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# A Conserved Negatively Charged Cluster in the Active Site of Creatine Kinase Is Critical for Enzymatic Activity*

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**Creatine kinase catalyzes the reversible transphosphorylation of creatine by MgATP. From the sequence homology and the molecular structure of creatine kinase isoenzymes, we have identified several highly conserved residues with a potential function in the active site: a negatively charged cluster ($Glu^{226}$, $Glu^{227}$, $Asp^{228}$) and a serine ($Ser^{280}$). Mutant proteins E226Q, E226L, E227Q, E227L, D228N, and S280A/S280D of human sarcomeric mitochondrial creatine kinase were generated by *in vitro* mutagenesis, expressed in *Escherichia coli*, and purified to homogeneity. Their overall structural integrity was confirmed by CD spectroscopy and gel filtration chromatography. The enzymatic activity of all proteins mutated in the negatively charged cluster was extremely low (0.002–0.4% of wild type) and showed apparent Michaelis constants ($K_m$) similar to wild type, suggesting that most of the residual activity may be attributed to wild-type revertants. Mutations of $Ser^{280}$ led to higher residual activities and altered $K_m$ values; S280A showed an increase of $K_m$ for phosphocreatine (65-fold), creatine (6-fold), and ATP (6-fold); S280D showed a decrease of $K_m$ for creatine (6-fold). These results, together with the transition state structure of the homologous arginine kinase (Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy G., Ellington, W. R., and Chapman, M. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 8449–8454), strongly suggest a critical role of $Glu^{226}$, $Glu^{227}$, and $Asp^{228}$ in substrate binding and catalysis and point to $Glu^{227}$ as a catalytic base.**

Creatine kinase (CK;[1] EC 2.7.3.2) catalyzes the interconversion of phosphocreatine (PCr) and ADP with ATP and creatine (Cr). The enzyme occurs as a family of tissue-specific isoenzymes, comprising dimeric cytosolic CK (MM-, MB-, and BB-CK) and mainly octameric mitochondrial CK (sMtCK and uMtCK), which can dissociate into dimers. These CK isoenzymes, together with easily diffusable Cr and PCr, maintain a unique cellular energy buffer and energy transport system, the CK/PCr circuit (for reviews see Refs. 1 and 2). Thus, the CK system plays a key role in energy metabolism of cells and tissues with high or fluctuating energy requirements like muscle or brain. Lately, the molecular structures of all four homooligomeric CK isoenzymes have been solved (3–6), as well as the transition state structure of monomeric arginine kinase (AK) from horseshoe crab (7). Both CK and AK belong to the larger guanidino kinase family. These new data allow a fresh look on amino acid residues with a critical role in substrate binding and catalysis.

The catalytic mechanism of CK has been studied extensively by numerous different techniques, yielding detailed information on kinetic and mechanistic aspects of the transphosphorylation reaction (8). It has been well documented that the γ-phosphoryl group is transferred via an associative in-line mechanism (9–11). At pH 8 and above, the CK reaction follows a rapid equilibrium random mechanism in both directions (12), whereas at pH 7, the kinetic mechanism is random only in the reverse direction (ATP synthesis) and equilibrium ordered, with ATP adding before Cr, in the forward direction (PCr synthesis) (13). By contrast, our knowledge about specific amino acid residues involved in substrate binding and catalysis is scarce. Especially the guanidino substrate-binding site of CK has not yet been characterized, because of the lack of specific mutants or a CK crystal structure containing creatine. Mutation of the highly reactive cysteine 278 (sMtCK numbering) and tryptophan 223 located near the active site led to severely decreased enzymatic activity (14, 15). C278 was implicated in substrate synergism (14) and may interact with the creatine substrate (7). Several arginines were identified to interact electrostatically with the negatively charged phosphate groups of the nucleotide (16). From earlier work, a histidine residue was proposed to act as an acid base catalyst in the transphosphorylation reaction (17). However, recent site-directed mutagenesis experiments (18, 19), as well as the AK structure (7), have clearly demonstrated that none of the conserved histidines is able to provide this function. It was speculated that CK might act as a "conzyme" without catalytic residue by just bringing the substrates into a close, favorable alignment (20). Alternatively, other residues may be involved to draw away partial positive charge from the reactive guanidinium-$N_{\eta 2}$ or even to act as a catalytic base. From the AK transition state structure, two glutamates were proposed as new candidates for acid base catalysis (7). In analogy to ATP hydrolysis at the myosin motor domain (21), also the γ-phosphate of ATP itself could act as a catalytic base, assisted by a serine that participates in hydrogen exchange by providing an energetically favorable geometry.

