Aggregation of creatine kinase during refolding and chaperonin-mediated folding of creatine kinase

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Abstract

The course of refolding and reactivation of urea-denatured creatine kinase (ATP; creatine N-phosphotransferase, EC 2.7.3.2) has been studied in the absence and presence of molecular chaperonin GroEL. The enzyme was denatured in Tris–HCl buffer containing 6 M urea for 1 h. In the refolding studies, the denatured enzyme was diluted 60-fold into the same buffer containing GroEL or not for activity, turbidity, fluorescence measurements and polyacrylamide gel electrophoresis. The results show that the reactivation process is dependent of creatine kinase concentration in the concentration range 2.5–4 μM. The levels of activity recovery decrease with increasing enzyme concentration because of the formation of wrong aggregates. The molecular chaperonin GroEL can bind the refolding intermediate of creatine kinase and thus prevent the formation of wrong aggregates. This intermediate is an inactive dimeric form that is in a conformation resembling the ‘molten globule’ state. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Creatine kinase (ATP; creatine N-phosphotransferase, EC 2.7.3.2) is an important enzyme in energy metabolism that has been studied extensively [1,2]. The activity and conformation changes of creatine kinase in urea and guanidine hydrochloride have been studied and it has been shown that the unfolding of creatine kinase is a multiple-step sequential process [3,4]. The refolding of guanidine- or urea-denatured creatine kinase has been well studied in the literature [5,6]. Under suitable conditions, both the native conformation and the activity of the enzyme unfolded in chemical denaturants can be quantitatively recovered. Both the reactivation and the refolding processes are independent of enzyme concentration...
within a certain range (0–2.6 μM) [7]. However, the refolding of creatine kinase in higher enzyme concentrations has not been studied. The present paper studies the refolding of creatine kinase over a wider concentration range (1–4 μM). The results show that the activity recovery of creatine kinase decreases as the concentration increases from 2.5 to 4 μM. At the same time, the aggregation of creatine kinase molecules increases at higher protein concentrations.

Molecular chaperonins are large multisubunit assemblies essential in mediating ATP-dependent polypeptide chain folding in a variety of cellular components [8]. Bacterial GroEL has been extensively studied. GroEL can assist the folding of denatured proteins of eukaryotic cells in vitro [9,10]. It can stabilize the polypeptide in a conformation resembling the ‘molten globule’ state and prevent misfolded aggregation from taking place [11–13]. The present paper considers the refolding of urea-denatured creatine kinase in the presence of GroEL. The results show that GroEL can bind the dimeric refolding intermediate of creatine kinase and thus decrease aggregation of creatine kinase during refolding upon dilution.

2. Materials and methods

2.1. Materials

The chaperonin GroEL was purified from the Escherichia coli over-producing strain pOF39-JM101 provided by Dr Jian-Guo Tang of the College of Life Science, Peking University. Cells were suspended in 20 ml lysis buffer (1 mM ethylenediamine tetraacetic acid, 0.5 M sucrose, 50 mM Tris–HCl; pH 8.0). After centrifugation, the cells were re-suspended in the same volume of 50 mM Tris–HCl buffer (pH 8.0), with 4 mg lysozyme added to the solution. After allowing the reaction to proceed for 45 min, 40 μl Triton X-100 was added to the mixture and the mixture was stirred until it changed to a ropy liquid. The mixture was centrifuged for 20 min, and the supernatant was filtered using filter paper. The filtrate was purified on a Sepharose CL-6B column. The collected GroEL sample was further purified by ion exchange chromatography on DEAE-cellulose (DE-32) with linear gradient elution (0–2.0 M NaCl in 50 mM Tris–HCl buffer; pH 8.0). Fluorescence spectroscopy measurements of the resulting product indicated that the purified GroEL did not contain tryptophan residues. The preparation of rabbit muscle creatine kinase was as described earlier [14]. Purified GroEL and creatine kinase were homogeneous on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. Ultra pure grade urea was obtained from Promega. Creatine and ATP were from Fluka. 1-Anilino-8-naphthalene sulfonate (ANS) was from Sigma. All other chemicals were local products of analytical grade.

2.2. Methods

The concentrations of GroEL and creatine kinase were determined using the absorption coefficients $E_{1	ext{cm}0.1\%}^{\text{nm}} = 0.25$ [15] and $E_{1	ext{cm}0.1\%}^{\text{nm}} = 0.88$ [16], respectively. The creatine kinase activity measurements were carried out as described by Yao et al. [17].

The enzyme was denatured at 25°C in a solution containing 6 M urea in 30 mM Tris–HCl buffer (pH 8.0) for 1 h. In the refolding studies, the enzyme denatured as already described was diluted 60-fold in the same buffer containing GroEL solutions of different concentrations without urea for activity and fluorescence measurements.

Fluorescence spectra measurements were made with a Hitachi 850 spectrofluorometer using an excitation wavelength of 295 nm. The excitation wavelength was 380 nm for the ANS-binding fluorescence spectrum. The activity was measured with a Perkin-Elmer Lambda Bio UV/VIS spectrophotometer.

