Evidence for Kinetic Intermediate States during the Refolding of GdnHCl-Denatured MM-Creatine Kinase. Characterization of a Trapped Monomeric Species†

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Received July 29, 1998; Revised Manuscript Received October 7, 1998

ABSTRACT: The kinetics of refolding of guanidinium chloride-denatured rabbit MM-creatine kinase was investigated. Recovery of enzymatic activity is biphasic, depending on the temperature but not on the protein or DTT concentration. Only 45% of the original, active dimeric form is recovered even after several hours of refolding. The reactivation yield is limited by the accumulation of a highly stable but nonproductive monomeric species. The ratio of "correct" to "incorrect" forms depends on the duration of exposure to the denaturant, which may be consistent with the existence of a heterogeneous population of unfolded states with regard to proline isomerization. The first fast reaction observed during renaturation results in the appearance of collapsed monomeric states, displaying features of a pre-molten globule state. These burst species are rapidly transformed into more structured monomers resembling a molten globule state possessing a partially folded C-terminal domain. A proportion of these latter transient intermediates (45%) associates into an active dimer, while the remainder (55%) is trapped by reshuffling in a monomeric dead-end product. Our results strongly indicate that (i) the dimeric state is a prerequisite for the expression of catalytic activity, (ii) the kinetic intermediates of refolding are very similar to those observed during equilibrium unfolding, and (iii) refolding of creatine kinase in these conditions is limited by the accumulation of inactive misfolded nondimerizable monomer.

One of the major aims of protein science is the elucidation of mechanisms and rules that drive an unfolded polypeptidic chain into its native three-dimensional structure. It has been clearly established that proteins fold along one, or more, defined pathways which involve intermediate structures that are only partly folded (*1*−*5*). The elucidation of a folding pathway requires the identification and the structural characterization of the intermediate states involved in the transition from the unfolded state to the final product. Kinetic intermediate species are difficult to study because of their short lifetime on the folding pathway and because they are less stable than the fully folded protein or the aggregated form. One can slow the rate of protein folding and stabilize the transient intermediates by using lower temperatures, and/or higher residual denaturant concentrations, and/or lower protein concentrations (*6*).

When kinetic intermediates are detected, their characteristics can be usefully compared with the energetic and structural properties of stable equilibrium intermediate states of refolding. Indeed, stable conformations of proteins that are not completely folded or unfolded are often observed under several non-native conditions (extreme pHs or organic solvent or mild denaturant concentrations). The majority of these equilibrium states retain an important native-like secondary structure but have a disrupted tertiary structure; these have been called molten globules (*2*, *7*, *8*) or compact intermediates (*9*) and could be the equilibrium counterpart of kinetic intermediates (*4*, *5*, *9*−*11*). The collapsed state formed within the first milliseconds of protein refolding (*12*) could correspond to another equilibrium intermediate (pre-molten globule) observed for monomeric β-lactamase and carbonic anhydrase B (*4*, *5*), for monomeric luciferase (*11*), and for dimeric cytosolic creatine kinase (CK)[1] (*13*).

Studies on the in vitro reactivation of proteins showed that correct refolding often competes with aggregation processes (*14*) or with the accumulation of misfolded species (*15*, *16*). The results reported in this paper shed new light on the refolding process of a homodimeric protein, cytosolic rabbit MM-creatine kinase (CK). This enzyme is made up of two identical subunits of 380 amino acids (43 kDa). It has been

† This work was supported by grants from the Association Française contre les Myopathies (AFM), the Région Rhône-Alpes, and the CNRS program "Physique et Chimie du Vivant".

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; CK, cytosolic MM-creatine kinase (EC 2.7.3.2); DTT, dithiothreitol; GdnHCl, guanidinium hydrochloride; λ_{max}, maximum fluorescence emission wavelength; PMSF, phenylmethanesulfonyl fluoride; NbS_2, 5,5′-dithiobis(2-nitrobenzoate); R_s, Stokes radius.

10.1021/bi981828p CCC: $15.00
Published on Web 11/21/1998

postulated that the CK monomer is composed of at least two domains, separated by a cleft, and that the catalytic site lays at the interface of these two domains (*17*).

Previous equilibrium denaturation studies determined that the use of different denaturants led to different unfolded structures with no detectable intermediate states in urea (*18*, *19*). This suggests that the CK behavior may differ during refolding. Indeed, renaturation of the urea-denatured CK indicates that full reactivation is usually obtained in the presence of DTT (*18*, *20*−*22*). In contrast, earlier studies of the refolding of GdnHCl-denatured CK have shown that reactivation yield is dependent on residual denaturant concentration but not on the presence of DTT (*23*). Our previous equilibrium denaturation studies demonstrated that, between 0 and 0.4 M GdnHCl, this enzyme keeps a native-like structure and activity. For higher denaturant concentrations, two intermediate species are detected which share some of the characteristics of a molten globule (*19*, *24*) and a pre-molten globule (*13*).

In this study, we report results from experiments undertaken to (i) identify kinetic folding intermediates, (ii) compare them with their equilibrium denaturation counterparts, and (iii) understand the mechanism of the off-pathway reaction which limits the reactivation yield in 0.3 M residual GdnHCl concentration. A model of the folding pathway of CK is proposed.

MATERIALS AND METHODS

Materials. ANS (hemimagnesium salt), NbS_2, trypsin, and creatine were purchased from Sigma. Tris, EDTA, DTT, PMSF, and ATP were obtained from Boehringer Mannheim, and GdnHCl was from Amresco. Rabbit muscle MM-CK was purchased from Boehringer Mannheim. The enzyme was desalted by chromatography on a PD10 column (Pharmacia) equilibrated in 20 mM Tris/acetate buffer (pH 7.4), and its purity was checked by SDS−polyacrylamide gel (12.5%) electrophoresis (*25*). The protein concentration was estimated according to the method of Lowry, as modified by Bensadoun (*26*), or using a molar extinction coefficient of 76 000 cm^{-1} M^{-1} at 280 nm.

Creatine Kinase Activity. The activity of 10−40 μL of enzyme, whether it had been previously incubated with denaturant (dimer CK concentration ranging from 0.58 to 5.8 μM), was estimated by the pH-stat method (30 °C and pH 8.8) using Mg-ATP and creatine as substrates (*27*). Native CK specific activity is 90 ± 10 units/mg. We verified that the maximal residual denaturant concentration in the pH-stat cell (3 mM GdnHCl) had no effect on the activity of the native enzyme and also that no further reactivation is observed during the measurements.

Refolding Procedure. Samples of 23−58 μM (2−5 mg/mL) dimeric CK were incubated in 20 mM Tris/acetate (pH 7.4), 0.1 mM EDTA, and 0.1 mM DTT (DTT was omitted for thiol group titration experiments) containing 3 M GdnHCl. No further variation of any of the biophysical parameters studied was observed when the protein was incubated overnight at 4 °C. CK refolding was initiated by dilution of these samples in 20 mM Tris/acetate (pH 7.4) and 0.1 mM EDTA with or without 0.1 mM DTT to a final GdnHCl concentration of 0.3 M. Aliquots were withdrawn at appropriate time intervals and assayed for activity as described above. Activities were compared with those observed in the 0.3 M GdnHCl-treated protein. Fitting of the kinetic reactivation data to a double-exponential curve was performed using Sigma Plot software (Jandel Scientific Software, San Rafael, CA).

