Protein Science (1998), 7:2631–2641. Cambridge University Press. Printed in the USA.

Unfolding and refolding of dimeric creatine kinase equilibrium and kinetic studies

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(RECEIVED March 6, 1998; ACCEPTED September 2, 1998)

Abstract

Equilibrium and kinetic studies of the guanidine hydrochloride induced unfolding-refolding of dimeric cytoplasmic creatine kinase have been monitored by intrinsic fluorescence, far ultraviolet circular dichroism, and 1-anilinonaphthalene-8-sulfonate binding. The GuHCl induced equilibrium-unfolding curve shows two transitions, indicating the presence of at least one stable equilibrium intermediate in GuHCl solutions of moderate concentrations. This intermediate is an inactive monomer with all of the thiol groups exposed. The thermodynamic parameters obtained by analysis using a three-state model indicate that this intermediate is similar in energy to the fully unfolded state. There is a burst phase in the refolding kinetics due to formation of an intermediate within the dead time of mixing (15 ms) in the stopped-flow apparatus. Further refolding to the native state after the burst phase follows biphasic kinetics. The properties of the burst phase and equilibrium intermediates were studied and compared. The results indicate that these intermediates are similar in some respects, but different in others. Both are characterized by pronounced secondary structure, compact globularity, exposed hydrophobic surface area, and the absence of rigid side-chain packing, resembling the "molten globule" state. However, the burst phase intermediate shows more secondary structure, more exposed hydrophobic surface area, and more flexible side-chain packing than the equilibrium intermediate. Following the burst phase, there is a fast phase corresponding to folding of the monomer to a compact conformation. This is followed by rapid assembly to form the dimer. Neither of the equilibrium unfolding transitions are protein concentration dependent. The refolding kinetics are also not concentration dependent. This suggests that association of the subunits is not rate limiting for refolding, and that under equilibrium conditions, dissociation occurs in the region between the two unfolding transitions. Based upon the above results, schemes of unfolding and refolding of creatine kinase are proposed.

Keywords: creatine kinase; dimerization of subunits; folding intermediate; kinetics; molten globule; protein folding

In spite of the accumulation of a large number of experimental studies, protein folding remains one of the most challenging subjects in structural biology. Characterization of folding intermediates is considered an important strategy for the elucidation of the mechanism of protein folding. A common equilibrium intermediate, the "molten globule" (MG) state, has been detected between the native (N) and the fully unfolded (U) states for many proteins (Fink, 1995; Ptitsyn, 1995). The MG state is characterized by pronounced secondary structure, compact globularity, exposed hydrophobic surface, and the absence of rigid side-chain packing (Ptitsyn, 1987, 1995; Kuwajima, 1989, 1996; Fink, 1995). Such an intermediate has been found not only in equilibrium unfolding-refolding transitions but also as a kinetic intermediate during the folding of proteins, both in vitro (Ptitsyn, 1995) and in vivo (Martin et al., 1991). However, most proteins for which a MG intermediate has been well characterized are small, monomeric, single domain proteins (Kim & Baldwin, 1990; Matthews, 1993; Ogasahara & Yutani, 1994; Kay & Baldwin, 1996; Arai & Kuwajima, 1996). Multidomain and oligomeric proteins remain relatively little explored (Jeanicke, 1991). Since the folding/unfolding of such proteins are accompanied by the association/dissociation of subunits, the processes are much more complicated than that of monomeric proteins and such studies can yield additional insight into the interdependence of folding and oligomerization processes (Price, 1994).

Creatine kinase (CK, EC. 2.7.3.2), an important enzyme in cellular energy metabolism, reversibly transfers a phosphoryl group from ATP to creatine. The cytoplasmic enzyme from rabbit muscle (MM isoenzyme) is a dimer composed of identical 43-kD polypeptide chains of known sequence (Putney et al., 1984). Although the three-dimensional structure of the isoenzyme has not yet been

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; CD, circular dichroism; CK, creatine kinase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GuHCl, guanidine hydrochloride; MG, molten globule; UV, ultraviolet.

reported, it has been postulated from biochemical and biophysical studies that the protomer of the enzyme is composed of two flexibly linked domains (Morris & Cartwright, 1990) and the subunits are assembled asymmetrically (Degani & Degani, 1980).

From studies of small proteins it has been proposed that early kinetic folding intermediates may be equivalent to equilibrium unfolding intermediates (Kuwajima, 1989; Kim & Baldwin, 1990, 1996; Ptitsyn et al., 1990; Sancho et al., 1992; Matthews, 1993; Evans & Radford, 1994; Ogasahara & Yutani, 1994; Roder & Elöve, 1994; Arai & Kuwajima, 1996; Kay & Baldwin, 1996). To understand the mechanism of the folding and assembly of oligomeric proteins, it is important to characterize the equilibrium unfolding intermediates at different stages of unfolding, as well as the relationship between them. The present study describes measurements of the equilibrium and kinetic GuHCl-induced unfolding and refolding of dimeric MM-CK, monitored by intrinsic fluorescence, far-UV CD, and binding with the extrinsic fluorescence probe ANS. The early events in the refolding process were investigated by stopped-flow techniques. The equilibrium and kinetic intermediates were characterized and compared. It was found that the kinetic intermediate, which is formed early, is similar to the equilibrium intermediate in dilute denaturant in some respects but not others. The dimerization of the subunits takes place after initial folding of the individual subunits to a certain conformation. Based upon the above results, pathways of unfolding and refolding of CK are proposed.

Results

Equilibrium studies

As shown in Figure 1A, with increasing GuHCl concentration, changes in both the fluorescence emission intensity and the red-shift of the emission maximum occur in multiple stages. At GuHCl concentrations lower than 0.3 M, the emission intensity decreases slightly with no change in the emission maximum. A marked increase in emission intensity takes place between 0.3–0.6 M GuHCl, together with a red-shift of the emission maximum from 333 to 340 nm. Further changes in the fluorescence properties occur at GuHCl concentrations between 1.0 and 3.0 M, with a decrease in emission intensity and a further red-shift of the emission maximum from 341 to 350 nm. To investigate the dissociation/association equilibrium of the dimeric enzyme during unfolding, the denaturation transition was determined at various protein concentrations. However, the results show that the unfolding equilibrium profiles are in-