In the present study, we have analyzed the available sequence and structural information for CK and AK to look for residues with a putative role in the active site of CK. Using site-directed mutagenesis with the recently characterized hu-

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[1] The abbreviations used are: CK, creatine kinase; PCr, phosphocreatine; Cr, creatine; AK, arginine kinase; uMtCK and sMtCK, ubiquitous and sarcomeric mitochondrial CK, respectively; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TSAC, transition state analog complex.

man sMtCK isoenzyme (22), we could identify four highly conserved residues, including a negatively charged cluster and a serine residue, which are critical for substrate binding and catalytic mechanism.

## EXPERIMENTAL PROCEDURES

*Site-directed Mutagenesis by Inverse Polymerase Chain Reaction*—The sequence of mature human sMtCK (GenBank™ J05401) has been cloned into a pET-derived expression vector (22, 23). Site-directed mutagenesis by inverse polymerase chain reaction was carried out on a Hybaid Omn-E Thermal Cycler (MWG-Biotech, Münchenstein, Switzerland), using 10 ng of double-stranded DNA (entire plasmid vector), 15 pmol of each oligonucleotide primer, and *Pfu* DNA polymerase (Stratagene, Zürich, Switzerland). The latter was added to the reaction mixture at 95 °C ("hot start") followed by 30 polymerase chain reaction cycles (95 °C, 0.5 min; 55 °C, 0.5 min; 72 °C, 6.5 min). All primers were 5′-phosphorylated for subsequent circularization of gel-purified polymerase chain reaction products with T4-DNA ligase. Plasmids were transformed into competent *E. coli* XL-1 blue using routine procedures (24) and sequenced with the dideoxy chain termination method (25) to verify *in vitro* mutagenesis and the absence of random mutations. Mutagenic primers (mismatches with the template are underlined): E226Q (huMib676$E_1$; 5′-CAGGAGGATCACACCAGGGT-3′, huMib654$R_1$; 5′-ATTTATCCAGATGAGAAATGTC-3′); E226L (huMib676$E_2$; 5′-CTGGAGGATCACACCAGGGTA-3′, huMib654$R_1$); E227Q (huMib676$E_3$; 5′-GAGCAGGATCACACCAGGGTAATC-3′, huMib654$R_1$); E227L (huMib676$E_4$; 5′-GAGCTGGATCACACCAGGGTAATC-3′, huMib654$R_1$); D228N (huMib682$D_1$; 5′-AATCACACCAGGGTAATCTCA-3′, huMib661$R_2$; 5′-CTCCTCATTTATCCAGATGAG-3′); D228L (huMib682$D_2$; 5′-CTTCACACCAGGGTAATCTCAA-3′, huMib661$R_2$); S280D (huMib838$S_1$; 5′-GATAACCTTGGAACAGGACTACG-3′, huMib817$R_3$; 5′-AGGACAGGTCAAAATGTATCC-3′); S280A (huMib838$S_2$; 5′-GCGAACCTTGGAACAGGACT-3′, huMib817$R_3$).

*Protein Expression and Purification*—Selected transformants of *E. coli* BL21(DE3)pLysS were grown at 37 °C in 2YT medium. Heterologous expression of sMtCK, induced at about 1 $A_{600}$ by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside, was continued for about 5 h. Soluble protein was extracted from bacterial pellets by sonication under the addition of 2 μl of Benzonase to digest nucleic acids. sMtCK was precipitated from lysate supernatant at an ammonium sulfate saturation of 33% (pH 6.5), resuspended in 20 mM Tris/HCl buffer (pH 9.0; 0.2 mM EDTA, 2 mM β-mercaptoethanol), and dialyzed overnight against resuspension buffer. sMtCK was further purified by high pressure liquid chromatography with the strong cation exchanger Poros HQ (4.6 × 100-mm column, PerSeptive Biosystems, Rotkreuz, Switzerland), equilibrated with HQ buffer (12 mM bis-Tris propane, 12 mM Tris/HCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, pH 9.0). sMtCK eluted in a linear NaCl gradient at about 100 mM salt, and peak fractions were pooled and concentrated with Centricon-30 (Millipore, Volketswil, Switzerland) to about 4 mg/ml protein. Final purification by gel filtration chromatography involved a HiPrep Sephacryl S-300 HR 16/60 column (Amersham Pharmacia Biotech) and was equilibrated and run with gel filtration buffer (50 mM sodium phosphate, pH 7.0, 2 mM β-mercaptoethanol, 0.2 mM EDTA, and 150 mM NaCl) at a flow rate of 0.4 ml/min. The purification process, as monitored by standard 12% SDS-polyacrylamide gel electrophoresis, yielded over 99% pure mutant sMtCKs. Protein concentration was determined according to Bradford (26), using bovine serum albumin as a standard.