Trypsin Susceptibility. At various time intervals after the initiation of refolding, protein aliquots were withdrawn and incubated with trypsin (0.1 μg/μg of protein) for 1 min at 20 °C (*28*). The activity of these samples was measured 30 s after inactivation by PMSF (1 mM) and compared with those of control refolding samples to which no trypsin had been added.

Size Exclusion Chromatography. Size exclusion chromatography was performed at room temperature with a HiPrep Sephacryl S100 16/60 column (Pharmacia) which was eluted at 0.7 mL/min. The elution buffer was 20 mM Tris/acetate, 0.15 M NaCl (to minimize nonspecific interactions with the column), and 0.3 M GdnHCl (pH 7.4). Elution profiles were monitored using a Waters diode array detector. The column was calibrated with proteins with known molecular masses and Stokes radii (R_s) (*13*, *29*). A calibration curve was obtained by plotting $\log(R_s)$ versus $1000/V_{el}$; $\log(R_s) = 4.2268 \times 10^{-2} (1000/V_{el}) - 0.32927$, where V_{el} is the elution volume (*30*).

At various refolding times, aliquots of CK were injected onto the column. After elution, the protein concentration was reduced by approximately 70-fold. An estimation of peak fraction was carried out using the "integrator" function of Waters HPLC software 991 with an accuracy of about 5−10%.

Intrinsic Fluorescence Measurements. Intrinsic fluorescence measurements were carried out as previously described (*13*). Recovery of the native emission fluorescence spectra (final dimer concentration of 2.3 μM) was recorded in less than 1 min between 300 and 400 nm using an excitation wavelength of 295 nm.

ANS Binding Measurements. They were carried out as previously described (*13*). The final dimeric CK concentration was 2.3 μM, and ANS was added in a 150-fold molar excess. We verified that addition of ANS, either in the renaturing buffer or in aliquots of protein after different times of refolding, leads to the same result. Thus, ANS simply binds transiently to partially folded intermediates without measurably altering their conformation or stability, and does not significantly perturb either the folding pathways or the kinetics.

Thiol Group Accessibility. Protein folding was initiated in spectrophotometer cuvettes at 20 °C. At various times, an aliquot of a 10 mM NbS_2 solution was added to the cuvette and the optical density at 412 nm was read after incubation for 10 min (final concentration of NbS_2 of about 0.1 mM). The number of accessible cysteines was calculated using 14 150 cm^{-1} M^{-1} as the thionitrobenzoate molar extinction coefficient in 0.3 M GdnHCl (*31*).

Second-Derivative Absorption Spectra. To estimate the degree of tyrosine exposure as a function of time, second-derivative absorption spectra of 2.3 μM refolded CK in 20 mM Tris/acetate (pH 7.4), 0.1 mM EDTA, and 0.3 M GdnHCl were recorded in less than 1 min at 20 °C between 270 and 300 nm using a Perkin-Elmer model 557 spectrophotometer (*32*).

Table 1: Physicochemical Properties of MM-Creatine Kinase Incubated at Equilibrium in 0.3 and 3 M GdnHCl[a]

	0.3 M GdnHCl	3 M GdnHCl
oligomeric form	dimer	monomer
Stokes radius (nm)	3.8	5.6
maximum fluorescence emission wavelength (nm)	332	352
iodide fluorescence accessible fraction (%)	25	90−100
no. of accessible tyrosines per monomer	6	10
no. of accessible thiols per monomer	1	4
ellipticity at 222 nm (deg cm^2 dmol^{-1})	−11200	−2400
ANS binding[b]	0.3	0.1
specific activity (units/mg)	90 ± 10	0

[a] Structural data are taken from Clottes et al. (*13*). [b] Normalized to the maximum intensity observed for the binding of ANS to the molten globule.

Circular Dichroism. Circular dichroism spectra of the denatured and renatured CK (2.3 μM) were recorded as previously described (*13*).

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed at 60 000 rpm at 20 °C in a Beckman XLA analytical ultracentrifuge, equipped with a UV scanning system, using a four-hole AN-60 Ti rotor with aluminum double-sector centerpieces with a 1.2 cm path length, on two renatured samples at 2.3 and 5.8 μM, and a native one at 5.8 μM. The profiles were analyzed using time derivatives (*33*). The corrected sedimentation coefficients s_w^{20} were obtained in the standard way from the maxima of the distribution function of the sedimentation coefficients, and using tabulated viscosity η and density ρ (*34*) and a value of 0.73 mL/g for ν, the specific volume of the protein. Equilibrium sedimentation profiles were obtained for 100 μL of the renatured samples, after 24 h at 20 °C and 10 000 rpm, and followed by sedimentation for 3 h at 60 000 rpm, to obtain an experimentally determined baseline. Data were analyzed using the softwares ideal 1 and ideal 2 supplied by Beckman. Molar masses M were estimated from the Svedberg equation $M = s_w^{20} N_A 6\Pi\eta R_s/(1 - \rho\nu)$ using the Stokes radii (R_s) from gel filtration experiments, where N_A is Avogadro's number.

RESULTS

In Table 1, some of the characteristics of CK, incubated in either 3 or 0.3 M GdnHCl, are summarized. When exposed overnight to 0.3 M GdnHCl, CK retains its active dimeric structure (R_s = 3.8 nm) (*13*). Exposure of CK to 3 M GdnHCl leads to a monomeric molecule (R_s = 5.6 nm) in which all cysteine, tyrosine, and tryptophan residues are exposed but where residual structures may persist (residual ellipticity at 222 nm). This state will be referred to as the "unfolded" state throughout this paper. If CK refolding was a completely reversible process, dilution of the enzyme, denatured overnight in 3 M GdnHCl to a final denaturant concentration of 0.3 M, would lead to the recovery of native-like biophysical characteristics.

Activity Recovery. The time course of the activity recovery for various protein concentrations at 18 °C is plotted in Figure 1A. For monomer concentrations between 1.16 and 11.6 μM, the reactivation curves can be superimposed, indicating that both yield and rate do not depend on protein concentration. The maximum specific activity recovered is only about 40 ± 10 units/mg, whereas the activity of the same concentration of CK incubated in 0.3 M GdnHCl is 90 ± 10 units/mg (Table 1). Thus, the recovered activity,

