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The active site histidines of creatine kinase. A critical role of His 61 situated on a flexible loop

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Abstract

A histidine residue with a pK_a of 7 has been inferred to act as a general acid-base catalyst for the reaction of creatine kinase (CK), catalyzing the reversible phosphorylation of creatine by ATP. The chicken sarcomeric muscle mitochondrial isoenzyme Mi_b-CK contains several histidine residues that are conserved throughout the family of creatine kinases. By X-ray crystal structure analysis, three of them (His 61, His 92, and His 186) were recently shown to be located close to the active site of the enzyme. These residues were exchanged against alanine or aspartate by in vitro mutagenesis, and the six mutant proteins were expressed in *E. coli* and purified. Structural integrity of the mutant proteins was checked by small-angle X-ray scattering. Kinetic analysis showed the mutant His 61 Asp to be completely inactive in the direction of ATP consumption while exhibiting a residual activity of 1.7% of the wild-type (wt) activity in the reverse direction. The respective His to Ala mutant of residue 61 showed approximately 1% wt activity in the forward and 10% wt activity in the reverse reaction. All other mutants showed near wt activities. Changes in the kinetic parameters K_m or V_{max}, as well as a significant loss of synergism in substrate binding, could be observed with all active mutants. These effects were most pronounced for the binding of creatine and phosphocreatine, whereas ATP or ADP binding were less severely affected. Based on our results, we assume that His 92 and His 186 are involved in the binding of creatine and ATP in the active site, whereas His 61 is of importance for the catalytic reaction but does not serve as an acid-base catalyst in the transphosphorylation of creatine and ATP. In addition, our data support the idea that the flexible loop bearing His 61 is able to move towards the active site and to participate in catalysis.

Keywords: acid/base catalysis; active site; creatine kinase; enzyme mechanism; guanidino kinases

Creatine kinase (CK[1], EC 2.7.2.3) is a key enzyme of the cellular energy metabolism, catalyzing the reversible phosphoryl transfer from phosphocreatine to ADP (for a review, see Wallimann et al., 1992). CK is present in vertebrate cells of high and fluctuating energy demand, for example, muscle fibres, neurons, photoreceptors, spermatozoa, or electrocytes, where it regenerates ATP from phosphocreatine during cellular work. Several cytosolic and mitochondrial isoforms of the enzyme have been reported. Three cytosolic isoenzymes exist in homo- or heterodimeric form (BB-, MB-, and MM-CK), and two mitochondrial CKs occur mainly as octamers (Mi_a- and Mi_b-CK), with Mi_b-CK being the sarcomeric muscle specific isoform, whereas Mi_a-CK is found in brain and most other tissues (Wallimann et al., 1992; Wyss et al., 1992).

At pH 8.0 and above, favoring the forward reaction in the direction of phosphocreatine (PCr) synthesis, early kinetic studies were consistent with a rapid equilibrium-random scheme (Morrison & Cleland, 1966) with phosphoryltransfer as the rate-limiting step (Kupriyanov et al., 1990). However, at pH 7.0, favoring the reverse reaction, the kinetic mechanism seems random only in the reverse direction and equilibrium ordered, with ATP adding before creatine (Cr), in the forward direction (Schimerlick & Cleland, 1973). At this lower pH, PCr is sticky, and its dissociation rate from the ternary enzyme–MgADP–PCr complex is significantly lower than the interconversion of enzyme-bound substrates and products (Cook et al., 1981), indicating that the rate-limiting step with the substrates MgATP and Cr is the product (PCr) release. Such an interpretation is in line with ^{31}P-NMR studies of CK (Rao & Cohn, 1981; Furumo & Viola, 1989; Kupriyanov et al., 1990) and with the kinetic features of many kinases. The reaction is accompanied by a conformational change of the enzyme (McLaughlin et al., 1976; Forstner et al., 1996) involving the movement of domains relative to each other.

Although the enzyme has been known for nearly 80 years, remarkably little has been known until recently about the details of the catalytic mechanism, mainly because of the lack of a high-resolution structure. Several amino acid residues were subjected to mutational analysis on the basis of either educated guesses or chemical modification results, for example, the active site cysteine

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Abbreviations: Ac, acetate anion; CK, creatine kinase; Cr, creatine; IPCR, inverse polymerase chain reaction; Mi_b-CK, chicken sarcomeric muscle mitochondrial creatine kinase; PCr, phosphocreatine; SAXS, small-angle X-ray scattering; wt, wild-type.

(C278), which was shown unequivocally not to be essential for catalysis while being important for synergism of substrate binding (Furter et al., 1993), or the tryptophane residues of Mi_b-CK (Gross et al., 1994) that by careful analysis using biophysical methods could be assigned to different roles in CK function and stability. pH studies suggested that a histidine residue was involved in the catalysis of the ATP creatine transphosphorylation, acting as a general acid-base catalyst by extracting a proton from the guanidine moiety of creatine, thereby facilitating the nucleophilic attack on the γ-phosphate. This residue, exhibiting a rather high pK_a of 7 in the absence of phosphate, instead of 5.7 to 6.3 as common for histidines in proteins (Sober, 1968; Antosiewicz et al., 1996), would have to be unprotonated for the phosphorylation of creatine and protonated in the reverse direction (Cook et al., 1981). Further NMR studies on the role of histidines in the active site of CK have revealed four such residues at distances of 12, 12, 14, and 18 Å from the Cr^{3+} in the paramagnetic β,γ-bidentate Cr^{3+}–ATP complex bound to the enzyme (Rosevear et al., 1981). However, lacking a high-resolution structure of the protein, the putative catalytic importance of a histidine could not be correlated with any of the histidine residues conserved in the creatine kinase family.

