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Subunit Interaction Slows the Unfolding of the N-Terminal Domain of Creatine Kinase in Urea

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Abstract—Fluorescence emission intensity changes with two different excitation wavelengths were used to measure the unfolding rate constants of different domains of muscle type creatine kinase (CK-MM) according to the heterogeneity of aromatic amino acid distributions in the crystal structure of CK-MM. The results were compared with those of brain type creatine kinase (CK-BB) and dithio-bis(succinimidyl propionate) cross-linked CK-MM. CK-BB differed greatly in its distribution of aromatic amino acids in each domain and the unfolding process of cross-linked CK-MM was not accompanied by the dissociation of the dimer. The N-terminal domain of CK-MM was shown to be well protected by subunit interaction during the unfolding of CK-MM in 4 M urea. Dissociating the CK dimer in high urea concentration (≥6 M) eliminated the subunit protection. Subunit interactions are also important in preserving secondary structure and forming contracted conformation at low urea concentration.

Key words: creatine kinase, N-terminal domain, dithio-bis(succinimidyl propionate) cross-link, subunit interaction

Creatine kinase (ATP:creatine N-phosphotransferase, EC 2.7.3.2) is widely distributed in highly energy-requiring tissues and it is crucial for cell energy metabolism [1]. There are four isoforms of creatine kinase: muscle type (MM), brain type (BB), hybrid type (MB), and mitochondria type (Mi). CK-MM is a dimer composed of two identical subunits, each with molecular mass of 43 kD. There is no interchain disulfide bond in the enzyme. The activity and conformational changes of creatine kinase in urea and guanidium chloride have been well documented in previous studies [2]. The unfolding of creatine kinase in urea solution is a multi-step sequential process. The crystal structure of rabbit muscle creatine kinase [3] shows that the CK-MM monomer consists of two domains: an N-terminal domain of approximately 100 residues and the larger C-terminal domain of approximately 250 residues, connected by a long linker. The C-terminal domain is rich in secondary structures with eight-strand anti-parallel β-sheets flanked by α-helices, while the N-terminal domain is mostly random-coil. Gross et al. [4] purified two isolated fragments of CK-Mi expressed in *Escherichia coli*: the isolated C-terminal fragment (168-380) refolded into a native-like conformation, whereas the N-terminal fragment was mostly evenly unfolded even under non-denaturing conditions. The C-terminal fragment of CK-Mi is an autonomous folding unit, and the folding of the C-terminal might precede conformational stabilization of the N-terminal moiety *in vivo* [4].

The model proteins that have been studied in the elucidation of the folding mechanism are small monomeric proteins. Denaturation of multi-subunit proteins is accompanied by subunit dissociation and the process is relatively complex.

In this study, the impact of subunit dissociation on the unfolding rates of different domains was the main focus. As a control to native CK-MM, some CK-MM were cross-linked between the two subunits with dithio-bis(succinimidyl propionate) (DTSP), thereby preventing dissociation of the enzyme during denaturation. Thus, the effect of dissociation on protein unfolding may become distinguishable. DTSP acylated the free amino group of each subunit. Previous studies showed that the unfolding transition of cross-linked CK-MM was also a two-stage process similar to that of native CK-MM [5].

MATERIALS AND METHODS

Tris (base), ATP, 2-mercaptoethanol, and urea were of ultra-pure grade from Sangon Ltd., Canada. All other reagents were local products of analytical grade.

Abbreviations: CK) creatine kinase; CK-BB, CK-Mi, and CK-MM) creatine kinase of brain type, mitochondria type, and muscle type, respectively; DTSP) dithio-bis(succinimidyl propionate).

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The CK-MM was prepared as described by Yao et al. [6] and CK-BB was prepared according to Epperberger et al. [7]. Enzyme concentration was determined using the absorption coefficient $A^{1\%}_{280,1cm}$ = 8.8 [8]. The specific activities were determined by the pH change indicator method [6]. DTSP cross-linked CK-MM was prepared according to Zhou et al. [5].

