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Relationship between Kinetic and Equilibrium Folding Intermediates of Creatine Kinase

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Creatine kinase (CK) is a dimeric enzyme important in ATP regeneration in cells where energy demands are high. The folding of CK under equilibrium and transient conditions has been studied in detail and is found to be complex. At equilibrium in 0.8 M GuHCl, 90% of CK molecules are in the form of a partially structured, monomeric intermediate. We exploit this property to measure kinetics of refolding and unfolding to and from this equilibrium intermediate (EI), using far-UV circular dichroism and intrinsic fluorescence as structural probes. We are thus able to compare the properties of EI and the kinetic intermediate formed during the burst phase in refolding. Native CK and EI unfold with rate constants in seconds and milliseconds, respectively. As is observed for refolding of fully-denatured CK, refolding from EI to the native state shows a burst phase followed by two exponential phases. The burst phase refolding intermediate is inferred to have more structure and greater stability than the equilibrium intermediate. When refolding from the fully-denatured state in 0.8 M GuHCl, the equilibrium intermediate is formed within the dead-time of mixing in the stopped-flow apparatus. The equilibrium intermediate may thus represent a kinetic intermediate formed early during folding.

Key Words: **creatine kinase; equilibrium intermediate; kinetic intermediate; molten globule; premolten globule.**

Proteins fold from random coil polypeptides to their specific three-dimensional structures along defined pathways that may involve transient, partly folded intermediates. The structures of intermediates formed in the early stages of folding are thought to play a pivotal role in orienting their folding pathways (1, 2). However, detailed characterization of the properties of folding intermediates has been impeded by the very short lifetimes of kinetic intermediates. One approach to overcoming this problem is to find equilibrium intermediates that exhibit similar structural properties to kinetic intermediates. Molten globules (MG), which are stable under certain conditions, such as extreme pH, high salt or in the presence of organic solvents, are common examples of partially folded forms (2, 3). They retain a substantial amount of secondary structure but have a disrupted tertiary structure. It is suggested from the studies of the molten globule state of a number of proteins that the equilibrium intermediate is indeed a relevant and perhaps universal species in the pathway of protein folding, at least for larger proteins (4). However, most proteins for which a molten globule intermediate has been well characterized are small, monomeric, single domain proteins (1, 5–7). The folding intermediates of multi-domain and oligomeric proteins remain relatively poorly characterized, since the unfolding/folding of such proteins is much more complex (8, 9).

Creatine kinase (CK; EC 2.7.3.2) from rabbit muscle (MM isoenzyme) is a dimeric enzyme composed of two identical 43-kDa polypeptide chains. In its large size and oligomeric structure, CK is thus representative of the majority of enzymes and hence a good model for protein folding studies. While a number of much smaller, monomeric proteins have been shown to fold by a simple two-state mechanism (10), the folding of CK is highly complex involving multiple phases and one or more kinetically significant intermediates (11–15).

A striking and unusual property of CK is the significant population of a partially folded intermediate at moderate concentrations (0.8 M) of guanidine hydrochloride (GuHCl) (15–19). It is thus possible to use this intermediate state as the starting point for measurement of kinetic rate constants for folding and unfolding. Measurement of discrete steps in folding and un-

Abbreviations used: CK, creatine kinase; EI, equilibrium intermediate; GuHCl, guanidine hydrochloride; MG, molten globule; N, native state; U, unfolded state.

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folding and comparing the results with the kinetics observed for the overall process provides insight into the relationship between equilibrium and kinetic intermediates and the relevance of equilibrium intermediates in the elucidation of protein folding pathways.

MATERIALS AND METHODS

Reagents and proteins. Creatine kinase was prepared and further purified as described previously (20). The absorbance (1%, 1 cm) value of 8.8 at 280 nm (21) was used for protein concentration measurements. GuHCl (ultra pure) was obtained from ICN Biomedicals. Other reagents were local products of analytical grade. Twice-deionized water was used throughout.

