Effect of Mg^{2+} on the thermal inactivation and unfolding of creatine kinase

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Abstract

The effect of Mg^{2+} on the thermal inactivation and unfolding of rabbit muscle creatine kinase has been studied for various temperatures and Mg^{2+} concentrations. Increasing the Mg^{2+} concentration in the denatured system significantly enhanced the inactivation and unfolding of creatine kinase during thermal denaturation. The analysis of the kinetic course of substrate reaction during thermal inactivation showed that at 47°C the increased free Mg^{2+} concentration caused the creatine kinase inactivation rate to increase. Increasing the temperature strengthened the effect of Mg^{2+} on the thermal inactivation. Control experiments showed that treating native creatine kinase with different concentrations of Mg^{2+} did not change the enzymatic activity. The fluorescence emission spectra showed that the emission maximum for creatine kinase red-shifted from 335 to 337 nm during thermal denaturation at 47°C for 10 min, while the presence of 3 mM Mg^{2+} caused the enzyme emission maximum to red-shift from 335 to 342.5 nm for the same thermal denaturation conditions. In addition, Mg^{2+} also enhanced the unfolding of the equilibrium state and decreased the time required to reach the equilibrium state of creatine kinase at 47°C. The potential biological significance of these results are discussed. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Creatine kinase; Thermal denaturation; Remaining activity; Conformation; Magnesium ion

1. Introduction

Magnesium, an alkaline earth metal, is essential for the regulation of a great variety of biological processes [1,2]. It is well known that

mammalian cells contain 10–30 mM total magnesium [3] and the concentration of free magnesium ions is in the range of 0.3–3 mM [4]. Many enzymes require magnesium for their activity and are activated through one of three general mechanisms. In the first mechanism, magnesium is a necessary component of the enzyme [5]. In the second, magnesium binds to a ligand, thereby making a suitable substrate (e.g. ATP–Mg) [6,7]. The third mechanism is through allosteric activation in which binding to one or

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more forms of the enzyme in the reaction course induces a change in its conformation [8,9].

Creatine kinase (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) is a dimeric enzyme (Mr86000). The reaction catalyzed by creatine kinase is considered to be bireactant [7]:

The enzyme plays an important role in energy metabolism and much information exists on the kinetic mechanism and on the nature and role of certain amino acid residues at or near the active site [10]. The unfolding of creatine kinase (CK) in denaturant solutions and the refolding after dilution have been extensively studied [11–16]. A recent study has also reported on the thermal unfolding of creatine kinase [17,18].

Free ATP is not a substrate for creatine kinase but the true substrate is an ATP–Mg complex. Therefore, creatine kinase requires Mg^{2+} for its activity. There is no indication of the involvement of additional Mg^{2+} in the reaction, but the metal is abundant in cells. Therefore, further investigation of the effect of magnesium ions on creatine kinase is of interest. Morrison and O'Sullivan [19] reported that high concentrations of free Mg^{2+} not only inhibited the reverse reaction, but also affected the binding of phosphocreatine to the enzyme. Lawson et al. [20] reported that free Mg^{2+} affects the creatine kinase equilibrium constants (K_{eq}). However, there have been no studies on the effect of free Mg^{2+} on the stability of creatine kinase. This study investigates the effects of Mg^{2+} on the thermal inactivation and unfolding of creatine kinase. The potential biological significance of these effects are discussed.

2. Materials and methods

2.1. Materials

ATP were obtained from Sigma. All other reagents were local products of analytical grade.

2.2. Enzyme concentration and activity assay

The preparation and further purification of rabbit muscle creatine kinase were as described by Yao et al. [21]. The purified enzyme was found to be homogenous by polyacrylamide gel electrophoresis and SDS polyacrylamide gel electrophoresis. The enzyme concentration was determined by absorbance at 280 nm with $A_{1\text{cm}}^{1\%} = 8.8$ [21]. The enzyme was assayed using the thymol blue–pH spectrophotometric method [16]. Concentration and activity of creatine kinase were measured with a Perkin-Elmer Lambda Bio UV/VIS Spectrometer.

2.3. Heat inactivation

The enzyme (0.087 mg/ml) was incubated for 10 min in 100 μl of 50 mM Glycine–NaOH buffer of pH 9.0 with $\text{Mg}(\text{Ac})_2$ (or NaAc) at the desired concentration and temperature. A 10 μl sample of this reaction mixture was added to a final volume of 1.0 ml of the assay mixture and initial rates were measured at 25°C.