*Enzyme Kinetics*—CK activity and kinetic constants were determined with a coupled enzyme photometric assay modified after Wallimann *et al.* (27). ATP production (reverse reaction) was coupled by hexokinase (300 units/ml) and glucose-6-phosphate dehydrogenase (150 units/ml) to NADPH production, using 4 mM ADP, 5 mM $MgCl_2$, 40 mM PCr, 40 mM D-glucose, and 1 mM NADP in 0.1 M triethanolamine buffer, pH 7.0. The production of PCr (forward reaction) was coupled by pyruvate kinase (160 units/ml) and lactate dehydrogenase (800 units/ml) to NADH oxidation, using 4 mM ATP, 4.5 mM magnesium-acetate, 20 mM creatine, 0.9 mM P-enolpyruvate, and 0.45 mM NADH in 0.1 M triethanolamine buffer, pH 8.0. Changes in the redox state of pyrimidine nucleotides were followed at 340 nm in a UNICAM UV4 (Unicam, Cambridge, UK) spectrophotometer thermostated at 25 °C. All substrates and enzymes for activity measurements were from Roche Diagnostics (Basel, Switzerland). Like in previous studies, the pH of standard reactions was chosen according to established pH optima (28). In addition, the pH dependence of enzymatic activity was determined with pH buffer (50 mM bis-Tris propane and 50 mM Tris, adjusted from pH 6 to 9) together with saturating activities of coupling enzymes. Apparent Michaelis-Menten constants ($K_m$) were determined by varying six to seven different concentrations of each substrate at a fixed, saturating concentration of the second substrate, always keeping the ratio $Mg^{2+}$/nucleotide constant. Calculation of kinetic constants was performed by least squares fitting of the substrate-dependent reaction velocities to the Michaelis-Menten equation.

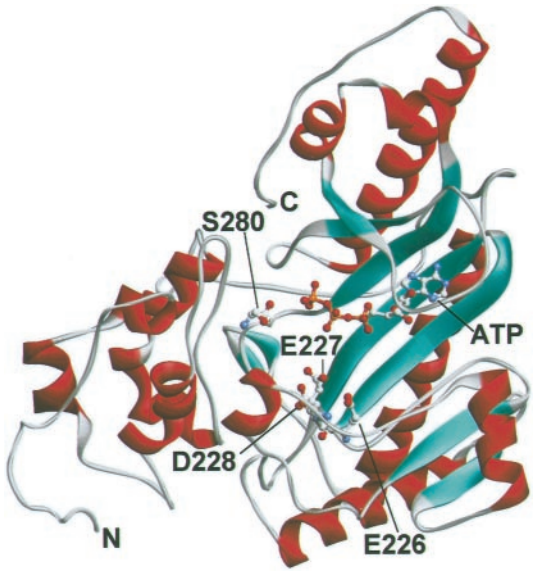


FIG. 1. **Location of mutated residues in the sMtCK structure.** Ribbon representation of the three-dimensional fold of a chicken sMtCK monomer (PDB code 1crk (3)). ATP, which marks the active site of CK, and amino acids mutated in this work ($Glu^{226}$, $Glu^{227}$, $Asp^{228}$, and $Ser^{280}$) are shown in a ball-and-stick representation with standard CPK colors (figure prepared with WebLabViewer V.3.1, MSI, San Diego, CA).

*Oligomeric State*—Quantitation of octameric and dimeric sMtCK by gel filtration chromatography and dissociation of sMtCK octamers by incubation with transition state analog complex (TSAC (9)) was carried out as described in Ref. 22.

*CD Spectroscopy*—Far and near UV CD spectra of human sMtCK wild-type and mutant protein were recorded on a JASCO J-715 dichrograph (Jasco, Great Dunmow, UK) at 25 °C and under constant nitrogen flow, using a quartz cell with a 1-cm optical path. sMtCK in 25 mM sodium phosphate (pH 6.75) was diluted in this buffer to about 0.2 mg/ml and sterile-filtered.

