Nucleotide binding to creatine kinase: an isothermal titration microcalorimetry study

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Abstract We investigated the binding of ATP in the presence and absence of Mg^{2+} to dimeric muscle creatine kinase (CK) by isothermal titration microcalorimetry as a function of pH and temperature. The thermodynamic parameters for these events show that (1) binding of nucleotide to the CK active site does not involve proton exchange with the buffer and (2) the active sites are the only nucleotide binding sites on CK. Interdependence of the active sites in the dimer could not be demonstrated. As CK undergoes major structural changes upon Mg-nucleotide binding, a thermodynamic cycle was employed to calculate the contributions of domain movements to the observed enthalpies.

Key words: Calorimetry; Creatine kinase; Domain movement; Thermodynamic cycle

1. Introduction

Creatine kinase (CK, EC 2.3.2.1) catalyzes the reversible transphosphorylation of creatine and ATP, forming ADP and phosphocreatine. Thereby, CK and the phosphocreatine system play a central role in aerobic metabolism, acting as a temporary and spatial energy buffer system (for a review see [1]). The X-ray structure of octameric chicken sarcomeric muscle mitochondrial CK (Mi-CK) has been solved in the substrate-free ('open') form [2] and recently the structure of the dimeric cytosolic muscle isoform M-CK has also been determined by X-ray crystallography [3]. Hitherto, no structure of any of the isoforms of CK in the substrate-bound ('closed') state has been solved. We have recently reported the structural changes upon substrate binding as observed by small-angle X-ray scattering [4] and shown that CK undergoes substantial structural changes upon binding of Mg-nucleotide or a transition state analogue complex (TSAC) consisting of creatine, ADP, $MgCl_2$ and KNO_3, whereas binding of creatine or Mg-free nucleotide does not lead to any major structural rearrangements. This behavior is in contrast to observations with other kinases, e.g. hexokinase, that undergo domain movements upon binding of their substrates (e.g. glucose), whereas nucleotide binding leaves them unaffected. Solution scattering studies on the monomeric enzyme arginine kinase (AK), a close relative of CK, have shown that the most likely primary change in structure is a domain movement of the two domains of the enzyme of about 9° relative to each other [4,5], assumptions which have recently been corroborated by the solution of the X-ray structure of AK in the presence of a TSAC [6].

Isothermal titration calorimetry (ITC) is the most direct method for the determination of heats of binding ($\Delta H_{binding}$) and binding constants (K_A) upon macromolecular ligand binding [7]. By performing ITC experiments, it is possible not only to determine those two values of a binding reaction, but also to quantify the pH and temperature dependence of the reaction, as well as the enthalpic and entropic contribution to the observed free energy change of binding. Furthermore, it is sometimes possible to determine whether binding sites in oligomeric proteins show independent binding behavior or whether possible cooperative interactions are reflected in the binding characteristics.

We report herein the results of ITC experiments, performed in order to quantify the energetic contributions of nucleotide and Mg-nucleotide binding to the active site and to get an estimate of the energetics of the structural rearrangement observed upon Mg-nucleotide binding. This is also the first report of a titration microcalorimetry study on substrate binding in a phosphagen kinase.

2. Materials and methods

2.1. Materials

Chicken M-CK was prepared as a recombinant protein in *Escherichia coli* and purified as described [8]. ATP was obtained from Sigma (St. Louis, MO, USA), all other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany). For the experiments, protein stock solutions were prepared at a concentration of 11 mg/ml (250 μM) in 10 mM buffer (see below) at pH 7.0 with 1 mM 2-mercaptoethanol added. Nucleotide and Mg-nucleotide solutions were prepared at a concentration of 10 mM in the appropriate buffers. Protein concentrations were determined by the Bio-Rad method [9] using bovine serum albumin as a standard.

2.2. Isothermal titration calorimetry

ITC was performed using an OMEGA titration calorimeter (MicroCal Inc., Northampton, MA, USA) as described by [10]. The calorimeter was calibrated with electrically generated heat pulses as recommended by the manufacturer. To improve baseline stability, the temperature of the system was kept about 5°C below the actual experimental temperature by means of a circulating water bath. Temperature equilibration prior to experiments was allowed for 10–12 h. All solutions were thoroughly degassed before use by stirring under vacuum. Protein samples were prepared in buffers of the same batch to minimize artifacts due to subtle differences in buffer composition and protein concentrations were varied between 10 and 200 μM. The reaction cell was loaded with 1.33 ml protein solution in buffer and the reference cell contained a 0.02% solution of sodium azide in water. The injection syringe was filled with nucleotide or Mg-nucleotide solution in buffer and was rotated at 350 rpm during equilibration and experiment. Ligand concentrations were varied between 4 and 20 mM. Injections were started after baseline stability had been achieved (rms noise < 5 ncal s^{-1}, baseline drift < 10 ncal min^{-1}).

