Role of C-Terminal Sequences in the Folding of Muscle Creatine Kinase

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ABSTRACT: Proteinase K selectively nicks the native homodimeric muscle creatine kinase (MM-CK) into two 37.1 kDa N-terminal (K1) and two 5.8 kDa C-terminal (K2) fragments that remain firmly associated in a nativelike, although inactive, heterotetrameric structure. This truncated protein has been named $(K1K2)_2$. To analyze the role of the C-terminal peptide in the protein structure acquisition, we studied in vitro refolding of the guanidinium chloride-denatured $(K1K2)_2$. Although they never reassociate with K2, in selected conditions the K1 fragments refold slowly to a dimeric state as shown by size exclusion chromatography data. This K1 dimer exhibits a fluorescence emission λmax of 335 nm, a high degree of tyrosine exposure, strongly binds ANS but not MgADP, a CK substrate, and according to these structural characteristics, could be a dimeric molten globule species. We propose a folding model that takes into account the existence of a new transient intermediate state in the MM-CK refolding process. Besides two monomeric premolten and molten globule kinetic intermediates and the active final dimeric form, an inactive dimer, with partly compacted monomers, must ephemerally exist. Our results strongly suggest that the C-terminal end of the protein accelerates folding and plays a critical role for monomer final packing into a nativelike conformation. The data also indicate that MM-CK catalytic efficiency is only acquired after dimerization.

Guanidino kinases form a highly conserved family of enzymes that catalyze the reversible transfer of the ATP γ-phosphate onto their guanidino substrate. Creatine kinase (CK[1], E. C. 2.7.3.2), the best-studied member of this family, is tissue-specifically expressed as three cytosolic MM, BB, and MB dimers made up of muscle- and/or brain-type 380-amino acid subunits, and as two ubiquitous and sarcomeric mitochondrial isoforms (*1*, *2*). These mitochondrial creatine kinases can exist and be active either as dimers or as membrane-binding octamers (*3*, *4*). Quaternary structure of creatine kinases has been shown to play a role in protein stability and to allow expression of the catalytic activity (*5*−*8*), although conflicting reports exist on that matter (*9*, *10*). For other phosphagen kinases, the arginine kinases, some isoenzymes exhibit activity as monomers, whereas others are active under a dimeric state (*11*).

Previous reports have shown that CK folding occurs through distinct intermediate states (*12*−*14*) of which the molten globule, a species with near native circular dichroism spectrum but fluctuating tertiary structure, is the most studied. It has also been shown that during refolding, the C-terminal part of the protein folds, while the N-terminal one is still loosely packed (*12*, *15*−*19*).

Limited CK proteolysis experiments have been described and interestingly, proteinase K specifically cleaves each M monomer of MM-CK (as well as other CK isoenzymes) at a discrete site of its C-terminal part. The cleavage does not lead to a dissociation of the proteolytic fragments but is accompanied by a total inactivation of the enzyme (*20*−*25*). The resulting protein is a heterotetramer we named $(K1K2)_2$, with K1 (37.1 kDa) being the N-terminal peptide and K2 (5.8 kDa) the C-terminal part of the M monomer (*26*). Except for the absence of activity for the $(K1K2)_2$ protein and a slight modification of its binding site for MgATP (*27*), no significant structural difference between the cleaved and uncleaved proteins has been measured, whereas denaturation experiments of the proteolyzed protein showed that the two K1 and K2 peptides are not independent unfolding entities (*28*).

The following work is a multiparameter equilibrium and kinetic study of GdmHCl-denatured $(K1K2)_2$ refolding aimed at identifying and characterizing the renaturation events and at comparing them with those occurring during folding of uncleaved MM-CK to get information about the role of the C-terminal end of the CK monomer in the enzyme folding mechanism.

EXPERIMENTAL PROCEDURES

Chemicals. BB-creatine kinase, TCEP, ADP, and creatine were purchased from Sigma. TRIS, EDTA, DTT, ATP, and MM-creatine kinase (MM-CK) from rabbit muscle were

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[1] ANS, 1-anilino-8-naphthalenesulfonate; BB-CK, cytosolic dimeric creatine kinase BB; DTT, dithiothreitol; GdmHCl, guanidinium hydrochloride; Mib-CK, mitochondrial creatine kinase; MM-CK, cytosolic dimeric creatine kinase MM; Rs, Stokes radius; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TCEP, tris(2-carboxyethyl)phosphine; Vs, Stokes volume.

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obtained from Roche. Proteinase K and GdmHCl were from Amresco.

Protein Purification. Before use, MM-CK was passed through a PD10 Sephadex G25 (Pharmacia) column equilibrated with 20 mM TRIS−AcOH, 0.1 mM EDTA, pH 7.4. Its purity was determined by SDS−polyacrylamide gel (12.5%) electrophoresis (*29*). Protein concentrations were estimated as described earlier (*30*) or using a molar extinction coefficient of 76000 cm^{-1} M^{-1} at 280 nm for dimeric MM-CK.

Creatine Kinase Activity. MM-CK activity was determined using a pH-stat method (*31*). Enzyme activity (monomer creatine kinase concentration about 4 μM) was measured at 30 °C and pH 8.8, using 4 mM MgATP and 40 mM creatine as substrates. Samples treated with GdmHCl were diluted at least 100-fold into the assay mixtures for determination of MM-CK activity. The residual denaturant was found not to affect the activity significantly (*32*).

Creatine Kinase Cleavage by Proteinase K. Cleavage was done as described earlier (*28*) and checked by high-resolution SDS−PAGE (*33*).

