

Microcalorimetric studies on the unfolding of creatine kinase induced by guanidine hydrochloride

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Abstract

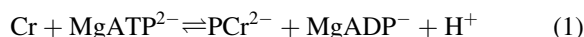
The unfolding of rabbit muscle-type creatine kinase (MM-CK) induced by guanidine hydrochloride (GuHCl) has been studied by isothermal microcalorimetry. It has been found that the decrease in the activity of MM-CK in dilute GuHCl solution is due to a slight perturbation of the active site conformation by dilute GuHCl, but not by a reversible inhibition by GuHCl binding at the active site or dissociation of the dimeric protein. The inactivation of MM-CK precedes the overall conformation change of this enzyme during denaturation by GuHCl, providing a thermodynamic evidence for the proposition that the active site of an enzyme is situated in a limited region more flexible than the enzyme molecule as a whole. The intrinsic enthalpy, Gibbs free energy, and entropy changes for formation of an intermediate state of MM-CK in the presence of moderate GuHCl concentrations at 25.00°C have been determined to be 260, 12.2 kJ mol⁻¹, and 830 J mol⁻¹ K⁻¹, respectively. Further unfolding of MM-CK is observed when GuHCl concentration is higher than 3.00 mol dm⁻³, and the protein is almost fully unfolded at 5.00 mol dm⁻³ GuHCl reached. The intrinsic enthalpy, Gibbs free energy, and entropy changes for formation of the unfolded state of MM-CK at 25.00°C have been measured as 8600, 23.0 kJ mol⁻¹, and 29 kJ mol⁻¹ K⁻¹, respectively. The experimental results indicate that the unfolding of MM-CK by GuHCl exhibits remarkable enthalpy–entropy compensation and the water reorganization is involved in the unfolding reaction. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Creatine kinase; Guanidine hydrochloride; Microcalorimetry; Protein unfolding; Thermodynamics

1. Introduction

Creatine kinase (ATP: creatine phosphotransferase, CK, EC 2.7.2.3) is a key enzyme for cellular energy metabolism and plays an important role in the transport

of high-energy phosphates via phosphocreatine (PCr) to sites of ATP utilization in vivo [1–7]. This enzyme catalyzes the reversible phosphoryl transfer between ATP and creatine (Cr) in the presence of Mg²⁺, and the release of an equimolar quantity of hydrogen ion



Cytosolic creatine kinase from rabbit muscle (MM-CK) is a dimer of two identical 43 kDa polypeptide chains of known sequence [8]. The recently published structure of the enzyme at 2.35 Å resolution has

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revealed that the dimeric interface of the enzyme is held together by a small number of hydrogen bonds [9].

Protein unfolding is crucial for dismantling protein complexes, transporting proteins between cellular compartments and preparing proteins for degradation when necessary [10]. Guanidine hydrochloride (GuHCl) is a chaotropic agent commonly used in the investigations of protein unfolding [11–13]. Although a significant number of experimental approaches have been used to elucidate the mechanism of the unfolding of CK induced by GuHCl in the past two decades [14–24], the detailed analysis of the unfolding in thermodynamic terms is lacking. The purpose of the present investigation is to provide thermodynamic data of the unfolding of MM-CK by GuHCl to furnish insights into the mechanism for the unfolding of this dimeric protein.

Microcalorimetry, including differential scanning calorimetry (DSC) and isothermal microcalorimetry, is an important tool for the study of both thermodynamic and kinetic properties of biological macromolecules by virtue of its general applicability and high precision, as shown by recent developments [25–27]. This method has yielded a large amount of useful thermodynamic data on protein folding/unfolding [12,28–39]. Among the vast amount of literature on the thermodynamics of protein unfolding, only a limited number of authors have paid some attention to the isothermal microcalorimetric investigations of the interaction of proteins with denaturants [12,28,29].

In a previous publication from this laboratory [7], the reversible phosphoryl transfer from ATP to creatine, catalyzed by rabbit muscle-type creatine kinase (MM-CK) in the presence of GuHCl, was investigated by isothermal microcalorimetry. In this study, isothermal microcalorimetry was used to conduct a thermodynamic investigation of the unfolding of MM-CK induced by GuHCl. The experimental results provide a thermodynamic evidence for the proposition that enzyme active sites may display more conformational flexibility than the enzyme molecules as a whole [15,16,20]. Thermodynamic models for formation of the intermediate and unfolded states of a protein by GuHCl were proposed and the intrinsic enthalpy, Gibbs free energy, and entropy changes for formation

of these states of MM-CK were reported for the first time.

