Limited Proteolysis of Creatine Kinase. Implications for Three-Dimensional Structure and for Conformational Substates[†]

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ABSTRACT: Proteinase K, subtilisin, pronase E, elastase, bactotrypsin, and thermolysin are all shown here to cleave native mitochondrial creatine kinase from chicken heart (Mi_b-CK) very specifically at a single site, either before or after Ala-323. In analogy with hen egg ovalbumin, where the same proteases all cleaved the polypeptide chain very specifically around Ala-352, Ala-323 of Mi_b-CK may be located in an exposed surface loop that is sensitive to protease attack. Gel permeation chromatography demonstrated that the two proteolytic fragments of Mi_b-CK with M_r's of ~37 000 and ~6000 remain associated with each other. Proteinase K cleavage did not influence the octamer to dimer ratio of Mi_b-CK, indicating that selective cleavage after Ala-323 has no direct effect on dimer–dimer interfaces within the octamer. However, upon addition of MgADP plus creatine and nitrate to induce a transition-state analogue complex of the enzyme, native Mi_b-CK dissociated much more readily into dimers than proteinase K-digested Mi_b-CK. Furthermore, proteinase K cleavage of Mi_b-CK resulted in 2–11-fold decreases in the V_{max} values, as well as in 6–23-fold increases in the K_m values for phosphocreatine, creatine, and MgATP, whereas the K_d values for both MgATP and creatine were unaffected. Consequently, proteinase K cleavage of Mi_b-CK does not affect substrate binding per se, but interferes with substrate-induced conformational changes which are essential for catalysis and which mediate the synergism in substrate binding as it is observed with the unmodified enzyme. Together with sequence comparisons and a variety of biochemical and biophysical similarities between 3-phosphoglycerate kinase and CK, the results strongly indicate that CK, like other phosphotransferases, is a hinge-bending enzyme composed of two domains undergoing conformational rearrangements upon substrate binding.

Creatine kinase (CK)[1] isoenzymes play a crucial role in the energy metabolism of tissues with high and fluctuating energy demands like heart, brain, skeletal muscle, spermatozoa, and retina [for reviews, see Wallimann et al. (1992) and Wyss et al. (1992)]. The three cytosolic CK isoenzymes are dimeric molecules composed of two types of subunits (MM-CK, MB-CK, and BB-CK, M standing for the "muscle" isoform, and B standing for the "brain" isoform). In contrast, the two Mi-CK isoenzymes (Mi_a-CK from brain and Mi_b-CK from heart) have the unique property of forming dimeric as well as octameric molecules which, depending on pH, ionic strength, protein and substrate concentrations, and other factors, are readily interconvertible. Electron microscopy revealed that the octameric Mi-CK molecules are highly ordered, cube-like structures displaying a 4-fold symmetry, a side length of

approx. 10 nm, and a central cavity or channel. However, the crystal structure is still unsolved, even though a number of groups reported on the crystallization of CK isoenzymes and on preliminary X-ray data [for references, see Schnyder et al. (1991)].

A fundamental property of kinases in general is that, in the transition state of the reaction, water has to be excluded from the surroundings of the bound substrates in order to avoid hydrolysis of the phosphoryl compounds and concomitant energy dissipation. In kinases for which the three-dimensional structure is already known [e.g., adenylate kinase, hexokinase, and 3-phosphoglycerate kinase; see Anderson et al. (1979), Harlos et al. (1992), and Gerstein et al. (1993)], this is achieved by an induced-fit mechanism of enzyme action (Koshland & Neet, 1968). The substrate binding sites of these enzymes are located on neighboring domains which, in the absence of substrates, are separated by a deep cleft. Substrate binding induces a movement of the domains relative to each other, resulting in a catalysis-competent orientation of the substrates, in the closure of the cleft between the two domains, and thus in the exclusion of water from the transition state of the enzyme.

For a variety of proteins that were or still are difficult to crystallize (e.g., actin, myosin, dynein, or kinesin), limited proteolysis proved to be a powerful tool to study structure–function relationships [Mornet et al., 1984; Mocz et al., 1988; for reviews, see Hambly et al. (1986), Vale (1990), and Vibert and Cohen (1988)]. Limited proteolysis of CK isoenzymes, however, has been described in just a few studies, and most of them were performed with proteinase K. This latter serine protease was shown to selectively "nick" the cytosolic CK isoenzymes, without dissociation of the two proteolytic fragments, and to cause a very pronounced or even complete

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[1] Abbreviations: CK, creatine kinase; Mi_b-CK, mitochondrial CK isoenzyme from chicken heart; BB-CK, cytosolic CK isoenzyme from chicken brain; Cr, creatine; PCr, phosphocreatine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PGK, 3-phosphoglycerate kinase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TSAC, transition-state analogue complex; EPR, electron paramagnetic resonance.

inactivation of the enzymes (Williamson et al., 1977; Price et al., 1981; Lough et al., 1985; Morris et al., 1985; Lebherz et al., 1986). In the present investigation, limited proteolysis of chicken Mi_b-CK was used to obtain new insights into (i) the structural organization of CK protomers and of octameric Mi-CK molecules, (ii) the relationships between substrate-induced conformational changes of Mi_b-CK and (reversible) dimer–octamer interconversions, and (iii) the mechanism of enzyme action. The results obtained strongly suggest that CK is a hinge-bending enzyme similar to 3-phosphoglycerate kinase.

MATERIALS AND METHODS

Materials. Chicken Mi_b-CK was purified as described previously (Schlegel et al., 1988), either from chicken heart or from *Escherichia coli* strains overexpressing this protein (Furter et al., 1992). Mi_b-CK from both sources did not display any qualitative or quantitative differences. $\Delta 1$–5 Mi_b-CK and C278S Mi_b-CK were kindly provided by P. Kaldis and R. Furter, respectively. Chicken BB-CK was purified according to Quest et al. (1989). Hen egg ovalbumin was obtained from Pharmacia (Dübendorf, Switzerland); subtilisin and PMSF, from Serva (Catalys SA, Wallisellen, Switzerland); bactotrypsin (porcine pancreas), from Difco (Chemie Brunschwig AG, Basel, Switzerland); PCr, from Calbiochem (Lucerne, Switzerland); pronase E, from Merck (Auer Bittmann Soulié AG, Basel, Switzerland); lobster tail muscle arginine kinase and Cr, from Sigma (Buchs, Switzerland); and ADP, ATP, proteinase K, elastase (porcine pancreas), trypsin (bovine pancreas), thermolysin, chymotrypsin A_4 (bovine pancreas), and yeast 3-phosphoglycerate kinase, from Boehringer Mannheim (Rotkreuz, Switzerland). All other chemicals were at least of reagent grade. Before the experiments, Mi_b-CK, BB-CK, arginine kinase, and PGK were extensively dialyzed against the appropriate buffers.