Renaturation Experiments. Samples, containing 80 μM (monomer concentration) of MM-CK or $(K1K2)_2$, were denaturated by incubation at 4 °C for 19 h with a 20 mM TRIS−AcOH buffer, pH 7.4, containing 100 μM EDTA, 3.5 mM DTT, and 4 M GdmHCl. The biophysical parameters we measured indicate no residual structure in both denatured proteins (*28*, *32*). Protein folding was induced by dilution of these samples in a 20 mM TRIS−AcOH buffer, pH 7.4 containing 100 μM EDTA, 5 mM DTT and various amounts of GdmHCl (final peptide concentration of 4 μM).

Intrinsic Fluorescence Measurements. Protein intrinsic fluorescence and iodide tryptophan fluorescence quenching measurements were carried out on a Hitachi F-4500 spectrofluorometer as previously described (*12*) with a 4 μM final peptide concentration.

MgADP Affinity. The affinity for the adenylic substrate MgADP was determined spectrofluorometrically as previously described (*32*), using AMP as an inner filter standard.

ANS Binding Measurements. ANS (310 μM final concentration) was added to the protein samples and ANS fluorescence was recorded at 495 nm with an excitation wavelength of 395 nm. All spectra were corrected for free ANS fluorescence.

Size-Exclusion Chromatography. Measurements were done using a size-exclusion Pharmacia Superdex 200 HR column (fractionation range 10−600 kDa), and the data were collected using a Waters diode array detector. The void and total volumes of the column were determined with Blue Dextran 2000 (8.5 mL) and ADP (21.3 mL), respectively, and it was calibrated with globular proteins of known Stokes radius (*34*): ferritin (450 kDa, Rs = 6.3 nm), catalase (240 kDa, Rs = 5.2 nm), aldolase (158 kDa, Rs = 4.7 nm), bovine serum albumin (68 kDa, Rs = 3.5 nm), ovalbumin (45 kDa, Rs = 2.8 nm), chymotrypsinogen (25 kDa, Rs = 2.09 nm), and cytochrome *c* (12.5 kDa, Rs = 1.7 nm) according to eq 1:

$$\log \mathrm{Rs} = 1.7413 - 0.0831 \times \mathrm{Vel} \quad (1)$$

where Rs is the Stokes radius of the molecule (nm), and Vel is its retention volume in milliliters (flow rate in the SEC column is 0.25 mL/min). The relationship between Stokes radii and molecular masses was obtained using eq 2:

$$\log \mathrm{Rs} = -0.1823 + 0.3788 \times \log(\text{molecular mass}) \quad (2)$$

where the molecular mass is expressed in kilodaltons.

Second Derivative Absorption Spectra. To estimate the degree of tyrosine exposure, second derivative absorption spectra at 270−300 nm were recorded at 20 °C using an Agilent 8453 spectrophotometer with 4 μM protein monomers in 20 mM TRIS−AcOH, 0.1 mM EDTA, pH 7.4, 2.5 mM TCEP, containing various amounts of GdmHCl (*35*−*36*). TCEP was used in both denaturation and renaturation buffers instead of DTT because it does not absorb in the UV range.

Cellulose Acetate Gel Electrophoresis. Active CK hybrids were identified by cellulose acetate gel electrophoresis as previously described (*3*). BB-CK (10 μg) was mixed with MM-CK or refolded samples (10 μg) in a final volume of 20 μL and submitted to four freezing-thawing cycles (one cycle: 20 min at −20 °C and 10 min at 30 °C).

K1 Purification. The nicked CK at 4.6 μM in a 10 mM TRIS−AcOH buffer pH 7.4 was exposed to increasing temperatures in a thermostatized UVikon 510 spectrophotometer cuvette. Medium turbidity changes were measured at 488 nm. Heated samples were then sedimented at 10 000*g* for 10 min, and the contents of both pellet and supernatant were checked on high-resolution SDS−PAGE (*33*). Over 58 °C, K1 fragments were found in the pellet, whereas K2 peptides were still soluble in the supernatant. K1 pellets were then solubilized for 19 h at 4 °C in a 10 mM TRIS−AcOH buffer pH 7.4 containing 3 M GdmHCl and 5 mM DTT. K1 refolding was induced by a 10-fold dilution in the same buffer without GdmHCl and allowed to refold at room temperature for 24 h.

RESULTS

Uncleaved and Proteinase K-Nicked MM-Creatine Kinase Refolding in Equilibrium Conditions. (a) Reactivation of GdmHCl Denatured MM-CK. Enzymatic activity is the most sensitive tool that can be used to monitor perturbations of CK structure (*32*). As described earlier (*28*, *32*, *37*), MM-CK incubated for 19 h at 4 °C with various concentrations of GdmHCl loses its activity above 0.4 M denaturant (Figure 1) and is fully inactivated above 0.9−1 M GdmHCl. To measure enzyme reactivation, MM-CK previously incubated at a monomer concentration of 80 μM for 19 h and at 4 °C with 4 M GdmHCl, was diluted to lower denaturant concentrations at a final monomer concentration of 4 μM and allowed to refold at room temperature for 24 h. Provided that reducing conditions are maintained, a significant CK activity is restored below 0.6 M denaturant and is maximum for a GdmHCl concentration of 0.2 M.

