Heat Capacity and Entropy Changes of the Two Major Isotypes of Bullfrog (*Rana catesbeiana*) Parvalbumins Induced by Calcium Binding†

Masaru Tanokura* and Kazuhiro Yamada*

Department of Physiology, Medical College of Oita, Hasama, Oita 879-56, Japan

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ABSTRACT: The possible structural changes of the two major isotypes (PA1 and PA2) of parvalbumins from bullfrog (*Rana catesbeiana*) skeletal muscle caused by Ca^{2+} binding have been analyzed by microcalorimetric titrations. Titrations of the parvalbumins with Ca^{2+} have been made in both the absence and presence of Mg^{2+} at pH 7.0 and at 5, 15, and 25 °C. The reactions of the parvalbumins with Ca^{2+} are exothermic in both the presence and absence of Mg^{2+} and at every temperature. But the contributions of enthalpy and entropy changes are variable; Mg^{2+}–Ca^{2+} exchange on PA1 at 25 °C is driven almost entirely by a favorable enthalpy change, whereas Ca^{2+} binding to PA2 at 5 °C is driven for the most part by a favorable entropy change. The magnitudes of the hydrophobic and internal vibrational contributions to the heat capacity and entropy changes of the parvalbumins on Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange have been estimated by the empirical method of Sturtevant [Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240]. Although PA1 (β) and PA2 (α) belong to genetically different lineages, the parvalbumins indicate very similar conformational changes to each other on both Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange. On Mg^{2+}–Ca^{2+} exchange, the vibrational as well as hydrophobic entropy is slightly increased in a parallel manner. In contrast, on Ca^{2+} binding, the hydrophobic entropy increases but the vibrational entropy decreases. The increase in the hydrophobic entropy indicates the sequestering of nonpolar groups from the surface to the interior of molecules, while the changes in the vibrational entropy suggest that the overall structures are tightened on Ca^{2+} binding but loosened on Mg^{2+}–Ca^{2+} exchange.

Parvalbumins are water-soluble, acidic, low M_r (10000–12000), Ca^{2+} binding proteins, which are ubiquitously present in the skeletal muscles of vertebrates [see Pechère et al. (1973) and Wnuk et al. (1982) for reviews]. They occur in high concentration in fish white and amphibian skeletal muscles and in lower concentrations in the muscles of higher vertebrates. Although their definite physiological role remains unknown (Gosselin-Ray et al., 1978), the physicochemical properties and structures of parvalbumins have been extensively studied because their primary and tertiary structures of Ca^{2+} binding sites are homologous to those of troponin C and calmodulin (Dedman et al., 1978; Babu et al., 1985; Herzberg & James, 1985; Sandaralingam et al., 1985).

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+} [see Wnuk et al. (1982) for review] but were distinguishable from each other with circular dichroism (Donato & Martin, 1974), ^{13}C nuclear magnetic resonance (Nelson et al., 1976), ^1H nuclear magnetic resonance (Birdsall et al., 1979), tryptophan fluorescence (Permyakow et al., 1980), and calorimetry (Tanokura et al., 1986b).

Parvalbumins from various sources are classified into two genetically different categories, i.e., α- and β-types (Goodman & Pechère, 1977; Goodman et al., 1979). Some muscles contain only α-parvalbumin(s) and others only β-parvalbumin(s). Frog skeletal muscle is especially interesting in that it contains both α- and β-parvalbumins (Capony et al., 1975; Gosselin-Rey & Gerday, 1977; Gillis et al., 1979; Jauregui-Adell et al., 1982; Tanokura & Yamada, 1985a; Tanokura et al., 1986a). The properties of α- and β-parvalbumins in Ca^{2+} and Mg^{2+} binding were compared by dialysis and ultraviolet spectroscopy (Haiech et al., 1979a) and by ^1H nuclear magnetic resonance (Williams et al., 1986). There is no report on the comparison of Ca^{2+} binding properties between α- and β-parvalbumins from the thermodynamic point of view.

Among the physicochemical techniques, calorimetry can provide unique information on conformational changes on the basis of thermodynamic properties. It has been used to examine possible conformational changes associated with Ca^{2+} binding to troponin C (Potter et al., 1977; Yamada, 1978; Yamada & Kometani, 1982; Kometani & Yamada, 1983; Imaizumi et al., 1987), calmodulin (Tanokura & Yamada, 1984), calmodulin in the presence of its inhibitor, trifluoperazine (Tanokura & Yamada, 1986), and parvalbumin (Moeshler et al., 1980).

In the present study, we carried out calorimetric titrations of the two major isotypes of parvalbumins (PA1[1] and PA2) from bullfrog, *Rana catesbeiana*, with Ca^{2+} in the presence and absence of Mg^{2+} at pH 7.0 and at 5, 15, and 25 °C. Enthalpy changes and binding constants were determined by analyzing the observed enthalpy titration profiles. The changes in heat capacity were obtained from the enthalpy changes at different temperatures, and the changes in entropy were obtained from the binding constants and enthalpy changes. On the basis of the results, the hydrophobic and the vibrational contributions to the heat capacity and entropy changes of bullfrog parvalbumins on Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange are discussed.