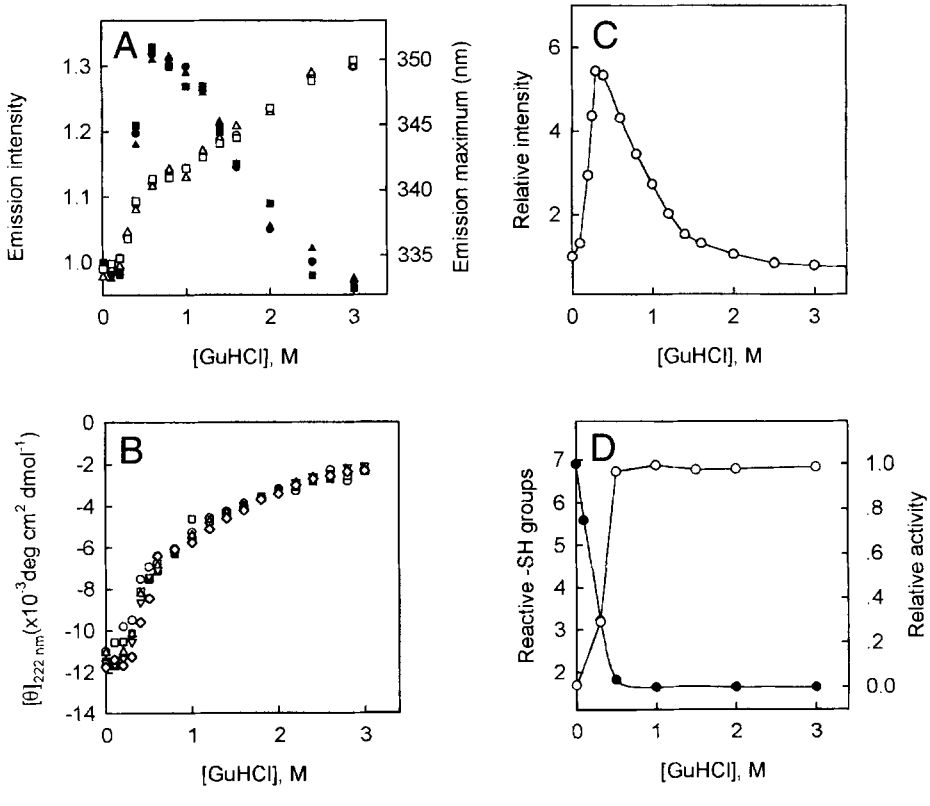

Fig. 1. Effect of GuHCl on equilibrium unfolding of creatine kinase. **A:** Intrinsic fluorescence of CK. The filled symbols represent the relative emission intensity at 335 nm (left ordinate) and the open symbols represent the emission maximum (right ordinate). The enzyme concentrations were 0.116 μM (△, ▲), 0.928 μM (○, ●), and 4.64 μM (□, ■). **B:** Ellipticity at 222 nm. The enzyme concentrations were 0.464 μM (△), 1.16 μM (○), 4.64 (▽), 11.6 μM (□), and 23.2 μM (◇). The cuvette pathlength was 3.0 mm for protein concentrations less than 1.16 μM and 1.0 mm for protein concentrations higher than 4.64 μM. **C:** ANS fluorescence in the presence of CK, 1.16 μM, in GuHCl solutions after incubation with 50-fold molar excess of ANS for about 2 h. The excitation and emission wavelengths were 350 and 470 nm, respectively. Emission values were relative to that of ANS in the presence of CK without GuHCl. **D:** The exposure of -SH groups (○, left) and the residual activity (●, right).

dependent of the enzyme concentration within 0.116–4.64 μM. The equilibrium refolding of 3 M GuHCl-denatured CK at different final concentrations of GuHCl was also monitored by the changes of intrinsic fluorescence (data not shown). The refolding profiles are superimpossible with those of unfolding, showing that the unfolding of CK is a reversible process, and that the transitions of both unfolding and refolding involve an identical, stable intermediate.

Measurement of far-UV CD was employed to monitor secondary structural changes of creatine kinase during denaturation in GuHCl (Fig. 1B). Similar to the fluorescence changes, at GuHCl concentrations lower than 0.2 M, there is very little change in the magnitude of ellipticity. The ellipticity decreases markedly between 0.2–0.6 M GuHCl, with the ellipticity at 222 nm being reduced by about 50%. The decrease in the far-UV CD signal is less steep between 1.0–3.0 M GuHCl. The transition curves are also independent of the enzyme concentration within 0.464–23.2 μM. We have recently reported a study of the unfolding of the enzyme by small angle X-ray scattering (Zhou et al., 1997). The unfolding curves obtained by small angle X-ray scattering at an enzyme concentration of about 80 μM are in good agreement with those described here, measured at lower protein concentration by optical techniques.

The conformational changes of CK induced by GuHCl have also been studied by binding with 1-anilino-naphthalene-8-sulfonate (ANS), a probe for apolar binding sites whose fluorescence is strongly dependent on the hydrophobicity of the environment. Free ANS fluoresces weakly in aqueous solution, and the fluorescence increase very little in the presence of native CK. A steep increase in fluorescence together with a blue shift of ANS emission takes place on addition of GuHCl, reaching a maximum at 0.6 M GuHCl. The emission at 470 nm increases by about 5.4-fold. With further increase of GuHCl concentration, the ANS fluorescence enhancement declines again to a constant emission intensity lower than that in the absence of GuHCl (Fig. 1C). The result indicates that the unfolding intermediate has a significant amount of exposed hydrophobic surface area. Creatine kinase contains eight SH groups, of which only two, believed to be situated at the active site of the enzyme, are accessible to reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Reddy & Watts, 1979). With increasing GuHCl concentration, the number of DTNB accessible SH groups increases to a maximum of 7 at 0.5 M GuHCl and above (Fig. 1D). The residual enzyme activity in GuHCl solutions, also shown in Figure 1D, corresponds closely with the extent of exposure of hidden SH groups and the initial changes in ANS fluorescence, but precedes the changes of intrinsic fluorescence and ellipticity at 222 nm. These results agree with previous reports from this laboratory (Yao et al., 1982a, 1982b) but differ from those reported by Couthon et al. (1995) who observed higher residual enzyme activity at the same GuHCl concentration. As pointed out by Tsou (1995), this may be due to reactivation of the denatured enzyme during activity determination, as the enzyme was assayed in the absence of the denaturant in the report by Couthon et al. (1995).