(b) Refolding Monitored by Fluorescence λmax Shift, Tryptophan Fluorescence Quenching by Iodide and ANS Binding. MM-CK or $(K1K2)_2$ were first denatured in 4 M GdmHCl, subsequently diluted to various residual denaturant concentrations (4−0.2 M for intact MM-CK and 4−0.3 M for the nicked enzyme), and allowed to refold for 24 h. In these denaturing conditions, the K1 and K2 peptides are dissociated and denatured (*28*). Both native proteins have a fluorescence emission λmax of about 332 nm when excited at 295 nm,

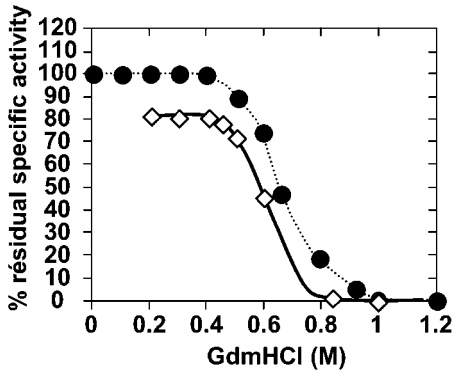

FIGURE 1: MM-CK activity measurements during equilibrium denaturation and renaturation. Activity measurements were performed using the pHstat method. The inactivation curve (black symbols) is obtained after incubation of MM-CK for 24 h at a subunit concentration of 4 μM in various concentrations of GdmHCl in 20 mM TRIS−AcOH, 100 μM EDTA, and 5 mM DTT, and pH 7.4 at room temperature. The reactivation curve (open symbols) is obtained by dilution to lower GdmHCl concentrations of the enzyme previously incubated in 4 M GdmHCl for 19 h at 4 °C in the presence of 3.5 mM DTT. Before activity measurement, the diluted protein (final subunit concentration of 4 μM) is allowed to refold at room temperature for 24 h in the above mentioned buffer.

indicating that most of the tryptophan residues are buried inside the protein structure (all four tryptophan residues are located in the K1 part of the monomer). To detect changes in side chains exposition, we monitored fluorescence emission λmax variations of equilibrium refolded, cleaved, and uncleaved CK in 0.2−0.3 to 4 M residual GdmHCl with an excitation wavelength of 295 nm (Figure 2A). As evidenced by 90°-light scattering measurements, with the nicked enzyme a protein precipitate prohibiting fluorescence measurements is observed with residual GdmHCl concentrations lower than 0.3 M. This does not occur with the uncleaved MM-CK and at 0.3 M or above for the cleaved enzyme.

At 4 M GdmHCl, both proteins show a λmax of 352 nm, which decreases below 2.5 M GdmHCl. A shoulder around 344 nm is observed for both proteins around 1.5 M denaturant indicating the presence of a stable intermediate as observed during denaturation of the two proteins (*28*). At lower denaturant concentrations, another shoulder is observed around 0.9 M GdmHCl, which is the sign of the molten globule intermediate presence (*38*). Below 0.8 M GdmHCl, the λmax of the MM-CK decreases and reaches 333 nm, a value close to the native protein one, whereas the $(K1K2)_2$ fluorescence emission λmax does not decrease below 335 nm at 0.3 M GdmHCl.

The Stern−Volmer quenching constant (Ksv) has been measured as described earlier (Figure 2B) using sodium iodide (*39*). In 4 M GdmHCl, Ksv is 4.9 M^{-1} for both proteins. For MM-CK, the Ksv value decreases with lower GdmHCl concentrations, and two shoulders are observed around 1.5 and 1 M GdmHCl. At 0.3 M denaturant, the Ksv value (0.7 M^{-1}) for refolded MM-CK is rather similar to the native MM-CK one. For the nicked protein, the Ksv value decreases in the same way as the MM-CK one, with a shoulder around 1.5 M GdmHCl, and remains stable below 1 M. At the lowest residual denaturant concentrations, the measured Ksv value indicates a higher iodide accessibility of tryptophan residues in the refolded cleaved protein as compared to the uncleaved one.

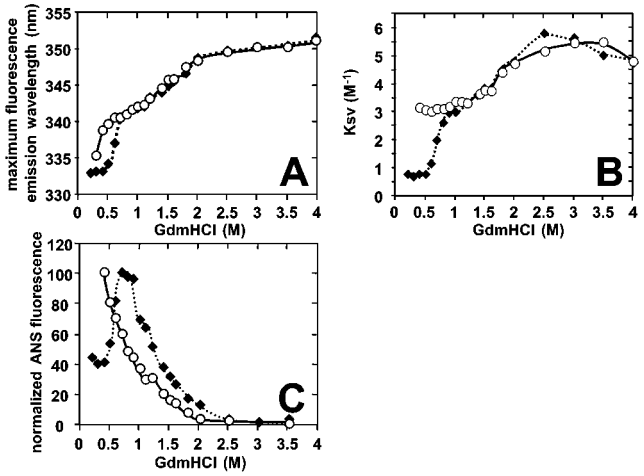

FIGURE 2: Equilibrium refolding of MM-CK (black diamonds) and nicked CK (open circles) monitored using spectroscopic techniques. The proteins, previously incubated for 19 h at 4 °C in 20 mM TRIS−AcOH, 100 μM EDTA, 3.5 mM DTT, pH 7.4 containing 4 M GdmHCl, were subsequently diluted to lower GdmHCl concentrations and allowed to refold at room temperature for 24 h. The final subunit concentrations were 4 μM in both cases. Panel A: The maximum fluorescence emission wavelength was determined using an excitation wavelength of 295 nm to selectively excite tryptophan residues. For each residual GdmHCl concentration, a blank was made to correct the protein fluorescence emission spectrum. Panel B: For each GdmHCl residual concentration, aliquots of a 5 M sodium iodide solution were added to a spectrofluorimeter cuvette containing equilibrium refolded proteins. The Stern−Volmer quenching constants (Ksv) for iodide were determined using an excitation wavelength of 295 nm, and the fluorescence values were measured at the protein maximum emission wavelength. Panel C: ANS binding measurements. Aliquots of a 25 mM methanolic ANS solution were added to the spectrofluorimeter cuvette (final ANS concentration of 310 μM). Samples were excited at 395 nm and the ANS fluorescence intensities were recorded at 495 nm. The values were corrected from the fluorescence of ANS alone.

