Isoleucine 69 and Valine 325 Form a Specificity Pocket in Human Muscle Creatine Kinase†

Walter R. P. Novak,‡ Pan-Fen Wang,§ Michael J. McLeish,§ George L. Kenyon,§ and Patricia C. Babbitt*,‡

Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California, 600 16th Street, San Francisco, California 94143, and College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, Michigan 48109

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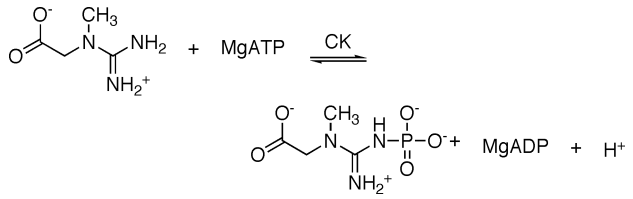
ABSTRACT: Creatine kinase (CK) catalyzes the reversible phosphorylation of creatine by ATP. From a structural perspective, the enzyme utilizes two flexible loop regions to sequester and position the substrates for catalysis. There has been debate over the specific roles of the flexible loops in substrate specificity and catalysis in CK and other related phosphagen kinases. In CK, two hydrophobic loop residues, I69 and V325, make contacts with the *N*-methyl group of creatine. In this study, we report the alteration of the substrate specificity of CK through the mutagenesis of V325. The V325 to glutamate mutation results in a more than 100-fold preference for glycocyamine, while mutation of V325 to alanine results in a slight preference of the enzyme for cyclocreatine (1-carboxymethyl-2-iminoimidazolidine). This study enhances our understanding of how the active sites of phosphagen kinases have evolved to recognize their respective substrates and catalyze their reactions.

Koshland originally suggested that conformational changes upon substrate binding could serve as a basis for substrate specificity; e.g., a good substrate induces an active conformation of an enzyme, and a poor substrate induces an inactive conformation (*1*). Frequently, these changes involve the movement of flexible loops which assist in substrate recognition and catalysis (*2*−*4*). While the contribution of induced fit to the substrate specificity of an enzyme has been greatly debated (*5*−*8*), it is clear that determinants of specificity can often be found on flexible loop regions (*4*, *9*, *10*). Here, we investigate the contributions of two flexible loop regions of creatine kinase (CK)[1] to the substrate specificity of the enzyme.

CK (EC 2.7.3.2) is a member of the phosphagen kinase superfamily of enzymes whose members catalyze the reversible phosphorylation of guanidino substrates by ATP. The CK reaction is shown in Scheme 1. The products of these reactions, phosphagens, act as reservoirs of high-energy phosphate which are utilized to rapidly regenerate ATP in cells with variable energy needs (e.g., muscle and nerve cells). In addition to their central role in energy homeostasis, the phosphagen kinases have been used as a paradigm for

Scheme 1



understanding the kinetics and mechanisms of bimolecular reactions (*11*−*13*).

The phosphagen kinases possess a unique structural fold that is only distantly similar to that of glutamine synthetase (*14*, *15*). High-resolution crystal structures of two phosphagen kinases, arginine kinase (AK) and CK, have been determined in the unbound form (*16*−*19*) and bound with a transition-state analogue complex (TSAC) (*20*, *21*). These structures indicate that large movements of two flexible loop regions (termed here N-terminal and C-terminal loops) occur upon substrate binding. The N-terminal loop region (N-loop, CK residues 60−73) is variable in length across the phosphagen kinase superfamily. The length of this loop shows an inverse relationship to the size of the cognate substrate, suggesting that it may be involved in determining specificity. In some studies, the N-loop has been termed the guanidino specificity region (*20*, *22*). The length of the C-terminal loop (C-loop, CK residues 323−332) is invariant across the phosphagen kinases, although the sequence motifs found in the loop are class (family) specific (e.g., there are differences between AKs and CKs, etc.). The gross movements of the N- and C-loops are similar in AK and CK, but consistent with the sequence differences among families, the specific interactions that occur between these two loops in the TSAC enzymes differ significantly. In the AK−TSAC structure, the N-loop is short and cannot make contacts with the C-loop.

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* To whom correspondence should be addressed. Telephone: (415) 476-3784. Fax: (415) 514-4260. E-mail: babbitt@cgl.ucsf.edu.

‡ University of California.

§ University of Michigan.

[1] Abbreviations: CK, creatine kinase; AK, arginine kinase; TSAC, transition-state analogue complex; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; TAPS, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl *β*-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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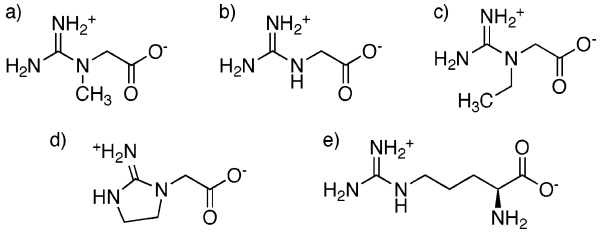

FIGURE 1: Natural (a, b, and e) and synthetic (c and d) guanidino substrates: (a) creatine, (b) glycocyamine, (c) *N*-ethylglycocyamine, (d) cyclocreatine, and (e) arginine.

In contrast, the CK−TSAC structure reveals significant interactions between the N- and C-loops (*21*).

Our examination of the structures of AK and CK suggests that CK residues I69 and V325 may act as a specificity-determining system in that enzyme. Accordingly, the roles of each of these residues in substrate recognition and catalysis have been investigated using site-directed mutagenesis. Several I69 and V325 mutants were constructed and assayed with creatine and creatine analogues (Figure 1a−d). Although the structural evidence suggests that both I69 and V325 make hydrophobic contacts with the substrate, our study found that single-site mutants at I69 are unable to direct the specificity of CK away from its natural substrate. In contrast, results from single-site mutations at V325 suggest that this residue can function as a "specificity switch". Changing the identity of the residue at this position alters the specificity of the enzyme among three guanidino substrates. The roles of I69 and V325 in the delivery of both substrate specificity and catalysis are discussed.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all chemicals and enzymes were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Microplate readings were taken on a SPECTRAmax 340 spectrophotometer from Molecular Devices (Sunnyvale, CA) using UV-transparent Costar 96-well plates from Corning (Corning, NY).

Synthesis of Cyclocreatine and N-Ethylglycocyamine. Cyclocreatine (1-carboxymethyl-2-iminoimidazolidine) and *N*-ethylglycocyamine (*N*-ethyl-*N*-amidinoglycine) were synthesized according to previously published methods (*23*).