The kinetic course of substrate reaction of creatine kinase was measured as described in Ref. [18].

2.4. Intrinsic fluorescence emission spectra measurements

The fluorescence emission spectra were measured with a Hitachi 850 spectrofluorometer. The excitation wavelength was 295 nm [6]. The course of the conformational changes was followed by rapidly mixing a small volume of the enzyme with a solution of 50 mM Glycine–NaOH buffer of pH 9.0 with $\text{Mg}(\text{Ac})_2$ at the desired concentration and preheated to the required temperature. The course of emission intensity change was followed at that temperature. All the measurements were corrected for the spectrum of the buffer solution.

Fig. 1. Effect of Mg^{2+} on the residual activity of creatine kinase. The enzyme was incubated for 10 min at 25°C (●) or 47°C (▲) in 50 mM Glycine–NaOH buffer pH 9.0 with $\text{Mg}(\text{Ac})_2$ of different concentrations.

3. Results

3.1. Effect of Mg^{2+} on the activity of native and thermally inactivated creatine kinase at 47°C

The activity change of CK as a function of $\text{Mg}(\text{Ac})_2$ concentration is shown in Fig. 1. The native CK activity was almost unchanged up to 3

Fig. 2. Effect of NaAc on the residual activity of creatine kinase. The enzyme was incubated for 10 min at 47°C in 50 mM Glycine–NaOH buffer pH 9.0 with NaAc of different concentrations.

mM $\text{Mg}(\text{Ac})_2$ at 25°C. However, the remaining activity of the enzyme incubated for 10 min at 47°C decreased greatly in the presence of $\text{Mg}(\text{Ac})_2$. Increasing the $\text{Mg}(\text{Ac})_2$ concentration caused greater decreases in the remaining activity. No residual activity was observed above 3 mM $\text{Mg}(\text{Ac})_2$. The midpoint value of the data obtained in the presence of Mg^{2+} (Fig. 1) occurred at about 0.73 mM $\text{Mg}(\text{Ac})_2$.

NaAc was used in the reaction to determine whether the effect of $\text{Mg}(\text{Ac})_2$ was due to the increased ionic strength or the increased Ac^- concentration. The effect of NaAc concentration, Fig. 2, shows that adding NaAc up to 6 mM has no significant effect on CK activity. Therefore, the change in the residual activity of the thermal inactivated CK in the presence of $\text{Mg}(\text{Ac})_2$ was not due to the Ac^- ions and the resulting change in the ionic strength, but due to the Mg^{2+} .

3.2. Effect of Mg^{2+} on the remaining activity of creatine kinase during thermal denaturation at different temperatures

Fig. 3 shows the remaining activity of the enzyme after thermal denaturation at tempera-

Fig. 3. Effect of Mg^{2+} on the remaining activity of creatine kinase. The enzyme was incubated for 10 min at different temperatures in 50 mM Glycine–NaOH buffers pH 9.0 in the absence (●) and presence (▲) of 3 mM $\text{Mg}(\text{Ac})_2$.

Fig. 4. Kinetic courses of substrate reaction during thermal inactivation at 47°C. Final enzyme concentration in assay system was 0.01087 mg/ml. The concentrations of free Mg^{2+} for curves 1–4 were 0, 1, 2 and 3 mM, respectively.

tures from 37 to 47°C in the absence and presence of 3 mM $Mg(Ac)_2$. The activity of CK decreased 46.1% between 37°C and 47°C in the absence of $Mg(Ac)_2$ and 91.8% in the presence of 3 mM $Mg(Ac)_2$. The data in Fig. 3 also shows that the remaining activity of CK in the absence of Mg^{2+} decreased rapidly when 3 mM $Mg(Ac)_2$ was added to the solution as the denaturation temperature increased between 37 and 47°C.

3.3. Rates of inactivity of creatine kinase

The effects of Mg^{2+} on the inactivation kinetics during thermal inactivation were also examined. The native CK activity was almost unchanged when up to 3 mM Mg^{2+} was added to the substrate (data not shown). The data in Fig. 4 shows the kinetic course of the substrate reaction during thermal denaturation in the presence different concentrations of Mg^{2+} . The results show that the kinetic course was a one phase reaction in the absence or presence of Mg^{2+} and that increased Mg^{2+} concentrations

caused the inactivation rates to increase (Fig. 5; Table 1).