The activity and absorption were measured with an Analytik Jena Specord 200 U/V spectrophotometer (Germany). CD spectra were recorded on a Jasco-715 spectropolarimeter (Japan). The sample cell path length was 2 mm. The fluorescence emission intensity spectra and changes were followed with a Hitachi F-2500 spectrofluorometer (Japan). Because all Trp residues are in the C-terminal domain, an excitation wavelength of 295 nm was used to measure the rate constants of the C-terminal domain unfolding according to [9]. An excitation wavelength of 280 nm was used to measure the mixed emissions of intrinsic tryptophans and tyrosines in CK [2]. The emission wavelength of 330 nm was used to make measuring conditions consistent among CK-MM, CK-BB, and DTSP cross-linked CK-MM. This was because other emission wavelengths would cause the fluorescence emission intensity change measurement for CK-BB to be disturbed by red shift.

RESULTS

Effect of different urea concentrations on CK fluorescence spectra and far-ultraviolet CD spectra. Two proper concentrations of urea were found to show the effects of subunit interactions on the unfolding rates of different domains. A relatively low urea concentration was used to show the unfolding of CK in dimer form. A relatively high urea concentration was used to show the unfolding of CK when subunit interactions disappeared, i.e., CK in monomer form. The intrinsic fluorescence of CK changed gradually when the urea concentration was higher in the low urea concentration and no red shift occurred. However, the dissociation of the dimer led to a relatively significant red shift (Fig. 1, a and b) [10]. No significant red shift occurred to the intrinsic fluorescence spectra of cross-linked CK-MM (Fig. 1c). The relationship between the red shift of CK intrinsic fluorescence spectra and urea concentrations is summarized in Fig. 2. Figures 1 and 2 and previous data [11] show that the CK-MM did not, for the most part, dissociate after being incubated in 4 M urea for 1 h at 25°C. For CK-BB, the concentration was approximately 3 M. However, in high urea concentration (6 M urea for CK-MM and 4 M urea for CK-BB) almost all of the dimers were dissociated.

Figures 3a and 3b show that low urea concentrations (for CK-MM ≤ 4 M and for CK-BB ≤ 3 M) only slightly affected the CK secondary structures by making the molecule more rigid, as was previously shown by urea gradient

