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Reconstitution of active octameric mitochondrial creatine kinase from two genetically engineered fragments*

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Abstract

Creatine kinase (CK) has been postulated to consist of two flexibly hinged domains. A previously demonstrated protease-sensitive site in M-CK (Morris & Jackson, 1991) has directed our attempts to dissect mitochondrial CK (Mi-CK) into two protein fragments encompassing amino acids [1–167] and [168–380]. When expressed separately in *Escherichia coli*, the two fragments yielded large amounts of insoluble inclusion bodies, from which the respective polypeptides could be purified by a simple two-step procedure. In contrast, co-expression of the two fragments yielded a soluble, active, and correctly oligomerizing enzyme. This discontinuous CK showed nearly full specific activity and was virtually indistinguishable from native Mi-CK by far- and near-UV CD. However, the positive cooperativity of substrate binding was abolished, suggesting a role of the covalent domain linkage in the crosstalk between the substrate binding sites for ATP and creatine. The isolated C-terminal fragment refolded into a native-like conformation in vitro, whereas the N-terminal fragment was largely unfolded. Prefolded [168–380] interacted in vitro with [1–167] to form an active enzyme. Kinetic analysis indicated that the fragments associate rapidly and with high affinity ($1/K_1 = 17\ \mu M$) and then isomerize slowly to an active enzyme ($k_2 = 0.12\ min^{-1}$; $k_{-2} = 0.03\ min^{-1}$). Our data suggest that the C-terminal fragment of Mi-CK represents an autonomous folding unit, and that the folding of the C-terminal part might precede the conformational stabilization of the N-terminal moiety in vivo.

Keywords: expression; fragment complementation; kinetics; protein folding

Many kinases have bilobal structures; for example, 3-phosphoglycerate kinase (Watson et al., 1982) is a popular model enzyme consisting of two well-separated, similar-sized globular domains. Previous biochemical and biophysical evidence (Morris & Cartwright, 1990; Dumas & Janin, 1983; Wyss et al., 1993) has suggested that the guanidino kinases (ATP: guanidinophosphotransferases), the most prominent members of this enzyme family being creatine kinase and arginine kinase, also exhibit such a two-domain architecture, with two well-separated, similarly sized structures being connected by a flexible "hinge." The existence of at least two domains can already be assumed from simple size considerations. The typical size of a domain is between 100 and 200 amino acids (Richardson, 1981); therefore, it is likely that the protomers of guanidino kinases, comprising about 350–380 amino acids, are subdivided into smaller structural units. Recent data on the equilibrium unfolding of guanidino kinases (Gross et al., 1995) also support the two-domain assumption, suggesting that these enzymes consist of at least two separate folding units.

* This paper is dedicated to the memory of the late Dr. Martin Gross, a respected colleague and dear friend, whose unexpected death brought to an end a promising career in the midst of a vibrant life.

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Abbreviations: BSA, bovine serum albumin; buffer A: 100 mM sodium phosphate, 5 mM 2-ME, 0.1 mM EDTA, pH 7.4; CK, creatine kinase; Cr, creatine; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; IPTG, isopropyl-β-D-thiogalactoside; 2-ME, 2-mercaptoethanol; Mi-CK, mitochondrial creatine kinase; Mi-CK(dc), discontinuous mitochondrial creatine kinase (reconstituted from fragments [1–167] and [168–380]); Mi_b-CK, chicken sarcomeric mitochondrial creatine kinase; PCr, phosphocreatine; R_S, Stokes' (hydrodynamic) radius; SEC-FPLC, size-exclusion fast protein liquid chromatography.

Because a two-domain architecture is supposed to have important implications for both the enzymatic function and for the folding of guanidino kinases, we intended to test the hypothesis by genetically engineering two fragments of mitochondrial creatine kinase (CK) that might correspond to the two domains of the enzyme. To date, no three-dimensional structure of any guanidino kinase is available; therefore, we had to rely mainly on biochemical data that might define the putative domain boundary. Morris and Jackson (1991) have found an endoproteinase-V8 cleavage site in rabbit M-CK and have suggested that this site may indicate an exposed flexible structure linking two globular domains of similar size. We therefore decided to cleave the cDNA of mitochondrial CK (Mi-CK) close to this putative domain boundary and to express the resulting two fragments in *Escherichia coli*. The purified polypeptides were analyzed for the ability to fold individually and in combination, to reconstitute a functional enzyme. The results obtained from this fragment complementation study yielded valuable information on both the folding mechanism of guanidino kinases and on the role of the putative two-domain structure in cooperative substrate binding.

Results

Engineering, overexpression, and purification of fragments [1–167] and [168–380]

Based on the assumption that CK may be a two-domain enzyme like many other kinases, we set out to produce two fragments of Mi-CK that might correspond to the two putative domains. Previous biochemical work (Morris & Jackson, 1991) has demonstrated an endoproteinase V8-sensitive site in rabbit M-CK that may indicate an exposed loop connecting two globular domains of the protein. In the Mi_b-CK cDNA, a *Sca* I restriction site is situated between the codons for amino acids 167 and 168, 18 bp downstream of the homologous region encoding the protease-V8 site in M-CK. We used this *Sca* I site to dissect the cDNA into two parts by recombinant means, producing separate genes for the two protein fragments. These fragments could then be expressed individually or in combination from a T7 promoter (Fig. 1; see Materials and methods).

When the two Mi-CK fragments were expressed individually in *E. coli* at 37 °C, they were recovered almost quantitatively as inclusion bodies in the insoluble cell fraction. Lowering the expression temperature to 30 °C significantly increased the proportion of soluble protein, but the largest amount of both fragments still remained in inclusion bodies (Fig. 2A). No CK enzyme activity was detected in any of the crude lysates. The two fragments were purified from the inclusion bodies by a simple washing procedure, employing a Triton X-100-containing buffer (see Materials and methods). The resulting crude inclusion body preparations did not show any significant proteinaceous contaminations (Fig. 2B); however, to remove residual *E. coli* lipids and pigments, as well as traces of detergent, the denatured fragments were further purified by cation exchange chromatography on a ResourceS column in the presence of 7 M urea (Fig. 2B). From 1 L of *E. coli* culture, ≥100 mg of pure protein were finally obtained. The two overexpressed polypeptides showed approximately the expected molecular weights of 18.6 and 24.7 kDa for [1–167] and [168–380], respectively, on SDS-polyacrylamide gels (Fig. 2B). Both fragments were detected by a polyclonal anti-

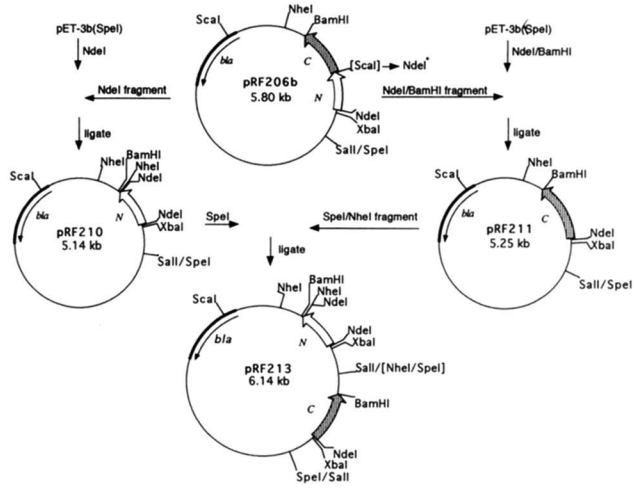

Fig. 1. Plasmid constructs used for expression of Mi-CK fragments. Mi-CK cDNA fragments encoded in plasmids pRF210, 211, and 213 are all expressed from the phage 10 promoter of expression plasmid pET-3b. Mi_b-CK protein fragment [1–167] is encoded by the sequence shown as open arrow (N). Protein fragment [168–380] is encoded by the sequence shown as stippled arrow (C). *bla* designates the gene conferring ampicillin resistance. *Nde* I*, the oligonucleotide inserted into the original *Sca* I restriction site in between the N- and C-terminal fragment, has the sequence GATAGCATATGT, encoding a stop codon and an initiating codon as part of the *Nde* I restriction site. [*Nhe* I/*Spe* I], [*Sca* I], restriction sites destroyed by the cloning procedure.