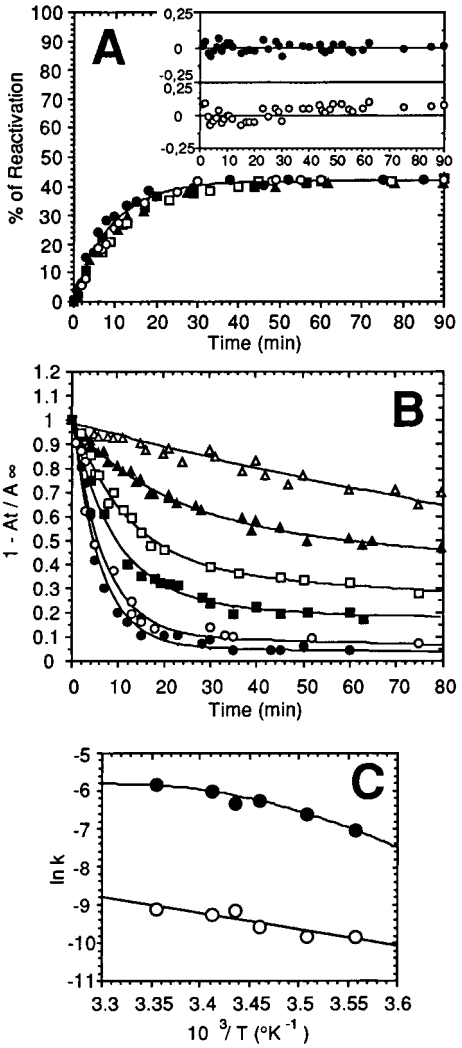


FIGURE 1: Kinetics of reactivation of CK. The protein was unfolded overnight in 3 M GdnHCl, 20 mM Tris/acetate (pH 7.4), 0.1 mM EDTA, and 0.1 mM DTT. Refolding was induced by a 10-fold dilution to 0.3 M GdnHCl. (A) Activity recovery at 18 °C for various final monomer concentrations was (■) 11.6, (○) 5.8, (●) 4.64, (□) 2.32, and (▲) 1.16 μM. The solid line indicates the best fit to eq 1 with the following values: a = 0.886, k_1 = 0.108 min^{-1}, b = 0.116, and k_2 = 0.0065 min^{-1}. As shown in the inset, the residuals show that the data were better fitted with the sum of two exponentials (●) than with a single one (○). (B) Activity recovery at various temperatures: (△) 0, (▲) 8, (□) 12, (■) 16, (○) 20, and (●) 25 °C. The final subunit concentration of CK is 4.64 μM. The data were best fitted (solid lines) to a single exponential at 0 °C (a = 0.964 and k_1 = 0.005 min^{-1}) or double exponential (8−25 °C) using the parameters given in Table 2. Each point is the ratio of the recovered activity at time t to the activity of 0.3 M GdnHCl-treated CK, measured under the same conditions of temperature and protein concentration. (C) Arrhenius plot of the temperature dependence of the first-order rate constants k_1 (●) and k_2 (○) for the fast and slow phase, respectively, of CK reactivation. The downward curvature of the k_1 plot is expected for a reaction that proceeds with a negative heat capacity change. The solid line is a nonlinear least-squares fit to the Chen equation (*35*), $y = A + B(293/T) + C \ln(293/T)$, where A = 493.44, B = −499.47, and C = 485.46.

Table 2: Values of the Parameters in eq 1 Determined at Temperatures of 8−25 °C

T (°C)	a	k_1 (10^{-3} min^{-1})	b	k_2 (10^{-3} min^{-1})
8	0.426	51	0.561	3.0
12	0.655	84	0.370	3.3
16	0.779	108	0.239	4.2
20	0.945	150	0.101	6.0
25	0.995	170	0.056	6.4

which does not increase even after renaturation for 48 h, represents only 45 ± 5% of the original specific activity. No critical dependence of the yield of reactivation was observed in the presence or absence of 0.1−10 mM DTT provided that 0.1 mM EDTA is included in the renaturing buffer. In the range of CK concentrations used for refolding, no aggregation can be evidenced by either light scattering or an increase of the turbidity at 410 nm (not shown).

In the time range of our experiments, refolding consists of two phases: a fast one and a slow one. The reactivation curves fitted well to the sum of two first-order reactions (see the inset in Figure 1A) according to eq 1:

$$1 - A_t/A_\infty = a e^{-k_1 t} + b e^{-k_2 t} \quad (1)$$

where A_t is the enzymatic activity at time t, A_∞ is the final recovered activity, a and b are the relative amplitude of the two phases, 0.886 and 0.116, respectively, and k_1 and k_2 are the rate constants, 0.108 and 0.0065 min^{-1}, respectively, at 18 °C.

Results from similar reactivation experiments, at various temperatures with a constant monomer concentration (4.6 μM), indicate that the refolding process is strongly temperature-dependent. Figure 1B shows the corresponding reactivation curves for temperatures ranging from 0 to 25 °C. Whatever the temperature, the final yield is 45%, even after reactivation for 2 days. Between 8 and 25 °C, the recovery of activity as a function of time is biphasic. At these temperatures, the data can be satisfactorily fitted to the sum of two exponentials according to eq 1. However, at 0 °C, data fitted to a single exponential give lower standard errors. This result suggests that, at this temperature, refolding proceeds via a single folding phase (where $a = 0.964$ and $k_1 = 0.005$ min^{-1}) or that the rate constants of the fast and slow phases become too close to be properly resolved. Reactivation rates strongly decrease above 37 °C (not shown).

Analysis of these data by eq 1 yields the values given in Table 2. The values for a and the rate constants of the fast and slow phases (k_1 and k_2, respectively) increase with increasing temperature, while b decreases. The solid lines in Figure 1B were drawn on the basis of eq 1, using the parameters given in Table 2.

Figure 1C is an Arrhenius plot of rate constants k_1 and k_2. During refolding, the rate constant of the slow phase (k_2) is slightly less temperature dependent than that of the fast phase (k_1). The plot is linear for k_2 and yields an apparent activation energy of 8.6 kcal/mol. However, the downward curvature of the fast phase rate constant plot is consistent with Chen's relationship (*35*), suggesting that the energy of activation (roughly 11.7 kcal/mol) changes with temperature and that this reaction proceeds with a negative heat capacity change.

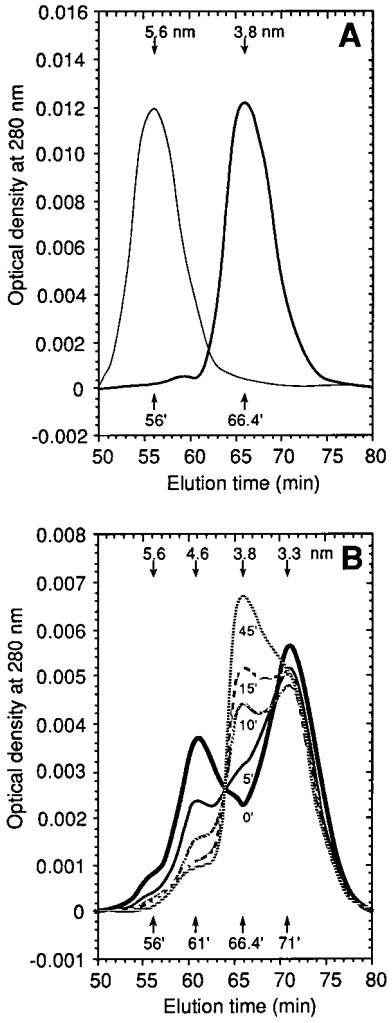


FIGURE 2: Representative size exclusion chromatographic profiles at 280 nm of CK folding. (A) Native dimeric CK was incubated overnight at 4 °C in 0.3 M GdnHCl (thick line) and loaded onto a HiPrep S-100 column equilibrated with 20 mM Tris/acetate (pH 7.4), 150 mM NaCl, and 0.3 M GdnHCl and had a retention time of 56 min. CK was incubated overnight at 4 °C in 3 M GdnHCl (thin line) and loaded onto the same column equilibrated with 20 mM Tris/acetate (pH 7.4), 150 mM NaCl, and 3 M GdnHCl and exhibited a retention time of 66.4 min. In both cases, the monomer concentration was 4.64 μM. (B) All profiles were obtained using a column equilibrated with 20 mM Tris/acetate (pH 7.4), 150 mM NaCl, and 0.3 M GdnHCl at room temperature. The refolding profiles were observed following injection of protein diluted to a final concentration of 0.3 M GdnHCl (the final monomer concentration being 4.64 μM) after incubation for 0, 5, 15, 30, and 45 min at 20 °C. Four molecular species were detected with retention times of 56, 61, 66.4, and 71 min. The R_s values calculated according to the empirical equation of Uversky (*30*) are indicated.

