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Relating Structure to Mechanism in Creatine Kinase

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ABSTRACT Found in all vertebrates, creatine kinase catalyzes the reversible reaction of creatine and ATP forming phosphocreatine and ADP. Phosphocreatine may be viewed as a reservoir of "high-energy phosphate" which is able to supply ATP, the primary energy source in bioenergetics, on demand. Consequently, creatine kinase plays a significant role in energy homeostasis of cells with intermittently high energy requirements. The enzyme is of clinical importance and its levels are routinely used as an indicator of myocardial and skeletal muscle disorders and for the diagnosis of acute myocardial infarction. First identified in 1928, the enzyme has undergone intensive investigation for over 75 years. There are four major isozymes, two cytosolic and two mitochondrial, which form dimers and octamers, respectively. Depending on the pH, the enzyme operates by a random or an ordered bimolecular mechanism, with the equilibrium lying towards phosphocreatine production. Evidence suggests that conversion of creatine to phosphocreatine occurs via the in-line transfer of a phosphoryl group from ATP. A recent X-ray structure of creatine kinase bound to a transition state analog complex confirmed many of the predictions based on kinetic, spectroscopic, and mutagenesis studies. This review summarizes and correlates the more significant mechanistic and structural studies on creatine kinase.

KEYWORDS energy homeostasis, guanidino kinase, myocardial infarction, phosphagen kinase, phosphoryl group transfer, transition-state analogue complex, X-ray structure

INTRODUCTION

Creatine kinase (CK; adenosine-5′-triphosphate:creatine phosphotransferase; creatine phosphokinase; phosphocreatine phosphokinase; creatine *N*-phosphotransferase; EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine (Cr), producing phosphocreatine (PCr) and MgADP (Figure 1).

Phosphocreatine was initially identified in muscle tissue (Eggleton & Eggleton, 1928). At that time, it was thought to be the chemical source for the energy required for muscle contraction. However, not long after, the enzyme now known as creatine kinase was first identified (Lohman, 1934), and it was subsequently shown that ATP was formed by transfer of a phosphoryl

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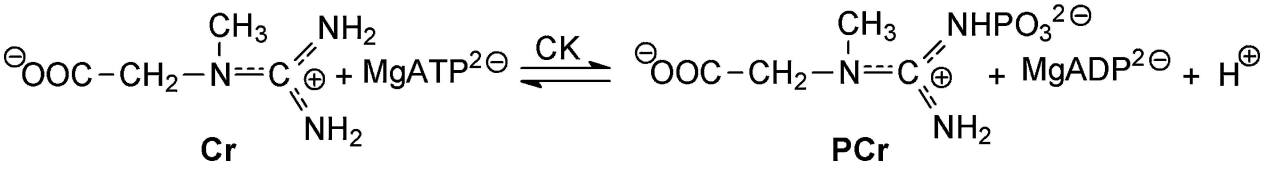

FIGURE 1 **Reaction catalyzed by creatine kinase.**

group from PCr (Lehmann, 1936). ATP, of course, is now known as the mediator of all energy changes within the cell.

There are four major CK isozymes, which have been named for the tissues from which they were historically isolated. The isozymes have been characterized on the basis of differences in gene and amino acid sequence, tissue localization and immunogenicity. There are two cytosolic forms, the muscle (MM-CK) and brain (BB-CK) forms, which exist as dimers under physiological conditions. Under some circumstances, cytosolic CK can exist as the MB heterodimer (Eppenberger *et al.*, 1967). Immunological studies show that antisera raised against MM-CK will not cross react with BB-CK, but there is cross reactivity with the same isozyme across several species (Chen *et al.*, 2000). There are also two mitochondrial forms of the enzyme, the ubiquitous (Mi_u-CK) and the sarcomeric (Mi_s-CK) forms which, based on their isoelectric point, are sometimes referred to as acidic (Mi_a-CK) and basic (Mi_b-CK) mitochondrial CK, respectively (Wyss *et al.*, 1992). The mitochondrial isoforms (MtCK) generally exist as octamers but can be readily dissociated into dimers (Wyss *et al.*, 1992).

For many years only the soluble form of creatine kinase was known, and the main physiological role ascribed to CK was the maintenance of energy homeostasis at sites of high energy turnover such as rapidly contracting skeletal muscle. The high levels of CK ensured that ADP and ATP levels remained almost constant, effectively buffering the cell against rapid depletion of ATP. The discovery of the mitochondrial isozymes showed that CK was located in individual "compartments" and the concept of a creatine-phosphocreatine shuttle was developed. Here distinct isozymes are associated with sites of ATP production and consumption, and they fulfil a role of a transport mechanism for high energy phosphates. Further discussion on the physiological role of CK, as well as the Cr-PCr shuttle, may be found in a special edition of *Molecular and Cellular Biochemistry* (Saks & Ventura-Clapier, 1994) as well as in the review by Wallimann *et al.* (1992).

Sequence Homology and Evolution

Creatine kinase is a member of the phosphagen (guanidino) kinase family. This family of enzymes is highly conserved and is found throughout the animal kingdom. Other members of the family include arginine kinase (AK), glycocyamine kinase (GK), taurocyamine kinase (TK), and lombricine kinase (LK). The structures of the various naturally occurring guanidino acceptors are shown in Figure 2. Creatine kinase is the only phosphagen kinase found in vertebrates, but it is also found in many invertebrates, including sponges, polychaetes, and echinoderms (Robin, 1964; Watts, 1968, 1971, 1975; Ellington, 2001). The phosphagen kinases are distributed along distinct phylogenetic and, sometimes, tissue-specific lines (Ellington, 2001). It has long been thought that AK is the most primitive phosphagen kinase and that the other members of the family arose by gene duplication followed by divergent evolution (Watts, 1971, 1975; Suzuki *et al.*, 1998). The fact that creatine kinase is present in sponges, the oldest of all multi-cellular animals, also suggests that the divergence of creatine kinase from an arginine kinase-like ancestral protein occurred very early (Sona *et al.*, 2004).

Four independent nuclear genes have been found to encode the individual isozymes of CK (Mühlebach *et al.*, 1994). Detailed analyses of the properties of these genes, including details of structure and regulation, can be found in reviews by Qin *et al.* (1998) and Suzuki *et al.* (2004). The full-length sequence of a mitochondrial isozyme is about 35 residues longer than that of its cytosolic counterpart. The additional residues belong to a leader peptide which is removed proteolytically, either during or after translocation across the mitochondrial membrane (Pfanner & Geissler, 2001). The mature gene products are between 40 and 44 kDa and, within each class of isozyme, amino acid sequence identities range from 85% to more than 99% (Mühlebach *et al.*, 1994; Qin *et al.*, 1998). The two cytosolic isozymes exhibit ca. 80% sequence identity, as do the two mitochondrial isozymes. However, the cytosolic and mitochondrial isozymes share only 60% to 65% sequence identity

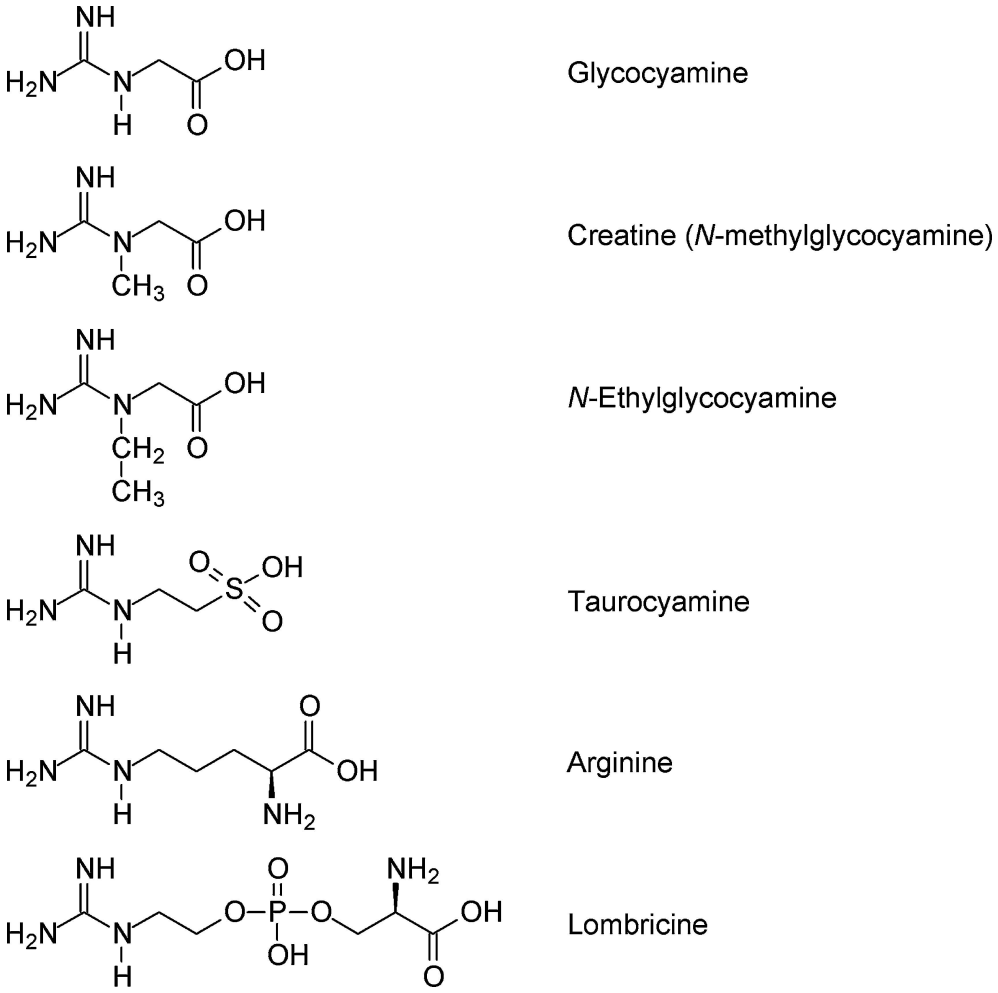

FIGURE 2 **Structures of naturally occurring substrates for phosphagen kinases.**

(Mühlebach *et al.*, 1994). Overall the sequence data confirm that the CKs are an evolutionarily conserved group of enzymes and suggest that the separation of the mitochondrial and cytosolic forms was due to a gene duplication event which occurred early in phylogeny. This, in turn, was followed by a second duplication event that gave rise to the two mitochondrial and two cytosolic isozymes (Mühlebach *et al.*, 1996; Qin *et al.*, 1998; Pineda & Ellington, 1999).