Site-Directed Mutagenesis. Amino acid substitutions in the human muscle CK isozyme were carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the pETHMCK vector (*24*) acting as the DNA template. Double mutants were created either by performing a second mutagenesis step on the appropriate mutant template or by digesting the appropriate mutant plasmids with *Nco*I and *Pvu*I and ligating the fragments. The forward primers are shown below with the lowercase letters indicating the base change from the wild type; the codon encoding the mutation is underlined: I69A, 5′-CAGGTCACCCCTTCgcCATGACCGTGGGC-3′; I69V, 5′-CCCAGGCCACCCCTTCgTCATGACCGTCGGCTGCG-3′; I69L, 5′-CCCAGGCCACCCCTTCcTCATGACCGTCGGCTGCG-3′; V325A, 5′-GGGGTACAGGTGGCGcGGACACAGCTGCCGTGGG-3′; V325E, 5′-GAGGGGTACCGGTGGCGaGGACACAGCTGCAGTGGGC-3′.

After treatment with *Dpn*I to remove template DNA, the PCR product was transformed into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA). The presence of the mutation and fidelity of the mutagenesis were confirmed by sequencing the entire gene.

Expression and Purification. Proteins were expressed and purified using previously described methods (*25*) with some minor modifications. The plasmids were transformed into *E. coli* strain BL21(DE3)pLysS (Stratagene), and the transformed cells were grown in LB medium at 37 °C to an A_{600} of 0.6−1.0. The cells were cooled to 25 °C, and protein expression was induced with 0.4 mM IPTG. After growing for 6 h, the cells were harvested by centrifugation and resuspended in MES buffer [10 mM MES, 20 mM KCl, and 1 mM DTT (pH 6.0)] containing 0.1 mM PMSF. The cells were lysed by freezing and thawing, followed by the addition of DNase to a final concentration of ∼60 units/mL. The suspension was centrifuged at 22000*g* for 30 min, and the supernatant was loaded onto a Blue Sepharose CL-6B column (Amersham Biosciences, Piscataway, NJ) as described previously (*25*). Mutant proteins were eluted with TES buffer [10 mM TES and 1 mM DTT (pH 8.0)] containing 20 mM KCl. The CK mutants were further purified on a HiPrep Q (Amersham Biosciences) column (*25*). Each purified mutant enzyme appeared as a single band on SDS−PAGE.

Protein Stability Measurements. Thermal denaturation profiles were performed on a Jasco J-715 spectropolarimeter (Easton, MD) equipped with a Jasco PTC-348WI Peltier-effect temperature control device and in-cell stirring. Native CK and mutant concentrations were 0.02 mg/mL in 50 mM KP_i, 200 mM KCl, and 38% ethylene glycol at pH 6.8. The CD spectrum at 223 nm was monitored in 0.2 °C increments from 20 to 70 °C. Melting temperatures for the gross unfolding of creatine kinase were determined using EXAM (*26*).

Kinetic Characterization. Steady-state kinetic analyses of CK were carried out using a previously described coupled assay (*27*, *28*) modified for a microplate format. Activity measurements were performed at 30 °C in 96-well UV-transparent microplates. Assay controls using the native enzyme found the results from the microplate procedure to be similar to those of previous studies (*24*, *25*, *29*). The final assay mixture contained 75 mM TAPS buffer (pH 9.0), 0.36 mM NADH, 0.36 mM phosphoenolpyruvate, 1 mM $Mg(OAc)_2$, 13 mM KOAc, and variable concentrations of MgATP, guanidino substrate, and CK in a final volume of 300 μL. Concentrations of the coupling enzymes, pyruvate kinase and lactic acid dehydrogenase, were 28 and 54 units/mL, respectively. The reaction was initiated by the addition of the guanidino substrate.

Activity was determined by monitoring the oxidation of NADH at 340 nm using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. The microplate assay uses a mean path length of 0.8 cm. Data points were collected every 12 s for 10 min, and the maximum rate was determined using at least five points sampled over 1 min. At pH 9.0, CK operates by a rapid equilibrium, random bi-bi mechanism (*11*). Data were fitted to eq 1, using SigmaPlot 8.0 with the Enzyme Kinetics module from SPSS (Chicago, IL)

$$v = (V_{max}[A][B])/[\alpha(K_{ia}K_{ib} + K_{ib}[A] + K_{ia}[B] + [A][B]] \quad (1)$$

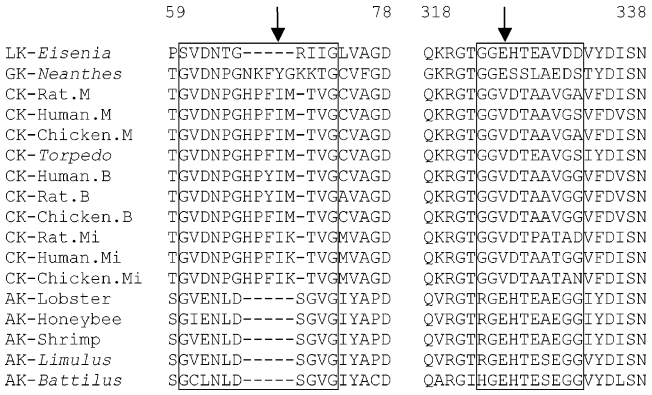

FIGURE 2: Alignment of the N- and C-loop regions of guanidino kinases. The sequence alignment was generated using Clustal W (*41*) and edited based on structural alignments of AK (PDB entry 1BG0) and CK (PDB entry 1N16) created with MinRMS (*42*). The numbering refers to human muscle CK. Loop regions are boxed. Hydrophobic specificity pocket residues I69 and V325 in CK are denoted with arrows on the multiple-sequence alignment. Swissprot accession numbers are as follows: O15991 for lombricine kinase (LK) *Eisenia*, P51546 for glycocyamine kinase (GK) *Neanthes*, P00564 for CK Rat.M, P06732 for CK Human.M, P00565 for CK Chicken.M, P00566 for CK *Torpedo*, P12277 for CK Human.B, P07335 for CK Rat.B, P05122 for CK Chicken.B, P25809 for CK Rat.Mi, P12532 for CK Human.Mi,; P70079, CK Chicken.Mi, P14208 for AK Lobster, O61367 for AK Honeybee, Q95V58 for AK Shrimp, P51541 for AK *Limulus*, and O15989 for AK *Battilus*.

where [A] and [B] are the substrate concentrations of the guanidino substrate (GS) and MgATP, respectively, K_{ia} and K_{ib} are the dissociation constants for the E−GS and E−MgATP complexes, respectively, and αK_{ia} and αK_{ib} are the dissociation constants for dissociation of GS and MgATP, respectively, from the E−GS−MgATP complex. Thus, the term α quantifies how the binding of one substrate affects the binding of the other.

