A calorimetric study of Ca^{2+} binding by the parvalbumin of the toad (*Bufo*): distinguishable binding sites in the molecule

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Microcalorimetric titrations of the major isotype of parvalbumin (tPA) from toad (*Bufo*) skeletal muscle, with Ca^{2+} in the presence and absence of Mg^{2+} and with Mg^{2+} in the absence of Ca^{2+}, have been carried out at 25°C and pH 7.0. The results indicate that the two binding sites in each molecule are distinguishable from each other for both Ca^{2+} binding and Mg^{2+} binding. Such a characteristic is distinctly different from those of other parvalbumins. The enthalpy changes determined are distinctly different from those of bullfrog parvalbumins on Ca^{2+} or Mg^{2+} binding, but are similar to those on Mg^{2+}-Ca^{2+} exchange. The results indicate that the reaction of Mg^{2+}-Ca^{2+} exchange is driven almost entirely by the large favorable enthalpy change.

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

1. INTRODUCTION

Parvalbumins are a family of water-soluble, acidic, low-molecular-mass Ca^{2+}-binding proteins [1,2]. They are ubiquitously present in the skeletal muscles of vertebrates, and in especially large quantities in fish white and amphibian skeletal muscles. Although their physiological role remains uncertain [3], the physicochemical properties and structures of these proteins have been extensively studied because their amino acid sequences and the spatial structures of the Ca^{2+}-binding sites are homologous to those of troponin C and calmodulin [4–7]. Parvalbumins contain two Ca^{2+}-binding sites per molecule, which also bind Mg^{2+} competitively (Ca^{2+}-Mg^{2+} sites). The two binding sites have been considered to be equivalent to each other with regard to affinity for Ca^{2+} and Mg^{2+} [2].

Parvalbumins from various sources are classified into two genetically different categories, i.e., α- and β-types [8,9]. The skeletal muscle of every frog which has been examined contains two major isotypes of parvalbumins; one α-parvalbumin and one β-parvalbumin [10–15]. Toad (*Bufo*) muscle is interesting in that it contains only one major isotype of parvalbumin (an α-type) (unpublished). Toads are genetically close to frogs and, like frogs, their muscles have often been used in physiological experiments.

Here, enthalpy titrations of parvalbumin from the toad, *Bufo bufo japonicus* with Ca^{2+} (or Mg^{2+}) were carried out, so that by comparing the results with those of bullfrog parvalbumins [14] species differences in the thermodynamic properties among parvalbumins could be elucidated. The results indicate that the two binding sites in the toad parvalbumin molecule are not equivalent to each other with regard to Ca^{2+} or Mg^{2+} binding,

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and that the thermodynamic properties of toad parvalbumin are distinctly different from those of bullfrog parvalbumins except for Mg^{2+}-Ca^{2+} exchange.

2. MATERIALS AND METHODS

The major isotype of parvalbumin, tPA, was extracted from the skeletal muscles of the Japanese giant toad, *B. bufo japonicus*, according to Yagi et al. [16] and purified by column chromatography as described by Haiech et al. [17] with a slight modification [15]. Isolated and decalcified toad parvalbumin was dialyzed against 1 mM $NaHCO_3$ and 0.2 mM dithiothreitol. The purity was confirmed by polyacrylamide gel electrophoresis with and without SDS [18–20], and by isoelectric focusing [21]. The proteins thus prepared were in the Ca-free form after trichloroacetic acid treatment. The amount of calcium contamination in the protein solutions was determined (using a Seiko SAS 727 atomic absorption spectrometer) to be less than 0.2 mol/mol protein. Protein concentrations were determined by the biuret method by assuming the coefficient to be the same as for bullfrog parvalbumins as described [15,22,23].

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 the successive addition of a small amount (4.1 μl) of 25 mM $CaCl_2$ (or 50 mM $MgCl_2$) solutions from a titration apparatus mounted on the outside of the calorimeter block [24,25]. The calorimeter cell contained 5 ml of 0.1–0.18 mM parvalbumin, 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 [25–27].

To obtain the heat attributable to Ca^{2+} (or Mg^{2+}) binding, the observed heat must be corrected for the heat caused by the interaction between the Pipes buffer and protons released when Ca^{2+} (or Mg^{2+}) binds. The latter was determined as in [22,25]; it was less than 0.05 mol/mol binding site. The observed enthalpy changes were not corrected, since the heat actually produced by the protons released was negligible, taking the enthalpy change of the protonation of Pipes as −11.46 kJ/mol [28,29].