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[1] Abbreviations: PA1, p*I* 4.78 isotype of parvalbumin from *R. catesbeiana*; PA2, p*I* 4.97 isotype of *R. catesbeiana* parvalbumin; Pipes, 1,4-piperazinediethanesulfonic acid.

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Materials and Methods

Materials. All chemicals were of reagent grade or the purest commercially available. PA1 and PA2 (Tanokura et al., 1986a) were extracted from the skeletal muscles of bullfrog, *Rana catesbeiana*, according to the method reported in earlier studies (Yazawa et al., 1980; Yagi et al., 1982) and purified by column chromatography as described previously (Haiech et al., 1979b) with a slight modification. Isolated parvalbumins were dialyzed against 1 mM $NaHCO_3$ and 0.2 mM dithiothreitol. The purity was confirmed by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate (Laemmli, 1970; Head & Perry, 1974; Tanokura et al., 1983) and by isoelectric focusing (Pechère et al., 1971). The proteins thus prepared were in the Ca-free form after trichloroacetic acid treatment (Tanokura & Yamada, 1983). The amount of calcium contamination in the protein solutions was less than 0.2 mol per mol of protein.

Methods. Protein concentrations were determined by the biuret method, in which the coefficients were obtained on the basis of amino acid analysis (Tanokura & Yamada, 1984; Ogawa & Tanokura, 1986a; Tanokura et al., 1986a). Calcium and magnesium were measured by atomic absorption spectrophotometry (Seiko SAS 727).

Calorimetric titrations were carried out at 5, 15, and 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 (Kodama & Woledge, 1976; Yamada & Kometani, 1982). The calorimeter cell contained 5 mL of 105–124 μM PA1 (or 89–113 μM PA2), 100 mM KCl, and 20 mM 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. Since the observed results were independent of the duration between the installation of protein solutions and the measurements for up to three days, it seemed unlikely that protein was absorbed to the walls of the cell or that metal ions leached from the cells. Further details of the calorimetric titrations were given in previous papers (Yamada & Kometani, 1982; Tanokura & Yamada, 1983, 1985b).

Amounts of Protons Released. 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 by measuring the amount of NaOH required for maintaining the pH at 7.0 when a Ca^{2+} (or Mg^{2+}) solution was mixed with a parvalbumin solution without Pipes buffer (Yamada & Kometani, 1982; Tanokura & Yamada, 1983, 1984).

Analysis of Calorimetric Data. Assuming that each of the PA1 and PA2 molecules has two independent Ca^{2+} binding sites, the oserved 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 change in kJ·(mol of site)$^{-1}$ (ΔH), and the apparent mole number (n) (Yamada & Kometani, 1982; Tanokura & Yamada, 1984, 1985a, 1986). The observed heat in kJ·(mol of protein)$^{-1}$ (Q) has the opposite sign from the enthalpy change and can be expressed as

$$Q = -\frac{(n - n_0)\Delta HK[\mathrm{Ca}]}{1 + K[\mathrm{Ca}]} + C \qquad (1)$$

where n_0, [Ca], and C denote the molar ratio of calcium contamination to protein, free Ca^{2+} concentration, and an arbitrary constant to compensate for any error in estimating the contamination of Ca^{2+} (or Mg^{2+}), respectively. [Ca] is obtained from the total concentrations of calcium ($[Ca]_T$) and protein ($[PA]_T$) by the equation

$$[\mathrm{Ca}]_T = [\mathrm{Ca}] + [\mathrm{PA}]_T \frac{nK[\mathrm{Ca}]}{1 + K[\mathrm{Ca}]} \qquad (2)$$

The standard deviations of the parameters were calculated as described in previous papers (Tanokura et al., 1976; Tanokura, 1983a,b).