Mi_b-CK serum on immunoblots (Fig. 2A). During expression, only insignificant proteolysis of the fragments seemed to have occurred (Fig. 2A).

In order to coexpress fragments [1–167] and [168–380], a construct was made in which the C-terminal fragment was inserted as a separate coding region upstream of the N-terminal fragment; each fragment contained its own initiating methionine and termination codon. When coexpressed in *E. coli* at 30 °C from the resulting plasmid pRF213, more than 50% of each fragment appeared in the soluble cell fraction (Fig. 2A), in contrast to the separate expression of [1–167] and [168–380]. Moreover, high CK activity was detected in the crude lysate, although no full-length Mi-CK protein was present (Fig. 2A), as ensured by the specific arrangement of the two cDNA fragments on plasmid pRF213 (Fig. 1). Upon coexpression at 37 °C, however, the fragments again mainly formed inclusion bodies, and very little enzyme activity was found in the lysate (not shown).

The active CK species obtained from coexpression of polypeptides [1–167] and [168–380] could be purified from the soluble *E. coli* fraction by essentially the same procedure as wild-type Mi_b-CK (Furter et al., 1992), using a Blue Sepharose affinity column, followed by cation exchange chromatography on an FPLC Resource S or Mono S column. The protocol was slightly modified by omitting the pH 7.0 washing step on the Blue Sepharose column, because the protein bound more weakly to the column than wild-type Mi_b-CK. The final product was homogeneous as judged from Coomassie-stained SDS-polyacrylamide gels, and contained [1–167] and [168–380] in a 1:1 stoichiometric ratio (Fig. 2B), confirming that it was a noncovalent in vivo-adduct of the two fragments. The final yield of pure protein was about 15 mg/L of culture, as compared with 30–50 mg for wild-type Mi_b-CK. After SDS-PAGE of the purified pro-

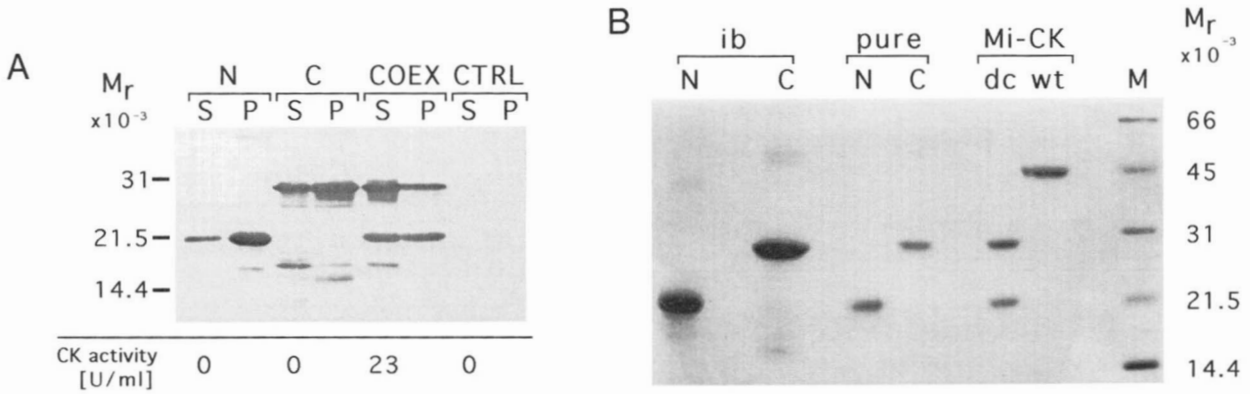

Fig. 2. A: Separate expression versus coexpression of [1–167] and [168–380] in *E. coli*. The two fragments were expressed either separately or together in *E. coli* at 30 °C, using the expression vectors pRF210 (N), pRF211 (C), or pRF213 (COEX). As a control, cells were transformed with an expression vector lacking the Mi-CK coding region (CTRL). Cells were harvested, sonicated, and centrifuged at 13,000 × *g*; supernatant (S) and pellet (P) fractions were electrophoresed in an SDS-polyacrylamide gel and blotted onto nitrocellulose. The blot was stained with a polyclonal anti-Mi_b-CK antiserum. In aliquots of the supernatant fractions, CK activity (reverse reaction) was determined. **B:** Inclusion bodies (N, C ib) formed upon separate expression of [1–167] and [168–380] at 37 °C were isolated by repeated washing with a Triton X-100 containing buffer (see Materials and methods). After dissolving them in 7 M urea, the inclusion bodies were further purified by cation exchange chromatography (N, C pure). Wild-type (wt) and discontinuous Mi-CK [Mi-CK(dc)], obtained by coexpression of [1–167] and [168–380], were purified by the standard Mi_b-CK protocol. All proteins were electrophoresed in an SDS-polyacrylamide gel and stained with Coomassie Blue. M, molecular weight markers.

tein and blotting onto PVDF membranes, the two bands were N-terminally sequenced, yielding the homogeneous amino acid sequences, TVHEKRKLFPPS and MYYSLTNMSERDQQ, for the smaller and the larger fragment, respectively. This confirmed that the N-terminal ends of the two Mi-CK fragments were intact, and showed that, in the case of [168–380], the additional initiation methionine had not been cleaved off as is the case for [1–167] and for wild-type Mi-CK (Furter et al., 1992).

Biochemical characteristics of the in vivo-adduct of [1–167] and [168–380]

SEC-FPLC of the purified, enzymatically active protein obtained by coexpression of Mi-CK fragments [1–167] and [168–380] revealed that it exists in two oligomeric forms of exactly the same sizes as octameric and dimeric wild-type Mi-CK (Fig. 3). Together with the observation that the protein contains the two fragments in a 1:1 molar ratio (see above), this shows that [1–167] and [168–380] associate in vivo to form a soluble, active, and correctly oligomerizing enzyme of the two interconvertible forms, $([1–167][168–380])_2$, and $([1–167][168–380])_8$. At a protein concentration of 6 mg/mL, the enzyme was more than 90% "octameric," similar to wild-type Mi-CK. Due to its Mi-CK-like properties, this reconstituted discontinuous protein will be termed Mi-CK(dc), and the hetero-tetrameric and -hexadecameric forms of Mi-CK(dc) will be named "dimeric" and "octameric," respectively.