Reactivation of the 3 M GdnHCl-denatured CK is far from complete. This could be due to the presence of at least two populations of protein. We therefore tried to obtain evidence supporting the existence of folding intermediates by size exclusion chromatography during CK renaturation.

Size Exclusion Chromatography (SEC). One can define the order of appearance of intermediate species as the protein folds from the random coil to the native structure by SEC experiments where the separation is based on differences in the hydrodynamic volume (*36*). The main drawback of the method is the length of the elution process as compared with the reactivation time course.

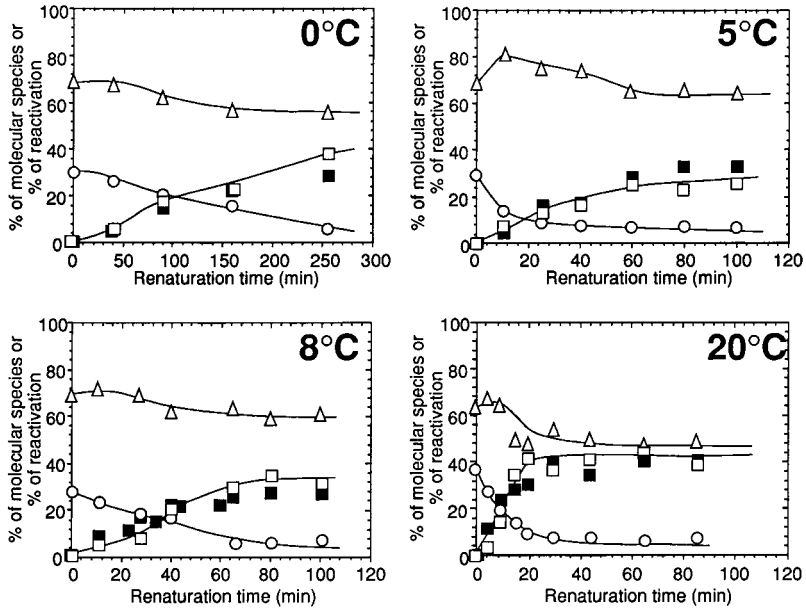


FIGURE 3: Influence of the incubation temperature on the percentage of CK folding species vs time, as determined by size exclusion chromatography. In all experiments, the CK final monomer concentration was 4.64 μM. The SEC column was equilibrated with 20 mM Tris/acetate (pH 7.4), 150 mM NaCl, and 0.3 M GdnHCl: (○) sum of the fractions of molecular species with retention times of 56 and 61 min (R_s = 5.6 and 4.6 nm, respectively), (□) fraction of the species with a retention time of 66.4 min (R_s = 3.8 nm), and (△) fraction of the species with a retention time of 71 min (R_s = 3.3 nm). Recovery of CK activity is also presented (■).

Elution profiles of native and GdnHCl-denatured enzyme are shown in Figure 2A. At various times, aliquots of the refolding mixtures were loaded onto the SEC column equilibrated in 20 mM Tris/acetate, 150 mM NaCl, and 0.3 M GdnHCl (pH 7.4). In all these experiments (Figure 2B), the CK monomeric concentration was 4.64 μM before injection onto the column, but was only about 0.05−0.10 μM in the eluted fractions. Immediately after dilution, two main peaks and a shoulder were observed which correspond to monomeric species exhibiting R_s's of 4.6, 3.3, and 5.6 nm, the least populated latter species being the unfolded monomer (Figure 2A and Table 1). After 5 min of refolding, another species appears with an R_s of 3.8 nm, identified with the CK dimer (Table 1). Its proportion increases with time (Figure 2B) at the expense of the three other intermediates.

Figure 3 represents the percentage of the various molecular species observed by SEC versus time at different temperatures. We have plotted the percentages of the species with R_s's of 4.6 and 5.6 nm together because their relative proportions are difficult to estimate. Whatever the refolding temperature, an almost complete disappearance of the species with a Stokes radii of 4.6−5.6 nm was observed, the percentage of the 3.3 nm form decreases to 50−60%, whereas that of the 3.8 nm species increases to 45−50%. The percentages of both the 3.3 and 3.8 nm species do not change for longer renaturation times.

Enzymatic activity, measured immediately after peak elution, shows that the 3.8 nm dimeric species is the only active form, whereas other chromatographically resolved populations are completely inactive. Plotting the abundance of the 3.8 nm dimeric population versus the recovery of enzymatic activity (Figure 3) demonstrates a close correlation between these two parameters, whatever the folding temperature. Thus, enzymatic activity recovery depends on the formation of the dimeric state. This conclusion is further strengthened by trypsin proteolysis experiments. Reactivation of the denatured enzyme was followed by adding trypsin to aliquots of the renaturing mixture so that any unfolded polypeptide chain in the sample would be rapidly degraded while folded dimeric molecules are not affected. The reactivation curves obtained in the presence or absence of trypsin can be superimposed (not shown), indicating that there is no accumulation of any active trypsin-sensitive intermediate.

Characterization of the Different Folding Species. SEC experiments were performed with a higher CK concentration (240 μM monomer) to elucidate the interconversion of the various species. The peaks corresponding to the different folding states were collected on ice, to slow the refolding process as much as possible, and then reinjected onto the SEC column. When the species with the retention time of 61 min was reinjected onto the SEC column, two peaks with retention times of 61 and 71 min were observed (Figure 4A). Whatever the renaturation length, reinjection of the 66.4 min dimeric species gave a single peak with an unchanged retention time, showing that this is the end product of the folding pathway (Figure 4B). Reinjection of the 71 min peak, collected from SEC runs performed with CK refolded for either 5 min or 12 h, gave different profiles. After renaturation for 5 min, the observed profile shows two peaks, one corresponding to the dimeric form and the second one being a 71 min peak (Figure 4C). For a longer renaturation (12 h), a single peak is obtained with the same retention time corresponding to a 3.3 nm monomeric species (Figure 4D). These latter observations clearly show that, under the 71 min peak, at least two species are present, one being able to fold into an active dimer and a second trapped one which is unable to dimerize.

