Rabbit Muscle Creatine Kinase: Consequences of the Mutagenesis of Conserved Histidine Residues†

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ABSTRACT: Creatine kinase (CK; EC 2.7.3.2) catalyzes the reversible conversion of creatine and MgATP to phosphocreatine and MgADP. In the absence of an X-ray crystal structure, we have used the sequence homology of creatine kinases and other guanidino kinases from a variety of sources to identify the conserved histidine residues in rabbit muscle CK, as well as to try to pinpoint a reactive histidine that has been implicated in the active site. This residue has been proposed to act as a general acid/base catalyst assisting in the phosphoryl transfer mechanism [Cook *et al*. (1981) *Biochemistry 20*, 1204−1210]. There are 17 histidine residues in rabbit muscle CK, and of these, only five have been conserved in all guanidino kinase sequences published to date [Mühlebach *et al*. (1994) *Mol. Cell. Biochem. 133*, 245−62]. In rabbit muscle CK, these residues are H96, H105, H190, H233, and H295. We have carried out site-specific mutagenesis of these five histidine residues, replacing each with an asparagine. Each of these mutants exhibited enzymatic activity but to varying degrees. The H105N, H190N, and H233N mutants displayed specific activities similar to that of the wild-type enzyme. H96N has high activity, but appears to be quite unstable, losing catalytic activity upon cell lysis by sonication and/or chromatographic steps involved in purification. H295N shows a significantly reduced catalytic activity relative to the native enzyme, due to marked decreases in k_{cat} and the affinities for both substrates. Each of the five mutants is inactivated by diethyl pyrocarbonate (DEP), and inactivation is reversible upon incubation with hydroxylamine. However, only H295N shows a dramatically reduced rate of inactivation relative to native CK, consistent with H295 being the residue modified by DEP in the native enzyme. These intriguing results indicate that four of the conserved histidines (H96, H105, H295, and H233) are not essential for activity, and while H295 may be at the active site of CK, it is unlikely to play the role of a general acid/base catalyst.

Creatine kinase (CK;[1] EC 2.7.3.2) continues to be one of the best-studied kinases for which no three-dimensional crystal structure is yet available. One of the major obstacles to obtaining X-ray quality crystals appears to be the presence of microheterogeneity in all of the enzyme preparations to date (Chen *et al*., 1991; Wood *et al*., 1995). Despite this handicap, there have been many studies to try to delineate the amino acid residues that may be involved in substrate binding and catalysis. These residues include a reactive cysteine whose function is still the subject of controversy (Kenyon & Reed, 1983; Zhou & Tsou, 1987; Furter *et al*., 1993; Hou & Vollmer, 1994), lysine and arginine residues that presumably interact electrostatically with the negatively-charged phosphates of the substrates (Kassab *et al*., 1968; Mahowald, 1969; James & Cohn, 1974; Borders & Riordan, 1975), a tryptophan that evidently stacks with the adenosine moiety of ATP/ADP (Kägi *et al*., 1971; Vasák *et al*., 1979; Messmer & Kägi, 1985; Zhou & Tsou, 1985; Gross *et al*., 1994), and a histidine that putatively acts as a general acid/base, abstracting a proton from the guanidino moiety of creatine thereby initiating the phosphoryl transfer reaction (Pradel & Kassab, 1968; Clarke & Price, 1979; Cook *et al*., 1981; Rosevear *et al*., 1981). Among these residues, the reactive cysteine has been shown to be at position 282 of the primary structure of rabbit muscle CK (Putney *et al*., 1984), and the "essential" tryptophan has been suggested to be Trp223 in mitochondrial CK (Gross *et al*., 1994), which corresponds to Trp210 in the rabbit muscle isozyme. None of the other residues mentioned above has been identified in the primary structure of CK.

Assuming that the evolutionary conservation of an amino acid residue is correlated with its importance in the structure and function of an enzyme, we have carried out a primary sequence alignment of the 43 creatine kinases and other structurally homologous guanidino kinases whose deduced amino acid sequences have been published in order to investigate the role(s) of the conserved histidine residues in rabbit muscle CK. This comparison is comprised of sequences previously aligned and published by Mühlebach

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[1] Abbreviations: CK, creatine kinase; LB/Amp, Luria−Bertani medium containing 50 μg/mL ampicillin or its analog, carbenicillin; DEP, diethyl pyrocarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB-derivatized CK, creatine kinase in which the reactive thiol of Cys282 has been reversibly modified by DTNB; H96N, histidine at position 96 of the rabbit muscle CK that has been replaced by asparagine.

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et al. (1994), as well as a compilation of more recent amino acid sequences present in the Genbank database as of June, 1995 (P. C. Babbitt and G. L. Kenyon, unpublished). Such an analysis reveals only five histidines conserved in all guanidino kinases. This report describes our investigation of the site-specific replacement of each of the five histidine residues in rabbit muscle CK with asparagine and the effects of each replacement on substrate binding and catalysis, the rate of inactivation by DEP, and the thermal stability of CK.

MATERIALS AND METHODS

Materials. Restriction and DNA modifying enzymes were purchased from Boehringer Mannheim, Gibco/BRL, and New England Biolabs. Reagents and enzymes for the polymerase chain reaction (PCR) were purchased from Perkin-Elmer/Cetus. Bacterial media were made from materials purchased from Difco. DNA sequencing was carried out by the dideoxy method (Sanger *et al*., 1977) with the Sequenase kit (U.S. Biochemical Corp.) and utilizing oligonucleotides synthesized by the Biomolecular Resource Center (University of California, San Francisco). Electrophoresis and protein chromatography reagents were from Bio-Rad and Pharmacia Biotech. All other reagents were of the highest quality available.