Where mutant activity was too low to perform detailed kinetic analysis or when the substrate solubility was poor, *V*/*K* conditions were used to estimate k_{cat}/K_m. The assay mixture was prepared as described above with the following changes. The guanidino substrate was varied at nonsaturating levels while the concentration of MgATP was maintained at 5 mM (saturating). Velocity values for at least three independent trials were plotted against substrate concentrations. Under these conditions, the slopes of the plots yield V_{max}/K_m values.

RESULTS

Sequence and Structural Similarities and Differences among Phosphagen Kinases. In many aspects, the functions of the flexible loop regions in the characterized phophagen kinases, AK and CK, are essentially identical. Each loop recognizes the appropriate substrate and assists in positioning the substrate for catalysis. In addition, the loops undergo substantial movement upon substrate binding, leading to stabilization of the active conformation. A multiple-sequence alignment of the flexible loop regions of a representative set of phosphagen kinases is shown in Figure 2.

The N-loops exhibit significant differences in the length and amino acid composition across the various phosphagen kinases (Figure 2) (*20*, *22*). In most[2] AKs, the amino groups of N-loop residues 63−65 (SGV) make contacts with the carboxylate of arginine and D62 stabilizes the N-loop through a hydrogen bond with R193 (Figure 3a) (*20*). Mutagenesis studies have shown that the mutation of either D62 or R193 to glycine reduces activity ~100-fold (*30*). The N-loop of the CKs is longer than that found in AKs and possesses a conserved residue, H66, which appears to interact with D326 from the C-loop, possibly acting to stabilize the active conformation (Figure 3b) (*21*). Recent studies (P.-F. Wang, A. J. Flynn, M. J. McLeish, and G. L. Kenyon, unpublished results) indicate that mutations at H66 or D326 have a significant effect on catalysis, with mutation of either residue resulting in greatly reduced activity. In CK, none of the conserved N-loop residues except I69 make specific contacts with the creatine substrate (*21*). In the CK−TSAC structure (Figure 3b), residue I69 makes hydrophobic contacts with both the *N*-methyl[3] of creatine and V325. The interaction of I69 with V325 forms a "specificity pocket" for the *N*-methyl group in CK (*21*).

Comparisons of phosphagen kinase C-loops reveal class specific sequence differences, although the lengths of the loops are identical across all superfamily members (Figure 2). All CKs have a conserved VD motif (CK residues 325 and 326) which aligns structurally with the EH motif (AK residues 314 and 315) conserved across all AKs and lombricine kinases and the ES motif of glycocyamine kinase (Figure 2). The AK−TSAC structure shows the C-loop EH motif contacts only the bound arginine substrate (*20*). E314 forms two critical hydrogen bonds with the guanidino terminus of arginine (Figure 3a) (*20*). The conservative E314Q mutation reduces the k_{cat} of the enzyme ~300-fold (*29*, *31*). H315 forms a single hydrogen bond with the carboxylate of arginine, and is too distant to interact with the AK N-loop (*20*). This is in contrast to CK, where C-loop residues V325 and D326 contact N-loop residues I69 and H66, respectively (Figure 3b) (*21*). V325 and I69 also make contacts with the *N*-methyl of the bound creatine substrate, whereas D326 and H66 do not make any substrate contacts but may stabilize the active, closed loop conformation (*21*). It is important to note that E314 of AK and V325 of CK are aligned in structural superpositions (Figure 3), although, prior to the determination of the CK−TSAC structure, it was often assumed that E314 of AK was homologous to, and would align with, D326 of CK (*29*, *32*).

Protein Expression and Stability. CK and all variants were routinely expressed and purified essentially as described previously (*25*). To confirm that the overall stability of these proteins is unaffected by the mutations, thermal denaturation profiles were obtained. The mutant enzymes exhibit profiles identical to that of native CK (data not shown).

Enzyme Activity and Kinetic Parameters. Kinetic parameters for the reaction of native and mutant CK enzymes in the forward direction (phosphagen formation) were determined in a microplate format (see Experimental Procedures). Creatine, glycocyamine, *N*-ethylglycocyamine, and cyclocreatine were employed as substrates (Figure 1a−d). Binding constants and α values are listed in Table 1, except for that

[2] In several AKs, the SGV (residues 63−65) motif and D62 are not conserved. This subclass of AKs presumably has a unique substrate recognition system (*31*).

[3] For clarity, *N*- refers to the N_γ position of creatine and the creatine analogues and the analogous N_ϵ of arginine, as applicable.