Kinetic measurements. All of the reactions and measurements were performed in 20 mM Tris–HCl buffer, pH 8.3, at 25°C. Rapid 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 1:6 volume ratio of the two solutions, driven pneumatically with nitrogen gas at a pressure of 4 to 5 kg/cm^2. The fluorescence emission intensity was monitored at wavelength above 320 nm using a 320 nm cut-off filter by excitation at 280 nm with a slit width of 5 nm. 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 Jasco J-720 spectropolarimeter. The dead times for both CD and fluorescence stopped-flow measurements were about 15 ms. For longer time intervals, kinetic measurements by fluorescence or CD were carried out on a Hitachi F-4010 spectrofluorimeter or a Jasco J-720 spectropolarimeter, respectively. A solution of the native or denatured protein was rapidly added manually to GuHCl solutions of various concentrations under stirring. An excitation wavelength of 280 nm was used for fluorescence measurement with both excitation and emission slits set at 5 nm. The emission intensity at 335 nm or CD ellipticity at 222 nm was recorded as a function of time. The dead time for this procedure was about 4 s. The kinetic data were analyzed by nonlinear least-squares methods, as described previously (15).

RESULTS

Calculation of Relative Proportions of Populated States with Varying GuHCl Concentrations

We have shown previously that the equilibrium denaturation curves obtained by incubation of CK with increasing concentrations of GuHCl and monitored by fluorescence intensity at 335 nm or ellipticity at 222 nm are non-coincident and biphasic (15). The results indicate that GuHCl induces a global unfolding reaction that simultaneously disrupts the secondary and tertiary structures of CK and that at least one equilibrium intermediate (EI) is populated. Fitting of the data to a three-state model (15) allows calculation of the relative populations of native (N), intermediate (EI) and unfolded (U) states at different concentrations of GuHCl (Fig. 1). The curves indicate that the monomeric equilibrium intermediate is maximally populated in 0.8 M GuHCl, under which conditions the fractions of N, EI and U are calculated to be 0.02, 0.89 and 0.09, respectively. Our previous results indicate

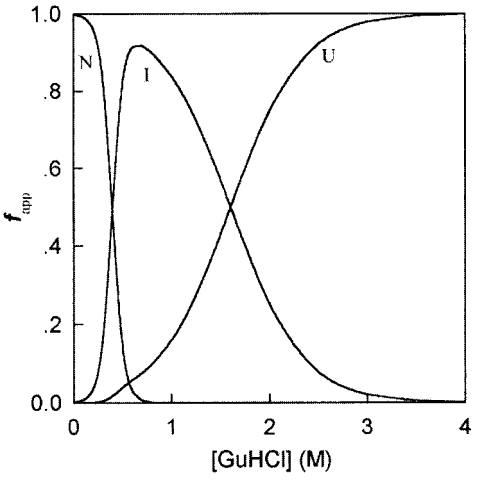

FIG. 1. Apparent fractions of native (N), intermediate (EI), and unfolded (U) states of CK as the function of GuHCl concentration. The mean values of thermodynamic parameters obtained from CD and fluorescence (see Ref. 15) were used to calculate the fractions.

that EI is a partially unfolded monomeric intermediate (18, 19). The structural properties of EI are summarized in Table 1 and compared with native and unfolded states.

Unfolding Initiated from the EI (0.8 → 3.0 M GuHCl)

In order to investigate whether unfolding of the native protein to the fully unfolded state goes through a kinetic intermediate that is similar to the EI, we compared unfolding of CK initiated from 0.8 M GuHCl (Fig. 2) and from the native state (15). Typical kinetic traces for unfolding of EI in a final GuHCl concentration of 3 M, monitored by changes of CD at 222 nm and fluorescence above 320 nm, are shown as curve 1 in Figs. 2A and 2B, respectively. Unfolding of native protein under the same conditions is monophasic and shows no burst phase (curve 2, Figs. 2A and 2B), however, the denaturant dependence of the rate constants shows extreme curvature reflecting that the 'denatured' state at low GuHCl concentrations is in fact a structured intermediate (15). The apparent rate constant for unfolding of the native enzyme in 3 M GuHCl ($0 \rightarrow 3$ M GuHCl) is 5 ± 1 s^{-1} (15). In contrast, unfolding of EI ($0.8 \rightarrow 3$ M GuHCl) is very fast, and is complete within the dead time of stopped-flow mixing (Fig. 2). The amplitudes obtained when unfolding from the native enzyme in different concentrations of GuHCl correspond to the appropriate equilibrium values, whether measured by fluorescence or far-UV CD (15). When unfolding from EI the final value is reached within the dead time of stopped-flow mixing and is the same as the value obtained for unfolding from the native state. This confirms that the transition from EI to the unfolded state is not rate-limiting for unfolding. This leaves open the possibility that unfolding to the