We measured the binding of the fluorescent hydrophobic probe ANS to partially refolded MM-CK and $(K1K2)_2$. For MM-CK, the ANS binding pattern is very similar to the one observed in equilibrium denaturing conditions (*28*, *32*, *37*). ANS fluorescence emission is low at high denaturant concentrations and increases with lower GdmHCl concentrations (Figure 2C). An ANS fluorescence emission peak is observed around 0.7−0.9 M GdmHCl, which decreases below 0.6 M denaturant. For the cleaved protein, ANS binding increases below 2 M GdmHCl, and a shoulder is also observed around 1.1 M. The maximum ANS fluorescence is measured at 0.3 M GdmHCl showing that the refolded nicked protein exhibits larger than normal accessible hydrophobic areas.

(c) Intermediate States Characterized by Size-Exclusion Chromatography. To separate and identify folding intermediates, samples of cleaved and uncleaved CK previously denatured in 4 M GdmHCl and subsequently refolded with residual denaturant concentrations ranging from 0.2 (0.3 M for the nicked CK) to 1.5 M were loaded onto a size-exclusion Superdex column equilibrated with the same denaturant concentrations. Stokes radii of the different species resolved were estimated using eq 1.

Typical elution profiles for uncleaved CK and nicked CK refolded 24 h in 0.3 M GdmHCl are shown in Figure 3. The refolded uncleaved CK exhibits a single peak with a

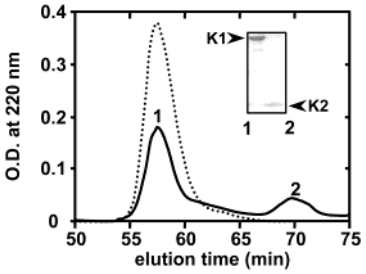

FIGURE 3: Size-exclusion chromatography elution profiles of intact (dashed line) and nicked CK (continuous line) refolded at equilibrium in 0.3 M residual GdmHCl. The two proteins were previously incubated for 19 h at 4 °C in 20 mM TRIS−AcOH, 100 μM EDTA, 3.5 mM DTT, pH 7.4, containing 4 M GdmHCl, then diluted to a final concentration of 0.3 M GdmHCl and allowed to refold at room temperature for 24 h at a final subunit concentration of 4 μM. They were injected onto a Superdex size-exclusion chromatography column equilibrated in 20 mM TRIS−AcOH, 150 mM NaCl, 0.3 M GdmHCl, pH 7.4. In both cases, 60 μg of protein was injected and the flow rate was 0.25 mL/min. The two peaks obtained after refolded nicked CK injection were collected, desalted, and then loaded onto a SDS−polyacrylamide 16.5% gel electrophoresis and stained with Coomassie blue (inset).

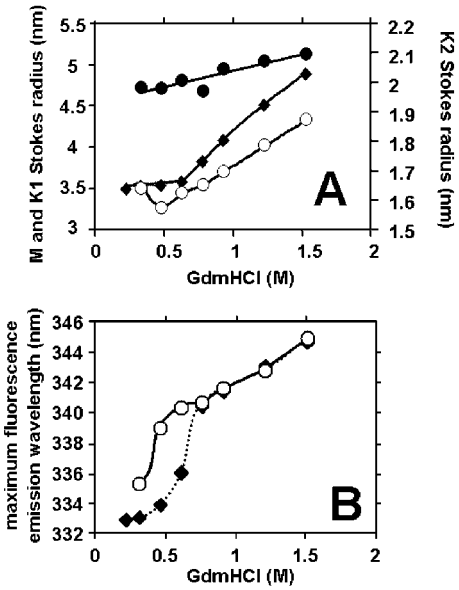

FIGURE 4: Size-exclusion chromatography of nicked (K1 and K2: open and black circles, respectively) and uncleaved CK (black diamonds) refolded at equilibrium. The two proteins were previously incubated for 19 h at 4 °C in a 20 mM TRIS−AcOH, 100 μM EDTA, 3.5 mM DTT, pH 7.4 buffer containing 4 M GdmHCl, and then diluted to lower GdmHCl concentrations and allowed to refold at room temperature for 24 h in the presence of 5 mM DTT. The final subunit concentration was 4 μM. Panel A: Aliquots of these samples were injected onto a Superdex column equilibrated in 20 mM TRIS−AcOH, 150 mM NaCl, pH 7.4 containing various GdmHCl concentrations. The flow rate was 0.25 mL/min. A total of 60 μg of protein was injected in both cases. The Rs estimation was done according to eq 1 (see Experimental Procedures). Panel B: K1 protein peaks were collected for each GdmHCl residual concentration, and their maximum fluorescence emission wavelength was determined.

calculated Stokes radius of 3.54 nm. For the nicked enzyme, two peaks are observed. SDS−PAGE analysis shows that the second one (Rs 2 nm) corresponds to the K2 peptide and that the first peak (Rs 3.57 nm) contains only K1 peptides (see Figure 3 inset).

Figure 4A shows Rs variations of the different species, renatured for 24 h at room temperature, as a function of residual denaturant concentrations. Between 1.5 and 0.3 M, uncleaved CK acquires a more and more compact structure as demonstrated by the decrease of its Rs from 4.9 to 3.5 nm. However, the dimerization step is not visible using these SEC conditions indicating that the folding monomer and the final dimeric state exhibit close Rs values. This strongly suggests that compaction of monomers is linked to dimerization.

