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Unfolding of creatine kinase induced by acid studied by isothermal titration calorimetry and fluorescence spectroscopy☆

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Abstract

Thermodynamics of the unfolding of rabbit muscle-type creatine kinase (MM-CK) induced by acid has been studied by isothermal titration calorimetry and fluorescence spectroscopy. The conformational transition between the native state and a partially folded intermediate of this protein occurs in the pH range 7.0–5.2, and the transition between the intermediate and the unfolded state of this protein occurs in the pH range 5.2–3.0. The protein is almost fully unfolded at pH 3.0. The intrinsic molar enthalpy changes for formation of the unfolded state of MM-CK induced by acid at 15.0, 25.0, 30.0 and 37.0 °C have been determined by isothermal titration calorimetry. A large positive molar heat capacity change of the unfolding, 36.8 kJ mol^{-1} K^{-1}, at all temperatures examined indicates that hydrophobic interaction is the dominant driving force stabilizing the native structure of MM-CK. Combining the results from 'phase diagram' method of fluorescence, we conclude that the acid-induced unfolding of MM-CK follows a 'three-state' model.

Keywords: Acid; Creatine kinase; Isothermal titration calorimetry; 'Phase diagram' method of fluorescence; Protein unfolding

1. Introduction

Creatine kinase (CK, EC 2.7.3.2) is a key enzyme for energy homeostasis in cells and plays a significant role in the transport of high-energy phosphates via phosphocreatine (PCr) to sites of ATP utilization in vivo [1–4]. This enzyme catalyzes the reversible phosphoryl transfer between ATP and creatine (Cr) in the presence of Mg^{2+}, and the release of an equimolar quantity of hydrogen ion:

$$Cr + MgATP^{2-} \rightleftharpoons PCr^{2-} + MgADP^{-} + H^{+} \quad (I)$$

Cytosolic creatine kinase from rabbit muscle (MM-CK) is a dimer of two identical 43 kDa polypeptide chains of known sequence [5]. The crystal structure of the enzyme at 2.35 Å resolution has revealed that the dimeric interface of the enzyme is held together by a small number of hydrogen bonds [6].

Chemical denaturants, such as guanidine hydrochloride and urea, have been widely used in the investigations of the unfolding of CK [7–15]. Because there is a direct interaction between a denaturant and a protein, some thermodynamic parameters may reflect protein–denaturant interaction rather than intrinsic parameters of the protein [14]. On the other hand, the heat of dilution of a denaturant may disturb seriously the measurement of heat accompanying the conformational change of a protein by the denaturant [14,16]. Acid-induced unfolding of proteins, however, obviates the above inconveniences, since the unfolding agent, H^{+}, is itself a part of the buffer used. Although some experimental approaches have been used to elucidate the mechanism of the unfolding of CK induced by acid in the past decade [17], thermodynamic information for the unfolding, which is necessary for a thorough understanding of the mechanism, is eagerly awaited. The purpose of this investigation is to provide detailed thermodynamic data for the acid-induced unfolding of MM-CK to furnish insights into the mechanism for the unfolding of this dimeric protein.

Isothermal titration calorimetry (ITC) 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

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developments [18–20]. This method has yielded a large amount of useful thermodynamic data on protein folding/unfolding [16,21–30]. Only a limited number of authors have, however, paid attention to the isothermal titration calorimetric investigations of the protein unfolding induced by acid [22,28].

In a previous publication from this laboratory [14], the unfolding of MM-CK induced by guanidine hydrochloride was investigated by isothermal calorimetry. In this study, isothermal titration calorimetry was used to conduct a thermodynamic investigation of the unfolding of MM-CK induced by acid. The intrinsic molar enthalpy changes for formation of the unfolded state of this protein induced by acid at different temperatures were reported for the first time. Combining the results from 'phase diagram' method of fluorescence, we concluded that hydrophobic interaction is the dominant driving force stabilizing the native structure of MM-CK and the acid-induced unfolding of this protein follows a 'three-state' model.

2. Experimental

2.1. Materials

Rabbit MM-CK (Sigma Chemical Co., St. Louis, MO) was used without further purification. The $A^{1\%}_{1\,cm}$ value of 8.8 [10] was used for protein concentration measurements. All chemicals used were made in China and of analytical grade. All reagent solutions were prepared in 0.01 mol dm^{-3} ammonium acetate buffers (pH 3.0–7.0) and 0.1 mol dm^{-3} citric acid–Na_2HPO_4 buffers (pH 3.0–7.0) for ITC and fluorescence experiments, respectively.