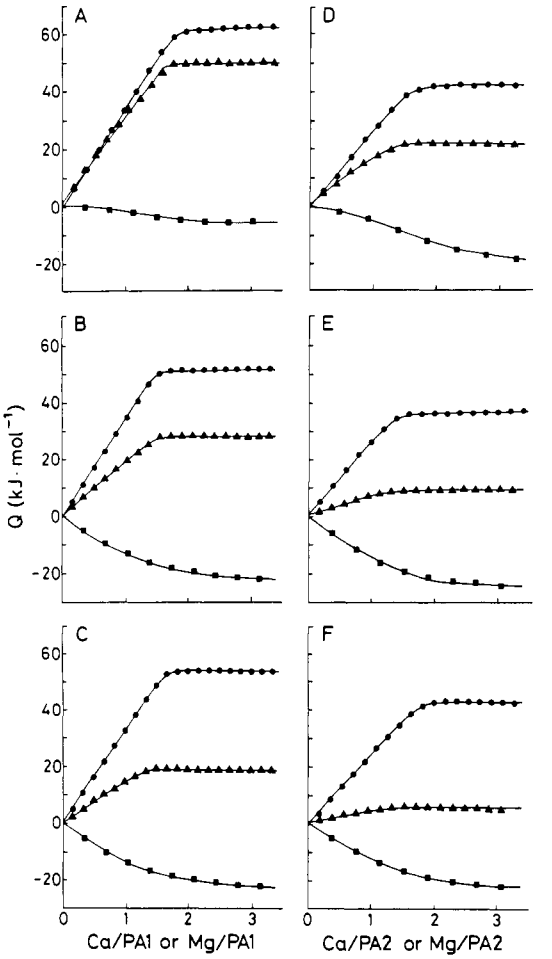

FIGURE 1: Enthalpy titration curves of PA1 and PA2 at various temperatures and at pH 7.0. The abscissas indicate the molar ratio of Ca^{2+} (or Mg^{2+}) added to parvalbumin and the ordinates the observed heat (Q). Titrations are (▲) with Ca^{2+} in the absence of Mg^{2+}, (●) with Ca^{2+} in the presence of 5 mM Mg^{2+}, and (■) with Mg^{2+} in the absence of Ca^{2+}. (A) PA1 at 25 °C; (B) PA1 at 15 °C; (C) PA1 at 5 °C; (D) PA2 at 25 °C; (E) PA2 at 15 °C; (F) PA2 at 5 °C.

Results and Discussion

Amounts of Protons Released on Ca^{2+} (or Mg^{2+}) Binding. The observed enthalpy changes were corrected for those associated with the amount of protons released, the enthalpy change in the protonation of Pipes taken as −11.46 kJ·mol^{-1} at every temperature mentioned here (Beres & Sturtevant, 1971; Kodama et al., 1984). The maximum of the correction values was 1.6 kJ per mol of Ca^{2+} binding site, for Ca^{2+} binding to PA1 at 5 °C.

Enthalpy Titration Curves of Bullfrog Parvalbumins. Figure 1 shows the results of enthalpy titrations of bullfrog parvalbumins at pH 7.0 and at various temperatures. All the reactions of Ca^{2+} binding are exothermic in both the presence

Table I: Analysis of Enthalpy Titration Curves—Numbers of Sites, Association Constants, and Enthalpy Changes Associated with the Ca^{2+} Binding to and Mg^{2+}–Ca^{2+} Exchange on the Ca^{2+} Binding Sites of Bullfrog Parvalbumins at pH 7.0[a]

	temp (°C)	n[b]	log K[c]	ΔH[c] (kJ·mol^{-1})
PA1				
Ca^{2+} binding	5	1.7 (2)	9.0[d]	−12.9 (2)
	15	1.8 (1)	8.1[d]	−17.5 (1)
	25	1.9 (2)	7.1 (8)	−33.2 (4)
Mg^{2+}–Ca^{2+} exchange[e]	5	2.0 (1)	7.4[d]	−31.8 (2)
	15	1.8 (1)	6.9[d]	−34.7 (2)
	25	2.0 (1)	6.5 (1)	−34.6 (2)
PA2				
Ca^{2+} binding	5	1.7 (5)	8.5[d]	−3.2 (2)
	15	1.7 (3)	7.6[d]	−5.9 (1)
	25	1.6 (3)	6.8 (6)	−16.2 (4)
Mg^{2+}–Ca^{2+} exchange[e]	5	2.0 (1)	6.6[d]	−23.8 (1)
	15	1.7 (1)	6.4[d]	−26.0 (2)
	25	1.8 (1)	6.2 (1)	−26.7 (1)

[a] PA1 is a β-parvalbumin and PA2 an α-parvalbumin. [b] Figures in parentheses are 100 times the standard deviations. [c] Figures in parentheses are 10 times the standard deviations. [d] The value has been calculated on the basis of the values at 25 °C and the temperature coefficient as found for bullfrog parvalbumins with tetramethylmurexide and a dual-wavelength spectrophotometer (Ogawa & Tanokura, 1986a). [e] Ca^{2+} titrations in the presence of 5 mM Mg^{2+}.

and absence of Mg^{2+}, whereas the reactions of Mg^{2+} binding are endothermic. Every titration curve reached a plateau at around 2 mol of Ca^{2+} (or Mg^{2+}) per mol of protein. These agree with the fact that parvalbumins bind 2 mol of divalent metal ions per mol of protein (Pechère et al., 1973; Wnuk et al., 1982). (1) As for plateau values, which indicate the overall enthalpy changes when the parvalbumins bind 2 mol of Ca^{2+} (or Mg^{2+}), the differences between those of Ca^{2+} binding in the presence and absence of Mg^{2+} are almost equal to those of Mg^{2+} binding. (2) No enthalpy changes were observed with the Mg^{2+} titrations in the presence of 0.4 mM Ca^{2+} for either PA1 or PA2 at any temperature examined in this work (data not shown). Therefore, Ca^{2+} displaced Mg^{2+} at each of the two binding sites for both PA1 and PA2. This agrees with the results of equilibrium and kinetics studies on bullfrog parvalbumins with dual-wavelength spectrophotomers and a metallo-indicator of Ca^{2+}, tetramethylmurexide (Ogawa & Tanokura, 1986a,b), and is in line with the results reported for other parvalbumins (Pechère et al., 1973; Wnuk et al., 1982).