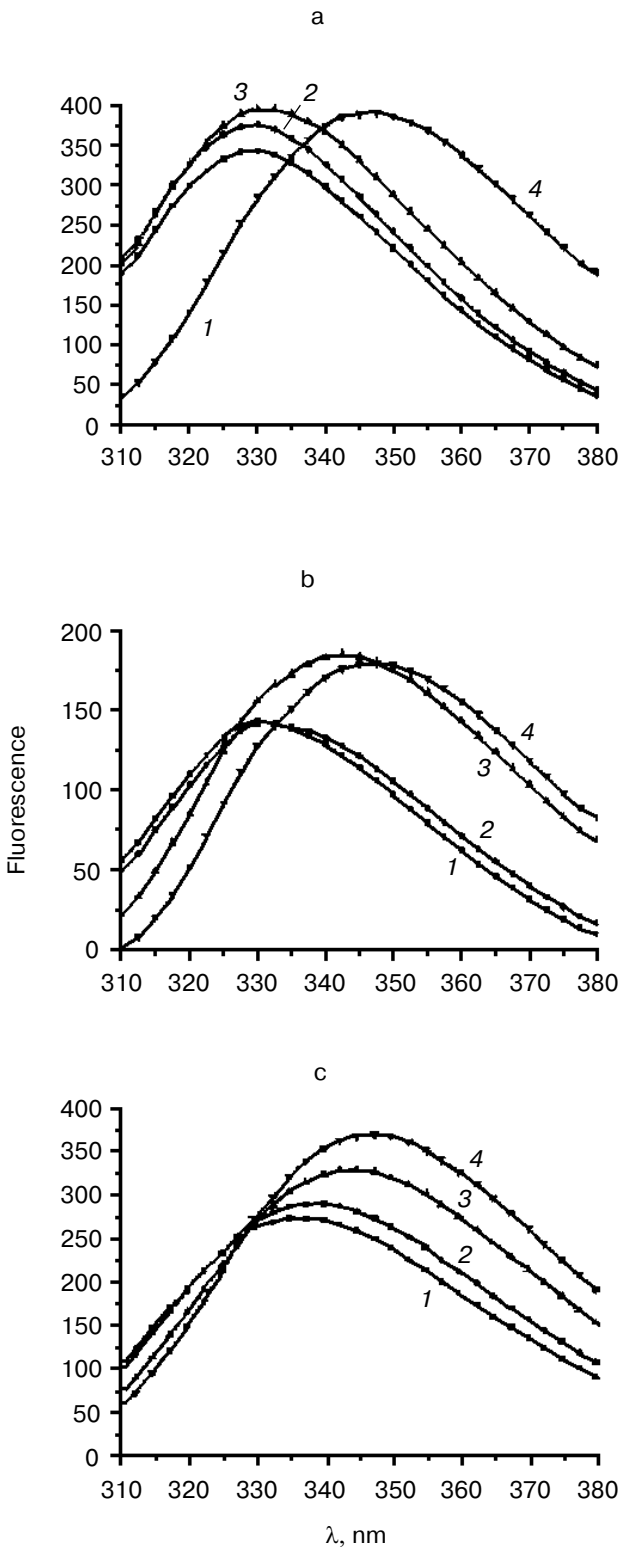

Fig. 1. Scan of the fluorescence emission spectra at 25°C after incubating the enzyme in solutions of different urea concentration at 25°C for 1 h: a) CK-MM; b) CK-BB; c) DTSP-cross-linked CK-MM. The urea concentrations were 1 (*1*), 2 (*2*), 4 (*3*), and 6 M (*4*). The final enzyme concentration was 2.6 μM. The buffer was 0.05 M Tris-HCl (pH 8.0) and the excitation wavelength was 295 nm.

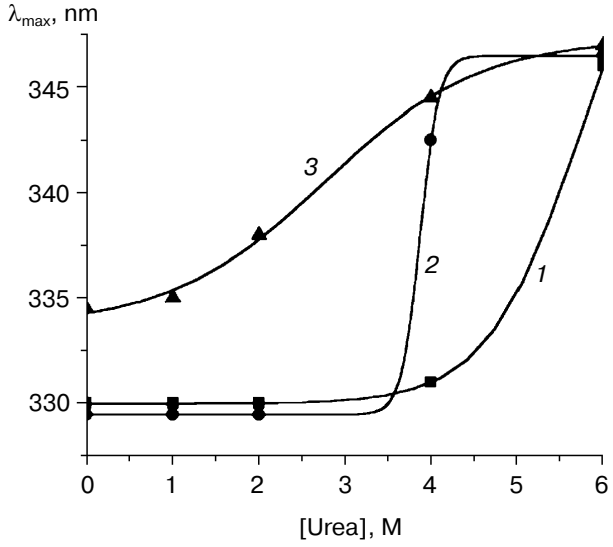

Fig. 2. Relationship between red shift (λ_{max}) and urea concentrations. The enzymes were CK-MM (*1*), CK-BB (*2*), and cross-linked CK-MM (*3*).

gel electrophoresis [12]. The molecule may have been in stable molten globular state at low urea concentration [13-15]. The CD spectra of cross-linked CK-MM differed from those of CK-MM and CK-BB. The secondary structure decreased gradually when the urea concentration increased (Fig. 3c). The relationship between ellipticity at 222 nm and urea concentration is summarized in Fig. 4.

Figures 1-4 show that in a 4 M urea solution CK-MM was in a similar unfolded state to CK-BB in 3 M urea solution, and CK-MM in 6 M urea solution was in approximately the same unfolded state as CK-BB in a 4 M urea solution. These similarities can be used to compare unfolding rate constants of CK-MM and CK-BB.

