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Thermodynamics of Ca^{2+} binding to calmodulin and its tryptic fragments

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The binding of Ca^{2+} to calmodulin and its two tryptic fragments has been studied using microcalorimetry. The binding process is accompanied by the uptake or release of protons, depending on the ionic strength. With no added salt, the total enthalpy change for the binding of four calcium ions to calmodulin is -41 kJ mol^{-1} but in the presence of 0.15 mM KCl ΔH_{tot} is $+17$ kJ mol^{-1}. The mode of binding of Ca^{2+} is also completely different with and without added salt. It is also shown that for the C-terminal fragment of calmodulin, TR_2C, the drastic reduction in ΔG_{tot} for the binding process on increasing the ionic strength is largely an enthalpic effect. Domain interactions in calmodulin are indicated by the fact that the sum of the enthalpies of calcium binding to the two tryptic fragments is not the same as the total binding enthalpy to calmodulin itself. The binding of Ca^{2+} to calmodulin has also been studied calorimetrically at different temperatures in the range 21–37 °C. ΔC_p is large and negative in this interval.

1. Introduction

The intracellular calcium-binding protein calmodulin (CaM) has been found in all eukaryotic cells. The protein binds four Ca^{2+} and undergoes conformational changes that expose hydrophobic sites on the surface of the protein which are involved in the interaction between CaM and a number of enzymes and other proteins [1–3].

CaM seems to function such that variations in the level of intracellular messenger Ca^{2+} are translated into metabolic responses. Because of its wide variety of interactions and functions, CaM has been the subject of extensive studies during the last few years. In particular, the use of different biophysical methods has been very important in clarifying the properties and physical mode of action of CaM [4]. In this connection, studies of the two tryptic fragments, TR_1C and TR_2C, of CaM have made decisive contributions [5,6], showing that the protein molecule is made up of two domains, approximately equal in size, and comprising the N-terminal and C-terminal halves of the molecule. This concept of the general structure of CaM has to a large extent been confirmed by the three-dimensional structure recently determined [7,8].

One of the keys to the understanding of the mode of action of a variety of biological macromolecules is the relationship between structure and thermodynamics [9]. Thermodynamic information for the binding of ligands to proteins like CaM is best obtained using microcalorimetry and some results for the case of Ca^{2+} binding to CaM have been published [10–15]. We have now studied by isothermal calorimetry the binding of calcium to CaM with particular reference to the effects of different salt concentrations since there has been

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considerable discussion on this point [16]. We have also included the two tryptic fragments in this work in view of the value previously shown in using these two halves of the CaM molecule. To our knowledge, this is the first time that the CaM fragments have been used directly in calorimetric studies of calcium binding.

2. Materials and methods

2.1. Materials

Bovine testis calmodulin and its tryptic fragments, TR_1C and TR_2C, as well as bovine brain CaM were prepared as described earlier [17,18]. Purities were checked by both SDS and agarose gel electrophoresis in the presence of excess EDTA or Ca^{2+}. Calcium-free proteins were prepared by passing the protein solution through a Chelex-100 (Bio-rad) column; the residual calcium content was determined by atomic absorption spectroscopy and was usually found to be less than 0.05 Ca^{2+}/protein. The absence of EDTA in the protein solutions was checked by ^{1}H-NMR at 360 MHz. Protein concentrations were determined using a Cary 219 spectrophotometer with the cells maintained at 25 °C.

All other chemicals used were of analytical grade. Doubly distilled water was used throughout and the solutions were stored in plastic containers. The buffer solutions used in the calorimetric experiments (Hepes and Tris) were also treated with Chelex-100 and the calcium contents checked as above.

2.2. Methods

The calorimetric measurements were made with an LKB batch microcalorimeter fitted with an LKB titration assembly 2107-350 [19]. The reaction cell typically contained between 4 and 5 ml of protein solution (protein dissolved in Hepes or Tris buffer; 0.1-0.5 mg/ml) and the reference cell was filled with the corresponding amount of buffer solution. The calcium concentration of the titrating solution ($CaCl_2$ dissolved in the appropriate buffer) ranged from 10 to 75 mM, depending on the heat effects to be measured and the protein concentration. Injection of the Ca^{2+} solution to the reaction cell was always accompanied by an equally large injection (5–15 μl) to the reference cell containing the buffer, thereby eliminating the need for separate dilution experiments. The calorimeter block was rotated in connection with each injection. Measurements were performed at different temperatures, in different buffers and at varying ionic strengths (see section 3).