Analysis of Enthalpy Titration Curves: Numbers of Sites, Association Constants, and Enthalpy Changes. The most probable values of binding parameters that account for the enthalpy titration curves of Figure 1 are summarized in Table I. The standard deviations of parameters are small enough to indicate that all the titration curves agreed well with the theoretical curves under the assumption that the two Ca^{2+} binding sites be equivalent to, and independent of, each other. The number of binding sites per molecule is approximately two in every observation. This agrees with the known fact that parvalbumins have two Ca^{2+}–Mg^{2+} sites in each molecule (Wnuk et al., 1982). Thus, the bullfrog parvalbumins have two Ca^{2+} binding sites equivalent to each other. This is of the same nature as carp pI 4.25 parvalbumin but is distinctly different from the toad parvalbumin as observed by calorimetry (Moeschler et al., 1980; Tanokura et al., 1986b).

Because of the relatively large temperature dependence of binding constants for Ca^{2+}, most of those at 5 and 15 °C are too large to be determined from the observed enthalpy titration curves. They were, therefore, calculated from the values at 25 °C determined in the present study, and the temperature

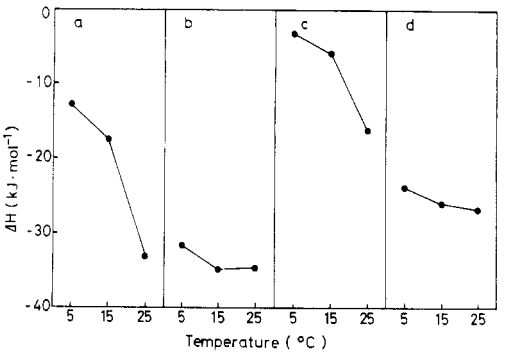

FIGURE 2: Temperature dependence of enthalpy changes associated with Ca^{2+} binding to and Mg^{2+}–Ca^{2+} exchange on bullfrog parvalbumins: (a) PA1 on Ca^{2+} binding; (b) PA1 on Mg^{2+}–Ca^{2+} exchange; (c) PA2 on Ca^{2+} binding; (d) PA2 on Mg^{2+}–Ca^{2+} exchange.

dependence was obtained for the same proteins at 10 and 20 °C by means of a metallo-indicator of Ca^{2+}, tetramethylmurexide, and a dual-wavelength spectrophotometer, where the ionic strength of sample solution was the same as that in the present study (Ogawa & Tanokura, 1986a).

For both PA1 and PA2, the temperature dependence of the enthalpy change was apparent for Ca^{2+} binding (in the absence of Mg^{2+}), but the reactions were almost independent of temperature for Mg^{2+}–Ca^{2+} exchange (in the presence of Mg^{2+}) (Table I and Figure 2). This is similar to skeletal and cardiac troponin C (Yamada & Kometani, 1982; Kometani & Yamada, 1983) and calmodulin–trifluoperazine complex (Tanokura & Yamada, 1986); for the calmodulin molecule temperature dependence was not clear because of small enthalpy changes associated with Ca^{2+} binding (Tanokura & Yamada, 1984). It may, therefore, be a common property among Ca^{2+} binding proteins that the enthalpy change associated with Ca^{2+} binding is independent of temperature in the presence of Mg^{2+}, whereas it is dependent on temperature in the absence of Mg^{2+}.

If enthalpy change is independent of temperature, it can be obtained from the temperature dependence of the binding constant according to the van't Hoff equation: $\mathrm{d} \ln K/\mathrm{d}T^{-1} = -\Delta H \cdot R^{-1}$, where T is the absolute temperature and R the gas constant. The enthalpy changes for PA1 and PA2 on Mg^{2+}–Ca^{2+} exchange are nearly constant over the temperature range studied, although they appear to become smaller in their absolute values at lower temperatures. According to the van't Hoff equation the enthalpy change is calculated to be −71 and −32 kJ per mol of binding site for PA1 and PA2, respectively, from the plot of ln K versus T^{-1}. For PA2 the observed enthalpy change is in fairly good agreement with that obtained according to the van't Hoff plot. For PA1, however, the van't Hoff enthalpy change is twice as large as that determined by calorimetry. This might indicate that the van't Hoff equation is not always applicable to proteins even if their enthalpy changes are independent of temperature.