Whatever the refolding conditions of the nicked enzyme, K2 peptide does not reassociate to K1 and elutes with an almost constant but higher than expected Rs, suggesting a lack of structural organization or a possible oligomerization. The larger peptide (K1) elutes as a species which Rs decreases with denaturant concentration from 4.4 to 3.3 nm for 0.45 M residual GdmHCl. At 0.3 M GdmHCl, the 3.3 nm species is transformed into a larger one (Rs 3.57 nm), which is the only large species observed.

In contrast with what has been reported for chicken Mib-CK (*25*), K1 molecules of GdmHCl-denatured nicked MM-CK can refold. However, as previously mentioned, for GdmHCl residual concentrations lower than 0.3 M, the denatured proteolyzed CK precipitates.

We checked that the fluorescence emission λmax values we measured with the refolded nicked protein (Figure 2A) monitored the structural properties of the K1 peptide (which contains the four tryptophan residues) and that in solution K2 was not interacting with K1 peptides. For this purpose, we collected K1 protein peaks obtained for various denaturant concentrations and measured their fluorescence emission λmax (Figure 4B). The curve we obtained is in good agreement with the results presented on Figure 2A.

One can point out that the rather important λmax decrease (339 to 335 nm) parallels the Rs value increase (3.3 to 3.57 nm), which can be interpreted as a K1 dimerization with a modification of tryptophan 210 surroundings at the K1 dimer interface (*8*, *38*).

Refolding Kinetics of Denatured (K1K2)$_2$ at a 0.3 M GdmHCl Residual Concentration. To check whether equilibrium intermediates also appear on the kinetic pathway, time variations of the spectroscopic properties and Rs were collected.

(a) Kinetics of Fluorescence Changes: λmax Monitoring and ANS Binding. As said above, below 0.3 M residual GdmHCl, (K1K2)$_2$ renaturation leads to protein precipitation. We therefore studied the refolding of the truncated protein at 0.3 M residual denaturant concentration. The nicked protein previously denatured in 4 M GdmHCl was diluted in the spectrofluorometer cuvette under constant stirring. The excitation wavelength was 295 nm and the maximum emission wavelength was determined every 30 s by rapid scanning (Figure 5A). Before refolding, the λmax is 352 nm and immediately upon dilution of the denatured sample, it reaches 340 nm indicating a fast tryptophan burying. Then, the λmax decreases very slowly to a final value of 335 nm. The λmax of the refolding uncleaved protein occurs within the same time scale: it rapidly reaches 335 nm and then slowly decreases to 333 nm. In similar experimental conditions, 310 μM ANS was added after various renaturation times (Figure 5B). After 30 s of renaturation, a strong increase of ANS fluorescence is observed for both cleaved and uncleaved proteins reflecting the formation of hydrophobic areas. Im-

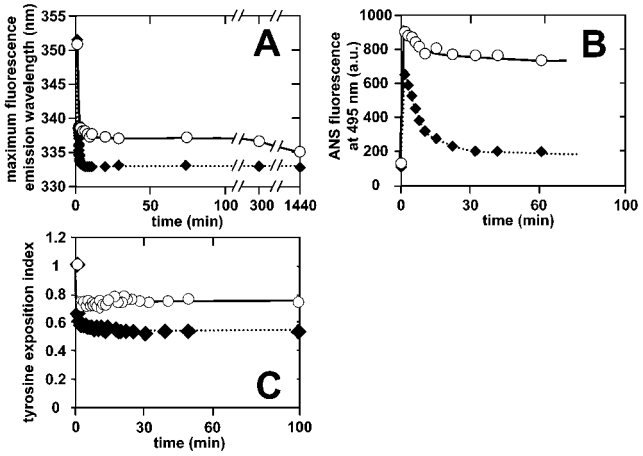

FIGURE 5: Kinetics of intact (black diamonds) and nicked CK (open circles) refolding in 0.3 M GdmHCl. Both proteins were previously incubated for 19 h at 4 °C in 4 M GdmHCl, and then diluted to 0.3 M GdmHCl in the spectroscopic cuvette under permanent stirring to a final subunit concentration of 4 μM. Panel A: The maximum fluorescence emission wavelength was measured using a fast scanning procedure every 30 s with an excitation wavelength of 295 nm. Panel B: ANS binding measurements. Aliquots of a 50 mM methanolic ANS solution were added to the spectrofluorimeter cuvette at various refolding times (final ANS concentration of 310 μM). Samples were excited at 395 nm, and the ANS fluorescence intensities were recorded at 495 nm. The values are corrected from the fluorescence of ANS alone. Panel C: Tyrosine accessibility was monitored using a second-derivative UV spectroscopy technique (*35*). In this case, 2.5 mM TCEP was used instead of 5 mM DTT because of TCEP lowest absorbance in the UV range.

mediately after, the ANS fluorescence decreases to a very low level with the uncleaved MM-CK but remains relatively high for the nicked enzyme. This shows that in the latter case protein folding does not proceed to completion.

(b) Kinetics of Tyrosine Burying. All 10 tyrosine residues of denatured cleaved or uncleaved CK are exposed to the solvent. Immediately upon dilution, the tyrosine accessibility index of both proteins strongly decreases and, after 2 to 3 min, reaches the mean values of 0.58 and 0.75 for MM-CK and $(K1K2)_2$, respectively, and thereafter remains constant with time (Figure 5C).

(c) Kinetics of Nicked Enzyme Refolding Followed by Size-Exclusion Chromatography. Despite potential dilution effects, SEC is a simple and reliable method to identify folding intermediates (*40*). Identical amounts of $(K1K2)_2$ refolded for various times at 0.3 M residual GdmHCl were injected onto the column equilibrated in the renaturation buffer. Figure 6A shows the elution profiles recorded at 220 nm. When the protein is injected 4 min after dilution, the elution profile reveals two main peaks with Stokes radii of 2.9 and 2 nm, respectively. As shown by SDS−PAGE and by the absence of 280 nm absorbance, the last peak to be eluted corresponds to the K2 peptide (absence of tryptophanyl or tyrosyl residues in K2), while the first one contains the K1 peptide. A shoulder around 56 min, possibly corresponding to a transiently populated folding intermediate of 3.8 nm, is also visible.

