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The N-Terminal Heptapeptide of Mitochondrial Creatine Kinase Is Important for Octamerization†

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ABSTRACT: Mitochondrial creatine kinase (Mi-CK) isoenzymes, in contrast to cytosolic CKs, form octameric molecules composed of four stable dimers. Octamers and dimers are interconvertible. Removal of the N-terminal pentapeptide of chicken cardiac Mi-CK (Mi_b-CK) by limited proteolysis drastically destabilized the octamer. The role of the charged amino acids within the N-terminal heptapeptide was studied in detail by progressively substituting the four charged residues by uncharged ones. In these altered proteins, the octamer/dimer ratio at equilibrium conditions was shifted toward the dimer. Also, the *in vitro* dissociation rate of octamers into dimers was increased in correlation to the number of charged residues eliminated. Point mutant E4Q, with only one positive charged amino acid removed, already displayed a 50-fold higher equilibrium constant and a 13-fold increased dissociation rate compared to wild-type Mi_b-CK. Mutant 4–7, having all four charged residues in the N-terminal heptapeptide substituted, showed a 100-fold higher equilibrium constant and a 146-fold increased dissociation rate. The corresponding values for double mutant E4Q/K5L were intermediate between the single and quadruple mutants. This strongly suggests that the charged amino acids in the N-terminal heptapeptide of Mi_b-CK, and therefore ionic interactions mediated by the N-terminal moiety, play an important role in forming and stabilizing the octameric molecule. The role of dimer–octamer interconversion *in vivo* as a possible regulator of contact site formation and of mitochondrial oxidative phosphorylation is discussed.

Creatine kinase (CK,[1] EC 2.7.3.2) isoenzymes catalyze the reversible transfer of the *N*-phosphoryl group of phosphocreatine to ADP [for a review, see Kenyon and Reed (1983)].

$$MgADP^- + PCr^{2-} + H^+ \leftrightarrow MgATP^{2-} + Cr$$

CK genes are expressed in tissues with high energy demands, e.g., in skeletal and cardiac muscle, brain, retina, and spermatozoa. In most tissues, cytosolic as well as mitochondrial CK isoenzymes are coexpressed. The three cytosolic CK isoenzymes always exist as dimeric molecules composed of two types of subunits (MM-CK, MB-CK, and BB-CK, with M standing for the muscle isoform and B standing for the brain isoform) and are situated either free in the cytosol or associated with subcellular structures, e.g., the myofibrillar M-line or the sarcoplasmic reticulum, where they are functionally coupled to the actomyosin ATPase (Wallimann et al., 1984) and the Ca^{2+}-ATPase (Rossi et al., 1990; Korge et al., 1993), respectively. On the other hand, the mitochondrial CK (Mi-CK) isoenzymes are bound to the outer side of the inner mitochondrial membrane (Jacobus & Lehninger, 1973). Studies on isolated mitochondria have shown that mitochondrial oxidative phosphorylation and the Mi-CK reaction are functionally coupled, that is, Mi-CK preferentially utilizes the ATP synthesized through oxidative phosphorylation for phosphocreatine synthesis [for reviews see Wallimann et al. (1992) and Wyss et al. (1992)].

It has been shown previously by several techniques that in solution Mi-CK isoenzymes form either dimeric or octameric molecules, with the dimer as the basic stable building block (Wyss et al., 1990). Both oligomeric forms were shown to be enzymatically active (Marcillat et al., 1987). Mi-CK dimers and octamers are readily interconvertible, with several factors influencing the dimer/octamer ratio *in vitro*. The octameric form is favored at high Mi-CK concentrations and at low pH (Schlegel et al., 1988b; Lipskaya et al., 1989; Wyss et al., 1990). Equilibrium substrate combinations (MgADP + MgATP + Cr + PCr) or formation of a transition-state analogue complex (so-called TSAC with MgADP, Cr, and nitrate) induce a fast dissociation of the octamers into dimers (Marcillat et al., 1987). Monomeric Mi-CK can only be generated under strongly denaturing conditions (Schnyder et al., 1988; Wyss et al., 1990).

Imaging of Mi-CK by electron microscopy revealed a banana-shaped structure for the dimer, while the octamer has a cubelike structure with $P42_12$ symmetry and a central cavity or channel running from top to bottom of the molecule along the 4-fold axis (Schnyder et al., 1988, 1990). Thus, due to its shape, the octameric molecule would be well suited to support metabolite channeling (Wyss & Wallimann, 1992) at the mitochondrial contact sites. This symmetric, cubelike structure may also be important in the association of Mi-CK with mitochondrial membranes, since it was shown that, *in vitro*, octameric chicken heart Mi-CK (Mi_b-CK) can induce contact formation between two membranes much better than dimeric Mi_b-CK does (Rojo et al., 1991).

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[1] Abbreviations: CK, creatine kinase; B-CK, cytosolic brain-type creatine kinase isoenzyme; M-CK, cytosolic muscle-type creatine kinase isoenzyme; Mi-CK, mitochondrial creatine kinase isoenzymes; Mi_a-CK, mitochondrial creatine kinase isoenzyme from chicken brain; Mi_b-CK, mitochondrial creatine kinase isoenzyme from chicken heart; Cr, creatine; PCr phosphocreatine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TSAC, transition-state analogue complex. Mutants with amino acid substitutions are denoted in the standard one-letter code by the wild-type residue and numbered position within the primary sequence, followed by the amino acid substitution; e.g., E4Q is a Mi_b-CK mutant with a glutamine residue at position 4 instead of a glutamic acid residue.

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Thus, the function of Mi-CK *in vivo* will most probably be dependent upon the proper oligomerization of the dimers into octamers. The aim of this study was to identify regions of the Mi_b-CK molecule that contribute to octamer formation and stabilization. After identification of such a region by limited proteolysis, the influence of different amino acid substitutions within this region on the stability of the octamer and its dissociation into dimers was studied *in vitro*.