The amino acid sequences of the CK isozymes show six regions of extensive homology which are flanked by seven more variable regions (Mühlebach *et al.*, 1994). Consequently, there have been several studies aimed at linking regions of conserved sequence with function. For example, it was thought that the residues responsible for the membrane binding of mitochondrial CK were probably located in the C-terminal region of the protein (Fritz-Wolf *et al.*, 1996; Kabsch & Fritz-Wolf, 1997), a proposal that has been recently confirmed by Schlattner *et al.* (2004). The residues likely to be important for octamer formation, on the other hand, are located in the N-terminal region (Kaldis *et al.*, 1993, 1994). Of the two cytosolic isozymes it has been shown that only the muscle isoform interacts with the sarcomeric M-line. The interaction has been traced to two lysine pairs, which are highly conserved in MM-CK but which are not present in BB-CK (Hornemann *et al.*, 2000b). From a mechanistic standpoint, there are two significant regions of highly conserved sequence. These are the negatively charged NEED-box (Eder *et al.*, 2000b; Cantwell *et al.*, 2001) and the region surrounding a cysteine residue, which is highly susceptible to chemical modification (Kenyon & Reed, 1983; Furter *et al.*, 1993). Both of these regions are conserved across all phosphagen kinases and play a major role in the catalytic mechanism (*vide infra*).

In addition to the cytosolic and mitochondrial isozymes, another gene coding for a unique flagellar isoform (fCK), consisting of three fused creatine kinase domains, is found in many protostome and deuterostome

invertebrates and in a protochordate (Suzuki *et al.*, 2004). It is thought that the gene arose as a result of a duplication/fusion process followed by an unequal crossing over resulting in gene triplication (Wothe *et al.*, 1990). Phylogenetic analysis suggests that the flagellar isozyme is more closely linked to the cytosolic forms, but that the data are somewhat equivocal (Suzuki *et al.*, 2004). Given that no fCK has yet been characterized, it is included in this discussion only for the sake of completeness, and will not be referred to again.

PHYSICAL AND CATALYTIC PROPERTIES

The earliest detailed review of the creatine kinase literature was that of Kuby and Noltmann (1962). Ten years later, the review of Watts (1973) appeared, to be followed another decade later by that of Kenyon and Reed (1983). While these three reviews have summarized the majority of the early studies on CK, many of the salient points, and some of the more recent work, are described below.

Purification, Assay and Physical Properties

The first purification of CK (Kuby *et al.*, 1954) as well as the majority of the early studies of the physical properties and structure of creatine kinase were carried out on the enzyme isolated from rabbit muscle (RMCK). In the presence of divalent cations, at high ionic strength and low temperature, the enzyme could be separated from other proteins by fractionation with ethanol (Kuby *et al.*, 1954). This became the most commonly used isolation method, although development of affinity chromatography using Blue Sepharose followed by ion-exchange has removed the necessity for ethanol precipitation (Chen *et al.*, 2000).

As creatine kinase is of major clinical interest, particularly as a marker for myocardial infarction, there have been many methods developed for the assay of CK activity. The rate of creatine formation could be followed by reaction with an α-naphthol-diacetyl reagent (Eggleton *et al.*, 1943), whereas that of phosphocreatine formation was followed colorimetrically as the acid-molybdate-labile phosphate (Kuby *et al.*, 1954). These stop procedures were later replaced by continuous spectrophotometric methods using coupled enzyme systems. Reaction in the forward direction (production of phosphocreatine) may be followed at 340 nm by coupling to pyruvate kinase and lactate dehydrogenase as described by Tanzer and Gilvarg (1959). Conversely, ATP production (the reverse reaction) may also be followed at 340 nm by coupling to hexokinase and glucose-6-phosphate dehydrogenase as initially described by Oliver (1955) and later by Rosalki (1967). Another routinely used continuous assay, particularly for the forward reaction, is the pH-stat assay of Mahowald *et al.* (1962), which takes advantage of the fact that a proton is generated when phosphocreatine is produced (Figure 1). One advantage of the latter method is that it shows less interference from other enzymatic activities and, consequently, can be used with crude enzyme preparations. However, if this assay is used for the reverse reaction it should be noted that, at pH values below 6.0, PCr transphosphorylation and H^+ consumption are no longer equimolar and pH-stat values must be corrected accordingly (Furter *et al.*, 1993).

Early sedimentation velocity studies suggested a molecular weight of 81,000, and that the molecule behaved as an unhydrated ellipsoid (Kuby & Noltmann, 1962). Later it was shown that creatine kinase consisted of two readily dissociable subunits, with no disulfide bridges, and a molecular weight of 82,600 Da. For other species, the molecular weight varied between 78,500 and 85,100 Da. Further sedimentation studies showed that denaturation with guanidinium chloride first dissociated and then unfolded the subunits into random coil configuration, whereas sodium dodecyl sulfate caused them to dissociate without any apparent loss of structural organization. Overall it was concluded that the enzyme comprised two cigar-shaped subunits lying side by side (Watts, 1973).

Although the cytosolic isozymes are found as dimers, their mitochondrial counterparts are generally octameric. However, when placed in a 'transition-state analog complex' (TSAC) mixture comprised of creatine, MgADP and planar anions such as nitrate, nitrite and formate (Milner-White & Watts, 1971), octameric Mi_b-CK dissociates into dimers (Gross & Wallimann, 1993). Consequently it appeared that the minimal catalytically active form of CK was the dimer, but this remained the subject of some conjecture.

Another of the continuing debates relates to the role of the individual subunits in catalysis. The early studies on the CK subunits, reviewed by Bickerstaff and Price (1978), focused on the behavior of the isolated

subunits. They established that there was a single active site per subunit, that the active sites were well separated, and further, that there was some indication that the monomeric form of the enzyme was active. Several studies examined the possible non-identical behavior of the subunits with evidence being provided to show that CK possesses either negative cooperativity or non-identical active sites (Price & Hunter, 1976; Bickerstaff & Price, 1978). Nevinsky *et al.* (1982), for example, showed that the subunits of rabbit muscle CK were functionally non-identical, whereas Degani and Degani (1979) suggested that the subunits were arranged asymmetrically and that a dimer was required for activity. More recent studies have demonstrated that the subunits act either independently (Wang *et al.*, 1990) or independently in the forward direction and cooperatively in the reverse reaction (Hornemann *et al.*, 2000a). In addition, a kinetic analysis has indicated that the two subunits may adopt different tertiary structures and behave as distinct entities (Wang & Pan, 1996).

There was an expectation that this question would be conclusively answered when the X-ray structure of substrate-bound creatine kinase became available. At first glance it was, with the crystallographic asymmetric unit of *Torpedo californica* CK (TcCK) found to contain two monomers that were not identical in either conformation or ligand binding state (Lahiri *et al.*, 2002). This enzyme was crystallized in the presence of a TSAC mixture comprised of MgADP, creatine and nitrate at concentrations well above the K_i of the TSAC. Each biological dimer was found to contain one monomer bound to MgADP and a second monomer bound to the TSAC (Lahiri *et al.*, 2002). Certainly this was suggestive of negative cooperativity yet, almost immediately, a mass spectrometric hydrogen/deuterium exchange experiment demonstrated conclusively that, in solution, both subunits of a dimer can bind substrates (Mazon *et al.*, 2003).

What has become clear is that an individual subunit of CK can catalyze the transphosphorylation reaction. This results from a study by Cox *et al.* (2003) in which site-directed mutagenesis was employed to remove several interactions at the dimer interface of RMCK. Several mutants were expressed in a soluble form and were purified by affinity chromatography. Size-exclusion chromatography and analytical centrifugation indicated that, at 1 mg/mL, two of them were monomeric. Kinetic analysis demonstrated that both these mutants were active for reaction in the direction of phosphocreatine synthesis, but the K_d values for both creatine and MgATP were about an order of magnitude higher than those of the WT enzyme. In addition, the data for the one of the mutants could only be fitted to the equation for an ordered mechanism wherein creatine binds first (Cox *et al.*, 2003). These experiments provide an unequivocal demonstration that, like the lobster arginine kinase, which catalyzes an analogous phosphorylation reaction in invertebrates (Morrison, 1973), creatine kinase can be active as a monomer.