When the protein is allowed to refold for longer periods before being injected onto the column, the 3.8 nm shoulder disappears and a new one with an Rs of 3.57 nm is observed. The proportion of this latter peak increases with time at the expense of the 2.9 nm one and reaches a maximal value after

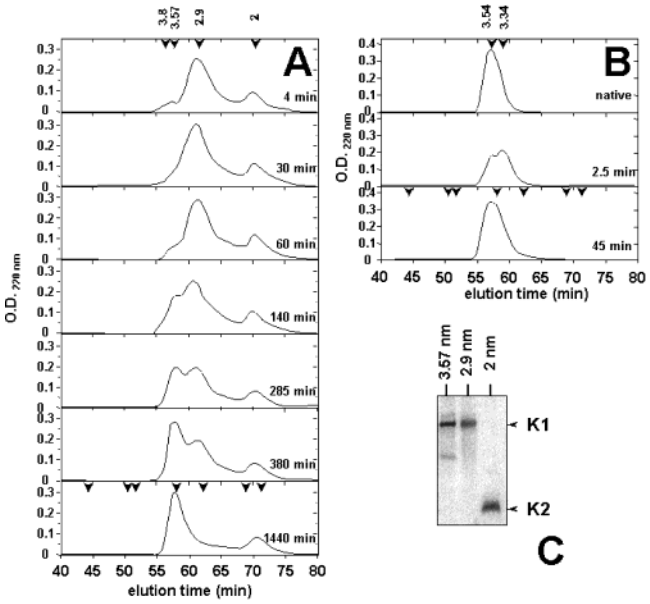

FIGURE 6: Size exclusion chromatography parameters measured during protein refolding in 0.3 M GdmHCl. All profiles were obtained using a column equilibrated at room temperature with 20 mM TRIS−AcOH, 150 mM NaCl, and 0.3 M GdmHCl, pH 7.4. The refolding profiles were recorded after injection of 60 μg of denatured protein diluted to a residual GdmHCl concentration of 0.3 M (final subunit concentration of 4 μM) after various times of incubation at room temperature. Panel A: The denatured nicked enzyme was allowed to refold 4, 30, 60, 140, 285, 380, and 1440 min at room temperature and then injected onto the SEC column (0.25 mL/min). Arrowheads indicate the Stokes radii corresponding to the protein peaks encountered during the protein refolding (from left to right: 3.8, 3.57, 2.9, and 2 nm). Panel B: Native MM-CK incubated for 24 h at room temperature in a buffer containing 0.3 M GdmHCl was injected onto the Superdex column. MM-CK, previously denatured for 19 h at 4 °C in 4 M GdmHCl, then allowed to refold for 2.5 or 45 min at room temperature in 0.3 M GdmHCl residual concentration, was injected onto the Superdex column in identical elution conditions (0.25 mL/min). Arrowheads indicate the Stokes radii of the protein peaks encountered during the protein refolding (3.54 and 3.34 nm). Panel C: Picture of a SDS−PAGE, loaded with the proteins found in the three peaks displayed on panel A (Rs = 3.57, 2.9, and 2 nm), and stained with Coomassie blue. For panels A and B, triangles in the last profile box indicate the elution times of calibration proteins.

24 h refolding. SDS−PAGE analysis of the protein content of these two peaks indicates that they contain only K1 peptides (Figure 6C); the increase of the Stokes radius, which corresponds to an almost doubling of the Stokes volume (*41*) of the molecule indicates a dimerization of K1 (Table 1). One can note that refolding to the most organized species is much slower for the truncated protein than for the uncleaved one, which in final form (3.54 nm) is already present in large amounts after 2.5 min of refolding (Figure 6B). According to these results and those previously obtained in different refolding conditions (*12*), the final species observed after $(K1K2)_2$ or MM-CK refolding appears to be dimeric.

Table 1 summarizes the molecular sizes of the different species as determined from their chromatographic behavior. Uncleaved CK refolds to its native active dimeric structure with 186 nm^3 Stokes volume (Rs 3.54 nm) via a partially reorganized and compacted monomeric species (*12*) with a 156 nm^3 Stokes volume (Rs 3.34 nm). As shown in Figure 4, dimerization of these incompletely refolded M monomers (between 0.5 and 0.3 M residual GdmHCl) does not lead to

Table 1: Analysis of the Dimensional Features of the Different Molecular Species Observed during Refolding of Proteolyzed and Intact MM-CK in 0.3 M Residual GdmHCl as Identified by Size Exclusion Chromatography

	MM-CK refolding		K1K2 refolding	
	first peak[a]	second peak	first peak	second peak
elution time (min)	58.6	57.4	61.5	57.2
measured[b] Rs (nm)	3.34	3.54	2.91	3.57
measured Vs (nm^3)	156	186	103	191
$Vs_{second\ peak}/Vs_{first\ peak}$[c]	1.19		1.85	
	MM-CK refolding[d]		K1K2 refolding	
	M monomer	MM dimer	K1 monomer	K1K1 dimer
molecular mass[e] (kDa)	42.982	85.964	37.064	74.128
predicted[f] Rs (nm)	2.73	3.55	2.58	3.35
predicted Vs (nm^3)	85	188	72	159
predicted $Vs_{dimer}/Vs_{monomer}$[g]	2.20		2.20	

[a] First and second peaks refer to the order of their appearance during the protein refolding process. The transient shoulder peak observed at the very beginning of the nicked CK refolding (Figure 6A) is not referenced in the table. [b] According to eq 1 and a flow rate in the column of 0.25 mL/min (see Experimental Procedures). [c] $Vs_{second\ peak}/Vs_{first\ peak} = 4/3\pi Rs^3_{second\ peak}/4/3\pi Rs^3_{first\ peak}$. [d] Theoretical SEC results in our conditions, according to the SEC column standard curve obtained with monomeric globular proteins (*34*). [e] From ref *26*. [f] According to eq 2 (see Experimental Procedures). These values are for globular and completely folded proteins. [g] According to eq 2 and $Vs = 4/3\pi Rs^3$.

an increase of the Stokes radius. This observation confirms a strong compaction of the refolding monomer inside the dimer (*8*).