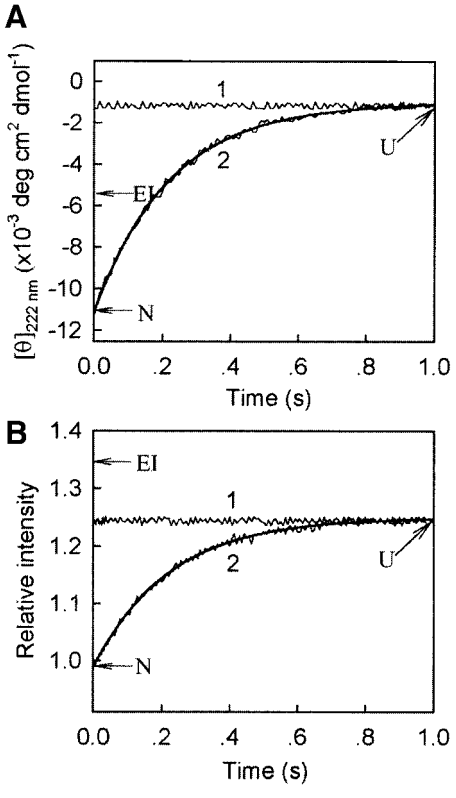

FIG. 2. Kinetics of CK unfolding initiated by a GuHCl concentration jump from 0.8 to 3 M as monitored by CD at 222 nm (A) and intrinsic fluorescence (B) using a stopped-flow apparatus (curve 1). The final enzyme concentration was 1.14 μM. Curve 2 in both (A) and (B) show the unfolding kinetic traces initiated from the native state for comparison (15).

fully-denatured state occurs via an intermediate that resembles EI, consistent with the unfolding model proposed (15).

Refolding Initiated from the EI (0.8 → 0.1 M GuHCl)

Figures 3A and 3B show typical kinetic curves for refolding of the EI (0.8 → 0.1 M GuHCl), monitored by far-UV CD and intrinsic fluorescence, respectively. Similar to refolding initiated from the 3 M GuHCl denatured state (15), there are three phases detected in the refolding of EI when monitored by either methods. The first phase is very fast and finishes within the dead time of the stopped-flow apparatus. Following this burst phase, the time course for refolding of EI fits well to a double exponential equation (inset 2, Figs. 3A and 3B). The apparent rate constants for these two phases (k_1 and k_2), whether measured by CD or fluorescence, are 0.021 ± 0.002 s^{-1} and 0.0032 ± 0.0008 s^{-1}, respectively. These rate constants do not vary within the protein concentration range 1.14–11.4 μM, suggesting that dimerization is not rate-limiting for refolding of EI. The rate constants are the same within error as those for refolding initiated from the 3 M GuHCl denatured state, which also show no protein concentration dependence (15). This provides direct experimental evidence that the burst phase intermediate observed during refolding from the fully denatured state is more structured and more stable than the equilibrium intermediate. If an intermediate resembling the EI is formed during refolding initiated from fully-unfolded state, its formation is therefore expected to occur prior to formation of the burst phase intermediate. Conversion between EI and the burst phase intermediate occurs rapidly, supporting the view of EI as a physiologically relevant denatured state, rather than a misfolded, trapped state. This confirms that the slowest step for refolding of CK occurs late in the folding process.