Bacterial Strains and Vectors. Protein overexpression was obtained using the commercially available pET expression system (Novagen), in particular, the pET17b expression vector. Early in the project, the pBAce expression vector and Sϕ606 bacterial cells [gifts from Dr. Sydney P. Craig III (University of California, San Francisco)] were used to express of some of the rabbit muscle CK variants, but this approach was abandoned in favor of the pET17b system because of the much greater level of protein expression in the latter. CJ236 and MV1190 bacterial cells and M13mp18 rfDNA were purchased from Bio-Rad.

Subcloning of the Genes for Rabbit Muscle CK and Its Histidine Mutants into the pBAce and pET17b Vectors. The expression vector pBAce contains a unique *Nde*I restriction site at an optimal distance from the *pho*A promoter, as well as a unique *Sal*I site downstream of the *Nde*I site (Craig *et al*., 1991). This construct has been shown to be a versatile vector for both eukaryotic and prokaryotic protein expression (Yuan *et al*., 1990; Craig *et al*., 1991). In order to utilize the optimal arrangement of transcriptional machinery, the polymerase chain reaction (PCR) was used to engineer an *Nde*I restriction site (CATATG) at the initiating ATG codon and a terminating *Sal*I site (GTCGAC) at the 3′ terminus of the rabbit muscle CK gene. The PCR was carried out on a Perkin Elmer/Cetus DNA Thermal Cycler 480 using 25 ng of dsDNA [pKTMCK (Chen *et al*., 1991)] and 25 pmol of each oligonucleotide primer. Initially, the DNA was denatured at 94 °C for 1 min, followed by a hybridization step at 30 °C for 1 min and then extension at 72 °C for 2 min. This was done for a total of 5 cycles, after which the hybridization temperature was elevated to 60 °C to increase the stringency of amplification. This latter amplification sequence was carried out for 30 cycles. At the end of the 30 cycles, the samples continued to be incubated at 72 °C for an additional 10 min to complete the extensions. The amplified products were purified with glass beads from the Geneclean Kit (*Bio*101, CA) as per the manufacturer's instructions. The rabbit muscle CK gene containing the *Nde*I and *Sal*I sites was then digested with these enzymes, purified, and ligated into the pBAce vector similarly digested. The resulting construct of 4.39 kb containing the rabbit muscle CK gene is designated pBAckrm7.

Conveniently, these *Nde*I and *Sal*I restriction sites that were engineered into the 5′- and 3′-flanking regions of the CK gene for subcloning into pBAce were also convenient for ligation into pET17b, thus allowing for the overexpression of the gene under the T7 promoter. This new construct is designated pET17/CK7.

Mutagenesis of the Rabbit Muscle CK Gene Subcloned into M13. The rabbit muscle CK gene was subcloned into M13mp18 from the expression vector pKTMCK; the M13 construct containing the rabbit muscle CK gene was designated M13CK718. Site-specific mutagenesis was then carried out by the method of Kunkel *et al*. (1985, 1987). Mutant constructs were then generated in the appropriate expression vectors by subcloning the fragments of the gene containing the mutation from M13 into the expression vector. The DNA sequence of the subcloned fragments of the gene was then verified by sequencing.

Expression and Purification of Rabbit Muscle CK and Its Variants. *Escherichia coli* cells were made competent with calcium chloride (Dagert & Ehrlich, 1979), transformed with the pBAckrm7 plasmid, and grown on LB/Amp plates at 37 °C. A fresh, isolated colony was picked from this plate and grown overnight in liquid LB/Amp medium at 37 °C, and an aliquot (100 μL) of the overnight growth was used to inoculate 1 L of low phosphate induction medium as described by Craig *et al*. (1991) and grown for 18 h at 30 °C before harvesting by centrifugation.

When the pET17b system was used to grow up wild-type and mutant CK, a fresh colony was used to inoculate LB/Amp medium as a starter culture. After overnight growth at 37 °C, the cells were washed with fresh media before inoculation of larger (1 L) cultures. The freshly inoculated cultures were grown at 37 °C until $A_{600} \sim 0.8-1$, at which time they were chilled on ice. After addition of IPTG to a final concentration of 0.4 mM, the cells were grown for an additional 16 h at 20 °C before harvesting.

The purification scheme for the isolation of rabbit muscle CK from pET17/CK7 was a modification of that described by Chen *et al*. (1991). The cells were resuspended in MES buffer (10 mM MES, 20 mM KCl, 0.1 mM PMSF, 1 mM DTT, pH 6.0), and cell lysis was achieved either by sonication or by mixing with glass beads in a Beadbeater apparatus (BioSpec Products, Bartlesville, OK). The lysed cells were centrifuged at 48000*g* for 30 min, and the supernatant was loaded directly onto a Blue-Sepharose 4B column (Pharmacia Biotech). After washing with 5 column volumes of MES buffer, the enzyme was eluted with TES buffer (10 mM TES, 20 mM KCl, 0.1 mM PMSF, 1 mM DTT, pH 8.0). The eluted enzyme was concentrated and dialyzed against 50 mM Tris·Cl, pH 8.8, before application to a MonoQ HR10/10 column (Pharmacia Biotech). A NaCl gradient in the same buffer was then used to elute >99% pure rabbit muscle CK at ~50 mM Tris·Cl, 30 mM NaCl, pH 8.8.