Quantitative analysis of denaturation parameters using a three-state model

The equilibrium unfolding-refolding profiles of CK detected by both intrinsic fluorescence and far-UV CD are multiphasic and reversible, indicating that there is at least one stable equilibrium intermediate at moderate GuHCl concentrations. Although cytoplasmic CK is a dimer, the positions of the denaturation transitions

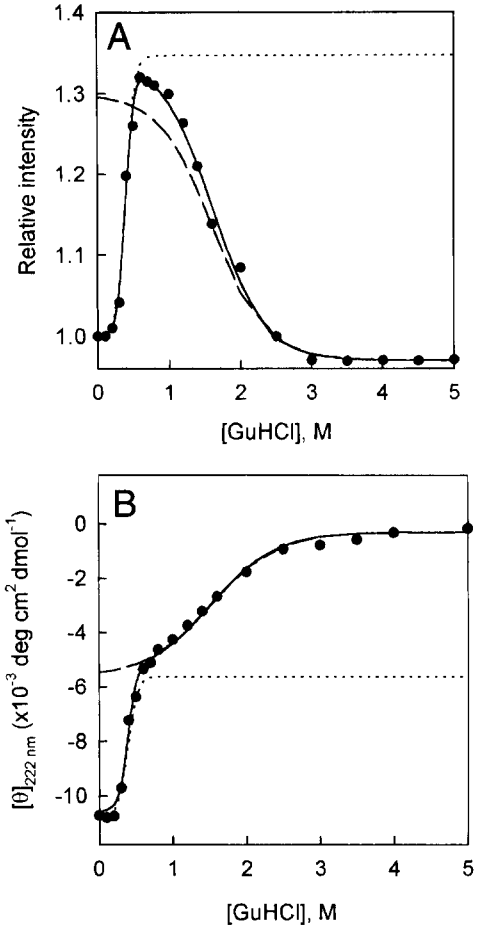

Fig. 2. Fitting of the unfolding profiles to a three-state model; ●, experimental values; ——, fit of the data to curves based on a 3-state model; · · · · ·, deconvoluted $N \rightarrow I$ transition; - - - -, deconvoluted $I \rightarrow U$ transition. Unfolding profile obtained by (**A**) intrinsic fluorescence emission and (**B**) CD at 222 nm. Details as in Figure 1.

are independent of protein concentration and can be treated as simple unimolecular reactions using a three-state model. Figures 2A and 2B show the results of the fluorescence and CD measurements and the fitting of the data to a three-state unfolding model. The thermodynamic parameters obtained are summarized in Table 1. The two-stage profiles are deconvoluted into two separate transitions using the parameters obtained from both the fluorescence and CD determinations, which are similar to each other. The free energy change for the $N \rightarrow I$ transition, ΔG^0_{NI}, is signif-

Table 1. *Thermodynamic parameters for the denaturation of creatine kinase*

	ΔG^0_{NI} (kJ)	m_{NI} (kJ M^{-1})	ΔG^0_{IU} (kJ)	m_{IU} (kJ M^{-1})
Fluorescence	16.4	41.5	10.9	6.8
CD	16.2	41.4	8.7	5.7

icantly greater than the value for the $I \rightarrow U$ transition, ΔG^0_{IU}, suggesting that the intermediate is energetically closer to the fully unfolded rather than the native state. The increase in solvent-accessible surface area in the $N \rightarrow I$ transition is much higher than that of $I \rightarrow U$, as can be seen by comparing the slopes and the transitions, m_{NI} and m_{IU}. The results suggest that the intermediate resembles a molten globule, as was found for tryptophan synthase by differential scanning calorimetry (Ogasahara et al., 1993). Gross et al. (1995) also applied a three-state model to the denaturation of guanidio kinase and obtained similar results, but as pointed by Eftink (1994), the use of the emission wavelength profile instead of emission intensity in their analysis is inappropriate.

Kinetic studies

The unfolding and refolding kinetics of CK were monitored by following changes of intrinsic fluorescence above 320 nm in a stopped-flow apparatus or at 335 nm by manual mixing, with excitation at 280 nm; and by changes in the ellipticity at 222 nm. The unfolding reaction of CK was initiated by concentration jumps to various concentrations of GuHCl, at pH 8.3 and 25 °C. Figures 3 and 4 show typical kinetic traces for unfolding of CK at a final GuHCl concentration of 1.0 M, monitored by CD at 222 nm and intrinsic fluorescence, respectively. With both methods, the unfolding kinetics at various concentrations of GuHCl fit well to a single exponential equation (Equation 6) with the same apparent rate constant. The dependence of the apparent rate constants on the final GuHCl concentration is shown in Figure 5. The amplitude of the monophasic unfolding kinetics determined by either intrinsic fluorescence or ellipticity at 222 nm covers the entire range of the equilibrium values at various final GuHCl concentrations (data not shown).

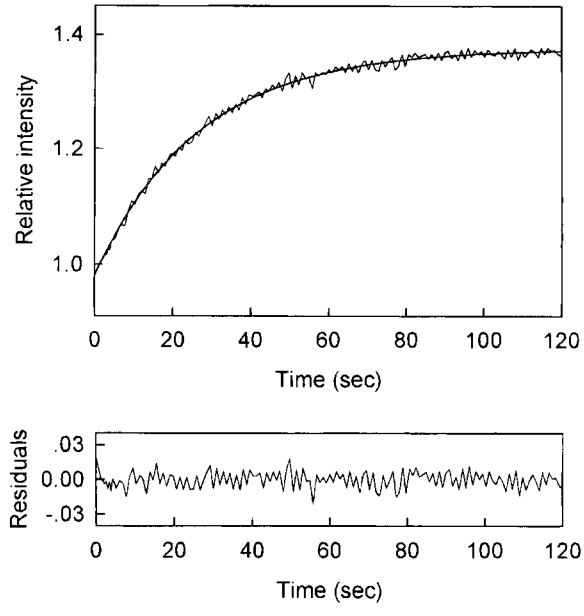

Fig. 4. Kinetics of CK unfolding as monitored by changes in intrinsic fluorescence. Details are as for Figure 3. The best-fit values of the parameters are $A_1 = -0.39$, $A_\infty = 1.38$, $k = 0.038\ s^{-1}$. The emission intensities are the values relative to that of the native state.

The refolding of CK was initiated by a concentration jump from 3 M GuHCl, where the protein is completely unfolded as shown above, to various GuHCl concentrations. The refolding curves of CK monitored by CD at 222 nm at various final GuHCl concen-

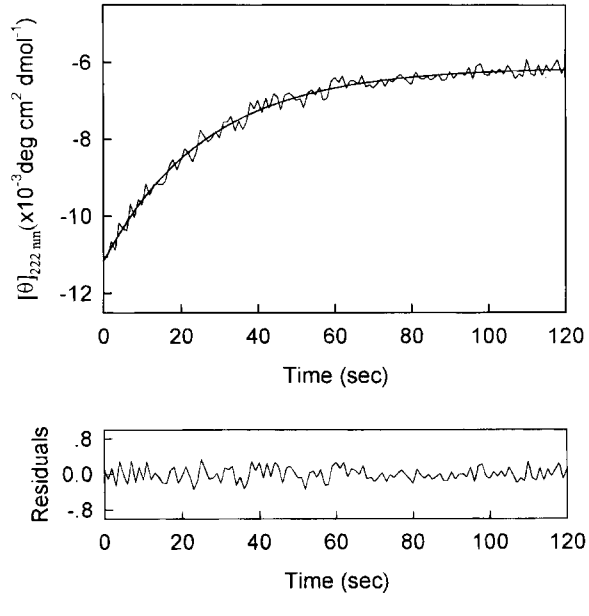

Fig. 3. Kinetics of CK unfolding as monitored by ellipticity changes at 222 nm. The final enzyme concentration was 1.14 μM. The thick line represents the theoretical curve for a single exponential function according to Equation 6. The lower panel shows the residual between the experimental points and the theoretical curve for parameter values $A_1 = -5.02 \times 10^3$ deg cm^2 dmol^{-1}, $A_\infty = -6.13 \times 10^3$ deg cm^2 dmol^{-1}, $k = 0.037\ s^{-1}$.