The differential calorimetric signal was amplified by a Keithley 148 nanovoltmeter. The output voltage-time curve was integrated (20 min) by a microprocessor which also controlled the injections from the syringes into the cells and the rotation of the calorimeter block. The calorimeter was calibrated electrically and also by using the standard procedure of protonation of Tris with HCl [20,21].

Separate experiments were made to determine the contribution of rotation of the calorimeter block to the measured heat effects.

Proton uptake or release accompanying the binding of Ca^{2+} to the proteins was determined in two ways. One method was to record the calorimetric titration curves in two different buffers, Hepes and Tris, with different enthalpies of protonation. In the other procedure, the pH changes associated with the binding of Ca^{2+} to the proteins were measured using standard titration equipment. Ca^{2+} solutions were added to unbuffered solutions of the proteins and the amounts of HCl or NaOH needed to compensate for the induced pH changes were used to determine the extent of proton exchange between the buffer and protein.

3. Results

3.1. Proton exchange

The results of the proton exchange experiments are summarized in table 1. Most of these results were obtained using standard titration techniques but in the cases where calorimetric titration was used (CaM, no added KCl; TR_1C with 0.10 M KCl) good agreement was found between the two methods. The observed enthalpies of reaction,

Table 1

Proton exchange between the proteins and buffer at 25 °C and pH 7.0

Positive values indicate a proton uptake by the protein, negative values a proton release process. The values are valid for a total of four Ca^{2+} bound to CaM and two to the fragments

Protein	Added KCl (M)	Proton exchange
CaM	0	+0.40
CaM	0.15	−0.27
TR_1C	0	+0.29
TR_1C	0.10	−0.14
TR_1C	0.15	−0.14
TR_2C	0	+0.10
TR_2C	0.10	−0.14
TR_2C	0.15	−0.14

ΔH_{Obs}, of Ca^{2+} with testis CaM at 25 °C and pH 7.0 in both Hepes and Tris buffers with no added KCl are shown as a function of Ca^{2+}/CaM in fig. 1. The differences in ΔH_{Obs} for the two buffer systems show that after the addition of about two equivalents of calcium there is a net change in protonation of the protein on binding Ca^{2+}. The enthalpy of protonation of Tris (−47.53 kJ mol^{-1} [20,21]) is more negative than that of Hepes (−20.9 kJ mol^{-1} [22]). As the ΔH_{Obs} values in Hepes buffer are more exothermic than those in Tris buffer, it follows that protons are taken up by the protein on binding Ca^{2+}. Using the protonation enthalpies of the two buffers and the results shown in fig. 1 the amount of protons taken up upon binding four Ca^{2+} per mole of CaM was calculated to be 0.4.

The result for CaM in a solution containing 0.15 M KCl is in excellent agreement with that obtained by Milos et al. [12].

The results in table 1 show that the proton exchange processes between the proteins and the buffers change direction when going from salt-free solutions to solutions with increasing ionic strength. It has also been shown that proton exchange, at least at high ionic strength, is strongly pH dependent [12]. It is obvious that the binding of Ca^{2+} to CaM and the tryptic fragments is a complicated process including coupled shifts in equilibria involving protons and one or more groups on the proteins.

3.2. Microcalorimetry

The observed enthalpy changes for the binding of Ca^{2+} to bovine testis calmodulin in 50 mM Hepes buffer at pH 7.0 and at all different temperatures are shown in fig. 2. These curves represent the direct experimental results and have not been corrected for proton exchange effects since the actual shapes of the curves are so important in

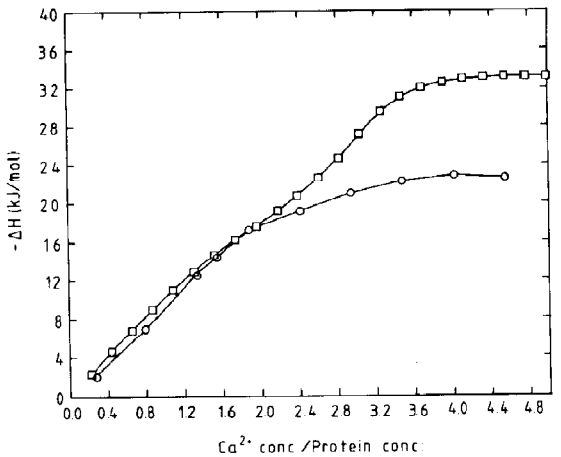

Fig. 1. Calorimetric titration curves for the binding of Ca^{2+} to bovine testis calmodulin at 25 °C and pH 7.0. (⊔——⊔) 50 mM Hepes; (○——○) 50 mM Tris.