Assuming that the toad parvalbumin molecule has two independent Ca^{2+}-binding sites, 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_i), the enthalpy change in kJ·(mol site)$^{-1}$ (ΔH_i), and the apparent mole number (n_i) of ith Ca^{2+}-binding sites [22,25]. The observed heat in kJ·(mol protein)$^{-1}$ (Q) has the opposite sign from the enthalpy change and can be expressed as follows:

$$Q = -\sum_{i=1}^{N} \frac{n_i \Delta H_i k_i [\mathrm{Ca}]}{1 + k_i [\mathrm{Ca}]} + C \qquad (1)$$

where [Ca] denotes free Ca^{2+} concentration, N the number of classes of binding sites, and C an arbitrary constant to compensate for any error in estimating the contamination of Ca^{2+} (or Mg^{2+}). N is assumed to be 1 for the titrations with Ca^{2+} in the presence of Mg^{2+} and to be 2 for those with Ca^{2+} in the absence of Mg^{2+} and with Mg^{2+} in the absence of Ca^{2+}. [Ca] is obtained from the total concentrations of calcium ($[Ca]_T$) and protein ($[tPA]_T$) by solving the following equation.

$$[\mathrm{Ca}]_T = [\mathrm{Ca}] + [\mathrm{tPA}]_T \sum_{i=1}^{N} \frac{n_i k_i [\mathrm{Ca}]}{1 + k_i [\mathrm{Ca}]} \qquad (2)$$

For reasons explained below the number of degrees of freedom is 7 for the Mg^{2+} titration (in the absence of Ca^{2+}), 4 for the Ca^{2+} titration in the presence of 5 mM Mg^{2+}, and 5 for the Ca^{2+} titration (in the absence of Mg^{2+}) after assuming the values of the binding constants.

3. RESULTS AND DISCUSSION

Fig.1 shows calorimetric records obtained during the titration of toad parvalbumin with Ca^{2+} in a Mg^{2+}-free solution. The amount of heat produced by each addition of Ca^{2+} was gradually decreased during the first part of the titration, increased during the next part, and decreased gradually again to zero during the remaining part of the titration. If the two Ca^{2+}-binding sites are equivalent to each other, it is expected as described

previously that the amount of heat produced by each addition of Ca^{2+} would not increase but decrease monotonically during a titration [22]. Therefore, the results indicate that the two Ca^{2+}-binding sites in the toad parvalbumin molecule are not equivalent to, but are distinguishable from, each other.

In fig.2, cumulative values of the heat produced ($-\Delta H$) are plotted vs the molar ratio of added Ca^{2+} (or Mg^{2+}) to toad parvalbumin. The titration curve with Ca^{2+} in the absence of Mg^{2+} shows an inflection at around 0.8–1.0 of the molar ratio of Ca^{2+} to protein.

The total enthalpy changes in Mg^{2+} binding to the two sites of toad parvalbumin in the absence of Ca^{2+} can be measured as the plateau value of the titration curve in fig.2. It explains well the difference between those in Ca^{2+} binding in the absence and presence of Mg^{2+}. No significant enthalpy changes were observed on the titration with Mg^{2+} in the presence of 0.4 mM $CaCl_2$ (not shown). These results indicate that Ca^{2+} displaces Mg^{2+} at each of the two binding sites of toad parvalbumin (Ca^{2+}-Mg^{2+} sites).

The titration curve of toad parvalbumin with Mg^{2+} in the absence of Ca^{2+} appears to reach a plateau at about 1.0 mol Mg^{2+} per mol protein, although this plateau is followed by a small amount of gradual heat absorption. To confirm that this is not due to the partial denaturation of the protein, the solution which had been titrated

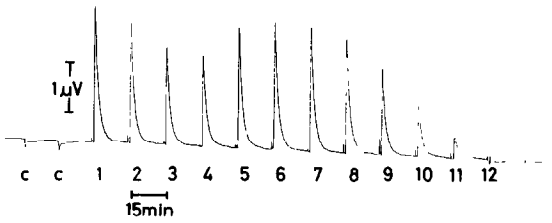

Fig.1. Part of the calorimetric record obtained during an enthalpy titration with Ca^{2+} of toad skeletal muscle parvalbumin (106 μM) in the absence of Mg^{2+} at 25°C. Upward displacement in the record indicates the production of heat. The calorimeter block was rotated, to mix the cell contents, marked by C. Later rotations were accompanied with an injection (4.1 μl) of Ca^{2+} solution (25 mM), denoted by numbers. The heat attributable to the reaction was obtained by integration of the calorimeter record and then by subtraction of the heat due to rotation without injection.