Chicken muscle Mi_b-CK has been crystallized in our laboratory (Schnyder et al., 1990) and its X-ray structure in the presence of bound ATP has been solved (Fritz-Wolf et al., 1996). Analysis of the ATP-binding region showed histidine residues that were close to the active site and might be involved in catalysis. These residues were His 92, His 291, His 229, His 61, and His 186, at distances of 12, 12, 14, 17, and 18 Å from the O7 atom of ATP, respectively. His 92 is closest to the γ-phosphate and to Cys 278. His 61, however, is sitting on a flexible loop with a high crystallographic temperature factor, i.e., more than 40 $Å^2$, that can be modeled to move towards the bound ATP and can be considered to be involved in structural reorganization of the active site upon catalysis. Whereas all other His residues mentioned above are conserved through the whole family of guanidino kinases, His 61 is a conserved residue in all known creatine kinase isoenzymes. It is, however, not conserved in the closely related arginine kinase from lobster, where this residue is replaced by serine, nor in guanidino kinases from *Caenorhabditis elegans* or *Schistosoma mansoni* where alanine or valine (*C. elegans*) or serine (*S. mansoni*) are found instead (Mühlebach et al., 1994). The histidine residues of rabbit MM-CK, corresponding to His 92, His 101, His 186, His 229, and His 291 in chicken Mi_b-CK were recently described not to be of importance for the transphosphorylation reaction catalyzed by creatine kinase (Chen et al., 1996). This study, however, lacks mutation of His 61 as well as kinetic analysis of the mutants made in the reverse direction of the reaction coordinate and is hampered also by the inability to purify the mutant of His 96 (corresponding to His 92 in Mi_b-CK) to homogeneity.

A detailed view of the ATP binding site with the three histidine residues studied here and His 291 explicitely shown is depicted in Figure 1. Table 1 shows the evolutionary conservation of histidine residues in the family of guanidino kinases.

From the analysis of distances we concluded that either His 92 or His 61 could function as the general acid-base catalyst. In order to analyze the importance of these residues for catalysis, as well as of His 186, which we considered to be important for nucleotide binding by forming an H-bond with the adenine, we performed site-directed mutagenesis studies exchanging the histidines versus alanine or aspartate residues, respectively.

Results

Mutation of residues and purification of mutant proteins

The amino acid residues His 61, His 92, and His 186 of chicken Mi_b-CK were successfully mutated by inverse PCR to yield their respective alanine or aspartate mutants. The recombinant proteins were expressed in *E. coli* under control of the T7-phage promoter in the expression vector pET-3b (Studier et al., 1990) and subsequently isolated as homogeneous proteins as described (Furter et al., 1992). Table 2 shows typical yields for the expression of the respective mutants. Most of the mutants were isolated in soluble form with the exception of His 92 Asp, which formed inclusion bodies and had to be purified by denaturation with 8 M urea and subsequent renaturation. This resulted in a lower yield compared to the other mutants. All mutant proteins were equally stable during storage at 4 °C, indicating that the substitutions of the histidines had no adverse effect on the proper folding and stability of Mi_b-CK. This is also indicated by the fact that His 92 Asp refolded properly upon renaturation from the urea-denatured state.

Small-angle X-ray scattering

To compare the overall structural parameters of the mutant proteins to those of the wt, small-angle X-ray scattering experiments (SAXS) were performed. The determination of the radii of gyration (R_g) and the maximum chord lengths (D_{max}) showed no significant differences to the values determined for the wt enzyme (Forstner et al., 1996). By fitting the linear parts of the scattering curves to straight lines in the Guinier representation, smeared R_g values of 56 ± 2.5 Å were determined for unliganded wt and mutant Mi_b-CKs. D_{max} values in the range of 154 ± 4 Å and desmeared R_g values slightly smaller than the respective smeared parameters were determined after data treatment as described in Materials and methods. The shapes of the radial distribution [$P(r)$] curves of wt and mutant Mi_b-CKs calculated by Fourier transformation of the scattering curves did not differ significantly in shape. The $P(r)$ curve of free Mi-CK showed the characteristics of a roughly spherical, more or less hollow particle, closely matching the $P(r)$ curve computed from the crystal coordinates (data not shown).

Analysis of mutant activities

The pH-dependencies of the reverse (ATP synthesis) and forward (ATP consumption) reactions were determined for each mutant by the pH-Stat method (Milner-White & Watts, 1971; Wallimann et al., 1984). The mutants of His 186 and His 92 showed the same pH-optima as the wt enzyme at pH 6.0 for the reverse reactions and at pH 9 for the forward reaction, whereas the pH-optimum for the His 61-mutants was shifted to the acidic side by 0.5 pH units to 5.5 for the reverse reaction. In the forward reaction His 61 Asp was completely inactive, whereas the His 61 Ala mutant showed a pH optimum largely similar to the wt enzyme, although the latter has to be taken with caution because activites were so low that large errors have to be taken into account. The activity profiles of the mutants with respect to pH are shown in Figure 2.

All specific activities were determined at pH 8.0 for the forward and pH 7.0 for the reverse reactions, respectively. The specific activities of the mutant and wt Mi_b-CKs are compiled in Table 3. As can be seen clearly, no mutant was completely inactive in the reverse reaction and only one mutant, His 61 Asp, was completely inactive in the forward reaction. The most dramatic loss of activity