Substrate Specificity

Creatine kinase possesses a very narrow substrate specificity. Of the naturally occurring substrates for guanidino kinases (Figure 2), only creatine (N-methylglycocyamine), glycocyamine (Tanzer & Gilvarg, 1959) and N-ethylglycocyamine (Ennor *et al.*, 1955) were found to be substrates. The list of substrates was later expanded (Figure 3) to include compounds such as cyclocreatine (1-carboxymethyl-2-iminioimidazolidine) and (*R*)-*N*-methyl-*N*-amidino-β-alanine (Rowley *et al.*, 1971). Cyclocreatine, in particular, proved to be useful in showing that creatine is phosphorylated on the nitrogen *trans* to the methyl group (Rowley *et al.*, 1971; Struve *et al.*, 1977), while other alternative substrates were useful in showing that the active site can tolerate only limited steric bulk, and that the planarity and orientation of the guanidino group is important for catalysis (McLaughlin *et al.*, 1972). More recently, Boehm *et al.* (1996) established that, when a porcine carotid artery, which contains a high percentage of cytosolic CK, was perfused with cyclocreatine, guanidinoacetic acid (glycocyamine), β-guanidinopropinic acid (Figure 3) or *N*-methylguanidinopropionic acid (Figure 3), the phosphorylated analogue was accumulated in all cases. Furthermore, all the analog could be dephosphorylated, albeit relatively slowly in the case of phosphocyclocreatine. Overall the results suggested that a poor *in vitro* substrate for cytosolic CK could be utilized effectively *in vivo* (Boehm *et al.*, 1996). Conversely, they found that the mitochondrial CK in intact mitochondria could only phosphorylate creatine and cyclocreatine, suggesting that MtCK may have a greater specificity than its cytosolic counterpart (Boehm *et al.*, 1996). Although the nucleotide triphosphate and nucleotide diphosphate are essential for the forward and reverse reactions, respectively, James and Morrison (1966) showed that ADP

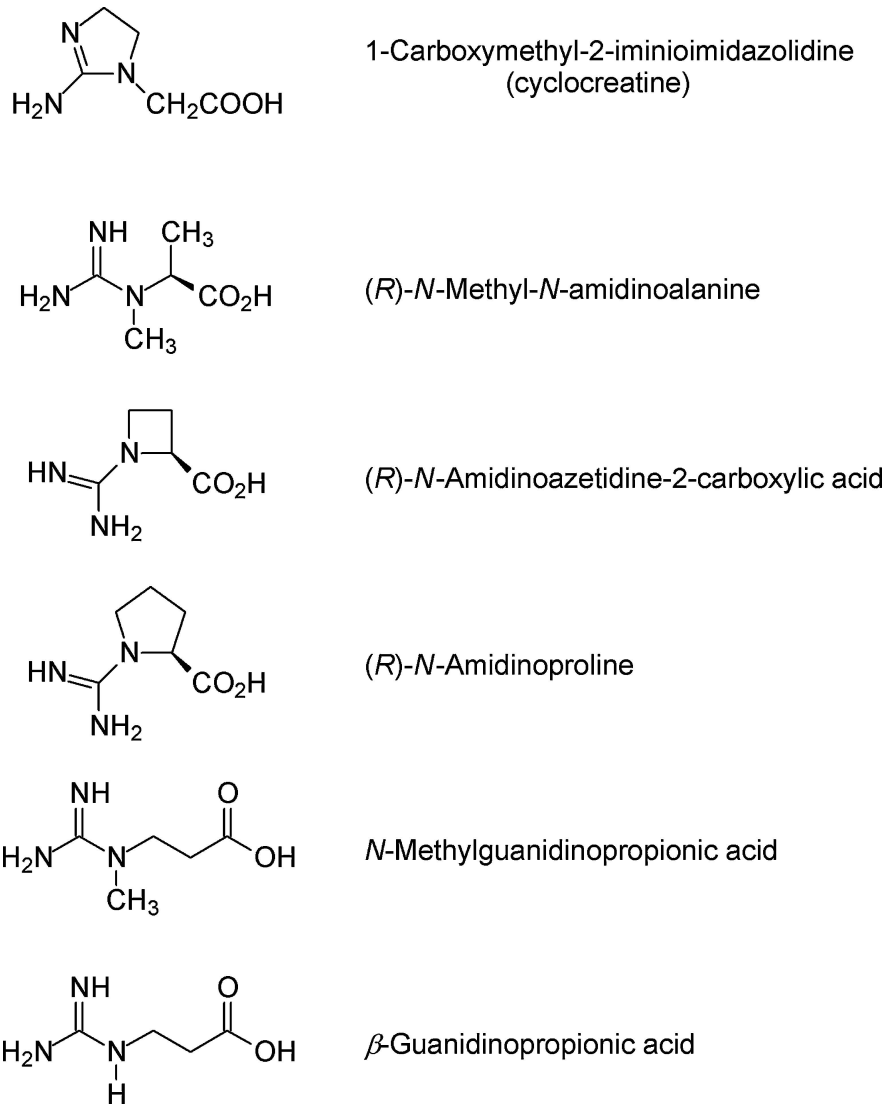

FIGURE 3 **Structures of alternative substrates for phosphagen kinases.**

could be effectively replaced by dADP. In fact, the rabbit muscle isozyme showed a broad specificity for the base (James & Morrison, 1966) that was not reflected in BB-CK and MM-CK from other species (Watts, 1973). It is also noteworthy that, in addition to Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} have been used as activators whereas Ni^{2+}, Cr^{2+}, and Cd^{2+} are either inactive or inhibitory (O'Sullivan & Morrison, 1963). Optimal activity was obtained when Mg^{2+}, provided as the acetate, was added in 1 mM excess over the nucleotide concentration (Cleland, 1967), suggesting that MgATP and MgADP were the true substrates for the reaction.

Kinetic Analyses

The initial kinetic studies undertaken on the rabbit muscle isozyme are summarized in Figure 4. The overall reaction is reversible and, at pH 8.0 and above, the enzyme operates by a rapid equilibrium random bimolecular, bimolecular mechanism (Figure 4A). No evidence has been obtained for any phosphorylated enzyme intermediate, and phosphoryl transfer was found to be the rate-determining step (Morrison & James, 1965; Morrison & Cleland, 1966; Morrison & White, 1967). At pH 7.0 and below, the situation is more complex (Schimerlik & Cleland, 1973). In the forward (creatine phosphorylation) direction, RMCK operates by an equilibrium ordered mechanism with ATP binding before creatine (Figure 4B), while in the reverse direction the reaction remains random (Schimerlik & Cleland, 1973).

Although there have been numerous measurements of kinetic constants for the soluble isozymes, data for the mitochondrial isozymes, particularly the ubiquitous

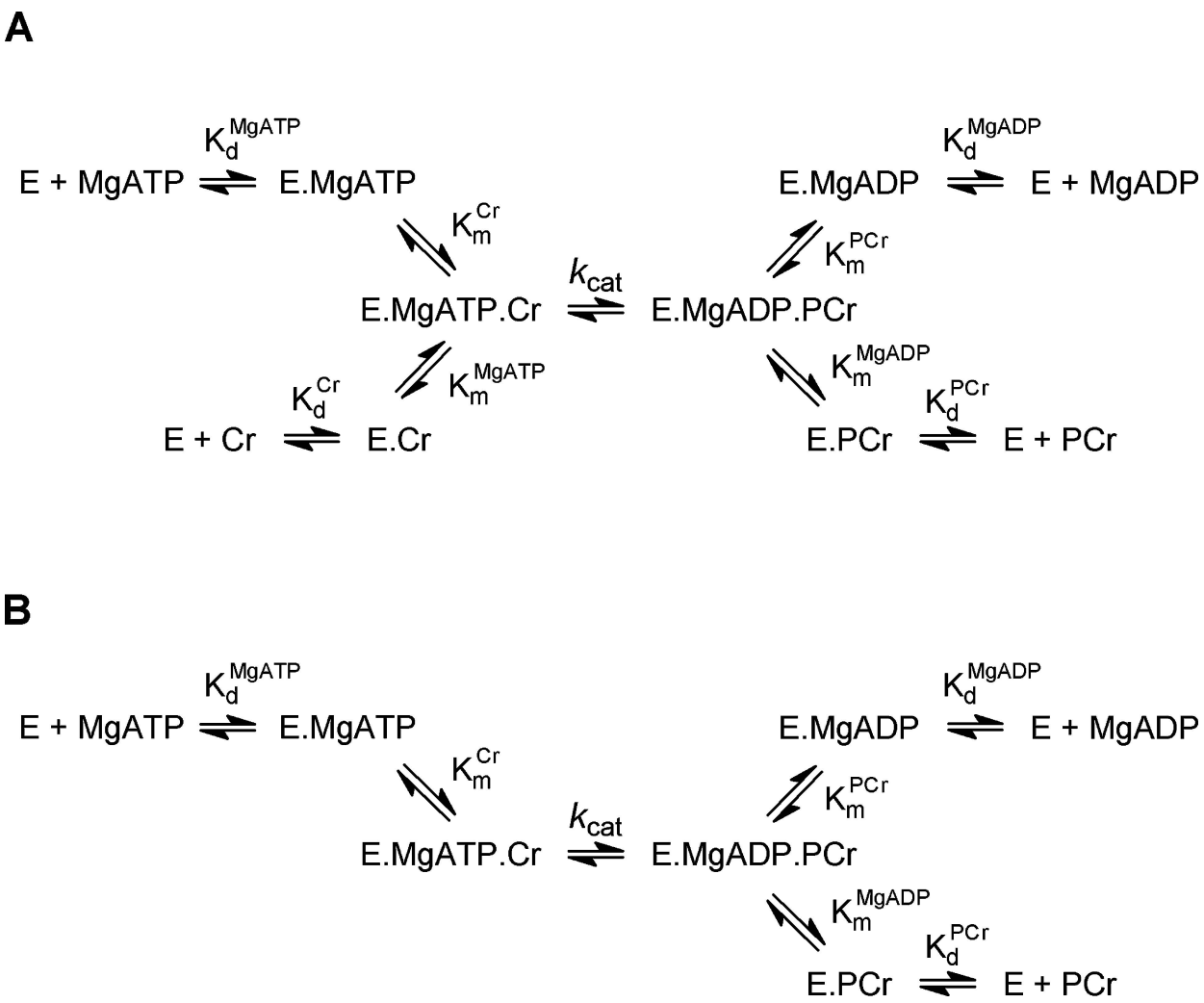

FIGURE 4 **Kinetic mechanism of rabbit muscle CK at (A) pH 8.0 and above (Morrison & James, 1965) and (B) pH 7.0 and below (Schimerlik & Cleland, 1973).**