The truncated K1 protein slowly refolds into a molecular species with a Stokes radius of 3.57 nm. It can be seen from Figure 6 that this final species ($Vs = 191\ nm^3$) derives from the 2.91 nm one ($Vs = 103\ nm^3$). This approximate doubling of the Stokes volume indicates that K1 peptides do refold to a dimeric state, but unlike intact M monomers, with no further compaction.

The K2 peptide is eluted with a retention time that corresponds to a protein with a Stokes radius value of about 2 nm. However, it has to be stressed that the Rs of this peptide cannot be determined with sufficient accuracy using this SEC column and these Rs standards.

Characterization of the Refolded Truncated Protein. (a) Is the K1 Dimer Specifically Dimerized or just Aggregated? This question was addressed using the MB hybridization technique. When a mixture of native MM-CK and BB-CK is subjected to four freezing-thawing cycles, it has long been shown that an active MB hybrid is formed that can be identified by cellulose acetate gel electrophoresis (*10*). A mixture of native $(K1K2)_2$ and BB-CK treated in identical conditions gives a (K1K2)B hybrid dimer that exhibits activity (*24*). After a 24 h refolding time, renatured $(K1K2)_2$ was injected onto the size-exclusion column, and the 3.57 nm K1 species fraction was collected and incubated with native BB-CK. After four freezing-thawing cycles, an active BK1 hybrid was observed by cellulose acetate electrophoresis, clearly indicating that the truncated enzyme folds in a dimerizable structure (Table 2).

(b) Is the Presence of K2 Important in K1 Refolding? K2 involvement in K1-folding was addressed by separating K1 and K2 peptides. For that purpose, $(K1K2)_2$ samples were incubated at high temperatures (beyond 58 °C). Protein aggregation was observed and after a 10000*g* sedimentation of precipitate, K1 peptide was recovered in the pellet, whereas the K2 peptide remained soluble in the supernatant (not shown). K1 peptides were therefore denatured at 4 °C for 19 h in a buffer containing 4 M GdmHCl and then allowed to refold 24 h in a buffer containing 0.3 M GdmHCl residual concentration. The protein sample was injected onto a SEC column, and a single peak was observed with a retention time, which corresponds to a K1 dimer (not shown). To determine its reorganization level, the 24 h K1 refolded species was collected after SEC, digested with proteinase K, and analyzed by SDS−PAGE. Three peptides were observed with apparent molecular masses of 7, 12, and 18 kDa (not shown). Then, only two cleavage sites were accessible to the protease active site in the refolded K1 dimer. This indicates an incomplete but still rather important structural organization of the K1 dimer.

Table 2: Detection by Cellulose Acetate Gel Electrophoresis of Quaternary Structure Acquirement

	protein mixtures tested				
	BB	MM	BB + MM	BB + $(K1K2)_2$[a]	BB + $(K1)_2$[b]
BB	+++		+	++	++
hybrid			+	+	+
MM		+++	+		

[a] $(K1K2)_2$ is the truncated MM-CK without any further treatment. [b] This molecular species, a dimeric form of K1, is the 3.57 nm Stokes radius species collected from the SEC column (see Table 1).

(c) Is K1 Dimer Capable of Binding Nucleotides? Putative binding of MgADP was measured on both refolded proteins by a nucleotide-induced fluorescence quenching technique (*32*, *42*). Affinities for MgADP of uncleaved and cleaved MM-CK are not significantly different (K_d about 60−100 μM). The K_d for the 24-h refolded MM-CK is somewhat increased to 300 μM. Nucleotide-induced fluorescence quenching is barely detectable on the refolded nicked protein, suggesting that either MgADP does not bind or does not induce a significant conformational change. This implies that the lack of compaction of K1 monomer within the dimeric final structure affects the substrate binding site conformation.

DISCUSSION

In the present study, we have considered the role of the C-terminal end in muscle creatine kinase folding. Proteinase K specifically cleaves each monomer of the native dimeric MM-CK leading to two N-terminal K1 peptides (1−325) and two C-terminal K2 peptides (328−380) (*26*). Except for the fact that the heterotetrameric protein is completely

inactive and that two amino acids are missing, the cleaved protein, we named $(K1K2)_2$, and uncleaved MM-CK have identical biochemical and biophysical properties (*27*).

A refolding study of both proteinase K cleaved and uncleaved proteins as a function of residual GdmHCl concentrations has been performed. MM-CK equilibrium unfolding (*28*, *32*) and refolding (ref *12* and this study) curves have similar profiles but appear slightly shifted toward lower residual denaturant concentrations for the refolding process. The discontinuous variations of the different parameters we studied (Figure 2) suggest the presence of intermediate states for GdmHCl concentrations of about 0.8−1 M and 1.1−1.5 M. These intermediates could correspond to the molten globule and premolten globule, respectively, that have already been described (*28*, *32*, *37*, *38*). Truncated MM-CK renaturation seems to follow a similar track with refolding intermediates populated at identical residual GdmHCl concentrations (Figure 2).