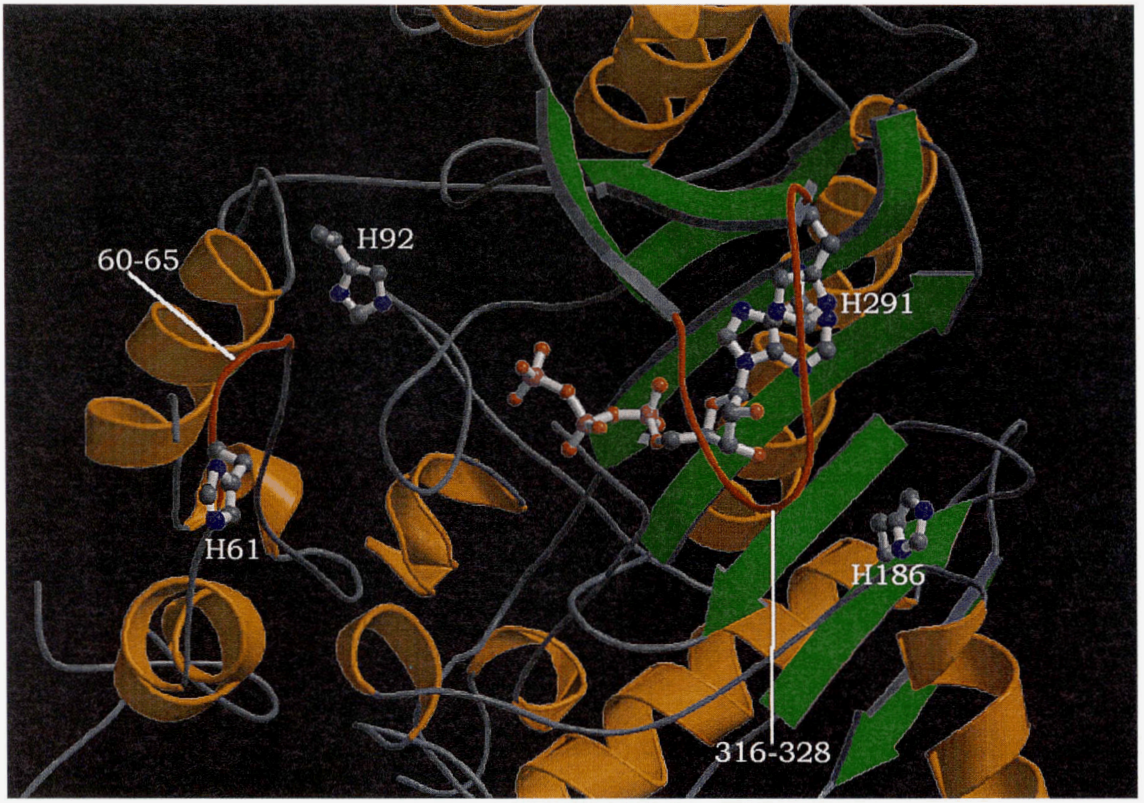

Fig. 1. The active site of CK. The active site region of chicken Mi_b-CK surrounding bound ATP as seen in the crystal structure is shown. ATP and the side chains of the histidine residues studied are drawn in a ball-and-stick representation, numbering is according to the position of the residues in the sequence of Mi_b-CK. His 291, which was studied by Chen et al. (1996), is included as well. The flexible parts of the structure, as indicated by temperature factors above 40 $Å^2$, i.e., the loop bearing His 61 (residues 60–65) and another flexible loop covering the ATP binding site (residues 314–331), are colored red and depicted by arrows. The figure was prepared from the X-ray coordinates of Mi_b-CK (Fritz-Wolf et al., 1996) using the program Molscript (Kraulis, 1991) and rendered with the use of the Raster3D software (Bacon & Anderson, 1988; Merritt & Murphy, 1994).

in the reverse reaction was observed for the same His 61 Asp mutant that exhibited only 1.6% of the wt activity.

Kinetic analysis

Kinetic analysis of the mutants was performed with their substrates ADP, ATP, creatine, and phosphocreatine, respectively, and the data were analyzed using software by W.W. Cleland (see Materials and methods). The percentage of increases or decreases in activity of the mutants are given with respect to the wt Mi_b-CK kinetic data that were also determined and were found to be comparable to

Table 1. *Evolutionary conservation of histidine residues in the active site of creatine kinase through the family of guanidino kinases*[a]

	His 61	His 92	His 186	His 295
CK	+	+	+	+
AK	−	+	+	+
GK	−	+	+	+

[a]CK represents the family of creatine kinases with their consensus sequence. AK is arginine kinase from lobster. GK is the guanidino kinase consensus sequence. A plus sign (+) denotes evolutionary conservation throughout the respective family, whereas a minus sign (−) means that this residue is not conserved in the consensus sequence. The numbers of the histidine residues denote their positions in the sequence of chicken Mi_b-CK. The consensus sequences for this table were taken from Mühlebach et al. (1994).

Table 2. *Expression yields of mutant proteins*[a]

Enzyme	Yield (mg/L)
wt	68.7
His 61 Ala	64.0
His 61 Asp	49.3
His 92 Ala	56.3
His 92 Asp	25.8
His 186 Ala	57.8
His 186 Asp	72.6

[a]All mutants were expressed in *E. coli* with the help of the T7-phage promoter in the expression vector pET-3b and purified as soluble proteins in a two-step procedure employing Blue-Sepharose affinity chromatography and ion-exchange chromatography, with the exception of His 92 Asp, which formed inclusion bodies that had to be dissolved by denaturation with urea, followed by dialysis against urea-free buffer and purification via ion-exchange chromatography.

