An Unusually Low pK_a for Cys282 in the Active Site of Human Muscle Creatine Kinase†

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ABSTRACT: All phosphagen kinases contain a conserved cysteine residue which has been shown by crystallographic studies, on both creatine kinase and arginine kinase, to be located in the active site. There are conflicting reports as to whether this cysteine is essential for catalysis. In this study we have used site-directed mutagenesis to replace Cys282 of human muscle creatine kinase with serine and methionine. In addition, we have replaced Cys282, conserved across all creatine kinases, with alanine. No activity was found with the C282M mutant. The C282S mutant showed significant, albeit greatly reduced, activity in both the forward (creatine phosphorylation) and reverse (MgADP phosphorylation) reactions. The K_m for creatine was increased approximately 10-fold, but the K_m for phosphocreatine was relatively unaffected. The V and V/K pH-profiles for the wild-type enzyme were similar to those reported for rabbit muscle creatine kinase, the most widely studied creatine kinase isozyme. However, the $V/K_{creatine}$ profile for the C282S mutant was missing a pK of 5.4. This suggests that Cys282 exists as the thiolate anion, and is necessary for the optimal binding of creatine. The low pK of Cys282 was also determined spectrophotometrically and found to be 5.6 ± 0.1. The S284A mutant was found to have reduced catalytic activity, as well as a 15-fold increase in K_m for creatine. The pK_a of Cys282 in this mutant was found to be 6.7 ± 0.1, indicating that H-bonding to Ser284 is an important, but not the sole, factor contributing to the unusually low pK_a of Cys282.

Creatine kinase (CK, EC 2.7.3.2)[1] catalyzes the reversible phosphorylation of creatine (Cr). The product, phosphocreatine (PCr), is considered to be a reservoir of "high-energy phosphate" which is able to supply ATP, the primary energy source in bioenergetics, on demand. Consequently, creatine kinase plays a major role in energy homeostasis of cells with intermittently high energy requirements, such as skeletal and cardiac muscle, neurons, photoreceptors, spermatozoa, and electrocytes (*1*−*3*).

The enzyme is most common in muscle and brain cells, although smaller amounts are found in other tissues. There are two cytosolic creatine kinase subunits, brain (B) and muscle (M), which associate to form the muscle (MM) and brain (BB) isozymes. In addition, there is a heterodimeric isozyme (MB), and it is elevated levels of this form of the enzyme which are used as a marker for myocardial infarction (*4*). Finally, there are two forms of the enzyme associated with the inner membrane of mitochondria. These are referred to as the sarcomeric (Mi_s) and ubiquitous (Mi_u) isozymes and may exist either as dimers or as octamers. Depending on the conditions, these forms are readily interconvertible (*5*), but, unlike the cytosolic CKs, no heteromers have been found in vivo.

Creatine kinase cDNAs, from species ranging from sea urchins to dogs, have been cloned and sequenced. Considerable sequence homology was observed, with each encoding a protein of about 40 kDa (*5*, *6*). In recent times, the X-ray structures of each of the four isomers of CK have been solved (*7*−*11*), as well as that of arginine kinase (AK, EC 2.7.3.3), another member of the guanidino kinase family (*12*). The CK structures were all solved in the absence of bound ligand, but the AK structure was solved with a bound transition-state analogue complex, arginine−nitrate−ADP (*12*). Not surprisingly, since AK and CK exhibit approximately 40% amino acid sequence identity, the structure of the liganded AK could be superimposed on that of the unliganded rabbit muscle creatine kinase (RMCK). For this superposition, the RMSD, over 236 of 361 total α-carbons, was only 1.25 Å, indicating that the AK-TSAC structure provides a reasonable model for liganded CK active site.

Prior to the X-ray data becoming available, there were extensive studies on kinetic and mechanistic aspects of the CK reaction (*13*, *14*). These studies implicated several amino acid residues as being important for binding and/or catalysis, including arginine (*15*), aspartate (*16*), cysteine (*17*), histidine (*18*), lysine (*19*), and tryptophan (*20*). With the aid of site-directed mutagenesis, and in light of the X-ray structures, many of these results are now being reevaluated. For

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[1] Abbreviations: CK, creatine kinase; Cr, creatine; PCr, phosphocreatine; HMCK, human muscle creatine kinase; RMCK, rabbit muscle creatine kinase; MtCK, mitochondrial creatine kinase; sMtCK, sarcomeric mitochondrial creatine kinase; uMtCK, ubiquitous mitochondrial creatine kinase; AK, arginine kinase; WT, wild-type; TSAC, transition-state analogue complex; LB medium, Luria−Bertani medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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example, early studies suggested that a histidine acted as an acid/base catalyst in the protonation/deprotonation of the phosphate-accepting nitrogen (*18*, *21*). However, inspection of the active site of the AK structure (*12*), as well as mutagenesis studies (*22*, *23*), now unequivocally attests that none of the conserved histidine residues is able to fulfill this role.

Historically, one of the more contentious aspects of the CK mechanism is the role of the so-called "essential" cysteine residue. To date, all creatine kinases, including both cytosolic (*17*) and mitochondrial (*24*, *25*) isozymes, have been found to contain one reactive sulfhydryl group per monomer. The cysteine residue, identified as Cys282 in RMCK (*26*), which corresponds to Cys278 in the mitochondrial isoforms, is conserved throughout the known CK sequences. Moreover, the creatine-based affinity label, epoxycreatine, has been shown to be attacked by Cys282 of RMCK, giving rise to a thioether linkage (*27*). Modification of Cys282 with many sulfhydryl-specific reagents leads to the complete abolition of catalytic activity. Conversely, modification of Cys282 with either a thiomethyl group (*28*) or a cyano group (*29*) appears to lead to some retention of activity, an indication that the cysteine may not be essential. These results were challenged by later studies, suggesting that the residual activity was due to loss of label, and that the cysteine was undoubtedly essential (*30*, *31*). Recently, in a more definitive experiment, site-directed mutagenesis was employed to investigate the role of Cys278 in mechanism of chicken mitochondrial CK (*32*). This study revealed that the reactive cysteine did not play a direct role in catalysis, but is important for the maximal activity of the enzyme. However, again, the waters have been muddied by additional site-directed mutagenesis experiments, this time on the human brain isozyme, which established that replacement of Cys283, by either serine or tyrosine, resulted in complete loss of activity (*33*).