Upon dilution of a concentrated stock solution of Mi-CK(dc), the octameric form dissociated slowly, i.e., within several days, into dimers, with the same rate as octameric wild-type Mi-CK (not shown). However, addition of a mixture of 20 mM Cr, 4 mM MgADP, and 50 mM nitrate, forming a transition state–analogue complex with Mi-CK (Milner-White & Watts, 1971), and known to induce a rapid dissociation of wild-type Mi-CK octamers within minutes (Marcillat et al., 1987; Gross & Wallimann, 1993; Gross & Wallimann, 1995), did not induce a fast decay of the Mi-CK(dc) octamers (not shown).

Wild-type Mi-CK binds its two substrates in a positively cooperative ("synergistic") fashion (Furter et al., 1993), which is

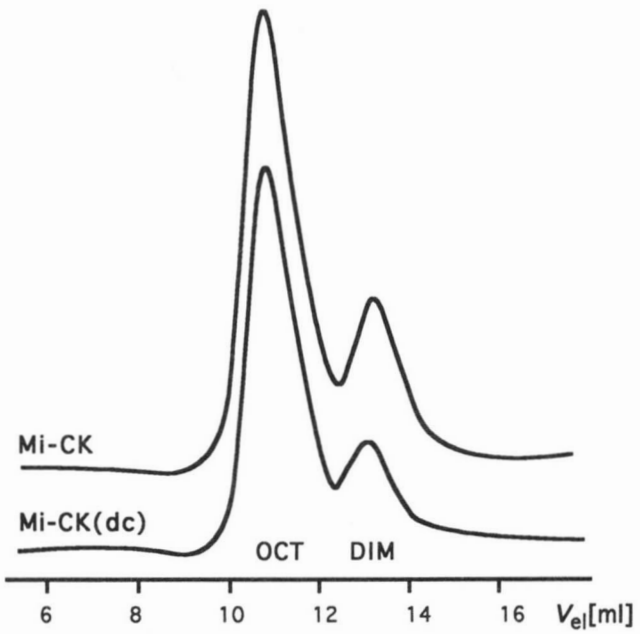

Fig. 3. Oligomeric forms of Mi-CK(dc). The concentrated stock solutions of purified wild-type and discontinuous Mi-CK [Mi-CK(dc)] were diluted in buffer A to a protein concentration of 50 μg/mL, incubated overnight at room temperature, and chromatographed on a Superose-12 SEC-FPLC column, using buffer A for elution. Elution profiles (O.D. at 280 nm) for both proteins are shown; both enzymes gave peaks at positions characteristic of octameric (OCT) and dimeric (DIM) Mi-CK, with no detectable difference in elution volumes (V_{el}) between the two proteins.

reflected by the fact that the K_m value for a given substrate is smaller than the corresponding K_d, the latter representing the affinity of the substrate to the "empty" enzyme. This cooperativity is speculated to be brought about by a conformational coupling of the two substrate binding sites, which might be located on two different domains. Therefore, extensive enzyme kinetic measurements on Mi-CK(dc) were performed, in order to detect an effect of the missing covalent domain linkage on the enzyme's substrate binding behavior. For the analysis of the enzyme kinetics data, it was assumed that the reaction mechanism of Mi-CK(dc) was the same as for native CK, namely a rapid equilibrium random mechanism (Morrison & Cleland, 1966; Lipskaya et al., 1989). The V_{max} and K_d(MgATP) were essentially unchanged for Mi-CK(dc) (Table 1); the K_d(Cr) was increased only twofold with respect to wild-type Mi-CK. Most interestingly, however, the two K_m values were no longer smaller than the corresponding K_ds, but more than twofold greater than the respective K_ds, indicating negative cooperativity of substrate binding for Mi-CK(dc).

Biophysical features of the two isolated fragments and of Mi-CK(dc)

Figure 4 shows the CD spectra of the isolated fragments in buffer A at 4 °C, together with the spectra of Mi-CK(dc) and of wild-type Mi-CK. The far-UV spectra (Fig. 4A) suggest a largely unfolded structure for [1–167], with traces of secondary structure, as indicated by some residual negative ellipticity around 222 nm. This weak Cotton effect completely vanished upon addition of 6 M GdnHCl, whereas the presence of 0.3 M GdnHCl or 0.5 M urea resulted in a decrease of only about 25% (not shown). In contrast, fragment [168–380] showed a far-UV CD spectrum (Fig. 4A) quite similar to wild-type Mi-CK, indicating that the C-terminal fragment formed a folded structure with a high proportion of helical secondary structure similar to the whole native enzyme. Addition of either 0.3 M GdnHCl or 0.5 M urea did not change the far-UV CD spectrum for fragment [168–380] (not shown). In the near-UV (Fig. 4B), the N-terminal fragment did not show any significant Cotton effects; in contrast, [168–380] exhibited a very large positive band below 290 nm, as opposed to the characteristic pattern of weaker negative bands in this region for wild-type Mi-CK. Above 290 nm, the C-terminal fragment showed some negative ellipticity, similar to the native wild-type enzyme.

The CD spectra of Mi-CK(dc) were nearly indistinguishable from wild-type Mi-CK (Fig. 4A,B), except for some subtle differences in the near-UV CD pattern below 295 nm (Fig. 4B, inset). The CD patterns suggest that Mi-CK(dc) formed a native-like structure with a nearly unaffected tertiary fold. This conclusion is strongly supported by the high specific enzyme activity that Mi-CK(dc) exhibited (Table 1).

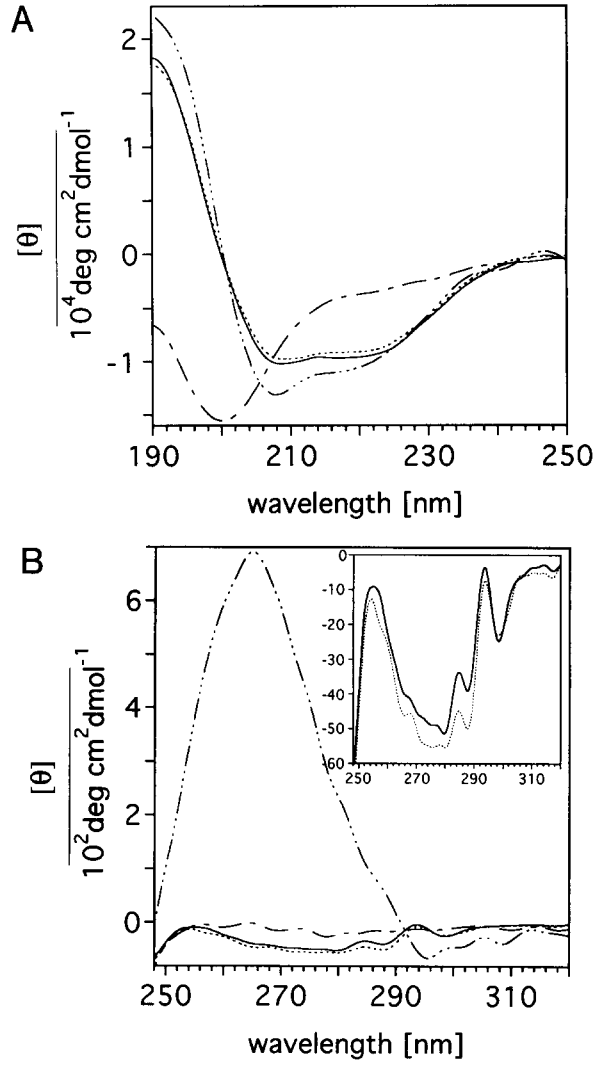

Fig. 4. CD spectra of fragments [1–167] and [168–380], of Mi-CK(dc) and of wild-type Mi-CK. All proteins were dialyzed against buffer A and measured at a protein concentration of 0.5 mg/mL at 4 °C, using an optical path of (**A**) 0.02 cm for far-UV and (**B**) 1 cm for near-UV spectra. ———, wild-type Mi-CK; - - - - - - -, Mi-CK(dc); —— – ——, fragment [1–167]; ———— —, fragment [168–380]. **Inset:** Enlargement of near-UV spectra of Mi-CK and Mi-CK(dc). Data are given as mean residue ellipticity, [θ].