MATERIALS AND METHODS

Escherichia coli Strains, Plasmids, DNA Manipulations, and Heterologous Mi_b-CK Expression. *E. coli* strain BL21-(DE3)pLysS and expression vector pET-3b have been described by Studier et al. (1990). Media and standard DNA manipulations were according to Sambrook et al. (1989). For the introduction of amino acid replacements at the N-terminus of the Mi_b-CK cDNA in expression vector pRF23 (Furter et al., 1992), a unique *Sac*I site was first created by changing nucleotide 22 from A into G (the first nucleotide of the initiating codon being 1) using site-directed mutagenesis (Nakamaye & Eckstein, 1986). After the resulting plasmid pPK22 was cut with *Nde*I and *Sac*I, the first 22 nucleotides were replaced by suitably designed double-stranded oligonucleotides. The complete coding sequence of Mi_b-CK (Hossle et al., 1988) in plasmid pPK22 was verified by dideoxy sequence analysis of the double-stranded expression plasmid. The plasmids constructed by insertion of oligonucleotide sequences were analyzed by sequencing within the changed region at the N-terminus. Wild-type Mi_b-CK and mutant Mi_b-CKs were expressed in *E. coli* and purified to homogeneity as described (Furter et al., 1992, 1993).

Enzyme Assays. CK activity was determined by the pH-stat method as described (Milner-White & Watts, 1971; Wallimann et al., 1984). One enzyme unit corresponds to 1 μmol of ATP or PCr transphosphorylated/min at 25 °C, respectively. For the reverse reaction (ATP synthesis), Mi_b-CK activity was determined in an assay mixture containing 65 mM KCl, 8.5 mM $MgCl_2$, 85 μM EGTA, 1 mM 2-mercaptoethanol, and 4 mM ADP at pH 7.0. [PCr] was varied from 0.5 to 20 mM to determine K_m(PCr) and $V_{max,rev}$. For the forward reaction (PCr synthesis), the assay mixture contained 45 mM Cr and 1 mM 2-mercaptoethanol at pH 8.0. In order to determine K_m(MgATP) and $V_{max,for}$, [ATP] was varied from 0.2 to 5 mM. The concentration of magnesium acetate always exceeded that of ATP by 1 mM. For the determination of K_m(PCr), K_m(MgATP), and V_{max} values, initial velocity data were analyzed using the program package written by W. W. Cleland (Cleland, 1979) as adapted for personal computers by R. Viola (the program was obtained through R. Viola, Akron University, Akron, OH). The values are given as mean ± standard error (SE) unless otherwise stated.

Proteolytic Digestions. Purified Mi_b-CK was digested with 21.5 × 10^6 units of endoproteinase Lys C (Boehringer Mannheim)/mol of CK in buffer A (50 mM NaH_2PO_4, 0.5 mM EDTA, pH 7.2 at 4 °C, 150 mM NaCl, and 1 mM 2-mercaptoethanol) at 37 °C in the presence of 1 mM PMSF for the time period indicated in the individual experiment. Digestion was stopped with 2000 Kallikrein inactivator units of Trasylol (Bayer, Leverkusen) per unit of endoproteinase Lys C added. Proteinase K (Boehringer Mannheim) digests of Mi_b-CK were performed at 30 °C for 90 min in buffer A, pH 9.0 at 4 °C. The ratio (w/w) of Mi_b-CK to proteinase K was chosen as 1:650, and digestion was stopped by adding PMSF to a final concentration of 2.5 mM.

Gel-Permeation Chromatography. Dissociation of Mi_b-CK octamers into dimers as a function of time was investigated by diluting concentrated samples of Mi_b-CK (9–24 mg/mL) to 0.1 mg/mL into buffer A at 4 °C and by subsequent analysis of aliquots of these diluted samples after 0, 1, 2, 4, 8, and 24 h and afterwards every 24 h by gel-permeation chromatography on a FPLC Superose 12 HR 10/30 column (Pharmacia), at 4 °C, using buffer A as eluent. The proportion of octamers and dimers was estimated by measuring the area of the absorption peaks (280 nm) with a digital planimeter (Placom KP-90, Japan). The proportion of octameric Mi_b-CK was plotted as a function of time, and the data points were subsequently fitted to the single-exponential equation

$$A(t) = A_\infty + (A_0 - A_\infty)e^{-k_1 t} \quad (1)$$

where A_0 represents the initial octamer content and A_∞ the octamer content after indefinite time, respectively. Half-lives of octamer dissociation were then calculated as $\tau_{1/2} = \ln 2/k_1$.

For the substrate-induced dissociation of Mi_b-CK octamers into dimers, experiments were done identically to those of the dilution-induced dissociation, except that the dissociation was induced by adding TSAC substrates (4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM nitrate). The time indicated in the individual experiments was measured from the addition of the TSAC substrates to the start of the gel permeation run.

The octamer/dimer ratios at different protein concentrations were determined by incubating Mi_b-CK protein samples in either the presence or absence of the TSAC substrates at the protein concentrations indicated in the individual experiments for a period of 2 weeks at 4 °C to ensure equilibration between the two oligomeric forms. Samples were then analyzed on a Superose column at 4 °C as described above. The proportion of octamers was plotted against the protein concentration.

case 1

$$1 \text{ octamer} \overset{K_1}{\leftrightarrow} 4 \text{ dimers}$$

case 2

$$1 \text{ octamer} \overset{K_4}{\leftrightarrow} 2 \text{ tetramers} \overset{K_3}{\leftrightarrow} 4 \text{ dimers}$$

The curves were fitted to the equation describing case 2 (see Results), where K_3 = [dimer]^2/[tetramer] and K_4 = [tetramer]^2/[octamer]. The total concentration of dimers is c = [dimer] + 2[tetramer] + 4[octamer], where [tetramer] was substituted and expressed in terms of [dimer] or [octamer]. In order to obtain the equilibrium constants (see Table 3), data points were analyzed by a computer program (kindly written by Dr. A. Wegner, University Bochum, Germany). The free energies (ΔG) were calculated from the equilibrium constants according to the equation $\Delta G = -RT \ln K$.