Since those experiments, the X-ray structures have appeared and confirmed that the "reactive" cysteine is indeed located within the CK active site. Further, the human muscle isozyme (HMCK) is now available in large quantities (*34*), and we thought it would be worthwhile to revisit this question, this time examining the human analogue of RMCK.

MATERIALS AND METHODS

Materials. Creatine, phosphocreatine, phosphoenolpyruvate, ADP, ATP, NADH, and NADP were purchased from Sigma Chemical Co. (St. Louis, MO). The coupling enzymes, pyruvate kinase, lactate dehydrogenase, hexokinase, and glucose-6-phosphate dehydrogenase, were also purchased from Sigma. All buffer salts and other reagents were of the highest quality commercially available.

Preparation of HMCK Variants. The mutants were prepared using *Pfu* DNA polymerase and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), using pETHMCK (*34*) as the DNA template. The forward primers used for the mutagenesis are shown below with the mutated codons underlined, with the lowercase letters indicating a base change from wild-type:

C282S: 5′-GCTACGTGCTgACCTcCCCgTCCAAC-CTGGGCAC-3′

C282M: 5′-GGCTACGTGCTgACCatgCCgTCCAAC-CTGGGCACTGG-3′

S284A: 5′-GCTACGTGCTCACtTGCCCAgCCAAC-CTGGGCAC-3′

In addition to creating the C282S and C282M mutations, a silent mutation was introduced which resulted in the gain of a *Drd*1 restriction site. For HMCK S284A, a silent mutation was also introduced, one which resulted in the loss of a *Bsp*M1 restriction site. Following mutagenesis, the template DNA was removed by treatment with *Dpn*1, and the remaining PCR products were transformed into *Escherichia coli* strain JM109 (Promega, Madison, WI). Single colonies were picked, and in each case their DNA was isolated and screened for the desired mutation by restriction analysis using either *Drd*1 (C282S, C282M) or *Bsp*M1 (S284A). For all mutants, the fidelity of the PCR amplification and the presence of the mutation were confirmed by sequencing. Plasmids containing the mutated HMCK were selected and denoted pETHMCKC282S, pETHMCKC282M, and pETHMCKS284A, respectively. For expression, the plasmids were transformed into *E. coli* strain BL21(DE3)pLysS (Novagen).

Expression and Purification of the Wild-Type Human Muscle Creatine Kinase (*HMCK*). Protein preparations were carried out as described previously (*34*) with some minor modifications. The transformed cells were grown in LB medium at 37 °C until OD_{600} reached ~0.6−0.8. The cells were cooled to 28 °C, and protein expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were grown for an additional 6 h at 28 °C prior to harvesting by centrifugation at 4000*g* for 8 min at 4 °C. The cell pellets were washed and resuspended in MES buffer (10 mM MES, 20 mM KCl, 1 mM DTT, pH 6.0) containing 0.1 mM PMSF. Cell lysis was achieved by freezing the cells at −20 °C followed by thawing at room temperature. After thawing, DNase was added to a final concentration of ~60 units/mL, and the lysed cells were centrifuged at 22000*g* for 30 min at 4 °C. The supernatant was loaded at room temperature onto a 2.6 × 14.5 cm Blue Sepharose CL-6B (Pharmacia Biotech) column which had been equilibrated in MES buffer. After further washing with MES buffer, the bound proteins were eluted with TES buffer (10 mM TES, 20 mM KCl, and 1 mM DTT, pH 8.0). The fraction containing creatine kinase activity was dialyzed at 4 °C against Tris buffer (50 mM, pH 8.8) containing 1 mM EDTA and 1 mM DTT. The dialyzed enzyme was then applied to a 1.6 × 2.5 cm HiTrap Q anion exchange column preequilibrated in 50 mM Tris buffer (pH 8.8) containing 1 mM DTT. The enzyme was eluted from the Q column with a linear gradient of 0−120 mM NaCl over 9 column volumes. The major fraction, which eluted around 30 mM NaCl, was more than 95% pure HMCK as judged by SDS−PAGE. The purified enzyme was concentrated and exchanged into 10 mM HEPES buffer containing 1 mM EDTA, pH 7.0, using an Amicon Centricon concentrator unit (Beverly, MA) before being stored in aliquots at −80 °C. The enzyme concentration was determined by using an absorbance at 280 nm of 0.88 for a 1 mg/mL sample (*35*), and checked using the Bradford assay.

Purification of HMCK C282S. The purification procedure for the C282S variant was essentially the same as that used

for the wild-type enzyme with the following adjustments. C282S HMCK was eluted from the Blue Sepharose column with TES buffer containing 500 mM KCl. C282S could be purified on the anion exchange column (HiTrap Q) in a manner similar to that of the WT enzyme. However, the maximum yield of enzyme was obtained when the gradient was run in 20 mM Tris at pH 8.4. Under those conditions, HMCK C282S eluted around 20 mM NaCl.