Modification with Diethyl Pyrocarbonate. Stock DEP concentration was made up by dilution of DEP into cold, anhydrous ethanol. The concentration was determined by the reaction of an aliquot of the DEP stock with 10 mM L-histidine hydrochloride, pH 7.5, to form carboethoxy-

histidine, which has an extinction coefficient of 3900 M^{-1} cm^{-1} (Choong *et al*., 1977). Creatine kinase subunits (23 μM wild-type or mutants) were incubated at 0 °C with 300 μM DEP in 50 mM MES, pH 6.1. At various time intervals, aliquots were removed, diluted into 100 mM L-histidine, pH 7.5, to quench the modification, and then assayed for activity. Control reactions, in which the modifying agent was absent, were also assayed under similar conditions. At the end of each series of modifications, the concentration of stock DEP was again determined to ensure that significant hydrolysis of the stock DEP had not occurred.

Treatment of DEP-Modified CK with Hydroxylamine. The treatment of modified enzyme with hydroxylamine was performed essentially as described by Miles (1977). Creatine kinase subunits (23 μM) were incubated at 0 °C with 300 μM DEP in 50 mM MES, pH 6.1, as before. At various time intervals, an aliquot (10 or 50 μL, depending on the variant in question) was removed and the modification reaction quenched by addition to an equal volume of 100 mM L-histidine, pH 7.5. After 10 min, one portion of the quenched reaction was assayed for remaining enzymatic activity. Another aliquot of the same quenched reaction was added to an equal volume of 1 M hydroxylamine, pH 8.0, and assayed after incubation for 30 min at room temperature to determine recoverable activity.

Sequential Modification of CK with DTNB and DEP. CK subunits (100 μM) were incubated with DTNB (600 μM) at room temperature for 5 min and assayed for enzymatic activity. All activity was lost within 1 min of incubation with DTNB. Excess DTNB and free thionitrobenzoate anions were removed by chromatography of the enzyme samples over a HiTrap desalting column (5 mL; Pharmacia Biotech) equilibrated in 50 mM MES, pH 6.1; the purified samples were stored on ice until use. For treatment with DEP, 25 μM CK subunits were incubated with 300 μM DEP in 50 mM MES, pH 6.1, at 0 °C. At desired time intervals, aliquots were removed and diluted into an equal volume of 10 mM DTT, 10 mM L-His, pH 7.5, incubated at room temperature for 5 min, and then assayed for residual CK activity. Samples containing $\leq$20% residual activity were further incubated with an equal volume of 1 M hydroxylamine, pH 8.1, for 30 min and then assayed for recoverable activity.

Thermal Denaturation. Thermal inactivation was carried out by monitoring the changes in molar ellipticity at 222 nm in a Jasco J-500A spectropolarimeter equipped with a constant temperature bath. In a 0.5 mm path length cell, 5 μM creatine kinase subunits, equilibrated in 10 mM PIPES, pH 7.0, were incubated at various temperatures. After the samples attained thermal equilibrium, 20 readings were obtained at $\lambda = 222$ nm and then averaged to produce the observed molar ellipticity. Analyses of the thermal denaturation profiles were performed using the program DENFIT, written and provided by Dr. Leslie A. Holladay (ALZA Corp., Palo Alto).

Protein Concentration. Protein concentration was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as the protein standard. For purified enzyme preparations, concentrations were determined using $A_{280} = 0.88$ for a 1 mg/mL sample (Noda *et al*., 1954). Within experimental error, the values obtained for protein concentration were equivalent for both methods.

Enzyme Assays and Other Methods. Creatine kinase activity was determined at 30 °C in the direction of phosphocreatine formation by the pH-stat method (Mahowald *et al*., 1962) using a Radiometer VIT90/ABU91. For the determination of specific activity or to follow the inactivation/reactivation experiments, each 3.0 mL assay, pH 9.0, contained the following: 40 mM creatine, 5.0 mM MgATP, 1.0 mM excess magnesium acetate, 0.1 mM EDTA, and 0.1% bovine serum albumin. The pH was maintained by titration of the released acid with 20 mM NaOH. Mg(II) ion was added as the acetate salt to avoid the inhibition that occurs with chloride. The kinetic analyses were also carried out at 30 °C and pH 9.0. Each 3.0 mL assay contained 2.0−100 mM creatine, 0.4−10.0 mM MgATP, 1.0 mM excess magnesium acetate, sufficient acetate to bring the total acetate concentration to 50 mM, 0.1 mM EDTA, and 0.1% bovine serum albumin. Five different concentrations of creatine and MgATP were run in all possible combinations, and the data were analyzed by the method of Cleland (1979) using software written by Dr. Ronald E. Viola at the University of Akron.

RESULTS AND DISCUSSION

Amino Acid Sequence Comparisons. We have utilized the sequence data presented in Mühlebach *et al*. (1994), as well as other independent observations (P. C. Babbitt and G. L. Kenyon, unpublished), to construct Table 1. This table shows the position of each of the seventeen histidinyl residues in rabbit muscle creatine kinase (column 1) and compares it to the corresponding residue at that position in the various guanidino kinases isolated from other tissues and organisms (columns 2 through 9). The organisms vary broadly over the evolutionary spectrum, ranging from invertebrates to primates. In Table 1, column 1 lists the seventeen histidine residues present in rabbit muscle CK. Column 2 shows whether or not each of these histidines is conserved in six additional muscle CKs (see Table 1 footnotes). Such an analysis reveals that all rabbit muscle CK histidine residues are conserved except for H123, which is present only in the rabbit muscle and human mitochondrial-sarcomeric CKs, and H300, which is present only in rabbit and human muscle CKs (Table 1 footnotes). Similarly, the rabbit muscle CK is compared with those of 10 different brain CK isozymes (column 3), and this analysis shows that H111, H123, H268, and H300 in rabbit muscle CK are not conserved in brain CK. The results from this type of analysis with the other guanidino kinases in columns 4 through 9 show that only *five* of the seventeen histidinyl residues present in rabbit muscle CK have been totally conserved in *all* of the ATP-guanidino kinases. These are located at positions 96, 105, 190, 233, and 295 of the rabbit muscle sequence.