Fluorescence Spectroscopy. Fluorescence measurements were done with a SPEX Fluorolog-2 instrument in the L-shaped configuration, using 10-mm quartz cuvettes with magnetic stirring. Excitation was at 295 nm, and the specific emission of tryptophan residues was measured at 340 nm. The standard excitation monochromator slit width was 1.7 nm. All experiments were done at 0.05 mg/mL protein concentration in buffer B (31 mM NaH_2PO_4, 75 mM NaCl, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol at pH 7.0) at 30 °C as described elsewhere (Gross & Wallimann, 1993). The dissociation of Mi_b-CK octamers into dimers was induced by adding the TSAC substrates (4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM nitrate).

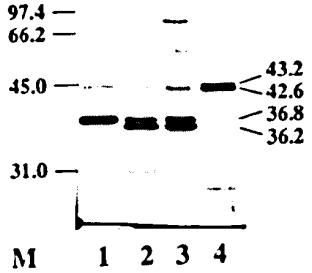

FIGURE 1: Mapping of the proteinase Lys C cleavage site at the N-terminus of Mi_b-CK. Single and double digests of Mi_b-CK, using proteinase Lys C and/or proteinase K, were separated by SDS–PAGE and visualized by silver staining. Lane 1: Mi_b-CK digested with proteinase K alone, yielding an N-terminal 36.8-kDa and a C-terminal 6.4-kDa fragment (the latter, not shown here, is only visible on Tricine–SDS–PAGE). Lane 2: Digestion of Mi_b-CK first with proteinase K and then with Lys C for prolonged time (≤40 h). Lane 3: Digestion of Mi_b-CK first with Lys C and then with proteinase K. Lane 4: Mi_b-CK digested with Lys C alone, yielding a 42.6-kDa fragment. The 43.2-kDa band represents undigested wild-type Mi_b-CK. M: Low molecular mass standards (Bio-Rad) with phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and bovine carbonic anhydrase (31 kDa). Note that proteinase Lys C removes a 0.6-kDa fragment from both native and proteinase K-digested Mi_b-CK, showing that the proteinase Lys C cleavage site is near the N-terminus.

Other Methods. SDS–PAGE was performed according to Laemmli (1970) and Tricine–SDS–PAGE according to Schägger and von Jagow (1987). Proteins were visualized by silver staining (Nielsen & Brown, 1984) or with Coomassie Brilliant Blue R-250. N-Terminal amino acid sequencing was done by automated Edman degradation on an Applied Biosystems 470A sequenator with on-line microbore phenylthiohydantoin detection. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad assay and bovine serum albumin as standard.

RESULTS

Effect of N-Terminal Truncation of Mi_b-CK on Octamer Formation. In solution, Mi_b-CK can be detected as an octameric and dimeric form, with the octameric form being favored in concentrated protein solutions. Upon dilution of concentrated Mi_b-CK to 0.1 mg/mL, slow dissociation of the octameric into the dimeric form can be observed (Schlegel, 1989). In an attempt to detect the regions in the protein that are involved in octamer formation, limited proteolysis of native octameric Mi_b-CK was performed with a variety of proteases like proteinase Lys C, proteinase Arg C, proteinase K, or trypsin, hoping to cleave off small peptide fragments that would result in a destabilization of the Mi_b-CK octamers. Curiously, the most promising condition found was digestion with proteinase Lys C in the presence of the unspecific serine protease inhibitor PMSF (see Material and Methods), which inhibited the protease only partially. By this treatment a small fragment of approximately 0.6 kDa was specifically removed from Mi_b-CK by the residual protease activity (Figure 1, lane 4). In contrast, treatment of Mi_b-CK with proteinase Lys C in the absence of PMSF led to total fragmentation of the protein. The N-terminal amino acid sequence (NH_2-TVHEKRKLF for wild-type Mi_b-CK, NH_2-RKLF for the digested enzyme) revealed that proteolytically truncated Mi_b-CK was shortened by the first five amino acids of the primary sequence. In double-digestion experiments, Mi_b-CK was digested first with Lys C and subsequently with proteinase K [which is known to selectively nick all CK isoenzymes

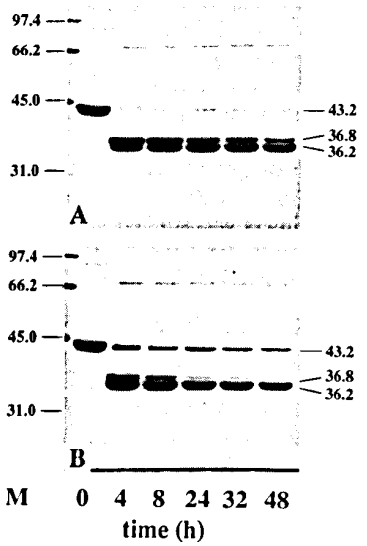

FIGURE 2: Preferential digestion of dimeric over octameric Mi_b-CK by proteinase Lys C. Octameric (A) or dimeric (B) Mi_b-CK (43.2 kDa) was digested with proteinase Lys C for the indicated periods of time (hours). After the proteinase Lys C digestion was stopped, all samples were additionally digested for 1.5 h with proteinase K in order to increase the difference in electrophoretic mobility between the two fragments generated by proteinase Lys C digestion (see Figure 1). After electrophoresis on an SDS–10% polyacrylamide gel, the proteins were visualized by Coomassie Blue staining. Twenty micrograms of protein was loaded per lane. Note that in Mi_b-CK octamers as compared to dimers, the N-terminus is partially protected against proteinase Lys C attack. The residual 43.2-kDa band seen in panel B is due to partial inhibition of proteinase K digestion of Mi_b-CK by the TSAC substrates (Wyss et al., 1993). M: For low molecular mass standards see Figure 1.

investigated so far near the C-terminus, yielding a 6.4-kDa and a 36.8-kDa fragment (Lebherz et al., 1986; Wyss et al., 1993)], or alternatively, first with proteinase K and then with Lys C. In both cases, SDS–PAGE revealed two bands with apparent molecular masses of 36.2 and 36.8 kDa (Figure 1, lanes 2 and 3), while Tricine–SDS–PAGE showed a single fragment of 6.4 kDa (data not shown). These results strongly suggest that proteinase Lys C truncates Mi_b-CK specifically and exclusively at the N-terminus. At comparable protein concentrations, Mi_b-CK truncated by Lys C formed substantially fewer octamers than wild-type Mi_b-CK (data not shown). In proteolysis time-course experiments, the progressive increase in the proportion of truncated Mi_b-CK was paralleled by a decrease in octameric enzyme species (data not shown). Additionally, octameric Mi_b-CK was digested by Lys C more slowly than dimeric Mi_b-CK (Figure 2), suggesting that in the octameric molecule the Lys C cleavage site is partially protected against Lys C attack and therefore the N-terminus might be located directly at the dimer–dimer interface.