Effects of different excitation wavelengths on the rate constants of unfolding. The change in protein intrinsic fluorescence during unfolding is a result of aromatic amino acid exposure. An excitation wavelength of 295 nm can be used to reflect the change in fluorescence emission intensity change due to the exposure of Trp only [9]. The effect on the fluorescence caused by Tyr is hard to follow in isolation. However, the effect of a mixed emission of Tyr and Trp on the fluorescence is possible to follow if the excitation wavelength is set to 280 nm [2]. So, by following fluorescence intensity change using the two excitation wavelengths, differences in the unfolding rates can be used to show changes of Tyr fluorescence.

Figure 5 shows the heterogeneity of different aromatic amino acids distributed in the crystal structure of CK-MM. Only Tyr was found in the N-terminal domain through which two subunits interacted with each other. In the C-terminal domain both Tyr and Trp were found. The Trp residues were mainly in the interior of the C-terminal

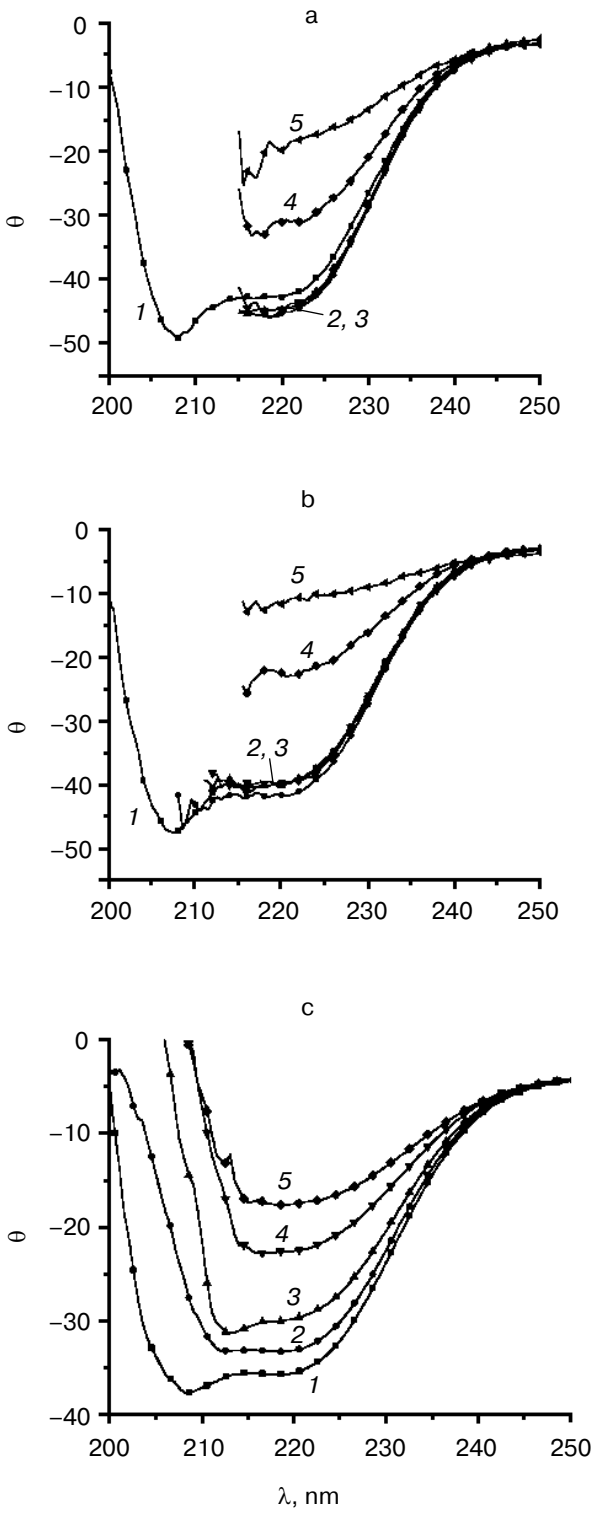

Fig. 3. Far-ultraviolet CD spectra of CK in solutions of different urea concentrations. The scan from 200 to 250 nm at 25°C was repeated ten times and the average taken: a) CK-MM; b) CK-BB; c) DTSP-cross-linked CK-MM. The urea concentrations were 0 (*1*), 1 (*2*), 2 (*3*), 4 (*4*), and 6 M (*5*). The enzyme was dissolved in 0.05 M Tris-HCl (pH 8.0) with different urea concentrations, with a final enzyme concentration of 2.6 μM.