Enthalpy changes associated with Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange have been examined for the parvalbumins from various sources: whiting pI 4.4, carp pI 4.25, frog (*R. temporaria*) pI 4.75, and toad pI 4.81 parvalbumins (Curtin & Woledge, 1978; Moeschler et al., 1980; Smith & Woledge, 1985; Tanokura et al., 1986b). For Ca^{2+} binding, the enthalpy changes of genetically α-type parvalbumins are diverse (−16 to −56 kJ per mol of site at 25 °C), whereas β-parvalbumins show enthalpy changes similar to one another (−33 to −37 kJ per mol of site at 25 °C) when the rather steep dependence on temperature is considered. For Mg^{2+}–Ca^{2+} exchange, the enthalpy changes are in a rather narrow range (−24 to −35 kJ per mol of site) for both of α- and β-parval-

Table II: Thermodynamic Functions of Bullfrog Parvalbumins Associated with Ca^{2+} Binding and Mg^{2+}–Ca^{2+} Exchange

	temp (°C)	$\Delta G°$ (kJ·mol^{-1})	$\Delta S°$ (J·mol^{-1}·K^{-1})	ΔS_u(PA) (J·mol^{-1}·K^{-1})	$\Delta C_p°$ (J·mol^{-1}·K^{-1})	ΔC_p(PA) (J·mol^{-1}·K^{-1})
PA1						
Ca^{2+} binding	5	−47.9	126	61	−460	−619
	15	−44.7	94	29	−1570	−1174
	25	−40.5	25	−40		−1729
Mg^{2+}–Ca^{2+} exchange	5	−39.4	27	90	−290	−344
	15	−38.0	12	74	10	−194
	25	−37.1	8	71		−44
PA2						
Ca^{2+} binding	5	−45.2	151	86	−270	−429
	15	−41.9	125	60	−1030	−809
	25	−38.8	76	11		−1189
Mg^{2+}–Ca^{2+} exchange	5	−35.1	41	103	−220	−274
	15	−35.3	32	95	−70	−199
	25	−35.4	29	92		−124

bumins at various temperatures between 5 and 25 °C.

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 (Curtin & Woledge, 1978; Woledge et al., 1985). In previous papers, we have proposed the typical value of enthalpy change associated with Mg^{2+}–Ca^{2+} exchange for the parvalbumins from various vertebrates (−31 kJ per mol of site) (Tanokura & Yamada, 1985a; Tanokura et al., 1986b). The enthalpy change of −30 kJ per mol of site for an "ideal" parvalbumin, which is an average of 10 observations in the present work (Table I) and previous studies, is close to the value given previously (Tanokura & Yamada, 1985a; Tanokura et al., 1986b). The expected amount of heat ($-\Delta H$) produced by Mg^{2+}–Ca^{2+} exchange on parvalbumins is calculated to be 24 mJ per g of wet weight of muscle. This is based on the additional assumptions that (1) frog muscles contain 0.4 μmol of parvalbumin per g of wet weight (Gosselin-Rey & Gerday, 1977; Haiech et al., 1979b; Tanokura et al., 1986a) and (2) all parvalbumin molecules are in Mg^{2+}-bound form in resting muscles and become Ca^{2+}-bound after an adequate time of tetanic contraction. The measured labile maintenance heat from various species of frogs ranges from 19 to 37 mJ per g of wet weight (Homsher et al., 1972; Curtin & Woledge, 1977; Homsher & Kean, 1978). Thus, at least more than two-thirds of labile maintenance heat may be accounted for by the heat of Mg^{2+}–Ca^{2+} exchange of parvalbumins. However, the parvalbumin concentration may differ from muscle to muscle, and in addition most parvalbumin molecules may not always be in Mg^{2+}-bound form in resting muscles due to the Ca^{2+} and Mg^{2+} binding constants of parvalbumins and intracellular free Ca^{2+} and Mg^{2+} concentrations (Ogawa & Tanokura, 1986a). Therefore, to discuss further the relation between the labile heat and Mg^{2+}–Ca^{2+} exchange on parvalbumins, it is necessary to measure the content of parvalbumin(s) and the concentration of Ca^{2+} and Mg^{2+} in the resting state in the muscle with which the labile maintenance heat is observed.

Changes in Free Energy, Entropy, and Heat Capacity. The standard free energy change ($\Delta G°$) associated with Ca^{2+} binding to or Mg^{2+}–Ca^{2+} exchange on a site was calculated from the value of log K listed in Table I according to the equation

$$\Delta G° = -2.3RT \log K \quad (3)$$

The entropy change associated with Ca^{2+} binding or Mg^{2+}–Ca^{2+} exchange on a site ($\Delta S°$) was then obtained from $\Delta G°$ and ΔH according to the equation

$$\Delta S° = (\Delta H - \Delta G°)T^{-1} \quad (4)$$

The values of $\Delta S°$ are summarized in Table II. The entropy changes are positive and hence make favorable contributions as the driving force of the reactions. Thus the reactions are driven by both favorable enthalpy and entropy changes. The entropy changes are smaller in PA1 than in PA2, for Mg^{2+}–Ca^{2+} exchange than for Ca^{2+} binding, and at higher temperatures than at lower temperatures. Therefore, the contribution of entropy changes to the progress of reactions becomes relatively smaller at higher temperatures than at lower temperatures and for Mg^{2+}–Ca^{2+} exchange than for Ca^{2+} binding.