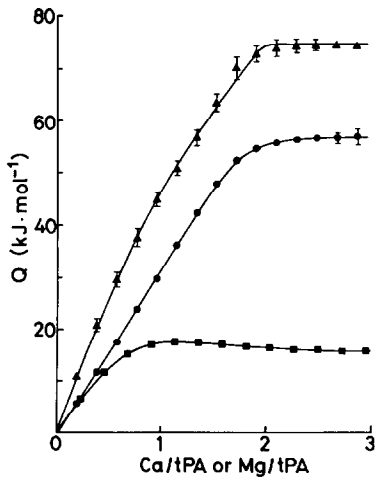

Fig.2. Enthalpy titration curves of toad parvalbumin (tPA) at 25°C and at pH 7.0. Titrations: (▲) with Ca^{2+} (in the absence of Mg^{2+}); (●) with Ca^{2+} in the presence of 5 mM Mg^{2+}; and (■) with Mg^{2+} (in the absence of Ca^{2+}). The abscissa indicates the molar ratio of Ca^{2+} (or Mg^{2+}) to protein and the ordinate the heat produced in kJ per mol protein. Solid lines in the titration profiles are the theoretical curves calculated according to eqns 1 and 2 described in section 2 by inserting the values listed in table 1. The standard deviations of individual data are indicated by error bars when they are greater than the size of the symbols.

with Mg^{2+} was subjected to a titration with Ca^{2+} (that corresponds to the titration with Ca^{2+} in the presence of about 0.8 mM Mg^{2+}). The observed titration curve (not shown) reached a plateau at about 2.0 mol Ca^{2+}/mol protein. This indicates that the protein in the solution used for the Mg^{2+} titration was intact and that Mg^{2+} binding to the second site of toad parvalbumin absorbs a small amount of heat. The titration curve with Ca^{2+} in the presence of 0.8 mM Mg^{2+} was superimposable on that in the presence of 5 mM Mg^{2+}. This agrees with the large binding constant of Mg^{2+} to parvalbumins [2,30–35].

Thus the titration curves of toad parvalbumin, with Ca^{2+} (in the absence of Mg^{2+}) and with Mg^{2+} (in the absence of Ca^{2+}), were analyzed in terms of two classes of binding sites. The titration curve with Ca^{2+} in the presence of 5 mM Mg^{2+} was fitted well to the theoretical curve calculated by assuming the two binding sites are equivalent to each other (fig.2).

The most probable values of the binding parameters accounting for the enthalpy titration curves of fig.2 are summarized in table 1. The number of binding sites per molecule is approx. 2.0 in every observation. This agrees with the known fact that parvalbumins have two Ca^{2+}-Mg^{2+} sites in each molecule [2]. The binding constants of Ca^{2+} in the absence of Mg^{2+} (k_{Ca}) are too large to be determined from the observed enthalpy titration curves. They are, therefore, calculated by using the observation that Ca^{2+} competes with Mg^{2+} for the same binding sites. The value of k_{Ca} is expressed according to the equation: $k_{Ca} = k_{Ca}' \cdot (1 + k_{Mg}[Mg])$ where k_{Ca}' denotes the binding constants of Ca^{2+} in the presence of Mg^{2+}, k_{Mg} the binding constant of Mg^{2+}, and [Mg] the molar concentration of Mg^{2+}. The binding constants are higher than those observed with bullfrog parvalbumins [14,23] but are in the range of values determined for parvalbumins from various other sources [2,30–35].

The enthalpy change associated with Ca^{2+} binding in the absence of Mg^{2+} is -56 kJ for the first binding site and -24 kJ/mol site for the second site as listed in table 1. These values are estimated within ± 5 kJ/mol site for Ca^{2+} binding (in the absence of Mg^{2+}) and Mg^{2+} binding (in the absence of Ca^{2+}) and within ± 1 kJ/mol site for Ca^{2+} binding in the presence of Mg^{2+}. When Mg^{2+} is present, the enthalpy change is -32 kJ/mol site for each site. The enthalpy change associated with Mg^{2+} binding is -33 kJ/mol site for the first binding site and 9 kJ/mol site for the second site. For the first binding site, the enthalpy change associated with Mg^{2+} binding is not in perfect agreement with the difference between those associated with Ca^{2+} binding in the presence and absence of Mg^{2+}. This might indicate that the two binding sites are distinguishable from each other even for the Ca^{2+} binding in the presence of Mg^{2+} or might be due to an error in estimation by the least-squares method. For the second site, the enthalpy change associated with Mg^{2+} binding agrees well with the difference between those associated with Ca^{2+} binding in the presence of Mg^{2+}. As a whole, therefore, the values are consistent with the idea that Mg^{2+} competes with Ca^{2+} for the same binding sites.