Partial Refolding Initiated from the EI (0.8 → x M GuHCl where 0 < x < 0.8)

Figures 4A and 4B show the amplitudes of CD signal and fluorescence intensity, respectively, for the ob-

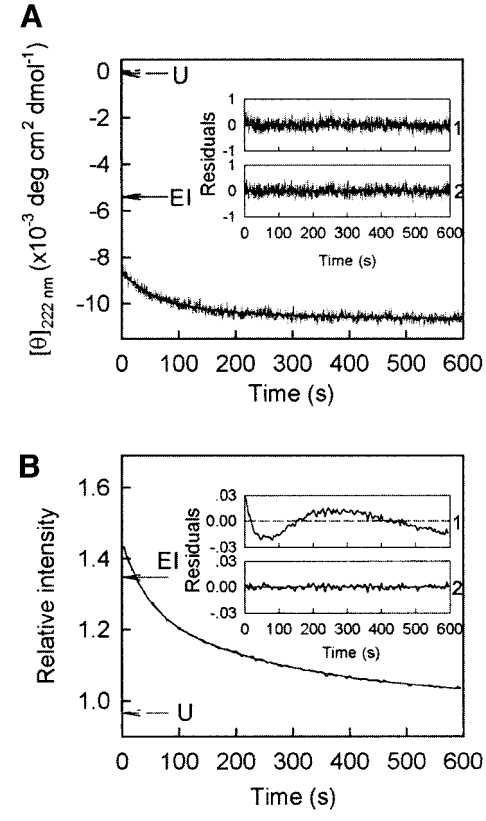

FIG. 3. Kinetics of CK refolding initiated by a GuHCl concentration jump from 0.8 to 0.1 M as monitored by CD at 222 nm (A) and intrinsic fluorescence relative to the native enzyme (B) using manual-mixing. The final enzyme concentration was 1.14 μM. The values for unfolded state in 3 M GuHCl and for intermediate in 0.8 M GuHCl at equilibrium are marked as 'U' and 'EI,' respectively. The thick continuous lines represent the theoretical double-exponential curves. Insets show the residuals between the experimental points and the theoretical curves, (1) fit to a single exponential and (2) to a double exponential function.

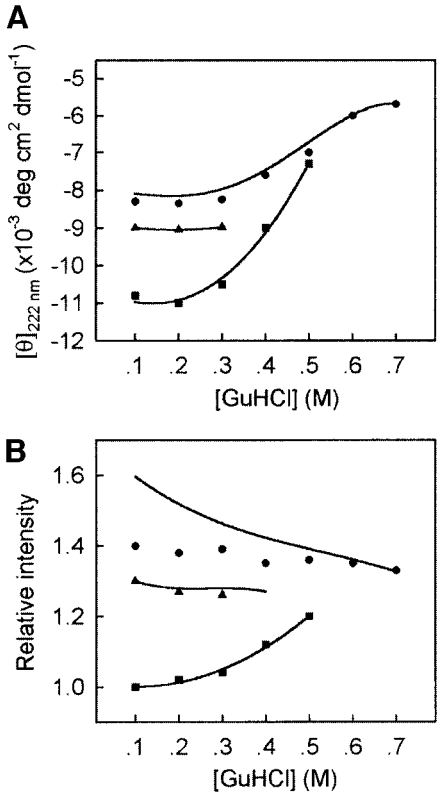

FIG. 4. The dependence of amplitudes in the terms of the CD at 222 nm (A) and intrinsic fluorescence intensity (B) on final GuHCl concentration for CK refolding initiated from 0.8 M GuHCl. The amplitude of the burst phase (●) was obtained by extrapolating the folding curves after the burst phase to zero time. The amplitudes of fast phase (▲) and slow phase (■) were obtained by curve fitting of Figs. 3A and 3B. The solid lines in both (A) and (B) show the corresponding amplitudes of refolding initiated from 3 M GuHCl for comparison.

served refolding kinetic phases initiated from the EI in various final GuHCl concentrations. The corresponding values for refolding from U, the 3 M GuHCl denatured state (15), are also shown in the figures as continuous curves for comparison. When monitoring CD, the amplitudes for the three phases, namely the burst phase, fast phase and slow phase, are the same whether refolding from the EI or from U (Fig. 4A). When monitoring fluorescence (Fig. 4B), the amplitudes for the fast and slow phases are also the same whether refolding from EI or U. However, the amplitude for the burst phase for refolding from these two denatured states shows some divergence. This suggests a slight difference in the tertiary structure of the intermediates formed when refolding from EI or U, resulting in different sensitivity of their side-chain packing to the denaturant concentration in the refolding buffer.