Purification of HMCK C282M. As with the C282S mutant, the binding of C282M on the Blue Sepharose column was tight, and this variant was also eluted with TES buffer containing 500 mM KCl. Conversely, the subsequent binding of C282M on the HiTrap Q column was very weak. As a consequence, the purification of C282M was completed by loading the protein onto a HiTrap Q column which had been equilibrated with 50 mM Tris/1 mM DTT at pH 8.4. Under these conditions, the enzyme did not bind to the column. However, all major impurities did bind, thereby permitting isolation of essentially pure C282M in the unbound fraction. The enzyme purified in this manner showed a single band on SDS−PAGE.

Purification of HMCK S284A. The purification procedure for the S284A variant was the same as that used for the wild-type enzyme. S284A was eluted from the column with about 40 mM NaCl.

Activity Assay and pH Studies. The activity of creatine kinase, in both directions, was measured at 30 °C. In the forward direction, i.e., the direction of creatine phosphorylation, a coupled assay, originally developed by Tanzer and Gilvarg (*36*), was used. Here, the reaction of creatine kinase is coupled to the reactions of pyruvate kinase and lactate dehydrogenase and the reaction followed by monitoring the absorbance decrease at 340 nm due to bleaching of NADH. The velocity of the reaction is calculated using an extinction coefficient of 6290 M^{-1} cm^{-1} for NADH.

Steady-state kinetic studies were carried out as described previously (*18*) with minor modifications. All assays were performed in a buffer system composed of 100 mM MES, 51 mM diethanolamine, and 51 mM *N*-ethylmorpholine. The buffer solutions were adjusted to the desired pH with acetic acid (for the pH range of 5.4−7.4) or potassium hydroxide (for the pH range of 7.4−9.8). This buffer system has major advantages in that both the buffer components and the ionic strength of the buffer remain constant over the whole pH range (*37*). The typical assay mixture contains 0.4 mM NADH, 0.4 mM phosphoenolpyruvate, 5 mM ATP, 6 mM magnesium acetate, and 13 mM potassium acetate, while the creatine concentration was varied from 10 to 100 mM. The concentrations for the coupling enzymes were 28−56 and 54−108 units/mL for pyruvate kinase and lactate dehydrogenase, respectively. To minimize degradation of NADH and the coupling enzymes, at pH values of 6.5 and below, NADH, lactate dehydrogenase, and pyruvate kinase were added immediately before the addition of creatine kinase. The concentrations of creatine kinase were 2.9−5.8 and 465−1860 nM for the wild-type enzyme and C282S variant, respectively.

In the reverse direction, i.e., the direction of ADP phosphorylation, the reaction was coupled to the reactions of hexokinase and glucose-6-phosphate dehydrogenase and followed by monitoring the increase in absorption at 340 nm due to the reduction of NADP. Again the assays were carried out essentially as described by Cook et al. (*18*) but with minor modifications. A typical assay mixture contained 15 mM glucose, 9 mM $Mg(OAc)_2$, 4 mM ADP, 9 mM DTT, and 1.3 mM NADP with variable phosphocreatine. As for the forward reaction, all assays were carried out in the Morrison buffer (*37*). The same levels of coupling enzymes, hexokinase (20 units) and glucose-6-P dehydrogenase (5 units), were employed over the entire pH range (*18*). The concentrations of creatine kinase were between 0.46 and 0.92 nM and between 168 and 838 nM for WT and C282S, respectively.

Data Processing. The kinetic data and pH profiles were analyzed using the method as described by Cook et al. (*18*). The kinetic parameters V_{max} and K_m (Cr or PCr) were determined at each pH by fitting the initial velocity data to eq 1, where v represents the initial velocity of the reaction observed from the coupled assay. Data for the pH profiles which showed a decrease in log V or log (V/K) with a slope of 1 as the pH was decreased were fitted to eq 2. Data for the $V/K_{creatine}$ profile, which decreased with a final slope of 2 as the pH was decreased, were fitted to eq 3. When log V and log (V/K) profiles decreased at both low and high pH, the data were fitted to eq 4. All curve fitting was carried out using SigmaPlot (Jandel Scientific).

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

$$\log y = \log[C/(1 + H/K_1)] \quad (2)$$

$$\log y = \log[C/(1 + H/K_1 + H_2/K_1K_2)] \quad (3)$$

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \quad (4)$$

In eqs 2−4, y represents V or V/K, C is the pH-independent value of y, K_1 and K_2 represent the dissociation constants for specific groups on the enzyme, and H is the proton concentration.

Spectrophotometric Measurement of the Ionization of the Thiol Group of Cys282. UV absorbance spectra were recorded at 30 °C on a Cary 100 spectrophotometer (Varian) using 1.0 cm path length cuvettes. The spectra were measured in a buffer containing 1 mM phosphate, 1 mM borate, 1 mM citrate, 0.1 mM EDTA, and 0.2 M KCl. Measurements were taken over a pH range from 4 to 9, and the buffer solutions were adjusted to the appropriate pH values with HCl or NaOH. The concentrations of HMCK, HMCK C282S, and HMCK S284A were between 11 and 14 μM. These were determined using the absorption at 280 nm, assuming an extinction coefficient (ϵ_{280}) of 37 840 M^{-1} cm^{-1} (*38*). The absorption difference (WT − C282S or S284A − C282S) at 240 nm, as a function of pH, was fitted to the Henderson−Hasselbalch equation:

$$\epsilon_{Exp} - \epsilon_{SH} = \frac{\epsilon_{S^-} - \epsilon_{SH}}{1 + 10^{pK_a - pH}} \quad (5)$$

in which ϵ_{Exp} represents the experimentally determined values of ϵ_{240} of the thiol group, and ϵ_{S^-} and ϵ_{SH} are the values of ϵ_{240} for the fully deprotonated form and the fully protonated form, respectively.