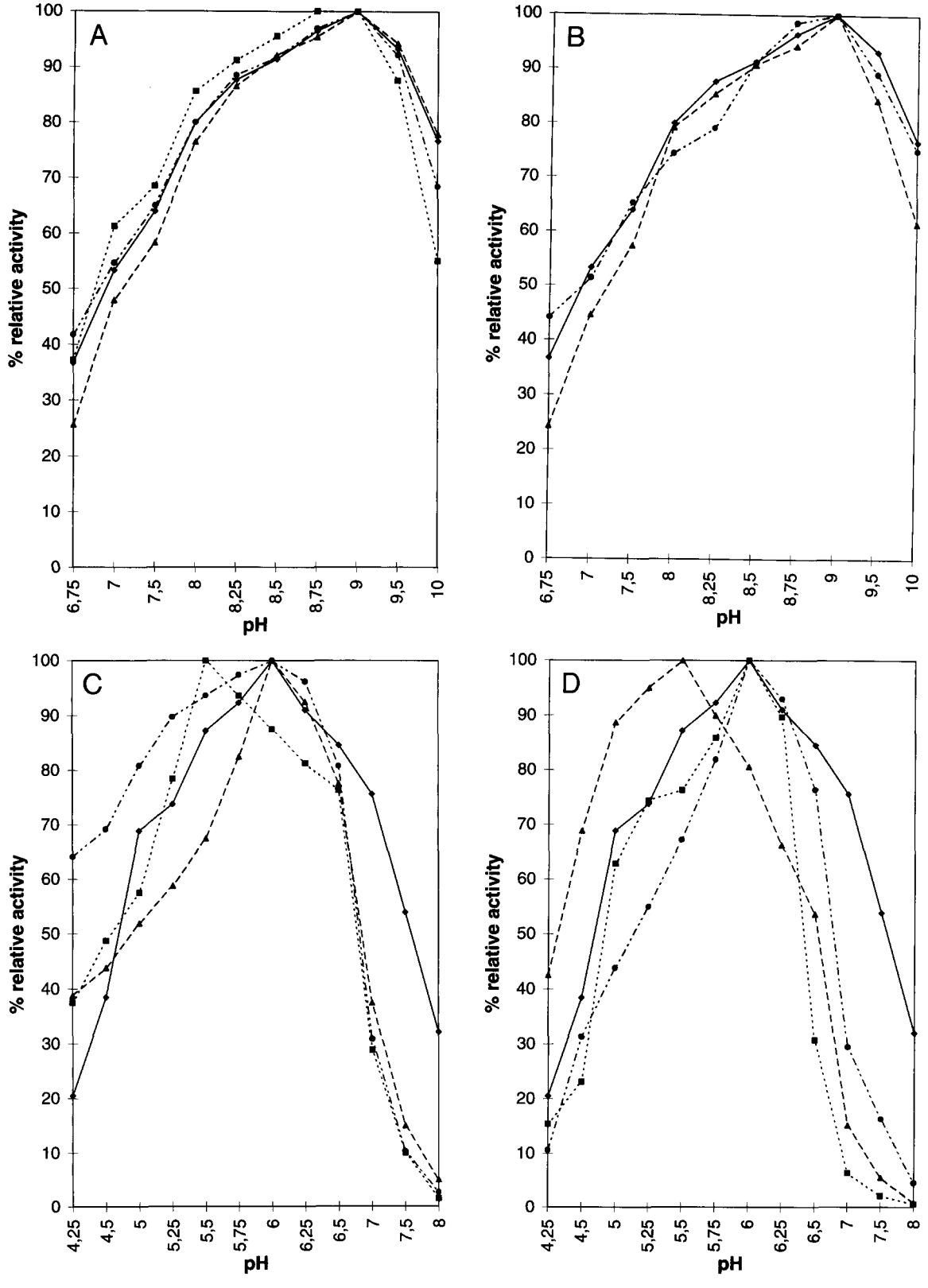

Fig. 2. pH optimum curves for wild-type and mutant Mi_b-CK enzymes. Relative activities (ratio of initial rates) in dependence of pH are shown. The maximum activity of each Mi_b-CK enzyme was set at a nominal value of 100%. However, the absolute specific activities of the mutant enzymes are lower than those of the wild-type (see Table 3)! **A:** Forward reaction (ATP consumption) for His to Ala mutants. Reaction conditions were 45 mM Cr, 5.4 mM ATP, 75 mM KCl, 0.1 mM EGTA, 16 mM $MgAc_2$, and 1 mM β-mercaptoethanol at 25 °C. **B:** Forward reaction for His to Asp mutants. **C:** Reverse reaction (ATP synthesis) for His to Ala mutants. Reaction conditions were 10 mM PCr, 1 mM ADP, 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM β-mercaptoethanol at 25 °C. **D:** Reverse reaction for His to Asp mutants (wild-type: solid diamond, solid line; mutants of His 61: solid square, dotted line; mutants of His 92: solid triangle, dashed line; mutants of His 186: solid circle, dashed-and-dotted line).

Table 3. *Specific enzyme activities*[a]

Enzyme	Forward reaction units/mg	Forward reaction % wt	Reverse reaction units/mg	Reverse reaction % wt
wt	76.1 ± 2.8	100	161 ± 3.5	100
His 61 Ala	0.84 ± 0.02	1.1	16.7 ± 1.0	10.4
His 61 Asp	No activity	No activity	2.57 ± 0.19	1.6
His 92 Ala	51.8 ± 1.1	68.1	105 ± 3.1	65.2
His 92 Asp	33.7 ± 1.3	44.3	74.8 ± 2.2	46.5
His 186 Ala	25.2 ± 1.3	33.1	51.5 ± 1.7	32
His 186 Asp	15.7 ± 0.89	20.6	23.7 ± 0.93	14.7

[a]Specific enzyme activities averaged for two independend protein preparations are shown. Activities were reproducible between individual preparations. The specific activities were determined under the following conditions: (A) Forward reaction (Cr + ATP → PCr + ADP + H^+): 90 mM Cr, 16 mM ATP, 16 mM $MgAc_2$, 75 mM KCl, 0.1 mM EGTA, and 1 mM β-mercaptoethanol at pH 8.0, 25 °C. (B) Reverse reaction (PCr + ADP + H^+ → Cr + ATP): 10 mM PCr, 10 mM ADP, 4 mM $MgCl_2$, 75 mM KCl, and 1 mM β-mercaptoethanol, pH 7.0, 25 °C.

One unit of enzyme activity is equal to 1 μmol of ATP or PCr transphosphorylated per minute, respectively, at 25 °C.

previously determined parameters (Furter et al., 1993; Chen et al., 1996). K_m values of ADP have to be handled with care, as very low concentrations of ADP are needed for the experimental determination of this parameter due to the low value of the wt K_m. The kinetic parameters K_m, K_d, and V_{max} are found compiled in Table 4 for the forward, K_m and V_{max} in Table 5 for the reverse reaction, respectively. (K_d refers to the dissociation constant of the reaction E + A → EA, or E + B → EB, and K_m refers to the Michaelis-Menten constant of the reaction EA + B → EAB or EB + A → EAB, whereby A and B represent either ADP and PCr or ATP and Cr, respectively. V_{max} denotes the maximal initial velocity as determined from Michaelis-Menten kinetics. Forward reaction is PCr synthesis, reverse reaction is ATP synthesis.)