A titration experiment consisted of one injection of 1 μl of titrant and 15 consecutive injections of 8 μl volume and 10 s duration each,

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with a 4.5 min interval between injections. The titration data were corrected for small heat changes observed in control titrations of buffer, nucleotide or $MgCl_2$ into buffer. Data analysis was carried out with the software supplied with the instrument [10]. The total apparent heat of binding, $\Delta H_{observed}$, and the binding constant K_A were obtained by non-linear least-squares fitting of the data to a 1:1 binding model utilizing the Marquardt algorithm. The software-provided stoichiometry estimate, n, was used as an internal control for accuracy and never deviated from the expected value of 1 by more than 5%.

The following buffers were used: 10 mM sodium cacodylate (pH 6.0), 10 mM HEPES (pH 7.0), 10 mM sodium phosphate (pH 7.0), and 10 mM Tris-HCl (pH 7.0 and pH 8.0). The pH values of the buffers were adjusted at the working temperatures of 25, 30 and 37°C, respectively. The ionization enthalpies of the buffers were taken from the literature [11,12].

3. Results and discussion

3.1. Microcalorimetric determination of the binding constants and the enthalpy of reaction dependence on the ionization heat of the buffer

ITC measures the calorimetric enthalpy change, ΔH, when a ligand binds to a protein [7]. Provided it is feasible to conduct the experiment in the appropriate range of protein and ligand concentrations, ITC also yields the association constant K_A, and thus ΔG of the protein:ligand complex according to the equation $\Delta G = -RT \ln K$. The entropy of the binding, ΔS, can then be calculated from the relation $\Delta G = \Delta H - T\Delta S$, with T being the absolute temperature. The course of a typical experiment is shown in Fig. 1.

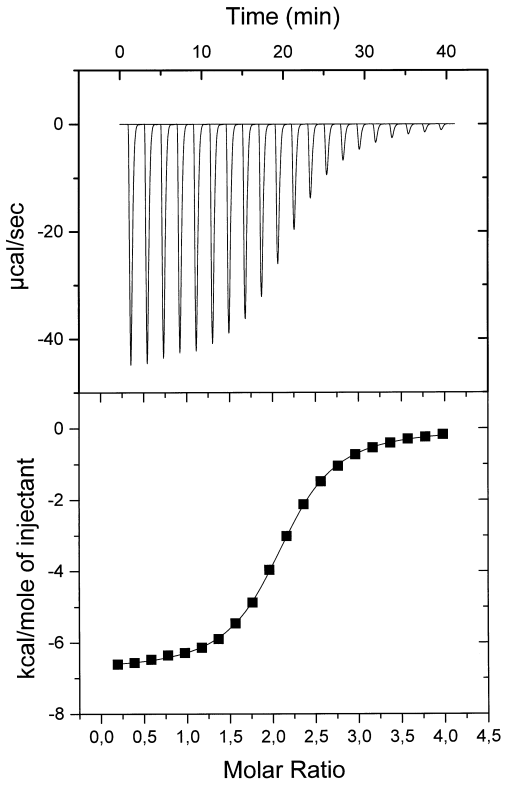

Fig. 1. The course of an ITC experiment for the interaction of creatine kinase with Mg-ATP at 25°C is shown, plotting in the top panel the measured enthalpy change vs. time and in the lower panel the titration curve normalized to molar concentrations. At the inflection point of the curve, the molar ratio in equilibrium can be deduced. The value of 2.0 found here corresponds to 2 mol of nucleotide per mol of CK, which means that both active sites in the dimer are occupied. The best fit of the data to a model with one set of binding sites obtained by non-linear regression is shown (solid line, lower panel).

Affinity constants of the Mg-nucleotide and nucleotide complexes with M-CK were determined in 10 mM Tris buffer at pH 7.0 at temperatures of 25, 30 and 37°C by ITC (Table 1). If binding involves a change in the protonation state of a titratable group in the active site, protons are exchanged with the buffered medium. To obtain the true binding enthalpy, $\Delta H_{binding}$, the observed heat of reaction, $\Delta H_{observed}$, must be corrected to account for the heat of ionization of the buffer, ΔH_{buffer}, according to