RESULTS

Sequence Homology. Figure 1 shows the primary sequence alignment for several species and isoforms of creatine and

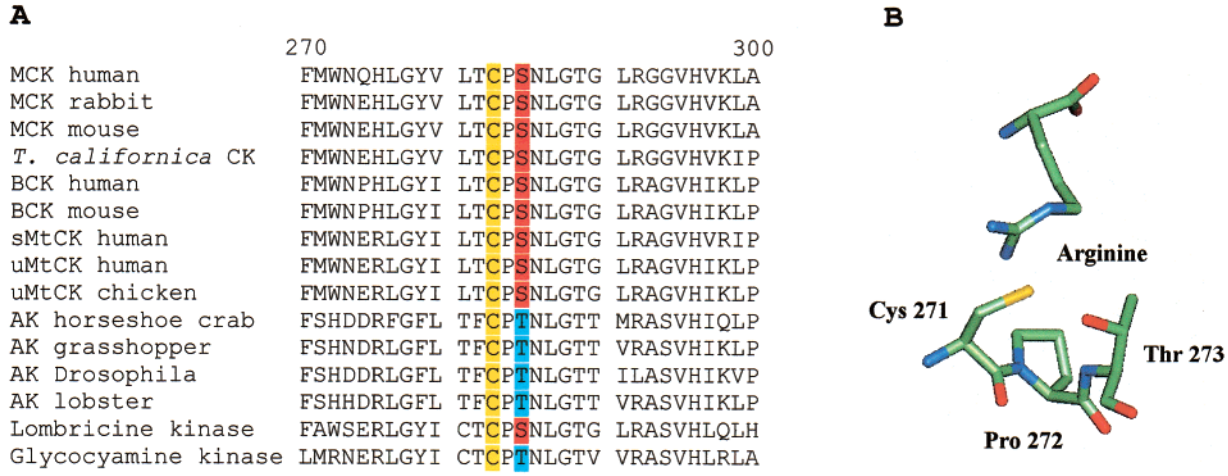

FIGURE 1: (A) Multiple sequence alignments of the regions of several guanidino kinases containing the conserved cysteine residue. The alignments were created using ClustalW (*55*). The cysteine is highlighted in yellow, and the serine and threonine residues described in the text are highlighted in red and cyan, respectively. Residue numbering is based on the HMCK sequence. MCK human, gi125305; MCK rabbit, gi125307; MCK mouse, gi125306; *T. californica* CK, gi125309; BCK human, gi125294; BCK mouse, gi417208; sMtCK human, gi125312; uMtCK chicken, gi2497494; uMtCK human, gi125315; AK horseshoe crab, gi1708613; AK grasshopper, gi1688218; AK *Drosophila*, gi1346366; AK lobster, gi585342; lombricine kinase, gi3183058; glycocyamine kinase, gi1730042. (B) The CPT motif and its spatial relationship to the arginine substrate in the active site of the arginine kinase-TSAC structure. Drawn using QUANTA (Molecular Simulations Inc.) and the coordinates in Protein Data Bank 1BGO (*12*).

arginine kinases in the region of Cys282, as well as for lombricine and glycocyamine kinases. As is usual for the guanidino kinases, there is a high degree of sequence similarity, and the "reactive" cysteine residue is fully conserved. On the C-terminal side, adjacent to the cysteine residue, is a fully conserved proline which is followed by either a serine (creatine and glycocyamine kinases) or a threonine (arginine and lombricine kinases). Examination of the X-ray structures of several CK isozymes shows that these are the only residues located within 4 Å of the sulfur atom of Cys282. The spatial relationship of the three residues, CPT, to the arginine in the active site of the AK-TSAC (*12*) is shown in Figure 1B.

Expression and Purification of the CK Variants. The WT and mutant proteins were expressed in *E. coli* using the expression vector pET17. Expression levels were, in all cases, approximately equal, and each protein was obtained in soluble form. The proteins were purified using a modification of the protocol developed for WT-HMCK (*34*), with similar binding and elution profiles being obtained for WT and the S284A variant. However, the two Cys282 mutants were found to bind more tightly to the Blue Sepharose column, necessitating a higher salt concentration (500 mM KCl) for elution. C282S binding to, and elution from, the HiTrap Q ion-exchange column was found to be most efficient at pH 8.4. In addition, although the C282M variant did not bind well to the ion-exchange column at pH 8.4, the contaminants remaining after Blue Sepharose chromatography did bind, and the C282M found in the unbound fraction was effectively purified. All the variants showed the same migration patterns on SDS−PAGE.

The presence of the mutations was confirmed by mass spectrometry, and the similarity of the CD spectra for the WT and mutant proteins indicated that the mutations had no significant effect on protein folding (data not shown).

Enzyme Activity and Kinetic Parameters. For comparative purposes, the activities of the wild-type and mutant enzymes in a standard assay mixture were determined at pH 9.0 and 7.0 for the forward (PCr production) and reverse (ATP production) direction, respectively. These were carried out using the coupled assay system. In addition, in the forward direction, the WT enzyme was assayed at pH 9.0 using a pH-stat method (*17*). The results obtained from the coupled assay and pH-stat assay were essentially identical (data not shown). The use of the latter method for pH studies is complicated by the fact that, at low pH values, PCr transphosphorylation and H^+ production are no longer equimolar (*32*). As a consequence, the coupled assay system became the method of choice for studies reported here.

