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# THERMODYNAMIC INVESTIGATIONS OF PROTEINS
# IV. CALCIUM BINDING PROTEIN PARVALBUMIN

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The conformational transitions of calcium binding protein parvalbumin III from carp muscle were studied by scanning calorimetry, potentiometric titration and isothermal calorimetric titration. Changes of Gibbs energy, enthalpy and partial heat capacity were determined. The removal of calcium ions by EDTA is accompanied by 1) a heat absorption of 75 ± 10 kJ per mole of the protein, 2) a decrease in the Gibbs energy of protein structure stabilisation of about 42 kJ $mol^{-1}$ and 3) a decrease in thermostability by more than 50 K. The protonation of the acidic groups leads to a loss of calcium followed by denaturation, while the pH of the transition strongly depends on calcium activity. The enthalpy and heat capacity changes at denaturation are comparable with the values observed for other compact globular proteins.

## 1. Introduction

Parvalbumins which were first isolated from white muscle of fish and amphibia represent a wide class of calcium binding globular proteins of low molecular weight. They have two homologous calcium binding structural domains which are found also in several other calcium binding proteins [1,2] and bind two calcium ions with relatively high binding constants ($K_a \sim 10^7$ $M^{-1}$) [3]. Though the functional role of parvalbumins in muscle is not yet clear, one can suppose that it is closely related to their calcium binding ability and sensitivity of their physical properties to calcium removal as shown by some methods [4–8]. It seems important to investigate the thermodynamics of calcium binding as well as the contribution of this process to the protein structure stabilisation. For this purpose the conformation transitions observed at heating, protonation and calcium removal were studied for parvalbumin III (carp) with a known three-dimensional structure [1].

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## 2. Methods

Parvalbumin III was isolated from white muscle of mirror carp as described by Pechère et al. [9], with slight modifications and stored lyophilized for a period not exceeding one month as prolonged storage leads to an unexplained increase of UV absorbance at 280 nm. The purity of the protein preparation was checked by disc electrophoresis and UV absorption spectra. The extinction ratio $E_{280}/E_{259}$ for protein solutions used in experiments did not exceed 0.03. The concentration determinations were performed spectrophotometrically using $E^{1\%}_{259} - E^{1\%}_{300} = 1.66$ [10]. The necessary solvent conditions were achieved by exhaustive dialysis against the solutions described in legends to figures. All chemicals were of an analytical grade.

Potentiometric titrations were done using 6.0 to 7.5 ml of $1.9 \times 10^{-4}$ to $3.3 \times 10^{-4}$ M protein solutions under $CO_2$ free nitrogen at 25 ± 0.02°C with precision microsyringes. For calorimetric titrations the LKB 10700 flow calorimeter was combined with a self made thermostated flow cell for measuring pH. The protein concentrations before mixing were from $4.5 \times 10^{-5}$ to $1.5 \times 10^{-4}$ M at flow rates $4.5 \times 10^{-3}$ or $9 \times 10^{-3}$ ml $sec^{-1}$.

Scanning calorimetric measurements were performed

in the adiabatic microcalorimeter DASM-1M with a 1.0 ml cell volume at protein concentrations of 0.1 to 0.25 percent and a heating rate of 1 K $min^{-1}$. The partial heat capacity of protein was determined from scanning microcalorimetric records according to the procedure described elsewhere [11], taking the value of 0.73 $cm^3$ $g^{-1}$ for the partial specific volume of parvalbumin. The transition enthalpy was determined from the heat sorption peak area above the lines extrapolating heat capacities of native and denatured states to the melting temperature, i.e. temperature of the maximal heat sorption. The Gibbs energy of protein structure stabilization was estimated by equation (see [11])

$$\Delta_{trs}G(T) = \Delta_{trs}H(T_{trs} - T)/T_{trs} - \Delta_{trs}C_p(T_{trs} - T) + T\Delta_{trs}C_p \ln T_{trs}/T . \quad (1)$$