Proteolysis Experiments. In experiments with trypsin and bactotrypsin, the concentration of chicken Mi_b-CK, chicken BB-CK, hen egg ovalbumin, or lobster arginine kinase was chosen to be 0.3 mg/mL, and with all other proteases, 1 mg/mL. Experiments with proteinase K, subtilisin, pronase E, elastase, and chymotrypsin A_4 were performed in buffer A (50 mM sodium phosphate, 150 mM NaCl, 0.2 mM Na_2-EDTA, 1 mM NaN_3, and 0.5 mM 2-ME), pH 9.0; experiments with trypsin and bactotrypsin, in buffer B (0.2 M sodium phosphate and 0.5 mM 2-ME, pH 7.0); and experiments with thermolysin, in buffer C (50 mM Tris, 10 mM $CaCl_2$, and 0.5 mM 2-ME, pH 8.0). After a 10-min preincubation of the samples at 30 °C, proteinase K (1:4000 w/w), subtilisin (1:100), pronase E (1:50–60), elastase (1:100), bactotrypsin (1:10), thermolysin (1:90), trypsin (1:10), or chymotrypsin (1:10) was added and the incubations were continued for the indicated periods of time at 30 °C. In experiments with proteinase K, subtilisin, pronase E, trypsin, and chymotrypsin, proteolysis was stopped by addition of an equivalent volume of solution D (5 mM PMSF in 20% ethanol) and a further 10-min incubation at 30 °C. These samples were subsequently used either for measurements of enzymatic activity by the pH-stat method (see below), for SDS–PAGE (Laemmli, 1970), or for Tricine–SDS–PAGE (Schägger & von Jagow, 1987). Control experiments demonstrated that PMSF at a final concentration of 2.5 mM completely inhibited proteolysis. In experiments with elastase, bactotrypsin, and thermolysin, proteolysis was stopped by directly transferring the samples into boiling SDS–PAGE sample buffer. The extent of cleavage of Mi_b-CK was quantified either by activity measurements or

by densitometric scanning of 10% SDS–polyacrylamide gels with a Shimadzu CS-930 scanner. Apparent first-order rate constants of cleavage of Mi_b-CK were calculated from the slopes of semilogarithmic plots (see Figure 2) derived from proteolysis time-course experiments (for example, Figure 1).

Gel Filtration Experiments. Gel permeation chromatography on a Superose 12 HR 10/30 column (FPLC, Pharmacia) was used for the determination of the octamer to dimer ratio of Mi_b-CK. Buffer A (containing 2 mM 2-ME), pH 7.2, served as elution buffer. To investigate the equilibrium octamer to dimer ratio at Mi_b-CK concentrations higher than 0.5 mg/mL (Figure 4), both uncleaved and proteinase K-digested Mi_b-CK were concentrated by Centricon 10 microconcentrators (Amicon). For Mi_b-CK concentrations below 0.5 mg/mL, uncleaved and "nicked" Mi_b-CK were diluted by a 1:1 mixture of buffer A, pH 9.0, and solution D. Control experiments demonstrated that, under these conditions, equilibration between dimeric and octameric Mi_b-CK rapidly occurs. Before gel permeation chromatography, all samples were dialyzed against buffer A (containing 2 mM 2-ME), pH 7.2, and the protein concentrations were subsequently determined according to Bradford (1976).

Kinetic Measurements. The enzymatic activity of Mi_b-CK was measured by the pH-stat method. One international unit (IU) corresponds to 1 μmol of PCr or ATP transphosphorylated/min at 25 °C and pH 7.00 or 8.00, respectively. Under standard conditions, Mi_b-CK activity was determined in the direction of ATP synthesis in an assay mixture containing 65 mM KCl, 8.5 mM $MgCl_2$, 85 μM EGTA, 4 mM ADP, and 10 mM PCr, pH 7.00. As titrant, 20 mM HCl was used. For the determination of K_m (PCr) and V_{max} in the direction of ATP synthesis, [PCr] was varied from 0.5 to 30 mM, while [ADP] and $[Mg^{2+}]$ were held constant at 4 and 5 mM, respectively.

Activity measurements in the direction of PCr synthesis were performed in an assay mixture containing 65 mM KCl, 85 μM EGTA, and $MgCl_2$ at pH 8.00, with $[Mg^{2+}]$ always exceeding [ATP] by 1 mM; 20 mM NaOH was used as titrant. For the determination of the K_d and K_m values for both MgATP and Cr as well as of the V_{max} value in the direction of PCr synthesis, six different MgATP concentrations (1.2, 1.7, 2.5, 3.5, 5, and 8 mM) were used, each at four different Cr concentrations (35, 50, 65, and 100 mM). All kinetic constants were determined by initial velocity measurements and analyzed using the program package written by Cleland (1979) as adapted for personal computers by R. Viola (Akron University, Akron, OH). The values are given as mean ± SE.

Other Methods. For N-terminal amino acid sequencing, SDS– and Tricine–SDS–polyacrylamide gels were directly semi-dry-blotted at a constant current of 1 mA/cm^2 for 105 min onto poly(vinyl difluoride) (PVDF) membranes (Immobilon-P^{SQ}; Millipore AG, Volketswil, Switzerland). Automated Edman degradation was done on an Applied Biosystems 470A sequencer with on-line microbore phenylthiohydantoin detection. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad reagent and bovine serum albumin as standard.

In order to investigate whether limited proteolysis of Mi_b-CK is paralleled by a transition from a stressed to a relaxed conformation as was previously shown for certain members of the serine protease inhibitor superfamily (Pemberton et al., 1988), uncleaved as well as proteinase K-digested Mi_b-CK (0.1 mg/mL) was incubated in buffer A (containing 2 mM 2-ME), pH 7.2, for 1 h at temperatures ranging from 30 to 90 °C. Denatured Mi_b-CK was subsequently pelleted by
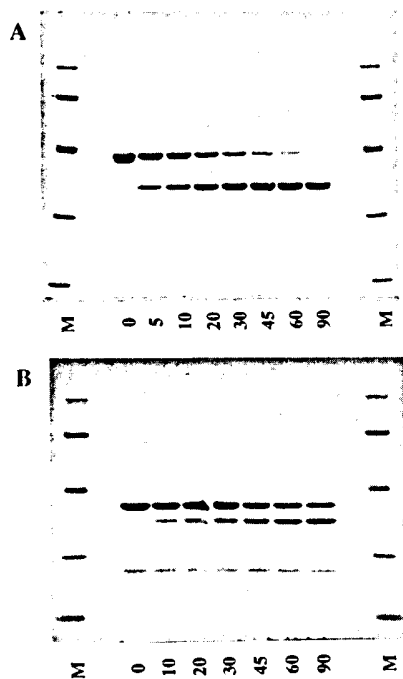
FIGURE 1: Time course of proteolysis of Mi$_b$-CK with proteinase K and of arginine kinase with pronase E. Chicken Mi$_b$-CK (A) and lobster arginine kinase (B) at a concentration of 1 mg/mL were digested with proteinase K and pronase E, respectively, for the indicated periods of time (min) as described under Materials and Methods. After electrophoresis on 10% SDS–polyacrylamide gels, the proteins (5–6 μg/lane) were visualized by Serva blue R. Note the highly selective fragmentation in both cases. Whereas native Mi$_b$-CK and arginine kinase have apparent M_r's of 43 100 ± 300 (43 181) and 38 900 ± 600 (39 844), the respective values for the proteolytic fragments are 36700 ± 300 (36 786) and 36 100 ± 600 (35 362) [mean ± SD; n = 5 for all values; numbers in parentheses were calculated from the cDNA sequences and from the experimentally determined cleavage sites (see Table I)]. M: low molecular weight standard proteins (Bio-Rad) comprising phosphorylase B (97 400), bovine serum albumin (66 200), ovalbumin (42 700), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400); the last is not visible on 10% SDS–polyacrylamide gels. The additional band with an apparent M_r of approx. 29 000 in (B) represents an impurity present in the commercial preparation of arginine kinase.