Table 1: Specific Enzyme Activities of Human Muscle CK Mutants[a]

enzyme	forward reaction[b] (units/mg)[d]	% wild-type	reverse reaction[c] (units/mg)[d]	% wild-type
wild-type	210 ± 8.0	100	469 ± 3.3	100
C282S	0.95 ± 0.03	0.45	0.84 ± 0.035	0.18
C282M	nd[e]		nd[e]	
S284A	22.0 ± 1.2	10.5	11.3 ± 0.51	2.4

[a] Specific activities are the mean of at least three individual experiments and are reproducible between individual enzyme preparations. [b] Activity in the forward direction (Cr + ATP → PCr + ADP + H^+) was determined in Morrison buffer (*37*) containing 100 mM creatine, 5 mM ATP, and 6 mM $Mg(OAc)_2$. [c] Activity in the reverse direction (PCr + ADP + H^+ → Cr + ATP) was determined in Morrison buffer containing 5 mM phosphocreatine, 4 mM ADP, and 9 mM $Mg(OAc)_2$. [d] 1 unit of enzyme activity equals 1 μmol of ATP or PCR transphosphorylated per minute at 30 °C. [e] Not detected.

The data in Table 1 show that, in the standard assay mixture used for either the forward or the reverse reactions, the specific activities of both Cys282 mutants were considerably reduced. Indeed, for the C282M variant, no activity could be observed in either direction. The S284A mutant showed the most residual activity, with about 10% of the wild-type activity in the forward direction.

The kinetic parameters for the active mutants, in the presence of saturating nucleotide, were also obtained at pH 9.0 and 7.0 for the forward and reverse reaction, respectively

Table 2: Kinetic Parameters for Wild-Type and Mutant HMCKs[a]

	V_{max} forward ($min^{-1} \times 10^{-3}$)	K_m^{Cr} (mM)	V_{max} reverse ($min^{-1} \times 10^{-3}$)	K_m^{PCr} (mM)
WT	10.9 ± 0.2	19.6 ± 1.5	31.9 ± 0.3	1.79 ± 0.13
C282S	0.13 ± 0.006	222 ± 13	0.06 ± 0.002	3.37 ± 0.18
S284A	4.27 ± 0.6	319 ± 58	0.68 ± 0.05	2.05 ± 0.15

[a] Reaction conditions: forward, pH 9.0, saturating ATP; reverse, pH 7.0, saturating ADP.

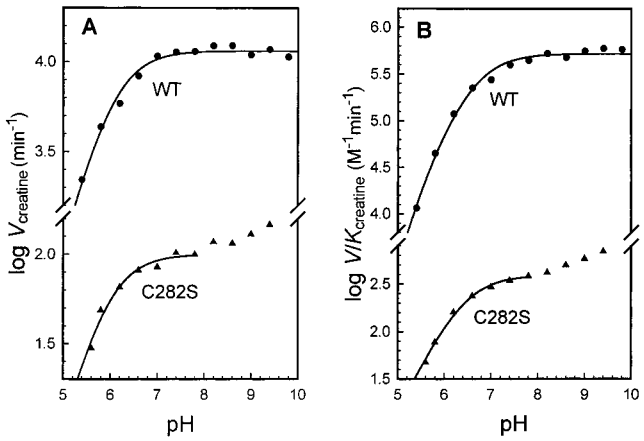

FIGURE 2: pH dependence of V_{max} (A) and V_{max}/K_m (B) for wild-type HMCK (●) and C282S HMCK (▲). The kinetic parameters were measured in the forward reaction by varying the level of creatine with MgATP saturated at 5 mM. All the assays were carried out in a buffer system containing 100 mM MES, 51 mM *N*-ethylmorpholine, and 51 mM diethanolamine (*37*). (A) The solid lines are theoretical curves calculated assuming single p*K* values of 6.1 and 6.0 for the wild-type enzyme and the C282S mutant, respectively. (B) The solid lines are theoretical curves calculated assuming two p*K*s of 6.7 and 5.4 for the wild-type enzyme, and one p*K* of 6.5 for C282S.

(Table 2). When compared to wild-type HMCK, the C282S mutant exhibits a drastically reduced V_{max} and significantly (>10-fold) increased K_m for creatine. The kinetic plots for the wild-type enzyme indicated some synergism in the binding of substrates (data not shown), as previously observed with rabbit muscle creatine kinase (*39*, *40*). The synergism was lost upon replacement of Cys282 with serine. Maggio et al. (*40*) reported a similar loss of synergism with CH_3S-blocked RMCK, as did Furter et al. (*32*) for the C278S and C278G mutants of the chicken mitochondrial enzyme. The S284A variant shows an even greater increase in K_m for creatine; however, the V_{max} was only reduced by about 60%. It should be noted that, due to the relatively low solubility of creatine, the kinetic constants for reaction in the forward direction were extrapolated from data obtained well under K_m.

pH Profiles of the Wild-Type HMCK and C282S HMCK. Figure 2 shows the pH dependence of log V_{Cr} and log (V/K_{Cr}) for the wild-type enzyme and the C282S mutant operating in the direction of creatine phosphorylation. Details of the p*K*s obtained from these data are provided in Table 3. V_{Cr} for both wild-type and C282S HMCK decreased below a single p*K*, almost certainly implicating the same ionizable group in the rate-determining step of the reaction. This is consistent with the observations of Cook et al. (*18*) for RMCK although the apparent pK_a of the ionizable group is marginally lower. The plot of log (V/K_{Cr}) against pH rose with a slope of 2 before reaching a plateau for both WT

Table 3: Summary of Data Obtained from pH−Rate Profiles of Wild-Type and C282S HMCK

	pK_1	pK_2
	Wild-Type	
$V_{creatine}$	6.1 ± 0.1[a]	
$V/K_{creatine}$	5.4 ± 0.2[b]	6.7 ± 0.1[b]
$V_{phosphocreatine}$	5.5 ± 0.2[c]	8.2 ± 0.1[c]
$V/K_{phosphocreatine}$	5.8 ± 0.2[c]	7.5 ± 0.2[c]
	C282S	
$V_{creatine}$	6.0 ± 0.1[d]	
$V/K_{creatine}$	−[e]	6.5 ± 0.1[d]
$V_{phosphocreatine}$	5.3 ± 0.1[c]	7.2 ± 0.1[c]
$V/K_{phosphocreatine}$	5.8 ± 0.1[c]	7.2 ± 0.1[c]

[a] pH−rate data fitted to eq 2. [b] Data fitted to eq 3. [c] Data fitted to eq 4. [d] Data from pH 5.4 to 8.2 fitted to eq 2. [e] This p*K* not observed.