In Figure 1, the primary structure of rabbit muscle CK is shown with the positions of these seventeen histidine residues highlighted. This figure demonstrates, first of all, that these residues are not clustered, but rather are distributed throughout the protein. In addition, the sequences of the highly conserved regions of this enzyme are underlined. It can be seen that several of the conserved residues fall into conserved regions, as expected. Of particular interest are the regions comprising amino acids 229−236 (NEED**H**LRV) and 281−296 (TCPSNLGTGLRGGV**H**V), which contain H233 and H295, respectively. The peptide sequence comprising amino

Table 1: Histidine Residues in Rabbit Muscle Creatine Kinase Compared with Other ATP-guanidino Kinases[a]

histidine no.	6 muscle[b]	10 brain[c]	2 ray[d]	sea urchin sperm[e]	6 mito[f]	trematode #1[g]	8 shellfish[h]	4 other[i]
6	♦	♦	♦	–	–	–	–	–
25	♦	♦	♦	♦	♦	–	–	–
28	♦	♦	♦	–	–	–	–	–
65	♦	♦	♦	♦	♦	–	–	–
96	♦	♦	♦	♦	♦	♦	♦	♦
105	♦	♦	♦	♦	♦	♦	♦	♦
111	♦	–	–	–	–	–	–	–
123	○[j]	–	–	–	○[k]	–	–	–
144	♦	♦	♦	–	–	–	–	–
190	♦	♦	♦	♦	♦	♦	♦	♦
218	♦	♦	♦	♦	♦	–	–	–
233	♦	♦	♦	♦	♦	♦	♦	♦
268	♦	–	–	–	–	–	–	–
275	♦	♦	♦	♦	–	–	–	–
295	♦	♦	♦	♦	♦	♦	♦	♦
300	○[l]	○[m]	♦	–	–	–	–	–
304	♦	♦	–	–	–	–	–	–

[a] (♦) indicates a histidine residue conserved at this position in the primary sequence; (○) indicates residues that are not fully conserved; (–) indicates that this histidinyl is not conserved. [b] 6 muscle CK sequences compared: mouse (GB Accession No. A23590), rat (PIR Accession: KIRTCM), rabbit (KIRBCM), dog (A24686), human (A26387), chicken (KICHCM). [c] 10 brain CK sequences: rainbow trout (S13164), *Xenopus leavus* (A60098, X64212), dog (A24227), mouse (A4207), human brain (KIHCB), rabbit (KIRCB), chicken (M33711, M35381), rat (M14400). [d] 2 ray sequences: *Torpedo californica* (KIRYCT), *Torpedo marmorata* (KIRYCM). [e] Sea urchin sperm (A43736): this protein is thought to be derived from a gene triplication (Wothe *et al*., 1990); type of guanidino kinase activity undetermined. [f] 6 mitochondrial sequences: mouse mitochondria (S24612), rat mitochondria/ubiquitous (S17189), rat mitochondria/sarcomeric (S17188), human mitochondria/ubiquitous (A31431), human mitochondria sarcomeric (A35756), chicken mitochondria (A22708). [g] The trematode *Schistosoma mansoni* (A35743): this protein is thought to be derived from a gene duplication (Stein *et al*., 1990); for the purposes of this comparison, only the first of the duplicate open reading frames is used. [h] Arginine kinases from various sources: European lobster (X68703); horseshoe crab (S36043); mollusk (D26104); shrimp (Suzuki, 1994); abalone *Sulculus diversicolor* (Suzuki, 1994); partial sequences from *Caenorhabditis elegans* (M79646, M79846, M79644). [i] Sequence derived from cDNA of glycocyamine kinase (D26103) and partial sequences of lombricine kinase (A08416), taurocyamine kinase (P11917), and *Bacillus subtilis* (D26185). [j] H124 is absent in all muscle sequences except for the rabbit muscle. [k] H124 is absent in all mitochondrial sequences except for the human mitochondria/sarcomeric CK. [l] H301 is present in only two muscle sequences: the rabbit and human muscle. [m] H301 is present in rabbit, mouse, rat, and chicken brain isoform B, but *not* in human, chicken isoform B, trout, or *Xenopus* sequences.

FIGURE 1: Primary sequence of rabbit muscle creatine kinase as deduced from the cDNA sequence (Putney *et al*., 1984). The 17 histidine residues are encircled in the sequence, and regions showing high degree of homology with other ATP-guanidino kinases have been underlined. Of the 17 histidine residues, only H96, H105, H190, H233, and H295 are conserved in all ATP-guanidino kinases; these residues lie in highly conserved regions of rabbit muscle creatine kinase (Mühlebach *et al*., 1994).

acids 229–236 has been entirely conserved in all guanidino kinases, even in the structural homologue glycocyamine kinase (Suzuki & Furukohri, 1994). In fact, this high degree of conservation has led to the design of a "universal guanidino kinase" oligonucleotide primer that was used in the isolation of the genes encoding the guanidino kinases from the polychaete *Neanthes diversicolor* (Suzuki & Furukohri, 1994), shrimp (Suzuki, 1994), and abalone (Suzuki & Furukohri, 1994).

