2+} (or Mg^{2+}) were performed by successive addition of aliquots (4.1 μl) of 25 mM $CaCl_2$ (or 50 mM $MgCl_2$) solutions using a titration apparatus mounted on the outside of calorimeter block [19,20]. The 5 ml of solution in the calorimeter cell contained 0.07–0.14 mM PA1 (or PA2), 100 mM KCl and 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes)–NaOH (pH 7.0). For titrations with Ca^{2+} in the presence of Mg^{2+}, the solution contained 5 mM $MgCl_2$ and 85 mM KCl instead of 100 mM KCl, to keep the ionic strength constant. Further details of the calorimetric titrations are given in [20,21].

To obtain exactly the heat ($-\Delta H$) attributable only to the binding of Ca^{2+} to parvalbumin, the observed heat must be corrected for the heat produced by the interaction between the buffer and protons released. The amount of protons released on the binding of Ca^{2+} (or Mg^{2+}) to parvalbumins was determined as in [18,20]; it was less than 0.06 mol/mol site for PA1 and less than 0.02 mol/mol for PA2. The observed enthalpy changes were not corrected, since the heat actually produced by the protons released was negligible, taking the enthalpy change on the protonation of Pipes as -11.46 kJ/mol [22,23].

The observed enthalpy titration curves were analyzed by the least-squares method to estimate the most probable values of the intrinsic binding constant in M^{-1} *(K)*, the enthalpy changes in $kJ \cdot mol^{-1}$ (ΔH) and the apparent mol number *(n)* of the Ca^{2+}-binding sites [20,21]. Assuming that PA1 and PA2 both have independent but equivalent Ca^{2+}-binding sites, the observed enthalpy change in $kJ \cdot mol^{-1}$ *(Q)* can be expressed as follows,

$$Q = \frac{n\Delta HK\,[\mathrm{Ca}]}{1 + K[\mathrm{Ca}]}$$

where [Ca] denotes free Ca^{2+} concentration. [Ca] is obtained from the total concentrations of Ca ($[Ca]_T$) and protein ($[PA]_T$) by solving the following equation.

$$[\mathrm{Ca}]_T = [\mathrm{Ca}] + [\mathrm{PA}]_T \frac{nK\,[\mathrm{Ca}]}{1 + K\,[\mathrm{Ca}]}$$

The standard deviations of the parameters were calculated as described [24–26].

3. RESULTS AND DISCUSSION

Fig.1 shows the enthalpy titration curves of bullfrog parvalbumins, PA1 and PA2. The reaction of Ca^{2+} binding in the absence of Mg^{2+} is exothermic for both PA1 and PA2, although Ca^{2+} binding to PA1 produces larger heat than that to PA2. The enthalpy titration curve of PA1 in the presence of 5 mM Mg^{2+} is almost identical with that in the absence of Mg^{2+}. This is in line with the observation that Mg^{2+} binding to PA1 produced little heat (fig.1a). On the other hand, the heat produced by Ca^{2+} binding to PA2 in the presence of 5 mM Mg^{2+} is larger than that in the absence of Mg^{2+}, being consistent with the heat absorbed by the binding of Mg^{2+} (fig.1b). The enthalpy titration curves in the presence of 1 mM Mg^{2+} were similar to those in the presence of 5 mM Mg^{2+} for both PA1 and PA2 (not shown), which would confirm the relatively large association constants of parvalbumins with Mg^{2+} [2,27,28].

The most probable values and standard deviations of binding parameters accounting for the enthalpy titration curves of fig.1 are summarized in table 1. Table 1 also lists the thermodynamic data derived from the binding parameters. The number of the binding sites *(n)* is near 2.0 in every case examined. This agrees with the fact that parvalbumin has two Ca^{2+}-binding sites in a molecule [2]. The association constants of Ca^{2+} are the smallest among parvalbumins from various sources [2,27–32] but agree with the values determined for those from bullfrogs by dual-wavelength spectrophotometry using a Ca^{2+} indicator (Ogawa, Y. and Tanokura, M., unpublished).