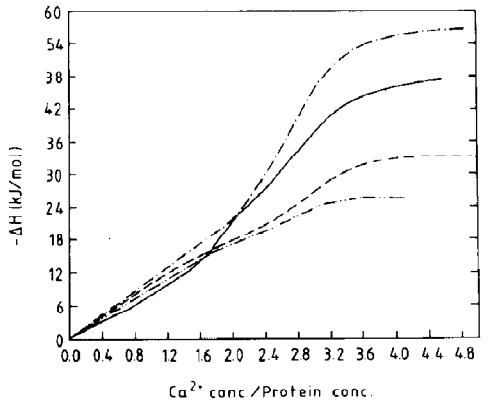

Fig. 2. Calorimetric titration curves for Ca^{2+} binding to calmodulin in 50 mM Hepes buffer, pH 7.0, and at different temperatures: (−·−) 37 °C, (——) 30 °C, (------) 25 °C, (−···−) 21 °C.

themselves (see section 4). In any case, the proton flux curves as a function of added Ca^{2+} are linear within the error limits over the range of calcium concentration in which proton exchange takes place (see also fig. 2 and ref. 12). Thus, corrections for proton exchange will not significantly affect the general appearance of the curves in fig. 2. Since previous studies had been made using bovine brain calmodulin [11,12], we carried out calorimetric experiments on Ca^{2+} binding at 25 °C to this protein as well. The results (not shown) were very similar to those given in fig. 1; there was a slight difference in the shapes of the curves but the total enthalpy change for the binding of four Ca^{2+} was the same within the limits of error. This is not surprising since it has been shown that bovine testis and bovine brain calmodulin have identical structures.

From the enthalpy values at the four different temperatures shown in fig. 2 it was possible to calculate the apparent heat capacity change on binding four Ca^{2+} to CaM. It is obvious that ΔC_p is non-linear in the temperature range studied. The binding curves at the different temperatures clearly indicate also that most of the changes in heat capacity occur on binding to the N-terminal part of the protein, the region containing the sites with lower affinity. The following apparent heat capacity changes can be estimated:

$$\Delta C_p(21\text{–}25\,^\circ\text{C}) = -1600 \pm 50 \text{ J K}^{-1} \text{ mol}^{-1}$$

$$\Delta C_p(30\text{–}37\,^\circ\text{C}) = -1300 \pm 50 \text{ J K}^{-1} \text{ mol}^{-1}$$

These values are not unusual for ligand binding to proteins [9] and include a major contribution from the proton exchange processes mentioned previously.

There are some striking differences between the results shown in fig. 2 and those presented in ref. 12 under high ionic strength conditions. The curves in fig. 2 show clearly that there are at least two classes of calcium binding sites on CaM and that under the prevailing conditions the sites are not equivalent.

Table 2 shows the observed enthalpy changes, ΔH_{Obs}, for the binding of four Ca^{2+} to the proteins at 25 °C, pH 7.0, 50 mM Hepes and at varying KCl concentrations. All the data have been corrected for proton uptake or release using the values given in table 1. The result for CaM in a solution containing 150 mM KCl is in acceptable agreement with the value of +19.9 kJ mol obtained by Milos et al. [12] under similar conditions but using Pipes buffer instead of Hepes. Another value, +20.8 kJ mol, in Pipes buffer and 100 mM KCl [13], is less reliable since it is based on fitting procedures which gave a total of only three calcium-binding sites.

Table 2

Observed enthalpy changes (ΔH_{Obs}), for the binding of Ca^{2+} to CaM and its tryptic fragments at 25 °C, pH 7.0 and in 50 mM Hepes and different concentrations of KCl

All the values have been corrected for proton exchange effects using the data in table 1

Protein	[KCl] (M)	ΔH_{Obs} (kJ mol^{-1})
CaM	0	−41
CaM	0.15	+17
TR_1C	0	−1
TR_1C	0.10	+13
TR_1C	0.15	+15
TR_2C	0	−13
TR_2C	0.10	0
TR_2C	0.15	+3

In table 3, the values of the total thermodynamic parameters for Ca^{2+} binding to the TR_2C fragment of CaM at different ionic strengths are shown. These results demonstrate once again the dramatic effects of increasing ionic strength on systems of the type studied here. The reduction in ΔG_{tot} of approx. 20 kJ mol^{-1} corresponds to a difference of about three orders of magnitude in the product of the two binding constants.