The sequence region 281–296 contains the reactive Cys282, which is also conserved in all ATP-guanidino kinases, as well as the signature sequence (GXGXXG) for a putative nucleotide binding site (Rossman *et al*., 1974). Recent studies by Hemmer *et al*. (1995) have also implicated this region as the site of autophosphorylation of various creatine kinase isozymes. In addition, mutagenesis of Arg291 to glutamine (R291Q) in rabbit muscle CK results in an inactive enzyme, whereas the mutant R291K has significantly lowered turnover and substrate binding (C. L. Borders, Jr., and G. L. Kenyon, in preparation). Similarly, it has been reported that, in human muscle CK, the R291K variant retains more than 40% of the wild-type activity (Lin *et al*., 1994). While these results suggest a possible role for this latter residue in substrate binding or catalysis, and it may indeed be the reactive arginine that is modified with phenylglyoxal (Borders & Riordan, 1975), the alteration of biochemical activity upon mutagenic perturbation of this region, as well as its evolutionary conservation, strongly suggest this region as important in maintaining the integrity of the active site.

Overexpression and Site-Specific Mutagenesis. Initially, the gene encoding the rabbit muscle creatine kinase was subcloned into a derivative of pKK233 (Pharmacia Biotech) and expressed in *E. coli* with a yield of 1 mg of pure enzyme/L of LB/Amp media (Chen *et al*., 1991). We have since subcloned the rabbit muscle CK gene into two other more efficient and productive expression vectors: the pBAce vector, which utilizes the bacterial alkaline phosphatase promoter (Craig *et al*., 1991), and the commercially available pET17b vector, which utilizes the T7 RNA promoter. The resultant constructs were designated pBAckrm7 and pET17/CK7, respectively. Mutagenesis of the rabbit muscle CK gene was carried as described in the Materials and Methods section, and an average mutation efficiency of >60% was observed in generating these mutants (data not shown).

Initial overexpression of the wild-type and mutant CKs were carried out with the pBAckrm7 construct, producing yields of 10–15 mg of pure enzyme/L of culture (data not shown). Utilizing this construct, however, significantly lower yields (<5 mg of pure enzyme/L of culture) of the H96N

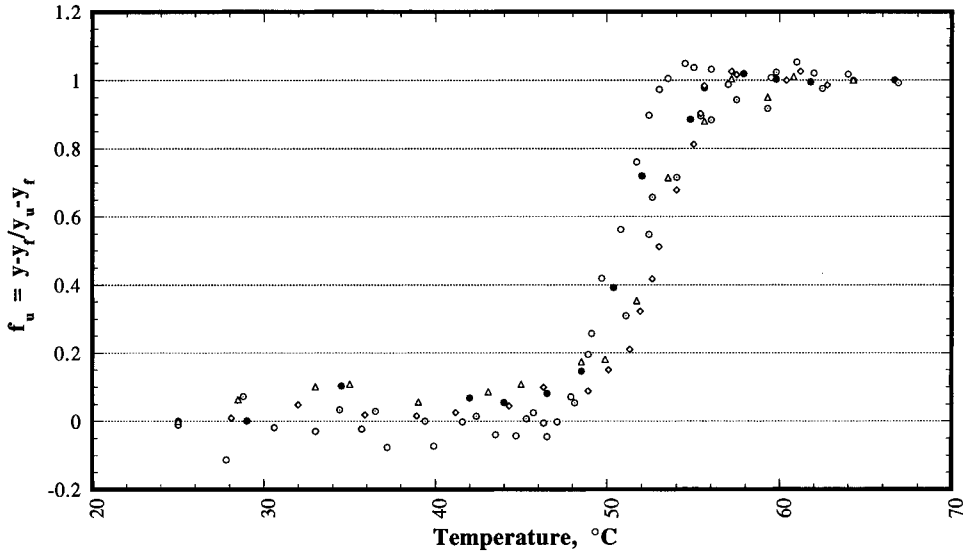

FIGURE 2: Irreversible thermal inactivation of native CK and its histidine mutants. CK subunits (5 μM) equilibrated in 10 mM PIPES, pH 7.0, were incubated at various temperatures. After temperature equilibration, 20 readings were acquired at $\lambda = 222$ nm and averaged to determine the molar ellipticity. f_u is the fraction of unfolded CK and is plotted as a function of temperature. (○) Native CK; (⊙) H105N; (◇) H190N; (●) H233N; (△) H295N. Based on these results, the calculated T_m values are native CK = 51.2 ± 1 °C; H105N = 52 ± 1 °C; H190N = 53.4 ± 1 °C; H233N = 50.4 ± 1 °C; H295N = 52.8 ± 1 °C.

and H233N mutants were obtained. Overexpression of wild-type enzyme from the pET17b/CK7 construct produced ~50 mg of pure enzyme/L of culture. The five mutants (H96N, H105N, H190N, H233N, and H295N) similarly induced with IPTG and grown at 20 °C gave similar yields of CK. In neither system were the proteins present as inclusion bodies as observed for wild-type creatine kinase in earlier experiments (Chen *et al*., 1991). Based on our results in the pBAckrm7 system, we conclude that the overall stability of the H96N and H233N mutants may be compromised, and under the growth conditions necessary for the pBAce expression system, these mutants may not be able to fold properly and are subsequently degraded *in vivo*.