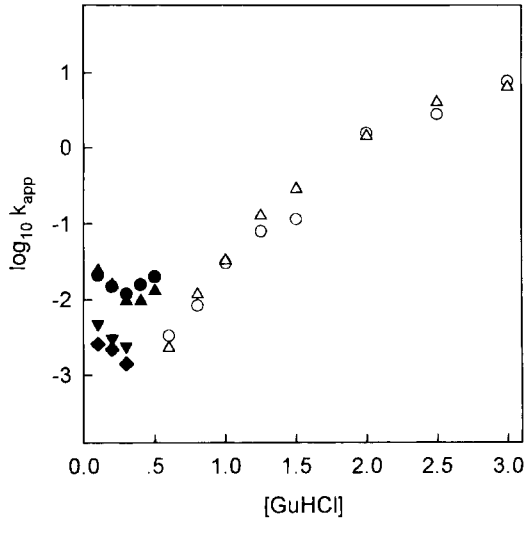

Fig. 5. Dependence of the apparent rate constants ($\log_{10} k_{app}$) on GuHCl concentration for unfolding and refolding at pH 8.3 and 25 °C. Initial GuHCl concentrations were 0 and 3 M for unfolding and refolding, respectively. (○) and (△), unfolding rate constants monitored by the CD signal at 222 nM and intrinsic fluorescence, respectively; (●) and (▲), rate constants for the fast phase of refolding obtained by measurement of CD at 222 nm and intrinsic fluorescence, respectively; (▼) and (♦), the rate constants for the slow phase of refolding obtained by measurement of the CD signal at 222 nm and intrinsic fluorescence, respectively.

trations are shown in Figure 6. It is clear that a substantial part of the ellipticity change from the unfolded state to the native state occurs within the dead time of the stopped-flow apparatus (15 ms) in a burst phase (Fig. 6A), indicating the formation of pronounced secondary structure at an early stage in refolding. The kinetic traces for refolding at different final GuHCl concentrations after the burst phase are shown in Figure 6B. The ellipticity values recovered in the burst phase at final GuHCl concentrations below 0.3 M show little dependence on GuHCl concentration, with a mean value of -8.0×10^3 deg cm^2 dmol^{-1}, 75% of that for the native molecule. Representative kinetic traces for refolding of CK at a final GuHCl concentration of 0.1 M, after the burst phase, were analyzed using both single and double exponential equations (Equation 6). As can be seen from the plots of the residuals in Figures 6C and 6D, the experimental results fit better to a biphasic function. However, at final GuHCl concentrations higher than 0.3 M, further refolding after the burst phase is a monophasic process, and at final GuHCl concentrations higher than 0.6 M, only the burst phase is observed, suggesting no further folding of the burst phase folding intermediate.

Using intrinsic fluorescence to follow the refolding kinetics of 3 M GuHCl-denatured CK gives essentially the same results (Fig. 7). There is also a burst phase that finishes within the dead time of the stopped-flow apparatus (Fig. 7A). The emission intensity increases dramatically in the burst phase to an amplitude of about 1.6 times that of the native enzyme at a final GuHCl concentration of 0.1 M. The intrinsic fluorescence of the rapidly formed intermediate is even greater than that of the stable equilibrium intermediate (see Fig. 1A). The refolding curves for CK at different final GuHCl concentrations, after the burst phase, are shown in Figure 7B. The emission intensity of the burst phase intermediate decreases with increasing final GuHCl concentrations. A representative kinetic trace for refolding of CK at a final GuHCl concentration of 0.1 M, after the burst phase, was analyzed by fitting to single and double exponential equations (Equation 6). Similar to the CD results, the experimental results fit better to a biphasic (Fig. 7C) rather than a monophasic (Fig. 7D) function. At final

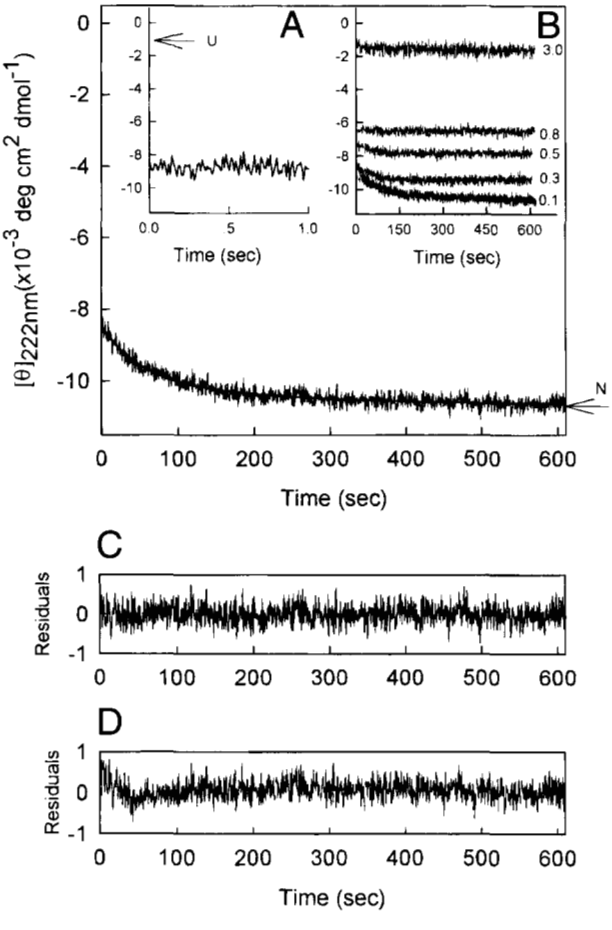

Fig. 6. Kinetics of CK refolding monitored by CD at 222 nm. Refolding was initiated by dilution of the unfolded enzyme at 3 M GuHCl into the refolding buffer at 25 °C. The enzyme concentration was 1.16 μM. The insets show: (**A**) the kinetic traces measured within 1 s using a stopped-flow apparatus and (**B**) the curves with different final GuHCl concentrations as indicated. *N* and *U* denote the values for the native and fully unfolded states at equilibrium, respectively. The thick continuous lines represent the theoretical double-exponential curves according to Equation 6. The lower panels show the residuals between the experimental points and the theoretical curves, (**C**) fit to a double-exponential and (**D**) to a single exponential. The best-fit values of the kinetic parameters are $A_1 = 1.76 \times 10^3$ deg cm^2 dmol^{-1}, $A_2 = 0.77 \times 10^3$ deg cm^2 dmol^{-1}, $A_\infty = -11.1 \times 10^3$ deg cm^2 dmol^{-1}, $k_1 = 0.021$, $k_2 = 0.0048$.