## RESULTS

*Choice of Residues with a Putative Role in the Active Site of CK*—Two highly conserved portions of the CK sequence (homology blocks 3 and 4 (30)) comprise large parts of the putative active site (3) and most residues involved in substrate binding or catalysis (*e.g.* $Cys^{278}$ (14) or $Trp^{223}$ (15), sMtCK numbering). The only negatively charged amino acids in this region form a cluster ($Glu^{226}$, $Glu^{227}$, and $Asp^{228}$) that is conserved among all creatine kinases, and even across the larger guanidino kinase family (30). It is located near the γ-phosphate of enzyme-bound ATP in chicken sMtCK (Fig. 1 (3)) and may be well positioned for coordinating $Mg^{2+}$ or binding guanidino substrates. The $Glu^{227}$ homologue of AK directly interacts with the guanidino group (7). Another highly conserved residue, $Ser^{280}$, which is conservatively replaced by threonine in some guanidino kinases (30), has close contact to the γ-phosphate of ATP and the "reactive" active site cysteine 278 (Fig. 1 (3)). It would be a good candidate for facilitating proton transfer during catalysis (21).

*Mutagenesis, Expression, and Purification of Mutant Protein*—Single amino acid replacement mutants of human sMtCK were generated by site-directed mutagenesis and validated by DNA sequencing. $Glu^{226}$, $Glu^{227}$, and $Asp^{228}$ were substituted with either their corresponding amides (E226Q, E227Q, and D228N) or aliphatic amino acids (E226L, E227L, and D228L). $Ser^{280}$ was replaced by aspartate or alanine (S280D, S280A). Except for D228L, all mutants could be ex-

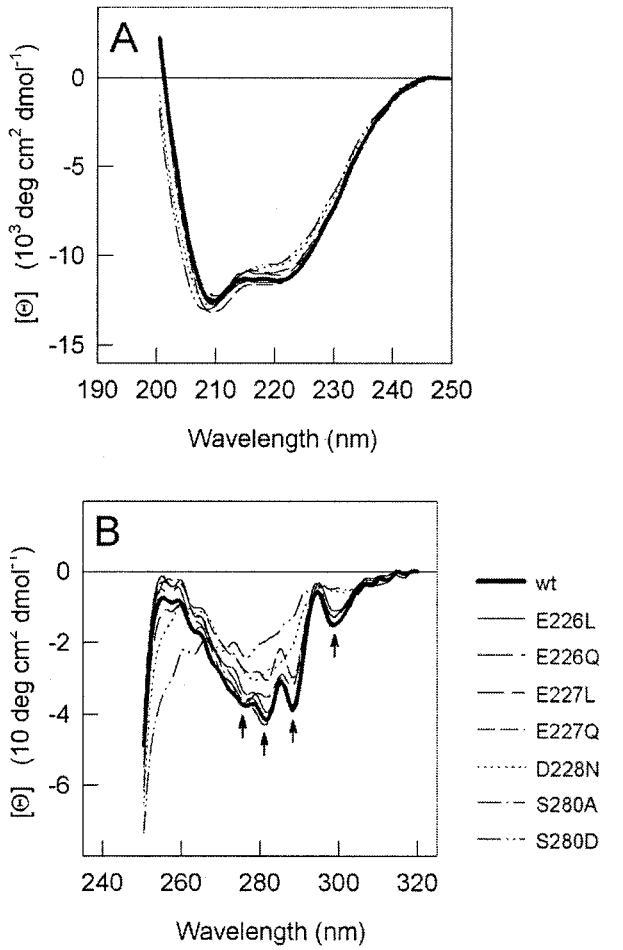


FIG. 2. **CD spectral patterns of wild type and mutant sMtCK.** (*A*) Far UV and (*B*) near UV CD spectra were taken with 0.2 mg/ml protein in 25 mM sodium phosphate (pH 6.75) and corrected with buffer spectra. *Arrows* in *B* indicate Cotton bands characteristic for CK at 275, 281, 288, and 300 nm. Note that mutant S280D was unstable under the chosen low ionic strength buffer conditions and was slowly denaturing during the measurement.

pressed in *E. coli* as soluble proteins. Because they did not bind to Blue Sepharose resin like wild-type MtCK, we used their low solubility at pH 6.5 for a quantitative precipitation at 33% ammonium sulfate saturation. This led to a substantial enrichment of 70–80% of total protein as judged from standard SDS-polyacrylamide gel electrophoresis (data not shown). Further purification by ion exchange and gel filtration chromatography yielded about 20–30 mg of homogeneous mutant protein/liter of bacterial culture, which is slightly less than for the wild-type enzyme.