Irreversible Thermal Denaturation. The irreversible denaturation of creatine kinase was measured by monitoring the changes in ellipticity at $\lambda = 222$ nm. This wavelength is used to monitor the loss of α-helical structure (Johnson, 1990). The results of these experiments are shown in Figure 2. We were unable to obtain purified H96N with significant activity, so the thermal denaturation of this mutant was not carried out. Overall, there appear to be small and apparently insignificant differences in the thermal denaturation curves of the remaining mutants when compared to the wild type; *i.e.*, T_m values within 1−2 °C of each other were obtained. The remarkable similarity of the mutants' T_m values to that of the wild type suggests that the inability to obtain significant overexpression of the H233N variant at elevated temperatures may not be due to the loss of conformational stability of this mutant. However, subtle conformational changes elicited by the removal of the conserved histidine in H233N may expose proteolytic sites that were previously inaccessible to *E. coli* intracellular proteases, resulting in the digestion of the nascent polypeptides. On the other hand, in the case of mutant H295N, the similarity of its T_m to that of the wild-type enzyme implies that the lower k_{cat} exhibited by H295N is not due to gross conformational changes in the enzyme which would lead to compromised stability.

Enzymatic Activity and Kinetic Analyses. Morrison and James (1965) showed that the kinetic mechanism for rabbit muscle CK can be described by a random order, rapid equilibrium, bi-bi mechanism. Additional studies by Maggio *et al*. (1977) further demonstrated synergism of substrate binding, whereby either substrate can bind first and the binding of the second substrate is enhanced by the binding of the first. Thus, a thorough kinetic analysis of any mutant needs to determine both the K_d for each substrate, that is, the dissociation constant for the formation of the binary complex between enzyme and substrate, as well as the K_m for each substrate, *i.e.*, the kinetic Michaelis constant that is a measure of the affinity of each substrate for its corresponding binary complex to give the catalytically-competent ternary complex (Maggio *et al*., 1977). In our kinetic analyses of the histidine mutants of creatine kinase, the K_d and K_m for MgATP are shown as K_d(ATP) and K_m(ATP), respectively, while the corresponding values for creatine are given by K_d(Cr) and K_m(Cr).

All five of the CK histidine mutants, H96N, H105N, H190N, H233N, and H295N, showed significant enzymatic activity, although H295N was of considerably lower activity than the rest. We carried out kinetic analyses of each histidine mutant (Table 2) and found that H105N, H190N, and H233N all had k_{cat} values indistinguishable from that of native CK. H96N also shows a significantly high turnover number, although this value is only approximate because of the instability of this mutant as described below, and H295N displays a >10-fold lower $k_{cat.}$ Examination of the K_d and K_m values for MgATP shows that only H295N displays significantly reduced affinity (higher K_d and K_m) and that the affinities for both MgATP and creatine are reduced between 3-fold and 10-fold.

The data strongly suggest that H96, H190, and H233 are not candidates for being the "essential" histidine at the active site of creatine kinase implicated in previous chemical modification (Pradel & Kassab, 1968; Clarke & Price, 1979), kinetic (Cook *et al*., 1981), and spectroscopic (Rosevear *et al*., 1981) studies. The most significant change in kinetic parameters was found with the H295N mutant, which has >10-fold reduction in both k_{cat} and binding affinities for

Table 2: Kinetic Constants for Rabbit Muscle CK and Various His→Asn Mutants[a]

RMCK mutants	MgATP, mM		creatine, mM		k_{cat}, (s^{-1})	relative k_{cat}/K_m[b]
	K_d (MgATP)	K_m (MgATP)	K_d (Cr)	K_m (Cr)		
WT	0.71 ± 0.10	0.25 ± 0.04	24.4 ± 5.5	8.61 ± 0.6	97.8 ± 2.1	1
H96N[c]	1.48 ± 0.29	1.83 ± 0.54	34.3 ± 6.7	42.5 ± 13.0	27.3 ± 2.0	0.06
H105N	1.15 ± 0.11	0.70 ± 0.21	1.75 ± 0.2	1.06 ± 0.3	91.5 ± 3.3	7.0
H190N	0.60 ± 0.07	0.70 ± 0.19	8.32 ± 0.9	9.75 ± 2.8	96.1 ± 3.2	0.9
H233N	1.04 ± 0.16	0.60 ± 0.12	33.9 ± 3.6	19.6 ± 4.8	98.3 ± 5.8	0.4
H295N	2.66 ± 0.40	1.80 ± 0.21	135 ± 15.3	91.1 ± 14.8	7.4 ± 0.5	0.007

[a] The initial rates were determined for the forward reaction using the pH-stat assay (Mahowald, 1962) under the following conditions: 0.40−10.00 mM MgATP, 2.0−100.0 mM creatine, 1.0 mM excess Mg^{2+} to avoid the inhibition of CK by chloride ions, and the total acetate was brought to 50 mM using sodium acetate. The data were analyzed for a random order rapid-equilibrium mechanism (Morrison & James, 1965) using software written by Dr. Ronald E. Viola, based on the programs of Cleland (1979) and distributed by Scitech, Inc. K_d(MgATP), K_m(MgATP), K_d(creatine), and K_m(creatine) are steady-state parameters defined in Maggio *et al.* (1977) and discussed further in the Results and Discussion. [b] Catalytic efficiency (k_{cat}/K_m) is calculated with respect to creatine. [c] Assays carried out in cell lysate. Total CK is estimated at ≥50% total protein concentration.

creatine, and a significant reduction in affinity for MgATP as well. Furthermore, the catalytic efficiency (k_{cat}/K_m) with respect to creatine is ≈150-fold lower for this mutant than for the native creatine kinase. The results would seem to be consistent with H295 playing a role in the active site of CK.