Table 1

Binding parameters and thermodynamic functions associated with binding of Ca^{2+} or Mg^{2+} to toad parvalbumin[a]

Reaction	Site	n	log$k$	ΔH (kJ/mol)	ΔG[b] (kJ/mol)	ΔS[b] (J/mol per K)
Ca^{2+} binding	1	1.0	9.5[c]	−56	−54.2	−8
	2	1.1	8.3[c]	−24	−47.4	80
Mg^{2+}-Ca^{2+} exchange[d]	1,2	2.0	6.1	−32	−34.8	10
Mg^{2+} binding	1	0.9	5.7	−33	−32.5	0
	2	0.9	4.5	9	−25.7	118

[a] Measured at 25°C and pH 7.0

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

nal assumption. See text
f Mg^{2+}

Data (unpublished) on the amino acid composition and isoelectric point of toad parvalbumin suggest that it is an α-parvalbumin. Thus it corresponds genetically to the following isotypes of frog parvalbumins: *Rana catesbeiana* (bullfrog) p*I* 4.97 (PA2), *R. esculenta* p*I* 4.88, and *R. temporaria* p*I* 4.97. In the previous paper, we described the possibility that the Mg^{2+} dependence of the enthalpy change associated with Ca^{2+} binding is small for β-parvalbumins and relatively large for α-parvalbumins [14]. This is the case for toad parvalbumin as well since there is a large difference between the enthalpy changes associated with Ca^{2+} binding in the absence and presence of Mg^{2+} (table 1).

The enthalpy changes of toad parvalbumin are quite different from those of bullfrog parvalbumins, at least for the Ca^{2+} binding to the first binding site (in the absence of Mg^{2+}) and for the Mg^{2+} binding to the first site (in the absence of Ca^{2+}) [14]. The enthalpy change associated with Ca^{2+} binding is -33.2 kJ for PA1 and -16.2 kJ per mol site for PA2. However, the enthalpy change associated with Ca^{2+} binding in the presence of Mg^{2+} is similar to those of bullfrog PA1 (-34.6 kJ/mol site) or PA2 (-26.7 kJ/mol site). This last fact may be important from the energetic point of view, because physiological reac-

tions occur in the presence of more than 1 mM Mg^{2+} [36]. It may also suggest that the enthalpy changes associated with Mg^{2+}-Ca^{2+} exchange are similar to one another among the various isotypes of amphibian parvalbumins: the average value (−31 kJ/mol site) of toad parvalbumin and bullfrog parvalbumins may be used as the enthalpy change associated with Mg^{2+}-Ca^{2+} exchange of amphibian parvalbumins.

The enthalpy changes associated with Ca^{2+} binding to (or Mg^{2+}-Ca^{2+} exchange on) parvalbumins have been measured only for β-parvalbumins other than bullfrog PA1 (β-type) and PA2 (α-type); (i) carp p*I* 4.25 [34], (ii) whiting p*I* 4.4 [37], and (iii) frog p*I* 4.75 (*R. temporaria*) [38]. The enthalpy changes are (i) −37.2 kJ (25°C) and (ii) −19 kJ (12°C) per mol site for Ca^{2+} binding, and (i) −25.1 kJ, (ii) −27 kJ (12°C) and (iii) −33 kJ per mol site for Mg^{2+}-Ca^{2+} exchange. For the parvalbumins, the enthalpy changes associated with Mg^{2+}-Ca^{2+} exchange are in the relatively narrow range (−25 to −35 kJ/mol site) and are less dependent on temperature ([39], unpublished). Therefore, the value described above (−31 kJ/mol site) may be taken to be typical for the parvalbumins from various vertebrates.

In the early stage of contraction, muscles are known to produce 'labile' maintenance heat, which could be related to the heat produced by Mg^{2+}-Ca^{2+} exchange on parvalbumin [36,37]. The value of the enthalpy change associated with Mg^{2+}-Ca^{2+} exchange on the 'ideal' amphibian parvalbumin proposed here is just the same as that used to estimate the amount of heat ($-\Delta H$) expected for Mg^{2+}-Ca^{2+} exchange of parvalbumins in intact frog skeletal muscles in a previous paper [14]. Therefore, a labile heat production of about 25 mJ/g wet wt muscle is expected for the Mg^{2+}-Ca^{2+} exchange of parvalbumins in intact muscles, indicating that more than two-thirds of the labile maintenance heat may be accounted for by the heat of Mg^{2+}-Ca^{2+} exchange of parvalbumins.

The changes in free energy (ΔG) and entropy (ΔS) calculated from the values of enthalpy changes and binding constants are also summarized in table 1. The entropy change associated with Ca^{2+} binding to the first site is unfavorably negative, indicating that the binding of Ca^{2+} to the first site of toad parvalbumin is driven solely by the favorable large negative enthalpy change. The entropy change associated with Ca^{2+} binding to the second site is favorably positive and the reaction is driven by both the enthalpy and entropy changes. The free energy change associated with Mg^{2+}-Ca^{2+} exchange (−34.8 kJ/mol site) is contributed almost entirely by the favorable enthalpy change (−32 kJ/mol site), indicating that the entropy change contributes little to driving the reaction. This is also the case for the Mg^{2+} binding to the first site. The Mg^{2+} binding to the second site is characterized by an unfavorable positive enthalpy change and hence the reaction is driven solely by the favorable entropy change.

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