Fragment [1–167] exhibited only weak intrinsic fluorescence with a tyrosine band below 300 nm (Fig. 5), due to the absence

Table 1. *Enzyme kinetic constants of Mi-CK(dc)*[a]

	V_{max} [U/mg]	K_d(Cr) [mM]	K_m(Cr) [mM]	K_d(MgATP) [mM]	K_m(MgATP) [mM]
Mi-CK[b]	60.7 ± 0.6	19.6 ± 1.6	8.9 ± 0.3	0.70 ± 0.04	0.32 ± 0.02
Mi-CK(dc)	54.7 ± 2.7	37.5 ± 8.6	87.7 ± 7.1	0.59 ± 0.10	1.37 ± 0.18

[a] Parameters were determined by measuring the forward CK reaction (PCr synthesis), varying the concentrations of both substrates, Cr and MgATP, as described in Furter et al. (1993). One unit (U) is defined as 1 μmol of substrate converted per minute.
[b] Data from Furter et al. (1993).

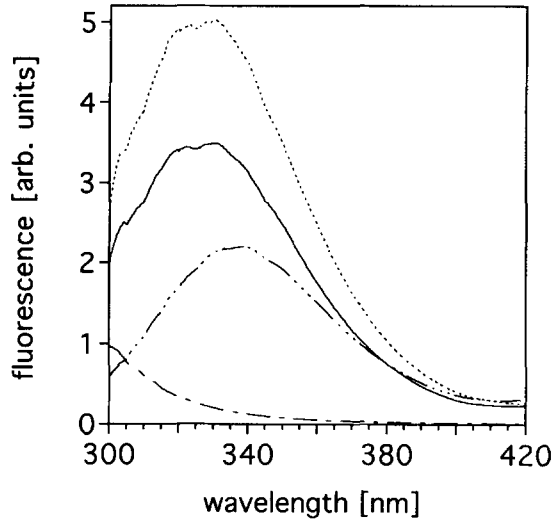

Fig. 5. Fluorescence spectra of fragments [1-167] and [168-380], of Mi-CK(dc), and of wild-type Mi-CK. All samples were measured at a protein concentration of 50 μg/mL at 4 °C in buffer A, using rectangular excitation at 280 nm. Arbitrary fluorescence units were normalized to equimolar protein concentrations. ———, wild-type Mi-CK; -------, Mi-CK(dc); —— – ——, fragment [1-167]; ——·——, fragment [168-380].

of indole side chains. The C-terminal fragment, on the other hand, showed indole emission centered at 338.5 nm, which is red-shifted by 9 nm with respect to native wild-type Mi-CK. The fluorescence spectrum of Mi-CK(dc) was characterized by the typical double peak of wild-type Mi-CK at 329.5 and about 320 nm; however, the fluorescence quantum yield was about 45% increased for Mi-CK(dc), which might be due to a dequenching of internally quenched indole side chains such as Trp-213 (Gross et al., 1994), arising from a disturbed domain interaction.

The hydrodynamic radii of both fragments at nondenaturing conditions (0.15 M GdnHCl in buffer A) were determined by size-exclusion chromatography on a Superose-12 column that had been calibrated with globular reference proteins (Table 2). The Stokes' radius of fragment [1-167] was much larger than that of [168-380], in spite of the higher molecular weight of [168-380]. The experimental Stokes' radius of [168-380] agrees well with the value that can be predicted for average globular proteins of the corresponding molecular weight by an empirical formula given in Uversky (1993), whereas the R_S of [1-167] is closer to the size expected for the unfolded state. This further supports the conclusion that [168-380] forms a compact globular structure, whereas [1-167] is largely unfolded.

Table 2. *Hydrodynamic radii of fragments [1-167] and [168-380]*[a]

	Measured R_S [Å]	R_S predicted for folded state [Å]	R_S predicted for unfolded state (6 M GdnHCl) [Å]
[1-167]	34	21	40
[168-380]	22	23	46

[a] The Stokes' radii of the two fragments in buffer A with 0.15 M GdnHCl were determined by SEC-FPLC on a Superose-12 column calibrated with globular reference proteins. Theoretical hydrodynamic radii for globular proteins of the same M_rs were calculated by the empirical formulas given in Uversky (1993).

In vitro-reconstitution of active enzyme

The reconstitution of active Mi-CK(dc) from the two isolated fragments was monitored by measuring CK activity upon addition of the unfolded fragment [1-167] to prefolded [168-380] in the presence of 0.5 M urea (see Materials and methods). Figure 6A shows the primary data obtained from reconstitution experiments at pseudo-first-order conditions (≥10-fold molar excess of [1-167]). The time-dependent proton consumption fit perfectly to Equation 8; however, not only the derived pseudo-first-order rate constants, k_{obs}, but also the final activity yields, k_{cat}^{obs}, varied systematically with the concentration of [1-167]. A plot of k_{cat}^{obs} against the total concentration of [1-167] (Fig. 6B) displayed the shape of a saturation curve, strongly suggesting that the association reaction is reversible. In fact, the data could be fit to an expression describing a simple association equilibrium,

$$k_{cat}^{obs} = \tfrac{1}{2} k_{cat}\big([C]_0 + [N]_0 + K_d - \sqrt{([C]_0 + [N]_0 + K_d)^2 - 4[N]_0[C]_0}\big), \quad (1)$$

yielding an apparent dissociation constant (K_d) of 3.92 μM (Table 3). A k_{cat} of 13.6 s^{-1} was determined as the saturation value of the curve; the corresponding value for octameric Mi-CK(dc), purified after fragment coexpression, and measured under the same conditions, was about 40 s^{-1}. Control experiments showed that the urea concentration of 0.5 M had no significant inhibitory effect either on Mi-CK or on Mi-CK(dc). Also, the presence of substrates did not affect the renaturation rate of wild-type Mi-CK (not shown).