2.2. Methods

2.2.1. Isothermal titration calorimetry

ITC measurements were carried out at 15.0, 25.0, 30.0 and 37.0 °C using a VP-ITC titration calorimetry (MicroCal, Northampton, MA). All solutions were thoroughly degassed before use by stirring under vacuum. Before each experiment, the ITC sample cell was washed several times with acetate buffer. The sample cell was loaded with 1.43 cm^3 of acetate buffer (pH 3.0–6.7) and the reference cell contained doubly distilled water. Titration was carried out using a 0.250 cm^3 syringe filled with the native MM-CK solution (pH 7.0), with stirring at 300 rpm. The concentrations of MM-CK were varied between 15 and 30 μmol dm^{-3}. Injections were started after baseline stability had been achieved. A titration experiment consisted of 28 consecutive injections of 0.0100 cm^3 volume and 20 s duration each, with a 6 min interval between injections. To correct for the heat effects of dilution and mixing, control experiments were performed in which an identical solution but without MM-CK (pH 7.0) was injected into acetate buffer (pH 3.0–6.7). The heat released by dilution of MM-CK is negligible. Calorimetric data were analyzed using MicroCal ORIGIN software supplied with the instrument. The enthalpy change for each injection was calculated by integrating the area under the peaks of recorded time course of change of power and then subtracted by that for the control titration. The molar enthalpy change accompanying the conformational change of MM-CK induced by acid, $\Delta_{conf}H_m$, was the average value of the enthalpy change for each injection, and the intrinsic molar enthalpy change for formation of the unfolded state of MM-CK induced by acid, $\Delta_U H^\circ_m$, was the average value of the molar enthalpy changes at pH values 3.0, 3.5 and 4.0 at each temperature. After the calorimetric experiment on the acid-induced unfolding, the pH value of the residual solution taken from the sample cell was almost the same as that of the acetate buffer used.

2.2.2. Fluorescence spectroscopy

Fluorescence spectroscopic experiments were carried out at 25 °C using an LS-55 luminescence spectrometer (Perkin-Elmer, Shelton, CT). Each MM-CK solution, at a final concentration of 0.29 μmol dm^{-3}, was incubated for 30–60 min at room temperature. The excitation wavelength at 295 nm was used for the intrinsic fluorescence measurements and the fluorescence spectra were recorded between 300 and 390 nm. The excitation and emission slits are both 10 nm and the scan speed is 1000 nm min^{-1}.

2.2.3. 'Phase diagram' method of fluorescence

The 'phase diagram' method of fluorescence is extremely sensitive for the accurate detection of unfolding/refolding intermediates of proteins [15,31,32]. The essence of this method is to build up the diagram of $I(\lambda_1)$ versus $I(\lambda_2)$, where $I(\lambda_1)$ and $I(\lambda_2)$ are the fluorescence intensity values measured on wavelengths λ_1 and λ_2, respectively, under different experimental conditions for a protein undergoing structural transformations. As fluorescence intensity is the extensive parameter, it will describe any two-component system by a simple relationship [15,31,32]:

$$I(\lambda_1) = a + bI(\lambda_2) \tag{1}$$

where a and b are defined by Eqs. (2) and (3):

$$a = I_1(\lambda_1) - \frac{I_2(\lambda_1) - I_1(\lambda_1)}{I_2(\lambda_2) - I_1(\lambda_2)} I_1(\lambda_2) \tag{2}$$

$$b = \frac{I_2(\lambda_1) - I_1(\lambda_1)}{I_2(\lambda_2) - I_1(\lambda_2)} \tag{3}$$

Here, $I_1(\lambda_1)$ and $I_2(\lambda_1)$ are the fluorescence intensities of the first and second components measured on wavelength λ_1, respectively, and $I_1(\lambda_2)$ and $I_2(\lambda_2)$ are those of the first and second components measured on wavelength λ_2, respectively.

Eq. (2) means that if with the change of denaturing factor, such as denaturant concentration, temperature and pH of the solution, the transition from the initial to the final state follows a 'two-state' or 'all-or-none' model without formation

of the intermediate states, the dependence $I(\lambda_1) = f(I(\lambda_2))$ must be linear. If the transition from the initial to the final state follows a 'three-state' or 'multi-state' model with the formation of one or several intermediate states, the dependence $I(\lambda_1) = f(I(\lambda_2))$ must be nonlinear and contains two or more linear portions. Moreover, each linear portion of the $I(\lambda_1) = f(I(\lambda_2))$ dependence will describe an individual all-or-none transition. In principle, λ_1 and λ_2 are arbitrary wavelengths of the fluorescence spectrum, but in practice such diagrams will be more informative if λ_1 and λ_2 will be on different slopes of the spectrum such as 320 and 365 nm [15,31,32].

3. Results

3.1. Molar enthalpy changes accompanying the conformational changes of MM-CK induced by acid

Fig. 1 shows the isothermal titration calorimetric curves of the unfolding of MM-CK induced by acid at 25.0 °C in acetate buffer at pH 3.0. As can be seen from Fig. 1, even though the apparent molar enthalpy change accompanying the conformational change of MM-CK induced by acid under such conditions is negative, the conformational change during the formation of the unfolded state of the protein is endothermic in practice.