isozyme, are scarce (Schlattner *et al.*, 2000). Specific activities for the muscle isozymes were similar across a number of mammalian species and not dissimilar to their brain counterparts (Watts, 1973). Tables 1 and 2 provide a comparison of the kinetic data for all four human isozymes in both the forward (phosphocreatine production) and reverse (ATP production) directions. The mitochondrial isozymes (MtCK) are generally 3 to 4 times slower than their cytosolic counterparts, and the reverse reaction is always faster than the forward reaction, with values of k_{cat}(for)/k_{cat}(rev) ranging from 0.32 (HMCK) to 0.85 (sMtCK). With the exception

TABLE 1 **Kinetic constants for reaction in the forward direction (*i.e.*, creatine phosphorylation).**

	HMCK[a]	HBCK[a]	uMtCK[b]	sMtCK[b]
k_{cat} (min^{-1})	9.21×10^3	12.9×10^3	3.1×10^3	4.5×10^3
K_d Cr (mM)	14.6 ± 1.2	6.0 ± 0.3	45.8 ± 11.2	43.9 ± 14.9
K_m Cr (mM)	9.5 ± 0.59	4.9 ± 0.4	1.01 ± 0.13	7.31 ± 1.27
k_{cat} /K_m Cr (mM/min^{-1})	9.7×10^2	2.6×10^3	3.1×10^3	6.2×10^2
K_d MgATP (mM)	1.2 ± 0.11	0.99 ± 0.05	4.04 ± 1.33	4.06 ± 0.78
K_m MgATP (mM)	0.89 ± 0.16	0.81 ± 0.10	0.11 ± 0.02	0.68 ± 0.21
k_{cat} /K_m MgATP (mM/min^{-1})	1.0×10^4	1.6×10^4	2.8×10^4	6.6×10^3

[a]From (Chen *et al.*, 2000). Reaction was at pH 9.0.
[b]From (Schlattner *et al.*, 2000). Reaction was at pH 8.0.

TABLE 2 **Kinetic constants for reaction at pH 7.0 in the reverse reaction (*i.e.*, ATP production).**

	HMCK[a,b]	HBCK[b]	uMtCK[c]	sMtCK[c]
k_{cat} (min^{-1})	2.9×10^4	2.1×10^4	4.7×10^3	5.4×10^3
K_d PCr (mM)	3.7 ± 1.0	0.22 ± 0.05	0.92 ± 0.13	2.87 ± 0.68
K_m PCr (mM)	1.33 ± 0.14	0.51 ± 0.06	0.55 ± 0.03	1.16 ± 0.14
k_{cat}/K_m PCr (mM/min^{-1})	2.2×10^4	4.1×10^4	8.6×10^3	4.7×10^3
K_d MgADP (mM)	0.07 ± 0.013	0.02 ± 0.002	0.22 ± 0.03	0.38 ± 0.09
K_m MgADP (mM)	0.03 ± 0.005	0.04 ± 0.002	0.13 ± 0.01	0.15 ± 0.02
k_{cat}/K_m MgADP (mM/min^{-1})	1.1×10^6	5.3×10^5	3.6×10^4	3.6×10^4

[a]From (Wang *et al.*, 2001).
[b]Wang, McLeish, Kenyon (unpublished results). Results are reported as ± S.E.M.
[c]From (Schlattner *et al.*, 2000).

of the HBCK in the reverse reaction, K_m values were significantly lower than K_d values, where K_m and K_d are the dissociation constants from the ternary and the binary complexes, respectively (Figure 4). A ratio of K_m/K_d (α-value) of less than one is indicative of substrate synergy wherein the binding of one substrate increases the affinity for the second substrate (Segel, 1975). At this time it is unclear why the reverse reaction with HBCK has an α-value of greater than unity but the degree of synergism in creatine kinases has been known to vary with reaction conditions (Morrison & James, 1965; Maggio *et al.*, 1977) and can also be affected by mutagenesis (Novak *et al.*, 2004). Overall the α-values were reasonably similar, the major exception being for uMtCK in the forward reaction where its α-value was almost an order of magnitude lower than those of the other isozymes, a discrepancy that was also manifest in the K_m values for both creatine and ATP. The value of k_{cat}/K_m is often used as a measure of the specificity of an enzyme for its substrates (Copeland, 2000). Tables 1 and 2 show that the sMtCK has the lowest specificity for any of its substrates and that the cytosolic isozymes are generally more efficient than the mitochondrial isozymes. This variation is more pronounced in the reverse reaction, where there is a ten-fold difference between the cytosolic and mitochondrial enzymes. At this point it is unclear whether the catalytic differences between the various isozymes have any important physiological significance.

In addition to the Michaelis-Menten kinetics there have been several determinations of the equilibrium constant for the reaction. These show that the equilibrium lies to the right (*i.e.*, favors PCr formation) and that the equilibrium constant greatly depends on both the concentration of free magnesium ions and the pH (Kuby & Noltmann, 1962; Watts, 1973; Lawson & Veech, 1979; Lerman & Cohn, 1980; Huddleston *et al.*, 1994).

Both sulfate and phosphate anions have been found to inhibit CK, competitively with respect to ATP and phosphocreatine and noncompetitively with respect to ADP and creatine. Based on these results, it was thought that small anions would occupy the same site on the enzyme as the transferring phosphoryl group, and their effectiveness as an inhibitor would be based on the extent to which they mimicked the phosphate group (Nihei *et al.*, 1961; Kumudavalli *et al.*, 1970). However, kinetic analyses showed that anions affected the initial velocities and the shape of the progress curves in a manner incompatible with that proposal (Milner-White & Watts, 1971). In addition there were reports that an equilibrium mixture of substrates protected the enzyme against alkylation by iodoacetamide (Watts & Rabin, 1962) and that a 'dead-end complex' of creatine-MgADP afforded even greater protection (O'Sullivan *et al.*, 1966). In a study that was alluded to briefly earlier, Milner-White and Watts (1971) showed that an anion was involved in addition to the substrates, that the level of substrate protection depended on the type of anion present, and that a planar anion or a halide was required for maximum inhibition. They noted that the CK reaction potentially involves a direct transfer of a phosphoryl group by an S_N2 type reaction, with the phosphoryl group forming an sp^3d hybrid in the transition state. On that basis they proposed that planar anions, such as nitrite and formate, mimicked the phosphoryl group in the transition state of the reaction and suggested that a quaternary complex (E–TSAC) comprising enzyme–creatine–anion–MgADP was formed (Milner-White & Watts, 1971). This study and its predictions, their accuracy confirmed by later spectroscopic (Reed & Cohn, 1972; Reed & Leyh, 1980) and X-ray (Lahiri *et al.*,

2002) experiments, not only addressed and explained the anomalous kinetic results, but also provided an experimental tool that would be used in a variety of subsequent investigations. It should also be noted that, from an entropic standpoint, it is much easier for the enzyme to bind its two substrates than to assemble the three components of the TSAC. Recently, Borders *et al.* (2002) used intrinsic fluorescence measurements to determine a dissociation constant of 4×10^{-10} M^3 for the TSAC into its individual components. This was possibly the first report of the dissociation constant for a ternary, let alone a quaternary, transition state analog complex.

THREE DIMENSIONAL (X-RAY) STRUCTURE

Almost since it was first isolated, attempts have been made to crystallize creatine kinase (Kuby *et al.*, 1954). Both the cytosolic (Keutel *et al.*, 1972; McPherson, 1973; Burgess *et al.*, 1978; Takasawa *et al.*, 1981; Gilliland *et al.*, 1983; Hershenson *et al.*, 1986) and mitochondrial (Schnyder *et al.*, 1990, 1991) isozymes have proved to be amenable to crystallization. However, although preliminary X-ray measurements on CK had been carried out by 1973 (McPherson, 1973), it was another two decades before the first X-ray structure of creatine kinase was published.

The Structure of Sarcomeric Mitochondrial Creatine Kinase (Mi_b-CK)

In 1996, Fritz-Wolf *et al.* reported the structure of the sarcomeric chicken cardiac Mi_b-CK, in the presence and absence of NaATP, at 3 Å resolution (Fritz-Wolf *et al.*, 1996). There were four crystallographically independent monomers with virtually identical structures. Each monomer consisted of a small (residues 1 to 112) N-terminal domain and a larger (residues 113 to 380) C-terminal domain, with the ATP binding site being located in a cleft between the two domains (Figure 5). In addition to the five N-terminal residues, two loops, comprising residues 60 to 65 and 316 to 326, were shown to be highly flexible. Small-angle X-ray scattering experiments (Forstner *et al.*, 1996) had demonstrated that Mi_b-CK exhibits a considerable decrease in the radius of gyration in the presence of MgATP or a TSAC.