In order to obtain homogeneously truncated Mi_b-CK preparations suitable for collecting quantitative information, a deletion spanning the first five N-terminal amino acids was constructed by site-directed mutagenesis (for details see Materials and Methods). The mutant protein encoded by this Mi_b-CK mutant allele (Δ1–5) was expressed in *E. coli* in soluble form (Table 1). The isolated mutant protein displayed nearly normal enzymatic activity (for kinetic parameters see Table 2). However, upon dilution of the enzyme to 0.1 mg/mL, mutant protein Δ1–5 dissociated 30 times faster into dimers than wild-type Mi_b-CK (see Table 4). Thus, experiments with both the N-terminally deleted Mi_b-CK expressed in *E. coli* as well as with the proteolytically

Table 1: Expression Levels and N-Terminal Amino Acid Sequences of Wild-Type and Mutant Mi_b-CKs[a]

protein	soluble CK activity/wet weight (units/g)[b]	experimentally determined N-terminal amino acid sequences							
		1	2	3	4	5	6	7	8
wild-type Mi_b-CK	3500	T	V	H	E	K	R	K	L
deletion mutant Δ1–5	4600					*M*	R	K	L
point mutant E4Q	8000	T	V	H	Q	K	R	K	L
double mutant E4Q/K5L	9500	T	V	H	Q	L	R	K	L
mutant 4–7	1100	T	V	H	Q	L	Q	Q	L

[a] All Mi_b-CK cDNAs were expressed in *E. coli* as described in Materials and Methods. Total CK activity contained in the supernatant of lysed cells was measured at pH 7.0 in the reverse direction of the reaction (ATP synthesis) and calculated per wet weight of bacteria as a measure for the efficiency of expression. The N-terminal amino acid sequences were determined after purification of each protein to homogeneity. The initiating methionine was lacking in all but the deletion mutant Δ1–5 Mi_b-CK. [b] One unit corresponds to 1 μmol of PCr transphosphorylated/min at 25 °C and pH 7.0.

Table 2: Enzyme Kinetic Parameters of Wild-Type and Mutant Mi_b-CKs[a]

protein	K_m(PCr) ± SE (mM)	$V_{max,rev}$ ± SE (units/mg)	K_m(MgATP) ± SE (mM)	$V_{max,for}$ ± SE (units/mg)
wild-type Mi_b-CK	1.31 ± 0.10 (n = 13)	114.09 ± 2.57 (n = 13)	0.41 ± 0.09 (n = 3)	100.67 ± 5.25 (n = 3)
deletion mutant Δ1–5	1.23 ± 0.08 (n = 3)	77.77 ± 1.40 (n = 3)	nd	nd
point mutant E4Q	1.20 ± 0.14 (n = 12)	99.30 ± 3.94 (n = 12)	0.30 ± 0.03 (n = 3)	73.58 ± 1.77 (n = 3)
double mutant E4Q/K5L	1.02 ± 0.08 (n = 12)	100.96 ± 2.65 (n = 12)	0.27 ± 0.04 (n = 3)	92.06 ± 2.91 (n = 3)
mutant 4–7	0.93 ± 0.07 (n = 12)	101.89 ± 2.18 (n = 12)	0.15 ± 0.02 (n = 3)	79.80 ± 2.19 (n = 3)

[a] To determine K_m(PCr) and $V_{max,rev}$ (V_{max} of the reverse reaction), [PCr] was varied between 0.5 and 20 mM at constant [ADP] of 4 mM and [$MgCl_2$] of 10 mM. To determine K_m(MgATP) and $V_{max,for}$ (V_{max} of the forward reaction), [ATP] was varied between 0.2 and 5 mM at constant [Cr] of 45 mM. The concentration of magnesium acetate always exceeded that of ATP by 1 mM. nd = not determined.

truncated Mi_b-CK strongly suggest that the N-terminus is important for the stability of the octamer.

Effect of N-Terminal Mutations on Enzyme Structure and Kinetics. The destabilization of the Mi_b-CK octamer caused by the deletion of the N-terminal pentapeptide was further investigated by site-directed replacement of certain amino acids at the N-terminus. Because dissociation of Mi-CK octamers can be induced by increasing the ionic strength (Marcillat et al., 1987), suggesting that the dimer–dimer interaction is at least in part ionic in nature, the charged amino acids at the N-terminus were chosen as targets for amino acid substitution and replaced with uncharged residues. One charged residue was replaced in E4Q, two in the double mutant E4Q/K5L, and four in the multiple mutant 4–7 (Table 1). The cDNAs encoding these substitutions were expressed in *E. coli* and the mutant proteins were purified to homogeneity. All mutant proteins were expressed in soluble form and at comparable amounts (Table 1). On SDS–PAGE, the mutants showed the expected mobility (data not shown). In addition, X-ray diffraction analysis of crystals of mutant Δ1–5 Mi_b-CK revealed the same space group ($P42_12$) with one Mi_b-CK octamer per asymmetric unit as for wild-type Mi_b-CK (T. Schnyder, personal communication).