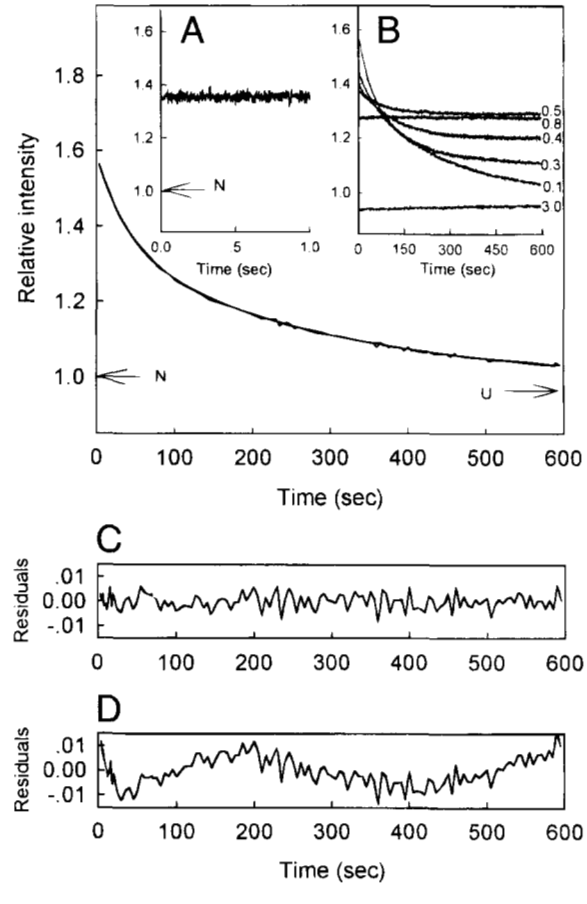

Fig. 7. Kinetics of CK refolding monitored by intrinsic fluorescence. Details are the same as in Figure 6, except the enzyme concentration was 1.16 μM. The best-fit values of the parameters are $A_1 = 0.35$, $A_2 = 0.24$, $A_8 = 0.99$, $k_1 = 0.024$, $k_2 = 0.036$. Fluorescence emission intensities were relative to that of the enzyme in the native state.

GuHCl concentrations above 0.4 M, however, only the fast phase of refolding is evident subsequent to the burst phase, and at 0.6 M GuHCl there is no more folding subsequent to the burst phase. The final fluorescence intensities reached at each GuHCl concentration are in complete accord with the values observed for the equilibrium state of unfolding.

As in the case of unfolding, following refolding by either CD at 222 nM or intrinsic fluorescence gives the same apparent rate constants, indicating that the changes in the tertiary and secondary structure proceed concurrently (Fig. 5). The rate constants for both the fast and slow phases decrease with increasing final GuHCl concentration, but the values for the single phase observed above 0.4 M GuHCl increase slightly with GuHCl concentration.

The rate constants obtained by analysis of the kinetic processes as first-order reactions at various enzyme concentrations are shown in Table 2. The rate constants are independent of enzyme concentration suggesting that the dimerization of the subunits during refolding of CK is not rate limiting.

Figure 8 shows the refolding process as monitored by fluorescence of ANS. A burst phase is also observed. The emission intensity after the burst phase depends on the final concentration of GuHCl, with an enhancement of about 11-fold at 0.1 M GuHCl compared with ANS added to native CK. The intensity is stronger than the maximal increase obtainable when ANS is added to the equilibrium unfolding intermediate at 0.8 M GuHCl, suggesting that the microenvironment of the ANS binding site is more hydrophobic in the burst phase than in the equilibrium intermediate state. There is subsequently only a slow and slight decrease in emission intensity in 0.1 M GuHCl and no further change in fluorescence intensity in GuHCl at final concentrations of 0.2–0.8 M. This might be due to tight ANS binding to the burst phase folding intermediate, which may prevent the ANS-bound molecule from folding further (Engelhard & Evans, 1995; Arai & Kuwajima, 1996).

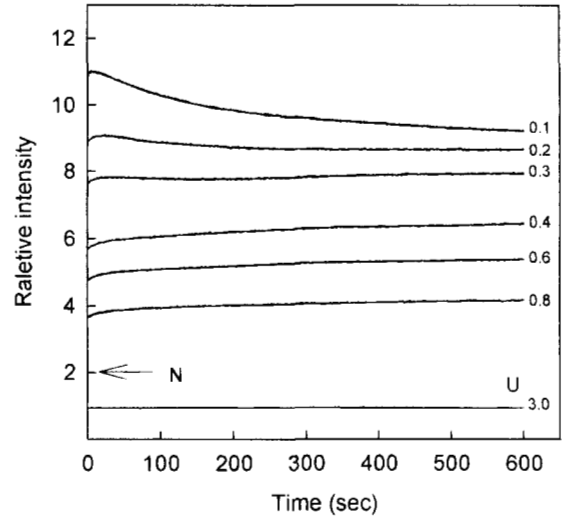

Fig. 8. Course of the enhancement of ANS fluorescence during refolding of CK. The refolding reaction was initiated by manual dilution of GuHCl from 3 M to various final concentrations as indicated, at pH 8.3 and 25 °C. The refolding buffer contained 50-fold molar excess of ANS and the final protein concentration was 1.16 μM. *N* and *U* denote the emission for the native and unfolded state, respectively.

CD and fluorescence spectra of the burst phase intermediate

In the refolding reaction, the stopped-flow measurements show that the time of formation of the burst phase intermediate must be less than 15 ms, but that of the subsequent folding is about 40–100 s. Because of a difference of at least three orders of magnitude, the burst phase intermediate can be kinetically separated from the subsequent folding intermediates. Thus, the ellipticity or the fluorescence intensity of the burst phase intermediate at various wavelengths can be obtained by extrapolating the folding curve after the burst phase to zero time (Ikeguchi et al., 1986; Arai & Kuwajima, 1996). The CD and fluorescence emission spectra of the burst phase intermediate are shown in Figures 9A and 9B, respectively, and are compared to those of the equilibrium unfolding intermediate at different GuHCl concentrations. The CD spectrum of the burst phase intermediate formed by dilution from 3 to 0.1 M GuHCl is most similar to that of the equilibrium intermediate at 0.4 M GuHCl, rather than that at 0.8 M GuHCl where the stable equilibrium intermediate is most highly populated.