For Ca^{2+} binding, $\Delta S°$ is the overall entropy change for the following reaction and hence contains the contributions of both parvalbumin and Ca^{2+}

$$PA + Ca^{2+} \rightleftharpoons PA\text{–}Ca \quad (5)$$

where PA denotes either PA1 or PA2. Thus

$$\Delta S° = S°(PA\text{–}Ca) - S°(PA) - S°(Ca^{2+}) \quad (6)$$

where $S°$ is the standard aqueous partial molar entropy of the species enclosed in parentheses. The partial molar entropy can be divided into two parts, the unitary and the cratic (Gurney, 1953; Kauzmann, 1959)

$$S° = S_u° - R \ln \chi \quad (7)$$

where $S_u°$ is the unitary part of entropy and χ is the mole fraction, which is equal to 0.018. Therefore, the unitary part of the entropy change of the parvalbumin molecule alone associated with Ca^{2+} binding to a site ΔS_u(PA) is obtained by the equation

$$\Delta S_u(PA) = S_u°(PA\text{–}Ca) - S_u°(PA) \quad (8)$$

From eq 6–8

$$\Delta S_u(PA) = \Delta S° + S°(Ca^{2+}) \quad (9)$$

The value of $S°(Ca^{2+})$ at 25 °C is −65.0 J·mol^{-1}·K^{-1} and is assumed to be equal at 5 and 15 °C (Noyes, 1964; Yamada & Kometani, 1982).

When Mg^{2+} is present, bound Mg^{2+} is displaced by Ca^{2+}.

$$PA\text{–}Mg + Ca^{2+} \rightleftharpoons PA\text{–}Ca + Mg^{2+} \quad (10)$$

Therefore, ΔS_u(PA) is expressed as follows, similarly to the case of the Mg^{2+}-free solution described above.

$$\Delta S_u(PA) = S_u°(PA\text{–}Ca) - S_u°(PA\text{–}Mg) = \Delta S° + S°(Ca^{2+}) - S°(Mg^{2+}) \quad (11)$$

The value of $S°(Mg^{2+})$ is −127.7 J·mol^{-1}·K^{-1} at 25 °C and is assumed to be equal at 5 and 15 °C (Noyes, 1964; Yamada & Kometani, 1982).

Table III: Estimation of Hydrophobic and Vibrational Contributions to the Changes in Heat Capacity and Entropy of Bullfrog Parvalbumins Associated with Ca^{2+} Binding and Mg^{2+}–Ca^{2+} Exchange

	temp (°C)	ΔC_p(hydro) (J·mol^{-1}·K^{-1})	ΔC_p(vib) (J·mol^{-1}·K^{-1})	ΔS_u(hydro) (J·mol^{-1}·K^{-1})	ΔS_u(vib) (J·mol^{-1}·K^{-1})
PA1					
Ca^{2+} binding	5	−269	−350	429	−368
	15	−812	−362	410	−380
	25	−1355	−374	352	−393
Mg^{2+}–Ca^{2+} exchange	5	−387	43	45	45
	15	−238	44	28	46
	25	−90	45	23	48
PA2					
Ca^{2+} binding	5	−216	−213	310	−224
	15	−588	−220	291	−231
	25	−961	−228	250	−239
Mg^{2+}–Ca^{2+} exchange	5	−317	42	59	45
	15	−243	44	49	46
	25	−170	45	44	48

The heat capacity change ($\Delta C_p°$) can be obtained from the temperature dependence of the enthalpy change according to Kirchhoff's formula:

$$\Delta C_p° = d\Delta H/dT \quad (12)$$

The temperature dependence of the enthalpy titration curves was found from observations at 5, 15, and 25 °C. The values of $\Delta C_p°$ were determined as described above in the temperature range from 5 to 15 °C; these values are not equal to the respective values obtained between 15 and 25 °C as listed in Table II. This may be because the heat capacity changes are not constant in the temperature range studied or because of errors in experimental determinations. For the analysis of the hydrophobic and vibrational contributions, therefore, the values of $\Delta C_p°$ determined in the temperature ranges from 5 to 15 °C and from 15 and 25 °C were used for 5 and for 25 °C, respectively, and the average of these two values was used for 15 °C. The results of analysis were essentially the same as those obtained by using the average value for 5, 15, and 25 °C.