The enzyme kinetic parameters of the different mutant enzymes were determined in the reverse and forward directions by the pH-stat method (see Materials and Methods). Reducing the number of charged residues at the N-terminus resulted in somewhat lower K_m(PCr) and K_m(MgATP) values, the lowest ones being observed for mutant 4–7 (Table 2). However, as with the K_m values, no dramatic change in V_{max} was observed for any of the mutant enzymes. To conclude, these control experiments confirm that all the mutant proteins have the expected amino acid sequence and molecular size and that they are properly folded and display normal catalytic properties.

Dimer/Octamer Ratio of N-Terminal Mutants at Equilibrium Conditions. The dimer/octamer equilibrium ratio is strongly dependent on the Mi_b-CK protein concentration (Schlegel et al., 1988a; Lipskaya et al., 1989; Wyss et al., 1990). To investigate the effect of the N-terminal amino acid substitutions on the octamer stability, mutant Mi_b-CKs

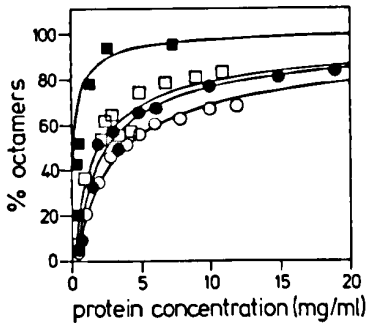

FIGURE 3: Octamer/dimer equilibrium as a function of the Mi_b-CK protein concentration. Point mutant E4Q (□), double mutant E4Q/K5L (●), and mutant 4–7 (○) were diluted from highly concentrated stock solutions to the protein concentrations indicated in the figure and incubated for 2–3 weeks at 4 °C to reach equilibration between dimers and octamers. Wild-type Mi_b-CK (■) was dissociated into dimers by adding the TSAC substrates, and then the samples were concentrated by Centricon ultrafiltration (Amicon) to the appropriate concentration and dialyzed against buffer A (see Materials and Methods). After equilibration of the samples, the dimer/octamer ratio was analyzed by gel-permeation chromatography at 4 °C. Symbols represent actual data points; lines connecting the symbols were derived from curves fitted to the data points by computer.

were adjusted to various protein concentrations and stored for 2–3 weeks at 4 °C to reach a dimer/octamer equilibrium. Since wild-type Mi_b-CK dissociated very slowly into dimers upon dilution, the dissociation was initially accelerated by adding the TSAC substrates. These samples were then concentrated to the appropriate protein concentrations by Centricon ultrafiltration, dialyzed against buffer A, and allowed to reach the dimer/octamer equilibrium for 2–3 weeks at 4 °C. Control experiments confirmed that, under these conditions, equilibration between octameric and dimeric Mi_b-CK occurs within hours. After equilibration, the dimer/octamer ratios were determined as a function of total Mi_b-CK concentration by gel-permeation chromatography (Figure 3). Data points were fitted to an equation describing either case 1 or case 2 (see Materials and Methods). The data of the wild type could be fitted well to both equations, but the data of the mutant enzymes could only be fitted well by using the equation corresponding to case 2. The poor fit of the mutant data to

Table 3: Thermodynamic Parameters of the Octamer/Tetramer/Dimer Interconversion[a]

protein	constant K_4[b] (μM)	ΔG_1 (kJ/mol)	constant K_3[c] (μM)	ΔG_2 (kJ/mol)
wild-type Mi$_b$-CK	0.1	37.1	8.0	27.0
point mutant E4Q	5.0	28.1	10.0	26.5
double mutant E4Q/K5L	5.0	28.1	5.0	28.1
mutant 4–7	10.0	26.5	10.0	26.5

[a] Wild type and mutants were incubated at different protein concentrations to reach stable dimer/octamer equilibria. After analysis of the samples by gel-permeation chromatography (as shown in Figure 3), equilibrium constants were calculated from the fitted curves. The free energy (ΔG) values were derived from these equilibrium constants (see Materials and Methods). [b] K_4 is the constant for the octamer–tetramer transition and ΔG_1 is the corresponding free energy. [c] K_3 is the constant for the tetramer–dimer transition and ΔG_2 is the corresponding free energy.

the case 1 equation and the subsequent need to postulate an intermediate, tetrameric species suggests that the dimer concentration was overestimated in the analysis of octamers and dimers by gel-permeation chromatography. The measured dimer concentration probably reflects both dimers and tetramers. Surprisingly, we never could detect a tetrameric species in gel-permeation chromatography, most likely due to the fact that the tetramer readily dissociates into dimers under these conditions. The equilibrium constants derived from the curve fitting and the free energies (ΔG) calculated from these constants are shown in Table 3. While values for K_4 of the mutant enzymes were increased 50–100-fold compared to values of the wild-type enzyme, corresponding to a change in free energy of 9–11 kJ/mol, values for K_3 were quite similar.

Mi$_b$-CK forms a transition state-analogue complex (TSAC), with the substrates MgADP, Cr, and the stabilizing anion nitrate, which mimics the transferable phosphate group in the transition state of the enzyme (Milner-White & Watts, 1971). The oligomeric equilibrium was previously shown to be shifted drastically toward the dimer in the presence of the TSAC substrates (Marcillat et al., 1987; Wyss et al., 1990) and therefore the mutants were examined under these conditions. Gel-permeation chromatography was used to determine the dimer/octamer ratios of each of the proteins at concentrations of 0.1 and 5 mg/mL. At a protein concentration of 0.1 mg/mL, wild-type Mi$_b$-CK and all mutants dissociated completely into dimers in the presence of these substrates. At a protein concentration of 5 mg/mL, however, this was the case only for mutant 4–7. Mutants E4Q/K5L and E4Q were only partially dissociated to 85% and 80%, respectively, while wild-type Mi$_b$-CK solutions at 5 mg/mL still contained 76% octamers. These results corroborate that formation of a TSAC shifts the dimer/octamer equilibrium toward the dimer for wild-type and mutant proteins. This shift can be counteracted more effectively for the wild type than the mutant enzymes by increasing the protein concentrations to 5 mg/mL.