$$\Delta H_{observed} = \Delta H_{binding} + n_H^+ \Delta H_{buffer} \quad (1)$$

with n_H^+ being the number of protons released into the buffer. Therefore, we performed ITC experiments in buffers of different heats of ionization at 25°C and analyzed the data according to Eq. 1. For both the binding of free nucleotide and of Mg-nucleotide the resulting slope is close to zero, indicating that binding of neither form of the nucleotide to M-CK involves net transfer of protons between the buffer and the whole protein-ligand complex. It cannot be stated whether there is any release or uptake of protons from the buffer to the active site of CK or vice versa as changes in pK outside the active site may well compensate for changes within the active site. The values do not differ beyond the error margin for the binding of the corresponding diphospho-nucleotides. ΔG varies only slightly with temperature, obviously because of enthalpy-entropy compensation. The binding enthalpy is negative over the whole temperature range, indicating an exothermic binding event. The entropy term $T\Delta S$ is relatively large in magnitude and positive in the physiological temperature range. A favorable ΔS can therefore be assumed to greatly contribute to the strength of nucleotide binding in CK.

3.2. Contributions of domain movements to the observed heats of reaction and sources for the observed thermodynamic quantities

As shown previously [4], CK undergoes large structural changes upon binding of Mg-nucleotide, whereas structural changes upon binding of free nucleotide are of local order only. The observed heats of binding $\Delta H_{binding}$ can be considered to contain a contribution stemming from structural rearrangements of CK. One can conceive a thermodynamic cycle, dissecting the contributions from the individual binding events and use this cycle to calculate the non-observable contribution of the domain closure from the observable values (Fig. 2). ΔG_5 can be calculated as $\Delta G_1 + \Delta G_2 - \Delta G_3 - \Delta G_4$. As the value ΔG_4 is found to be much smaller than the other values, its contribution can be ignored and the calculation can be simplified to the calculation of the difference of $\Delta G_1 + \Delta G_2 - \Delta G_3$. A value for ΔG_2 of -35.3 kJ mol^{-1} at 25°C has been assumed, which is the standard free energy of formation of Mg-ATP^{2-} from ATP^{4-} and Mg^{2+} [13]. The same thermodynamic cycle can be performed for ΔH with similar results, assuming ΔH_2 to be 22.9 kJ mol^{-1} [13]. We thus arrive at a value of -38.2 kJ mol^{-1} for the ΔG of domain closure of CK at 25°C. The corresponding ΔH value is 26.9 kJ mol^{-1} at 25°C, from which a value for $T\Delta S$ of 65.1

kJ mol^{-1} can be calculated. These values can naturally not be considered to be very accurate but yield at least a semi-quantitative idea about the enthalpic and entropic contribution of the domain rearrangement in CK to the observed heats of binding.

Interestingly, if one calculates $\Delta\Delta G$ for the complex formation, the formation of the Mg-ATP-CK complex is 2.9 kJ mol^{-1} more favorable than the formation of the ATP-CK complex. It has been suggested that the binding of Mg-nucleotide to CK, characterized by a large structural change [4], results in an increase in domain interaction [14] which would be characterized by a more favorable ΔG of the complex formation. These results are corroborated by our findings.

The observed overall positive entropy of the binding reaction (see Table 1) stems to a large extent from the structural rearrangement during the domain closure, with the free nucleotide having a substantially lower positive entropy of binding due to the lack of pronounced structural changes of CK upon nucleotide binding. There are still several rearrangements observed in the CK active site upon binding of the free nucleotide [2], which may contribute to the observed entropic change while most of the latter might well be related to solvent molecules. The overall negative binding enthalpy, on the other hand, is indicative of strong electrostatic interactions governing the binding of nucleotide in the CK active site, probably including major desolvation of charged side chains. It should further be kept in mind that the structural changes in the dimer most probably not only contain the contributions from domain closure but that there are further rearrangements of the domains relative to each other. This assumption is corroborated by the finding that applying the domain movement found in the TSAC structure of AK upon the dimeric structure of M-CK, the solution scattering curve observed experimentally cannot be satisfactorily modeled from this structure (M. Forstner, unpublished observation). As ligand binding is stabilized not only by direct interactions at the binding site but also by changes in the pattern of domain interactions resulting from ligand binding, the tighter binding of Mg-ATP than free ATP to the CK active site can probably be attributed directly to the effects of the structural change.