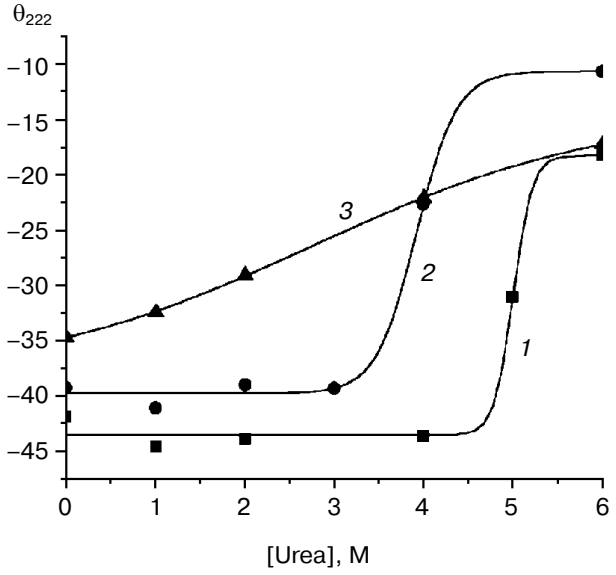

Fig. 4. Relationship between θ_{222} and urea concentration. The enzymes were CK-MM (*1*), CK-BB (*2*), and cross-linked CK-MM (*3*).

domain while the Tyr residues were mainly outside. The distribution is summarized in Table 1. Only Tyr affected the fluorescence emission change in the unfolding of the N-terminal domain, while Trp had a much greater effect on the fluorescence emission intensity change in the unfolding of the C-terminal domain than Tyr. Therefore, unfolding of the C-terminal domain can be recorded by following Trp fluorescence intensity change and unfolding of N-terminal domain can be recorded by following Tyr fluorescence intensity change.

The very fast phase of CK unfolding in urea was too fast to be followed using manual mixing because the CK inactivation was not due to obvious conformational changes [2]. However, the subsequent changes were mainly due to the conformational changes. These changes were recorded by following the fluorescence emission intensity change from the exposure of internal aromatic residues (Fig. 6a). The fast phase rate constants of CK unfolding in urea solutions with different concentrations were obtained by subtracting the contribution of the slow phase according to Yao et al. [2] and Ou et al. [16] (Fig. 6b). The results summarized in Table 2 show that the rate constant of CK-MM unfolding in 4 M urea with an excitation wavelength of 295 nm ($k_{295}^{4\,M}$) was much greater than the one with an excitation wavelength of 280 nm ($k_{280}^{4\,M}$). Also, $k_{295}^{6\,M}$ increased approximately 31.4% over $k_{295}^{4\,M}$, while $k_{280}^{6\,M}$ increased 130.9% over $k_{280}^{4\,M}$.

CK-BB was used in the same experiments to confirm that the differences between k_{295} and k_{280} were due to the heterogeneity of different aromatic amino acid distributions. Though CK-BB and CK-MM are highly congeneric, there were differences in the aromatic amino acid distributions between these isoforms: only two Tyr residues in the N-terminal domain of CK-BB and three in the C-terminal domain. Trp was quite conserved in both the CK-BB and CK-MM primary and tertiary structures [3]. The differences are summarized in Table 3. The fast phase rate constants for CK-BB unfolding in solutions of different urea concentrations (3 and 4 M) were obtained as for CK-MM and are shown in Table 2. Both the rate constants (k_{280} and k_{295}) and the increments of rate constants (16.8% for k_{280} and 17.5% for k_{295}) were approximately equal.

The unfolding rate constants of cross-linked CK-MM were measured to show the role of the dimer form in the unfolding process and the results are shown in Table 2. During the unfolding process the dimer could not be destroyed. So, it was then compared to the unfolding process of native CK-MM, in which the dimer dissociated when the urea concentration was over 4 M. The rate constants (k_{295} and k_{280}) were approximately equal and the increments of the rate constants (46% for k_{280} and 67% for k_{295}) were also similar.