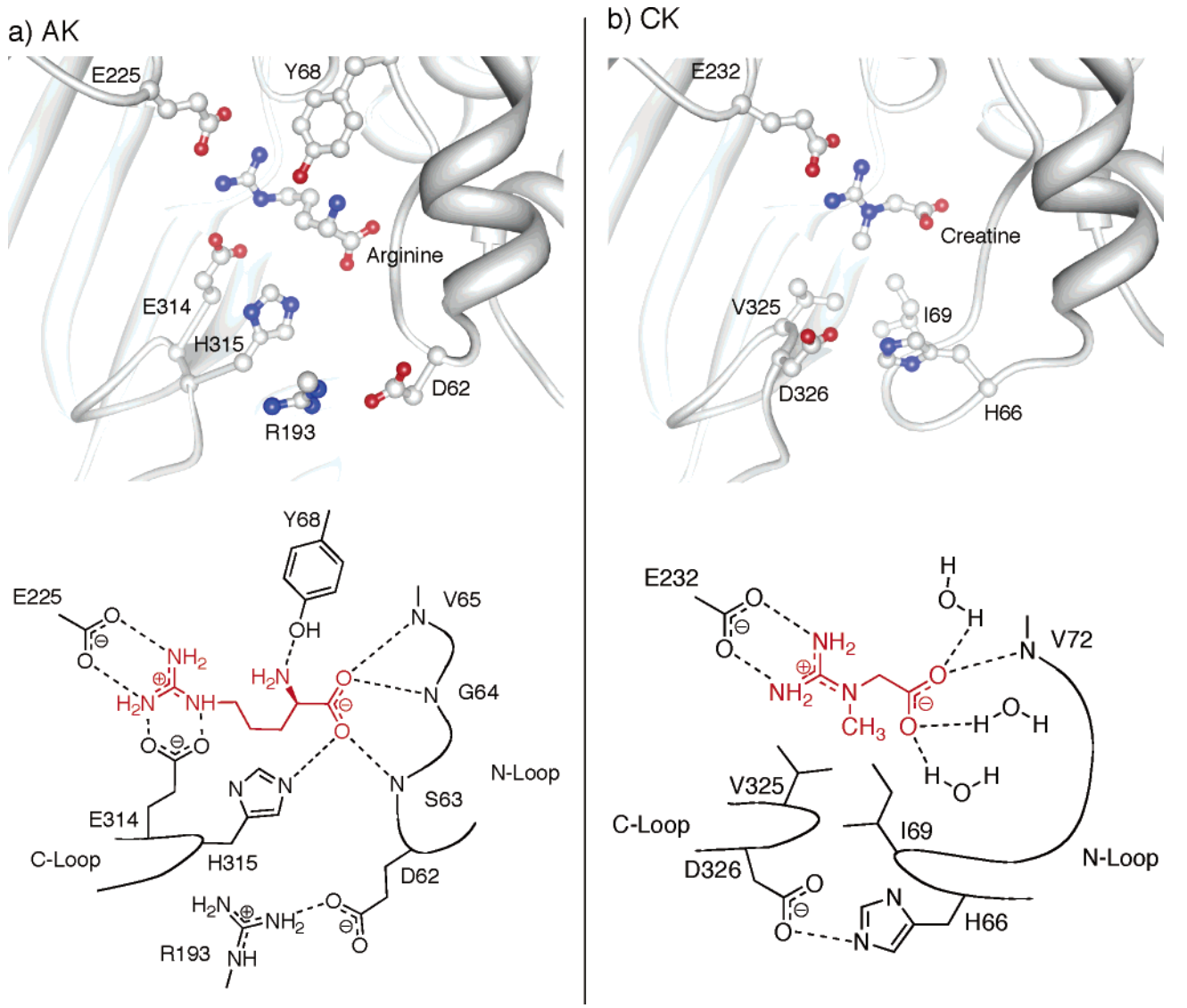

FIGURE 3: Active site comparisons of AK and CK. Ribbon diagrams with ball-and-stick ligands and side chains generated with Chimera (*15*) and ChemDraw representations of the active sites of AK (1BG0) and CK (1N16). Requirements of the flexible loop regions for both enzymes include precise positioning of the guanidino substrate and stabilization of the loops in the active conformation. Only the glutamate at position 225 in AK or position 232 in CK is strictly conserved between these active sites. (a) In the AK−TSAC structure, the guanidino terminus of arginine is positioned through C-loop residues E314 and H315 while the amino terminus is positioned through interactions with the backbone nitrogens of N-loop residues 63−65. The N-loop of AK is stabilized by the interaction of D62 with R193. (b) In the CK−TSAC structure, the guanidino terminus of creatine is positioned through the hydrophobic interaction of I69 and V325 with the methyl group of the creatine substrate. The carboxy terminus of CK is stabilized primarily through interactions with conserved, buried waters. The CK loops are stabilized by the interaction of H66 with D326, from the N- and C-loops, respectively.

of glycocyamine, which, due to its poor solubility, could not be fully characterized kinetically. These substrates were selected on the basis of their similarity to creatine, and their ability to probe specific enzyme−substrate interactions. For example, glycocyamine lacks the *N*-methyl group of creatine, while cyclocreatine and *N*-ethylglycocyamine have bulkier rigid and flexible N-substituents, respectively. The k_{cat} and specificity constants (k_{cat}/K_m) are listed in Table 2. None of the mutations we made significantly perturb the K_d value for MgATP. Although the I69A/V325A and I69V/V325A double mutants were also prepared and tested, the activities of these mutants were too low to allow accurate interpretation (data not shown).

I69 Mutations. The effects of mutagenesis at I69 vary significantly depending on the mutation and substrate utilized. The I69A mutant possesses the highest activity with creatine as the substrate; however, the k_{cat} is reduced 20-fold in comparison to that of the native enzyme. The K_d for creatine is increased approximately 2-fold over that of the native enzyme, while the K_m for creatine is increased more than 25-fold, indicative of a change in the synergy (α value[4]) of the enzyme. Binding synergy has been previously observed for some CK isozymes, although the degree of synergy varies and may be affected by many factors (*11*, *33*). Under our conditions, native CK exhibits synergy with creatine and has an α value of 0.24. In contrast, the I69A mutation results in a greater than 15-fold increase in α with

[4] An α value equal to unity indicates that the binding of the first substrate has no effect on the binding of the second. A value of less than unity signifies an increased affinity for the second substrate, and a value of greater than unity indicates a decrease in the affinity of the enzyme for the second substrate.

Table 1: α Values and Dissociation and Michaelis Constants for CK Mutants with Several Guanidino Substrates[a]

			MgATP		guanidino substrate	
enzyme	substrate[b]	α	K_d^{MgATP} (mM)	K_m^{MgATP} (mM)	K_d^{GS} (mM)	K_m^{GS} (mM)
WT	Cr	0.24	0.90 ± 0.12	0.22 ± 0.05	38.6 ± 6.3	9.3 ± 2.5
	CyCr	0.37	0.79 ± 0.09	0.29 ± 0.07	67 ± 12	24.5 ± 7.1
	EG	0.68	0.91 ± 0.07	0.62 ± 0.12	103 ± 14	70 ± 16
I69A	Cr	3.7	0.86 ± 0.09	3.17 ± 1.02	69 ± 11	250 ± 90
	CyCr	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
	EG	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
I69V	Cr	0.49	1.11 ± 0.10	0.54 ± 0.11	75 ± 10	36.4 ± 8.5
	CyCr	0.51	0.95 ± 0.10	0.47 ± 0.14	130 ± 28	66 ± 22
	EG	1.7	1.00 ± 0.09	1.66 ± 0.38	81 ± 11	135 ± 34
I69L	Cr	0.52	0.84 ± 0.06	0.44 ± 0.09	137 ± 20	72 ± 17
	CyCr	0.71	0.89 ± 0.11	0.64 ± 0.17	75 ± 13	53 ± 16
	EG	1.3	0.85 ± 0.10	1.1 ± 0.3	58.2 ± 9.1	76 ± 21
V325A	Cr	0.63	1.01 ± 0.07	0.64 ± 0.12	113 ± 15	71 ± 15
	CyCr	0.46	0.95 ± 0.07	0.44 ± 0.08	109 ± 14	50 ± 10
	EG	0.83	0.85 ± 0.09	0.70 ± 0.20	100 ± 19	83 ± 27

[a] Values are shown ± the standard error. K_d and K_m values are discussed in Experimental Procedures. Each data point is an average of at least three individual measurements. GS is the guanidino substrate. [b] Cr, creatine; CyCr, cyclocreatine; EG, *N*-ethylglycocyamine. [c] Activity is too low for full kinetic characterization.