The 71 min peak was characterized by a Stokes radius that was smaller than that of the native dimeric enzyme, suggesting a monomeric form. Analytical ultracentrifugation experiments were performed to support this hypothesis. The distribution function of the sedimentation coefficient obtained for the dimeric 0.3 M GdnHCl-treated CK shows one peak

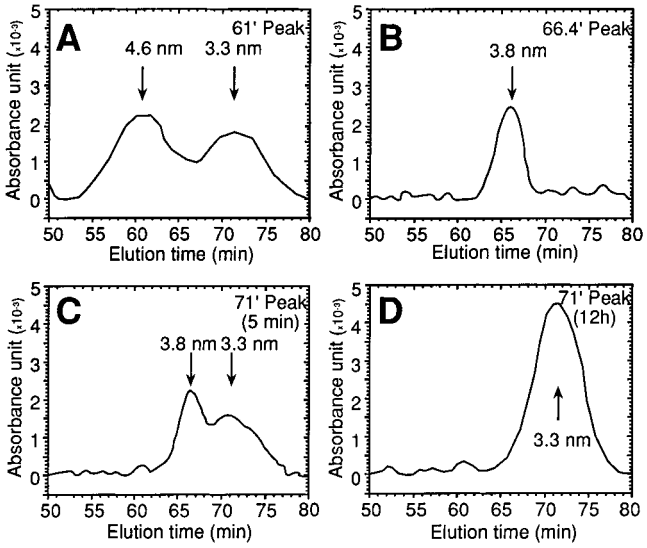


FIGURE 4: Reinjection onto the SEC column of the different molecular species observed during CK folding. The protein, denatured overnight at 4 °C in 3 M GdnHCl, was diluted 10-fold to a residual denaturant concentration of 0.3 M. After various times of renaturation, the refolding CK, at a final concentration of 240 μM, was injected onto a SEC column equilibrated as described in Materials and Methods. Before reinjection, the collected fractions were maintained on ice to slow, as much as possible, the refolding process. (A) Reinjection of the 61 min peak obtained after refolding for about 15 s, i.e., the time for manual mixing. (B) Reinjection of the 66.4 min peak regardless of the renaturation time. (C and D) Reinjection of the 71 min peak collected after refolding for 5 min or 12 h, respectively.

characterized by an s_w^{20} value of 5.5 S, whereas the 0.3 M GdnHCl-renatured CK (2.3 or 5.8 μM) shows two peaks, the first one overlapping the dimeric one and the second corresponding to a species with an s_w^{20} of 2.9 S. Combining theses values with the Stokes radii obtained from gel filtration of 3.8 and 3.3 nm in the Svedberg equation gave values for the molar masses of 89 and 41 kDa, respectively, which were thus close to that of a dimer and a monomer. Equilibrium sedimentation was also performed on the renaturated species at creatine kinase concentrations of 2.3 and 5.8 μM (data not shown). This leads to values for the weight-average molar mass of 56 kDa, which were well fitted considering a mixture of about 35% dimer and 65% monomer. The 71 min peak in Figure 2B corresponds thus to a monomeric species.

For each peak, intrinsic tryptophan fluorescence was analyzed immediately after collection using an excitation wavelength of 295 nm. The results are reported in Table 3 along with those of the intermediates detected during equilibrium denaturation. The λ_{max} of the 61 min peak (R_s = 4.6 nm) is 343 nm which, compared with the value of 352 nm measured for the unfolded state, implies the presence of some organized structure in the vicinity of tryptophan residues. As described above, the 71 min peak (R_s = 3.3 nm) contains at least two different species (panels C and D of Figure 4) which may exhibit different spectral properties with an average λ_{max} value of 338 nm. For longer renaturation, when only one stable species is present, the λ_{max} value is 336 nm. This characterizes a monomeric state with properties intermediate between those of the molten globule and a compact monomer, which should have an R_s of about 2.8−2.9 nm according to the SEC column calibration curve, log(R_s) versus log(MW) (*30*). Then, the λ_{max} of the 66.4 min species (R_s = 3.8 nm) is 333 nm, which is very similar to the native dimer 332 nm value.

Biophysical and Biochemical Changes during Creatine Kinase Refolding. Tryptophan Microenvironment. MM-CK possesses four tryptophan residues per monomer, all located in a restricted part of the protein sequence (positions 210, 217, 227, and 272). λ_{max} variation during renaturation is a multiphasic process (Figure 5A). After dilution, during the time required for manual mixing, a hypsochromic shift is observed from 352 nm (denatured CK) down to 341 nm, indicating the rapid masking of some tryptophan residues inside a hydrophobic structure. A second slower phase is observed up to 20 min, leading to a λ_{max} of 336−337 nm, and during the much slower third phase, the λ_{max} approaches 335 nm. These decays are consistent with slow rearrangements of the overall structure of the protein.

ANS Binding. ANS is a probe of accessible hydrophobic areas at the surface of a protein and weakly binds to unfolded chains. Immediately after the 10-fold dilution of the denatured protein, its fluorescence dramatically increases (Figure 5A). This increase reflects the transient formation of hydrophobic clusters leading to a molten globule state. After 1 min of refolding, the ANS fluorescence intensity decreases rapidly, reflecting ANS desorption from the protein. However, at about 8−12 min, it reproducibly increases, indicating a structural reorganization of a fraction of the monomeric species, leading to the reexposure of previously buried hydrophobic areas. ANS fluorescence then decreases, reaching a value close to that of the native species.

Titrable Cysteine Residues. The GdnHCl-unfolded CK exhibits four titrable thiol groups per monomer (positions 73, 145, 253, and 282). One sulfhydryl group per monomer is rapidly buried during the hydrophobic collapse; the number of accessible thiol groups then decreases slowly to one residue per monomer, as in the native enzyme (Figure 5B).

Tyrosine Exposure. At time zero, 100% of the tyrosine residues are exposed to the solvent (10 tyrosine residues per monomer). Then, at the outset of refolding, two or three tyrosines are rapidly buried (Figure 5B), but between 2 and 10 min, the extent of tyrosine exposure transiently increases, indicating a reorganization of the refolding intermediates. The extent of tyrosine exposure finally reaches a value of six to seven, close to that measured with native enzyme.

As shown above, CK refolding leads to a mixture of dimeric protein and nonproductive species. Thus, measured parameters (panels A and B of Figure 5) are a weighted mean of those of each species present at a given time. To estimate the biophysical properties of the trapped species as a function of time, experimental data were corrected to eliminate the contribution of the active dimer (panels C and D of Figure 5). Since the dimer is the only active species, the amount of active enzyme can be calculated according to eq 2:

$$A_t = [1 - (ae^{-k_1 t} + be^{-k_2 t})]A_\infty \qquad (2)$$

where the values of a, b, k_1, and k_2 at 20 °C are given in Table 2 and A_∞ is the maximal activity recovered in that particular experiment. The characteristics of the inactive misfolded species are calculated according to eq 3:

$$y = (P_t - A_t D_p)/(1 - A_t) \qquad (3)$$

Table 3: Biophysical Characteristics of the Main Species Identified by Size Exclusion Chromatography during CK Refolding Compared with Those Observed during Equilibrium Denaturation

species	kinetic refolding					equilibrium denaturation[c]			
						U	PMG	MG	D
retention time (min)	56	61	66.4	71*[b]	71**[b]	56	60	67.3	66.4
Stokes radius (nm)[a]	5.6	4.6	3.8	3.3	3.3	5.6	4.8	3.7	3.8
λ_{max} (nm)	352	343	333	338	336	352	345	341	332
activity	0	0	+++	0	0	0	0	0	+++

[a] Parameter calculated from the SEC column calibration curve (see Materials and Methods). [b] The peak at 71 min was collected (*) at the beginning of the refolding process (mixture of species with similar R_s but different λ_{max} values) or (**) 24 h after the enhancement of refolding (only one species is present). [c] U, unfolded state; PMG, pre-molten globule species; MG, monomeric molten globule state; D, dimeric form (*13*).