All measurements were carried out in 30 mM Tris–HCl buffer (pH 8.0) at 25°C.
3. Results

3.1. Effect of enzyme concentration on the courses of reactivation of urea-denatured creatine kinase

The effect of enzyme concentration on the courses of reactivation of creatine kinase has been studied by Zhou and Tsou [7]. The results show that, within the range studied (0.4–2.6 μM), the process of reactivation is independent of enzyme concentration, showing that the dimerization of the enzyme molecules is not rate-limiting. The results from the present experiments (Fig. 1) show that, for enzyme concentrations less than 2.5 μM, the concentration changes have no effect on the creatine kinase reactivation process in accordance with previous results. For enzyme concentrations higher than 2.5 μM, the reactivation, which was about 75% at an enzyme concentration of 2.5 μM, decreased sharply with increasing enzyme concentration to only 34% of the original activity at 4.0 μM enzyme concentration.

Aggregation (turbidity) of creatine kinase at different concentrations was measured as absorbance at 320 nm [9]. The results (Fig. 2) show that creatine kinase is prone to aggregation during refolding when the enzyme concentration is quite high. Aggregation increased strongly at higher enzyme concentration in conjunction with a decrease in the $t_{1/2}$ values where one-half of the maximum $A_{320}$ was attained (see Fig. 2). This was accompanied by a sharp decrease in spontaneous reactivation, suggesting that the association of the aggregation-prone species of the folding intermediates competes with its correct folding and thus partially prevents the recovery of the native structure.

3.2. Effect of molecular chaperonin GroEL on the courses of refolding of creatine kinase

When creatine kinase denatured in 6 M urea at 25°C for 60 min was diluted 60-fold into a 50 mM Tris–HCl buffer containing different concentrations of GroEL without urea, the enzyme activity gradually recovered (Fig. 3A,B). The levels of creatine kinase activity recovery decreased as the GroEL concentration increased. An equimolar concentration of GroEL tetradecamer reduced the yield of reactivated creatine kinase dimer from 35% to less than 3% for a creatine kinase concentration of 4 μM, and from 75% to less than 10% for a creatine kinase concentration of 2.4 μM. The existence of Mg-ATP caused no changes in the
levels of creatine kinase activity recovery during refolding in GroEL solutions, showing that unfolded creatine kinase binds to GroEL independent of the presence of ATP (data not shown).

When the molecular ratio of GroEL tetradecamer versus creatine kinase dimer was greater than one, the levels of creatine kinase activity recovery changed little with increasing GroEL concentrations. As with other denatured proteins [18,19], GroEL tetradecamer binds one molecule of creatine kinase dimer.

As shown in Fig. 4, dilution of urea-denatured creatine kinase resulted in a rapid increase in absorbance at 320 nm indicating marked aggregation, which approached a constant value within 5 min. In the presence of GroEL, both the rate and the extent of aggregation decreased with increasing GroEL concentration in the dilution buffer.

Fig. 5A shows the fluorescence emission spectra measured at different time intervals after dilution of the urea-denatured creatine kinase. With the gradual refolding of the molecule, the emission intensity decreased accompanied by a blue-shift of the emission maximum. If the renaturation time was long enough, the emission maximum would blue-shifted to 334 nm, indicating the full renaturation of the unfolded creatine kinase. Fig. 5B shows the changes in the fluorescence emission spectrum during creatine kinase renaturation in the presence of equimolar concentration of GroEL. After renaturation for a very short time,
the emission maximum of creatine kinase blue-shifted to 340 nm, then the emission intensity decreased slowly with almost no blue-shift of the emission maximum, indicating that its conformation continued to change but changed a little.

3.3. Characteristics of the complex of GroEL and the refolding intermediate of creatine kinase

The intrinsic tryptophan fluorescence of GroEL-bound creatine kinase was examined to measure the tertiary structure. The GroEL component interferes only minimally with the analysis because it lacks Trp residues. The fluorescence of creatine kinase changed markedly as the protein unfolded: the emission maximum shifted from 334 to 355 nm accompanied by an increase in the fluorescence intensity. The emission maximum of GroEL-bound creatine kinase was 340 nm, between the fluorescence maxima of the native and unfolded proteins (Fig. 6). Apparently, the Trp residues in GroEL-bound creatine kinase are already in a more hydrophobic environment than the completely unfolded protein, as indicated by the 70% blue-shift of the emission. The fluorescence emission of ANS is known to increase when the dye binds to the hydrophobic regions of a protein [20]. Fig. 7 shows the ANS fluorescence of free and GroEL bound proteins. The GroEL-stabilized creatine kinase shows strong ANS fluorescence compared with the native or urea-unfolded creatine kinase or with the GroEL tetradecamer alone. These results indicate that GroEL stabilizes unfolded creatine kinase as a flexible tertiary structure with an internal hydrophobic core, as in the 'molten globule' state.