The dependence of the apparent pseudo-first-order rate constant, k_{obs}, on $[N]_0$ (Fig. 6C) did not follow the linear relationship that would be expected if the association step were rate-limiting, suggesting that a fast-forming, inactive primary adduct, *NC**, slowly isomerizes in a second, rate-determining reaction step to form the active enzyme, *NC*:

$$N + C \underset{k_{-1}}{\overset{k_1[N]}{\rightleftharpoons}} NC* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} NC. \quad (2)$$

In this case of a bi-uni reaction sequence, and under the given pseudo-first-order conditions ($[C]_0 \ll [N]_0$), k_{obs} should depend on $[N]_0$ according to

$$k_{obs} = k_{-2} + k_2[K_1/(1 + K_1)] \quad (3)$$

(Bernasconi, 1976), with K_1 being the association equilibrium constant for the isolated first reaction step. In fact, a good fit of the data to this expression was achieved (Fig. 6C), yielding the $1/K_1$, k_2, and k_{-2} values listed in Table 3. The overall ap-

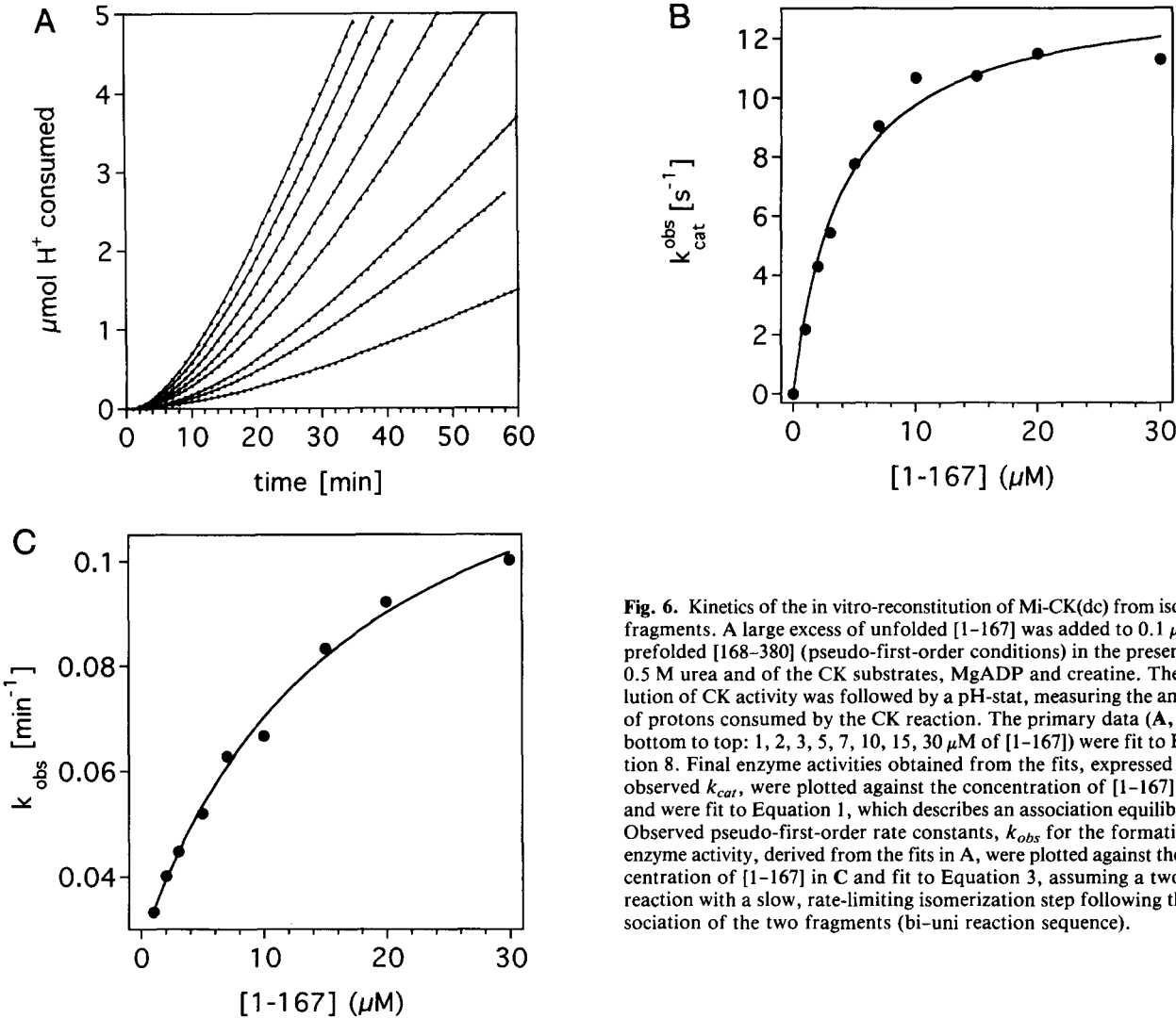

Fig. 6. Kinetics of the in vitro-reconstitution of Mi-CK(dc) from isolated fragments. A large excess of unfolded [1–167] was added to 0.1 μM of prefolded [168–380] (pseudo-first-order conditions) in the presence of 0.5 M urea and of the CK substrates, MgADP and creatine. The evolution of CK activity was followed by a pH-stat, measuring the amount of protons consumed by the CK reaction. The primary data (**A**, from bottom to top: 1, 2, 3, 5, 7, 10, 15, 30 μM of [1–167]) were fit to Equation 8. Final enzyme activities obtained from the fits, expressed as an observed k_{cat}, were plotted against the concentration of [1–167] in **B**, and were fit to Equation 1, which describes an association equilibrium. Observed pseudo-first-order rate constants, k_{obs} for the formation of enzyme activity, derived from the fits in A, were plotted against the concentration of [1–167] in **C** and fit to Equation 3, assuming a two-step reaction with a slow, rate-limiting isomerization step following the association of the two fragments (bi–uni reaction sequence).

parent dissociation equilibrium constant, K_d, could be calculated as

$$K_d = k_{-2}/(K_1 k_2), \quad (4)$$

under the assumption of small intermediate concentrations at equilibrium. This K_d value of 3.95 μM (Table 3) was virtually identical to the one determined independently from the $[N]_0$-dependent reaction amplitudes (Fig. 6B), confirming that the data are consistent with the proposed reaction sequence.

The in vitro reconstitution of the two fragments was performed and analyzed under the assumption of pseudo-first-order reaction conditions and the data was analyzed using this assumption (see above). In order to assure that the reconstitution kinetics were not complicated by dimerization and octamerization events, the oligomerization pattern of the enzyme formed during the reconstitution experiment was analyzed. Samples of the two fragments were incubated under conditions of the reconstitution assay [i.e., at a concentration of 0.1 μM Mi-CK(dc)] for 2 h up to one week, and subsequently analyzed by SEC-FPLC on a Superdex-75 column, using buffer A + 0.3 M GdnHCl for elution (not shown). In these chromatograms, only the separate fragments, but no dimers or octamers, were detected. To confirm that dimers and octamers would be stable under the chosen chromatographical conditions, the following control experiment was performed. Purified Mi-CK(dc), consisting of dimers and octamers, was an-

Table 3. *Kinetic and equilibrium constants for the in vitro-reconstitution of Mi-CK(dc)*[a]

K_d*	3.92 ± 0.50 μM
K_d**	3.95 μM
$1/K_1$	17.2 ± 3.0 μM
k_2	0.118 ± 0.009 min^{-1}
k_{-2}	0.027 ± 0.002 min^{-1}
k_{cat}	13.6 ±0.6 s^{-1}

[a] The overall macroscopic association constant, K_d, was either (*) determined by fitting the k_{cat}^{obs} values to Equation 1 (Fig. 6B), or (**) calculated from K_1, k_2, and k_{-2} using Equation 4. The dissociation constant of the primary complex, $1/K_1$, and the two first-order rate constants for the isomerization step, k_2 and k_{-2}, were obtained from the fit to Equation 3 shown in Figure 6C. k_{cat} was determined as the saturation value of the curve in Figure 6B.

alyzed on a Superdex-75 column under the same conditions as the reconstituted samples. In these control chromatograms, no separate fragments were detected, showing that dimers and octamers, once formed, are not dissociating during the SEC-FPLC analysis. These two observations, taken together, strongly suggest that only monomeric, active species was formed during the reconstitution assay. These monomers readily dissociated into the observed individual fragments during the SEC-FPLC analysis. The fact that the k_{cat} of the enzyme in the reconstitution assay was about threefold lower than that of the purified octameric and dimeric Mi-CK(dc) is another indication of the monomeric state. Only under at least 50-fold higher fragment concentrations than those used in the reconstitution assay (i.e., at 5 μM) could dimers and octamers be detected during reconstitution experiments.