Histidine186

In the reverse reaction a 33-fold increase of K_m for PCr was observed for the His 186 to Ala mutant, whereas the respective V_{max} was only slightly reduced. The corresponding His 186 to Asp mutant showed a 21-fold increase in K_m for this substrate with an approximately 10-fold decrease of V_{max}. For ADP, both mutants showed roughly a doubling of K_m (see Table 5). In the forward reaction, a doubling of K_m for Cr could be observed with both mutants while the V_{max} values were slightly decreased. The K_m values for ATP were also roughly doubled in both mutants (see Table 4). Furthermore, mutation of histidine 186 led to a loss in synergism of substrate binding (see below), with the K_d values for creatine and ATP being approximately equal to the respective K_m values.

Histidine 92

The His 92 to Ala mutant did not show significant changes in activity in either direction of the reaction, whereas the K_m values for PCr and Cr were increased approximately 10-fold. The His 92 to Asp mutant exhibited an approximately 15-fold increase in K_m for Cr and a 27-fold increase of the K_m for PCr. The determination of the K_m values for the nucleotides showed no changes for ADP, whereas the affinities for ATP of both mutants were decreased as seen by a fivefold increase of K_m with both enzymes (see Tables 4 and 5). Remarkably, mutants of this residue also revealed the most severe loss of synergism of all the mutants studied (see below). The His 92 to Ala mutant further showed an increase in V_{max} in the reverse reaction, although the affinity for phosphocreatine was markedly decreased, which we, however, do not consider significant.

Histidine 61

In the forward direction the mutant His 61 Asp was completely inactive, whereas the respective His 61 to Ala mutant exhibited about 1% residual activity (see Table 3). No activity of His 61 Asp was observed upon changing the pH of the reaction in either direction by 1.5 units in steps of 0.5 pH units, to check for a possible change in the pH optimum of the reaction. In the reverse direction the His 61 to Asp mutant showed 1.7% residual activity, whereas the His 61 to Ala mutant still had 10% wt activity under standard conditions (see Table 3). The affinities of Cr and PCr to the His 61 Ala mutant were decreased, as can be seen from the significantly increased K_m values (20-fold for PCr or Cr), whereas the affinities

Table 4. *Kinetic constants for the enzymes, forward reaction (PCr synthesis)*[a]

Enzyme	K_m(ATP) (mM)	K_d(ATP) (mM)	K_m(Cr) (mM)	K_d(Cr) (mM)	V_{max} (units/mg)
wt	0.31 ± 0.02	0.72 ± 0.04	8.80 ± 0.31	20.4 ± 3.2	61.7 ± 0.82
His 61 Ala	0.74 ± 0.03	0.73 ± 0.14	148.3 ± 2.7	142.1 ± 11.2	2.54 ± 0.04
His 61 Asp	n.d.[b]	n.d.	n.d.	n.d.	n.d.
His 92 Ala	1.45 ± 0.23	1.22 ± 0.27	87.0 ± 1.1	66.3 ± 8.5	43.2 ± 0.73
His 92 Asp	1.55 ± 0.22	1.13 ± 0.34	129.1 ± 1.8	93.2 ± 7.8	24.1 ± 0.68
His 186 Ala	0.73 ± 0.04	0.72 ± 0.11	21.9 ± 0.67	22.0 ± 2.7	45.6 ± 0.67
His 186 Asp	0.78 ± 0.02	0.75 ± 0.19	22.5 ± 0.52	21.5 ± 3.3	48.2 ± 0.77

[a]Reaction conditions were 75 mM KCl, 0.1 mM EGTA, 16 mM $MgAc_2$, and 1 mM β-mercaptoethanol at pH 8.0, 25 °C. Cr concentrations were varied between 7 and 45 mM for the wt enzyme and between 40 and 90 mM for the mutant enzymes. ATP concentrations were varied between 0.155 and 2.33 mM for wt and mutant enzymes.

[b]n.d.: not done, due to lack of activity.

Table 5. *Michaelis-Menten constants for the enzymes, reverse reaction (ATP synthesis)*[a]

Enzyme	K_m(ADP) (mM)	K_m(PCr) (mM)	V_{max} (units/mg)
wt	0.166 ± 0.037	1.21 ± 0.11	92.2 ± 1.0
His 61 Ala	0.160 ± 0.049	23.3 ± 0.48	3.20 ± 0.19
His 61 Asp	0.175 ± 0.051	19.0 ± 1.6	0.93 ± 0.03
His 92 Ala	0.140 ± 0.044	33.2 ± 1.9	136.5 ± 1.1
His 92 Asp	0.090 ± 0.038	11.7 ± 0.35	71.6 ± 3.7
His 186 Ala	0.320 ± 0.043	40.0 ± 2.6	57.4 ± 0.98
His 186 Asp	0.430 ± 0.047	25.3 ± 1.7	13.7 ± 2.4

[a]The assay mixture for the wild-type and the mutants contained 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM β-mercaptoethanol at pH 7.0, 25 °C. For the determination of K_m values of PCr, the concentration of ADP was 4 mM. PCr concentrations were varied between 30 and 0.2 mM for wt and 30 and 3 mM for mutant enzymes. For the determination of K_m values of ADP, the concentration of PCr was 10 mM and the ADP concentration was varied between 1 and 0.1 mM for wt and mutant enzymes.

for nucleotide binding are less affected (a twofold increase in K_m for ATP and no significant change for ADP). The same effects were observed for ADP and PCr with the His 61 Asp mutant in the reverse reaction (see Tables 4 and 5). Furthermore, the mutants of His 61 were the only ones with a significant change in their pH-optima in the reverse direction. Both mutants showed a decrease of their pH optima from 6.0 for the wild type to 5.5 (see Fig. 2C, D). A loss of synergism was also observed for His 61 Ala, albeit the effect was not as large as with the mutants of His 92.