3.4. Effect of Mg^{2+} on the fluorescence emission spectra of creatine kinase during thermal denaturation

The fluorescence emission spectra of CK during thermal denaturation at 47°C are plotted in Figs. 6 and 7. The results show that increased concentrations of free Mg^{2+} caused the intrinsic fluorescence intensity of CK to increase with a red shift of the emission maximum. The maxi-

Table 1

Rate constants of inactivation of creatine kinase during thermal denaturation at 47°C

Mg^{2+} concentration (mM)	Inactivation ($\times 10^4$ s $^{-1}$)
0	6.51
1	13.06
2	17.25
3	26.57

Fig. 5. Semilogarithmic plots of Fig. 4. The concentrations of free Mg^{2+} in curves 1–4 were 0, 1, 2 and 3 mM, respectively.

imum emission wavelength (λ_{max}) red-shifted from 335 nm for native CK to 337 nm for CK thermally denatured after 10 min at 47°C. Further increasing the Mg^{2+} concentration (0–3 mM) caused λ_{max} of CK to red-shift to 342.5 nm in the presence of 3 mM Mg^{2+} during thermal denaturation at 47°C. Mg^{2+} also affect the equilibrium state maximum emission of creatine kinase after thermal denaturation at 47°C (Fig. 7). Increasing the concentration of Mg^{2+} (0–3 mM) caused the emission maximum of creatine kinase to red-shift from 341.5 nm (0 mM Mg^{2+}) to 343 nm (1 mM Mg^{2+}), 343.7 nm (2 mM Mg^{2+}) and then 345 nm (3 mM Mg^{2+}). In addition, the time required to reach the equilibrium state decreased from 45 min (0 mM Mg^{2+}) to about 35 min (1 mM Mg^{2+}), 30 min (2 mM Mg^{2+}) and then 25 min (3 mM Mg^{2+}) (data not shown). 6 mM NaAC had no significant effect on the CK emission maximum (data not shown). Thus, the results suggest that the enzyme underwent faster and further unfolding with the ad-

dition of Mg^{2+} during thermal denaturation at 47°C.

4. Discussion

Although Mg^{2+} concentrations up to 3 mM had no effect on the native enzyme, unfolding studies using thermal denaturation have clearly indicated that Mg^{2+} strongly enhanced the inactivation and unfolding of CK when CK was denatured at high temperatures. Higher Mg^{2+} concentrations resulted in faster rates of inactivation and lower stability. In addition, increasing the denaturation temperature increased the effect of Mg^{2+} on the remaining activity of creatine kinase.

The intrinsic fluorescence of proteins is a sensitive method for investigating conformational changes of proteins. During denaturation, the emission maximum for proteins containing tryptophan is usually shifted from shorter wave-

Fig. 6. Fluorescence emission spectra of creatine kinase during thermal denaturation at 47°C. Curve 1 shows the spectrum of the native enzyme at 25°C and curves 2–5 are spectra in the presence of 0, 1, 2, 3 mM $\text{Mg}(\text{Ac})_2$.

lengths to longer wavelengths [22,23]. Although, the unfolding of CK during thermal denaturation is significantly affected by the presence of Mg^{2+} (Fig. 6), free Mg^{2+} did not affect the structure of native CK [24] as seen in the data that showed that free Mg^{2+} did not affect the λ_{max} of CK (Fig. 7). Thus, further decreases in the catalytic activity of CK in the presence of Mg^{2+} during thermal denaturation occurred because Mg^{2+} enhanced the conformational changes of CK and enhanced the denaturation of CK for those conditions.

The modification of protein structure and activity by millimolar amounts of divalent cations was reported for high molecular weight oligomeric hemoglobins [25], hemocyanins [26], Groel oligomer [27], coagulation factor IX [28] and protein tyrosine kinases [6]. Some factors affecting cell surface receptors were found to significantly increase the Mg^{2+} influx and intracellular con-

Fig. 7. Effect of Mg^{2+} on the intrinsic fluorescence emission maximum wavelength of creatine kinase after thermal denaturation at 47°C. The values for curve ($\blacktriangle$) were taken from the curves in Fig. 4. Curve ($\blacklozenge$) shows the emission maximum wavelength of native creatine kinase incubated for 10 min at 25°C. Curve ($\bullet$) shown the emission maximum wavelength at equilibrium state of CK incubated at 47°C.

centration of free Mg^{2+} in a variety of cells [6]. This raises the possibility that Mg^{2+} plays an essential role in the activity and the stability of the structures of many oligomer proteins in the cells.