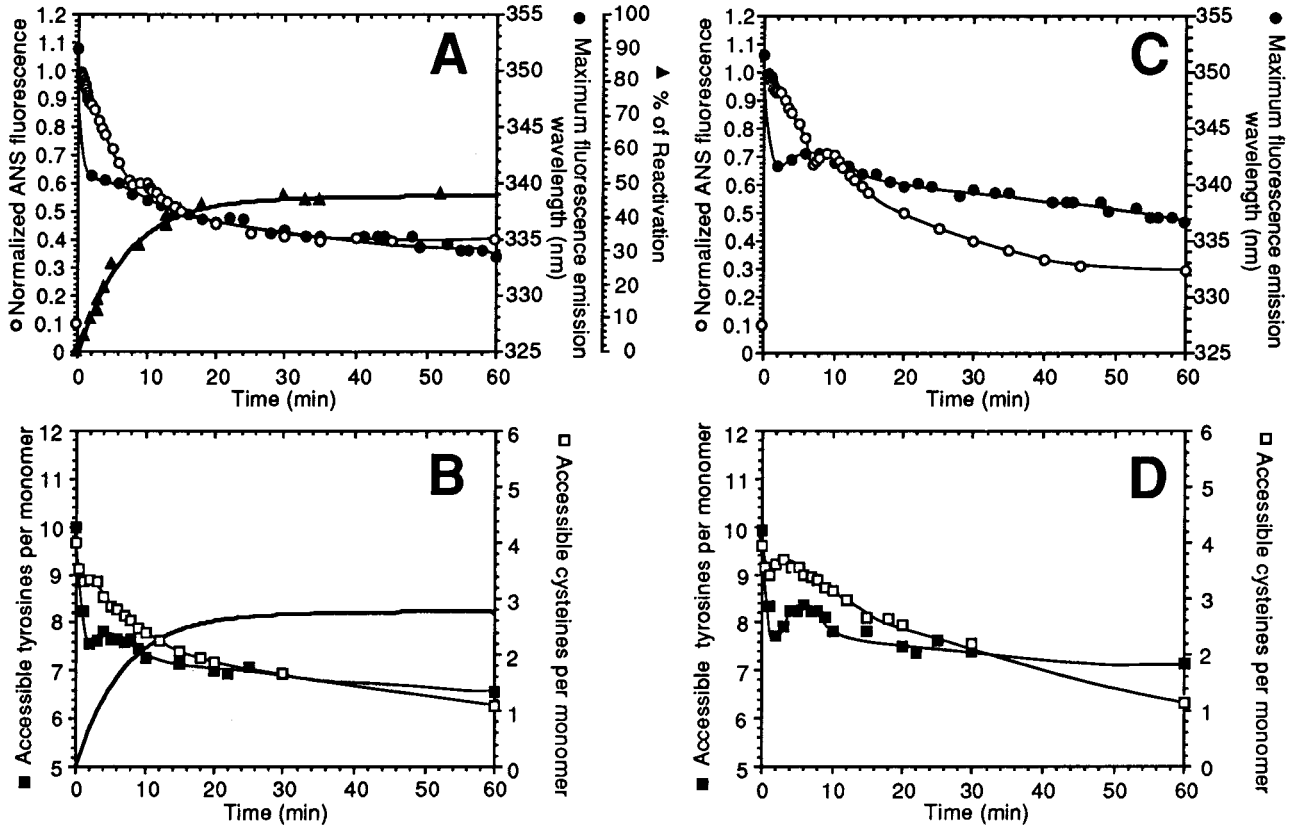


FIGURE 5: CK kinetic folding. (A) Maximum fluorescence emission wavelength measurements (●) and ANS binding (○). These experiments have been reproduced three to six times as described in Materials and Methods. (B) Thiol group (■) and tyrosine residues (□) accessibility. Each point is the average of at least 10 individual experiments as described in Materials and Methods. The enzyme concentration was identical in each experiment (4.64 μM in the monomer), and the folding temperature was 20 °C. The bold lines show the theoritical fit to the experimental data (▲) of CK reactivation. Panels C and D represent the same experiments corrected from the contribution of the native and active dimer according to eq 3.

where P_t is the measured parameter at time t, A_t the amount of native dimer at time t, and D_p the value of the parameters for the native dimer (one Cys and six Tyr accessible per monomer, $\lambda_{max} = 332$ nm, normalized ANS fluorescence = 0.4).

Those calculations were performed for cysteine and tyrosine accessibilities, ANS fluorescence, and λ_{max} (panels C and D of Figure 5). In each case, a significant reshuffling of the molecule is evidenced between 2 and 10 min, leading to an inactive stable misfolded monomeric species that is unable to dimerize. After 60 min of refolding, this relatively compact misfolded monomer ($R_s = 3.3$ nm) weakly binds ANS and is characterized by seven accessible tyrosine residues, one accessible thiol, and a λ_{max} of 336 nm.

Determination of the molar ellipticities of native or renatured CK in 0.3 M GdnHCl from the CD spectra shown in Figure 6 gives values of −12000 and −9550 deg cm^2 dmol^{-1}, respectively, at 222 nm. The ellipticity of the misfolded state, calculated according to eq 3 (−7450 deg cm^2 dmol^{-1}), is more negative than that measured with 1 M GdnHCl-denatured CK (−5500 deg cm^2 dmol^{-1}), indicating that the nonproductive species is more structured than the molten globule state.

Effect of the Denaturation Length on the Extent of Renaturation. Reactivation experiments were performed at 20 °C with CK samples denatured in either 1 M GdnHCl (accumulation of inactive monomeric intermediates exhibiting some properties of the molten globule state) or 3 M GdnHCl (accumulation of a denatured form), after various denaturation periods (Figure 7). After denaturation for 1 h, activity is fully recovered with monoexponential kinetics for the enzyme denatured in 1 M GdnHCl ($k = 0.198$ min^{-1}),

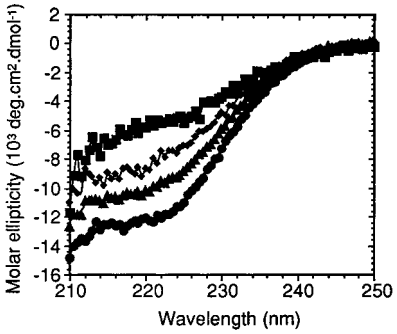


FIGURE 6: Far-UV circular dichroism spectra of 2.3 mM native CK or 0.3 M GdnHCl-treated CK in 20 mM Tris/acetate and 0.1 mM EDTA (pH 7.4) (●), CK incubated overnight in 1 M GdnHCl (■), and CK incubated for 12 h in 3 M GdnHCl and diluted to 0.3 M 12 h before measurements (▲). The spectrum of the misfolded species has been calculated according to eq 3 (◆).

whereas only 80% of the activity is restored through a biexponential process for the 3 M GdnHCl-denatured CK (Figure 7A). In contrast, after denaturation for 12 h and whatever the denaturant concentration, reactivation kinetics are superimposable. Figure 7B shows that renaturation of the GdnHCl-denatured protein depends on the duration of exposure to guanidine. Renaturation after short periods (up to 90 min) of exposure to 1 M GdnHCl allowed full reactivation, while longer exposures decreased the yield to 45%. In contrast, with the 3 M GdnHCl-denatured protein, the yield decreases progressively to the value observed with 1 M GdnHCl.