Synergism

Creatine kinase isoenzymes were shown to exhibit synergism of substrate binding, thereby facilitating the binding of one substrate if the other is already bound to the active site of the enzyme (Maggio et al., 1977). Mutations of several amino acid residues of Mi_b-CK, for example, the reactive cysteine (Cys 278), have been shown to lead to a decrease or complete loss of synergism of substrate binding (Furter et al., 1993). Therefore, we also determined the K_d values, i.e., the dissociation constants for the formation of binary enzyme–substrate complexes, for Cr and ATP. In the case of synergism, K_d is expected to be significantly higher than the Michaelis constant K_m, representing the affinity of each of the substrates for its corresponding binary complex of reaction partners to form the catalytically active ternary complex. All active mutant proteins studied showed a significant decrease of synergism of substrate binding, with His 92 exhibiting a complete loss of synergism for both substrates (see Table 4).

Discussion

A histidine residue with a pK_a of approximately 7 has been proposed to play a crucial role in the catalysis of the phosphorylation of creatine by ATP, mediated by creatine kinase. We have performed in vitro mutagenesis studies of those histidine residues (61, 92, and 186) of Mi_b-CK, which were found to be closest to the active site determined by X-ray crystallography (Fritz-Wolf et al., 1996) by exchanging them against an alanine or an aspartate residue. Several other histidine residues that are conserved throughout the family of the guanidino kinases were omitted from this study due to their location, either close to the purine ring of adenine or too far away from the putative active site, making them unsuitable as canditates for the active site histidine. While this article was written, His 291, also located close to the ATP binding site, was shown by Chen and co-workers (1996) in studies of the highly homologous rabbit muscle CK not to serve as the catalytic base in the reaction of creatine kinase. These authors, however, did not study the histidine residue, His 65, in MM-CK, which corresponds to His 61 in chicken Mi-CK, the mutation of which herein is shown to have most severe effects on CK activity. Unfortunately, they were also unable to purify the mutant protein of His 96 (corresponding to His 92 here) nor did they perform kinetic analysis in both directions of the reaction coordinate. Their results for the histidine residues 92 (96 in MM-CK) and 186 (190 in MM-CK), however, correlate very well with our findings.

All mutant Mi_b-CKs could be isolated in homogeneous form after expression in *E. coli*. The refolding of mutant His 92 Asp to an enzymatically and structurally intact protein after denaturation with 8 M urea as well as the determination of overall structural parameters by SAXS showed that the mutations did not adverseley affect the folding behavior of Mi_b-CK. We, therefore, conclude that none of the observed activity effects was due to misfolding of any of the mutant enzymes, although local changes in fold cannot be excluded.

From enzyme activity analysis and determination of the kinetic parameters we concluded that His 92 and His 186 were unsuitable candidates for the postulated acid-base catalyst in the CK active site; they both, however, have to be involved in substrate binding, as suggested by the decrease of synergism observed upon mutation of either of the residues and the changes in the K_m values for the substrates. A mutation of His 92 has major effects on the creatine/phosphocreatine binding site of Mi_b-CK and affects the binding of both substrates, whereas His 186 is of primary importance for the binding of phosphocreatine, while binding of creatine is less affected. Without a high-resolution structure of the enzyme in the presence of creatine or phosphocreatine these results obviously cannot be interpreted unequivocally. The fact, however, that His 92 is located rather close to the γ-phosphate of ATP (Fig. 1), lets us conclude that this residue is likely to be located close to the creatine/phosphocreatine binding site of the enzyme. We can tacitly assume that altering the electrostatic properties of the region in the ATP/ADP–creatine/phosphocreatine interface should have effects on the catalytic process, even if the mutated residue is not directly involved in catalysis. As His 186 is also located close to the adenine ring of ATP in the crystal structure of Mi_b-CK, an explanation for the observed effects on creatine or phosphocreatine binding is more difficult to find. At the moment, we have no satisfactory explanation why a mutation of this residue primarily effects the latter substrates and not the binding of nucleotide; however, we assume that a mutation of His 186 might lead to a change in the conformation of bound nucleotide, which in turn, could affect the binding properties of phosphocreatine or creatine.

His 61 turned out to be a more suitable candidate for the postulated acid-base catalyst. This residue is located on a highly flexible loop, as suggested by high crystallographic B-values of the loop structure compared to the rest of the molecule. This loop could also be modeled by simulated annealing (Kirkpatrick et al., 1983; Brünger, 1991) to undergo a movement towards the active site, thereby bringing His 61 from a distance of originally 17 Å in closer proximity to the γ-phosphate of ATP (data not shown; de-

tails will be published elsewhere. The histidine to alanine mutant shows residual activities of 10% and 1% in the back- and forward reactions, respectively, compared to wt creatine kinase. The histidine to aspartate mutant, however, which exhibits only 1/60 of the wildtype activity in the ATP synthesis reaction, is enzymatically inactive in the direction of ATP consumption.

We are now confronted with the question whether His 61 is indeed the catalytic base in Mi_b-CK. There are several caveats that lead us to assume that, while the amino acid residue His 61 is certainly important for catalysis, it is not essential for catalysis, very much like the active site cysteine Cys 278 (Cys 282 in MM-CK; see Furter et al., 1993).