In the case of PA1, the enthalpy change associated with Ca^{2+} binding in the absence of Mg^{2+} is -33 kJ/mol site for PA1. When 5 mM Mg^{2+} is present, the enthalpy change is -35 kJ/mol site, only slightly different from that in the absence of Mg^{2+}. As for PA2, on the other hand, the enthalpy change is -16 kJ/mol site in the absence of Mg^{2+} and is approximately twice as large as that in the presence of 5 mM Mg^{2+}. The amino acid compositions of PA1 and PA2 respectively correspond to those of p*I* 4.50 and p*I* 4.88 parvalbumins from frog, *Rana esculenta* (unpublished). Since p*I* 4.50 and p*I* 4.88 respectively correspond to β- and α-parvalbumins [5,6], PA1 may be of the β-lineage and PA2 of the α-lineage. Therefore, the enthalpy

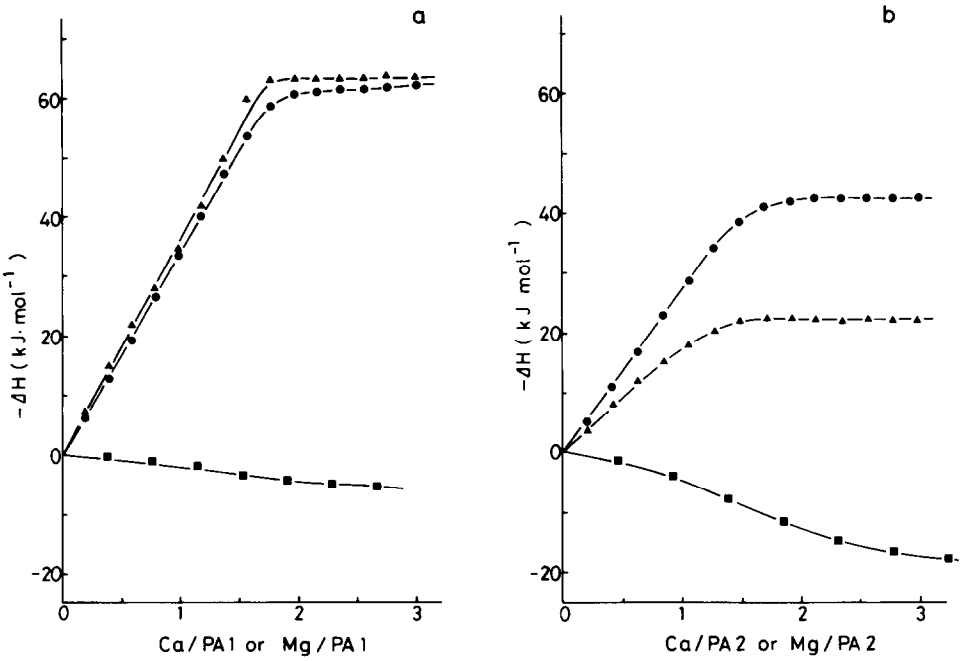

Fig.1. Enthalpy titration curves of (a) PA1 and (b) PA2 at 25°C and at pH 7.0. Titrations are: (▲) with Ca^{2+} in the absence of Mg^{2+}; (●) with Ca^{2+} in the presence of 5 mM Mg^{2+}; and (■) with Mg^{2+} in the absence of Ca^{2+}. Sample solutions were made by mixing the concentrated solution of parvalbumin (15–25 mg/ml), 0.5 M KCl (or mixture of 0.425 M KCl and 25 mM $MgCl_2$), and 0.1 M Pipes-NaOH (pH 7.0). The abscissas indicate the molar ratios of Ca^{2+} (or Mg^{2+}) to protein.

change of β-parvalbumins associated with Ca^{2+} binding might show a very small dependence on Mg^{2+} and that of α-parvalbumins a relatively large dependence on Mg^{2+}. In the absence of Mg^{2+}, the enthalpy change associated with Ca^{2+} binding to PA1 is twice as large as that of PA2, whereas the enthalpy change associated with Mg^{2+}-Ca^{2+} exchange of PA1 is similar to that of PA2.

The enthalpy change associated with Ca^{2+} binding to parvalbumin has been measured only for β-

Table 1

Thermodynamic parameters associated with binding of Ca^{2+} to bullfrog parvalbumins[a]

		n[b]	log$K$[c]	ΔH[c] (kJ/mol)	ΔG (kJ/mol)	ΔS[d] (J/mol per K)
PA1	Mg-free	1.9(2)	7.1(8)	−33.2(4)	−40.5	28
	5 mM Mg	2.0(1)	6.5(1)	−34.6(2)	−37.1	8
PA2	Mg-free	1.6(3)	6.8(6)	−16.2(4)	−38.8	76
	5 mM Mg	1.8(1)	6.2(1)	−26.7(3)	−35.4	29