3.3. On the interdependence of the nucleotide binding sites in M-CK

It has been shown [15] that substrate binding to CK exhibits a cooperative behavior of substrate binding in the form of synergism, i.e. if one substrate is bound, the binding of the other is facilitated. There has been a great amount of speculation whether there is also some sort of cooperativity between the two binding sites in the M-CK dimer or the eight

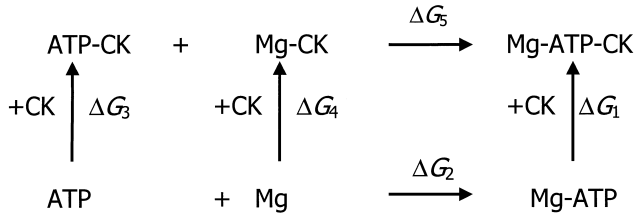

Fig. 2. A thermodynamic cycle has been constructed to determine the not experimentally observable free energy of domain closure (ΔG_5) from the experimentally observable free energies of binding of Mg-ATP (ΔG_1) and free ATP (ΔG_3) to the active site of creatine kinase and from the free energy of Mg-ATP formation (ΔG_2). The value for the binding of Mg^{2+} ions (ΔG_4) is substantially lower than the other values and can be ignored in the calculation. Note that the equations shown in the figure must not be interpreted as stoichiometric chemical equations.

active sites in the Mi-CK octamer and whether the binding sites are identical [16,17]. Enzyme kinetic studies do not show any signs of allostery, so this kind of cooperativity can be ruled out. There are, however, other possibilities for cooperative substrate binding and it seems at least possible that oligomeric CK shows such behavior. It was also unclear whether all active sites in CK have to be occupied or whether there might be partial occupancy. We found a 1:1 stoichiometry of nucleotide and CK active sites, suggesting either that both active sites are occupied or that for one active site in the dimer there exists a second nucleotide binding site other than the second active site. It can be assumed that this second site, if it exists, should show substantially different binding characteristics than the active site. The analysis of the binding thermodynamics by ITC offers a possibility of distinguishing between independent binding sites (i.e. the same binding characteristics for every binding site) or alternative models with different binding characteristics for the individual binding sites. We have therefore fitted our obtained data to different models, assuming either independent or interacting binding sites in the M-CK dimer and varying the values for K_A and ΔH. When the fit was restrained assuming equivalent sites, we obtained an almost perfect fit to the experimental curve as shown in Fig. 1 (χ^2 fit in the software distributed with the ITC instrument [10]). The χ^2 value obtained was 699.2 with a stoichiometry of 1:1 and values for K_A and ΔH as shown in Table 1. Optimizing the fit with a model for independent non-equivalent sites yielded a good fit (χ^2 value 781.2) with almost equivalent values for K_A and ΔH of the individual sites. When we optimized for two interacting nucleotide binding sites, we obtained a less good fit (χ^2 value 3256.3) with the values for K_A and ΔH almost equal to each other and the values ob-

Table 1
Thermodynamic parameters of ATP and Mg-ATP binding to CK in 10 mM Tris buffer (pH 7.0)[a]

T	Nucleotide	$K_A \times 10^{-5}$	ΔG	ΔH	TΔS
298	ATP	0.8 ± 0.24	−28.0 ± 0.74	−17.36 ± 1.80	10.6 ± 1.06
298	Mg-ATP	2.56 ± 0.47	−30.9 ± 0.89	−13.37 ± 1.54	17.5 ± 0.65
303	ATP	0.78 ± 0.44	−28.4 ± 0.78	−17.41 ± 1.95	11.0 ± 1.17
303	Mg-ATP	2.43 ± 0.35	−31.2 ± 0.41	−13.05 ± 1.66	18.2 ± 1.25
310	ATP	0.64 ± 0.23	−28.5 ± 0.69	−16.96 ± 1.85	11.5 ± 1.16
310	Mg-ATP	1.6 ± 0.33	−30.9 ± 0.77	−12.30 ± 1.47	18.6 ± 0.70

[a]Numbers are means ± mean experimental error, determined from four or five individual measurements. Temperatures are in K, the K_A values are in units of (l mol^{-1}), all other values are in (kJ mol^{-1}). Values of ΔG were calculated from K_A. Measured ΔH values were corrected for small heat changes observed in control titrations of buffer, nucleotide or $MgCl_2$ into buffer.

tained in the other fits. The thermodynamic parameters shown in Table 1 were therefore determined assuming equivalent, non-interacting nucleotide sites. From the observed 1:1 stoichiometry of nucleotide binding and the fact that the nucleotide binding sites do not differ at all or at least not significantly in their binding characteristics, we conclude that there are no nucleotide binding sites on the CK molecule other than the active sites. This is corroborated by crystallographic observations of ATP binding to the Mi-CK, which showed density for the nucleotide only in the active sites but not anywhere else [2].

On the other hand, we cannot conclude from the present data whether the active sites in dimeric CK are independent or whether they interact. Although the quality of the fit is better for a model assuming non-interacting, equivalent sites, a model for interacting nucleotide binding sites yields a plausible fit to the experimental data. If there is cooperative behavior, the effects are very subtle and probably only significant under physiological conditions but not observable under the conditions applied in these microcalorimetric studies.

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