Table 2: Velocity and Specificity Constants of CK Mutants[a]

enzyme	substrate[b]	k_{cat} (s^{-1}) (% of WT)	k_{cat}/K_m (s^{-1} M^{-1}) (% of WT)	$(k_{cat}^{GS}/K_m^{GS})/(k_{cat}^{Cr}/K_m^{Cr})$
WT	Cr	85.3 ± 1.5 (100)	9190 (100)	1
	CyCr	35.2 ± 0.8 (100)	1440 (100)	0.16
	EG	14.5 ± 0.5 (100)	207 (100)	0.02
	Gly	nd[c]	21[d] (100)	0.002
I69A	Cr	4.2 ± 0.5 (5)	17 (0.2)	1
	CyCr	nd[c]	0.5[d] (0.03)	0.03
	EG	nd[c]	0.5[d] (0.2)	0.03
	Gly	nd[c]	nd[c]	nd[c]
I69V	Cr	82.7 ± 2.2 (97)	2270 (25)	1
	CyCr	4.4 ± 0.2 (13)	67 (5)	0.03
	EG	12.0 ± 0.7 (83)	89 (43)	0.04
	Gly	nd[c]	1.4[d] (7)	0.001
I69L	Cr	45.0 ± 1.3 (53)	627 (7)	1
	CyCr	1.5 ± 0.1 (4)	28 (2)	0.04
	EG	0.55 ± 0.02 (4)	7.2 (3)	0.01
	Gly	nd[c]	0.3[d] (1)	0.0005
V325A	Cr	15.4 ± 0.5 (18)	216 (2)	1
	CyCr	18.0 ± 0.4 (51)	359 (25)	1.66
	EG	4.3 ± 0.2 (30)	52 (25)	0.24
	Gly	nd[c]	1.7[d] (8)	0.008
V325E	Cr	nd[c]	0.14[d] (0.002)	1
	CyCr	nd[c]	nd[c]	nd[c]
	EG	nd[c]	nd[c]	nd[c]
	Gly	nd[c]	15[d] (71)	104

[a] Each data point is an average of at least three individual measurements. Unless otherwise noted, k_{cat} and k_{cat}/K_m values are determined from Table 1. Values are shown ± the standard error. GS is the guanidino substrate. [b] Cr, creatine; CyCr, cyclocreatine; EG, *N*-ethylglycocyamine; Gly, glycocyamine. [c] Activity is too low for determination. [d] Values were determined under pseudo-first-order conditions discussed in Experimental Procedures. The standard error is estimated to be ±10%.

creatine (α = 3.7), indicating negative synergy with creatine. The increase in this α value also dramatically affects the K_m value, resulting in an enzyme with a 500-fold reduction in its specificity for creatine.

The I69V mutant exhibits a k_{cat} similar to that of the native enzyme with both creatine and *N*-ethylglycocyamine as substrates, while the k_{cat} for cyclocreatine is reduced 8-fold. While the k_{cat} values for creatine and *N*-ethylglycocyamine are near wild-type (WT) values, the K_m values for these substrates are significantly higher, thereby also affecting the specificity of the enzyme. The K_m for cyclocreatine is lower than that for *N*-ethylglycocyamine; however, this tighter binding does not result in an increase in the k_{cat} value with the cyclocreatine substrate. Instead, the decrease in K_m is accompanied by a decrease in k_{cat} for cyclocreatine. This phenomenon may indicate nonproductive binding with cyclocreatine as the substrate (*34*). Despite the slight changes in activity and binding for *N*-ethylglycocyamine, the I69V mutation results in an increase in α from 0.7 in the native enzyme to 1.7 in the mutant. This negative synergy is similar to that observed for I69A with creatine, albeit less pronounced.

In addition to mutations which generate a larger substrate binding pocket at I69, we also investigated the I69L mutant. Although this change reduces the size of the binding pocket and would be expected to decrease the affinity for creatine and bulkier substrates, it was designed to probe whether the extra methyl group at this position could alter the enzyme and direct it to prefer glycocyamine by mimicking the *N*-methyl group of the creatine substrate. However, the results indicate that the k_{cat}/K_m value for I69L with glycocyamine is not enhanced, but rather reduced ~100-fold in comparison to that of the WT enzyme. As expected, the k_{cat} value with this mutant is reduced 2-fold with the creatine substrate, and 25-fold with cyclocreatine and *N*-ethylglycocyamine in comparison to the values for the WT enzyme with these substrates. The K_d for cyclocreatine is similar to that for WT, and the K_d for *N*-ethylglycocyamine is almost 2-fold lower than the WT value, despite the added bulk nearer the substrate binding pocket. The specificity constants (k_{cat}/K_m) for each of the substrates are significantly reduced, with values for all substrates ranging from 1 to 7% of those for the WT enzyme. The α values for I69L are similar to those for I69V for all substrates, including the negative synergy observed with *N*-ethylglycocyamine.

V325 Mutations. While I69 mutations affect both the synergy and specificity of the enzyme, mutations at this position are unable to switch the overall preference of CK away from its natural substrate, creatine. In contrast, both V325 mutations alter the specificity of the enzyme away from creatine and toward one of its analogues. Mutation of V325 to alanine creates a slightly larger substrate binding pocket, which we speculated would direct the enzyme to prefer bulkier substrates such as cyclocreatine and *N*-ethylglycocyamine. However, the V325A mutant fails to improve the K_d or K_m values (Table 1) for either of the bulkier substrates. In fact, the mutation of V325 to alanine results in increased K_m values, accompanied by decreased activities, for all substrates. The ability of V325A CK to bind and catalyze the reaction with creatine as the substrate is weakened more than with the cyclocreatine substrate. This results in a modest 1.7-fold preference for the cyclocreatine substrate over creatine.

Mutation of V325 to glutamate results in a striking preference for glycocyamine over creatine. As shown in Table 2, comparisons of the specificity constants of V325E for creatine and glycocyamine reveal that the enzyme prefers glycocyamine by ~100-fold. The k_{cat}/K_m value with creatine as the substrate is reduced nearly 70000-fold relative to that of the wild type, while the specificity constant with glycocyamine remains essentially unchanged in comparison to that of the native enzyme. Examination of the sequences and structures suggests an explanation for this preference. Both the larger size and negative charge of the glutamate at this position should interfere with the binding of the hydrophobic *N*-methyl group of creatine (see the Discussion). In addition, the glutamate may be able to accept a hydrogen bond from the glycocyamine substrate, a potentially stabilizing interaction which the native valine at that position cannot provide.