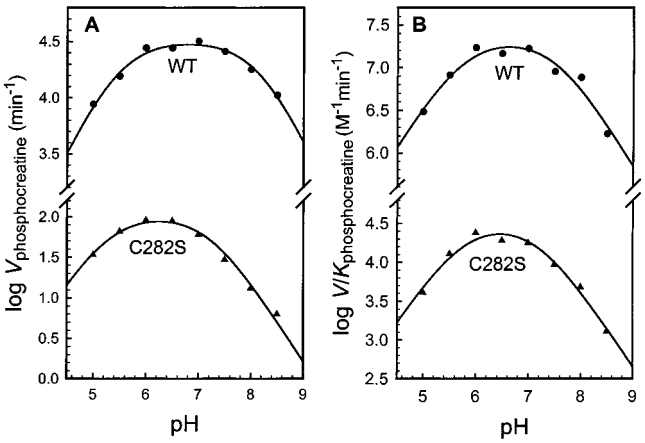

FIGURE 3: pH dependence of V_{max} (A) and V_{max}/K_m (B) for wild-type HMCK (●) and C282S HMCK (▲). The kinetic parameters were measured in the reverse reaction by varying the level of phosphocreatine, with MgADP saturated at 4 mM. Again, assays were carried out in the constant ionic strength buffer of Ellis and Morrison (*37*). (A) The solid lines are theoretical curves calculated assuming p*K*s of 8.2 and 5.5 for the wild-type enzyme, and p*K* values of 7.2 and 5.3 for C282S. (B) Theoretical curves were calculated assuming p*K* values of 7.5 and 5.8 for the wild-type enzyme, and p*K*s of 7.2 and 5.8 for C282S.

HMCK and RMCK and could be fitted to eq 3, providing two p*K*s. However, unlike either wild-type HMCK or RMCK, the decrease of V/K_{Cr} for the C282S mutant at low pH can only be fitted to a single p*K* of 6.5. In another difference between C282S and the wild-type enzyme, both log V_{Cr} and log (V/K_{Cr}) for C282S increase slightly in the region above pH 8.2. Therefore, only data obtained below pH 8.2 were fitted to eq 3.

As shown in Figure 3, the pH profiles of V_{PCr} and V/K_{PCr} are both bell-shaped. The V_{PCr} profile for RMCK was also bell-shaped, but the V/K_{PCr} profile at 12 and 25 °C was found to contain a "hollow" which was not seen at 35 °C (*18*). No hollow was observed for HMCK, but, as the experiments described here were carried out at 30 °C, it is not clear whether the lack of a hollow for HMCK is a function of the temperature at which these studies were undertaken or a property of the enzyme itself.

Table 3 also shows the pK_a results obtained from the data in Figure 3. The V_{PCr} data for the mutant and wild-type enzyme provided a pK_a around 5.5, indicative of a group which must be deprotonated for maximal enzyme activity. pK_as of 8.2 and 7.2 for the wild-type and C282S mutant,

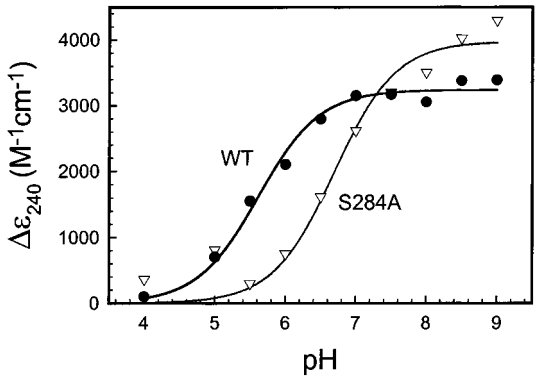

FIGURE 4: Determination of the pK_a of the thiol group of Cys282. The absorbance spectra were obtained for each protein (11−14 μM) in a buffer containing 1 mM citrate, 1 mM borate, 1 mM phosphate, 0.2 M KCl, and 0.1 mM EDTA. The difference in molar extinction coefficient at 240 nm ($\Delta\epsilon_{240}$) between the wild-type enzyme and C282S (●) and between S284A and C282S variants (▽) is shown as a function of pH. The solid line is a theoretical curve calculated assuming p*K*s of 5.6 and 6.7 for the WT and S284A enzymes, respectively.

respectively, indicated the presence of a second ionizable group, which must be deprotonated for maximal activity. Unlike those observed for the forward reaction, the V/K_{PCr} profiles for the reverse reaction were almost identical for the wild-type and mutant HMCK, giving rise to apparent pK_a values of ca. 5.8 and 7.4.

Spectrophotometric Determination of the pK_a Value of Cys282. The dissociated form of a thiol, the thiolate anion, is characterized by an UV absorption band centered around 240 nm with an extinction coefficient about 4000 M^{-1} cm^{-1}, whereas the undissociated form has negligible absorbance at 240 nm (*35*, *41*). This difference makes it possible to determine the ionization state of a thiol group spectrophotometrically. Because many groups on a protein absorb at this wavelength, and some of that absorbance may be pH-dependent, it is necessary to compare the UV spectrum of a protein containing the thiol group of interest to the spectrum of the same protein in which the thiol group is absent (*42*, *43*). Any absorbance observed in the difference spectrum should arise only from the thiolate anion.