Hydrophobic and Vibrational Contributions to Heat Capacity and Entropy Changes. The changes in heat capacity and entropy associated solely with molecules, which are obtained as described above, may be analyzed in terms of the two principal contributions, the hydrophobic effect and the internal vibrational modes (Sturtevant, 1977; Yamada & Kometani, 1982). Therefore

$$\Delta C_p(\text{PA}) = \Delta C_p(\text{hydro}) + \Delta C_p(\text{vib}) \quad (13)$$

$$\Delta S_u(\text{PA}) = \Delta S_u(\text{hydro}) + \Delta S_u(\text{vib}) \quad (14)$$

where ΔC_p(hydro) and ΔC_p(vib) are the hydrophobic and vibrational contributions to the heat capacity change, respectively, and ΔS_u(hydro) and ΔS_u(vib) are the respective contributions to the unitary entropy change. The empirical relations proposed by Sturtevant (1977) are

$$\Delta S_u(\text{hydro}) = -0.26\Delta C_p(\text{hydro}) \quad (\text{at } 25\ °\text{C}) \quad (15)$$

$$\Delta S_u(\text{vib}) = 1.05\Delta C_p(\text{vib}) \quad (\text{at } 0\text{–}100\ °\text{C}) \quad (16)$$

From eq 13–16, the values of ΔC_p(hydro), ΔC_p(vib), ΔS_u(hydro), and ΔS_u(vib) can be calculated for 25 °C. The hydrophobic and vibrational contributions at temperatures other than 25 °C are obtained with eq 16 and the relationship (Sturtevant, 1977)

$$\ln [\Delta S_{u2}(\text{vib})\Delta S_{u1}(\text{vib})] = 0.95 \ln (T_2/T_1) \quad (17)$$

where ΔS_{u1}(vib) and ΔS_{u2}(vib) are the vibrational contributions to the unitary entropy change at temperatures T_1 and T_2, respectively. The detailed results are listed in Table III. Figure 3 shows ΔS_u(hydro) and ΔS_u(vib) in PA1 and PA2 associated with Ca^{2+} binding to and Mg^{2+}–Ca^{2+} exchange on each of the two sites at 25 °C.

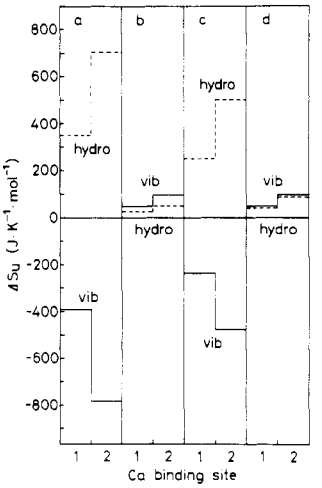

FIGURE 3: Hydrophobic (hydro) and vibrational (vib) contributions to the entropy changes associated with Ca^{2+} binding to and Mg^{2+}–Ca^{2+} exchange on bullfrog parvalbumins. Entropy changes are ascribed to the binding sites involved as indicated at the bottom of the figure. Entropy changes are drawn in a cumulative way, so that the final levels indicate the overall entropy changes when parvalbumin binds 2 mol of Ca^{2+} per mol of protein. (a) PA1 on Ca^{2+} binding; (b) PA1 on Mg^{2+}–Ca^{2+} exchange; (c) PA2 on Ca^{2+} binding; (d) PA2 on Mg^{2+}–Ca^{2+} exchange.

For both Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange, PA1 and PA2 show hydrophobic and vibrational entropy changes similar to each other. This indicates, therefore, that essentially the same conformational changes occur in the α- and β-parvalbumins associated with Ca^{2+} binding or Mg^{2+}–Ca^{2+} exchange. The changes in both hydrophobic and vibrational contributions to the unitary entropy of parvalbumins are similar to those found in skeletal and cardiac troponin C (Yamada & Kometani, 1982; Kometani & Yamada, 1983) and the complex of calmodulin and its inhibitor, trifluoperazine (Tanokura & Yamada, 1986), but are distinctly different from those for calmodulin alone (Tanokura & Yamada, 1984).

The Ca^{2+} binding to each of the binding sites of bullfrog parvalbumin (PA1 and PA2) causes a gradual decrease in the vibrational entropy, making the molecules less mobile as a whole. With regard to the hydrophobic entropy, the binding of Ca^{2+} causes a gradual increase in the bullfrog parvalbumin molecules, the hydrophobic effect. In other words, the total area of nonpolar regions is decreased on the surface of the parvalbumin molecules due to either (1) a sequestering of nonpolar groups from the surface into the interior of the molecules or (2) an assembling of scattered small nonpolar

regions exposed to solvent water to form a large mass, which makes the total surface area smaller.

For Mg^{2+}–Ca^{2+} exchange, the absolute values of both hydrophobic and vibrational entropy changes in each of PA1 and PA2 became much smaller than those of Ca^{2+} binding. This indicates that the conformational changes are smaller for Mg^{2+}–Ca^{2+} exchange compared with those for Ca^{2+} binding. For the Ca^{2+} binding in the presence of Mg^{2+}, the vibrational entropy changes are positive with calmodulin and the calmodulin–trifluoperazine complex, while the vibrational entropy changes are mostly negative with skeletal and cardiac troponin C (Yamada & Kometani, 1982; Kometani & Yamada, 1983; Tanokura & Yamada, 1984, 1986). Because Mg^{2+}–Ca^{2+} exchange on each of the two sites causes gradual increases in both hydrophobic and vibrational entropy, Mg^{2+}–Ca^{2+} exchange makes PA1 and PA2 more mobile as a whole.