Fig. 2A shows the molar enthalpy changes accompanying the conformational changes of MM-CK in acetate buffers at different pH values and at 15.0, 25.0, 30.0 and 37.0 °C, measured by isothermal titration calorimetry, and Fig. 2B displays the temperature dependence of the intrinsic molar enthalpy change for formation of the unfolded state of MM-CK induced by acid. As shown in Fig. 2A, the molar enthalpy change for the unfolding of MM-CK induced by acid depends on the temperature at which unfolding occurs, which is varied by adjusting pH. The intrinsic unfolding enthalpy is negative at lower temperature (15.0 °C) but increases rapidly with temperature, becoming positive at higher temperatures (25.0, 30.0 and 37.0 °C). As can be seen from Fig. 2B, the molar heat capacity change associated with the unfolding of MM-CK, $\Delta_U C_{P,m}$, was 36.8 kJ mol^{-1} K^{-1}

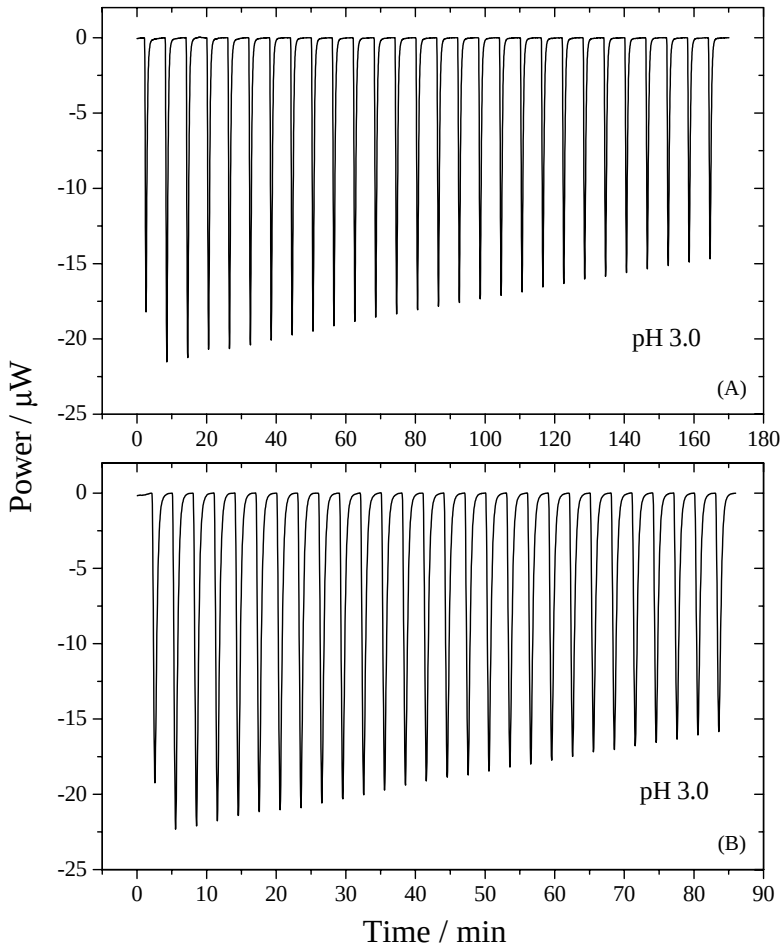

Fig. 1. Isothermal titration calorimetric curves of the unfolding of MM-CK induced by acid at 25.0 °C in 0.01 mol dm^{-3} ammonium acetate buffer (pH 3.0). (A) Typical calorimetric titration of the buffer with native MM-CK (pH 7.0). A volume of 0.01 cm^3 of native MM-CK was added for each injection and the protein concentration was 24.7 μmol dm^{-3}. (B) Control titration in the absence of the protein.