Refolding to the EI (3.0 → 0.8 M GuHCl)

Refolding initiated by a GuHCl concentration jump from 3 M to 0.8 M shows only a burst phase and the recovered amplitude when monitoring fluorescence or CD is the same as the corresponding equilibrium value (not shown). This indicates that the equilibrium intermediate is formed within the dead time of mixing in the stopped-flow apparatus and provides further experimental evidence that the slow steps in CK refolding occur subsequent to formation of this intermediate.

DISCUSSION

Rate-Limiting Steps in the Folding/Unfolding of CK

We have shown previously that unfolding of native CK initiated by GuHCl concentration jump, whether the final GuHCl concentration is above or below 0.8 M, is a monophasic process with half-life time in seconds (15). The results presented here show that unfolding initiated from the equilibrium intermediate (EI) is much faster, with half-life time in milliseconds. Earlier findings from this laboratory (22) show that the inactivation rate of CK in either GuHCl or urea is about three orders of magnitude faster than the rate of unfolding detected by global molecular changes under identical conditions. The conformational change in the inactivation step cannot be detected by intrinsic fluorescence or far UV-CD, but can be detected using a covalently attached fluorescence probe at the active site (23). These results taken together indicate that the enzyme unfolds via an inactivate, native-like intermediate and that there may be a further kinetic intermediate, structurally similar to EI, formed on the unfolding pathway of CK. The observed unfolding rate may correspond to the transition between these two intermediates, consistent with the scheme for unfolding proposed earlier (15).

Similar to CK, equilibrium intermediates have been observed at low to moderate urea or GuHCl concentration for the α-subunit of tryptophan synthase (24). Matthews and coworkers recently showed using an unfolding double-jump assay, that the rate-limiting step in the unfolding of α-subunit of tryptophan synthase is the conversion of the native-like form to its corresponding intermediate (25). It seems that the conversion of native or native like structure to an intermediate state is likely to be the rate-limiting step during the unfolding of multi-subunit and multi-domain proteins.

Similarly, refolding of CK is observed to involve multiple kinetic intermediates (14, 15), which apparently occur subsequent to formation of a state that resembles EI. The rapid transition between EI and both the fully-denatured state (Fig. 2) and the burst phase refolding intermediate (Fig. 3) contradicts the idea that this intermediate is a trapped, misfolded state, but suggest that it may represent a physiologically relevant denatured state.

TABLE 1

Comparison of Conformation Properties of Creatine Kinase in Different States

	$[\theta]_{222\ nm}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})	Intrinsic fluorescence λ_{max} (nm)	Intrinsic fluorescence Relative intensity at 335 nm	Quarternary structure
Native state[a]	−10.6	331	1	Dimer
Inactived state (0.4 M GuHCl)[a]	−8.2	337	1.2	Dimer[b]
Equilibrium intermediate (0.8 M GuHCl)[a]	−5.5	341	1.35	Monomer[b]
Burst intermediate[a] (3.0 → 0.1 M GuHCl)	−8.1	339	1.61	Fast dimerization upon formation
Burst intermediate (0.8 → 0.1 M GuHCl)	−8.3	338	1.4	Fast dimerization upon formation
Fully unfolded state[a]	0	355	0.96	Monomer

[a] Data taken from Ref. 15.
[b] Data from Ref. 12 or 14.

Differences between the Kinetic and Equilibrium Intermediates

Reconstruction of the fluorescence and CD spectra of the burst phase intermediate shows that it most closely resembles the equilibrium state populated in 0.4 M GuHCl, rather than the EI populated in 0.8 M GuHCl (Table 1; 15). The burst phase intermediate has the properties of a molten globule state (15). As shown in Figs. 3 and 4, the burst phase intermediates formed when refolding from the fully-denatured state (3 → 0.1 M GuHCl) or from EI (0.8 → 0.1 M GuHCl) show only minor differences. When refolding in 0.8 M GuHCl (3 → 0.8 M GuHCl), EI is formed during the dead-time of mixing. This implies that a kinetic intermediate with the properties of EI may be formed during refolding prior to formation of the more structured and more stable kinetic burst phase intermediate. EI may thus correspond to a premolten globule state (2, 26, 27, 28). In the case of CK, the study of its properties provides insight into the earliest steps of protein folding.

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