The data for H96N are tempered by the fact that this mutant is extremely unstable under the conditions of purification. The mutant is greatly overexpressed in the pET17b system, with a qualitative evaluation by SDS−PAGE suggesting that up to 50% of the total soluble protein in the cell-free extract is the mutant (data not shown). Purification of H96N according to procedures used for the native enzyme resulted in total loss of creatine kinase activity. Even relatively benign purification procedures such as size-exclusion chromatography, affinity chromatography, and ion exchange chromatography result in total loss of enzymatic activity. We could obtain activity in crude-cell lysates, but short ultrasonic treatment of such extracts resulted in significant loss of activity. To obtain the kinetic data for H96N shown in Table 2, we used crude cell-free lysates for the activity and estimated a k_{cat} by assuming that 50% of the total protein was the mutant enzyme. If H96N is less than 50% of the total protein, the value calculated for k_{cat} would be higher than that shown (Table 2). The values of K_d and K_m would not be affected by the purity of the enzyme and are assumed to be accurate.

Interestingly, the H105N mutant of creatine kinase has a greater catalytic efficiency than the native enzyme (Table 2). While the turnover number for this mutant remains unchanged, the K_m and K_d for creatine are *decreased* 8- to 14-fold; thus, k_{cat}/K_m is approximately 10-fold *greater* than the corresponding value for the wild-type enzyme. We offer no explanation of this observation at this point, but it might be revealing to examine the local environment of H105N when the X-ray crystal structure of creatine kinase becomes available.

Finally, all the histidine mutants examined showed a dramatic loss of the synergism of substrate binding exhibited by the wild-type enzyme. H96N and H190N display a total loss of synergism, while any synergism displayed by H105N, H233N, and H295N is deemed insignificant. Maggio *et al.* (1977) suggested that the observed synergism may be related to the conformational changes exhibited by CK upon substrate binding. Indeed, it has been suggested that the observed loss of synergism upon mutation of the reactive cysteine equivalent to C282 in rabbit muscle CK is an indicator of the role of the cysteine in the active site (Furter *et al.*, 1993); that is, the reactive cysteine may reside in a hinged loop whose movement is required for substrate binding, even though the cysteine residue may not in fact participate directly in catalysis (Furter *et al.*, 1993). It may be that the replacement of any of the five histidine residues examined in our study has also affected the ability of the enzyme to elicit these conformational changes upon substrate binding. Our observation of the alterations in apparent substrate binding in these mutants, however small, is consistent with this premise.

Table 3: Inactivation of Rabbit Muscle CK Wild-Type and Histidine Mutants with Diethyl Pyrocarbonate[a]

RMCK mutants	k_{inact}, min^{-1}	$t_{1/2}$, min
wild-type	0.126 ± 0.008	5.5 ± 0.4
H96N[b]	0.126 ± 0.004	5.5 ± 0.2
H105N	0.118 ± 0.005	5.9 ± 0.2
H190N	0.126 ± 0.002	5.5 ± 0.1
H233N	0.147 ± 0.002	4.7 ± 0.1
H295N	0.039 ± 0.002	17.7 ± 0.4

[a] Creatine kinase (23 μM) was incubated at 0 °C with 0.3 mM DEP in 50 mM MES, pH 6.1. Aliquots were removed and quenched in 10 mM L-histidine before being assayed for remaining activity by the pH-stat method (Mahowald *et al.*, 1962). k_{inact} represents the pseudo-first order rate constant for the inactivation of CK in the presence of 0.3 mM DEP. The $t_{1/2}$ value was derived from the k_{inact} and related by $t_{1/2} = (0.693)/k_{inact}$. [b] H96N cell lysate supernatant was used in these inactivation experiments; protein concentration estimates were as described in the kinetic analyses (Table 2).

Chemical Modification with Diethyl Pyrocarbonate. All the mutants of creatine kinase are inactivated by treatment with DEP (Table 3). In addition, the pseudo-first order rates of inactivation by 0.3 mM DEP of the native enzyme and all variants except H295N are very similar, with half-lives of inactivation of ranging from 4.7 to 5.9 min. H295N is less susceptible to inactivation, with $t_{1/2} = 17.7$ min in this experiment.

Since the kinetic analyses (*vide supra*) suggest that H295 is more likely to be present at the active site of creatine kinase than H96, H105, H109, or H233, and since the results of chemical inactivation of the native and mutant enzymes seem to support this assumption, we further examined the inactivation of H295N by DEP. The single reactive cysteine group of creatine kinase can be rapidly and reversibly blocked by treatment of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) (Borders & Riordan, 1975; Price & Hunter, 1976), and