As shown in Figure 9B, the maximum emission of the burst phase intermediate is at about 340 nm, the same as the equilibrium unfolded state at 0.8 M GuHCl, but the emission intensity of the burst phase intermediate is stronger. The results indicate that the microenvironment of the tryptophan residues is similar in the burst phase intermediate and in the equilibrium unfolding intermediate. The difference in the emission intensity may be due to a slightly different microenvironment of packing of the side chains.

Table 2. *Rate constants of refolding of creatine kinase after the burst phase at various protein concentrations at a final GuHCl concentration of 0.1 M*

Protein concentration (μM)	Refolding constants (s^{-1})			
	Intrinsic fluorescence		CD at 222 nm	
	k_1	k_2	k_1	k_2
1.16	0.024	0.0036	0.021	0.0048
4.64	0.018	0.0032	0.021	0.0017
11.6	0.019	0.0041	0.023	0.0020

Discussion

Secondary and tertiary structural changes occur simultaneously during unfolding and refolding of CK

There are four tryptophan residues per subunit of rabbit muscle creatine kinase. From the recently published high resolution crystal structure of the mitochondrial CK isoforms (Fritz-Wolf et al., 1996), the high sequence homology between the cytosolic and mitochondrial isoforms of CK, and the similar denaturation curves of the two isozymes (Gross et al., 1995), it appears that the tryptophan residues in cytosolic CK, as in mitochondrial CK, are not located within elements of secondary structure of the molecule. Thus, the

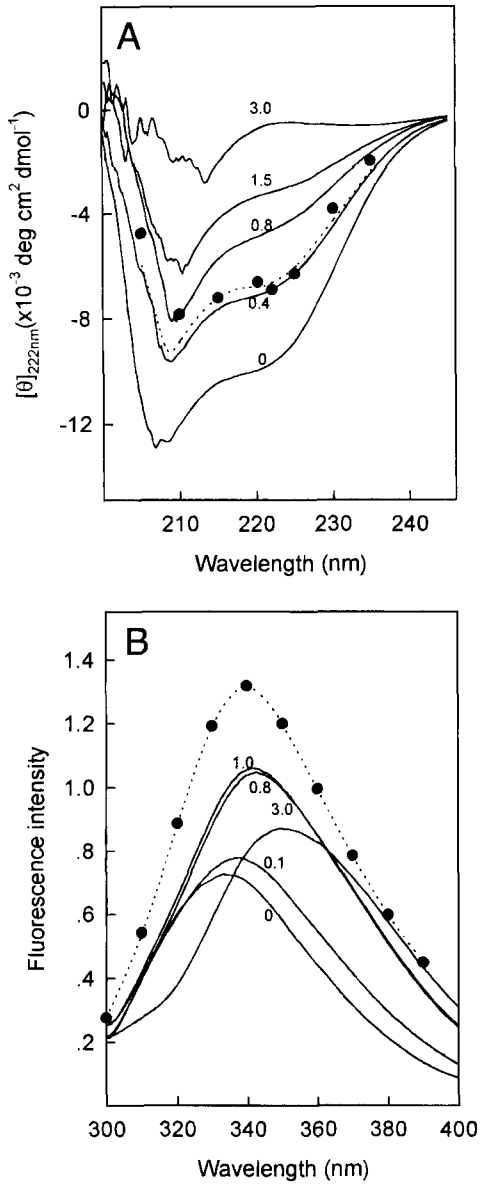

Fig. 9. Comparison of CD and intrinsic fluorescence spectra of the burst phase refolding intermediates of CK with that of CK in GuHCl at different concentrations. **A:** CD spectra of the burst phase refolding intermediate (···●···) formed by a concentration jump from 3 to 0.1 M GuHCl. Each value was obtained by the method described in the text. The solid lines are CD spectra of CK in different GuHCl concentrations as indicated, measured after incubation of the enzyme with GuHCl at the desired concentration in 50 mM Tris-HCl buffer at pH 8.3 and 25 °C for 12 h. The protein concentration was 1.16 μM. **B:** Intrinsic fluorescence spectra. The symbols for the curves and the method of measurement were the same as in **A**.

changes in the intrinsic fluorescence during unfolding and refolding of the enzyme probably principally reflect changes in its tertiary structure. The positions of the equilibrium unfolding transitions measured by intrinsic fluorescence and far-UV CD are the same. This, and the coincidence of the kinetic parameters for the folding and unfolding reactions measured by the two methods, suggests that during the folding and unfolding of CK changes in secondary structure are concurrent with changes in tertiary structure.

The early folding intermediate

The reconstructed CD and fluorescence spectra show pronounced secondary structure and buried tryptophan residues for the burst phase intermediate indicating a sudden collapse of the unfolded chain within 15 ms to form this intermediate. A comparison of the properties of the burst phase intermediate, equilibrium intermediate, native and fully unfolded forms of the enzyme are summarized in Table 3. It is evident that both the stable equilibrium and burst phase intermediates are characterized by pronounced secondary structure, compact globularity, exposed hydrophobic surface area, and the absence of rigid side-chain packing, resembling the characteristics described for the molten globule state (Kuwajima, 1989). However, in reactivation experiments, there is no burst phase recovery of activity, showing that regain of the molecular conformation required for enzyme activity is a slow process.

The identity or close similarity between a molten globule state observed during equilibrium unfolding and a rapidly formed kinetic intermediate observed during refolding has been reported for single domain proteins, such as α-lactalbumin (Kuwajima, 1989), tryptophan synthase α-subunit (Ogasahara et al., 1993; Ogasahara & Yutani, 1994), lysozyme (Ptitsyn, 1987), and ribonuclease A (Kim & Baldwin, 1990). Results obtained in this paper show that the equilibrium and burst phase intermediates of CK are similar in some respects, but differ significantly in other respects. The burst phase intermediate shows more secondary structure, exposed hydrophobic surface area, and more flexible side-chain packing than the equilibrium intermediate. Whether the differences between the equilibrium intermediate of CK and its early-formed counterpart is a property common to other multi-subunit proteins is of interest for further study.