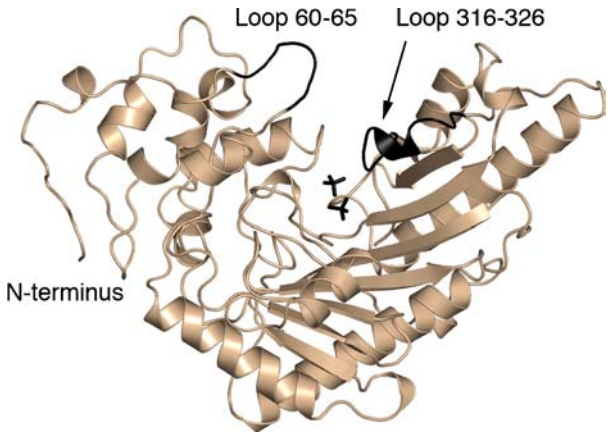

FIGURE 5 **Ribbon diagram of sarcomeric chicken creatine kinase (Mi_b-CK) monomer. The two flexible loops are highlighted. The ATP binding site is identified by the two phosphates in the cleft between the N-and C-terminal domains. Coordinates used were those from PDB 1CRK (Fritz-Wolf *et al.*, 1996).**

Conversely neither ATP nor ADP alone had any effect on the scattering curves. Given the position of the flexible loops it was postulated that, when creatine and a magnesium ion were bound, these loops would move to close over the active site and exclude water during catalysis (Fritz-Wolf *et al.*, 1996; Kabsch & Fritz-Wolf, 1997).

As expected, the six conserved regions of high sequence homology (Mühlebach *et al.*, 1994) were found to form the core of the structure, covering the active site of the enzyme. The active site itself was found to contain many of the residues implicated by earlier studies as being either located in the active site or important catalytically. Nuclear magnetic resonance (NMR) studies, for example, had shown that four histidine residues were located within 18 Å of the Cr^{3+} of a Cr^{3+}-ATP complex bound to the enzyme (Rosevear *et al.*, 1981). One of these histidines was also postulated to have a $pK_a \sim 7$, and to act as an acid/base catalyst (Cook *et al.*, 1981). However, none of the histidines was within 12 Å of the γ-phosphate of ATP and, intriguingly, mutagenesis of each of these histidine residues showed that none were essential for catalysis (Chen *et al.*, 1996; Forstner *et al.*, 1997). The pH studies which implicated the histidine residue also indicated that an ionized carboxylic acid group was required for the binding of both creatine and phosphocreatine (Cook *et al.*, 1981). The X-ray structure of Mi_b-CK showed that two glutamic acid residues, Glu226 and Glu227, as well as an aspartic acid residue, Asp228, were located in the active site, any of which could fulfil this role. Note that, unless stated

otherwise, the numbering of residues is that of the CK isozyme under immediate discussion.

Chemical modification studies had shown that rabbit muscle CK was fully inactivated by the arginine specific reagent, phenylglyoxal (Borders & Riordan, 1975), whereas NMR had shown that an arginine residue was located near the ATP binding site (James, 1976). Consequently it was not surprising when the X-ray structure revealed that the phosphate group of ATP interacted with four arginine residues and that two more arginine residues are within 5 Å of the γ-phosphate. Also, not unexpectedly, a tryptophan residue was found in the active site. This residue, Trp223, was deduced by earlier NMR and fluorescence measurements (Vasák *et al.*, 1979; Messmer & Kagi, 1985), as well as by mutagenesis studies (Gross *et al.*, 1994), as being important for catalysis.

Over the years, there have been numerous studies implicating a cysteine residue in the CK active site. In both the cytosolic (Mahowald *et al.*, 1962) and mitochondrial (Fedosov & Belousova, 1988; Wyss *et al.*, 1993) isoforms, one highly reactive cysteine residue per monomer is able to react with sulfhydryl-specific reagents. Marletta and Kenyon (1979) used the creatine-based affinity label, epoxycreatine, to completely inactivate rabbit muscle CK, thereby confirming that the reactive group, possibly a cysteine or a carboxylate, must be located adjacent to the creatine binding site. Subsequently, tryptic digestion combined with mass spectrometry was used to show that it was a cysteine residue, identified as Cys282, that was labeled (Buechter *et al.*, 1992). This was one of the two fully conserved cysteine residues in the CKs, and the only cysteine residue to be conserved across all guanidino kinases. The X-ray structure of Mi_b-CK showed that Cys278 (analogous to Cys282 in RMCK) was indeed located within the active site and was positioned near the γ-phosphate of ATP and near the acidic residues thought to be involved in creatine binding.

Although the well-conserved residues formed a compact core containing the active site, it was possible also to identify residues in the less-conserved regions of sequence that could be linked to isoform specific properties. For example, each monomer was shown to contain four contact regions, two of which could be linked to octamer formation, and three to dimer formation. A detailed analysis of the structure and its relationship to the cellular functions of creatine kinase may be found in a review by Schlattner *et al.* (1998).

Comparison of Structures from All Four Isozymes

Following the publication of the Mi_b-CK structure (Fritz-Wolf *et al.*, 1996) the X-ray structures of the rabbit muscle isozyme (Rao *et al.*, 1998), the cytosolic chicken brain (BB-CK) isozyme (Eder *et al.*, 1999), the human ubiquitous mitochondrial (Mi_a-CK) isozyme (Eder *et al.*, 2000a), the bovine muscle (MM-CK) isozyme (Tisi *et al.*, 2001), and the human muscle (MM-CK) isozyme (Shen *et al.*, 2001) followed in rapid succession. Not surprisingly, given the level of sequence identity, the overall structures were all very similar, particularly in the region of the active site. Nonetheless, comparison of these structures was able to provide a rationale for some of the functional differences observed among the various CK isozymes.

Amino acid sequence alignments had suggested that isozyme-specific sequences were localized at the N- and C-termini, the linker region between two domains, and in parts of three helices. The structural comparisons showed that, with the exception of the N-terminal region, the three dimensional structure was conserved in the regions of divergent sequence although there were changes in the electrostatic surface of the protein. Before any CK structures were available, it had been predicted that the residues likely to be important for octamer formation would be located in the N-terminal region (Kaldis *et al.*, 1993, 1994). Comparison of the X-ray structures of the two cytosolic BB-CK isoforms (Eder *et al.*, 1999; Tisi *et al.*, 2001) showed that the N-terminal region was important for dimer formation. It was also found to be important for octamer formation in the mitochondrial isoforms (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 2000a) but, unfortunately, the seven N-terminal amino acids are disordered in the structures of the MM-CK isoforms (Rao *et al.*, 1998; Shen *et al.*, 2001). However, there are still significant differences between the human muscle isoform residues 8 to 15 and residues 1 to 10 of the human mitochondrial isoforms which are presumably related to octamer formation (Shen *et al.*, 2001). It is also interesting to note that the contact surface area between monomers in a chicken BB-CK dimer is almost 50% greater than the contact area between two MtCK monomers, presumably reflecting the greater stability of the dimer in the cytosolic isoforms (Eder *et al.*, 1999, 2000a). In addition, there are differences in the stability of the octamers that can also be related to contact surface areas, this time

of the dimer-dimer interface. For example, the human Mi_a-CK isoform forms a more stable octamer than the Mi_b-CK and has more than twice the contact surface between its dimers (Eder *et al.*, 2000a). It must also be noted that, although these N-terminal interactions are important for monomer-monomer and dimer-dimer interactions, other regions of the structure are of equal or greater import (Fritz-Wolf *et al.*, 1996). Finally, the C-terminal region of the mitochondrial isozymes contains 3 to 4 additional residues, two of which are positively charged. At present it is unclear whether the CK octamer binds only to the membrane surface (Cheneval *et al.*, 1989) or enters the lipid bilayer (Rojo *et al.*, 1991; Vacheron *et al.*, 1997). It has been postulated that these charged groups may bind to the negatively charged head groups of the cardiolipin of the mitochondrial membrane (Fritz-Wolf *et al.*, 1996; Eder *et al.*, 1999), but it has also been suggested that the predominantly hydrophobic character of the five C-terminal residues of mitochondrial CKs are also well suited for membrane insertion (Eder *et al.*, 2000a).

Whereas these structures were useful in that they identified many active site residues and provided significant information on those residues involved in dimer and octamer formation, they were of less use in attempts to relate structure with mechanism. The foremost problem was that, with the exception of that of the Mi_b-CK isoform, none of these structures was obtained in the presence of either substrates or inhibitors. Indeed, even the structure of Mi_b-CK liganded to ATP was obtained in the absence of Mg^{2+}, and each ATP molecule was found at a slightly different position in its monomer (Fritz-Wolf *et al.*, 1996). *In toto*, the structures provided no details about creatine binding, nor did they provide details about the binding of any substrate in the ternary enzyme complex. This was a serious limitation in that EPR (McLaughlin *et al.*, 1976) and small angle X-ray scattering (Forstner *et al.*, 1996, 1998) studies, as well as later infrared difference (Granjon *et al.*, 2001) and hydrogen-deuterium exchange (Mazon *et al.*, 2003) experiments, have all indicated that substrate binding brings about a considerable conformational change in CK. As alluded to earlier, small-angle X-ray scattering experiments had demonstrated that CK, in the presence of MgATP and the TSAC mixture, shows a decrease in radius of gyration (Forstner *et al.*, 1996; Forstner *et al.*, 1998). This decrease was similar to that observed for the closely related enzyme, arginine kinase, in the presence of MgADP or MgATP (Dumas & Janin, 1983; Forstner *et al.*, 1998). In AK it was proposed that this decrease was consistent with the hinge rotation of two domains (Dumas & Janin, 1983). Later comparison of the X-ray structure of the substrate-free AK (Yousef *et al.*, 2003) with the structure of AK liganded to a TSAC comprising arginine, nitrate and MgADP (Zhou *et al.*, 1998) confirmed the domain movement.