2. Theory and method

2.1. A thermodynamic model for formation of the intermediate state of a protein in moderate concentrations of GuHCl

For the intermediate state of a protein in moderate concentrations of GuHCl, we have [30]

$$\Delta_{\text{conf}}H_{\text{m,a}}^{\text{I}} = \Delta_{\text{I}}H_{\text{m}}^{\circ} + \Delta n_{\text{I}}\Delta_{\text{b}}H_{\text{m}}^{\circ} \frac{K_{\text{b,I}}a_{\text{GuHCl}}}{1 + K_{\text{b,I}}a_{\text{GuHCl}}} \quad (2)$$

where $\Delta_{\text{conf}}H_{\text{m,a}}^{\text{I}}$ is the apparent molar enthalpy accompanying the conformational change of the protein by intermediate concentrations of GuHCl, which can be measured by isothermal microcalorimetry, $\Delta_{\text{I}}H_{\text{m}}^{\circ}$ the intrinsic enthalpy change for the intermediate state (I), Δn_{I} the difference in the number of binding sites for GuHCl between the intermediate and native states (N), $\Delta_{\text{b}}H_{\text{m}}^{\circ}$ the intrinsic binding enthalpy of GuHCl, $\Delta n_{\text{I}}\Delta_{\text{b}}H_{\text{m}}^{\circ}$ the total binding enthalpy of GuHCl binding to I, $K_{\text{b,I}}$ the intrinsic binding constant of GuHCl binding to I, and a_{GuHCl} is the activity of GuHCl which can be calculated using the mean ion activity coefficients published by Pace and co-workers [11,12,31]. The above equation can be used to perform a non-linear least-squares analysis of $\Delta_{\text{conf}}H_{\text{m,a}}^{\text{I}}$ as an explicit function of a_{GuHCl} . Then three unknown thermodynamic parameters, $\Delta_{\text{I}}H_{\text{m}}^{\circ}$, $\Delta n_{\text{I}}\Delta_{\text{b}}H_{\text{m}}^{\circ}$, and $K_{\text{b,I}}$, can be obtained by fitting the experimental data ($\Delta_{\text{conf}}H_{\text{m,a}}^{\text{I}}$ and a_{GuHCl}) to the model using the Origin software provided by Microcal Software Inc. (version: 6). Thus, the intrinsic Gibbs free energy change, $\Delta_{\text{I}}G_{\text{m}}^{\circ}$, and the intrinsic entropy change, $\Delta_{\text{I}}S_{\text{m}}^{\circ}$, for the intermediate state can be calculated by the following formulae, respectively

$$\Delta_{\text{I}}G_{\text{m}}^{\circ} = \Delta_{\text{NI}}G_{\text{m}}^{\circ} \quad (3)$$

$$\Delta_{\text{I}}S_{\text{m}}^{\circ} = \frac{\Delta_{\text{I}}H_{\text{m}}^{\circ} - \Delta_{\text{I}}G_{\text{m}}^{\circ}}{T} \quad (4)$$

where $\Delta_{\text{NI}}G_{\text{m}}^{\circ}$ is the intrinsic Gibbs free energy change of the transition N → I.

2.2. A thermodynamic model for formation of the unfolded state of a protein by high concentrations of GuHCl

For the unfolded state of a protein by high concentrations of GuHCl, we get [30]

$$\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}} = \Delta_{\text{U}}H_{\text{m}}^{\circ} + \Delta n_{\text{U}}\Delta_{\text{b}}H_{\text{m}}^{\circ} \frac{K_{\text{b,U}}a_{\text{GuHCl}}}{1 + K_{\text{b,U}}a_{\text{GuHCl}}} \quad (5)$$