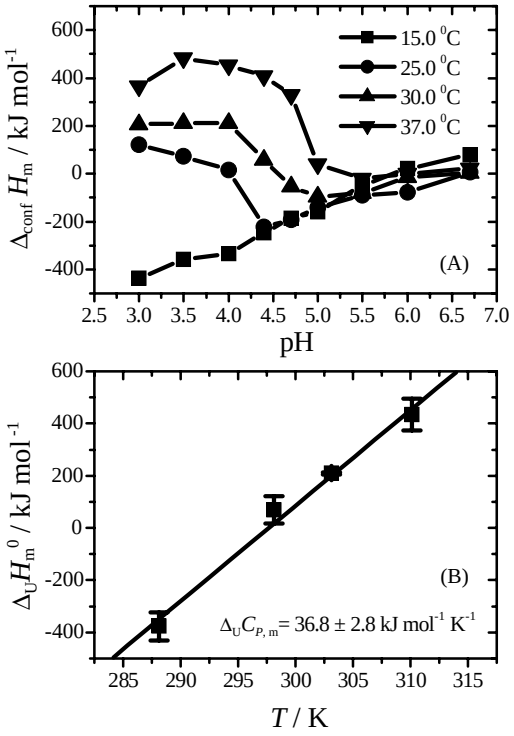

Fig. 2. (A) Molar enthalpy changes accompanying the conformational changes of MM-CK in ammonium acetate buffers at different pH values and at (■) 15.0, (●) 25.0, (▲) 30.0 and (▼) 37.0 °C, measured by isothermal titration calorimetry. (B) Temperature dependence of the intrinsic molar enthalpy change for formation of the unfolded state of MM-CK induced by acid. The intrinsic molar enthalpy change was the average value of the apparent molar enthalpy changes at pH values 3.0, 3.5 and 4.0 at each temperature. The molar heat capacity change associated with the unfolding of MM-CK, $\Delta_U C_{P,m}$, was determined by linear regression analysis of the plot by $\Delta_U H^\circ_m = \Delta_U C_{P,m} T + \Delta H^\circ$ using the data in A. The data with error bars were expressed as mean ± S.D. ($N = 3$).

with a linear correlation coefficient of 0.9942, indicating that the molar heat capacity change of this unfolding was independent of temperature in the range studied.

3.2. 'Phase diagram' analysis of MM-CK fluorescence data

Fig. 3 shows a phase diagram representing the unfolding of MM-CK induced by acid in citric acid–phosphate buffer, designed using the 'phase diagram' method of fluorescence. As can be seen from Fig. 3, the conformational transition between the native state (N) and a partially folded intermediate (I) of this protein occurs in the pH range 7.0–5.2, and the transition between the intermediate and the unfolded state (U) of this protein occurs in the pH range 5.2–3.0. The protein is almost fully unfolded at pH 3.0. This reflects the existence of two independent transitions separating three different conformational states, N, I and U, during the unfolding of MM-CK induced by acid.

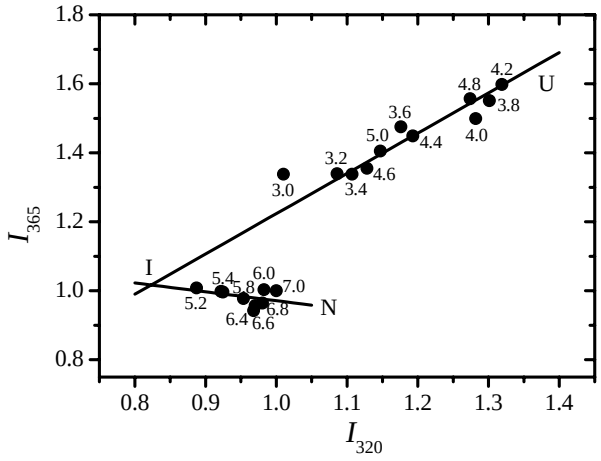

Fig. 3. Phase diagram representing the unfolding of MM-CK induced by acid in 0.1 mol dm^{-3} citric acid–Na_2HPO_4 buffer. The pH values are indicated in the vicinity of the corresponding symbol. Each straight line represents an all-or-none transition between two conformers of MM-CK, denoted as N (the native state), I (the intermediate state) and U (the unfolded state). The excitation wavelength was 295 nm and the protein concentration was 0.29 μmol dm^{-3}. Measurements were carried out at 25 °C.

4. Discussion

It is well known that the unfolding reactions of different proteins display certain common properties [33,34]. The positive molar heat capacity change associated with the unfolding of a protein, for example, is commonly attributed to the hydrophobic interaction, although other factors may contribute to $\Delta_U C_{P,m}$ [23,33]. A large positive molar heat capacity change of the unfolding of MM-CK induced by acid, 36.8 kJ mol^{-1} K^{-1}, at all temperatures examined indicates that hydrophobic interaction is the dominant driving force stabilizing the native structure of this protein. $\Delta_U C_{P,m}$ is the thermodynamic term which has been most often scaled to structural feature of a protein [34]. The value of $\Delta_U C_{P,m}$ per amino acid residue for MM-CK, 48.4 ± 3.7 J K^{-1} mol^{-1} residue, is the same order of magnitude as those for monomeric proteins (58 ± 2 J K^{-1} mol^{-1} residue [34]) and homodimeric pea lectin (47.8 J K^{-1} mol^{-1} residue [35]), suggesting that $\Delta_U C_{P,m}$ is approximately proportional to the size of a protein. Combining the results from 'phase diagram' method of fluorescence, we conclude that the acid-induced unfolding of MM-CK follows a 'three-state' model.

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