Considering the distribution of charges, for the CK reaction a potential acid-base catalyst would have to be unprotonated for the phosphorylation of creatine and protonated for the reverse reaction, a condition that His 61 indeed could fulfill under physiological conditions, provided that this residue had a pK_a of around 7. Mutation to an aspartate, with a pK_a of approximately 4.5 then yields a residue that is unprotonated in the optimal pH regions of both forward and reverse reactions of CK. Thus, aspartate could indeed serve as a weak base, thereby still potentially being able to convey residual activity for the phosphorylation of creatine. On the other hand, alanine can definitely not be protonated or unprotonated under physiological conditions and the respective mutant should be completely inactive. Both theoretical predictions were not observed in our experiments. However, the histidine to aspartate mutant was inactive in the forward reaction while having some activity in the reverse reaction, whereas the alanine mutants still showed residual activity both in the forward and reverse reactions (see above). While mutation of an catalytically essential residue is supposed to lead to inactive proteins, we did not observe complete inactivation with any of the mutants of His 61. This alone is evidence enough for concluding that His 61 cannot be an essential residue for CK function. However, His 61 could, nevertheless, still be a catalytic residue providing elctrostatic interactions (e.g., hydrogen bonds) with the reaction partners in the CK reaction. This would explain very well the effects observed upon mutation. While an exchange of His 61 against aspartate can be expected to result in dramatic alterations of the electrostatic environment of the active site by introducing a net negative charge, the mutation to alanine should not lead to such pronounced effects. This would then explain why His 61 Ala has residual activity in both directions of the reaction. The inactivity of His 61 Asp in the forward reaction with some residual activity in the reverse direction can be described by a primary involvement of this residue in the breakage of the β-γ phosphoryl bond of ATP in the forward reaction (see below) but with less importance for the reverse reaction. There is also the possibility of some "redundancy" being built into the protein structure, i.e., His 61 is the catalytic residue but, upon mutation, some compensatory mechanism might work, as recently suggested for tRNA-amino acid synthases (First & Fersht, 1995).

A very strong argument against the role of His 61 as the catalytic base in Mi_b-CK comes from evolution by comparing the primary structures of the guanidino kinases (Table 1). Whereas His 186 and His 92 are conserved throughout all guanidino kinases, His 61 is conserved through all creatine kinases but not in the closely related arginine kinase from lobster, where it is replaced by a serine. In a guanidino kinase from *Schistosoma mansoni* an alanine residue is found at the position of His 61. Because acid-base catalysis involves the guanidino moiety of the phosphagens, one would expect nature to have conserved the catalytic residues of the otherwise highly conserved guanidino kinases throughout evolution. This, in our opinion, indicates that His 61 cannot be the catalytic base in CK, provided that the catalytic pathway of transphosphorylation has been conserved throughout the family of guanidino kinases. We can also conclude that a mutation to a residue that already occurred in evolution (His to Ala, see above) has less dramatic effects on catalytic activity than a corresponding mutation to an inversely charged amino acid.

Obviously, the combined results of this study and of Chen et al. (1996) lead to the conclusion that no histidine residue serves as an acid-base catalyst in the transphosphorylation of ATP and creatine catalyzed by Mi_b-CK. This, in turn, opens the question of an alternative pathway for the reaction to proceed. As recently pointed out by R.M. Stroud (1996), creatine kinase might well belong to a group of enzymes for which he has coined the name "conzymes." This class of enzymes are thought to primarily align their substrates and to exclude excess water from the active site, to allow the enzymatic reaction to take place. In our case, this would mean that creatine kinase has the primary function of aligning the substrates in a way to facilitate phosphate transfer and catalysis would take place more or less spontaneously, once all surplus water has been excluded from the active site and the reaction partners have found their proper orientations. In such a model there is no need for an "essential residue," with all the amino acids in the active site just providing the electrostatic environment to make the substrates favorably adopt a conformation that allows the reaction to proceed. This theory is intriguing, as it is still in line with the hypothesis originally stated by Linus Pauling (Pauling, 1946) that enzymes work by stabilizing the transition state of a reaction, which since has been confirmed by different studies (Lolis & Petsko, 1990). Again, our assumptions made above that changing the potential in the active site by exchanging histidine against aspartate should lead to more dramatic changes in the enzyme's properties than the respective exchange against an alanine residue, would hold true with this theory. The "conzyme" theory can also very well explain why mutations of several amino acid residues that previously have been thought to be essential for CK reactivity did not lead to severe changes in enzymatic activity. However, it still holds open the question whether a catalytic base, which is theoretically needed for the transphosphorylation of ATP and creatine to take place, indeed exists.

Recently, similar considerations about the need for a catalytic base in the p21-Ras protein were investigated by the group of Arieh Warshel (Schweins et al., 1994, 1995). Several mutational studies by different groups were not successful in detecting the postulated catalytic base needed for GTP hydrolysis by this protein. Thorough theoretical calculations applying the protein dipoles/Langevin dipoles (PDLD) model (Warshel & Russel, 1984; Russel & Warshel, 1985) revealed that indeed no amino acid residue was playing that role, but that the γ-phosphate of GTP itself acted as catalytic base in that reaction. We are currently investigating the possibility for a similar role of ATP in the transphosphorylation of ATP and creatine. A potential role for His 61 in the creatine kinase reaction could in this case come from interaction with the nucleophile, creatine, by stabilizing it or, alternatively, by stabilizing the transition state of the reaction. Upcoming X-ray data of diffraction experiments from recent crystallization work (M. Forstner & W. Kabsch, unpubl. obs.) should provide us with more detailed information to further investigate the reaction pathway of the CK reaction.

We conclude that His residue 61 of Mi_b-CK certainly plays a critical role in the catalysis of transphosphorylation of creatine and

ATP, most probably by its contribution to the electrostatic environment of the active site. It cannot, however, be considered essential as shown by significant residual activites of the mutants. The strong effects on catalysis observed by mutation of His 61 also give indirect evidence for the importance of the highly flexible loop for the active site structure. The other residues investigated, His 92 and His 186, are both of importance for substrate binding, mainly the binding of creatine and phosphocreatine, but neither of these residues is essential for the function of Mi_b-CK as well. Our observations also lead us to the conclusion that the ATP-creatine transphosphorylation reaction by creatine kinase is not catalyzed by a histidine serving as an acid-base catalyst. Other reaction pathways have to be taken into account that will, however, have to be further investigated by more thorough theoretical studies of the active site of CK based on high-resolution structures of the active site in the presence of all substrates, once they are available.