## 3. Results

### 3.1. Scanning calorimetry

The typical calorimetric records obtained at neutral pH and different salt and calcium content in solution are shown in fig. 1. The calcium-bound protein exhibits a considerable thermostability: its melting temperature at $10^{-4}$ M $CaCl_2$ in solution reaches 90°C, and the enthalpy of transition is 500 ± 30 kJ $mol^{-1}$. The protein has the same high stability in the pH range from 6 to 10. A further increase of pH up to 12.5 results in a very notable decrease in melting temperature, but the protein is still native at room temperature. In the whole pH range of protein stability the heat denaturation in presence of $Ca^{2+}$ is characterized by an asymmetric melting curve with the $\Delta H_{cal}/\Delta H_{v.H.}$ ratio exceeding 1.1 (precise value depends on pH), hence this process cannot be considered as a two-state one. The complexity of the denaturation process becomes evident at pH lower than 6, where two superimposed peaks of the heat sorption are clearly visible. The second drawback is the low reversibility (usually less than 50 percent) of the melting process in the presence of calcium. All these facts should be taken into account in considering the thermodynamic functions since they can be calculated only approximately. Nevertheless, insofar as we calculate these functions (and primarily the enthalpy) from the calorimetric and not indirect equilibrium data, the error of their estimation could not be large.

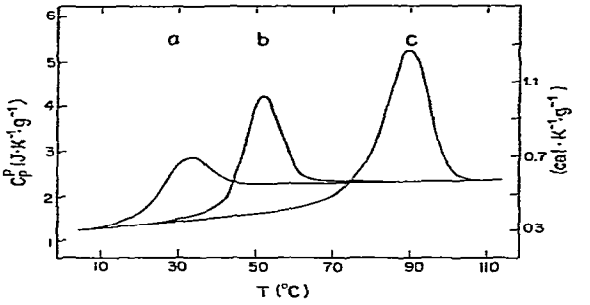


Fig. 1. Temperature dependencies of the partial specific heat capacity of parvalbumin. Curve a: in 10 mM sodium phosphate, 5 mM $Na_2$EDTA pH 7.5. Curve b: in 10 mM sodium phosphate, 10 mM $Na_2$EDTA, 1 M NaCl pH 7.5. Curve c: in 10 mM sodium cacodylate, $10^{-4}$ M $CaCl_2$ pH 7.0.

The removal of tightly bound $Ca^{2+}$ ions by dialysis against millimolar EDTA solutions at pH higher than 7 results in a drastic decrease of the protein structure stability and enthalpy of denaturation. At the same time, the heat induced unfolding becomes a fully reversible two-state transition as it follows from the comparison of calorimetric and van 't Hoff enthalpy. The latter was calculated either from calorimetric curves (see [11]), or by van 't Hoff treatment of temperature dependencies of the specific optical rotation. The destabilizing effect of calcium loss can be partially suppressed by increase of monovalent ion concentration in the solution, so that the melting temperature which in presence of only 10 mM sodium phosphate, 5 mM EDTA pH 7.5 is 32°C rises up to 55°C after adding 2M NaCl to the solution. The similar stabilizing effect could be achieved after replacement of the water by $D_2O$ at pD > 8. Again, as in the presence of calcium, in water solutions containing EDTA at pH < 6 complex melting curves were observed. This is most likely due to the impossibility to remove one of the tightly bound $Ca^{2+}$ ions even at rather high EDTA concentrations.