centrifugation at 15 000g and 4 °C for at least 35 min in a Heraeus Christ Biofuge A (Heraeus AG, Zürich, Switzerland). The proportion of Mi$_b$-CK in supernatants (native) and pellets (denatured) was measured either by SDS–PAGE, followed by densitometric scanning of the lanes, or by determination of the protein concentration.

RESULTS

Time Course of Proteolysis of Mi$_b$-CK and Arginine Kinase. Chicken Mi$_b$-CK was selectively cleaved (nicked) by proteinase K (1:4000, w/w) in a time-dependent manner (Figure 1A), resulting in two fragments with apparent M_r's of 36 700 and ~6400; the latter is only visible on Tricine–SDS–polyacrylamide gels (see the band marked by a star in Figure 3B). Likewise, lobster tail muscle arginine kinase was selectively and time-dependently cleaved by pronase E (Figure 1B), resulting in two fragments with apparent M_r's of 36 100 and ~4500. The lack of additional minor degradation bands suggests that cleavage in both cases is highly specific and that, after scission of the sensitive peptide bond, no additional cleavage sites become available to the proteases, even though proteinase K and pronase E are in general rather unspecific proteases.
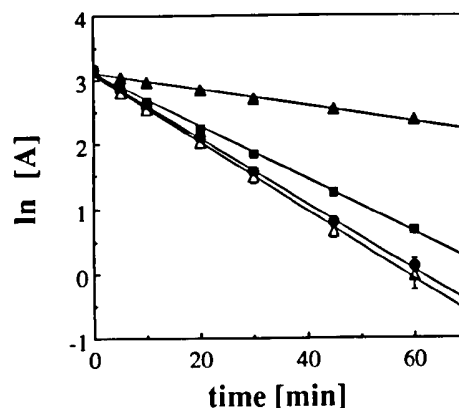

FIGURE 2: Effect of CK substrates on the rate of proteinase K cleavage of Mi$_b$-CK. Chicken Mi$_b$-CK (1 mg/mL) was digested with proteinase K for the indicated periods of time as described under Materials and Methods either in the absence (●) or in the presence of the following ligands: (△) 20 mM PCr; (■) 4 mM ADP and 5 mM MgCl$_2$; or (▲) 4 mM ADP, 5 mM MgCl$_2$, 20 mM Cr, and 50 mM KNO$_3$. Proteolysis of Mi$_b$-CK was followed by SDS–PAGE and densitometric scanning of the lanes. [A] represents the concentration of native, undigested Mi$_b$-CK and is given in μM. From the semilogarithmic plot, the following apparent first-order rate constants were calculated (mean ± SD; n = 3): without competitor, (8.43 ± 0.39) × 10⁻⁴ s⁻¹; 20 mM PCr, (8.83 ± 0.55) × 10⁻⁴ s⁻¹; 4 mM ADP and 5 mM MgCl$_2$, (6.82 ± 0.25) × 10⁻⁴ s⁻¹; 4 mM ADP, 5 mM MgCl$_2$, 20 mM Cr, and 50 mM KNO$_3$, (2.05 ± 0.10) × 10⁻⁴ s⁻¹. When cleavage of Mi$_b$-CK was followed by measurements of enzymatic activity, a plot very similar to the one shown here was obtained only under the assumption that fully digested Mi$_b$-CK under the standard pH-stat conditions retains 16% of the enzymatic activity of the undigested enzyme.

In similar time-course experiments, Mi$_b$-CK was digested with proteinase K in the presence of various substrates and was subsequently analyzed by SDS–PAGE (not shown). The concentration of native, uncleaved Mi$_b$-CK was calculated from densitometric scanning of the respective SDS–PAGE lanes. Plotting these values against time (Figure 2) allowed the determination of apparent first-order rate constants of proteinase K cleavage of Mi$_b$-CK and thus also the quantitation of the effect of substrates on the cleavage rate. Similarly to previous experiments on rabbit MM-CK (Williamson et al., 1977), the rate of proteinase K cleavage of Mi$_b$-CK was not influenced by 20 mM PCr, whereas it was decreased 20% by 4 mM ADP plus 5 mM MgCl$_2$, or even 75% by formation of a transition-state analogue complex of Mi$_b$-CK (Mi$_b$-CK, 4 mM ADP, 5 mM MgCl$_2$, 20 mM Cr, and 50 mM nitrate). Under all conditions tested, proteinase K cleavage of Mi$_b$-CK proved to be a monophasic process, in contrast to previously published, weak evidence suggesting that formation of a transition-state analogue complex of rabbit MM-CK results in biphasic degradation and inactivation by proteinase K (Williamson et al., 1977).