Materials and methods

Inverse PCR (IPCR)

Plasmid pRF23, coding for the wt Mi_b-CK (Furter et al., 1992) was used as a template in all IPCR experiments. To amplify whole plasmids as templates while avoiding random mutations, DNA polymerase from *Pyrococcus furiosus* (Stratagene) was used. The reactions were initiated by adding the enzyme to the denatured DNA template at 95 °C. Routinely, 25 cycles of PCR were performed (95 °C, 1 min.; annealing, 1 min; 72 °C, 7 min). The following primers and annealing temperatures (T_{ann}) were used to introduce point mutations: His 61 Ala ($Mi171H_1$: 5′-GGACAAT CCTGGCGCTCCTTTCATTA-3′, Mi170Z: 5′-ACCCCAGTCTGG ATGCACTGGT-3′, T_{ann} = 54 °C); His 61 Asp ($Mi171H_2$: 5′-GGACAATCCTGGCGATCCTTTCATTA-3′, Mi170Z, T_{ann} = 54 °C); His 92 Ala ($Mi264H_1$: 5′-TAAAGCAAGAGCTAACGGC TATG-3′, Mi263Z: 5′-ATGACAGGGTCAAAAATCTCAGC-3′, T_{ann} = 45 °C); His 92 Asp ($Mi264H_2$: 5′-TAAAGCAAGAGAT AACGGCTA-3′, Mi263Z, T_{ann} = 52 °C); His 186 Ala ($Mi559H_1$: 5′-GCCTTTCTCTTTGACAAGCCGG-3′, Mi558Z: 5′- ATCATC AATAAGCTGCTGCTGATC-3′, T_{ann} = 45 °C); His 186 Asp ($Mi559H_2$: 5′-GACTTTCTCTTTGACAAGCCG-3′, T_{ann} = 45 °C). All PCR primers were phosphorylated on their 5′-ends to allow subsequent ligation and recircularization of the products. The linear PCR products were circularized using T4-DNA ligase, XL1-blue competent *E. coli* cells were transformed with the plasmids and plasmids were prepared and analyzed using routine procedures (Ausubel et al., 1992). All mutated plasmids were checked routinely for the success of in vitro mutagenesis by sequence analysis using the dideoxy-chain termination method (Sanger et al., 1977; Tabor & Richardson, 1987).

Protein purification

E. coli cells of strain BL-21(DE3)lys S were transformed with plasmids coding for the mutant and wt enzymes and proteins were expressed as described previously (Furter et al., 1992). The proteins were purified from the cell extracts as described (Furter et al., 1992) or from inclusion bodies in the case of His 92 Asp. In this case, the cells were harvested by centrifugation, resuspended in ice-cold NaCl (0.9%), lysed by ultrasonification, and pelleted again. The pellets, containing the inclusion bodies, were then dissolved in 8 M urea (U-8 buffer: 8 M urea, 10 mM Na-phosphate, 5 mM EDTA, 10 mM β-ME, pH 8.0), and incubated at room temperature for 12 h. The solution was subsequently dialyzed against buffer A (40 mM Na-phosphate, 50 mM NaCl, 0.2 mM EDTA, 1 mM β-ME, pH 7.0) with PMSF added, upon which most proteins in solution precipitated, whereas nearly 80% of the desired mutant protein refolded and remained soluble thereafter. Finally, the renatured protein was purified by cation exchange chromatography (Mono-S, Pharmacia, Sweden) employing a linear gradient of NaCl ranging from 50 to 250 mM in buffer A as described (Furter et al., 1992). All protein preparations were homogeneous as judged by Coomassie Blue-stained SDS-polyacrylamide gels, which were also used to monitor the purification steps. Protein concentrations were determined by the BioRad method (Bradford, 1976) using BSA as a standard.

Measurement of protein activity, enzyme kinetics

Protein activities were measured by the pH-stat method (Millner-White & Watts, 1971; Wallimann et al., 1984) in a buffer containing 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM β-ME by measuring the H^+-consumption in the reverse reaction (PCr + ADP + H^+ → Cr + ATP). The H^+-release in the forward reaction (Cr + ATP → PCr + ADP + H^+) was determined using a buffer containing 75 mM KCl, 0.1 mM EGTA, 16 mM $MgAc_2$, and 1 mM β-ME. For routine checks of enzyme activity, phosphocreatine and ADP were added to a final concentration of 10 mM each and the reaction was performed at pH 7.00 in the reverse direction. The pH-dependence of the reaction for wt and mutant enzymes was checked in steps of 0.25 pH units in the peak area and of 0.5 pH units elsewhere in the pH profile. For performing enzyme kinetics studies, the concentrations were varied for each of the substrates holding the other one constant. For details of the reaction mixtures for each kinetic experiment, see the respective Tables 4 and 5. For the determination of kinetic parameters K_m, K_d, and V_{max}, a software package by W.W. Cleland (Cleland, 1979) and adapted for personal computer by R. Viola was used (the program was obtained through R. Viola, Akron University, Akron, OH). Standard errors are given for the calculated values. Each set of constants represents the mean of three or four independent measurements.

Small-angle X-ray scattering (SAXS)

All SAXS experiments were performed using a Kratky camera (Kratky, 1982) with a thermostated sample holder at 20 °C using a quartz capillary with a volume of 60 μL and an internal diameter of 1.0 mm. The primary X-ray beam of 1.542 Å wavelength was produced by a water-cooled rotating anode generator. The camera was equipped with a position-sensitive proportional detector at a sample to detector distance of 26.7 cm and an associated data acquisition system (MBraun, Munich, Germany). After background subtraction, the resulting data were deconvoluted with the slit width and slit length profiles of the primary beam and subsequently subjected to Fourier transformation using the program ITP (Glatter, 1977) to calculate radii of gyration (R_g) and maximum chord lengths (D_{max}) of the samples.

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