Unfolding and refolding pathways of creatine kinase

Previous results from this laboratory (Zhou et al., 1993; Liu & Zhou, 1995) have shown that at low GuHCl concentrations, the enzyme unfolds to an inactive dimeric intermediate with conformational changes that cannot be detected by either far-UV CD or intrinsic fluorescence, but can be detected by extrinsic fluorescent or spin probes introduced at the active site of the enzyme. The rate constant of exposure of this covalently labeled fluorescent probe measured in 1 M GuHCl is 2.9 s^{-1}, which is of the same order of magnitude as the inactivation rate constant, but is about 2 orders of magnitude higher than the unfolding rate constant measured by intrinsic fluorescence or CD. It has been shown by size exclusion chromatography (Zhang et al., 1998) and sedimentation studies (Couthon et al., 1995) that the stable equilibrium unfolding intermediate is monomeric. The position of the equilibrium unfolding transitions are independent over a 200-fold change in protein concentration, indicating that unfolding does not coincide with dissociation of the dimer. The results suggest that dissociation occurs subsequent to unfolding of the enzyme to a dimeric intermediate, but before this intermediate begins to unfold to the fully denatured state, and that the dissociation reaction is not detectable by the spectroscopic methods employed. Based on these results, a possible mechanism for the unfolding of CK in GuHCl is suggested in Scheme 1:

$$N_2 \rightarrow I_2^a \xrightarrow{k_1} I_2^b \xrightarrow{fast} I^c \rightarrow 2U \qquad \text{(Scheme 1)}$$

Table 3. *Comparison of conformational properties of creatine kinase in different states*

	Intrinsic fluorescence				
	Wavelength (nm)	Intensity	CD $[\theta]_{222\,nm}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	Exposure of SH groups	Fluorescence of ANS
Native state	331	1.0	−10.6	1.7	1
Equilibrium intermediate	341	1.35	−5.5	6.9	5.4
Burst phase intermediate	341	1.61	−7.5	—	11
Denatured state	355	0.96	−1.5	6.8	0.5

where N_2 is the native dimer, I_2^a and I_2^b are partially unfolded dimeric intermediates, I^c is the monomeric intermediate, and U is the fully unfolded monomer.

The burst phase intermediate, with characteristics resembling the molten globule, forms within the dead time of stopped-flow mixing (15 ms). The subsequent folding after the burst phase is a biphasic process leading to a final state indistinguishable from the native state by intrinsic fluorescence and far UV CD. However, in spite of the 100% recovery of CD and intrinsic fluorescence, the enzymatic activity is only about 80% of the native enzyme. Full reactivation occurs many hours after any detectable conformational changes have come to an end, similar to the results reported previously for the urea denatured enzyme (Yao & Tsou, 1985; Zhou & Tsou, 1986).

It has been suggested that in the initial stages of folding, a protein molecule collapses to a compact denatured state driven by nonlocal hydrophobic interactions, with secondary structure formation being followed by slower rearrangements of side-chain packing in the subsequent stages (Dill, 1985, 1990; Ikeguchi et al., 1986; Arai & Kuwajima, 1989; Dill et al., 1995). Others have proposed, however, that isolated secondary structure elements form very early in folding (S state) followed by assembly into the compact "molten globule" state (Ptitsyn, 1995). In the present study, the reconstructed CD and fluorescence emission spectra of the burst phase intermediate suggest that both the collapse to form a relatively compact structure and formation of secondary structure elements take place before the dead time of mixing, i.e., within 15 ms. The sudden dilution of the denaturant results in the collapse of the extended peptide chain mainly by nonlocal hydrophobic interactions and by formation of hydrogen bonds between neighboring residues to form ordered secondary structure elements. For multidomain proteins, this collapse may lead to the formation of domain or subdomain structures before assembly to form the native structure (Fersht, 1995).

Neither the equilibrium nor the kinetic refolding curves are dependent on protein concentration, showing that the association of the monomeric subunits to form the dimer is not the rate-limiting step on the refolding pathway of CK. This result implies that after formation of the burst phase intermediate, the molecule rearranges to form subdomain structural elements, including additional secondary structure, and there is rearrangement of side-chain packing. This allows subunit recognition and assembly to form a native-like structure. The independence of the refolding on protein concentration suggests that the association of the subunits is very fast and may be a diffusion controlled process. During the final step of the reactivation, a fine adjustment of the arrangement of the side-chain groups occurs, particularly at the active site region, to form the fully active molecule. These changes could be too subtle, or limited only to a small region of the molecule, and so escape detection by the methods employed. It is not known why the regain of full activity is so slow. One possibility is that the rate of one of the steps may be limited by cis/trans isomerization of peptidylproline. A possible refolding model is given in Scheme 2:

$$2U \xrightarrow{burst\ phase} 2MG \xrightarrow{k_1} 2I^d \xrightarrow{fast} I_2^g \xrightarrow{k_2} N_2 \xrightarrow{very\ slow} N_2$$

(Scheme 2)

where U is an extensively unfolded monomer; MG is the molten globule burst phase intermediate; I^d is the intermediate subsequent to the MG state; I_2^g is the dimerized intermediate; N_2' is the dimeric intermediate with essentially the same conformation as the native state, but without full recovery of activity; N_2 is the native state; and k_1 and k_2 are the kinetic rate constants determined by CD or intrinsic fluorescence measurements.

The lack of protein concentration dependence is one of the interesting features of this system. Folding of some well-studied small dimers (reviewed by Neet & Timm, 1994), such as the Arc repressor (Bowie & Sauer, 1989; Robinson et al., 1997), Trp repressor (Gloss & Matthews, 1997), and neurotrophins (Timm & Neet, 1992; Timm et al., 1994) are concentration dependent. Folding of larger dimeric proteins are much more complicated and involve several intermediates. In some cases, the native to molten globular transition is concentration dependent, e.g., aspartate, aminotransferase (Herold & Kirschner, 1990). However, Blond and Goldberg (1985) observed that folding of the β_2 subunit of tryptophan synthase, like CK, is concentration independent and suggested that this is due to the very fast rate of the dimerization step.

Comparison of denaturation by urea and GuHCl

Urea and GuHCl are the most commonly used denaturants for unfolding studies of proteins, and they are believed to have a similar mode of action (Hibbard & Tulinsky, 1978; Yao & Bolen, 1995), except that GuHCl is a stronger denaturant (Pace, 1986). It is interesting that differences are observed in the folding of CK, depending on the denaturant used (Yao & Tsou, 1985; Zhou et al., 1997). The pathway of protein folding and assembly in vitro may depend on the initial unfolded states. As the unfolded states in the two denaturants differ significantly (Pace et al., 1990), it is not unexpected that this would lead to differences in the folding. There is no burst phase observed by fluorescence in the kinetic refolding of urea-denatured CK (Zhou & Tsou, 1986), and dimerization of

the subunits takes place at an earlier stage in refolding (Wang et al., 1995). These differences can likely be attributed to differences in the GuHCl and urea induced denatured states (Yao & Tsou, 1985); however, the additional properties of GuHCl as a salt may also affect folding and dimerization.

Materials and methods

Materials

Creatine kinase was prepared and further purified as described previously (Yao et al., 1982a). The absorbance (1%, 1 cm) value of 8.8 at 280 nm (Noda et al., 1955) was used for protein concentration measurements. The final preparations used typically had a specific activity of 130–160 μmol min^{-1} mg^{-1} (Mahowald et al., 1962) and showed only one band by polyacrylamide gel electrophoresis. GuHCl (ultrapure) were obtained from ICN Biochemicals (Cleveland, Ohio) and Nacalai Tesqre, Inc. (Kyoto, Japan). Other reagents were local products of analytical grade. Twice-deionized water was used throughout.