### 3.2. Potentiometric titration

To investigate the pH-induced conformation transi-

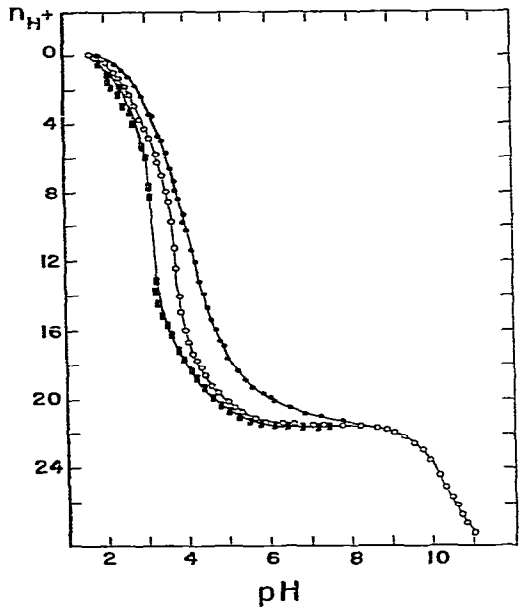


Fig. 2. Direct and reverse potentiometric titration curves of parvalbimun at 25°C. Filled circles: in 8 M GuHCl, $10^{-4}$ M $CaCl_2$; open circles: in 0.1 M KCl, $10^{-4}$ M $CaCl_2$; filled rectangles: in 0.033 M $CaCl_2$.

tion we have performed potentiometric titrations of the protein at various solvent conditions (fig. 2). Judging by the CD-spectrum (see also [6]) and the absence of an extra heat absorbed in the scanning calorimeter, the protein conformation in 8 M guanidine hydrochloride (GuHCl) should be considered as close to the completely unfolded one. At these conditions the titration is reversible, while electrostatic interactions are absent judging by the Linderstrøm-Lang plot. The amount of titratable groups between pH 8 and 1.5 is 21 ± 0.5 in agreement with the amino acid content of the protein (14 Asp, 6 Glu, 1 His). The most interesting feature of the titration curve obtained in the presence of $CaCl_2$ is the jump in the proton uptake beneath the isoelectric point. After the transition the curves converge to the GuHCl curve. The titration is fully reversible in the ranges of pH from 11 to 4 and from 3 to 1.5, while at pH 3–4 slight turbidity appeared giving rise in experimental error. The titration curves obtained at the same ionic strength, but different calcium concentrations, differ either in shape or in the pH of transition, reflecting a competition between protons and calcium ions for the binding sites.

The difference in Gibbs energy between native parvalbumin and that completely unfolded by GuHCl can be estimated at a fixed $Ca^{2+}$ activity using the following equation [12]:

$$\Delta_{trs}G(\mathrm{pH}) = 2.3\,RT \int_{\mathrm{pH_m}}^{\mathrm{pH}} \Delta\nu(\mathrm{pH})\,\mathrm{dpH} \qquad (2)$$

where $\Delta\nu$(pH) is the difference in charge between two states at any pH and $\mathrm{pH_m}$ is the point of half-convertion, i.e. 3.7 at 0.1 mM $CaCl_2$ and 3.2 at 33 mM $CaCl_2$. The results of these calculations are shown in fig. 5.

### 3.3. Isothermal calorimetric titration

The conformational transition at protonation was also studied by isothermal calorimetric titration at various temperatures (fig. 3). From pH 7 to lower values the curves are characterized by a small initial slope followed by a rather sharp endothermal transition. The sharpness of the transition enables determination of the apparent heat of the process $\Delta_{trs}H^{app}$ by tangents without large errors. The slopes of the potentiometric and calorimetric curves at 25°C near pH 7 are not striking since the N-terminal amino group is acetylated and pK of a single His-26 seems to be abnormal as an exothermal effect of the order of 25–33 kJ $mol^{-1}$ typical for histidine protonation is not observed at pH 6–7. To calculate the enthalpy of conformation transition $\Delta_{trs}H$ from $\Delta_{trs}H^{app}$ the heats of ionization of the acidic groups which take part in calcium binding were taken into account, assuming that they do not differ from those for free amino acids [13].

### 3.4. Enthalpy of denaturation and calcium binding

The dependencies of $\Delta_{trs}H$ on melting temperature obtained by different calorimetric techniques for calcium-bound and calcium-free protein are shown in fig. 4. In order to decrease the melting temperature, scanning experiments were carried out at neutral and alkaline values of pH. It follows from the slope of the upper line in fig. 4 and direct calorimetric observations that $\Delta_{trs}C_p(+\mathrm{Ca}) = 4.6 \pm 0.5$ kJ $K^{-1}$ $mol^{-1}$. Nearly