DISCUSSION

It has been reported that the reactivation yield of GdnHCl-denatured CK was limited by the residual denaturant concentration (*23*). Since low concentrations have no or a limited effect on the enzyme structure, we investigated the refolding pathway of GdnHCl-denatured CK with a residual denaturant concentration of 0.3 M.

Time Course of Formation of Native Molecules during Refolding. Under the conditions used, a partial reactivation of CK is observed which is independent of the dimer concentration in the range of 0.58−5.8 μM. The absence of concentration dependence of reactivation rate and yield agrees with previous results (*23*) and indicates that dimerization is not the rate-limiting step of refolding. The reactivation kinetics are well fitted to the sum of two exponentials (eq 1). This biphasic process is consistent with the presence, at time zero, of a mixture of at least two different populations (fast and slow folding molecules) that refold via parallel pathways. The reactivation yield and rate both depend on the length of exposure to the denaturant; the longer the exposure to GdnHCl, the lower the yield and rate of reactivation. These data could be consistent with the involvement of cis−trans isomerization of X−proline bonds (*37*).

When CK is refolded in 0.3 M GdnHCl, only 45% of the activity is recovered. Previous urea refolding studies determined that reactivation is also a biphasic process which depends on DTT concentration and length of exposure to urea but not on protein concentration. The fast phase rate constant is similar to the value that we obtained in GdnHCl (*18*, *20*). The dissimilarity of the reactivation yields can be explained by the different experimental conditions and different modes of interaction of these denaturants with proteins, leading to different unfolded states.

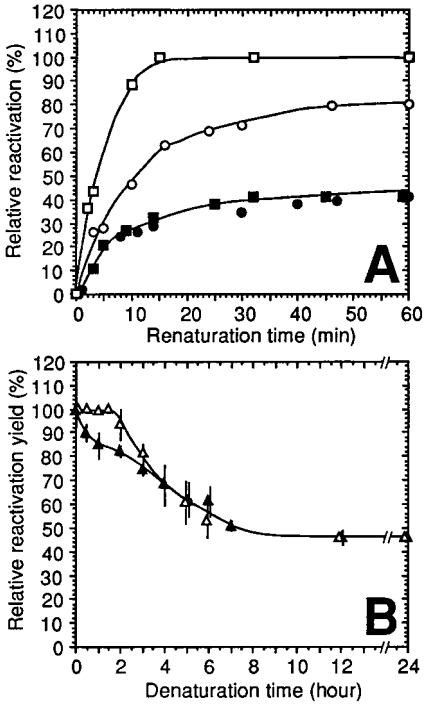


FIGURE 7: Comparison of the reactivation of CK from the molten globule or the denatured states. (A) Time course of activity recovery. CK was denatured for 1 (white symbols) or 12 h (black symbols) in either 1 (□ and ■) or 3 M GdnHCl (○ and ●). Refolding at 20 °C was induced by dilution of the denatured samples to a final GdnHCl concentration of 0.3 M and a final subunit concentration of 4.64 μM. (B) Effect of length of exposure to either 1 (△) or 3 M (▲) GdnHCl denaturing buffer. Each point corresponds to the relative reactivation yield after refolding for 60 min in 0.3 M GdnHCl at 20 °C (4.64 μM monomer).

In contrast with data reported with urea-denatured protein, a strong correlation between the recovery of the native dimeric structure and that of the activity is demonstrated by SEC and trypsin proteolysis experiments. This indicates that the dimeric form is the only structure expressing enzymatic activity and confirms our previous conclusions pointing out that final conformational stability is ensured through quaternary interactions (*38*).

As only 45% of the activity is recovered, competing side reactions occur which could lead to accumulation of either inactive misfolded species or protein aggregates. Protein aggregation is frequently a major side reaction of protein folding and could involve intermolecular disulfide bond formation (*18*, *24*, *39*). However, under our experimental conditions, DTT has no effect on the refolding yield (provided EDTA is present) and there is no evidence for the formation of aggregates during the refolding process. Furthermore, a kinetic competition between folding and aggregation is unlikely since reactivation is not protein concentration dependent (*14*). The 55% inactive species corresponds thus to soluble but misfolded molecules.

Sequential Mechanism of Refolding. Detection of Fast Folding Molecules. The temperature dependence of CK reactivation demonstrates that hydrophobic interactions play an important role in the rate-limiting step. CK reactivation is slowed at low temperatures; the slow phase of reactivation

(k_2) is slightly less temperature dependent than the fast phase (k_1). The downward curvature of the Arrhenius plot for the fast phase indicates that changes in heat capacity occur during the refolding process. These changes are consistent with the burial of a considerable amount of hydrophobic surface between the unfolded ground state and the folding transition state, indicating that some of the activation energy could be required for disruption of water organized around such surfaces prior to burial (*35*, *40*).

Three well-resolved stages can be distinguished in the course of CK refolding. The first one, immediately after manual mixing, is evidenced by the large increase in ANS fluorescence and the large λ_{max} blue shift (352 to 341 nm), which demonstrate the hydrophobic changes occurring in the environment of tryptophan residues. This fast contraction of the polypeptidic chain in hydrophobic clusters reflects a collapsed state appearance. The second stage, characterized by intramolecular rearrangements and desolvation of hydrophobic clusters (λ_{max} fluorescence decay and release of bound ANS), provides evidence for the conversion of the collapsed state into a more organized structure. This step is followed by the rapid reassociation of the subunits, leading to enzymatic activity recovery.