X-Ray Structures of Arginine Kinase

The AK-TSAC structure, initially reported at 1.86 Å resolution (Zhou *et al.*, 1998) and now refined to 1.2 Å (Yousef *et al.*, 2002), provided an immediate boost to the study of CK. Arginine kinase and creatine kinase share a relatively high level (38% to 44%) of sequence identity (Mühlebach *et al.*, 1994) and have almost superimposable far-UV CD spectra (Oriol & Landon, 1970; Mühlebach *et al.*, 1994). As with CK, AK possesses a reactive active site cysteine residue (Der Terrossian *et al.*, 1969) and has similar overall kinetic properties (Morrison, 1973). Now it was possible to compare the various CK structures with that of the AK-TSAC structure (Zhou *et al.*, 1998) and confirm that there was considerable structural homology as well. Given those similarities it was not unreasonable to model the transition-state conformation of CK by superimposing the structures of the "open" (substrate-free) forms of CK on the "closed" AK-TSAC structure (Eder *et al.*, 1999; Zhou *et al.*, 2000). The superimposition identified two flexible loops that would presumably undergo large conformational changes during catalysis. These were the loops containing residues 60 to 65 and 316 to 326 (Figure 5) which were characterized by either high temperature (B-) factors or were disordered. This model of the closed form of the enzyme was sufficient to direct a number of mutagenesis/mechanistic studies on CK (Eder *et al.*, 2000b; Cantwell *et al.*, 2001; Wang *et al.*, 2001), as well as on AK itself (Pruett *et al.*, 2003; Azzi *et al.*, 2004; Gattis *et al.*, 2004; Uda & Suzuki, 2004). Nonetheless, the precise details of the involvement of individual CK residues in particularly substrate binding and specificity remained elusive.

Structure of Substrate-Bound Creatine Kinase and Correlation with Mechanistic Studies

Recently, the X-ray structure of *Torpedo californica* creatine kinase (TcCK), a MM-CK isozyme, liganded

to a TSAC complex of creatine-nitrate-MgADP, was published (Lahiri *et al.*, 2002). As expected from the sequence similarity, TcCK shows considerable overall fold similarity with the other known creatine kinase structures. Fortuitously, a structure of TcCK bound to MgADP was also obtained from the same crystal (Lahiri *et al.*, 2002), which permitted a direct comparison of the open (CK-MgADP) and closed (CK-TSAC) forms of the enzyme. As shown in Figure 6, the residues involved in nucleotide binding did not move greatly on going from the open to the closed form. However, two residues, Ile69 and Val325, moved into the active site

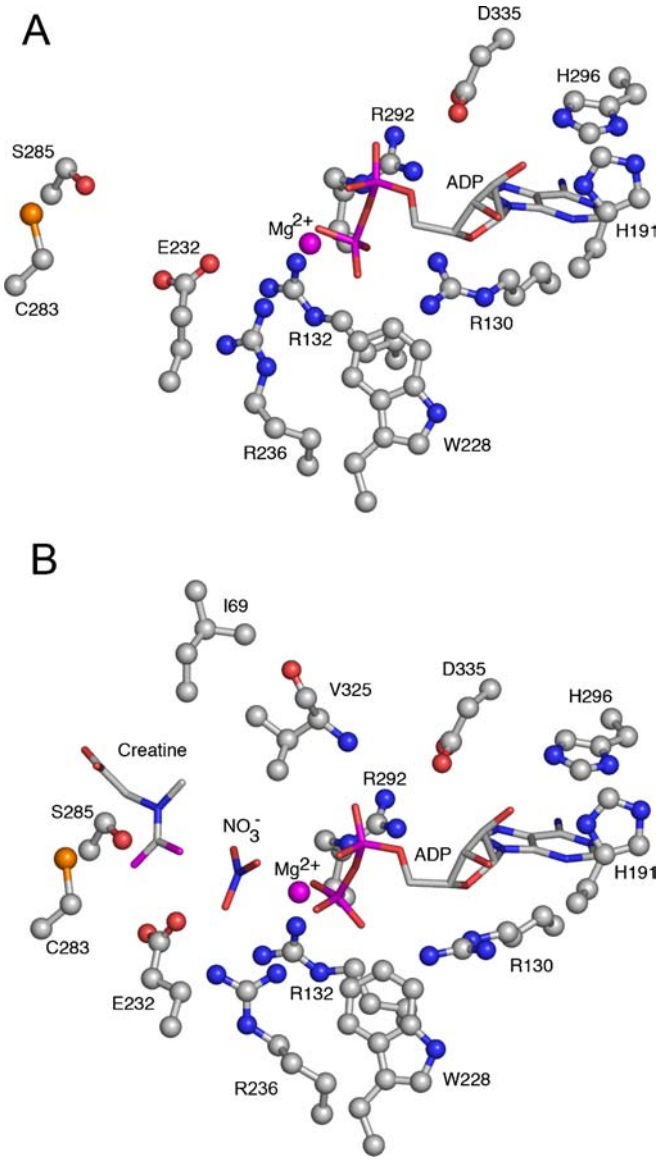

FIGURE 6 **Active site of *Torpedo californica* creatine kinase bound to (A) MgADP and (B) a TSAC comprised of creatine, NO_3^- and MgADP. For clarity, the water molecules bound to Mg^{2+} and Arg320 which interacts with the phosphates of MgADP are omitted. Coordinates used were those from PDB 1N16 (Lahiri *et al.*, 2002).**

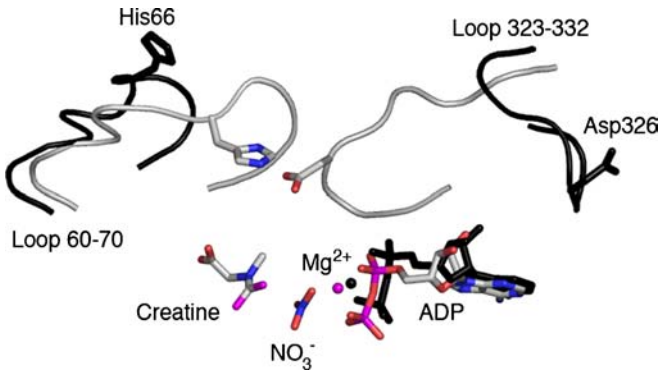

FIGURE 7 **Overlay of the structures of *Torpedo californica* creatine kinase bound to MgADP (dark) and to the TSAC (light). The movement of His66 and Asp326 are highlighted. More than 25 Å apart in the MgADP-liganded enzyme, they approach to within 3 Å in the TSAC structure, forming a "latch" over the active site. Conversely, there is little movement of either the Mg^{2+} ion, or ADP. Again, the coordinates used were those from PDB 1N16 (Lahiri *et al.*, 2002).**

possibly to provide a binding pocket for the N-methyl group of creatine. These two residues were brought into place by the movement of the flexible loops as they closed over the active site in the transition state. The loops also brought together His66 and Asp326, which appear to form a "latch" locking the flexible loops in place (Figure 7). Concomitantly, the flexible loops become more ordered with B-factors similar to those of the average main chain B-factor (Lahiri *et al.*, 2002).

It is somewhat intriguing that, in the same biological dimer, open and closed complexes can be observed. The fact that the TcCK-MgADP complex is in an open conformation is even more surprising, given that small-angle X-ray scattering showed a decreased radius of gyration when MgATP was added to CK (Forstner *et al.*, 1996; Forstner *et al.*, 1998) or when MgADP was added to AK (Dumas & Janin, 1983). It is conceivable that crystal packing forces might constrain asymmetrically the conformation changes required for substrate binding, thus giving rise to differences between the subunits in the crystal. Such a rationale is necessary as hydrogen-deuterium exchange experiments demonstrated that, in the presence of a TSAC mixture, both subunits of a dimer can bind substrates (Mazon *et al.*, 2003).

The Nucleotide Binding Site

In both complexes the MgADP groups are virtually superimposable, indicating that there is little or no strain involved in catalysis (Figures 6 and 7). As predicted by previous NMR experiments (Rosevear *et al.*, 1981) the adenine base in the ADP is in the *anti*

conformation with respect to the ribose ring, and the adenosine rings are held in place by stacking interactions with His296 and hydrogen bonds to His191 as well as to the main chain and to a few water molecules. His296 has been demonstrated to be particularly important by studies showing that even the conservative replacement with asparagine resulted in a considerable decrease in both substrate binding affinity and catalytic activity (Chen *et al.*, 1996).

As described earlier for the Mi_b-CK isozyme, the nucleotide phosphate binding pocket is formed primarily by five highly conserved arginines (Arg130, Arg132, Arg236, Arg292, and Arg320) that stabilize the negatively charged nonbonding oxygens of phosphate groups by a series of monodentate and bidentate interactions. The arginines are in the same position in both the CK-TSAC and CK-MgADP structures, except Arg320. In the E-MgADP structure, this residue interacts only with the nonbonded oxygen of the α-phosphate, but, in the E-TSAC complex, Arg320 moves closer and changes to bidentate ligand geometry, forming an additional hydrogen bond to an oxygen of the nitrate group. It is conceivable that Arg320, which is located at the base of one of the flexible loops, may be involved in conformational switching (Forstner *et al.*, 1998). In addition to the arginine residues Trp228 must form part of the binding pocket for, although it does not interact directly with the substrate, mutagenesis of this residue leads to inactivation of the enzyme (Gross *et al.*, 1994).