DISCUSSION

Though urea unfolding of creatine kinase has been extensively studied, the exact conformational changes of

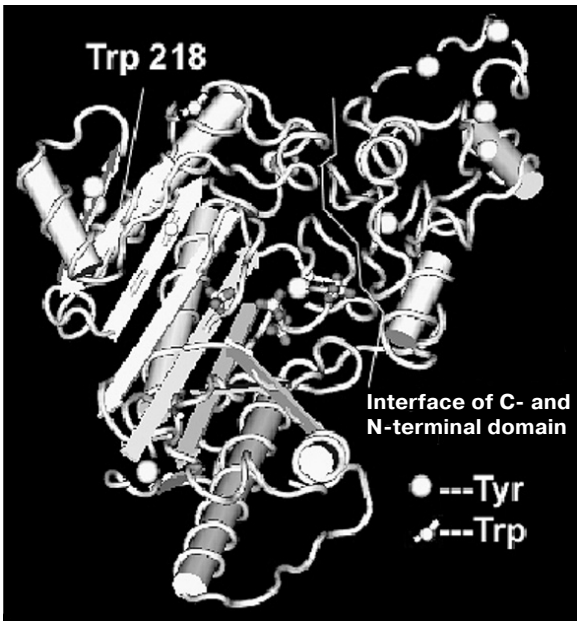

Fig. 5. Spatial distribution of aromatic amino acids in the crystal structure. Trp is labeled with a ball and stick model and Tyr with a ball model. The crystal structure of CK-MM was downloaded from www.ncbi.nlm.nih.gov. The N-terminal domain is the small section above the line. The larger region represents the C-terminal domain.

Table 1. Distribution of aromatic amino acids in the monomer crystal structure of CK-MM

Aromatic amino acid	Total number	N-terminal domain	C-terminal domain	
			inside	outside
Tyr	10	5	2	3*
Trp	4	0	3	1

* Two of which are located in the interface of the N-terminal and C-terminal domain.

each domain and the relationship between these changes and dimer dissociation have not been significantly reported. In most of the previous studies the entire monomer was considered to be unfolded as a whole and the unfolding details of each domain were unclear. The crystal structure of CK-MM provided useful information for further research [3]. Figure 5 and Table 1 show that Tyr and Trp distribution is not symmetrical in the level of tertiary structure of CK-MM. There are five Tyr residues in the N-terminal domain, another two Tyr residues on the interface between the N-terminal domain and the C-terminal domain, one on the surface of the C-terminal domain, and one in the interior of the C-terminal domain. All of the four Trp residues are located in the C-terminal domain, with three inside and one outside. Thus, it is possible to follow the conformational changes of different domains by monitoring the intrinsic fluorescence intensity changes different aromatic amino acids.

The results shown in Figs. 1-4 illustrate the differences between k_{295} and k_{280} in Table 2: CK-MM dimer dissociated in 6 M urea but not in 4 M urea, as was also verified by Wang et al. [11]. Thus, CK-MM was unfolded mainly as a dimer with the two subunits still interacting in 4 M urea. The N-terminal domain, the linker region between subunits, was well protected in the interior of the dimer. The outside region C-terminal domain was directly exposed to urea solution. So the unfolding rate of the C-terminal domain was much faster than that of the N-terminal domain. In 6 M urea CK-MM dimer was completely destroyed and the subunit interactions disappeared. So, the interactions could no longer protect the N-terminal domain and the rate constant (k_{280}) increased