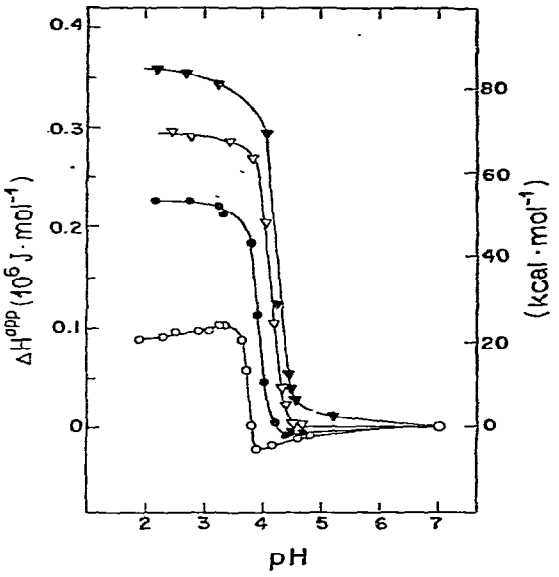


Fig. 3. Isothermal calorimetric titration curves of parvalbumin in 0.1 M KCl at various temperatures. Open circles, 25°C; filled circles, 40°C; open triangles, 50°C; filled triangles, 59°C.

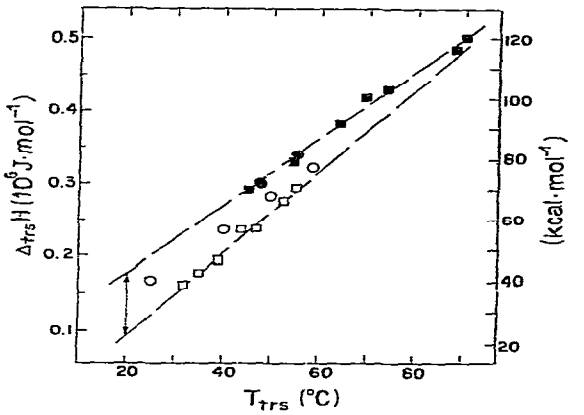


Fig. 4. Dependencies of $\Delta_{trs}H$ of parvalbumin on melting temperature. Filled squares: scanning calorimetry data in neutral and alkaline pH-ranges, $CaCl_2$ concentration from $10^{-4}$ to $10^{-5}$ M; filled circles: isothermal $CaCl_2$ and EDTA titration data at pH 8–7.5; open circles: $\Delta_{trs}H$ values replotted from fig. 3 after correction for ionization enthalpy; open squares: values for calcium-free protein in 10 mM sodium phosphate, 5–10 mM $Na_2EDTA$ pH 7.5, and various NaCl concentration (from zero to 2 M).

the same value corresponds to the temperature dependence of molar heat sorption at pH induced transition, though these heats are somewhat lower than those observed in scanning experiments at corresponding melting temperatures. A greater and more reliable difference both in $\Delta_{trs}H$ and $\Delta_{trs}C_p$ exists between calcium-bound and calcium-free proteins. From the slope of the lower line in fig. 4 and from calorimetric records $\Delta_{trs}C_p(-Ca)$ was found to be equal to 5.6 ± 0.5 kJ $K^{-1}$ $mol^{-1}$. From a comparison of the two sets of scanning data it follows that calcium release must be accompanied by heat sorption whose magnitude depends on temperature. Extrapolating both the $\Delta_{trs}H$ to 20°C we get the value of 80 ± 10 kJ $mol^{-1}$ for $\Delta_{bind}H$. In order to check the reality of this value, isothermal calorimetric measurements were performed by titration of calcium-free protein with $CaCl_2$ as well as reverse titration of calcium-bound form with EDTA. Both titration procedures gave close results when the enthalpy of proton transfer between the EDTA and buffer was taken into account. This value which corresponds only to the binding of $Ca^{2+}$ ions to the high-affinity sites, is equal to 75 ± 10 kJ $mol^{-1}$ at 20°C and low ionic strength, and is in a good agreement with the value mentioned above. To check the correctness of scanning data at alkaline pH, where the contribution of ionization heats of lysines to $\Delta_{trs}H$ might be high, we have performed isothermal experiments at neutral pH in the temperature range of 47–60°C. From the consideration of the melting curves obtained at low ionic strength in absence of $Ca^{2+}$, it is seen that at these temperatures calcium-free protein is completely denatured. Therefore the treatment of calcium-bound protein by EDTA at these temperatures must lead to protein structure denaturation. The results obtained in these experiments are also plotted in fig. 4 to demonstrate their coincidence with scanning data.