Discussion

In vivo-reconstitution of Mi-CK from two fragments

Although the Mi-CK fragments [1–167] and [168–380] formed inclusion bodies predominantly when expressed separately (Fig. 2A), they were capable of reconstituting an active, correctly oligomerizing enzyme at high yield upon coexpression from a single plasmid at 30 °C. The reconstituted, discontinuous enzyme was termed Mi-CK(dc). Bacterial chaperones are most likely to be involved in shielding the two separate fragments from aggregation before they recombine and form the fully folded enzyme (Wynn et al., 1992). At higher temperature (37 °C), aggregation appears to become kinetically favored over the proper association of the two fragments. In spite of the additional N-terminal methionine in fragment [168–380], Mi-CK(dc) formed a structure virtually indistinguishable from wild-type Mi-CK (Fig. 4A,B). This suggests that the engineered cleavage point between amino acids 167 and 168 lies within some exposed surface loop where an additional bulky residue can be tolerated without interfering with tertiary structure formation. This is particularly important to note, because the cleavage point was not constructed at the position corresponding exactly to the protease V8-sensitive site of M-CK (Morris & Jackson, 1991), but was instead located six amino acids downstream.

The missing covalent linkage between the two Mi-CK fragments appears to be nonessential for the basic functions of the enzyme. Mi-CK(dc) exhibited nearly full specific activity (Table 1) with high-affinity binding of MgATP, and formed stable dimeric and octameric structures like wild-type Mi-CK (Fig. 3). However, the reconstituted protein showed some more subtle deviations from wild-type behavior; in particular, the positive cooperativity in the binding of the two substrates Cr and MgATP was abolished and even turned into a negative cooperativity (Table 1). The loss of substrate binding cooperativity has also been observed for mutations of the reactive Cys-278 (Furter et al., 1993). A second difference between Mi-CK(dc) and the wild-type enzyme is that the characteristic dissociation of wild-type octamers into dimers upon addition of the transition state–analogue complex mixture was not found in the case of Mi-CK(dc). The abolishment of this induced octamer decay has also been observed with Mi-CKs containing major active-site alterations, e.g., with Cys-278 modified or mutated (unpubl. results), or with a mutation of the active-site tryptophan residue, Trp-223 (Gross et al., 1994). All these findings suggest that the formation of the proper transition-state conformation, which is inherently linked to a destabilization of the octameric structure (Gross & Wallimann, 1995), depends both on the integrity of the active site structure itself and on a functional domain linkage. The role of the latter might be to mechanically transmit substrate binding information from one binding site to the other, thereby inducing a major conformational change that results in both the positive cooperativity of substrate binding and octamer dissociation in wild-type Mi-CK.

Properties of the isolated fragments

Fragment [1–167] is in a largely unfolded conformation even under nondenaturing conditions. This is indicated by the far-UV CD spectrum (Fig. 4A), by the absence of near-UV ellipticity (Fig. 4B), and by the Stokes' radius of the fragment, which is much larger than predicted for a folded conformation (Table 2). The far-UV CD spectrum of [1–167] (Fig. 4A) suggests the presence of a very small fraction of residual secondary structure; likewise, the hydrodynamic radius of [1–167] is significantly smaller than expected for a fully unfolded polypeptide (Table 2). Fragment [1–167] eluted as a single peak in SEC-FPLC experiments. Such a unimodal distribution implies that the fragment either occurs as a single population of molecules containing only few, possibly fluctuating, secondary structure elements, or alternatively, that a small population of mostly folded molecules is in rapid exchange with a large unfolded fraction.

In contrast to [1–167], fragment [168–380] clearly folded into a compact structure, with a high proportion of α-helical secondary structure, similar to wild-type Mi-CK (Fig. 4A), which has been predicted to contain 37% α-helix, 30% antiparallel β-sheet, and no parallel β-sheet (Gross, 1994; Mühlebach et al., 1994). The compactness of fragment [168–380] is further corroborated by the SEC-FPLC data (Table 2), which revealed a hydrodynamic radius typical for a native globular protein. Additional experiments (unpubl. results) showed that [168–380] is digested by proteinase K at an about 10-fold lower rate than [1–167]. However, fragment [168–380] also showed significant differences relative to the structure of native wild-type Mi-CK. Its near-UV CD spectrum was characterized by a very intense positive Cotton effect in the region below 290 nm, in contrast to the much weaker pattern of negative bands for Mi-CK, suggesting the presence of tight, but probably non-native tertiary interactions. In the region around 300 nm, where wild-type Mi-CK has a negative CD band caused by a putative hydrophobic-core tryptophan [Trp-206 (Gross, 1994)], also [168–380] showed negative ellipticity, which might be an indication of nativelike hydrophobic core packing. The fluorescence spectrum of fragment [168–380] (Fig. 5), which contains all tryptophan residues of Mi-CK, was red-shifted by 9 nm relative to the native enzyme, showing that the indole groups within [168–380] are, on average, in a more polar environment, but yet not fully exposed. In addition, fragment [168–380] exhibited a very high surface hydrophobicity, as indicated by the strong tendency to aggregate both in vivo and in vitro, and by its high capacity for ANS binding (unpubl. data). From these observations, we conclude that [168–380] probably has a tertiary structure slightly different from its conformation in the native protein. Its exposed hydrophobic surface could be partly due to the altered tertiary fold, and partly due to the exposure of a putative hydrophobic domain interface. Irrespective of whether fragment [168–380] exactly corresponds

to a true C-terminal domain of Mi-CK or not, the data presented here clearly define it as an independent folding unit of Mi-CK.

In vitro-complementation of [1–167] and [168–380]

Even after long separate incubation at nondenaturing conditions, the two fragments were capable of forming the active enzyme upon mixing (see Materials and methods). This clearly indicates that [1–167] is recognized and bound by the folded fragment [168–380], whereupon the complex rearranges into the native Mi-CK(dc) structure. Most importantly, the finding also implies that the two fragments should not be strongly intertwined in the native structure; thus, it is consistent with the hypothesis that fragments [1–167] and [168–380] may correspond to distinct structural parts of Mi-CK. The kinetic experiments suggest that the actual association step is very fast, because even at the highly dilute conditions used in the assay, it never became rate-limiting (Fig. 6C), and the primary complex is relatively stable ($K_d = 1/K_1 = 17$ μM, see Table 3). The low equilibrium constant for the isomerization step ($K_2 = k_2/k_{-2} = 4.4$) following association suggests that already in the primary adduct, [1–167] might be in a rather folded state. The value for K_2 corresponds to a free energy difference between *NC** and *NC* of 3.7 kJ/mol, which amounts to only 12% of the total conformational free energy of wild-type Mi-CK, determined by GdnHCl unfolding experiments, and to even less when comparing with urea unfolding data (Gross et al., 1995). This low energy value is therefore rather unlikely to reflect the transition of a largely unfolded [1–167] to the fully native state. Hence, we suggest that the solution structure of free [1–167] consists of a small folded population that is in a highly dynamic equilibrium with the larger unfolded fraction.