where $\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}}$ is the apparent molar enthalpy accompanying the conformational change of the protein by high concentrations of GuHCl, which can also be measured by isothermal microcalorimetry, $\Delta_{\text{U}}H_{\text{m}}^{\circ}$ is the intrinsic enthalpy change for the unfolded state (U), Δn_{U} is the difference in the number of binding sites for GuHCl between the unfolded and native states (N), $\Delta n_{\text{U}}\Delta_{\text{b}}H_{\text{m}}^{\circ}$ is the total binding enthalpy of GuHCl binding to U, and $K_{\text{b,U}}$ is the intrinsic binding constant of GuHCl binding to U. The above equation can be used to perform a non-linear least-squares analysis of $\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}}$ as an explicit function of a_{GuHCl} . Then three unknown thermodynamic parameters, $\Delta_{\text{U}}H_{\text{m}}^{\circ}$, $\Delta n_{\text{U}}\Delta_{\text{b}}H_{\text{m}}^{\circ}$, and $K_{\text{b,U}}$, can also be obtained by fitting the experimental data ($\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}}$ and a_{GuHCl}) to the model using the Origin software. The intrinsic Gibbs free energy change, $\Delta_{\text{U}}G_{\text{m}}^{\circ}$, and the intrinsic entropy change, $\Delta_{\text{U}}S_{\text{m}}^{\circ}$, for the unfolded state can be calculated by the following relations, respectively

$$\Delta_{\text{U}}G_{\text{m}}^{\circ} = \Delta_{\text{N}}G_{\text{m}}^{\circ} + \Delta_{\text{IU}}G_{\text{m}}^{\circ} \quad (6)$$

$$\Delta_{\text{U}}S_{\text{m}}^{\circ} = \frac{\Delta_{\text{U}}H_{\text{m}}^{\circ} - \Delta_{\text{U}}G_{\text{m}}^{\circ}}{T} \quad (7)$$

where $\Delta_{\text{IU}}G_{\text{m}}^{\circ}$ is the intrinsic Gibbs free energy change of the transition $\text{I} \rightarrow \text{U}$.

3. Experimental

3.1. Reagents

Rabbit MM-CK was prepared and purified as described previously [1]. The $A_{1\text{cm}}^{1\%}$ value of 8.8 [24] was used for protein concentration measurements. The specific activity of this enzyme was determined by the method developed in this laboratory [19,40] to be 130 U mg^{-1} . Ultra-pure GuHCl was from ICN Biomedicals Inc., USA. The CK-GuHCl solution at each denaturant concentration

was prepared by incubation of the enzyme with GuHCl at different concentrations at 4°C overnight. Creatine, glycine and the disodium salt of ATP were Sigma products with purity $\geq 98\%$. Magnesium acetate (MgAc_2) and other chemicals used were domestic and of A.R. grade. All reagent solutions were prepared in 0.1 mol dm^{-3} glycine-NaOH buffer ($\text{pH} = 9.0$). ATP solution was always freshly prepared.

3.2. Instrumentation

The heats of the unfolding of rabbit MM-CK induced by GuHCl were determined at 298.15 K (25.00°C) using a LKB-2107 batch microcalorimeter equipped with a microtitrator unit, which consists of a microbatch reactor with a heat-conduction isothermal calorimeter. The first of this type of microcalorimeter was designed and built by Wadsö [41]. One of the main components of the instrument consists of twin calorimeter cells, one of which is the reaction cell and the other a reference cell, each cell being divided into two parts [7,41–43]. Compartment I of reaction cell contained 0.0250 cm^3 (or 0.0500 , 0.100 , 0.200 cm^3 , respectively) of MM-CK solution, and compartment II contained 0.500 cm^3 (or 1 , 2 , 4 cm^3 , respectively) of GuHCl solution. To avoid the influence of the heat effects of diluting and mixing, etc. on the results, the contents and quantities in both cells were made as identical as possible except that CK was not added to the reference cell. After thermal equilibrium of the microcalorimetry system and a steady baseline on the recorder had been obtained, the reaction run was initiated by starting rotation of the calorimeter 360° clockwise and then anti-clockwise, in order to mix the two compartments thoroughly. The output signal was recorded in the form of a calorimetric curve by means of the LKB-2210 dual-pen integration recorder. The heat released by dilution of CK is negligible.