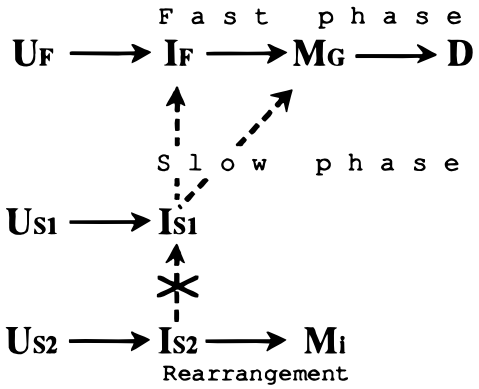
With respect to the high sequence homology between cytosolic and mitochondrial isoenzymes (*41*), the CK monomer can be considered a protein with two domains: an N-terminal one (residues 1−112) and a second C-terminal domain (residues 113−380) (*42*). The rapid λ_{max} decay detected during the hydrophobic collapse indicates that the restricted C-terminal area containing the tryptophan residues refolds rapidly. Kinetic measurements of cysteine masking showed that one cysteine per monomer is rapidly buried during the collapse, two others are slowly hidden during reorganization, and the fourth one remains accessible in the final state. As the only accessible cysteine is Cys 282, one can assume that the rapidly masked one is Cys 253, localized in the tryptophan area of the collapsed C-terminal domain. Thus, Cys 145 and 73 could be the slowly buried residues. This pattern agrees with our previous findings indicating that Cys 253 is the least accessible cysteine residue during salt unfolding of CK (*43*). Kinetic masking of the tyrosine residues (more evenly distributed over the N-terminal part of the polypeptidic chain) shows that two or three of them are rapidly buried, with the six or seven other Tyr residues remaining accessible. In accordance with other studies using antibodies, proteolysis, and protein engineering (*44*−*48*), these results indicate that the C-terminal part of the protein rapidly collapses and is the first well-packed domain that appears during refolding, slow changes occurring essentially in the N-terminal domain.

Detection and Characterization of the Kinetic Intermediates of Refolding. Size exclusion chromatography experiments have been carried out to detect the different intermediates involved in the refolding process. Immediately after initiation of CK folding, two molecular species, different from the denatured form (R_s = 5.6 nm), are recovered (Table 3). The first one exhibits a retention time corresponding to a protein with an R_s of 4.6 nm and a measured λ_{max} of 343 nm. This collapsed state, more organized than the unfolded monomer (Table 1), could correspond to the pre-molten globule state detected at equilibrium in GdnHCl (*13*). It can be supposed that this intermediate state has a condensed or partially condensed C-terminal domain and a less folded N-terminal domain.

This intermediate is then transformed into a second more compact species whose characteristics are very close to those of the molten globule state observed at equilibrium in GdnHCl (R_s = 3.3 nm and λ_{max} = 339 nm) (*19*, *24*). Only 45% of this transient monomeric state has a properly folded dimerization site and can dimerize to the active dimeric form. No peak or shoulder, indicating the presence of a compact folded monomer (R_s = 2.8 nm), can be observed, indicating that the dimerization step is a rapid process. The remaining population of molecules consists of a long-lived intermediate which becomes kinetically trapped in a soluble misfolded monomeric state lacking the necessary structural elements for dimer formation.

Trapped Intermediate. Local structures formed in the early stages of refolding are thought to play a pivotal role in orienting its folding pathway (*49*). When corrected for the accumulation of the dimeric state (panels C and D of Figure 5), our results show that significant re-exposition of hydrophobic areas, tyrosine and cysteine residues, occurs, making this the yield-limiting step in attaining the native conformation. Since λ_{max} is only slightly affected, it can be postulated that the reshuffling occurs in the partially folded N-terminal part of the monomer [domain containing the dimerization site (*42*, *50*)]. Then, the contact surface required to form the dimerization site could be hindered either by domain swapping (*51*) or by interface reshuffling to protect exposed hydrophobic areas from the solvent and give a thermodynamically more stable monomeric but misfolded state. This inactive trapped species is more structured and more compact than the equilibrium molten globule state but less than a theoretical compact monomer (*13*). Rearrangement of this species, preventing a normal dimerization, could be the consequence of the presence of incorrect X−Pro peptide bonds (see discussion below).

Creatine Kinase Refolding Pathway. The following scheme is a tentative interpretation of the CK folding pathway in 0.3 M residual GdnHCl:



where U_F and U_{S1} (R_s = 5.6 nm) are the fast and slow refolding isomers of the unfolded monomer, respectively, I_F and I_{S1} (R_s = 4.6 nm) are the fast and slow folding isomers of the early intermediate, respectively (monomeric inactive form with pre-molten globule state features), and M_G is the association-competent molten globule-like monomer with a correct subunit interface leading to the native and enzymatically active dimer D (R_s = 3.8 nm). U_{S1} and I_{S2} are the unfolded and intermediate precursors, respectively, of a

nonproductive misfolded monomeric species M_i (R_s = 3.3 nm). The distribution between U_F and U_S states does not depend on protein concentration or temperature of refolding but only on the length of exposure to GdnHCl. U_F and U_{S1} (about 45% of the overnight-denatured state) are converted at different rates (k_1 and k_2, respectively) and in a temperature-dependent manner to a dimer which is the only active state, while U_{S2} (55%) misfolds into a trapped monomer that limits the refolding yield. The collapsed intermediate I_{S2} is probably very similar to I_{S1} and I_F in structure but is less stable; a reshuffling process, possibly in its N-terminal part, prevents the correct tertiary packing of the side chains and the formation of a normal subunit interface eventually leading to M_i. It has been observed that wrong domain interactions cause partial irreversibility of the GdnHCl denaturation (*52*) and, more recently, that some specific sequences can be stabilized by GdnHCl (*53*).

Possible Involvement of Proline Isomerization. For several proteins, the presence of partially folded species or multiphasic reactivation curves have been explained by isomerization of X−Pro peptide bonds in the unfolded states (*37*, *54*, *55*). The possible involvement of proline isomerization in CK refolding has already been suggested (*20*−*22*). Proteins for which proline isomerization is known to be a rate-limiting step show activation energy values in the range of 16−20 kcal/mol (*37*). The activation energy value of the CK slow reactivation phase (8.6 kcal/mol) is low and suggests that this phase does not correspond to a simple proline isomerization. However, as suggested by Jackson and Fersht (*40*), such a low value could be observed when the isomerization takes place in a partially structured state such as the collapsed intermediate.

The decrease in reactivation yield requires longer incubation in 1 M GdnHCl than in 3 M GdnHCl, consistent with the fact that proline isomerization is more difficult in a partially structured state than in a less structured one and could preferentially take place in flexible regions rather than in highly organized areas. The U_F form would retain the native-like X−proline peptide bond configuration, while U_S isomers would contain the non-native nonessential (U_{S1}) or essential (U_{S2}) proline residues. We have performed preliminary reactivation experiments with proline isomerase which show no significant improvement of the reactivation yield and rate. This cannot exclude the possibility that incorrect X−Pro bonds are rapidly buried during refolding and become inaccessible to proline isomerase active site. Proline mutants should provide more information about the implication of these residues in the refolding process.

This work shows that, unlike what has been shown for urea-denatured CK, renaturation of GdnHCl-denatured enzyme occurs through distinct intermediate states that resemble those which have been described during equilibrium denaturation. Formation of a nonproductive trapped species is a new example of a misfolded kinetic intermediate that hampers direct folding of a protein to its native state (*15*, *49*, *56*−*58*). It remains unclear why the residual GdnHCl concentration-dependent divergence of the productive and nonproductive renaturation pathways is an early event, whereas 0.3 M GdnHCl has no measurable effect on the native protein. One can hypothetize that transient GdnHCl stabilization of a part of the structure leads to a nondimerizable intermediate.

ACKNOWLEDGMENT

We are grateful to E. Quéméneur (CEA-Saclay) and F. Cretin (Université de Grenoble, Grenoble, France) for the gift of human recombinant proline isomerase.

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BI981828P