DISCUSSION

The use of flexible loops in the binding of and discrimination between substrates as well as catalysis is common among many enzyme families. In these cases, a conformational change, such as the closure of flexible loops, occurs upon ligand binding and is required for the proper orientation of the catalytic machinery and substrates for catalysis. There is substantial precedent in several enzymes that the alteration of specificity may be achieved through variations in flexible loops (*4*, *9*, *10*). Inspection of the unliganded (*16*−*19*) and TSAC (*20*, *21*) structures for both AK and CK indicates that these phosphagen kinases also utilize flexible loop regions to orient active site elements and substrates for catalysis. These structures also suggest that some determinants of substrate specificity are present on these loop regions (*30*, *35*, *36*). However, the precise roles of many of the residues in the loops have remained unclear, and prior to this study, researchers have been unable to alter the specificity of either of these enzymes by making changes in these loops (*36*).

Literature reports of the alteration of substrate specificity have generally been limited to sites distant from or only indirectly associated with the residues involved in catalysis (*4*, *10*, *37*). In contrast, the specificity determinants in the phosphagen kinases are close to the site of chemistry, and may even assist in catalysis through the optimal positioning of the substrate. Thus, the phosphagen kinases present a unique system in which to examine the delivery of substrate specificity through flexible loop regions.

The reasons for a dual role of the flexible loop residues both in determining specificity and in mediating catalysis can be rationalized by examination of the TSAC structures of AK and CK. First, the loop residues interacting with the substrates are important for catalysis (*20*, *31*). The sp^2 hybridization of the guanidino nitrogens in all guanidino kinase substrates results in a rigid, planar substrate at the site where chemistry occurs. This planarity allows the precise alignment of the guanidino group necessary for productive catalysis to be controlled through the positioning of any rigid N-substitutions at the guanidino group, or by the positioning of the guanidino group itself. Therefore, creatine may be optimally aligned for catalysis through interactions with its rigid *N*-methyl substitution at the guanidinium ion (Figure 1a), while the other naturally occurring guanidino substrates (Figure 1b,e) may be positioned through direct interactions with the guanidino group. The importance of precise substrate positioning has been demonstrated in AK where structures of AK−TSAC mutants reveal that subtle perturbations in the substrate alignment lead to a loss of activity (*31*). Second, these same residues are important in the discrimination of substrates. In AK, the arginine substrate appears to be precisely positioned for catalysis, at least in part, through the interaction of E314 with the guanidino group (Figure 3a) (*20*). In CK, precise alignment of the substrate is partially achieved through the interaction of the *N*-methyl group of creatine with hydrophobic residues I69 and V325 (Figure 3b) (*21*). E314 of AK and V325 of CK align structurally, and our results show that the substrate specificity of CK may be altered through the mutagenesis of V325. Thus, in CK, and possibly in other phosphagen kinases, we see that specific flexible loop residues may play a role in both catalysis and substrate recognition.

The results reported here are consistent with the interpretation that precise positioning of the substrates is important in catalysis and help to explain results from previous studies of AK and CK. These studies have generated much debate over the contributions of acid−base chemistry (*25*, *29*, *38*, *39*), strain, and optimal substrate alignment to catalysis (*16*, *20*, *29*, *31*, *32*). Prior to the solution of the CK−TSAC structure, the candidates for the putative catalytic base were identified as either the strictly conserved E232 (E225 in AK) or C-loop residue D326 (E314 in AK) (Figure 3a,b) (*20*, *29*, *32*). A study by Cantwell et al. (*29*) found that in CK the functionally conservative E232D mutation results in a 500-fold decrease in activity compared with a modest 3-fold decrease in activity for the D326E mutation. This suggests that E232 is more likely to act as a catalytic base; in AK, a parallel result was found for the mutation of E225 (*31*).

Pruett et al. (*31*) have suggested that in AK, the flexible loop residue which interacts with the guanidino terminus, E314, is not acting as a catalytic base. Rather, its role lies in the precise positioning of the substrate. Our results support this interpretation. Examination of the CK−TSAC structure shows that there is no candidate on the C-loop to act as a catalytic base. V325 of CK aligns both structurally (Figure 3a,b) and in the multiple-sequence alignment (Figure 2) with E314 of AK. Clearly, valine is unable to act as a catalytic base. Instead, V325 is important in determining the specificity of CK. The V325E mutation severely restricts the ability

of the enzyme to turn over creatine, as determined from the k_{cat}/K_m, while the k_{cat}/K_m value with glycocyamine as a substrate is essentially unaffected. This results in an ~100-fold preference of V325E CK for glycocyamine over creatine. Our results also show that by increasing the size of the binding cavity at this position (V325A) we reduce the k_{cat}/K_m value for creatine to 2% of that of WT, whereas the k_{cat}/K_m value with cyclocreatine is only reduced to 25% of that of WT. Thus, we obtain a modest (1.7-fold) preference for the rigid substrate, cyclocreatine.

In comparison to that of V325, the role of I69 is more subtle with respect to its contribution to either catalysis or specificity. While I69 mutations do affect substrate specificity, mutations at this position alone fail to alter the preference of the enzyme for a substrate other than creatine. Even the I69L mutation, which might be expected to increase activity with glycocyamine by compensating for the loss of the *N*-methyl group, fails to direct the enzyme to prefer this substrate. Thus, the low activities of I69L and I69A may largely be associated with the fact that specificity in CK is conferred through the interaction of V325 and I69 with the *N*-methyl group in creatine, in a composite system whose precise interactions do not easily tolerate changes. These interactions appear to be unnecessary in homologous enzymes such as AK and GK, whose substrates lack the *N*-methyl group. We speculate that in the V325E CK mutant, glycocyamine may prefer a hydrogen bond acceptor at the V325 position for optimal positioning and/or catalysis. (Note that an H-bond acceptor is not strictly required as WT CK has activity with glycocyamine.)

I69 mutations also affect the binding synergy. The loss of synergy resulting from the I69A, I69V, and I69L mutations is similar to that recently reported in an I69G mutant of the *Danio* CK isozyme (*35*). Taking all of the mutation results together, we propose the roles of I69 and V325 in CK are both to act as a specificity filter and to aid in the optimal positioning of the guanidino substrate for catalysis.

Importance of Differences in Loop Interactions in AK and CK. Although much has been learned about phosphagen kinase structure−function relationships from comparative studies on the homologous CK and AK enzymes, transfer of insight about catalysis from either AK to CK or *vice versa* should be approached with some caution due to important differences in the nature and behavior of their flexible loops. The specific roles of the flexible loop residues in AK appear to differ significantly from those in CK. AK is considered a more primitive phosphagen kinase than CK (*40*), and its flexible loops do not interact. In the study by Pruett et al. (*31*), the C-loop in AK was substituted with a CK-like C-loop (R312G/E314V/H315D/E317A/E319V). This mutant has near-wild-type activity with arginine, leading those investigators to question the role of the interactions of the C-loop with the substrate in the AK reaction. By contrast, in the more evolved CK, the loop residues have significant interactions. These interactions may place additional functional constraints on the CK enzyme not present in AK and may help in rationalizing the differences between the results of the Pruett study (*31*) and those reported here. A more informative understanding of these results may require a TSAC structure of the AK mutant described above and more detailed studies of the effects of the individual mutations in its C-loop.