The Metal Ion

Cleland (1967) suggested that MgATP and MgADP were the true substrates for the reaction. Exchange-inert Cr(III) nucleotide complexes were initially used to determine the stereochemistry of the interaction with CK (Dunaway-Mariano & Cleland, 1980), and a mechanism was proposed in which the Mg(II) has β,γ-coordination to ATP prior to phosphoryl transfer and then migrates to form an α,β-bidentate complex with the product, ADP. A later study suggested that α,β,γ-coordination to ATP is also feasible (Burgers & Eckstein, 1980). As described earlier, creatine kinase was found to bind tightly to a 'transition-state analog complex' mixture comprised of creatine, MgADP and planar anions such as nitrate, nitrite and formate (Milner-White & Watts, 1971). Infrared spectroscopy showed that the anions were directly coordinated to the metal ion at the active site (Reed *et al.*, 1978), while EPR studies using Mn(II) and ^{17}O-labeling showed that the metal ion is bound to the α- and β-phosphates of ADP, to the anion, and to three water molecules (Reed & Leyh, 1980). A final EPR study suggested that an α,β,γ-tridentate complex of Mn(II) ATP is a substrate for the forward reaction and, in the product complex, Mn(II) remains coordinated to the α- and β-phosphates of MgADP as well as to phosphocreatine (Leyh *et al.*, 1985).

The CK-TSAC structure provided conclusive evidence that the earlier studies were indeed correct. In general, Mg(II) prefers a coordination number of six, and a preference for oxygen atoms as electron donating ligands (Katz *et al.*, 1996). In the TSAC structure the Mg^{2+} ion has octahedral coordination geometry involving three water molecules, two non-bridging oxygens from the α- and β-phosphates of ADP and an oxygen of the nitrate group. In the CK-MgADP structure, a water molecule replaces the nitrate ion (Lahiri *et al.*, 2002) while, in a subsequent structure with CK bound to MgAMPPNP, a non-hydrolyzable MgATP analogue, the Mg^{2+} ion interacts with three water molecules and the oxygens of the α,β,γ-phosphates of AMPPNP (Lahiri, 2004). The Mg^{2+} is liganded to the pro-*R* oxygen of the α-phosphate; thus not only did the early EPR studies correctly identify the coordination geometry of the Mg^{2+}, but the predictions of the stereochemical configuration of the metal nucleotide complex were also accurate (Reed & Leyh, 1980; Leyh *et al.*, 1982, 1985).

The Creatine Binding Site

Not surprisingly, the creatine binding site is much smaller than that of the nucleotide. The carboxylate of creatine forms a hydrogen bond to the main chain nitrogen of Val72 with the remainder of its interactions occurring via water molecules held in place by protein side-chains. Studies with alternative substrates had shown that replacement of the *N*-methyl group of creatine with the smaller hydrogen group (glycocyamine) or the bulkier ethyl group (*N*-ethylglycocyamine) led to a 43-or 11-fold reduction in binding affinity for the substrate, respectively (McLaughlin *et al.*, 1972). This specificity for creatine appears to be achieved by use of a binding "pocket" formed by Ile69 and Val325 (Figure 6B). These two hydrophobic residues are found on the two loops that close over the active site in the CK-TSAC structure and are not part of the active site in the CK-MgADP structure (Figure 6A). Mutagenesis studies with HMCK have subsequently revealed that

replacement of Ile69 by alanine results in greatly decreased enzymatic activity and complete loss of synergy (Novak *et al.*, 2004). Replacement of Ile69 by glycine in *Danio* MM-CK provided a similar result (Uda & Suzuki, 2004). In the same study, it was shown that replacement of Val75 with glycine led to an unstable enzyme, and the authors speculate that Val75 may be involved in stabilizing the flexible loop structure (Uda & Suzuki, 2004). In the C-terminal loop the substitution of Val325 in HMCK with glutamic acid results in a 100-fold preference for glycocyamine while replacement with alanine results in a slight preference for cyclocreatine (Novak *et al.*, 2004). Interestingly, in the AK structure Val325 is replaced by a negatively charged glutamic acid residue, Glu314, which interacts with the ε-nitrogen of the bound arginine. Sequence alignments had suggested that Asp326 was the CK homolog of Glu314, and replacement of Asp326 by alanine had resulted in a significant reduction in the value of k_{cat} for the forward reaction and an increase in the value of K_m for creatine (Cantwell *et al.*, 2001). Further, slight preference for cyclocreatine as a substrate was now apparent. At the time those results were discussed in terms of creatine binding but, when the CK-TSAC structure became available, it was clear that Asp326 in fact forms a salt bridge to His66 effectively "latching" the two loops into the closed position (Figure 7). The importance of His66 in catalysis by CK has also been demonstrated (Forstner *et al.*, 1997; Mourad-Terzian *et al.*, 2000), although it does not appear to be the acid-base catalyst identified in pH-rate profile studies (Cook *et al.*, 1981; Wang *et al.*, 2001). It is possible that the latch contributes to the electrostatic environment of the active site and assists in maintaining the conformation of the two loops so that the substrates are optimally aligned for catalysis. In any event, it is noteworthy that, from their respective positions in the MgADP structure, His66 and Asp326 move more than 25 Å to form the latch in the CK-TSAC structure. It had also been shown that at least one residue per monomer is labeled with diethyl pyrocarbonate, a reagent selective for histidine residues (Pradel & Kassab, 1968; Clarke & Price, 1979). The labeling occurred even if the thiol groups were reversibly blocked and there was no evidence for substrate protection by creatine (Pradel & Kassab, 1968). These results could be explained if His66 was the residue being labeled for, unless the loops are closed as in the CK-TSAC structure, His 66 is readily accessible to chemical modification agents and is located at some distance from the active site cysteine residue. That said, the studies of Chen *et al.* (1996) suggest that His296 is the likely target for the diethyl pyrocarbonate modification although the possibility that His66 is candidate was not addressed.

Studies with the conformationally restricted creatine analog, cyclocreatine, had indicated that the guanidino nitrogen cis to the methyl group was stereoselectively phosphorylated (Struve *et al.*, 1977; Phillips *et al.*, 1979). The CK-TSAC structure provides the structural basis for the stereospecificity. Not only does Glu232 form a bidentate salt bridge with the guanidino group, thereby stabilizing its positive charge, but it is also correctly positioned to act as a general base to remove a proton from the nucleophilic nitrogen. Glu232 is part of the NEED box that is conserved throughout the guanidino kinases and mutagenesis of this residue results in a severely impaired enzyme (Eder *et al.*, 2000b; Cantwell *et al.*, 2001). Even the most conservative replacement, by an aspartate, results in a 500-fold decrease in activity (Cantwell *et al.*, 2001). Similar results were obtained for arginine kinase, although the loss of activity was not as dramatic (Pruett *et al.*, 2003).

In addition to its interaction with Glu232, the guanidino group of creatine is also held in place through the interaction of its non-nucleophilic η1-nitrogen with Cys283. This is the 'reactive' cysteine described earlier, which is conserved throughout the guanidino kinases. Cys283 has a relatively low pK_a value of 5.4 suggesting that the optimal binding of creatine occurs when Cys283 is in the form of a thiolate anion (Wang *et al.*, 2001). Ser285 provides two interactions with Cys283, through its backbone carbonyl group and through its hydroxyl group, both of which contribute to its low pK_a value (Wang *et al.*, 2001; Naor & Jensen, 2004). In AK the serine is replaced by a threonine (Zhou *et al.*, 1998). Historically, one of the most contentious subjects for CK studies has been the importance of the reactive or as it was often termed, the "essential" cysteine residue. The controversy has arisen because of conflicting results from chemical modification studies with a variety of cysteine-specific reagents. In some instances the modified enzymes were completely inactive (Mahowald *et al.*, 1962; Zhou & Tsou, 1987; Wu *et al.*, 1989), whereas in others there was some residual activity (Smith & Kenyon, 1974; Der Terrossian & Kassab, 1976; Maggio *et al.*, 1977). Mutagenesis experiments have now demonstrated unequivocally that, while important, neither Cys283 nor Ser285 are essential for catalysis (Furter *et al.*, 1993; Wang *et al.*, 2001). Further, X-ray and kinetic

studies on mutants of the analogous cysteine residue in arginine kinase, also support this conclusion (Strong & Ellington, 1996; Gattis *et al.*, 2004). Taken together, the kinetic and mutagenesis data suggest that Cys283 is not indispensable, but it does help to keep creatine anchored and positioned for nucleophilic attack on the γ-phosphorus of MgATP (Lahiri *et al.*, 2002). In addition, it is possible that the basic character of the thiolate anion perturbs the resonance of the guanidinium by drawing positive charge toward the non-reactive $N_{\eta}1$, thereby increasing the nucleophilicity of the reactive nitrogen in the forward reaction and enhancing the leaving group properties of creatine in the reverse reaction (Gattis *et al.*, 2004).

The Nitrate Ion and Phosphoryl Group Transfer

Following the discovery of creatine kinase, it did not take long to recognize that the enzyme catalyzes the transfer of a phosphoryl, not a phosphate, group from ATP to creatine, and hence the early use of ATP-creatine transphosphorylase as a preferred name (Kuby & Noltmann, 1962). No evidence has been found for a phosphorylated enzyme intermediate, nor is there any exchange of phosphate between [^{32}P]ADP and ATP in the absence of guanidino substrates (Noda *et al.*, 1960). Equilibrium isotope exchange was used to demonstrate that the rate of phosphoryl transfer between ADP and ATP is similar to that between phosphocreatine and creatine (Morrison & Cleland, 1966), while quenched flow kinetics showed that phosphoryl group transfer is rate-limiting in both directions (Engelborghs *et al.*, 1975).