## 4. Discussion

Though the enthalpy of pH induced conformation transition is lower than the values obtained in scanning and EDTA treatment experiments, there is still a satisfactory agreement between all these three sets of data.

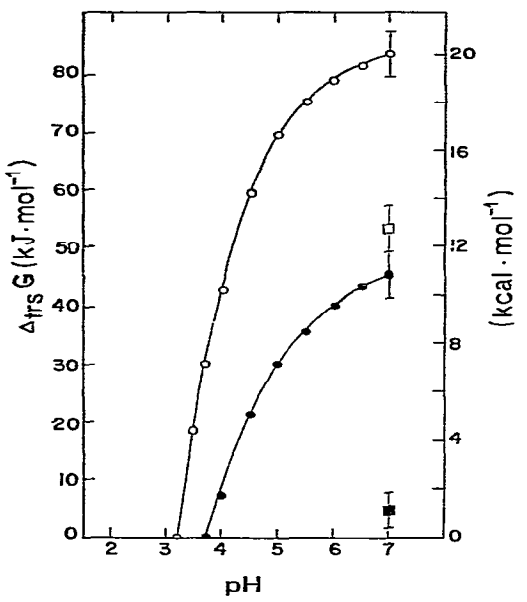


Fig. 5. pH dependence of the apparent Gibbs energy of protein structure stabilization. $\Delta_{trs}G$ values calculated using eq. (2) from scanning data are given for comparison. Open circles: in 0.033 M $CaCl_2$ solution, potentiometric titration; filled circles: in 0.1 M KCl, $10^{-4}$ M $CaCl_2$, potentiometric titration; open square: in $10^{-4}$ M $CaCl_2$, scanning calorimetry; filled square: in 5 mM $Na_2$ EDTA, scanning calorimetry.

We have a similar concordance for Gibbs energies of protein structure stabilization at 25°C and $10^{-4}$ M of $CaCl_2$ calculated from the calorimetric data using eq. (1) and from the potentiometric titration data using eq. (2) (see fig. 5). The existing small difference both in the enthalpy and Gibbs energy changes for calcium-bound form melting might arise either from imperfection of the system, i.e. irreversibility and complexity of the heat denaturation in presence of $Ca^{2+}$ and also from aggregation phenomena at acid induced transition, or from incompleteness of this transition. The latter possibility is real since the optical data [6,7] could be interpreted in terms of existence of some residual structure at pH 2. However, our attempts to detect an additional heat effect in the scanning calorimeter at pH 2, as well as by GuHCl treatment of acid denatured protein in isothermal experiments were not successful. Thus, though we can not rule out the possibility of residual structure existence at acidic pH, it could be concluded that protein structure after acid denaturation does not differ noticebly from the heat denatured state, while they both do not differ from a random coil in the enthalpy scale. In other words, the observed thermodynamic function changes could be considered as true parameters reflecting native structure stabilization as compared to a completely disordered state.

If this be so, it would be interesting to compare the results of this work with those for other small compact globular proteins. The value of specific $\Delta_{trs}C_p$ for parvalbumin is of the same order as for other proteins studied before [11]. Moreover, the extrapolation of specific enthalpy of denaturation to 110°C gives exactly the same value that was found for other globular proteins, i.e. 50 J $g^{-1}$ [11], both for calcium-bound and calcium-free forms of the protein. This implies that they are typical representatives of small globular proteins.