Samples of urea-unfolded creatine kinase refolded in the presence of different GroEL concentrations were used for native polyacrylamide gel electrophoresis (Fig. 8). The results show that creatine kinase was bound to equimolar GroEL tetradecamer. When the molecular ratio of

Fig. 5. Changes in the fluorescence emission spectra during renaturation of creatine kinase in the absence and presence of GroEL. (A) The fluorescence emission spectra were recorded 1, 3, 5, 7, 10, 15, 25, 40, 50 and 60 mins (from top to bottom) after dilution of the creatine kinase denatured in 6 M urea 60-fold. The final creatine kinase concentration was 2.0 μM. (B) The fluorescence emission spectra were recorded 1, 3, 5, 7, 10, 15, 25 and 40 min (from top to bottom) after dilution. The creatine kinase and GroEL concentrations were both 2.0 μM.
GroEL tetradecamer versus creatine kinase dimer was greater than one, the free creatine kinase band in the gel disappeared, indicating that all the creatine kinase molecules were bound with GroEL. The electrophoresis results also show that GroEL cannot bind with native creatine kinase.

4. Discussion

The refolding of urea- or guanidine-denatured creatine kinase has been extensively studied in the literature. We proposed a possible model for the refolding of creatine kinase based on previous studies [7,21,22]:

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**Fig. 6.** Trp fluorescence of creatine kinase unfolding in 6 M urea (1), native creatine kinase (2), refolded creatine kinase (3), and GroEL-bound creatine kinase refolding intermediate (4). The final creatine kinase concentrations were all 2.0 μM.

**Fig. 7.** ANS fluorescence of free and GroEL-bound creatine kinase. Various forms of creatine kinase were incubated for 20 min at 25°C with a 20-fold molar excess of ANS. The fluorescence emission intensity were recorded at 480 nm for ANS (1), native creatine kinase (2), creatine kinase unfolded in 6 M urea (3), GroEL-bound creatine kinase refolding intermediate (4), and GroEL (5). The concentrations of the various forms of creatine kinase and GroEL were 2.0 μM. Data are the means ± S.D. of three independent experiments.

**Fig. 8.** Native polyacrylamide gel electrophoresis of creatine kinase, GroEL and the complex of GroEL and creatine kinase. Urea-denatured creatine kinase was diluted 60-fold in the presence of 0, 0.375, 0.75, 1.125, 1.5 and 1.875 μM GroEL (lanes 4–9, respectively) and then separated on native polyacrylamide gel consisting of 3.75% polyacrylamide for the stacking gel and 6.5% polyacrylamide for the separating gel. The final creatine kinase concentration was 1.5 μM. Lane 1, native creatine kinase; lane 2, native GroEL; and lane 3, mixture of native creatine kinase and GroEL. The native creatine kinase and GroEL concentrations were 1.5 μM.
where U is an extensively unfolded CK subunit, U’ is an inactive, monomeric folding intermediate, U₂ is an inactive dimeric folding intermediate, U₂% is a misfolded dimer, N₂% is a partially active dimeric folding intermediate, and N₂ is an active dimer.

According to this refolding model, the unfolded creatine kinase is faced with two alternatives of either folding properly to form the active dimer structure or interacting with other creatine kinase molecules to form aggregates. When the creatine kinase concentration is low during refolding, the concentration of refolding intermediate U₂ is quite low and only a few intermediates (U₂) are changed into misfolded dimers (U₂%). The low concentration of misfolded dimers (U₂%) decreases the probability of aggregation and most enzyme molecules undergo the proper folding process to form the active dimers (N₂). When the creatine kinase concentration is high (> 2.5 μM), the probability of aggregation increases as the U₂ and U₂% concentrations increase. The dynamic equilibrium between U₂ and U₂% is then broken and more dimeric refolding intermediates (U₂) are changed into misfolded dimers (U₂%), bringing about the decrease of the yield of reactivated creatine kinase.

It has been generally considered that the molecular chaperonin GroEL can assist the correct folding of polypeptides by preventing their misfolding and/or aggregation [23,24]. Here, we observed that GroEL binds with some kind of refolding intermediate of creatine kinase to decrease the extent of aggregation. The interaction between GroEL and the refolding intermediate is very strong and cannot be broken by weak physical and chemical forces. After binding with GroEL, the conformation of creatine kinase continues to change but the change is very small. The results of polyacrylamide gel electrophoresis prove the existence of the complex of GroEL and the refolding intermediate of creatine kinase. By analyzing the polyacrylamide gel electrophoresis results and the time courses of reactivation for creatine kinase during refolding in solutions containing different concentrations of GroEL, we propose that GroEL binds with the inactive dimeric folding intermediate (U₂). Jorg et al. [9] previously reported that the conformation of GroEL-bound polypeptides resembles that of the ‘molten globule’ or ‘compact intermediate’, an early folding state that rapidly interconverts with the fully unfolded form. It contains all or part of the secondary structure of the protein in conjunction with a relatively compact, but flexible tertiary structure with an internal ‘molten’ hydrophobic core. The GroEL-stabilized intermediate of creatine kinase fulfils these criteria. Thus, we propose the inactive dimeric folding intermediate (U₂) that binds with GroEL is one kind of ‘molten globule’-like intermediate.

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References