In conclusion, magnesium ion appears to be an important regulator of the activity and stability of CK. Free Mg^{2+} may perhaps modulate the activity and stability of CK in cells by affecting the unfolding of CK, to suit different physiological conditions.

References

- [1] R.H. Himes, P.R. Burton, J.M. Gaito, *J. Biol. Chem.* 252 (1977) 6222–6228.
- [2] B.M. Altura, A. Gebrewold, A. Zhang, B.J. Altura, R.K. Gupta, *Biochim. Biophys. Acta* 1358 (1) (1997) 1–5.
- [3] M.E. Maguire, *Metal Ions Biol. Syst.* 26 (1990) 135–153.
- [4] B.M. Altura, J. Durlach, M.S. Seelig, 1987. In: B.M. Altura, J. Durlach, M.S. Seelig (Eds.), *Magnesium in Cellular Processes and Medicine*. pp. 1–4.
- [5] C.B. Black, H.W. Huang, J.A. Cowan, *Coordination Chemistry Reviews* 135/136 (1994) 165–202.
- [6] G. Sun, R.J.A. Budde, *Biochemistry* 36 (8) (1997) 2139–2146.

- [7] J.F. Morrison, E. James, *Biochem. J.* 97 (1965) 37–57.
- [8] F. Sekiya, J. Yamashita, H. Atoda, Y. Komiyama, T. Morita, *J. Biol. Chem.* 270 (24) (1995) 14325–14331.
- [9] C.X. Zhu, C.J. Roche, Y.C. Tse-Dinh, *J. Biol. Chem.* 272 (26) (1997) 16206–16210.
- [10] P.R. Rosevear, V.M. Powers, D. Dowhan, A.S. Mildvan, G.L. Kenyon, *Biochemistry* 26 (17) (1987) 5338–5344.
- [11] Q.Z. Yao, M. Tian, C.L. Tsou, *Biochemistry* 23 (1984) 2740–2744.
- [12] H.M. Zhou, X.H. Zhang, Y. Yin, C.L. Tsou, *Biochem. J.* 291 (1993) 103–107.
- [13] Z.F. Wang, M.Q. Huang, X.M. Zou, H.M. Zhou, *Biochim. Biophys. Acta* 1251 (1995) 109–114.
- [14] G.F. Bickerstaff, C. Paterson, N.C. Price, *Biochim. Biophys. Acta* 621 (1980) 305–314.
- [15] H.M. Zhou, C.L. Tsou, *Biochim. Biophys. Acta* 869 (1986) 69–74.
- [16] Z.F. Wang, Y. Yang, H.M. Zhou, *Biochimie* 77 (1995) 953–956.
- [17] Y.N. Kuang, Q.Z. Yao, C.L. Tsou, in: T.P. Li, Z.X. Lu, Y.S. Zhang (Eds.), *Retrospect and Prospect of Protein Research*, World science press, Singapore, 1991, pp. 195–201.
- [18] J.H. Bai, S.Y. Zheng, H.M. Zhou, *Biochem. Mol. Biol. Int.* 45 (1998) 941–951.
- [19] J.F. Morrison, W.J. O'Sullivan, *Biochem. J.* 94 (1965) 221.
- [20] J.W.R. Lawson, R.L. Veech, *J. Biol. Chem.* 254 (14) (1979) 6528–6537.
- [21] Q.Z. Yao, L.X. Hou, H.M. Zhou, C.L. Tsou, *Sci. Sin. (Engl. Ed.)* 25 (1982) 1296–1302.
- [22] M.R. Eftink, *Biophys. J.* 166 (1994) 482–501.
- [23] F.X. Schmid, in: T.E. Creighton (Ed.), *Protein Structure: A Practical Approach*, IRL Press, Oxford, 1989, pp. 251–284.
- [24] M. Forstner, M. Kriechbaum, P. Laggher, T. Wallimann, *Biophys. J.* 75 (1998) 1016–1023.
- [25] O.H. Kappo, G. Polidori, M.G. Mainwaring, A.V. Crewe, S.N. Vinogradov, *J. Biol. Chem.* 259 (1984) 628–639.
- [26] T.H. Vannoppen-Ver Eecke, R. Lontie, *Contemp. Biochem. Physiol.* 45B (1973) 945–954.
- [27] A. Azem, S. Diamant, P. Goloubinoff, *Biochemistry* 33 (21) (1994) 6671–6675.
- [28] F. Sekiya, T. Yamashita, H. Atoda, Y. Komiyama, T. Morita, *J. Biol. Chem.* 270 (24) (1995) 14325–14331.