Very recently, Azzi et al. (*36*) investigated the roles of the N-loop residues as specificity determinants in AK and showed that the specificity of AK is not altered by substituting a longer CK-like N-loop for the short AK N-loop. In our study of CK, the results for the V325E mutant provide a possible explanation for the lack of change in specificity in the AK study. Our results show that the bulk and/or negative charge of the glutamate at position V325 has a critically negative impact on the catalytic efficiency of the enzyme with creatine as the substrate (an ~70000-fold decrease). Pertinent to these observations, Azzi et al. (*36*) also obtained a structure of AK with creatine and ADP bound. In this structure, creatine binds similarly to its native conformation in CK, except, as expected, there is a distortion in the guanidino plane of the substrate, presumably caused by a steric clash of the methyl group of creatine with E314. We note that the loop mutants of Azzi et al. (*36*) all contain E314. On the basis of results reported here, it is possible these AK loop mutants have no detectable activity with creatine simply because E314 interferes with the optimal positioning of creatine. On the basis of observations from our study, and the Pruett (*31*) and Azzi (*36*) studies, we suggest that the conversion of AK to CK will not be achieved solely through N-loop mutations and, minimally, will require the additional mutation of E314 to a small hydrophobic residue such as valine or alanine.

While it is now clear there are significant differences between the flexible loops of AK and CK, early interpretation of the roles of these loops in recognition and catalysis in CK was compromised by the incorrect assumption that the residue homologous to E314 of AK is D326 of CK (*20*, *29*, *32*). The CK−TSAC structure allows the correction of this mistake and suggests that E314 of AK has been replaced in CK with the hydrophobic pocket formed by I69 and V325. Our mutagenesis results support this suggestion. Moreover, we now postulate that the specific role of V325 in CK is to recognize and align the substrate for catalysis. We therefore have termed V325 a "specificity switch", where the identity of the amino acid at this position determines the enzyme's preference for creatine, cyclocreatine, or glycocyamine.

Evolution of Specificity in Phosphagen Kinases. Given the results of this and earlier studies, we may now suggest a new idea for how the phosphagen kinase superfamily of enzymes has evolved to recognize a variety of substrates and catalyze their reactions. Phosphagen kinase substrates may be described in relative terms as long (arginine and lombricine), short (creatine and glycocyamine), having an *N*-methyl group (creatine) and lacking an N-substitution (arginine and glycocyamine). Similarly, the cognate enzymes of these substrates have evolved accordingly. Although the study by Azzi et al. (*36*) raises questions about the importance of loop length relative to substrate size, at least in nature, long substrates are catalyzed by enzymes with short N-loops, while short substrates are catalyzed exclusively by enzymes with long N-loops. Substrates with an *N*-methyl group such as creatine may only be catalyzed by enzymes possessing a small hydrophobic group on the C-loop, while substrates lacking this N-substitution such as arginine and glycocyamine require an acidic group, e.g., a glutamate, at the analogous position. Only one of the four possible substrate−enzyme combinations (a long, N-methylated substrate) does not occur naturally. It may be that the interactions between the N- and

C-loops are required for recognition of an N-methylated substrate (note the reduced activity of the I69A mutant), and therefore, limitations in the size of the associated binding pocket may preclude the use of a substrate such as *N*-methylarginine.

Conclusion. In the context of induced fit theories, our results suggest that specificity factors presented on flexible loops in the phosphagen kinase superfamily are also important in catalysis. Thus, in addition to proton abstraction, precise positioning of the substrates is required for optimal catalysis. Here, we have investigated the specificity system unique to CK involving interactions between the N-loop and C-loop residues I69 and V325 with the *N*-methyl group of the natural creatine substrate. One of these residues, V325, may be altered to direct the enzyme to prefer an alternative substrate.