4. Results

4.1. Comparison of inactivation with conformational changes of MM-CK at different GuHCl concentrations

Fig. 1 shows a comparison of the activity of MM-CK with the apparent molar enthalpy accompanying

Table 1

Intrinsic thermodynamic parameters for the intermediate (I) and the unfolded state (U) of MM-CK at different concentrations of the protein and at 298.15 K and pH 9.0

State	$[E]_0$ ($\mu\text{mol dm}^{-3}$)	ΔH_m° (kJ mol^{-1})	ΔG_m° (kJ mol^{-1})	ΔS_m° ($\text{J K}^{-1} \text{mol}^{-1}$)	K_b	$-\Delta n \Delta_b H_m^\circ$ ^a (kJ mol^{-1})
I	1.73	250	11.8	800	1.8	7200
	3.47	260	12.5	830	2.0	6400
	6.94	270	12.3	860	2.3	5700
Average values		260 ± 10	12.2 ± 0.4	830 ± 30	2 ± 0.3	6400 ± 800
U	1.73	8300	22.4	28000	0.45	65500
	3.47	9100	23.4	30000	0.48	65300
	6.94	8400	23.3	28000	0.50	60600
Average values		8600 ± 400	23 ± 0.6	29000 ± 1000	0.48 ± 0.03	63800 ± 2800

^a $\Delta n_U / \Delta n_I \approx 10$.

At intermediate GuHCl concentrations (0.400–1.00 mol dm⁻³), the intermediate state is maximally populated [17,21,23,24]. Therefore, the thermodynamic model for the intermediate (Eq. (2)) can be used to perform a non-linear least-squares analysis of the calorimetric data ($\Delta_{\text{conf}} H_{m,a}^I$) as a function of GuHCl activity in this range of GuHCl concentration. Table 1 summarizes the intrinsic thermodynamic parameters for the intermediate state of MM-CK at different concentrations of the protein and at 298.15 K and pH 9.0, in which the values of $\Delta_I H_m^\circ$, $\Delta n_I \Delta_b H_m^\circ$, and $K_{b,I}$ are obtained by fitting the experimental data (the hollow circles in Fig. 3B, in part) to Eq. (2). Here, the experimental data are calculated from the peak height and peak area of the calorimetric curves (Fig. 3A) by Tian's equation [25,43]. The $\Delta_I G_m^\circ$ is obtained from the observed fractional change in $\Delta_{\text{conf}} H_{m,a}^I$ in this range of GuHCl concentration (Fig. 1) according to a three-state model as described [18]. Then the remaining intrinsic thermodynamic parameter for the intermediate state, $\Delta_I S_m^\circ$, is calculated by Eq. (4). The solid line in Fig. 3B is the apparent molar enthalpy change accompanying the conformational change of the enzyme in moderate concentrations of GuHCl as predicted by the parameters in Table 1. As can be seen from Table 1, even though the apparent molar enthalpy change accompanying the conformational change of MM-CK in moderate concentrations of GuHCl is negative, the conformational change during the formation of the intermediate state of the protein is endothermic in practice. As can also be seen from Table 1, the value of $\Delta_I H_m^\circ$ is independent of the concentrations of MM-CK, implying that the