*Structural Integrity of Mutant sMtCK*—All purified sMtCK mutant proteins remained soluble and enzymatically active after storage at 4 °C, suggesting that stability and proper folding were not significantly affected. Far UV CD spectra of all mutants were superimposable to those of wild-type enzyme, confirming the overall structural integrity (Fig. 2*A*). CD spectra in the near UV range (≥240 nm, Fig. 2*B*) revealed, except for the mutation S280D, only small differences to the wild-type enzyme with a conservation of the Cotton band pattern characteristic for CK (15). Hence, changes in the microenvironment of aromatic residues were neglectably small and probably because of minor conformational alterations introduced by the charge shift mutations. The octamer content of mutant proteins at 0.1 mg/ml protein was at least 81% (Table I). This is

TABLE I

*TSAC-induced octamer dissociation of human sMtCK mutants*

Relative octamer content of sMtCK before and after TSAC incubation for 72 h at room temperature as determined from gel filtration elution profiles. Protein concentrations were adjusted to 0.1 mg/ml in 50 mM potassium-phosphate, 150 mM NaCl, pH 7.0.

| | Octamer content of sMtCK | | Octamer dissociation |
|---|---|---|---|
| | Control | TSAC-incubated | |
| | % | | % |
| Wild-type | 98 | 17 | 83 |
| E226Q | 91 | 79 | 13 |
| E226L | 86 | 64 | 26 |
| E227Q | 90 | 82 | 9 |
| E227L | 89 | 89 | 0 |
| D228N | 96 | 68 | 29 |
| S280A | 86 | 78 | 9 |
| S280D | 81 | 62 | 23 |

comparable to wild-type protein,[2] indicating that the mutations had no severe effect on the oligomeric state either.

*Enzyme Kinetic Parameters of Mutant sMtCK*—Enzymatic activities and kinetic parameters for wild-type and mutant enzymes were determined in the forward reaction (PCr production) at pH 8.0, as well as in the reverse reaction (ATP production) at pH 7.0 by a coupled enzyme assay. E226Q, E226L, E227Q, E227L exhibited residual catalytic activities as small as 0.002–0.036% of wild-type activity in the forward as well as in the reverse reaction (Table II). Residual enzymatic activities of D228N and S280D were higher but still extremely low as compared with wild-type human sMtCK (0.10–0.46% of wild-type activity). S280A showed the highest residual activities, especially in the reverse reaction (1.1–6.0% of wild-type activity). We could exclude that mutations of the acidic residues shifted the pH optimum of the CK reaction, because none of the mutant proteins showed significantly increased residual activity at other pH values than those used for the standard protocol (data not shown). Apparent Michaelis-Menten parameters ($K_m$) of each mutant were determined for all substrates (Table III). Mutations of anionic residues led to $K_m$ values comparable to wild-type (only $K_m$ for PCr, if any, was somewhat higher). By contrast, S280A showed a 65-fold increase of $K_m$ (PCr), together with a 6-fold increase of $K_m$ (Cr) and $K_m$ (MgATP), whereas S280D revealed a decrease of $K_m$ (Cr). Because mutation of anionic residues may potentially alter the complexation of cations, we also varied the $Mg^{2+}$ concentration at fixed nucleotide concentrations. There was no difference in $Mg^{2+}$ dependence as compared with wild-type protein, thus excluding a $Mg^{2+}$ limitation in our assays (data not shown). Exact dissociation constants of the binary complexes ($K_d$) could not be determined, because nonsaturating concentrations of both substrates gave residual activities at or below the detection limit of the assay system.

*Octamer Dissociation of Mutant sMtCK*—MtCK octamers are known to dissociate rapidly into dimers upon the addition of substrates that form a TSAC (29, 31). This is because of large conformational changes induced by the binding of TSAC compounds (32) weakening MtCK dimer-dimer interactions. We found that the susceptibility of octameric mutant sMtCK for TSAC-induced dissociation was severely reduced, especially in the case of E227Q and E227L (Table I). Either these mutants have a decreased affinity for TSAC substrates or bound TSAC is unable to induce the necessary conformational changes (2).

## DISCUSSION

Using a site-directed mutagenesis approach, we have identified four residues in the active site pocket of human sMtCK

[2] U. Schlattner and T. Wallimann, unpublished results.