Limited Proteolysis of Mi$_b$-CK with Various Proteases: Location of the Cleavage Site and Effect on the Overall Three-Dimensional Structure. Since it was surprising that Mi$_b$-CK and arginine kinase were cleaved by nonspecific proteases (proteinase K and pronase E, respectively) at a single site, a number of other proteases were examined. Limited proteolysis of native Mi$_b$-CK by proteinase K, subtilisin, pronase E, elastase, bactotrypsin, and thermolysin resulted in highly selective cleavage of the polypeptide chain (Figure 3A,B). Each of these proteases produced only two fragments which showed seemingly identical gel migration patterns, indicating that Mi$_b$-CK contains a single site that is hypersensitive to protease attack. This conclusion was corroborated by N-terminal sequencing of all fragments (Table I), proving that
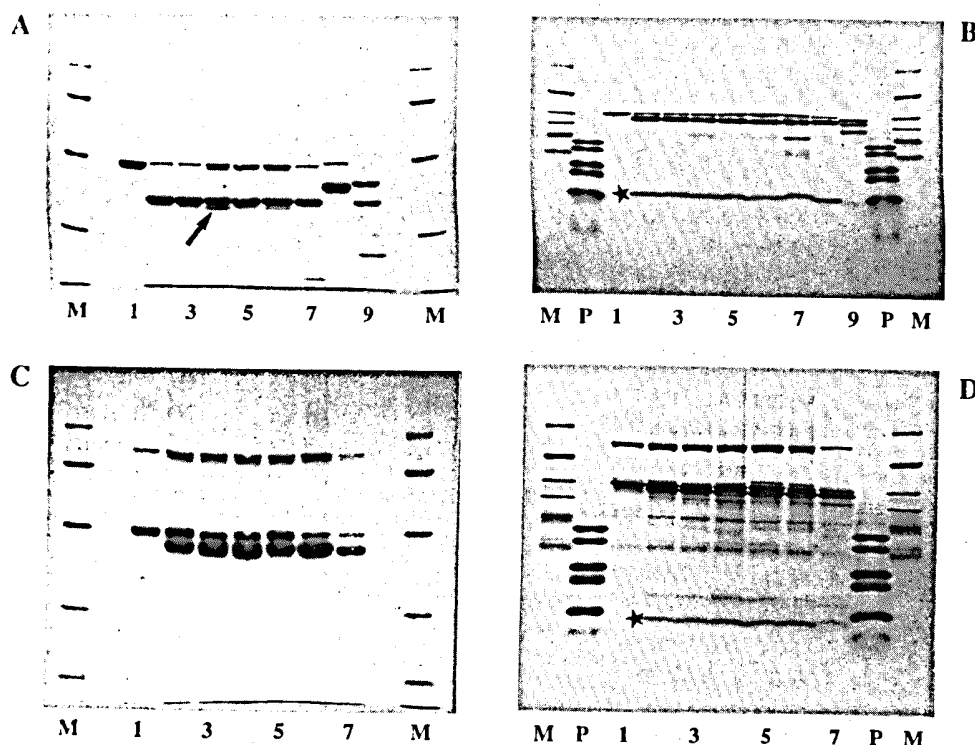
FIGURE 3: Cleavage of Mib-CK (A,B) and ovalbumin (C,D) with various proteases. Chicken Mib-CK and hen egg ovalbumin (lanes 1) were digested with proteinase K (lanes 2; 90 min for Mib-CK, 2 h for ovalbumin), subtilisin (3; 20 min), pronase E (4; 90 min), elastase (5; 90 min), bactotrypsin (6; 90 min), and thermolysin (7; 4 h) as described under Materials and Methods. Lanes 8 and 9 in panels A and B contain BB-CK digested for 90 min with proteinase K and lobster arginine kinase digested for 60 min with pronase E, respectively. Proteins were separated either by SDS–PAGE (A,C) or by Tricine–SDS–PAGE (B,D). Note that all six proteases apparently generated the same two fragments. The small fragment is only visible on Tricine–SDS–polyacrylamide gels and is marked by a star. The band labeled by an arrow in panel A is due to the combination of cleavage at the protease-hypersensitive site and removal of the first six N-terminal amino acids (see the text). The prominent band in panels C and D with an apparent M_r of 77 000 represents an impurity present in the commercial preparation of ovalbumin. The additional bands between the two proteolytic fragments of ovalbumin in panel D most probably represent minor degradation products. M, low molecular weight standards (Bio-Rad; see Figure 1); P, peptide markers (Pharmacia-LKB) comprising native horse heart myoglobin and cyanogen bromide fragments thereof with M_r's of 16 900, 14 400, 10 000, 8200, 6200, and 2500 (the last is only barely visible).

Table I: Elution Volumes of "Nicked" Mib-CK from a Gel Filtration Column, and Amino-Terminal Sequences of the Proteolytic Fragments of Mib-CK, BB-CK, Arginine Kinase, and Ovalbumin[a]

	elution position of octameric Mib-CK (mL)	elution position of dimeric Mib-CK (mL)	amino-terminal sequence		
			large proteolytic fragment	small proteolytic fragment	small fragment of ovalbumin
native protein	10.22 ± 0.05	12.70 ± 0.05	TVHEKRKL[b]		
proteinase K	10.19 ± 0.03	12.73 ± 0.02	TVHEKRKL	AVADVYDI	XVSEEFR
subtilisin	10.24 ± 0.02	12.74 ± 0.04	TVHEKRK	AVADVYD	XVSEEFR
pronase E	10.27 ± 0.09	12.73 ± 0.06	TVHEKRK	AVAXVY	XVSEEFRAD
elastase	10.20 ± 0.02	12.68 ± 0.04	TVHEKRK	AVADVYD	XVSEEF
bactotrypsin	10.22 ± 0.04	12.68 ± 0.01	TVHEKR(K)	AVAXVY	(S)VSEEFR
thermolysin	10.19 ± 0.03	12.68 ± 0.05	TVHEKR(K)	AAVADVY[c]	(AAS)VSEEF
				AVADVYD	
BB-CK/proteinase K			PFSN(S)HN	AVGGVFD	
AK/pronase E			d	AEGGIYDISN	

[a] Limited proteolysis of chicken Mib-CK, chicken BB-CK, lobster arginine kinase, and hen egg ovalbumin was performed as described under Materials and Methods. A Superose 12 gel filtration column (FPLC, Pharmacia) was used to determine the elution volumes of octamers and dimers of both uncleaved and "nicked" Mib-CK. Amino-terminal sequencing of the proteolytic fragments of Mib-CK and ovalbumin revealed that the proteins were nicked by all six proteases at a single site. [b] The amino terminus of Mib-CK deduced from the cDNA sequence (Hossle et al., 1988). [c] Amino-terminal sequencing of the small proteolytic fragment of thermolysin-digested Mib-CK revealed two species. The peptide with the upper amino terminus represented approx. 2/3, and that with the lower one approx. 1/3, of the sample. [d] The amino termini of both the native arginine kinase and the large proteolytic fragment thereof were blocked.

proteinase K, subtilisin, pronase E, elastase, and bactotrypsin cleave Mib-CK between Ala-323 and Ala-324. Limited proteolysis by thermolysin is an exception to the rule, in as far as the major cleavage site was identified between Thr-322 and Ala-323. In addition, N-terminal sequencing of the band marked by an arrow in Figure 3A [(KL)FPP(S)A] revealed that the satellite bands of both the native, uncleaved protein and the large fragments of pronase E- and bactotrypsin-treated Mib-CK (Figure 3A, lanes 4 and 6) are likely due to

(additional) cleavage at the very N-terminus, in accordance with proteinase Lys-C experiments on chicken Mib-CK that resulted in very selective removal of the first five amino acids (Kaldis et al., 1993).

Chicken BB-CK was selectively cleaved by proteinase K between Ala-328 and Ala-329, and lobster arginine kinase was selectively cleaved by pronase E between Glu-316 and Ala-317 [Figure 3A,B, lanes 8 and 9; amino acid numbering is deduced from the respective cDNA sequences (Hossle et

al., 1988; Dumas & Camonis, 1993)]. These cleavage sites coincide in location within the primary structure exactly with the protease-hypersensitive site of Mi_b-CK. Since in addition native rabbit MM-CK, chicken MM-CK, and chicken BB-CK were previously shown to be selectively cleaved by proteinase K and pronase E (Williamson et al., 1977; Price et al., 1981; Lough et al., 1985; Morris et al., 1985; Lebherz et al., 1986), these data indicate that a protease-hypersensitive site may be a general phenomenon of all phosphagen kinases.