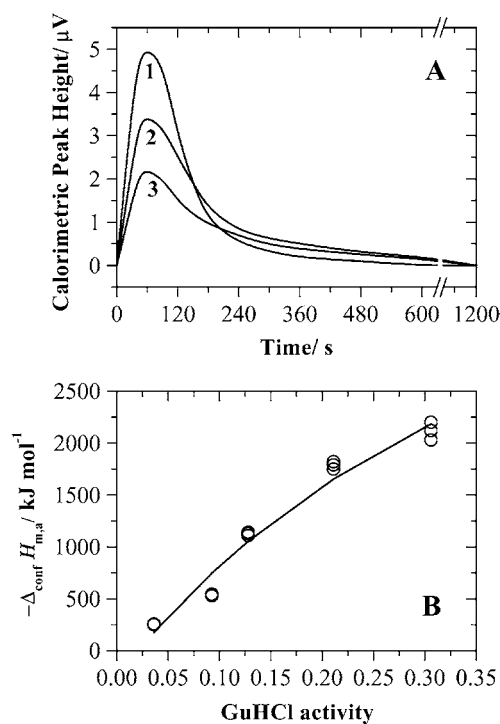


Fig. 3. (A) Calorimetric curves for the unfolding of MM-CK at moderate concentrations of GuHCl; (B) plot of the apparent molar enthalpy change accompanying the conformational change of MM-CK measured by isothermal microcalorimetry at moderate concentrations of GuHCl against GuHCl activities. The experimental conditions were $T = 298.15$ K, $\text{pH} = 9.0$, and the initial concentration of enzyme was $3.47 \mu\text{mol dm}^{-3}$. In (A), the initial concentrations of GuHCl are 1.00, 0.800 and $0.600 \text{ mol dm}^{-3}$ for curves 1, 2 and 3, respectively. The total volume of the reaction system is 2.10 cm^3 in the present case. In (B), the hollow circles are the experimental data, and the solid line is the theoretical curve predicted by the thermodynamic model (Eq. (2)) and the parameters in Table 1.

dissociation of the dimer is independent of the concentrations of the protein.

4.4. Intrinsic thermodynamic parameters for the unfolded state of MM-CK

From the isothermal microcalorimetric experiments (Fig. 1), further unfolding of MM-CK in GuHCl concentration higher than 3.00 mol dm^{-3} involves no further changes in intrinsic fluorescence, UV absorbance, and CD spectra [1,24]. The protein is almost fully unfolded at a concentration of GuHCl of $75.00 \text{ mol dm}^{-3}$, in agreement with the ESR results [19].

At high GuHCl concentrations ($1.50\text{--}8.00 \text{ mol dm}^{-3}$), the unfolded state becomes the most significantly populated state [23,24]. Hence, the thermodynamic model for the unfolded state (Eq. (5)) can be used to perform a non-linear least-squares analysis of the calorimetric data ($\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}}$) as a function of GuHCl activity in this range of GuHCl concentration. Table 1 also lists the intrinsic thermodynamic parameters for the unfolded state of MM-CK at different concentrations of the protein and at 298.15 K and $\text{pH } 9$, in which the values of $\Delta_{\text{U}}H_{\text{m}}^{\circ}$, $\Delta n_{\text{U}}\Delta_b H_{\text{m}}^{\circ}$, and $K_{\text{b,U}}$ are obtained by fitting the experimental data (the hollow circles in Fig. 4B, in part) to Eq. (5). Here, the experimental data are calculated from the peak height and peak area of the calorimetric curves (Fig. 4A) by Tian's equation. The $\Delta_{\text{U}}G_{\text{m}}^{\circ}$ is obtained from the observed fractional change in $\Delta_{\text{conf}}H_{\text{m,a}}^{\text{U}}$ in this range of GuHCl concentration (Fig. 1) according to the three-state model reported [18]. Then the remaining intrinsic thermodynamic parameter for the unfolded state, $\Delta_{\text{U}}S_{\text{m}}^{\circ}$, is calculated by Eq. (7). The solid line in Fig. 4B is the apparent molar enthalpy change accompanying the conformational change of the enzyme by high concentrations of GuHCl as predicted by the parameters in Table 1. As can be seen from Table 1, the standard relative errors of the intrinsic thermodynamic data for the intermediate and unfolded states are small, therefore validating the thermodynamic models used. As we can also see from Table 1, the value of Δn_{U} is about 10 times as that of Δn_{i} , indicating that the buried hydrophobic side chains become exposed gradually during the unfolding of MM-CK with increasing concentration of GuHCl. This suggests that the number of exposed amino acid