TABLE II
*Specific enzyme activities of human sMtCK mutants*
Specific enzyme activities are averaged from at least three independent measurements, using a coupled enzyme assay (22). wt, wild-type.

| | Forward reaction[a] | | Reverse reaction[b] | |
|---|---|---|---|---|
| | $V_{max}$[c] | % wt | $V_{max}$[c] | % wt |
| | *units/mg* | | *units/mg* | |
| Wild-type | 105 ± 3.5 | 100 | 130 ± 2.8 | 100 |
| E226L | 0.038 ± 0.0011 | 0.036 | 0.004 ± 0.0003 | 0.003 |
| E226Q | 0.008 ± 0.0003 | 0.008 | 0.004 ± 0.0002 | 0.003 |
| E227L | 0.002 ± 0.0001 | 0.002 | 0.002 ± 0.0001 | 0.002 |
| E227Q | 0.003 ± 0.0005 | 0.003 | 0.002 ± 0.0002 | 0.002 |
| D228N | 0.11 ± 0.003 | 0.10 | 0.6 ± 0.02 | 0.46 |
| S280A | 1.16 ± 0.02 | 1.10 | 7.8 ± 0.15 | 6.0 |
| S280D | 0.15 ± 0.005 | 0.14 | 0.55 ± 0.012 | 0.42 |

[a] Forward reaction: Cr + ATP → PCr + ADP + $H^+$, pH 8.0.
[b] Reverse reaction: PCr + ADP + $H^+$ → Cr + ATP, pH 7.0.
[c] One unit of enzyme activity is equal to 1 μmol of ATP or PCr transphosphorylated/min at 25 °C.

TABLE III
*Enzyme kinetic constants of human sMtCK mutants*
The initial rates were determined using a coupled enzyme assay (22). Kinetic constants were calculated by fitting the data from at least three independent measurements to the Michaelis-Menten equation.

| | Forward reaction | | Reverse reaction | |
|---|---|---|---|---|
| | $K_m$ (MgATP) | $K_m$ (Cr) | $K_m$ (MgADP) | $K_m$ (PCr) |
| | *mM* | *mM* | *mM* | *mM* |
| Wild-type | 0.7 ± 0.1 | 7.3 ± 0.5 | 0.15 ± 0.02 | 1.2 ± 0.14 |
| E226L | 1.8 ± 0.2 | 12.2 ± 0.7 | 0.5 ± 0.09 | 8.4 ± 0.4 |
| E226Q | 2.2 ± 0.2 | 14.1 ± 0.6 | 0.4 ± 0.08 | 10.7 ± 0.5 |
| E227L | 1.9 ± 0.7 | 6.5 ± 1.0 | 0.35 ± 0.09 | 7.4 ± 2.9 |
| E227Q | 1.5 ± 0.5 | 7.3 ± 0.7 | 0.2 ± 0.07 | 3.4 ± 0.5 |
| D228N | 0.6 ± 0.05 | 1.5 ± 0.1 | 0.35 ± 0.02 | 3.2 ± 0.1 |
| S280A | 4.6 ± 0.6 | 45 ± 3.2 | 0.37 ± 0.1 | 79.5 ± 5.2 |
| S280D | 0.4 ± 0.04 | 1.3 ± 0.1 | 0.21 ± 0.02 | 1.8 ± 0.15 |

that are important for substrate binding and catalysis: $Glu^{226}$, $Glu^{227}$, $Asp^{228}$, and $Ser^{280}$ (sMtCK numbering). Mutation of these residues yielded proteins with dramatically reduced enzymatic activities (mostly between 0.002 and 0.4% of wild-type), together with a well conserved overall protein structure. The latter is indicated by identical binding properties to anion exchange columns, stability at 4 °C, nearly indistinguishable near and far UV CD spectra, and proper octamer formation. The residual enzymatic activity of the CK mutants could not be improved by elevating $Mg^{2+}$ concentrations or varying the pH in the applied assay. We can therefore exclude subsaturating cation binding or a shift in pH optimum as responsible for the strongly reduced enzymatic activities, such as was observed in certain cysteine mutants (14).