The fragment complementation data on Mi-CK presented here have important implications concerning the folding pathway of guanidino kinases. It has been postulated previously, based on studies with conformationally sensitive monoclonal antibodies, that the C-terminal moiety of CK folds prior to the N-terminal segment (Morris, 1989). In that study, it was also shown that, during the refolding process, the protein acquires an intermediate state in which the C-terminal part is already resistant against trypsin digestion, whereas the N-terminal segment is still readily hydrolyzed by the protease. These data are now confirmed by our observations; because isolated [168–380] can fold into a nativelike structure, whereas [1–167] is unable to acquire a stably folded conformation on its own, one can suggest that also in the full-length protein, the C-terminal part has to fold first, in order to serve as a scaffold for the subsequent stable folding of the N-terminal moiety. Moreover, it has been observed previously (Furter et al., 1992) that the deletion of as little as 30 C-terminal amino acids from Mi-CK leads to a completely insoluble protein; similarly, removal of the 57 C-terminal residues by proteinase K cleavage (Wyss et al., 1993) produces a protein unable to refold (unpubl. obs.).

Materials and methods

E. coli strains, plasmids, DNA manipulations

E. coli strain BL21(DE3)pLysS and expression vector pET-3b have been described (Studier et al., 1990). DNA manipulations were done according to standard procedures (Sambrook et al., 1989). The T7 promoter-containing Mi_b-CK expression plasmid pRF23, encoding the full-length, mature Mi_b-CK sequence, has been described (Furter et al., 1992). Plasmid pET-3b(SpeI) was constructed by inserting a double-stranded oligonucleotide into the *Sal* I site of pET-3b, creating a new *Spe*I site.

The *Xba* I/*Bam* HI fragment encoding Mi_b-CK and part of the phage 10 promoter was isolated from plasmid pRF23 and subcloned into the *Xba* I/*Bam* HI sites of plasmid pET-3b(*Spe* I), creating pRF206a. A double-stranded oligonucleotide encoding a translational stop signal and an *Nde* I restriction site was then inserted into the *Sca* I site of pRF206a located within the Mi_b-CK coding sequence, to create plasmid pRF206b (Fig. 1). The *Nde* I/*Nde* I fragment from pRF206b was isolated and inserted into the *Nde* I site of pET-3b(SpeI), creating pRF210. This plasmid allows the expression of the N-terminal portion of Mi_b-CK (amino acids 1–167). The 3′-half of the gene encoded on plasmid pRF206b was isolated as an *Nde* I/*Bam* HI fragment and inserted into the *Nde* I/*Bam* HI sites of pET-3b(*Spe* I), creating pRF211. This plasmid allows the expression of the C-terminal portion of Mi_b-CK (amino acids 168–380); due to the need of an initiating methionine, this C-terminal polypeptide contains a methionine that is not encoded in the original Mi_b-CK sequence.

For coexpression of the C-and N-terminal Mi_b-CK portion, plasmid pRF213 (Fig. 1) was constructed. Plasmid pRF210 was linearized with *Spe* I and the *Spe* I/*Nhe* I fragment of pRF211 was inserted such that translation of the N-and C-terminal polypeptide proceeds in the same direction. Note that the gene for the C-terminal polypeptide is located upstream of the gene for the N-terminal polypeptide.

Overexpression and purification of proteins

The plasmids pRF23, pRF210, pRF211, and pRF213 were transformed into the *E. coli* strain BL21(DE3)pLysS and expressed as described previously (Furter et al., 1992). The cells were harvested 5 h after induction with 0.4 mM IPTG.

Wild-type Mi-CK and the discontinuous Mi-CK obtained by coexpression of fragments [1–167] and [168–380] (=Mi-CK(dc)) were purified according to the standard Mi-CK protocol (Furter et al., 1992), using a Blue Sepharose affinity column, followed by FPLC cation exchange chromatography. Instead of a Mono S column (Pharmacia), a 6-mL ResourceS column (Pharmacia) was used because of its higher binding capacity and operating flow rates.

To isolate the inclusion bodies formed by separate overexpression of fragments [1–167] and [168–380], the following procedure was designed. The *E. coli* pellet obtained from a 1.6-L culture grown at 37 °C was washed with 70 mL buffer P (20 mM PBS, pH 7.2, 5 mM EDTA) and centrifuged for 10 min in two SS-34 tubes (Sorvall) at 5,000 rpm. The cells were resuspended in 70 mL ice-cold distilled water and kept on ice for 10 min to allow for osmotic swelling. After centrifugation for 10 min at 10,000 rpm (SS-34), the clear supernatant containing the periplasmic material was discarded, and the cells were resuspended in 20 mL buffer P containing 10 units of benzone nuclease (Merck). The suspension was divided into two portions and sonicated on ice, using a Branson sonifier equipped with a micro tip (3 × 30 s, maximum performance, 50% duty cycle). The SS-34 tubes were filled up with buffer P and incubated for 30 min at 37 °C to digest nucleic acids. The lysate was subsequently centrifuged (4 °C, 12,000 rpm, 15 min), and the supernatant con-

taining the soluble cytosolic material was discarded. The pellets were resuspended with the help of a plastic spatula in 15 mL of buffer W (buffer P including 25% sucrose and 1% Triton X-100) and sonicated for 1 min (same conditions as above). After filling up each tube to 30 mL with buffer W, the inclusion bodies were pelleted for 10 min at 16,000 rpm. This washing procedure (i.e., sonication in buffer W) was repeated twice; the pellets were then washed in the same way with distilled water to remove residual detergent. Finally, the inclusion bodies were dissolved in 15 mL buffer U (7 M urea [ICN UltraPure], 10 mM sodium phosphate, pH 8.0, 10 mM 2-ME, 2 mM EDTA) by stirring overnight at room temperature.

The denatured fragments were further purified by cation exchange chromatography on an FPLC ResourceS column, using buffer U (4 °C) for binding and washing. The solubilized inclusion bodies were applied to the column in 2-mL aliquots (≤ 20 mg protein) and eluted with 150 mM NaCl in buffer U. Both fragments were purified by the same procedure. The pooled fractions were stored in the denatured state at −20 °C. Protein concentrations were determined by the Lowry assay (Lowry et al., 1951), and purity was checked on SDS-polyacrylamide gels and by immunoblotting, using polyclonal rabbit-anti-Mi_b-CK sera with horseradish peroxidase detection. N-terminal sequencing of the two fragments was performed with PVDF membrane-blotted material by automated Edman degradation in the Protein Sequencing Facility of the ETH (Dr. P. James).