Potentially, the CK reaction involves a direct transfer of a phosphoryl group by an S_N2 type reaction, with the phosphoryl group forming an sp^3d hybrid in the transition state. It was proposed that the planar anions, such as nitrite and formate, mimicked the phosphoryl group in the transition state of the reaction (Milner-White & Watts, 1971). On that basis phosphoryl group transfer must occur via an 'in line' process as the planar anion is incompatible with an 'adjacent' transfer mechanism wherein the guanidine nitrogen of creatine forms part of the equatorial plane and the oxygen atoms of the phosphoryl group are not planar with the phosphorus atom (Milner-White & Watts, 1971). Although NMR data too, suggested that the phosphoryl group is transferred in a direct, in-line process (McLaughlin *et al.*, 1976), an S_N1 mechanism also would have a stable PO_3^- anion as a stable intermediate and, consequently, this mechanism could not be ruled out. Further evidence suggesting that the reaction proceeded via an associative (S_N2) rather than dissociative (S_N1) mechanism was found by Lowe and Sproat (1980) who determined that, in the absence of creatine or the presence of competitive inhibitors, there was no ^{18}O scrambling in adenosine 5′-[$\alpha\beta$-^{18}O, β-$^{18}O_2$]triphosphate. The fact that P_{γ}-OP_{β} bond cleavage followed by rotation around the P_{β}-OP_{α} bond and reformation of ATP would lead to scrambling, it was clear that the P_{γ}-OP_{β} bond must not be not broken in the absence of creatine. Subsequently, Hansen and Knowles (1981) used adenosine [γ-(*S*)-^{16}O, ^{17}O, ^{18}O]triphosphate to show that the reaction proceeds with inversion of configuration. Taken together, all the evidence is consistent with, but not definitive of, phosphoryl transfer occurring via an associative (S_N2) mechanism.

In the CK-TSAC structure, the nitrate anion is held in place by interactions with the Mg^{2+} ion, a water molecule which is also liganded to the Mg^{2+} ion, as well as with Arg236 and Arg320 which are located on either side of the anion. In general, divalent metal ions interact only weakly with anions, such as nitrate, and it is the interactions with the arginine residues that really stabilize this interaction. It is thought that, in the TSAC, the nitrate ion mimics the γ-phosphoryl group being transferred in the state intermediate between a dissociated metaphosphate and a pentavalent form (Reed & McLaughlin, 1973). In the CK-TSAC structure, the nitrate is not constrained by partial bonds to the β-phosphate and the guanidino nitrogen of creatine, as it would be for the γ-phosphate group in the true transition state. However, as shown in Figure 8, if a line is drawn which connects the guanidino $\eta 2$ nitrogen and the oxygen of the β-phosphate group, while passing through the center of the nitrate group, the plane of the anion is almost perpendicular to that line. The deviation

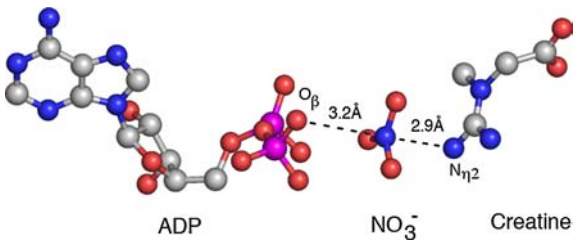

FIGURE 8 **Ball and stick representation of the transition state complex formed by ADP, NO_3^- and creatine in the active site of TcCK. The nitrate group mimics the planar phosphoryl group during an in-line transfer between the ADP O_{β} and $N_{\eta 2}$ of creatine.**

from perpendicular, ~6°, is slightly greater than those observed for the analogous atoms in the refined AK-TSAC structure (Yousef *et al.*, 2002). Nonetheless, the distance between the guanidino $N_{\eta 2}$ and the ADP O_{β}, through the nitrate anion, is 6.1 Å, which is close to the 6 Å expected of the transition state for an associative in-line transfer mechanism (Yousef *et al.*, 2002).

Borders *et al.* (2003) noted that, in the AK-TSAC structure, the nitrate ion has an interaction with an asparagine residue as well as the arginine residues. The corresponding residue in the CK-TSAC structure, Asn285, is also positioned for a similar interaction (Lahiri *et al.*, 2002). Replacement of Asn285 in RMCK by aspartic acid led to a 15,000-fold reduction in k_{cat} for the mutant enzyme (Borders *et al.*, 2003). However, the values of K_m for both ATP and creatine were virtually unchanged, suggesting that Asn285 plays an important role in transition-state stabilization. The fact that no Asn285 mutant was able to form a TSAC lent further credence to that suggestion.

FUTURE DIRECTIONS

In general, this review has focused primarily on creatine kinase. Much of the forthcoming work on creatine kinase will need to be placed in context with studies on other guanidino (phosphagen) kinases. Consequently, in this brief summary, as yet unanswered questions about creatine kinase will also be considered in that framework.

One of the intriguing aspects of creatine kinase catalysis is the lack of any clearly indispensable residue. Glu232 appeared to be a prime candidate to act as a catalytic base but, while mutagenesis studies have shown that it is obviously important, the enzyme can get by without it (Brooks & Suelter, 1987; Eder *et al.*, 2000b; Cantwell *et al.*, 2001). The negatively charged NEED cluster is also conserved throughout the guanidino kinases and the X-ray structure of arginine kinase bound to a TSAC shows that the Glu232 homolog, Glu225, occupies the same position relative to the guanidino group (Zhou *et al.*, 1998). However, as with Glu232 in creatine kinase, mutagenesis of Glu225 results in reduced enzyme activity but the decrease is much less than may be expected for removal of a catalytic base (Pruett *et al.*, 2003). What is the precise role of Glu232, in particular, and the other residues in the NEED cluster?

For many years Cys283 was also thought to be essential although mutagenesis studies have now ruled that out (Furter *et al.*, 1993). As with Glu232 it is conserved throughout the guanidino kinases and has a homolog occupying the same position in the AK-TSAC structure (Zhou *et al.*, 1998), and mutants of the AK homolog also retain partial activity (Gattis *et al.*, 2004). Why does this cysteine residue have such a low pK_a? Is the presence of the thiolate anion mechanistically important for guanidino kinases?

Stroud has suggested that, for reactions involving two substrates, orientation of the substrates may be the most important element in catalysis. Further, the mechanism whereby substrates are aligned may be more important than any single catalytic residue (Stroud, 1996). Given the precise alignment of the components of the TSAC in both creatine (Lahiri *et al.*, 2002) and arginine (Yousef *et al.*, 2002) kinase, and the observation that even small deviations in the correct alignment drastically reduce arginine kinase activity (Pruett *et al.*, 2003), Stroud's comments are almost certainly appropriate for all the guanidino kinases. It is likely that both Glu232 and Cys283 play a significant role in the alignment of the substrates and that any involvement in acid-base catalysis is secondary. This, of course, has yet to be shown conclusively.

Although the recent structural data have answered many questions, they have raised many more. For example, precisely how do the guanidino kinases recognize their substrates? Using Table 1, it is possible to describe guanidino kinase substrates in relative terms as long (arginine, lombricine), short (creatine, glycocyamine), having an *N*-methyl group (creatine) and lacking an *N*-substituent (arginine, glycocyamine). As predicted by early sequence alignments (Suzuki *et al.*, 1997; Cantwell *et al.*, 2001), the comparison of open and closed structures of CK (Lahiri *et al.*, 2002) and AK (Yousef *et al.*, 2003) shows that much of the substrates specificity resides in the flexible loops. Sequence alignments would suggest that phosphorylation of long substrates is catalyzed by enzymes with short N-terminal loops, whereas that of short substrates is catalyzed exclusively by enzymes with long N-terminal loops (Suzuki *et al.*, 1997). Further, substrates with an *N*-methyl group such as creatine may require small hydrophobic residue (*e.g.*, Val325) on the C-terminal loop, while substrates lacking this *N*-substituent such as arginine and glycocyamine require an acidic group, *e.g.*, a glutamate, at the analogous position (Novak *et al.*, 2004). Unfortunately the initial results with AK/CK chimeras (Azzi *et al.*, 2004) are inconclusive as the lack of activity of

all the chimeras may be explained by the presence of Glu314 which interferes with the optimal positioning of creatine (Novak *et al.*, 2004). However, it is clear that such an approach will lead to a greater understanding of the specificity determinants of CK in particular, and the guanidino kinases in general.

The conzyme philosophy (Stroud, 1996) requires that the loops not only recognize the substrates, but help align the substrates for catalysis. A recent examination of the effects of mutations in the methyl specificity pocket in creatine kinase indicates that Ile69 in the N-terminal loop is important for synergism whereas Val325 in the C-terminal loop has a greater influence on substrate specificity (Novak *et al.*, 2004). In light of the conzyme proposal this is not surprising, but exactly what triggers the conformational changes necessary for correct substrate alignment and catalysis is a question that is yet to be addressed.

Other fundamental questions include why does CK prefer to be a dimer and AK a monomer? Can we make a stable and fully functional CK monomer? Is there crosstalk between the CK active sites? Of all enzymes, creatine kinase is one of the most intensively studied. However, despite more than 70 years of detailed investigation, a litany of questions remain to be answered.

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