Below 0.5 M residual GdmHCl and in reducing conditions, MM-CK activity is restored (Figure 1). A fraction (15−20%) of the original activity is however lost, indicating that some molecules did not refold properly; this misfolding might be due to proline isomerization as suggested earlier (*12*). SEC experiments indicate that refolded MM-CK (0.3 M residual denaturant concentration) is recovered as a 3.54 nm Rs species identical to the native dimer (Figure 6B).

Experiments with nicked CK in equilibrium conditions show that, whatever the residual denaturant concentration, the two K1 and K2 peptides never reassociate (Figure 3), but that the large K1 protein fragment refolds (Figure 4A) with its Rs decreasing with denaturant concentration down to 0.6 M. At 0.3 M, a soluble species with a Stokes radius of 3.57 nm is obtained, but below 0.3 M denaturant the folding peptides precipitate.

Kinetic analysis of K1 refolding followed by SEC (Figure 6A) indicates the transient formation of a monomeric 3.8 nm Rs intermediate, which is then converted into a 2.9 nm one. This 2.9 nm species is afterward transformed into a 3.57 nm Rs one. It is tempting to speculate that the transient 3.8 nm species is the counterpart of the burst phase intermediate species identified for the uncleaved CK (*12*, *13*). The 2.9 nm species has a Stokes volume of 103 nm^3, whereas the 3.57 nm species volume is almost twice as much (191 nm^3), thus strongly suggesting that this species is a K1 dimer. Other evidence of such K1 dimerization is brought by emission fluorescence λmax shift measurements (Figure 4B and 5A). At 0.45 M GdmHCl (Figure 4A), K1 peptides are most likely monomeric with an Rs of 3.3 nm, whereas in 0.3 M denaturant, these peptides are recovered with an Rs of 3.57 nm. In the same residual denaturant concentration range, λmax at 0.45 M is 339 nm and reaches 335 nm at 0.3 M GdmHCl. This 4 nm blue shift could be at least in part attributed to tryptophan 210 hiding at the K1K1 interface (*8*, *38*, *43*). As shown in Figure 6A, K1 dimerization is a slow process that can also be monitored by a λmax decrease over time (Figure 5A). A λmax decrease over time mirrors the appearance of the 3.57 nm Rs species. As opposed to tryptophan hiding, tyrosine burying is achieved before the 3.57 nm Rs form appearance, which means that dimerization does not lead to a higher burying of the tyrosine residues. One may conclude that no tyrosine residue is located nearby the K1K1 interface.

Scheme 1

$$2\ U \underset{}{\overset{very\ fast}{\rightleftharpoons}} 2\ I_{PMG} \rightleftharpoons 2\ I_{MG} \overset{fast}{\rightleftharpoons} D_{MG} \overset{fast}{\rightleftharpoons} D_A$$

Even if the 3.57 nm K1 species is not fully structured (Table 1; Figure 2 and 5), it is able to form active hybrids with B-CK monomers (Table 2), again proving that it has a functional dimerization site and a dimeric structure. This possibility of obtaining active K1B hybrids suggests that the final K1 state is not off-pathway. However, adenylic substrate binding experiments show that a nucleotide-binding site is not functional in the K1K1 dimeric structure.

In summary, the K1 dimer exhibits a fluorescence emission λmax of 335 nm, a high degree of tyrosine exposure, strongly binds ANS, does not bind MgADP, and according to the structural features deduced from SEC measurements, could be a dimeric molten globule species.

It has already been shown that the C-terminal part of the MM-CK folds before the N-terminal one (*12*, *15*−*19*). Moreover, expression of domains [1−167] and [168−380] of the highly similar mitochondrial creatine kinase isoform has revealed that the C-terminal domain of the protein can refold autonomously and stabilizes the N-terminal one (*18*). Since, in selected conditions, the K1 peptide can fold, this further reduces the C-terminal folding initiating region to amino acids 168−325.

K2 is not part of the dimerization site (*44*), and since it is not involved in the dimerization site appearance, this very C-terminal part of the creatine kinase M monomer could play a role in the final packing of the tertiary structure leading to fully functional nucleotide-binding sites, for instance. Furthermore, the nicked CK refolds very slowly compared with the uncleaved protein (Figures 5 and 6), indicating that the C-terminal end is acting as an intramolecular accelerator of protein folding in a manner reminiscent of what has been described for the proregion of α-lytic protease (*45*) or phosphatidylinositol transfer protein (*46*).

We show that the nicked-enzyme folding is very slow compared to MM-CK renaturation, but both refolding processes share similar features, at least for the first events. Taking advantage of the slow nicked CK refolding pathway, we try to clarify the MM-CK one. One can assume that MM-CK refolding follows the track described in Scheme 1. The unfolded species (U) collapses into an intermediate we called premolten globule (I_{PMG}), and then this species rapidly reorganizes to give a monomeric molten globule state (I_{MG}). Two monomeric molten globules join to give an inactive molten globule dimer (D_{MG}), which very rapidly compacts and becomes active (D_A). The presence of an inactive CK dimer has been shown in a previous study performed with high hydrostatic pressures (*47*). The nicked CK can go through these steps more slowly than the uncleaved CK, but the refolding process is blocked at the D_{MG} state because of its missing C-terminal end.

From our experiments, we conclude that MM-CK is only active as a dimer because during the protein folding, the monomer can finish its compaction exclusively if it is associated with another monomer, the [328−380] region of the protein being involved in this final structure acquisition. Thus, dimerization is needed for CK activity and as recently

shown allows cooperativity appearance between the two subunits (*48*).

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