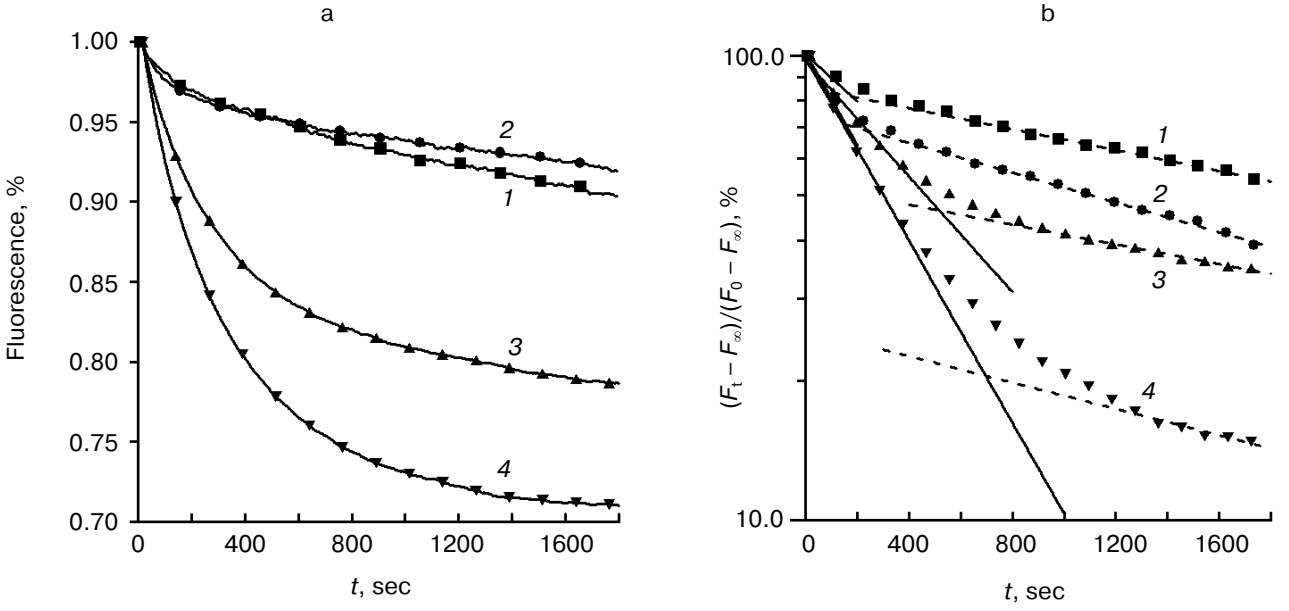

Fig. 6. a) Kinetic course of fluorescence emission intensity during unfolding of CK-MM in urea solution. The experiment was carried out at 25°C with 0.05 M Tris-HCl (pH 8.0) as the buffer. Enzyme concentration was 2.6 μM. Urea concentrations and excitation wavelengths were 4 M and 280 nm (*1*), 4 M and 295 nm (*2*), 6 M and 280 nm (*3*), and 6 M and 295 nm (*4*). The fluorescence emission intensity change was recorded at 330 nm 10 sec after mixing. b) Semilogarithmic plots for determination of the rate constants. F_0, F_t, and F_∞ are the initial, current, and limiting values of the intensity of fluorescence.

more significantly than the increment of k_{295}, and the final unfolding rates ($k_{280}^{6\,M}$ and $k_{295}^{6\,M}$) were approximately equal. CK-BB was used to repeat the experiments. Urea at 3 and 4 M concentrations were used because in 3 M urea the dimer dissociation was still incomplete while 4 M urea led to complete dissociation (graph for CK-BB in 3 M urea solution is very similar to that in 2 M urea solution). Figures 1 and 2 show that the states chosen for CK-BB were similar to those of CK-MM. Table 2 shows that the rate constants (k_{280} and k_{295}) and their increments were approximately equal. That is because, although CK-MM and CK-BB were highly congeneric with very similar crystal structures and folding modes [17-19], the aromatic amino acids distribution of CK-BB was symmetrical. So, the above stated differences of CK-MM really came from heterogeneity of Tyr and Trp distribution in the tertiary structure reporting different domain unfolding. Cross-linked CK-MM was also used as a control. Cross-linked CK-MM did not dissociate in urea, so the role of subunit interaction in the N-terminal domain unfolding can be obtained through a comparison with native CK-MM. There were no great differences between the increments of rate constants like native CK-MM, this being in accordance with previous suppositions. However, the rate constants $k_{295}^{4\,M}$ and $k_{280}^{4\,M}$ did not differ significantly like those of the native CK-MM. The reason may be the inter-subunit cross-link. The covalent bonds formed between subunits prevented the contracted conformation forming (Fig. 3c). In 4 M urea the N-terminal domain of native CK-MM can be embedded into the interior of the contracted dimer while the N-terminal domain of DTSP-cross-linked CK-MM cannot, so the unfolding rate is relatively high.