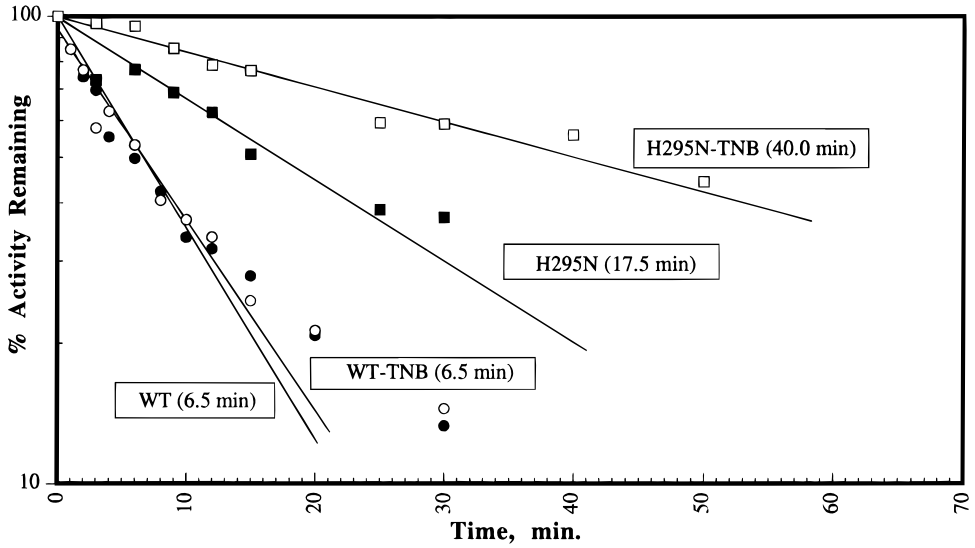

FIGURE 3: Chemical modification of wild-type and H295N creatine kinase. CK samples (25 μM) were incubated with 300 μM DEP in 50 mM MES, pH 6.1, at 0 °C. At various times, aliquots were removed and quenched before assaying for residual activity by the pH-stat method (Materials and Methods). Generation of the thionitrobenzoate-derivatized CK was as described in Materials and Methods. (●) Native wild-type rabbit muscle CK; (○) thionitrobenzoate-derivatized wild-type CK; (■) native H295N rabbit muscle CK; (□) thionitrobenzoate-derivatized H295N CK.

the thionitrobenzoate blocking group can be rapidly and completely removed by treatment with dithiothreitol (Borders & Riordan, 1975). The reactive cysteine is presumably C282, which has been shown to be at or near the creatine binding site by its reaction with the creatine analog, epoxycreatine (Marletta & Kenyon, 1979; Buechter *et al*., 1992). We thus treated both unblocked and thiol-blocked native and H295N creatine kinases with DEP under conditions very similar to those for the experiment described in Table 3. The results (Figure 3) show that native creatine kinase, irrespective of whether the reactive thiol is free ($t_{1/2}$ = 6.5 min) or blocked with the thionitrobenzoate group ($t_{1/2}$ = 6.5 min), is inactivated at indistinguishable rates. H295N, on the other hand, shows different rates of inactivation depending on whether the reactive thiol is free ($t_{1/2}$ = 17.5 min) or blocked ($t_{1/2}$ = 40 min).

Conclusions. There are only five histidine residues totally conserved throughout the entire family of guanidino kinases, corresponding to H96, H105, H190, H233, and H295 in rabbit muscle creatine kinase. Having mutated each of these five residues to asparagines, we have shown that all variants demonstrated significant enzymatic activity, implying that none of these five conserved histidines is absolutely required for catalysis. Indeed, the H105N mutant has a 10-fold greater catalytic efficiency than the native enzyme. All five mutants also show a great decrease in, or total loss of, the synergism of substrate binding that is found with native creatine kinase (Maggio *et al*., 1977). Kinetic analyses of the five histidine mutants, however, showed that the kinetic constants of H295N are altered most dramatically. This mutation reduces k_{cat} as well as the affinities for both substrates.

Knowing that creatine kinase activity is lost on modification of one histidine residue per subunit with DEP (Pradel & Kassab, 1968; Clarke & Price, 1979), we have further demonstrated that all five histidine mutants are inactivated by DEP (Table 3). Four are inactivated at the same rate as the native enzyme under identical conditions, while the fifth, H295N, shows decidedly altered kinetics of inactivation (Figure 3). DEP inactivation of all five mutants is reversed upon addition of hydroxylamine, suggesting that the inactivation of each mutant is due to the modification of a histidine residue. The data in Table 3 and Figure 3 suggest that the single histidine of native creatine kinase (and presumably of the H96N, H105N, H190N, and H233N mutants) that is modified by DEP to cause inactivation is H295, and that H295 is spatially removed from C282, for it is modified at the same rate whether or not the thiol is free or blocked with the bulky thionitrobenzoate group (Figure 3). On replacement of H295 with asparagine, the mutant enzyme is still inactivated by DEP, but at a significantly slower rate, and the rate of inactivation is decreased further yet when the thiol is blocked. The DEP inactivation of thiol-free and thiol-blocked H295N is >90% reversed by treatment with hydroxylamine (data not shown), so it is likely due to the modification of a different histidine. This second histidine may be closer than H295 to C282 in the three-dimensional structure of the enzyme, since blocking the sulfhydryl with the bulky thionitrobenzoate group reduces the rate of inactivation, or the mutation of H295 to asparagine may cause a perturbation of the active site that leads to the observed results.

Four of the fully conserved histidines in rabbit muscle creatine kinase, H96, H105, H190, and H233, evidently play no essential role in binding or catalysis, nor is any of them the residue modified on treatment with DEP. H295N, on the other hand, shows a significant decrease in catalytic efficiency; however, this decrease is not due to perturbation of a single kinetic parameter, but to decreases in k_{cat}, the affinity for MgATP, and the affinity for creatine (Table 2). We conclude that while H295 may be involved in some way in catalysis, it does not play the critical role suggested by Cook *et al*. (1981), *i.e.*, that of a general base that extracts a proton from the guanidinium group of creatine to promote nucleophilic attack on the γ-phosphate of MgATP. If it played such a role, replacement of H295 by a neutral asparagine residue would be expected to cause a much greater decrease in k_{cat} than that observed. A more plausible explanation is that replacement of H295 by asparagine gives

rise to a small perturbation of the active site that affects both substrate binding and chemical catalysis. The role of H295 at the active site remains an open question and will have to await the solution of the X-ray crystal structure of the enzyme.

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