The UV absorbance spectra of wild-type HMCK and the C282S mutant have been measured over a pH range between 4 and 9. The differences between the molar extinction coefficients at 240 nm for the two proteins ($\Delta\epsilon_{240}$) are presented in Figure 4 as a function of pH. These data, when fitted to the Henderson−Hasselbalch equation (eq 5), provided a pK_a for Cys282 (in the wild-type enzyme) of 5.62 ± 0.05. In a similar manner, the $\Delta\epsilon_{240}$ values for the S284A and C282S variants were determined over the same pH range and also plotted in Figure 3. Following analogous treatment of data, the pK_a of Cys282 in the HMCK S284A mutant was found to be 6.70 ± 0.12.

DISCUSSION

Chemical modification studies, using sulfhydryl-specific reagents as well as the affinity label epoxycreatine, have shown that rabbit muscle creatine kinase has one reactive sulfhydryl group per monomer. This was identified as Cys282 (homologous to cysteine residue 278 in the mitochondrial isozymes). The majority of these studies concluded that modification of the cysteine led to complete abolition of activity [reviewed by Kenyon and Reed (*14*)]. There were, on the other hand, some studies using modification agents with small uncharged groups that demonstrated only partial loss of activity (*28*, *29*), and concomitant loss of synergism (*40*). It was suggested that the chemical modification may not have been complete, or that there may have been intramolecular transfer of the thiol-modifying group that could lead to restoration of activity (*30*, *31*). With the publication of a seemingly definitive site-directed mutagenesis study on the mitochondrial CK carried out by Furter et al. (*32*), it appeared that the controversy may have been resolved, and that the reactive cysteine, probably in the form of the thiolate anion, was required for synergism but not essential for catalysis. However, a subsequent site-directed mutagenesis study, this time on the human brain isozyme, suggested that, for this isozyme at least, the cysteine was essential for activity (*33*).

Sequence alignments of several creatine kinases and arginine kinases, as well as lombricine and glycocyamine kinases, show that Cys282 is conserved across all guanidino (phosphagen) kinases (Figure 1). The affinity labeling studies of Buechter et al. (*27*), as well as the X-ray structures of the various CKs (*7*−*11*), show that Cys282 is indeed found in the active site. Perhaps more informative is the structure of the arginine kinase transition-state analogue complex (*12*) which suggests that the cysteine interacts with the nonreactive guanidinyl nitrogen (Figure 1B). In addition, it forms a hydrogen bond with a conserved threonine residue, and is consistent with the proposal that CK is most active with the cysteine as the thiolate anion.

Our results for human muscle CK show that even the conservative substitution of serine for Cys282 results in a marked effect on the rate of both the forward and reverse reactions. However, there is still at least 0.2% residual activity, indicating that Cys282 is *not* essential for catalysis. The binding of creatine is considerably affected by this substitution, as evidenced by the 10-fold increase in K_m, whereas the binding of phosphocreatine, already tighter than that of creatine, is less affected. Undoubtedly the additional phosphoryl group plays a significant role in binding of this substrate. The overall effects on the kinetic parameters brought about by the C282S substitution in HMCK are very similar to those found for the C278S mutant of Mi_b-CK (*32*), with the main difference being that the latter mutation showed a slightly greater decrease in affinity for phosphocreatine.

The total lack of reactivity of the C282M mutant was perhaps more surprising. Maggio et al. (*40*) had observed about 20% residual activity when RMCK Cys282 was blocked which CH_3S, following reaction with methyl methanethiosulfonate. It was thought that the replacement of cysteine with methionine in the C282M variant would provide a reasonable mimic of the chemically modified enzyme and similar kinetic results would be obtained. The close (3.3 Å) interaction of the "essential" cysteine and the nonreactive guanidinyl nitrogen observed in the AK-TSAC structure suggests that there may be steric conflict if this residue is mutated to larger substituents or modified with bulky groups (*12*). Certainly the C278D and C278N mutants of Mi_b-CK showed a significantly greater decrease in activity than did the C278S or C278G mutants (*32*). In our case, we

were not able to see any activity with the C282M variant, and, in light of this, it is surprising that the CH_3S-modified RMCK had any activity. In fact, close examination of the kinetic constants obtained in that study reveals that the blocking group has little effect on the K_m for creatine (*40*). This is at odds with all mutational studies on the "essential" cysteine, and suggests that the CH_3S-modified RMCK had, in some undetermined manner, lost some of its label, leading to the observation of significant residual activity.

The pH dependence of V_{max} provides information about the ionization state of amino acid residues involved in the rate-determining step in the reaction. As V_{max} describes the reaction of the enzyme−substrate complex, the profiles provide an apparent p*K* (i.e., they are not necessarily the intrinsic p*K*s that would be found on the free enzyme). In this instance, the V_{max}vs pH profiles of the wild-type enzyme and C282S variant, shown in Figures 2 and 3, are quite comparable, and indicate that HMCK possesses an ionizable group with an apparent p*K* around 7 which must be deprotonated in the direction of creatine phosphorylation and protonated in the direction of MgADP phosphorylation. In addition, there is a second group, with a p*K* below 6, which must be unprotonated for activity in either direction. The p*K*s are relatively unaffected by the replacement of Cys282 with serine and are not dissimilar to those observed for RMCK (*18*).