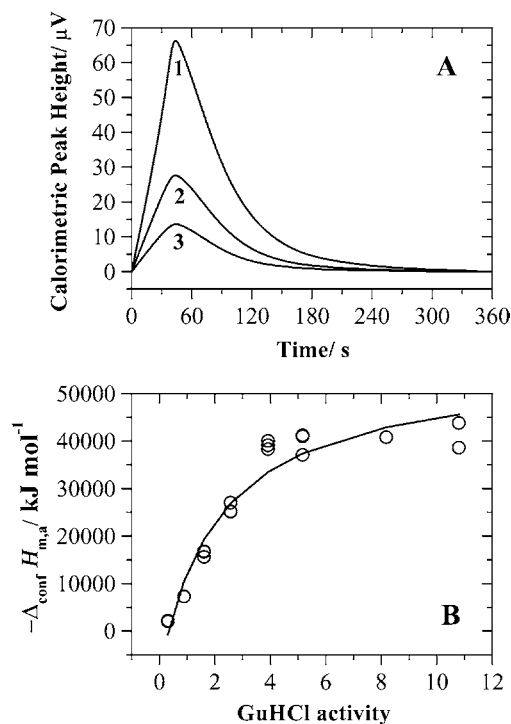


Fig. 4. (A) Calorimetric curves for the unfolding of MM-CK at high concentrations of GuHCl; (B) plot of the apparent molar enthalpy change accompanying the conformational change of MM-CK measured by isothermal microcalorimetry at high concentrations of GuHCl against GuHCl activities. The experimental conditions were $T = 298.15 \text{ K}$, $\text{pH} = 9.0$, and the initial concentration of enzyme was $3.47 \text{ } \mu\text{mol dm}^{-3}$. In (A), the initial concentrations of GuHCl are 5.00 , 3.00 and 2.00 mol dm^{-3} for curves 1, 2 and 3, respectively. The total volume of the reaction system is 1.05 cm^3 in the present case. In (B), the hollow circles are the experimental data, and the solid line is the theoretical curve predicted by the thermodynamic model (Eq. (5)) and the parameters in Table 1.

residues in the unfolded state far exceeds that in the intermediate state. Recently, Lyubarev et al. [36] investigated the thermal denaturation of rabbit MM-CK using DSC. The intrinsic molar enthalpy accompanying the thermally-induced conformational change of the protein was determined by this method to be 1079 kJ mol^{-1} , ranging between the intrinsic enthalpy change for the intermediate state (260 kJ mol^{-1}) and that for the unfolded state (8600 kJ mol^{-1}). This suggests that the denatured monomer formed during thermal unfolding of MM-CK should be a pre-molten globule state provided the intermediate state in this study is a molten globule state, therefore supporting

the mechanism for the unfolding of MM-CK proposed by Vial and co-workers [21].

It is noted that the observed $\Delta_U H_m^\circ$ per residue for the GuHCl-induced unfolding of MM-CK is one order of magnitude higher than those for the thermal unfolding reactions of other proteins of this size [34]. This may result from the difference between denaturant and temperature-induced unfolding of proteins.

5. Discussion

5.1. About the heat of dilution of GuHCl

The dilution of GuHCl at moderate and high concentrations (0.300–8.00 mol dm⁻³) is a largely endothermic process, and hence the heat of dilution of the denaturant will disturb seriously the measurement of heat accompanying the conformational change of a protein by GuHCl as recognized by Makhatadze and Privalov [12]. In this paper, we have taken two measures against the influence of this type of dilution heat to reduce the experimental errors effectively. The first measure is that the contents and quantities in both cells were made as identical as possible except that MM-CK was not added to the reference cell. The second measure is that the volume ratio of MM-CK to GuHCl is very small (in this paper, the ratio is 0.0500) so that the heat of dilution of the denaturant can be avoided as much as possible.

5.2. Unfolding of MM-CK induced by GuHCl exhibits remarkable enthalpy–entropy compensation

Liu et al. [44] have proposed that the intriguing phenomenon of enthalpy–entropy compensation in protein unfolding results from the water molecule reorganization. As shown in Table 1, the intrinsic molar enthalpy change for the GuHCl-induced unfolding of MM-CK was almost compensated by a corresponding change in entropy resulting in a smaller net Gibbs free energy increase. That is, remarkable enthalpy–entropy compensation occurred in the unfolding of the protein induced by GuHCl, suggesting that the water reorganization is involved in the unfolding reaction.

5.3. Decrease in the activity of MM-CK in dilute GuHCl solutions is due to a slight conformational perturbation of the active site

To demonstrate that the active site conformation of MM-CK is indeed perturbed in dilute GuHCl solutions leading thus to inactivation of the enzyme, the real molar enthalpy change accompanying the slight conformational perturbation of the protein, excluding the binding enthalpy of GuHCl, has been obtained from the calorimetric data.