In contrast to the results presented so far, native Mi_b-CK proved to be highly resistant to (pure) trypsin and chymotrypsin A_4, even at high concentrations of these proteases (1:10, w/w), thus corroborating previous studies which also revealed that CK is resistant to trypsin digestion (Price & Stevens, 1982; Morris, 1989). These findings may be explained by the lack of basic and aromatic residues in close proximity to Ala-323. Native lobster arginine kinase was insensitive to digestion by both proteinase K and trypsin (not shown).

Gel permeation chromatography did not reveal any shifts in the elution positions of Mi_b-CK with any of the proteases (Table I). In addition, upon selective cleavage of the Mi_b-CK polypeptide chain either before or after Ala-323, no additional sites became susceptible to proteolytic attack (Figure 3A,B), suggesting that the overall three-dimensional structure of Mi_b-CK is not affected by limited proteolysis (see also Discussion). The same holds true for proteinase K-digested rabbit MM-CK, where native PAGE, gel permeation chromatography, and cross-linking experiments also revealed no dissociation of the fragments under non-denaturing conditions (Williamson et al., 1977; Price et al., 1981).

Comparison with Ovalbumin. A specificity in protein cleavage analogous to the one observed for Mi_b-CK (Figure 3A,B) was also found for hen egg ovalbumin (Figure 3C,D). The same six proteases as in the case of Mi_b-CK selectively cleaved ovalbumin at a single site, either between Ala-352 and Ser-353 or between Asp-350 and Ala-351 (Table I). These findings agree with previous investigations showing that subtilisin BPN', elastase, thermolysin, and bromelain cleave ovalbumin at a single site, and that the cleavage site for both elastase- and subtilisin BPN'-digestion is located between Ala-352 and Ser-353 (Wright, 1984).

Ovalbumin (385 amino acids, M_r 42 750) belongs to the serine protease inhibitor (serpin) superfamily, all the members of which are selectively cleaved near the C-terminus by a variety of proteases. Upon limited proteolysis, the "real" serine protease inhibitors (α_1-antitrypsin, antithrombin, etc.) undergo a transition from a native, stressed (S) to a relaxed (R) conformation that is associated with a considerable increase in thermal stability (Pemberton et al., 1988). Ovalbumin and angiotensinogen, however, which display no protease inhibitor activity, lack such a pronounced conformational rearrangement (Stein et al., 1989). X-ray crystallography and biochemical experiments on ovalbumin, α_1-antitrypsin, and other serpins have shown that, upon selective "nicking" of the polypeptide chain, the two proteolytic fragments, as in Mi_b-CK, remain associated with each other and that the protease-hypersensitive site is located in an exposed loop on the surface of the molecule (Wright, 1984; Pemberton et al., 1988; Huber & Carrell, 1989; Wright et al., 1990). Since in addition the amino acid sequence around Ala-323 of Mi_b-CK displays considerable similarity to the protease-hypersensitive stretch of ovalbumin and other serpins (not shown), it is tempting to postulate that Ala-323 of Mi_b-CK is also part of an exposed surface loop that is easily accessible to proteases.
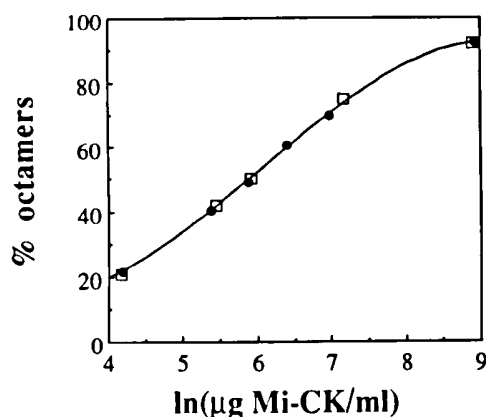
FIGURE 4: Effect of proteinase K cleavage on the octamer to dimer ratio of Mi_b-CK. Chicken Mi_b-CK (1 mg/mL) was digested with proteinase K for 90 min as described under Materials and Methods. Control (uncleaved) Mi_b-CK was treated identically, except that proteinase K was omitted from the incubation. Subsequently, aliquots of the incubation mixtures were brought to the appropriate Mi_b-CK concentrations, and equilibration between dimeric and octameric Mi_b-CK molecules was allowed to occur. Finally, the octamer to dimer ratios of both uncleaved (□) and proteinase K-digested (●) Mi_b-CK were analyzed by gel permeation chromatography.

Proteinase K Cleavage and the Octamer to Dimer Ratio of Mi_b-CK. Compared to the cytosolic CK isoenzymes MM-, MB-, and BB-CK which exclusively form dimeric molecules, the mitochondrial CK isoenzymes have the unique property of forming both dimeric and octameric molecules [for a review, see Wyss et al. (1992)]. The two oligomeric forms are readily interconvertible, depending on Mi-CK concentration, salt concentrations, pH, and other conditions. For example, formation of a transition-state analogue complex of Mi_b-CK (0.1 mg/mL in buffer A, pH 7.2) with MgADP, Cr, and nitrate (where the latter simulates the transferable phosphoryl group in the transition state of the reaction) results in rapid and almost complete dissociation of the octamers into dimers. Under in vivo conditions, however, Mi-CK is thought to be predominantly octameric.

As already stated above, selective nicking of the Mi_b-CK polypeptide chain by six different proteases did not influence the elution positions of dimeric and octameric Mi_b-CK from a gel filtration column (Table I). Furthermore, in the absence of substrates, uncleaved and proteinase K-digested Mi_b-CK displayed indistinguishable dependencies of the octamer to dimer ratio on the Mi_b-CK concentration (Figure 4), thus corroborating that, under these conditions, limited proteolysis affects neither the overall three-dimensional structure nor the dimer–dimer interfaces within the octamer.