The pH profile of V_{max}/K_m provides information on which residues are important for both binding and catalysis. Unlike the V and V/K_{Pcr} profiles, the pH profiles of V/K_{Cr} exhibit significant difference between the wild-type HMCK and the C282S variant (Figure 2). For the wild-type enzyme, two p*K*s (6.7 and 5.4) were observed for the pH dependence of V/K_{Cr}. This is very similar to previous observations for wild-type RMCK (*18*), where the profile of $V/K_{creatine}$ also exhibited two p*K*s (7.40 and 5.57). It was suggested that the lower p*K* of 5.57 may belong to a neutral acid, possibly a carboxyl group, which must be ionized for binding of creatine. Given the close sequence similarity of RMCK and HMCK, the p*K* of 5.4 seen in the HMCK V/K_{Cr} vs pH profile would, by analogy, also belong to a carboxylate. Reinforcing these conclusions are recent studies showing that glutamic acid residues are critical for the activity of both human muscle (*44*) and mitochondrial (*45*) CK. However, unexpectedly, the V/K_{Cr} profile of C282S only exhibits a single p*K* of 6.5. *This provides clear evidence that the pK of 5.4 observed for wild-type HMCK belongs to Cys282 and, for the optimal binding of creatine, the cysteine should be in the form of the thiolate anion.*

The thiol group of a free cysteine generally has a pK_a value of 8.5 ± 0.5 (*46*). While the p*K* of 5.4 suggested for Cys282 is quite low, it is by no means unique. Enzymes as diverse as papain (*47*), DsbA (*42*, *48*), protein-tyrosine phosphatase (*49*), pyruvate decarboxylase (*50*), and glutathione *S*-transferase (*43*, *51*) all have cysteine residues with a p*K* below 6. A number of methods have been used to determine the p*K* of these thiol groups. Since thiols are alkylated in their anionic form (*46*), a loss of reactivity is expected with increasing pH. This permits the determination of a thiolate p*K* by examination of the pH dependence of alkylation using reagents such as iodoacetamide and iodoacetic acid (*48*, *52*). FT-IR and isoelectric focusing have also been employed to identify cysteines with low pK_a (*50*).

Here we have taken the most common approach, that of UV difference spectroscopy (*41*, *43*, *48*, *51*), to obtain an independent determination of the p*K* of Cys282. The extinction coefficient of a thiolate anion at 240 nm is around 4000 M^{-1} cm^{-1} whereas that of the un-ionized thiol is essentially zero (*35*, *41*). A plot of the difference in extinction coefficient ($\Delta\epsilon_{240}$) between WT and C282S HMCK provided a p*K* of 5.62 ± 0.05 for Cys282. This is almost identical to the p*K* of 5.4 that is "missing" in the V/K_{Cr} vs pH profile of the C282S mutant, and about 3 pH units below that of a normal cysteine.

What are the factors that can contribute to the unusually low pK_a value of Cys282? Ion-pairing with a histidine residue as is observed for papain (*47*), thiosubtilisin (*41*), arsenate reductase (*53*), and glutathione *S*-transferase (*43*) is probably the most common mechanism, with pK_a values being lowered by up to 5 units. Yet there are no histidine residues within 5 Å of Cys282 (or its homologues) in any of the X-ray structures of either CK or AK-TSAC. Simple electrostatic effects brought about by the proximity of positive charges to the sulfur atom can also be ruled out since, with the exception of bound substrate as in the AK-TSAC structure (*12*), there are no positively charged residues located near Cys282. The thioredoxin fold has been shown to use an electrostatic mechanism to provide a similar decrease in pK_a values (*54*). For this to occur, the cysteine residue must be located at or near the N-terminus of a helix, so that the cysteine thiol may be specifically oriented with respect to the helix dipole (*54*). In creatine kinase, Cys282 is located on a loop.

Hydrogen bond formation may also assist in the deprotonation of a thiol group. Examination of the X-ray structures suggests that Ser284 may be capable of interacting with Cys282. This residue is fully conserved in all creatine kinases and is also present in glycocyamine kinase. In lombricine kinase, as well as all arginine kinases, the serine has been conservatively substituted by threonine. In the X-ray structure of AK-TSAC, there is ca. 3 Å between the sulfur of the thiolate and the threonine oxygen, indicative of a hydrogen bond between these residues (Figure 1B). Similar distances are found between Cys282 and Ser284 in all published X-ray structures of the CK isozymes. Serine 284 therefore may play a dual role in both lowering the pK_a of Cys282 as well as helping to maintaining its position for efficient binding of the guanidinium of the substrate.

To explore this possibility, we prepared the S284A mutant and examined its kinetic properties as well as its contribution to the pK_a of Cys282. In a standard assay, this mutant showed considerably reduced activity in both forward and reverse directions, although the residual activity was an order of magnitude greater than that of C282S. More detailed kinetic analysis showed that the K_m for creatine was greatly increased (>15-fold) whereas the K_m for phosphocreatine was almost unchanged from the wild-type value. It should be noted that creatine is relatively insoluble and, for C282S and as well as S284A, the K_m values for creatine were 2−3 times higher than the maximum concentrations possible in the assay. This means that all the observed V_{max} and K_m values for the forward reaction have been extrapolated from data obtained below the K_m for creatine. However, similar values were obtained from at least three experiments, and the values are in line with those observed for Cys282 mutants in an earlier

study (*32*). Given those constraints, these data also indicate that, for creatine phosphorylation, the S284A mutant has a V_{max} almost 40% that of the wild-type. It would appear that, if the enzyme could be saturated with substrate, the loss of Ser284 will have negligible effect on the overall reaction. The same cannot be said for the reverse reaction where, although the K_m for phosphocreatine is unaltered, the V_{max} is only 2% of that of the wild-type HMCK. In any event, these data confirm that Ser284 plays an important role in CK catalysis.

As seen clearly in Figure 4, the S284A mutation produces a significant increase in the pK_a of Cys282. However, the magnitude of the shift, about 1.1 pH units, indicates that Ser284 alone does not account fully for the abnormally low pK_a of Cys282. Other factors contributing to the low pK_a await clarification.

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