Mutations of glutamate residues $Glu^{226}$ and $Glu^{227}$ in the negatively charged cluster showed the most striking decrease of enzymatic activity by 4–5 orders of magnitude, yielding almost inactive enzymes. Turnover numbers of aspartate mutant D228N were an order of magnitude higher but still heavily reduced compared with wild-type (see Table II). These very low residual enzymatic activities of recombinant MtCK may result from molecules that have fortuitously restored the correct wild-type residue by error during protein synthesis (20, 33). Although we have minimized such misincorporation by avoiding rare *E. coli* codons in our mutagenic primers, the basic translational error rate in *E. coli* ($2 \times 10^{-2}$ to $2 \times 10^{-3}$/codon) is further increased under the burden of heterologous protein expression (33, 34) and could account for residual enzymatic activities as high as 0.2% of wild type. For comparison, mutants of the "active site" tryptophan 223 and cysteine 278 showed much higher residual activities in the order of 0.5–4% and 2–5% at optimal pH, respectively (14, 15). Mutations of $Ser^{280}$ also yielded a more active enzyme, especially with S280A (1.1–6% of wild-type). Earlier, it was speculated that mutations near or at the active site of CK do not necessarily obliterate catalysis, and that CK is therefore a "robust" enzyme (35). Our data clearly establish that this is not the case and strongly suggest a critical role of $Glu^{226}$, $Glu^{227}$, and $Asp^{228}$ in substrate binding and/or catalysis of CK.

Altered substrate binding is at least one consequence of the mutations that we have introduced. This is clearly indicated by the very weak binding of mutant proteins to Blue Sepharose resin, which mimics a nucleotide-like substrate, and their poor octamer dissociation upon the addition of TSAC compounds. On the other hand, apparent Michaelis-Menten parameters ($K_m$) of mutations in the negatively charged cluster were unchanged compared with wild-type. This observation, however, may reflect the contribution of wild-type revertants to the very low residual activity of these preparations. The situation is entirely different for serine mutants. Here, higher residual enzymatic activities and apparent $K_m$ values different from wild-type confirm the presence of a partially active mutant protein. In addition, the changes in apparent $K_m$ were dependent on the replacing amino acid; $K_m$ increased for MgATP, Cr, and PCr in S280A but decreased for Cr in S280D.

The recently reported transition state structure of AK (7) allows a more subtle functional analysis of the mutated CK residues. Because the overall three-dimensional fold of AK follows closely that of all known CK structures (3–6) and residues in the active site are highly conserved (5, 7, 30), it is very likely that all guanidino kinases share a similar reaction mechanism. The AK transition state structure shows that the $Glu^{226}$ and $Asp^{228}$ homologues have no direct contact with substrates but provide important hydrogen bonds that finally lead to substrate binding and alignment. $Glu^{226}$ is hydrogen-bonded to two water molecules, which are part of the octahedral coordination sphere of the essential magnesium ion (Fig. 3). $Mg^{2+}$ is further ligated with a third water molecule and the oxygen of the α-, β-, and γ-phosphate of ATP. However, hydrogen bonding alone cannot be the only function of $Glu^{226}$ in CK, because

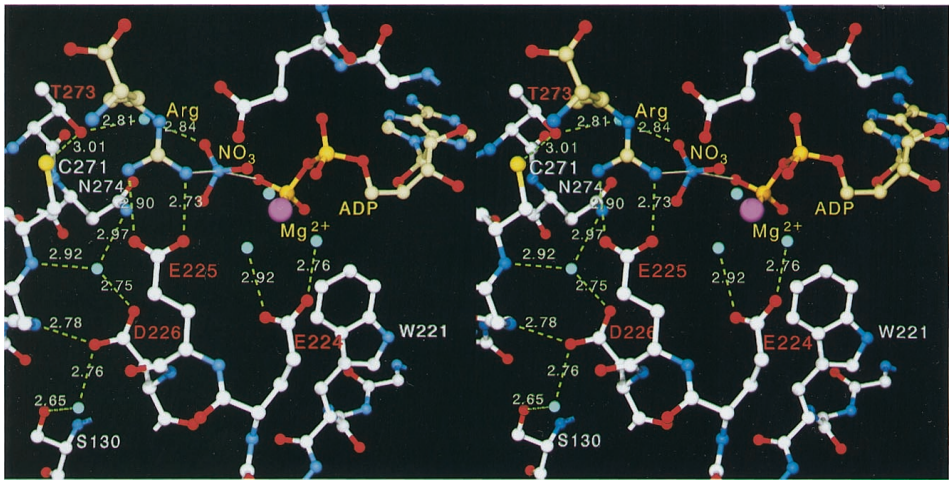


FIG. 3. **The active site of arginine kinase in the presence of transition state analog complex components.** Stereo view representation of AK with TSAC (MgADP + $NO_3^-$ + arginine; PDB code 1bg0 (7)), showing the TSAC components (*yellow label*) and the amino acid homologues of CK residues mutated in this work (*red label*) with residues 224–226 (AK) corresponding to the negatively charged cluster 226–228 (human sMtCK) and $Thr^{273}$ (AK) conservatively replacing $Ser^{280}$ (human sMtCK). The reactive cysteine 278 (human sMtCK) is located at AK position 271 (*white label*). Several water molecules in close proximity to the substrates are drawn in *blue*. Distance monitors are shown in *green* (figure prepared with SwissPDB-Viewer V.3.1 and POVRAY V.3.02).