Dissociation of Octameric into Dimeric Mi$_b$-CK upon Dilution of the Enzyme. Concentrated Mi$_b$-CK solutions (up to 24 mg/mL) were diluted to 0.1 mg/mL, and the proportion of octamers and dimers was subsequently analyzed in appropriate intervals in order to investigate the effect of the N-terminal mutations on the rate of octamer dissociation. Figure 4 shows that amino acid substitutions at the N-terminus of Mi$_b$-CK drastically accelerate the dissociation of octamers into dimers. The data points obtained for each investigated protein were fitted to eq 1. In each case, the curve so derived fits well to the experimental data; the good fit to a single-exponential function is an indication that octameric Mi$_b$-CK dissociates in one step into dimers, without any long-lived

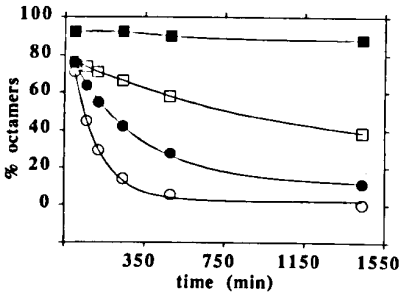

FIGURE 4: Dilution-induced dissociation of Mi$_b$-CK octamers into dimers. Wild-type Mi$_b$-CK (■), point mutant E4Q (□), double mutant E4Q/K5L (●), and mutant 4–7 (○) were concentrated so that the initial octamer content in all preparations was higher than 70%. Dissociation was started by diluting the enzyme to a protein concentration of 0.1 mg/mL. At the indicated time points, aliquots were analyzed by gel-permeation chromatography to determine the dimer/octamer ratios. Symbols in this graph represent measured values; lines were derived using equation $A(t) = A_\infty + (A_0 - A_\infty)e^{-k_1 t}$. Half-lives calculated from these curves are shown in Table 4.

Table 4: Kinetics of Dilution-Induced Dissociation of Mi$_b$-CK Octamers[a]

protein	half-life $\tau_{1/2}$[b] (min)	k_1 (min^{-1})	increase of rate constant relative to wild-type Mi$_b$-CK (x-fold)
wild-type Mi$_b$-CK	13281	5.2×10^{-5}	1
point mutant E4Q	1037	6.7×10^{-4}	≈13
deletion mutant Δ1–5	451	1.5×10^{-3}	≈30
double mutant E4Q/K5L	240	2.9×10^{-3}	≈55
mutant 4–7	91	7.6×10^{-3}	≈146

[a] Dissociation of Mi$_b$-CK octamers was induced by dilution of the proteins to 0.1 mg/mL in buffer A. The decay curves shown in Figure 4 were fitted to eq 1 (see Materials and Methods). The standard error of k_1 of the curve fit was below 8%. [b] The half-lives shown were calculated as $\tau_{1/2} = \ln 2/k_1$.

intermediates. The number of charged amino acids at the N-terminus greatly affects the dissociation rate upon dilution of the enzyme. Removal of a single negative charge (E4Q) increased the dissociation rate approximately 13-fold, removal of a negative and a positive charge (E4Q/K5L) increased the dissociation rate 55-fold, and removal of four charges (mutant 4–7) increased the dissociation rate 146-fold (Table 4). In deletion mutant Δ1–5, Glu-4 and Lys-5 are removed along with three additional, uncharged amino acids. The fact that substitution of these two charged residues by uncharged residues (E4Q/K5L) had a higher destabilizing effect than the combined changes made in Δ1–5 strongly suggests that the destabilizing effect is predominantly governed by substitution of the charged amino acids and that the three uncharged amino acids in the N-terminus play no major role in octamer stability.

Substrate-Induced Dissociation of Octameric into Dimeric Mi$_b$-CK. The dissociation rate of Mi$_b$-CK can be drastically increased by adding TSAC substrates. Several authors have presented strong evidence for a conformational change in dimeric, cytosolic M-CK induced by the TSAC substrates (Cohn, 1970; Milner-White & Watts, 1971). It is intriguing to assume that Mi$_b$-CK undergoes a similar substrate-induced conformational change (Wyss et al., 1993) and that this structural change facilitates the dissociation of octamers into dimers (Marcillat et al., 1987). Since upon simple dilution of the enzyme no conformational change is assumed to take place, the mechanisms of dissociation induced by dilution or by adding TSAC substrates might be quite different. To investigate this possibility, the effect of TSAC formation on

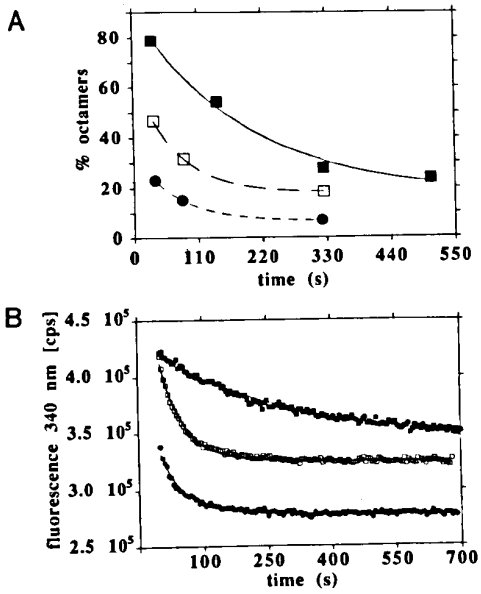

FIGURE 5: Substrate-induced dissociation of Mi_b-CK octamers into dimers. (A, top panel) Wild-type Mi_b-CK (■), point mutant E4Q (□) and double mutant E4Q/K5L (●) were diluted to 0.1 mg/mL in buffer A containing the TSAC substrates (see Materials and Methods) at 4 °C to start dissociation. Aliquots were analyzed by gel-permeation chromatography on a Superose 12 column, and the octamer/dimer ratio was determined. (B, bottom panel) Wild-type Mi_b-CK (■, upper curve), point mutant E4Q (□, middle curve), and double mutant E4Q/K5L (●, lower curve) were diluted to 0.05 mg/mL in buffer B at 30 °C (see Materials and Methods). Dissociation was started by the addition of TSAC substrates, and the decrease in intrinsic tryptophan fluorescence was determined as a measure of octamer dissociation. For both panels, data points were fitted to eq 1 (see Materials and Methods). Symbols represent actual data points and lines connecting the symbols represent the curves fitted to eq 1.