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables giving the amount of protons released on Ca^{2+} and Mg^{2+} binding, enthalpy changes associated with Ca^{2+} binding and Mg^{2+}–Ca^{2+} exchange on parvalbumins from various sources, and a comparison of the expected heat produced by Mg^{2+}–Ca^{2+} exchange on parvalbumins with the amount of labile maintenance heat in frog muscle cells (3 pages). Ordering information is given on any current masthead page.

Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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Involvement of a Carboxyl Group in the Interaction between Succinate Dehydrogenase and Its Membrane-Anchoring Protein (QPs) Fraction†

Jian-Xing Xu, Linda Yu, and Chang-An Yu*
Department of Biochemistry, OAES, Oklahoma State University, Stillwater, Oklahoma 74078
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ABSTRACT: The involvement of the carboxyl groups in the membrane-anchoring protein (QPs) in reconstitution of succinate dehydrogenase to form succinate–ubiquinone reductase is studied by using a carboxyl group modifying reagent, dicyclohexylcarbodiimide (DCCD). Inactivation of QPs by DCCD is found to be dependent on the temperature, pH, detergent, and DCCD concentration used. When QPs is treated with 300 molar excess DCCD at room temperature for 10 min, about 90% of the original reconstitutive activity is lost. When intact or reconstituted succinate–ubiquinone reductase formed from reconstitutively active succinate dehydrogenase and QPs is treated with DCCD under the same conditions, no loss of succinate–ubiquinone reductase activity is observed. However, when a mixture of reconstitutively inactive succinate dehydrogenase and QPs is treated with DCCD before being reconstituted with active succinate dehydrogenase, an inactivation behavior similar to that with QPs alone is observed. These results indicate that DCCD modifies the carboxyl groups of QPs which are essential for the interaction with succinate dehydrogenase to form succinate–ubiquinone reductase. Inactivation of QPs by DCCD parallels the incorporation of DCCD into QPs. About two carboxyl groups per molecule of QPs are essential for the interaction with succinate dehydrogenase. These essential carboxyl groups are located in the smaller subunit (M_r 13 000) of QPs. Modification of QPs by DCCD also alters the heme environment of cytochrome b_{560}.

Mitochondrial succinate–ubiquinone (succinate–Q)[1] reductase (Ziegler & Doeg, 1962), which catalyzes the electron transfer from succinate to Q, is composed of four protein subunits with molecular weights of 70 000, 27 000, 15 000, and 13 000. The isolated complex can be resolved into two reconstitutively active fractions: a soluble succinate dehydrogenase (Keilin & King, 1958; Davis & Hatefi, 1971; Ackrell et al., 1977; Yu & Yu, 1980a) composed of the two larger subunits and a membrane-anchoring fraction, generally known as QPs (Yu & Yu, 1980b), protein fraction (Vinogradov et al., 1980), CII-$_{3,4}$ (Ackrell et al., 1980), or a cytochrome b_{560} fraction (Hatefi & Galenta, 1980), containing the two smaller subunits. Further resolution of succinate dehydrogenase into M_r 70 000 and 27 000 subunits has not yet been achieved. To date, attempts to dissociate QPs into the M_r 15 000 and 13 000 subunits resulted in a significant loss of ability to reconstitute with succinate dehydrogenase. Isolation of the M_r 13 000 protein, practically devoid of the M_r 15 000 protein, has been reported (Yu & Yu, 1980b; Vinogradov et al., 1980), but the reconstitutive activity of this preparation is only 20% compared to that obtained with two-subunit QPs. Isolation of a single-polypeptide cytochrome b_{560} has not yet been reported.

The interaction between succinate dehydrogenase and QPs is not only functionally necessary but also structurally important. Succinate dehydrogenase is stable in its membrane-bound form, such as in submitochondrial particles, isolated succinate–cytochrome *c* reductase, or succinate–Q reductase. However, it becomes very labile when detached from its membrane-anchoring frction; the half-life is about 20 min at 0 °C under aerobic conditions. The close physical relationship between succinate dehydrogenase and its membrane-anchoring proteins has been recently demonstrated

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[1] Abbreviations: Q, ubiquinone; DCCD, dicyclohexylcarbodiimide; DCIP, dichlorophenolindophenol; PMS, phenazine methosulfate; DATA, *N,N'*-diallyltartardiamide; DMG, decanoyl-*N*-methylglucamide; SDS, sodium dodecyl sulfate; QPs, a two-subunit protein fraction that converts succinate dehydrogenase into succinate–Q reductase; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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