As is shown by the results in tables 2 and 3, the changes in the free energies when increasing the

Table 3

Thermodynamic parameters for the binding of two Ca^{2+} to the TR_2C fragment of CaM at 25 °C and pH 7.0

[KCl]/M	ΔG_{tot}[a] (kJ mol^{-1})	ΔH_{tot} (kJ mol^{-1})	ΔS_{tot} (J K^{-1} mol^{-1})
0	−83.8	−13	238
0.10	−65.0	0	218
0.15	−63.2	+3	222

[a] S. Linse, unpublished results.

salt content are dominated by an enthalpic effect. In particular for the TR_2C fragment, the only example here where a reliable value for ΔG_{tot} at zero added salt is known, the enthalpic contribution is nearly 80% of the total change in ΔG_{tot} when going from zero to 0.15 M KCl. The entropy term, on the other hand, is relatively insensitive to ionic strength changes.

4. Discussion

Calorimetric titration of CaM with Ca^{2+} shows that the total binding process is very complex. Apart from the induced conformational changes the binding process is accompanied by proton exchange and is very sensitive to variations in certain parameters such as temperature and ionic strength (undoubtedly pH changes will also be important). One very important point in this connection concerns the treatment of calorimetric data in the type of system studied here, where the whole CaM molecule binds four Ca^{2+}. At least eight parameters, four enthalpy changes and four equilibrium constants, must be included in any curve-fitting procedures, and this coupled with problems in the determination of protein concentrations and also with the probable existence of cooperativity between sites makes such procedures extremely difficult if not meaningless [23].

We therefore decided to present our calorimetric binding curves in the form of raw experimental data (see fig. 2), thus avoiding any perturbation of the results by complicated fitting schemes. In any case, no reliable binding constants for Ca^{2+} to CaM are known at very low ionic strength. A comparison of the results in fig. 2 with those under high ionic strength conditions [12] is very revealing. In general terms, in processes where electrostatic effects are of importance, going to low ionic strengths will increase binding constants due to the reinforcement of the electrostatic interaction between a metal ion and polar groups at or near a protein binding site (a 10-fold increase has been suggested in the case of Ca^{2+} and CaM [3]). The magnitude of the effect for the TR_2C fragment can be seen in table 3. The important conclusion is that not only the binding constants but also the mode of binding of Ca^{2+} is extremely sensitive to the ionic strength. This demonstrates concisively that caution should be exercised when discussing and comparing binding results from measurements at even slightly different ionic strengths.

Another difference between the work reported here and that already published is the study of the two tryptic fragments of CaM. It is interesting that the sum of the ΔH values for calcium binding to the two fragments is not equal to the total enthalpy change for the binding of four Ca^{2+} to the whole CaM molecule (see table 2). This is probably an indication of significant domain interactions in the intact protein.

Returning to the results shown in table 3, the domination of the enthalpic term in the free energy changes induced by changes in salt content and the fact that entropy term is rather insensitive to such changes are of interest. It seems probable that the effect of added salt is mainly of electrostatic character and does not affect the conformational changes induced in the proteins by calcium binding. The ΔS values would be expected to be dominated by changes in the degree of hydration of the Ca^{2+} on binding to a protein. The fact that ΔS_{tot} for CaM and for the TR_2C fragment are much lower than expected [24] for the binding of four and two Ca^{2+}, respectively, is another piece of evidence that significant conformational changes take place in the proteins during the Ca^{2+}-binding process. A more quantitative analysis of the situation in the CaM-Ca^{2+} system must await determination of accurate Ca^{2+}-binding constants to CaM at zero salt content.

The conformational changes that occur in CaM in connection with the binding of Ca^{2+} are also probably involved in the proton exchange effects noticed in this work. CaM is negatively charged and it seems that the charges are moved closer together when Ca^{2+} binds to the protein, resulting in an uptake of protons to reduce the unfavourable electrostatic effects. The addition of salt leads to a screening of the charges and actually a proton release from the protein.

A comparison of the thermodynamics of Ca^{2+} binding to CaM and other calcium binding proteins of the EF-hand type, all at relatively high

ionic strength, has been made by several authors [24,25]. The balance between enthalpy and entropy effects is different for the different proteins and there seems to be no direct correlation between the thermodynamic parameters for Ca^{2+} binding per site and the number of carboxylate groups involved in the binding. Such a correlation has been suggested previously [26]. The entropies of binding are positive, as is also the case for binding of Ca^{2+} to EDTA and EGTA [27], showing the importance of solvation effects. It is worth pointing out, however, that a comparison between the TR_2C fragment of CaM and calbindin D_{9k} [28] at zero added salt shows some similarities. For these two proteins of very similar size and homology, albeit that one of the sites in calbindin is somewhat modified, the average enthalpy changes per site are -6.5 and -8 kJ mol^{-1}, respectively, while the corresponding entropy values are 119 and 136 J K^{-1} mol^{-1}. It will be interesting to see if such similarities exist between these and other EF-hand proteins under low ionic strength conditions.

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