The important role of tightly bound calcium ions in the maintenance and stabilization of the native parvalbimun structure was demonstrated by a number of techniques including X-ray data [1], NMR [5,8] and optical methods [4,6,7]. Therefore it is not surprising that we have observed gross changes in thermostability and Gibbs energy at calcium removal. But the striking feature is that the stabilising effect of $Ca^{2+}$ is of an enthalpy nature. It is known that binding of divalent ions by the chelating agents like EDTA or EGTA is accompanied by negligible heat effects and high entropy changes. Thus, since the binding sites in parvalbumin are formed mainly also by carboxyl groups, one can assume that the observed heat effect should be ascribed to a conformation transition in protein. Indeed, the existence of changes in protein structure was shown by various techniques [7,8]. Donato and Martin [7] have observed the change in CD absorption at $Ca^{2+}$ removal which was interpreted as a decrease in the $\alpha$-helical content corresponding to the disruption of one of the six helical regions. It is difficult to say, however, which part of the observed heat effect reflects the conformation transition itself. Recently a similar heat effect has been reported [14] for $Ca^{2+}$ binding by troponin C. At the same time it has been shown that the heat effect per each binding site has the same magnitude of 32 kJ $mol^{-1}$, though detectable changes in protein structure as shown by a number of methods occur at calcium binding to only two of the four high

affinity sites. This fact could mean that the main part of the heat effect of $Ca^{2+}$ binding either to the parvalbumin or to troponin C is connected with a change of $Ca^{2+}$ environment, while the heat effect of secondary structure change is compensated essentially by heat of the rearrangement of the tertiary structure or protein-solvent interactions.

Considering the thermodynamic properties of the calcium-free state, it should be noted that this structure is under electrostatic strain due to the loss of four positive charges and the stabilizing effect of monovalent cations can be explained by the screening of electrostatic repulsion in the negatively charged clusters which are binding sites. Since the interactions with monovalent ions are not usually accompanied by heat effects in absence of a conformation change, the observed dependence of $\Delta_{trs}H(-Ca)$ on $T_{trs}$ corresponds to the real dependence of the enthalpy of protein structure stabilization on temperature, i.e. to the real $\Delta C_p$ value. According to the existing hypothesis, the heat capacity change at denaturation arises from the exposure of apolar groups to water. If this is so and all the heat effect as well as the increase of $\Delta C_p$ at calcium release are bound with a conformation change, a larger number of apolar groups can be protected from water in the calcium-free form in comparison with the calcium-bound one. In any case, the interception of the enthalpy dependencies at 110°C must imply a compensation of a possible loss in hydrogen bond content by other interactions having a higher temperature dependence of the enthalpy.

The knowledge of Gibbs energies of protein structure stabilization in the presence and absence of calcium allows us to estimate calcium binding constants for high affinity sites. To this end it is better to use the value of $\Delta_{trs}G(+Ca)$ at $10^{-4}$ M $CaCl_2$, as the contribution of low affinity sites to $\Delta G$ can be neglected at this calcium activity [3].

Assuming a noncooperative binding of two $Ca^{2+}$ ions [3,7] with equivalent binding constants $K_a$, the following equation can be written for the equilibrium constant $K_e$ for transition between calcium-free and calcium-bound states:

$$K_e = [PrCa_2]/[Pr] = K_a^2[Ca^{2+}]^2 , \quad (3)$$

which corresponds to $\Delta G_e$

$$\Delta G_e = -RT\ln K_e = -2RT\ln(K_a[Ca^{2+}]) . \quad (4)$$

The total change in Gibbs energy in the process of denaturation of calcium bound protein can be presented as a sum

$$\Delta_{trs}G(+Ca) = -\Delta G_e + \Delta_{trs}G(-Ca) . \quad (5)$$

Combining equations (4) and (5) we get for $K_a$

$$K_a = \frac{1}{[Ca^{2+}]}\exp\left(\frac{\Delta_{trs}G(+Ca) - \Delta_{trs}G(-Ca)}{2RT}\right). \quad (6)$$

The substitution of numerical data at 25°C and $Ca^{2+} = 10^{-4}$ M (see fig. 5) gives the value of order $3 \times 10^7$ $M^{-1}$ for $K_a$. This value is in a reasonable agreement with those published for other parvalbumins [3].

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