the rate of octamer dissociation was followed both by gel-permeation chromatography (Figure 5A) and additionally by monitoring the change in intrinsic tryptophan fluorescence as a direct measure of change in octamer content (Figure 5B; see Materials and Methods). The latter measurements were performed at different experimental conditions (higher temperature, lower protein and salt concentration) and allowed on-line detection of the octamer dissociation, without the time delay inherent with gel-permeation chromatography. The data points derived from both of these techniques were fitted to eq 1; the resulting dissociation rate constants are shown in Table 5. As with the dissociation in the absence of the TSAC substrates (Figure 4), the mutant Mi_b-CKs dissociated much faster than wild-type Mi_b-CK in the presence of TSAC. However, the formation of a TSAC increased the rate of octamer dissociation by 600–4000-fold compared to that seen upon dilution in the absence of substrates (compare $\tau_{1/2}$ in Table 4 to those in Table 5). Most interestingly, hardly any difference between the dissociation rates of point mutant E4Q and double mutant E4Q/K5L was found after formation of the TSAC (Table 5). This is in contrast to the results obtained for dilution-induced octamer dissociation where the dissociation rates for these two mutants differed more than 4-fold (Table 4). This suggests that the rate-limiting step in the substrate-induced dissociation is probably different from the one for the dilution-induced dissociation. For mutant 4–7, the rate of substrate-induced octamer dissociation was too fast to be monitored by either method.

DISCUSSION

Mi_b-CK was cleaved very specifically at the N-terminus by partially inhibited proteinase Lys C, which removed the first five amino acids. Cleavage was paralleled by partial dissociation of Mi_b-CK octamers into dimers. The unique and specific cleavage by proteinase Lys C of Mi_b-CK after Lys-5 is striking, since Mi_b-CK contains a number of other lysine residues as possible target sites for proteinase Lys C, and indicates that the N-terminal end of a Mi_b-CK subunit is accessible for the protease on the surface of the molecule. The N-terminus may act in octamer formation by providing a charged patch for ionic interactions between dimers, since the first seven amino acids of Mi_b-CK (TVHEKRK) contain a number of charged amino acids. To investigate this, a number of mutant Mi_b-CKs were constructed. In one mutant, the first five amino acids were deleted; in other mutants, one, two, and four of the charged residues were replaced by uncharged ones. Biochemical characterization of the expressed and purified mutant proteins strongly suggested that none of these changes in the N-terminal heptapeptide altered the correct folding and function of the mutant Mi_b-CKs.

Substitution of charged by uncharged amino acids at the N-terminus significantly influenced the octamer/dimer ratio under equilibrium conditions. At any given protein concentration the equilibrium for the mutant Mi_b-CKs was shifted towards the dimeric form (see Figure 3). The equilibria of wild-type and mutant Mi_b-CKs could be best described with the equation for case 2 (see Materials and Methods). The equilibrium constant K_4 was increased 50-fold in the single and double mutants and 100-fold in the quadruple mutant 4–7, compared to wild type. A cooperativity of octamer formation from Mi_b-CK dimers can be defined as the ratio K_3/K_4; this ratio was significantly decreased from 80 for the wild-type Mi_b-CK to 2 for point mutant E4Q and double mutant E4Q/K5L and to 1 for mutant 4–7. Interestingly, the data pertaining to the equilibrium condition were fitted

Table 5: Kinetics of Substrate-Induced Dissociation of Mi_b-CK Octamers[a]

	gel-permeation chromatography[b]		fluorescence spectroscopy[c]	
protein	half-life $\tau_{1/2}$ (s)	increase of rate constants relative to wild-type Mi_b-CK (x-fold)	half-life $\tau_{1/2}$ (s)	increase of rate constants relative to wild-type Mi_b-CK (x-fold)
wild-type Mi_b-CK	193.3	1	236.9	1
point mutant E4Q	29.0	6.7	39.2	6
double mutant E4Q/K5L	25.7	7.5	38.5	6.2

[a] The data points obtained from the time-dependent dissociation of octameric Mi_b-CK induced by TSAC substrates as shown in Figure 5 were fitted to eq 1 (see Materials and Methods). The half-lives shown were calculated as $\tau_{1/2} = \ln 2/k_1$. [b] Dissociation was induced at a protein concentration of 0.1 mg/mL in buffer A, at 4 °C, by the addition of TSAC substrates and monitored by gel-permeation chromatography. [c] Dissociation was induced at a protein concentration of 0.05 mg/mL in buffer B at 30 °C with TSAC substrates and monitored by measuring the change in intrinsic tryptophan fluorescence. Although the absolute $\tau_{1/2}$ values differ somewhat, due to the different conditions optimized for each individual method, the quantitative trend is very similar. The standard error of the half-lives of the curve fit was below 8%, except for the double mutant E4Q/K5L in the gel-permeation experiment, where it was 26%.

best when a tetrameric species was postulated. The proportion of tetramers computed by this model (case 2) for the wild-type Mi_b-CK was never significant over the whole range of protein concentration. In contrast, for the mutant Mi_b-CKs, the calculated proportion of tetramers reached up to 40% of the total oligomeric species. However, we could never detect experimentally a tetrameric species in gel-permeation chromatography, probably due to the fact that the tetramer readily dissociates into dimers under these conditions.