Table 2. Fast phase rate constants of unfolding in solutions of different urea concentrations

Enzyme	Urea concentration, M	$k_{280} \times 10^3$, sec^{-1}	$k_{295} \times 10^3$, sec^{-1}
CK-MM	4	0.85 ± 0.06	2.04 ± 0.05
	6	1.96 ± 0.02	2.68 ± 0.02
	change, %	130.9	31.4
CK-BB	3	3.39 ± 0.11	5.71 ± 0.52
	4	3.96 ± 0.27	6.71 ± 0.44
	change, %	16.8	17.5
DTSP-cross-linked CK-MM	4	3.46 ± 0.10	5.14 ± 0.16
	6	5.06 ± 0.22	8.59 ± 0.30
	change, %	46.2	67.1

The above discussion suggests that the subunit interactions offer strong protection to the N-terminal domain which has only two short α-helices but is rich in loops and turns. Far ultraviolet CD spectra show that the secondary structure did not significantly change, even after 1 h unfolding in solution of 4 M urea. Dissociation in 6 M urea was accompanied by a significant loss of secondary structure. The subunit interactions helped to maintain a rather stable and contracted conformation in dilute denaturant [13, 14]. Cross-linked CK-MM did not undergo contraction in conformation, which suggests that the formation of a contracted dimer is related to distance change between the two subunits. After the flexible subunit interactions were replaced by rigid covalent bonds, the dimer was fixed and the contraction was unable to occur.

The method introduced in this paper gives a way to follow the unfolding of different domains of CK-MM. The C-terminal domain is a relatively independent region whose core structure seems to be invariant. This well-conserved structural motif must be common to CK as well as guanidine-kinase structures. However, the N-terminal domain did not show as much sequence similarity among CK enzymes [3]. The folding of the C-terminal domain showed much independence while the N-terminal domain could not fold independently [4]; rather, it relied greatly on other factors such as the interaction between the two subunits and the interaction between the C-terminal domain and the N-terminal domain. When the N-terminal domain was left alone, no conformation in the native state was obtained [4], but after being folded and stabilized with the help of the interactions, even 4 M urea

Table 3. Distribution of Tyr of CK-MM and CK-BB

Enzyme	Number of residues	N-terminal domain	C-terminal domain	
			inside	outside
CK-MM	10	5	2	3
CK-BB	5	2	2	1

could not fully unfold it and the unfolding rate was slower than that of the C-terminal domain. If the concentration was increased to 6 M, the interactions were destroyed quickly and the N-terminal domain could not retain its conformation any longer, so the unfolding rate constant (here, rate constants obtained by using the excitation wavelength of 280 nm are used to compare with those obtained by using 295 nm as the excitation wavelength) increased greatly compared with that of the C-terminal domain. This experiment suggests that although the senior structures were determined by the primary structure of the protein, they were strengthened by the interactions between subunits and the interaction between domains.

Subunit interaction often amplifies even minimal changes caused by different concentrations of substrates or conformational factors [20]. Also, the formation of oligomers may diminish errors in translation and transcription. In this case, for muscle type creatine kinase, subunit interactions helped to preserve conformation of the N-terminal domain and secondary structure. Therefore, the formation of a dimer may offer CK some advantages in conformational stability to its close partner in invertebrates—arginine kinase (EC 2.7.3.3). Arginine kinase is found mainly as a monomer and shows high sequence and structure similarities to CK, as well as a similar substrate synergism [21], but the distance separating active sites which communicate with each other is still unclear [3].

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