substitution with the equally sized glutamine, also able to form the hydrogen bonds, resulted in a virtually inactive enzyme. Possibly, at some stage of the domain movements induced by the binding of magnesium nucleotide (32), the negatively charged glutamate interacts directly with $Mg^{2+}$.

The AK homologue of $Asp^{228}$ forms a dense hydrogen bond network at the active site (Fig. 3), which links the negatively charged cluster 226–228 to a loop region containing the homologue of cysteine $Cys^{278}$. In AK, this cysteine interacts with the nonreactive guanidyl $N_{\eta 1}$ and is not only important for substrate binding but also enhances catalytic activity by positioning the guanidinium group and drawing a partial positive charge away from the reactive $N_{\eta 2}$ atom (7). This may explain the failure to obtain native D228L protein, because the aliphatic amino acid at this position is unable to form hydrogen bonds and may cause a critical perturbation of the enzyme fold. In mutant D228N, although capable of providing hydrogen bonds, a distortion of local geometry, as well as the change in the electrostatic environment at the active site, could explain the lack of activity.

The third residue in the negatively charged cluster, $Glu^{227}$, is most probably in direct contact with the substrate guanidinium. The AK homologue of $Glu^{227}$ locks the guanidinium group in an optimal position for phosphoryl transfer (Fig. 3). Moreover, it draws away a partial positive charge from the substrate guanidinium group to increase the nucleophilicity of the reacting $N_{\eta 2}$ atom. A failure to correctly position the guanidino group could also account for the pronounced inability of octameric $Glu^{227}$ mutant protein to dissociate into dimers upon TSAC addition. A correct alignment of TSAC compounds is likely to be essential for triggering the conformational changes that lead to octamer dissociation. For AK, two glutamates were proposed as candidates for acid base catalysis, including the homologue of $Glu^{227}$ (7). The properties of $Glu^{227}$ mutations in CK are fully consistent with such a function.

$Ser^{280}$ is conservatively replaced by $Thr^{273}$ (AK numbering) in the transition state structure of AK (Fig. 3). However, this residue is not adequately positioned to facilitate an intrinsic proton transfer as proposed. By contrast, $Thr^{273}$ is in hydrogen bond distance (3.0 Å) to the AK homologue of $Cys^{278}$, which in turn is 3.3 Å away from the substrate guanidinium (7). In analogy, $Ser^{280}$ may also fix the relative orientation of the thiol group of $Cys^{278}$. This is consistent with the low $K_m$ of S280D for creatine, because the additional negative charge of aspartate at this location may attract the positively charged guanidinium group during initial binding. However, one has to consider also the less stable protein structure of S280D as seen with near UV CD spectra and octamer formation. The increased $K_m$ of S280A for different substrates is not obvious from the AK structure.

Taken together, we have identified several key residues in the active site of CK. Based on properties of mutant proteins and the recently published transition state structure of AK (7), we could also deduce the putative functions of these amino acids. Our study clearly demonstrates that even residues that are not found in direct contact with the substrates can contribute to efficient catalysis. CK obviously uses a complicated network of interactions with several residues involved rather than a single, catalytic residue. Overall geometry and charge distribution at the active site may be sufficient to achieve the high turnover rates of guanidino kinases through perfect alignment of both substrates and an optimal electrostatic environment for the transphosphorylation reaction. The AK transition state structure (7) suggests a multifaceted catalytic mechanism of guanidino kinases, including restriction of freedom of the substrates (36), orbital steering (37), strain toward the transition state, partial charge withdrawing, and possibly also acid base catalysis. For the latter function, based on the AK structure (7), our data propose $Glu^{227}$ as the most likely candidate. A multidisciplinary approach, combining structural biology, biophysics, and biochemistry will be necessary to finally reveal the exact catalytic mechanism of guanidino kinases at a molecular level.

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