In preliminary renaturation attempts, aliquots of the two separate Mi-CK fragments, purified and stored under denaturing conditions (buffer U), were dialyzed against various buffers (PIPES, sodium phosphate, Tris) at pH values ranging from 6 to 9, at different ionic strengths and temperatures (4 and 22 °C), and with optional additions of up to 20% sucrose or ethylene glycol. DTT (20 mM) was routinely added to the denatured fragments prior to dialysis, to completely reduce the thiol groups. Under all conditions, a large proportion of both fragments aggregated. The highest recovery of soluble protein was achieved by employing a low concentration (0.3 M) of GdnHCl in buffer A (100 mM sodium phosphate, pH 7.4, 5 mM 2-ME, 0.2 mM EDTA) at 4 °C; about 3 mg/mL of [1–167] and 0.7 mg/mL of [168–380] remained in solution for several weeks at these conditions. At room temperature, however, both fragments precipitated rapidly. Fragment [1–167] could alternatively be stored in 0.5 M urea, in which it remained soluble up to at least 1 mg/mL even at 25 °C.

Measurement of enzyme activity

CK enzyme activity was determined by the pH-stat method (Wallimann et al., 1984), which measures the amount of protons consumed or produced by the CK reaction. For routine measurements during enzyme purifications and for monitoring the time course of fragment association in vitro, the reverse CK reaction (ATP production, one proton consumed per reaction cycle) was measured at pH 7.0 and 25 °C, with 10 mM PCr and 4 mM ADP in the pH-stat assay mix (75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 2 mM 2-ME). The enzyme kinetic constants, V_{max}, K_m(MgATP), K_m(Cr), K_d(MgATP), and K_d(Cr), were determined by measuring the forward CK reaction (PCr production, one proton liberated per reaction cycle) at pH 8.0 and 25 °C, employing varying initial concentrations of both MgATP and Cr (Furter et al., 1993); the initial velocity data were analyzed according to a rapid equilibrium random mechanism, using the program package KINETICS (Cleland, 1979), adapted for personal computer by R. Viola (program obtained from R. Viola, Akron University, Akron, Ohio).

Size-exclusion chromatography

The hydrodynamic (Stokes') radii (R_S) of proteins and fragments were determined by size-exclusion chromatography (SEC-FPLC) at 4 °C on a Superose-12 column (Pharmacia), calibrated with the globular reference proteins, ferritin (61.0 Å), aldolase (48.1 Å), ovalbumin (30.5 Å), chymotrypsinogen A (20.9 Å), and RNase A (16.4 Å). Buffer A was used for elution, unless indicated otherwise. The resolution of the Superose-12 column covered the size range from the isolated fragment [168–380] up to octameric Mi-CK. For the analysis of the active enzyme formed in in vitro-reconstitution experiments (see below), a calibrated Superdex-75 column (Pharmacia) was used at 4 °C, with buffer A containing 0.3 M GdnHCl as the eluent. Under these conditions, monomeric CK species can be detected, while at the same time, coexisting octamers or dimers are not dissociated (Gross et al., 1995).

Spectroscopic methods

Steady-state protein fluorescence was measured on a SPEX Fluorolog-2 spectrometer, equipped with a 450-W xenon arc lamp as excitation source. One-centimeter quartz cuvettes in a thermostatted cell holder were used, and fluorescence emission was measured with rectangular excitation. Emission spectra were acquired in 0.5-nm steps in the ratio mode, using a rhodamine-B reference quantum counter, and were corrected using the instrument's correction factor files. The excitation wavelength was centered at 280 nm, and the excitation and emission slit widths were each set at 1 mm.

CD was measured on a Jasco J-710 instrument, using cylindrical, water-thermostatted quartz cells. Optical paths of 0.02 and 1 cm were used for far-UV and near-UV measurements, respectively, at a protein concentration of 0.5 mg/mL. Mean residue ellipticities, $[\theta]$, were calculated using a mean residue M_r of 113.7 for Mi-CK, and of 111.4 and 115.5 for the N- and C-terminal fragments, respectively.

In vitro-reconstitution experiments

The capability of the two Mi-CK fragments to reconstitute an active enzyme in vitro was assayed under various conditions in preliminary experiments. Equimolar amounts of [1–167] and [168–380] were mixed in the presence of 0.15–3 M GdnHCl and subsequently dialyzed against buffer A. Regardless of the initial denaturant concentration, always the same activity yield (around 7.5 U per mg of protein at 0.5 mg/mL total protein concentration, reverse CK reaction measured) was obtained. Even after separate overnight preincubation in buffer A, the fragments rapidly (within minutes) formed an active enzyme after mixing at 25 °C. Unfortunately, a large portion of both fragments aggregated at these conditions, such that the setup had to be changed for quantitative kinetic measurements. At 0 to 4 °C, on the other hand, where the solubility of the separate fragments was significantly higher (see above), the development of enzyme activity occurred only in a time scale of days. The di-

lemma regarding the optimal conditions was finally resolved by employing a very low concentration (0.1 μM) of [168–380] at 25 °C in a solution containing 0.5 M urea, which allowed the use of high concentrations of [1–167] without precipitation. The kinetic assay was performed directly in the pH-stat assay vessel in the presence of the CK substrates, MgADP and Cr, which additionally provided the advantage of monitoring the appearance of CK activity in a continuous, on-line fashion. To allow for prior folding of [168–380] alone, this fragment was added to the assay mixture 5 min before addition of excess [1–167]. Increasing the delay time up to 20 min did not significantly affect the kinetics of reconstitution.

The final assay conditions were chosen as follows: 0.1 μM of [168–380] (stock solution in buffer U) was added to 3 mL (final volume) of pH-stat assay mix (see above), pH 7.0, containing 0.5 M urea, 4 mM ADP, and 10 mM PCr (final concentrations). After a preincubation period of 5 min at 25 °C, a defined large excess (1–30 μM) of [1–167] (stock solution in buffer U) was added, and the consumption of protons by the reverse CK reaction was monitored with the pH-stat for 20–80 min, until no more than 5 μmol of each substrate had been consumed. This ensured a continuous substrate saturation of the enzyme during the experiment.

In the case of an irreversible reaction, the association of the two fragments (N = [1–167], C = [168–380]) is described by the rate law,

$$\frac{d[NC]}{dt} = k_1[N][C], \tag{5}$$

where k_1 is the second-order rate constant. Under the given experimental conditions (large excess of N over C), the concentration of N remains virtually constant during the course of the reaction, such that a pseudo-first-order rate law of the form

$$\frac{d[NC]}{dt} = k_{app}[C], \tag{6}$$

with $k_{app} = k_1[N]$ can be applied. Integration yields

$$[NC] = [C]_0(1 - \exp(-k_{app}t)), \tag{7}$$

where $[C]_0$ represents the concentration of C at $t = 0$ (total concentration of C). Assuming enzymatic activity for the monomeric species, NC, the concentration of NC should be proportional to the measured enzyme activity, with the turnover number, k_{cat}, being the correlation factor. Another integration over time gives an expression describing the time-dependent consumption of protons during reconstitution of the enzyme (V_{assay} represents the assay volume):

$$n(H^+consumed) = V_{assay} \cdot k_{cat} \cdot \left\{ [C]_0 t + \frac{[C]_0}{k_{app}} \cdot (e^{-k_{app}t} - 1) \right\}. \tag{8}$$

After including an additional linear term to account for the slow baseline drift of the pH-stat, the data from the reconstitution experiments fit perfectly to Equation 8; however, it was found that the association reaction itself was both reversible and nonrate-limiting, in contrast to the assumptions underlying the above equations (see Results). Therefore, the experimentally measured k_{app} and k_{cat} were renamed k_{obs} and k_{cat}^{obs}, respectively.

All curve fittings in this work were performed on an Apple Macintosh computer, using the software KaleidaGraph.

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