[a] Measured at 25°C and at pH 7.0

[b] Figures in parentheses are 100-times the standard deviations

[c] Figures in parentheses are 10-times the standard deviations

[d] The free energy changes (ΔG) and the entropy changes (ΔS) were calculated from: $\Delta G = -2.3RT\log k = \Delta H - T\Delta S$ where R is the gas constant and T the absolute temperature

parvalbumins; carp p*I* 4.25 [31], whiting p*I* 4.4 [11] and frog p*I* 4.75 *(R. temporaria)* [33]. The enthalpy changes are −37.2 kJ (carp p*I* 4.25 at 25°C) and −18.5 kJ (frog p*I* 4.75 at 12°C) per mol site in the absence of Mg^{2+}, and −25.1 kJ (carp p*I* 4.25), −33 kJ (frog p*I* 4.75) and −22 kJ (whiting p*I* 4.4 at 12°C) per mol site in the presence of 1 mM Mg^{2+}. The enthalpy changes of carp p*I* 4.25 were measured at the same temperature as that in the present study to allow exact comparison. The enthalpy change associated with Ca^{2+} binding is similar for PA1 and carp p*I* 4.25 in the absence of Mg^{2+}. In the presence of Mg^{2+}, however, the amount is slightly different between PA1 and carp p*I* 4.25. This might be due to a species difference between the parvalbumins.

In the early stage of contraction, muscles are known to produce 'labile' maintenance heat, which could be related to the heat produced by the Mg^{2+}-Ca^{2+} exchange of parvalbumins [11]. The expected amount of heat ($-\Delta H$) produced by the Mg^{2+}-Ca^{2+} exchange of parvalbumins is calculated to be 25 mJ/g wet wt muscle. This is based on the following assumptions that (i) frog muscles contain 0.4 μmol parvalbumin/g wet wt muscle [7], (ii) parvalbumin has two metal-binding sites in a molecule and (iii) on average the heat of 31 kJ/mol site is produced by Mg^{2+}-Ca^{2+} exchange for both PA1 and PA2. In table 2 the measured labile maintenance heats from various species are summarized for comparison. The enthalpy changes of bullfrog parvalbumins associated with Mg^{2+}-Ca^{2+} exchange depend only slightly on temperature (unpublished). Therefore, more than two-thirds of the labile maintenance heat may be accounted for by the heat of Mg^{2+}-Ca^{2+} exchange of parvalbumins. However, the concentration of parvalbumin was determined with the mixture of various muscles from one species *(R. temporaria)* [7]. The concentration may differ from muscle to muscle. To discuss further the relation between the labile heat and Mg^{2+}-Ca^{2+} exchange of parvalbumins, it is necessary to measure the content of parvalbumin in the muscle with which the labile maintenance heat is observed.

Table 2

Comparison of the expected heat produced by Mg^{2+}-Ca^{2+} exchange of parvalbumins with the amount of labile maintenance heat in frog muscle cells

Muscles[a]	Heat ($-\Delta H$) (mJ/g)	Temperature (°C)	Reference
Expected heat for parvalbumin			
catesb	25	25	This work
Labile maintenance heat			
temp (sart)	37 ± 12	0	[34]
temp (sart)	22 – 30	0	[35]
temp (sart)	29 – 39	17	[35]
pip (sart)	26	0	[36]
pip (semi)	19	0	[36]

catesb, temp and pip: *R catesbeiana, R temporaria* and *R pipiens*, respectively. Sart and semi: sartorius and semitendinosus muscles, respectively

The entropy changes *(ΔS)* calculated from values of enthalpy changes and binding constants are summarized in table 1. The entropy changes obtained are positive for both PA1 and PA2 irrespective of the presence of Mg^{2+}. Thus the Ca^{2+}-binding reactions of both PA1 and PA2 are driven by favorable enthalpy and favorable entropy changes in every case examined here. The driving force for the Ca^{2+} binding to PA1 is the same as that of carp p*I* 4.25 parvalbumin. However, in both cases the entropy changes contribute little to the reactions in the presence of Mg^{2+} [31]. In accordance with PA1 and PA2, the Ca^{2+}-binding reactions to skeletal and cardiac troponin C are driven by both enthalpy and entropy changes [20,37,38]. On the other hand, the driving force for parvalbumins is separate from that for calmodulin [18]. The Ca^{2+} binding to calmodulin is driven solely by entropy changes in the absence of trifluoperazine and is driven solely by enthalpy changes in the presence of trifluoperazine. Thus, both α- and β-parvalbumins are similar in thermodynamic properties to skeletal and cardiac troponin C but are distinctly different from calmodulin.

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