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REFERENCES

1. Koshland, D. E., Jr. (1958) Application of a theory of enzyme specificity to protein synthesis, *Proc. Natl. Acad. Sci. U.S.A. 44*, 98−104.
2. Joseph, D., Petsko, G. A., and Karplus, M. (1990) Anatomy of a conformational change: hinged "lid" motion of the triosephosphate isomerase loop, *Science 249*, 1425−1428.
3. Gerstein, M., and Chothia, C. (1991) Analysis of protein loop closure. Two types of hinges produce one motion in lactate dehydrogenase, *J. Mol. Biol. 220*, 133−149.
4. Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992) Converting trypsin to chymotrypsin: the role of surface loops, *Science 255*, 1249−1253.
5. Fersht, A. R. (1974) Catalysis, binding and enzyme−substrate complementarity, *Proc. R. Soc. London, Ser. B 187*, 397−407.
6. Fersht, A. R. (1985) in *Enzyme Structure and Mechanism*, pp 331−333, W. H. Freeman and Co., New York.
7. Herschlag, D. (1988) The role of induced fit and conformational changes of enzymes in specificity and catalysis, *Bioorg. Chem. 16*, 62−96.
8. Post, C. B., and Ray, W. J., Jr. (1995) Reexamination of induced fit as a determinant of substrate specificity in enzymatic reactions, *Biochemistry 34*, 15881−15885.
9. Graf, L., Jancso, A., Szilagyi, L., Hegyi, G., Pinter, K., Naray-Szabo, G., Hepp, J., Medzihradszky, K., and Rutter, W. J. (1988) Electrostatic complementarity within the substrate-binding pocket of trypsin, *Proc. Natl. Acad. Sci. U.S.A. 85*, 4961−4965.
10. Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., and Holbrook, J. J. (1988) A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework, *Science 242*, 1541−1544.
11. Morrison, J. F., and James, E. (1965) The mechanism of the reaction catalyzed by adenosine triphosphate creatine phosphotransferase, *Biochem. J. 97*, 37−52.
12. Morrison, J. F., and Cleland, W. W. (1966) Isotope exchange studies of the mechanism of the reaction catalyzed by adenosine triphosphate: creatine phosphotransferase, *J. Biol. Chem. 241*, 673.
13. Cleland, W. W. (1967) Enzyme Kinetics, *Annu. Rev. Biochem. 36*, 96−99.
14. Kabsch, W., and Fritz-Wolf, K. (1997) Mitochondrial creatine kinase: A square protein, *Curr. Opin. Struct. Biol. 7*, 811−818.
15. Huang, C. C., Novak, W. R., Babbitt, P. C., Jewett, A. I., Ferrin, T. E., and Klein, T. E. (2000) Integrated tools for structural and sequence alignment and analysis, *Pac. Symp. Biocomput.*, 230−241.
16. Yousef, M. S., Clark, S. A., Pruett, P. K., Somasundaram, T., Ellington, W. R., and Chapman, M. S. (2003) Induced fit in guanidino kinases: comparison of substrate-free and transition state analog structures of arginine kinase, *Protein Sci. 12*, 103−111.
17. Fritz-Wolf, K., Schnyder, T., Wallimann, T., and Kabsch, W. (1996) Structure of mitochondrial creatine kinase, *Nature 381*, 341−345.
18. Rao, J. K., Bujacz, G., and Wlodawer, A. (1998) Crystal structure of rabbit muscle creatine kinase, *FEBS Lett. 439*, 133−137.
19. Eder, M., Schlattner, U., Becker, A., Wallimann, T., Kabsch, W., and Fritz-Wolf, K. (1999) Crystal structure of brain-type creatine kinase at 1.41 Å resolution, *Protein Sci. 8*, 2258−2269.
20. Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W. R., and Chapman, M. S. (1998) Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions, *Proc. Natl. Acad. Sci. U.S.A. 95*, 8449−8454.
21. Lahiri, S. D., Wang, P. F., Babbitt, P. C., McLeish, M. J., Kenyon, G. L., and Allen, K. N. (2002) The 2.1 Å structure of *Torpedo californica* creatine kinase complexed with the ADP−Mg^{2+}−NO_3^-−creatine transition-state analogue complex, *Biochemistry 41*, 13861−13867.
22. Suzuki, T., Kawasaki, Y., Furukohri, T., and Ellington, W. R. (1997) Evolution of phosphagen kinase. VI. Isolation, characterization and cDNA-derived amino acid sequence of lombricine kinase from the earthworm *Eisenia foetida*, and identification of a possible candidate for the guanidine substrate recognition site, *Biochim. Biophys. Acta 1343*, 152−159.
23. Rowley, G. L., Greenleaf, A. L., and Kenyon, G. L. (1971) On the Specificity of Creatine Kinase. New Glycocyamines and Glycocyamine Analogs Related to Creatine, *J. Am. Chem. Soc. 93*, 5542−5551.
24. Chen, L. H., White, C. B., Babbitt, P. C., McLeish, M. J., and Kenyon, G. L. (2000) A comparative study of human muscle and brain creatine kinases expressed in *Escherichia coli*, *J. Protein Chem. 19*, 59−66.
25. Wang, P. F., McLeish, M. J., Kneen, M. M., Lee, G., and Kenyon, G. L. (2001) An unusually low pK_a for Cys282 in the active site of human muscle creatine kinase, *Biochemistry 40*, 11698−11705.
26. Kirchhoff, W. (1993) *EXAM*, National Institute of Standards and Technology, Gaithersburg, MD.
27. Tanzer, M. L., and Gilvarg, C. J. (1959) Creatine and Creatine Kinase Measurement, *J. Biol. Chem. 234*, 3201−3204.
28. Wang, P. F., Novak, W. R., Cantwell, J. S., Babbitt, P. C., McLeish, M. J., and Kenyon, G. L. (2002) Expression of *Torpedo californica* creatine kinase in *Escherichia coli* and purification from inclusion bodies, *Protein Expression Purif. 26*, 89−95.
29. Cantwell, J. S., Novak, W. R., Wang, P. F., McLeish, M. J., Kenyon, G. L., and Babbitt, P. C. (2001) Mutagenesis of two acidic active site residues in human muscle creatine kinase: implications for the catalytic mechanism, *Biochemistry 40*, 3056−3061.
30. Suzuki, T., Fukuta, H., Nagato, H., and Umekawa, M. (2000) Arginine kinase from *Nautilus pompilius*, a living fossil. Site-directed mutagenesis studies on the role of amino acid residues in the guanidino specificity region, *J. Biol. Chem. 275*, 23884−23890.
31. Pruett, P. S., Azzi, A., Clark, S. A., Yousef, M. S., Gattis, J. L., Somasundaram, T., Ellington, W. R., and Chapman, M. S. (2003) The putative catalytic bases have, at most, an accessory role in the mechanism of arginine kinase, *J. Biol. Chem. 278*, 26952−26957.
32. Zhou, G., Ellington, W. R., and Chapman, M. S. (2000) Induced fit in arginine kinase, *Biophys. J. 78*, 1541−1550.
33. Maggio, E. T., and Kenyon, G. L. (1977) Properties of a CH_3-blocked creatine kinase with altered catalytic activity. Kinetic consequences of the presence of the blocking group, *J. Biol. Chem. 252*, 1202−1207.
34. Fersht, A. R. (1998) *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*, W. H. Freeman and Co., New York.
35. Uda, K., and Suzuki, T. (2004) Role of amino acid residues on the GS region of *Stichopus* arginine kinase and *Danio* creatine kinase, *Protein J. 23*, 53−64.
36. Azzi, A., Clark, S. A., Ellington, W. R., and Chapman, M. S. (2004) The role of phosphagen specificity loops in arginine kinase, *Protein Sci. 13*, 575−585.
37. Venekei, I., Szilagyi, L., Graf, L., and Rutter, W. J. (1996) Attempts to convert chymotrypsin to trypsin, *FEBS Lett. 383*, 143−147.

38. Cook, P. F., Kenyon, G. L., and Cleland, W. W. (1981) Use of pH studies to elucidate the catalytic mechanism of rabbit muscle creatine kinase, *Biochemistry 20*, 1204−1210.
39. Eder, M., Stolz, M., Wallimann, T., and Schlattner, U. (2000) A conserved negatively charged cluster in the active site of creatine kinase is critical for enzymatic activity, *J. Biol. Chem. 275*, 27094−27099.
40. Suzuki, T., Kawasaki, Y., and Furukohri, T. (1997) Evolution of phosphagen kinase. Isolation, characterization and cDNA-derived amino acid sequence of two-domain arginine kinase from the sea anemone *Anthopleura japonicus*, *Biochem. J. 328* (Part 1), 301−306.
41. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res. 22*, 4673−4680.
42. Jewett, A. I., Huang, C. C., and Ferrin, T. E. (2003) MINRMS: an efficient algorithm for determining protein structure similarity using root-mean-squared-distance, *Bioinformatics 19*, 625−634.

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