According to the amino acid sequence of 760 amino acid residues and the structure of MM-CK [8,9], 10% of the protein are believed to be exposed to solvents. For the unfolded state of MM-CK, it can be supposed that all the residues are exposed to solvents. Combining the intrinsic thermodynamic data in Table 1, we easily get that the values of Δn_U , $\Delta_b H_m^\circ$, Δn_I , and n_I are close to 700, -90 kJ mol⁻¹, 70, and 150, respectively. The above analysis indicates that about 20% of the amino acid residues become exposed in the intermediate of MM-CK.

The real molar enthalpy change accompanying the slight conformational perturbation of MM-CK in GuHCl of low concentrations (0.0100–0.100 mol dm⁻³), $\Delta_{\text{conf}} H_m$, excluding the total apparent molar binding enthalpy of GuHCl, $\Delta_b H_{m,a}$, can be calculated by the following equations, respectively

$$\Delta_{\text{conf}} H_m = \Delta_{\text{conf}} H_{m,a} - \Delta_b H_{m,a} \quad (8)$$

$$\Delta_b H_{m,a} = n_N \Delta_b H_m^\circ \frac{K_{b,N} a_{\text{GuHCl}}}{1 + K_{b,N} a_{\text{GuHCl}}} \quad (9)$$

where $\Delta_{\text{conf}} H_{m,a}$ is the apparent molar enthalpy change accompanying the slight conformational perturbation of the protein in GuHCl at low concentrations, which has been measured by isothermal microcalorimetry directly (see Fig. 1), n_N the number of binding sites of the native state (N) by GuHCl, the value of $n_N \Delta_b H_m^\circ$ approximates to -7000 kJ mol⁻¹, and $K_{b,N}$ is the intrinsic binding constant of GuHCl binding to N, whose value is close to that of $K_{b,I}$. The calculated results of these thermodynamic data are shown in Fig. 5. From Fig. 5, it can be found that at the same GuHCl concentration, the changes in $\Delta_{\text{conf}} H_m$ and in the activity of MM-CK at the concentration range of 0.0100–0.100 mol dm⁻³ GuHCl occur to the same extent. Sedimentation velocity and size-exclusion chromatography experiments [17,21,23] show that

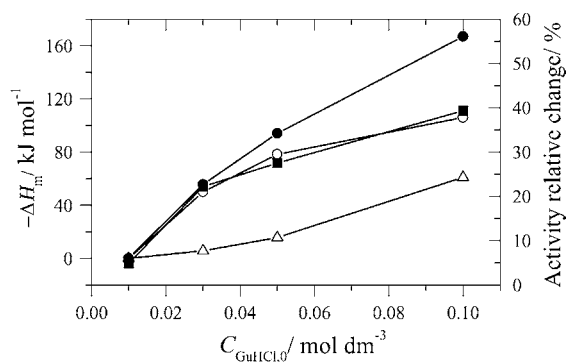


Fig. 5. The apparent (●, left) and real (○, left) molar enthalpies accompanying the conformational change of MM-CK measured by isothermal microcalorimetry, the total apparent molar binding enthalpy of GuHCl (△, left), and the activity relative change percent (■, right) of MM-CK in dilute GuHCl solutions. The experimental conditions were $T = 298.15$ K, $\text{pH} = 9.0$, and the initial concentration of enzyme were 3.47 (●, ○, △) and $0.607 \mu\text{mol dm}^{-3}$ (■).

at such low GuHCl concentrations, MM-CK remains in a dimeric state. GuHCl concentrations for the loss of the activity of MM-CK are much lower than those required to dissociate the dimer, suggesting that the active site is inherently flexible compared with the remainder of the protein, and is able to unfold in a co-operative manner. As GuHCl does have the same structural feature as creatine (*viz.* the guanidino group), it could indeed act as a competitive inhibitor of the enzyme [14]. However, it is known that the binding of a reversible competitive inhibitor to the enzyme usually has a half-time in the microsecond range whereas the half-time for the rapid inactivation of the enzyme in GuHCl has been found to be in the range of 0.1 – 1 s [14,45]. There are several orders of magnitude slower than the expected binding rate for a reversible competitive inhibitor. It is therefore clear that the decrease in the activity of MM-CK in dilute GuHCl solutions is due to a slight perturbation of the active site conformation by dilute GuHCl, but not by a reversible inhibition by GuHCl binding at the active site or dissociation of the dimeric protein.

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