In order to compare the cleavage rates of octameric and dimeric Mi_b-CK, octameric wild-type Mi_b-CK (87% octamers), dimeric wild-type Mi_b-CK (89% dimers), octameric Δ1-5 Mi_b-CK (85% octamers; Δ1-5 Mi_b-CK is a mutant expressed in *E. coli* that lacks the first five amino acids and displays a decreased octamer stability, but has normal kinetic properties), and dimeric Δ1-5 Mi_b-CK (93% dimers) were digested with proteinase K (1:1150, w/w) at a concentration of 0.1 mg/mL in buffer A, pH 7.2, for various periods of time. Control gel filtration runs demonstrated that during the experiment the proportion of octamers and dimers in any case changed only little. SDS-PAGE showed that dimeric and octameric Mi_b-CK were selectively cleaved at the same single site (not shown). Together with the indistinguishable cleavage rates observed for Mi_b-CK octamers and dimers [determined as exemplified in Figures 1 and 2; apparent first-order rate constants of $(3.51 \pm 0.16) \times 10^{-4}, (3.27 \pm 0.11) \times 10^{-4}, (3.54$
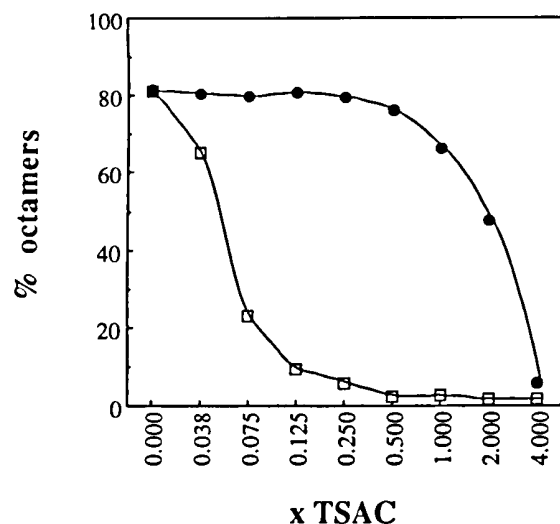
x TSAC

FIGURE 5: Effect of proteinase K cleavage on substrate-induced dissociation of Mi$_b$-CK octamers. Chicken Mi$_b$-CK (1 mg/mL) was digested with proteinase K for 90 min as described under Materials and Methods. Control (uncleaved) Mi$_b$-CK was treated identically, except that proteinase K was omitted from the incubation. Both native, uncleaved (□) and proteinase K-digested (●) Mi$_b$-CK were incubated for 24 h at 4 °C in buffer A (containing 2 mM 2-ME), pH 7.2, with the respective concentrations of substrates. 1× TSAC (transition-state analogue complex inducing substrates) corresponds to 4 mM ADP, 20 mM Cr, and 50 mM nitrate. [Mg^{2+}] always exceeded [ADP] by 1 mM. At the end of the incubation, the proportion of Mi$_b$-CK octamers was determined by gel permeation chromatography.

± 0.28) × 10^{-4}, and (3.77 ± 0.10) × 10^{-4} s^{-1}, respectively; mean ± SD; n = 3], this suggests that formation of octamers out of Mi$_b$-CK dimers does not impose steric constraints on the accessibility of the protease-hypersensitive stretch for proteinase K. Inother words, the cleavage site is exposed on the surface in both Mi$_b$-CK dimers and octamers.

A different picture emerges in the presence of substrates. When Mi$_b$-CK was incubated for 24 h at 4 °C with increasing concentrations of the transition-state analogue complex inducing substrates (MgADP, Cr and nitrate), native, un-cleaved Mi$_b$-CK proved to be much more sensitive to the substrates and more readily dissociated into dimers than proteinase K-digested Mi$_b$-CK (Figure 5). Therefore, pro-teinase K cleavage may either affect substrate binding itself or prevent substrate-induced conformational changes which lead to destabilization of the Mi$_b$-CK octamers.

Effect of Proteinase K Cleavage on the Kinetic Properties of Mi$_b$-CK. Activity measurements under standard pH-stat conditions revealed that limited proteolysis of chicken Mi$_b$-CK is paralleled by a pronounced inactivation of the enzyme. As can be seen in Figure 6, degradation and inactivation of Mi$_b$-CK by proteinase K were almost linearly related. Extrapolation to zero concentration of uncleaved enzyme indicates that quantitatively nicked Mi$_b$-CK under these experimental conditions retained approx. 16% of the original enzymatic activity, thus contrasting with previous investiga-tions on rabbit MM-CK, chicken MM-CK, and chicken BB-CK where proteinase K cleavage was paralleled by complete or almost complete inactivation of the enzyme (Williamson et al., 1977; Lough et al., 1985; Lebherz et al., 1986; Morris, 1989).

More extensive kinetic analysis (Table II) showed that the K_d values of proteinase K-digested Mi$_b$-CK for both MgATP and Cr were virtually indistinguishable from the K_d values of native, uncleaved Mi$_b$-CK (Furter et al., 1993). This indicates that substrate binding per se is not influenced by proteinase
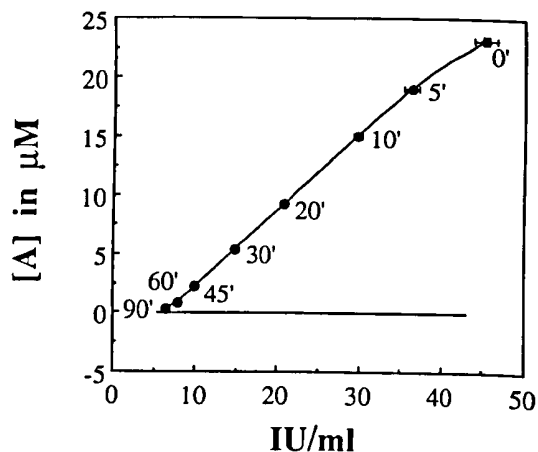
FIGURE 6: Correlation between degradation of Mi$_b$-CK by proteinase K and loss of enzymatic activity. Chicken Mi$_b$-CK (1 mg/mL) was digested with proteinase K for the indicated periods of time as described under Materials and Methods. After addition of PMSF to irreversibly inactivate proteinase K, the samples were used both for measurements of enzymatic activity and for SDS-PAGE. In the latter case, the concentration of undigested Mi$_b$-CK ([A]) was determined by densitometric scanning of 10% SDS-polyacrylamide gels stained with Serva blue R (see Figure 1A).

K cleavage, in general agreement with the data of Price et al. (1981) who found that, in the absence of other substrates, native and proteinase K-digested rabbit MM-CK have the same affinity for ADP. Binding of Mg^{2+} to Mi$_b$-CK was also not affected by proteinase K cleavage (not shown). In contrast, proteinase K-digested Mi$_b$-CK displayed 11- and 2-fold-decreased V_{max} values in the direction of PCr synthesis and ATP synthesis, respectively, as well as a 6-fold-increased K_m(Cr), a 6-fold-increased K_m(MgATP), and a 23-fold-increased K_m(PCr) compared to native, uncleaved Mi$_b$-CK. Consequently, the K_m values of proteinase K-digested Mi$_b$-CK for both MgATP and Cr were higher than the respective K_d values, whereas the opposite is true for uncleaved Mi$_b$-CK (Furter et al., 1993).

To conclude, these results demonstrate that proteinase K cleavage of Mi$_b$-CK prevents synergism in substrate binding. Such synergism has been observed for all native CK isoenzymes investigated so far [Morrison & James, 1965; for reviews, see Kenyon and Reed (1983), Seraydarian and Vial (1986), and Furter et al. (1993)]. Most likely, proteinase K cleavage impedes substrate-induced conformational changes which facilitate binding of the second substrate. In addition, these substrate-induced conformational changes obviously desta-bilize the octameric Mi$_b$-CK molecules, resulting in facilitated dissociation of the latter into dimers (Figure 5).