Determination of dilution-induced dissociation rates of all N-terminal mutants (see Table 4) showed that the single substitution of Glu-4 by Gln resulted in a mutant enzyme with a 13 times faster dissociation rate. The substitution of an additional residue (double mutant E4Q/K5L) or a total of four substitutions (mutant 4–7) resulted in a 55-fold and 146-fold increase, respectively. These results suggest either that, once the first charged residue within the N-terminus is substituted, the major part of octamer stability is lost, with additional substitutions showing a progressively smaller effect, or alternatively that Glu-4 is the most important of the four charged residues for octamer stabilization. The idea that a single charged residue may play a predominant role in the octamer stability is supported by the thermodynamic parameters derived from analysis of the octamer/dimer equilibrium (Table 3). A difference in 9–11 kJ/mol in free energy for octamer dissociation was calculated between wild-type Mi_b-CK and the mutant Mi_b-CKs; this energy value corresponds to about one or two ionic interactions (Cantor & Schimmel, 1980). A similar predominant role is played by a charged amino acid in the N-terminus of the GroEL protein; replacement of an N-terminal lysine by a glutamine completely blocks the formation of an oligomer (Horovitz et al., 1993).

Two possibilities for the octamer-stabilizing effect mediated by the N-terminus are conceivable: the two N-termini of a dimer may directly interact with regions of an adjacent dimer, or alternatively, the N-termini may influence neighboring regions within its "own" dimer, thus conveying to the dimer a structure competent for octamer formation. In the first case, deletion of the N-terminus would remove an important interaction domain, whereas in the second case, removal of the N-terminus would induce a conformational state of the dimers that would no longer be competent for oligomerization. Some evidence for an N-terminus-mediated intermolecular dimer–dimer interaction comes from the results seen in the initial protease digestion experiments, in which the octameric Mi_b-CK was digested much more slowly than the dimeric enzyme (Figure 2). One explanation for this phenomenon is that the N-terminal proteinase Lys C target site is fully protected in the native octamer against protease attack, while the same site is accessible in the small proportion of dimers present in all Mi-CK octamer solutions. Proteolytic cleavage of the N-terminus in the dimers then is shifting the octamer/dimer equilibrium toward the formation of more dimers, since the cleaved dimers are not contributing to the regular octamer/dimer equilibrium. The argument supporting this is the very long time needed (several days) to cleave Mi_b-CK. Preliminary data for X-ray analysis of octameric Mi_b-CK (W. Kabsch and T. Schnyder, unpublished information) also support the notion that the N-terminus may be located at the dimer–dimer interface. This proposed role of the Mi_b-CK N-terminus is also in agreement with the observation that terminal regions of oligomeric proteins are often located in the subunit interfaces and are involved in links that stabilize protein quaternary structure (Thornton & Sibanda, 1983); e.g., the N-terminal domain contributes to the tetrameric structure of lactate dehydrogenase (Holbrook et al., 1975).

The primary sequences of the mitochondrial creatine kinases, Mi_a-CK and Mi_b-CK, share approximately 80% identity, with their N-termini being conserved least (Haas & Strauss, 1990). Since it has been found that Mi_a-CK from chicken brain dissociates faster into dimers upon dilution than Mi_b-CK (Schlegel et al., 1988a) and that the dimer/octamer ratio at equilibrium is shifted more toward the octamer compared to Mi_b-CK (Wyss et al., 1990), the N-terminus may represent an important domain for octamer stability and its regulation in an isozyme-specific manner. Also consistent with the role of the N-terminus in the octamer formation is the fact that the N-termini of the dimeric, cytosolic CKs differ substantially from the mitochondrial ones.

While the destabilization of the octamer by the N-terminal mutations implies an important role for an ionic interaction, the ability of all the mutants to still form octamers to a variable extent suggests that additional regions of the molecule are involved in octamer stabilization. Indeed, combining the N-terminal mutant E4Q/K5L with the point mutant K191G (which also was noted to display a decreased octamer stability; R. Furter, unpublished result) further destabilized the octamer. At 16.4 mg/mL, this combined mutant was only able to form 13% octamers, and upon dilution to 0.1 mg/mL, the enzyme dissociated within 1 h completely into dimers (not shown). Furthermore, a chimera fused at amino acid 187 consisting of the N-terminal half of Mi_b-CK and the C-terminal half of cytosolic B-CK did not form octamers either, indicating an essential contribution toward octamer stability of yet another region in the C-terminal half of Mi_b-CK (R. Furter, unpublished). Recent results in our group showed that hydrophobic interactions contribute also to the octamer stability (Gross & Wallimann, 1993).

The question of whether the dimeric form of Mi_b-CK is present *in vivo* is still not resolved. The majority of Mi-CK is released from native mitochondrial membranes in its octameric form, independent of the releasing agent used (Marcillat et al., 1987). It is likely that the small proportion of dimeric molecules (≈10%; Schlegel et al., 1990) represents molecules that are also dimeric *in vivo*, since the release of Mi-CK from the membrane occurs rapidly, with a time scale that would not permit significant amounts of octamers to dissociate in solution (Schlegel et al., 1990). Despite the inability to recover large amounts of dimeric Mi-CK from intact mitochondria, however, a regulated dynamic octamer/dimer equilibrium and a differential interaction of octamers and dimers with mitochondrial membranes (Schlegel et al., 1990), governed by pH and nucleotide concentration, may still have to be considered as important (Wallimann et al., 1992).

Contact sites seem to be dynamic structures (Bücheler et al., 1991). Octameric Mi_b-CK was shown to mediate the formation and stabilization of contacts between two membranes *in vitro* much better than dimeric Mi_b-CK (Rojo et al., 1991). In addition, octameric Mi_b-CK displays features of a central channel leading from top to the bottom of the molecule that could be very well suited for a transport of substrates from inner to outer mitochondrial membrane while dimeric Mi_b-CK does not form such a channel. Therefore, it might be possible that octamerization is one of the inducers of contact site formation and/or stabilization, whereas dimerization may lead to a breakup of contact sites.

In this study, we identified an important octamerization domain of Mi_b-CK through a functional approach. The exact structural arrangement of the N-termini within the octamer

will only be solved once a high-resolution electron density map becomes available (Schnyder et al., 1990). In the meantime, the N-terminal mutants characterized here are valuable tools to study intrinsic structural and functional differences between the two oligomeric species of Mi-CK.

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