The activity of creatine kinase was routinely determined at 25 °C by following proton generation during the reaction of ATP and creatine with the indicator thymol blue at 597 nm (Yao et al., 1982b). The reaction mixture contained: ATP (4 mm), creatine (24 mM), magnesium acetate (5 mM), and thymol blue (0.008%) in 5 mM glycine buffer, pH 9.0.

Equilibrium measurements

For the equilibrium studies, samples were incubated for about 12 h to fully equilibrate at the appropriate final GuHCl concentrations at 25 °C before spectra were taken. For intrinsic fluorescence measurements, the emission spectra were recorded on a Hitachi F-4010 spectrofluorometer at 25 °C. Unless otherwise specified, an excitation wavelength of 280 nm was used, and the emission intensities were measured at 335 nm, with both excitation and emission slits set at 5 nm. For the 1,8-anilinonaphthalenesufonic acid (ANS) binding studies, samples denatured in GuHCl were incubated with a 50-fold molar excess of ANS for about 2 h at 25 °C, after which the fluorescence emission spectra were measured with excitation at 370 nm and the excitation and emission slits set at 5 and 7 nm, respectively. Circular dichroism spectra were measured on a Jasco J-720 spectropolarimeter, using a cuvette of 1.0 mm pathlength, thermostatted at 25 °C.

Determination of the number of reactive SH groups

The determination of thiol groups was carried out using DTNB (5, 5′-dithiobis(2-nitrobenzoic acid)) as described by Ellman (1959) with a Shimadzu UV-250 spectrophotometer at 25 °C. Both the reference and sample cells contained 0.5 mM DTNB in 50 mM Tris-HCl buffer, pH 8.3, 1 mM EDTA, with 4 μM CK added to the sample cell. The number of reactive -SH groups was evaluated using a molar absorbance coefficient of 13,600 M^{-1} cm^{-1}.

Analysis of the equilibrium unfolding profile using a three-state model

A stable equilibrium intermediate can be detected during the denaturation of creatine kinase in GuHCl solutions. A three-state unfolding process is described as follows: $N \leftrightarrow I \leftrightarrow U$, where N, I, and U are native, intermediate, and unfolded states, respectively. If y represents the experimental variable being used to follow the transition, and y_N, y_I, y_U are the values of y for N, I, and U, respectively; f_I and f_U are the fractions of I and U, respectively (Tanford, 1968), we have

$$y = y_N + f_I(y_I - y_N) + f_U(y_u - y_N) \tag{1}$$

$$f_I = \frac{K_{NI}}{1 + K_{NI} + K_{NI}K_{IU}} \tag{2}$$

$$f_U = \frac{K_{NI}K_{IU}}{1 + K_{NI} + K_{NI}K_{IU}} \tag{3}$$

with the equilibrium constants (Pace, 1986):

$$k_{NI} = \exp((m_{NI}\cdot[D] - \Delta G^0_{NI})/RT) \tag{4}$$

$$K_{IU} = \exp((m_{IU}\cdot[D] - \Delta G^0_{IU})/RT) \tag{5}$$

where m_{NI} and m_{IU} may be interpreted to reflect changes in solvent-accessible surface area in the transitions; ΔG^0_{NI} and ΔG^0_{IU} are the free energies of the transitions of $N \rightarrow I$ and $I \rightarrow U$, respectively; and $[D]$ is the concentration of the denaturants. By substituting Equations 4 and 5 into Equations 2 and 3, respectively, then substituting Equations 2 and 3 into Equation 1, an equation relating y vs. $[D]$ can be obtained. By fitting the experimental data to this equation, we can obtain the following parameters: ΔG^0_{NI}, m_{NI}, ΔG^0_{IU}, m_{IU}, and y_I.

Kinetic measurements of unfolding and refolding

The reaction of unfolding and refolding, induced by concentration jumps of GuHCl at pH 8.3, 25 °C, was followed by both CD and fluorescence measurements. Fast kinetic processes were followed in a stopped-flow apparatus. Fluorescence experiments were carried out with an Unisoku type USP-539 stopped-flow spectrophotometer equipped with a mixing device using a 1:6 volume ratio of the two solutions, driven pneumatically with nitrogen gas at a pressure of 4 to 5 kg/cm^2. Fluorescence measurements were performed by excitation at 280 nm with a slit width of 5 nm. The emission intensity was monitored at wavelengths above 320 nm using a 320 nm cut-off filter. CD stopped-flow experiments were performed using an Unisoku stopped-flow apparatus with a cell of 0.4 cm pathlength and a mixing device using a volume ratio of 1:9 at a driving pressure of 4 atm installed in the cell compartment of the J-720 spectropolarimeter (Aria & Kuwajima, 1996). The dead times for both the CD and fluorescence stopped-flow measurements were about 15 ms. For longer time intervals, kinetic measurements were carried out using a Hitachi F-4010 spectrofluorimeter. A solution of the native or denatured protein was rapidly added manually to GuHCl solutions of various concentrations in a cell of 1 cm light pathlength under stirring by a 4-fin spinning mixer with a magnetic stirrer, and the emission intensity was recorded as a function of time. The dead time for this procedure was about 4 s. All of the kinetic measurements were performed in 20 mM Tris-HCl buffer, pH 8.3 at 25 °C.

The kinetic data were analyzed by nonlinear least-squares fitting to the equation:

$$A(t) = \sum_i A_i \exp(-k_i t) + A_\infty \tag{6}$$

where $A(t)$ is the value of fluorescence intensity or CD signal at a given time t, A_i is the amplitude corresponding to each individual phase (i), k_i is the associated rate constant, and A_∞ is the amplitude at infinite time.

CD and fluorescence spectra of the creatine kinase burst phase intermediate state

The CD and fluorescence spectra of the folding intermediates were obtained by following the refolding at different wavelengths kinetically by GuHCl concentration jumps from 3 M or 0.8 to 0.1 M at pH 8.1 and 25 °C. The kinetic curves after the burst phase measured by either CD or fluorescence at each individual wavelength fit well to a double exponential equation. The spectra were reconstructed from the ellipticity and emission intensity values of the burst phase folding intermediates at each wavelength as obtained by extrapolation of the measured post burst phase values to zero time.

Acknowledgments

This paper was supported in part by the Pandeng project of the China Commission of Science and Technology. We would like to thank Prof. K. Kuwajima of the Department of Physics, School of Science, University of Tokyo for the use of stopped-flow CD instruments. We also thank Miss L. Zhu for her assistance in some of the experiments.

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