DISCUSSION

Mitochondrial creatine kinase from chicken heart (Mi$_b$-CK) was shown in the present investigation to be selectively cleaved by six different proteases, resulting in only two fragments with M_r's of 36 700 and 6400 (Figure 3). With all of these proteases (proteinase K, subtilisin, pronase E, elastase, bactotrypsin, and thermolysin), cleavage occurred either before or after Ala-323 (Table I). Several lines of evidence indicate that the overall three-dimensional structure of Mi$_b$-CK is not or is only minimally affected by limited proteolysis. Both uncleaved and nicked Mi$_b$-CK form dimeric as well as octameric molecules. Moreover, the dependence of the proportion of octamers on the Mi$_b$-CK concentration is indistinguishable for uncleaved and nicked Mi$_b$-CK (Figure 4). Consequently, limited proteolysis neither directly nor

Table II: Comparison of K_m, K_d, and V_{max} Values of Uncleaved and Proteinase K-Digested Mib-CK[a]

	K_m (PCr) (mM)	V_{max} for ATP synthesis (IU/mg)	K_m(MgATP) (mM)	K_d(MgATP) (mM)	K_m(Cr) (mM)	K_d(Cr) (mM)	V_{max} for PCr synthesis (IU/mg)
native Mib-CK	1.61 ± 0.07	94.8 ± 4.3	0.32 ± 0.02[b]	0.70 ± 0.04[b]	8.9 ± 0.3[b]	19.6 ± 1.6[b]	60.7 ± 0.6[b]
proteinase K-digested Mib-CK	36.4 ± 2.3	48.0 ± 3.0	1.96 ± 0.31	0.69 ± 0.03	57.3 ± 7.5	20.1 ± 8.3	5.69 ± 0.41

[a] Kinetic experiments and calculations of K_m, K_d, and V_{max} values were performed as described under Materials and Methods. For the determination of K_m(PCr) and V_{max} in the direction of ATP synthesis, [PCr] was varied from 0.5 to 30 mM, while [ADP] and [Mg^{2+}] were held constant at 4 and 5 mM, respectively. For the determination of the K_m and K_d values for both MgATP and Cr, as well as of the V_{max} value in the direction of PCr synthesis, initial velocity measurements were performed at 1.2, 1.7, 2.5, 3.5, 5, or 8 mM MgATP and at either 35, 50, 65, or 100 mM Cr. Each value is based on 30–66 individual activity measurements and is given as mean ± SE. [b] Data from Furter et al. (1993).

indirectly affects the dimer–dimer interfaces within the octamer, at least in the absence of substrates. Gel permeation chromatography revealed that the elution positions of both Mib-CK octamers and dimers are not influenced by limited proteolysis (Table I). This finding suggests that the overall shape of the molecule (Stokes radius) is conserved and that the two fragments generated by selective cleavage of the polypeptide chain remain associated with each other. Furthermore, the far-UV CD spectra of native, uncleaved Mib-CK and of proteinase K-digested Mib-CK were almost identical (M. Gross, personal communication), thus indicating no significant change in secondary structure elements. Finally, uncleaved and proteinase K-digested Mib-CK were shown to be equally sensitive to temperature denaturation (not shown; this experiment is described under Materials and Methods).

A comparison with ovalbumin, a member of the serine protease inhibitor (serpin) superfamily, may give a hint about the location of the protease-hypersensitive region in the three-dimensional structure of Mib-CK. Ovalbumin has been shown previously to be selectively cleaved near Ala-352 by a variety of proteases (Wright, 1984). X-ray crystallography revealed that this cleavage site is located in an exposed loop on the surface of the molecule and that the two proteolytic fragments remain associated with each other (Wright et al., 1990). In the present investigation, both Mib-CK and ovalbumin were selectively cleaved by the same six proteases (Figure 3; Table I), whereas they were highly resistant to trypsin digestion [not shown; see also Wright (1984)]. Furthermore, the protease-hypersensitive stretches of Mib-CK on one hand and of ovalbumin and other serpins on the other hand display some sequence homology (not shown), indicating that Ala-323 of Mib-CK may also be part of an exposed, probably rather flexible surface loop.

Several lines of evidence suggest that this inferred surface loop overlaps neither with monomer–monomer interfaces within the dimer nor with dimer–dimer interfaces within the octamer. Mib-CK dimers are very stable and, like the dimeric cytosolic CK isoenzymes, can only be dissociated into monomers by denaturing agents. Accordingly, in all gel filtration experiments, no indication at all for a decay of Mib-CK octamers and dimers into monomers was observed (not shown), making it highly unlikely that the protease-hypersensitive site is located at monomer–monomer interfaces. Furthermore, dimeric and octameric Mib-CK were selectively cleaved by proteinase K at the same single site (not shown), and the cleavage rates were not significantly different for octameric and dimeric Mib-CK molecules. Finally, the octamer to dimer ratio of Mib-CK was not influenced by proteinase K cleavage (Figure 4).

Despite the lack of effects on the overall structure, limited proteolysis drastically affected the kinetic properties of Mib-CK (Figure 6; Table II). Proteinase K cleavage considerably decreased the V_{max} values in both directions of the reaction catalyzed by Mib-CK and substantially increased the K_m values

for MgATP, Cr, and PCr. The K_d values for both MgATP and Cr, however, were unchanged. As a result, the K_m values of proteinase K-digested Mib-CK for these latter two substrates were higher than the respective K_d values, whereas the opposite is true for native, uncleaved Mib-CK (Furter et al., 1993). Consequently, proteinase K digestion abolishes synergism in substrate binding as it is observed for native Mib-CK. A variety of previous studies, using EPR, NMR, and fluorescence spectroscopy as well as experiments on the protection by substrates from modification and/or inactivation by alkylating agents, have implicated that substrate binding results in conformational changes of the CK molecules, and that these substrate-induced conformational changes are essential for catalytic activity and for synergism in substrate binding (Cohn, 1970; Milner-White & Watts, 1971; Quiocho & Olson, 1974; Haugland, 1975; McLaughlin et al., 1976; Milner-White & Kelly, 1976; Markham et al., 1977; Keighren & Price, 1978). Therefore, the loss of synergism in substrate binding observed for proteinase K-digested Mib-CK may reflect the inability of the nicked enzyme to undergo substrate-induced conformational changes. This interpretation is favored by two additional lines of evidence: (i) The presence of conformational substates of Mib-CK was corroborated in the present investigation by the finding of a 75% reduction in the rate of selective cleavage of the Mib-CK polypeptide chain by proteinase K upon formation of a transition-state analogue complex of the enzyme (Figure 2). (ii) It has been suggested previously that substrate-induced conformational changes result in dissociation of Mi-CK octamers into dimers [Marcillat et al., 1987; see also Wyss et al. (1992)]. Accordingly, for proteinase K-digested Mib-CK relative to native Mib-CK, much higher concentrations of substrates were needed to induce octamer dissociation (Figure 5).

Recent low-resolution maps of chicken Mib-CK (T. Schnyder and W. Kabsch, personal communication) and lobster arginine kinase (C. Dumas and J. Janin, personal communication) may give a hint about the mechanism of the conformational changes induced by substrates. They clearly showed that the protomers of both phosphagen kinases are composed of two domains separated by a cleft, a conclusion that is corroborated by limited proteolysis experiments on (re)folding intermediates of chicken MM-CK that were indicative of two domains with M_r's of 20 000 and 23 000 (Morris & Cartwright, 1990; Morris & Jackson, 1991). This three-dimensional organization is reminiscent of the structure of other phosphokinases, like hexokinase, adenylate kinase, or 3-phosphoglycerate kinase (Anderson et al., 1979; Watson et al., 1982; Gerstein et al., 1993; see also Figure 7). For these three enzymes, X-ray crystallography, small angle X-ray scattering, NMR, and sedimentation experiments revealed that substrate binding induces a relatively large movement of the two domains relative to each other, thereby resulting in the closure of the cleft between the domains (Tanswell et al., 1976; Anderson et al., 1979; Pickover et al., 1979; Roustan

FIGURE 7: Schematic drawing of the structure of the open form of the yeast 3-phosphoglycerate kinase molecule. The helices are denoted by cylinders, and the sheet strands are denoted by arrows. The binding position for MgATP is shown behind helices VI and IX. The 3-phosphoglycerate binding site is behind helix XIII. β-Strand M, which seems to be structurally related to the protease-hypersensitive site of Mi$_b$-CK, is shown in black. Reprinted from Watson et al. (1982) with kind permission of Oxford University Press, England.

et al., 1980; Sinev et al., 1989; Harlos et al., 1992; Gerstein et al., 1993). Since the two substrate binding sites in all three cases are located on opposite sides of the cleft (for example, see Figure 7), substrate-induced closure of the latter was suggested to bring the two substrates together, in order to allow a direct in-line transfer of a phosphoryl group and to discriminate against water as a substrate, thus avoiding hydrolysis of the substrates and concomitant energy dissipation. In accordance with these results, small angle X-ray scattering of lobster arginine kinase revealed that the radius of gyration decreases by 1.20 ± 0.25 Å upon binding of MgADP and arginine, thus comparing reasonably well with the substrate-induced decreases in the radii of gyration of hexokinase and 3-phosphoglycerate kinase of 0.95 ± 0.24 and 1.09 ± 0.34 Å, respectively (Dumas & Janin, 1983). In addition, EPR spectra of rabbit MM-CK revealed that, upon formation of a transition-state analogue complex of the enzyme (MM-CK, MnADP, Cr, and nitrate), all the water molecules were removed from the first coordination sphere of the paramagnetic Mn^{2+} ion (Reed & Cohn, 1972).

As evidenced by a variety of structural, biochemical, and kinetic parallelisms (preliminary low-resolution maps, sizes of the domains, susceptibility of the domains to denaturation, reaction mechanism, effect of anions on catalytic activity, effect of substrates on the enzyme's conformation, and others), CK and arginine kinase seem to resemble most pronouncedly, among the phosphokinases for which the three-dimensional structure is known, 3-phosphoglycerate kinase (PGK; Figure 7), a monomeric enzyme with approx. 415 amino acids that is involved in glycolysis and gluconeogenesis. Most importantly, the primary sequences of CK and PGK can be aligned reasonably well, especially in the C-terminal halves of the molecules (not shown). The highest degree of homology was found between residues 177–206 of Mi$_b$-CK and residues 215–241 of yeast PGK (corresponding to helix VI, β-strand H, and part of helix VII in Figure 7). In addition, residues 301–341 of Mi$_b$-CK obviously correspond to residues 343–384 of PGK (helices XI and XII and β-strand M). As a matter of fact, these secondary structure elements make up a large part of the adenine nucleotide binding site of PGK and are therefore most likely to share homology with CK sequences. According to this sequence alignment, the protease-hypersensitive site of

Mi$_b$-CK (Ala-323) would be part of the ATP binding domain and would correspond to a residue near β-strand M of PGK (Figure 7; shown in black) which is in close spatial proximity to the hinge region of the molecule. This structural comparison serves as a "feedback control" for the interpretation of the results presented in this investigation, in as far as disturbances of the hinge region of Mi$_b$-CK exerted by proteinase K cleavage may explain quite well the observed loss of synergism in substrate binding and of substrate-induced conformational changes.

In conclusion, the present study has shown that phosphagen kinases in general and chicken Mi$_b$-CK in particular have a protease-hypersensitive site which, in Mi$_b$-CK, is located most probably in a loop that is exposed on the surface of both octameric and dimeric molecules. Selective cleavage of the Mi$_b$-CK polypeptide chain at this site, i.e., after Ala-323, interferes with substrate-induced conformational changes and thus prevents synergism in substrate binding as it is observed for native, uncleaved Mi$_b$-CK. In addition, the V_{max} values are decreased while substrate binding per se is not influenced by limited proteolysis. From a comparison with 3-phosphoglycerate kinase, it is suggested that CK protomers are composed of two domains separated by a cleft and that selective cleavage of Mi$_b$-CK after Ala-323 interferes with a substrate-induced hinge motion of the two domains relative to each other, thereby impeding closure of the cleft.

Chemical modification (Cohn, 1970; Maggio et al., 1977; Markham et al., 1977; Keighren & Price, 1978) and site-directed mutagenesis (Furter et al., 1993) of the highly reactive sulfhydryl group of CK (Cys-278 in chicken Mi$_b$-CK) were also shown to result in markedly decreased V_{max} values, in a decreased ability to undergo substrate-induced conformational changes, and in loss of synergism in substrate binding. A similar mechanism of action of proteinase K cleavage on one hand and of manipulations of the highly reactive sulfhydryl group on the other hand is further suggested by the finding that proteinase K cleavage, site-directed mutagenesis of Cys-278 to a Ser residue, and reversible modification of Cys-278 by oxidized glutathione all impede substrate-induced dissociation of Mi$_b$-CK octamers into dimers (not shown).

In the future, the determination of detailed crystal structures of Mi$_b$-CK (in the absence and presence of substrates) is hoped to explain how the stretches around Ala-323 and Cys-278 are involved in the substrate-induced conformational changes, and how these latter affect the dimer–dimer interfaces within the octamer so as to decrease octamer stability. In addition, it seems highly attractive to further investigate whether selective cleavage of the Mi$_b$-CK polypeptide chain is, in analogy to most or all of the serpins, physiologically relevant, e.g., for irreversible inactivation or for initiation of biodegradation of Mi$_b$-CK in vivo.

ACKNOWLEDGMENT

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