Crystal structure of rabbit muscle creatine kinase

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Abstract The crystal structure of rabbit muscle creatine kinase, solved at 2.35 Å resolution by X-ray diffraction methods, clearly identified the active site with bound sulfates surrounded by a constellation of arginine residues. The putative binding site of creatine, which is occupied by a sulfate group in this analysis, has been tentatively identified. The dimeric interface of the enzyme is held together by a small number of hydrogen bonds.
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Key words: Creatine kinase; Rabbit muscle; Enzyme; Crystal structure

1. Introduction

Creatine kinase [1–3] (CK; adenosine 5′-triphosphate-creatine phosphotransferase, EC 2.7.3.2), a member belonging to the subclass of guanidino-kinases, reversibly catalyzes the reaction:

Creatine + MgATP ↔ creatine phosphate + MgADP.

CK plays an important role in the rapid regeneration of ATP in cells where such demands are high. It functions as an 85-kDa dimer, and there are three cytosolic forms of the dimers as well as a mitochondrial (Mt) form that exists both as a dimer and an octamer. The cytosolic forms are MM, BB and MB, where M and B denote muscle and brain, respectively, the preponderant organ sites of the enzyme occurrence. The elevated level of CK in human blood is an important diagnostic indicator for diseases of the nervous system and the heart muscle, for malignant hypothermia, and for certain tumors. Metals such as Mg, Mn and Ca are cofactors for CK, whereas several nucleotide phosphates as well as thyroxine have been shown to be inhibitors [1]. Phosphocreatine (PCr), the product of an enzymatic reaction catalyzed by CK, inhibits the replication of HIV in macrophages [4]. It has also been shown that CK is a substrate of protein kinase C, an important component of several signaling pathways of cell growth [5]. Cyclocreatine, a substrate analog of PCr, has been shown to inhibit the growth of a broad spectrum of tumors [6].

The crystal structure of chicken MtCK was solved by Kabsch et al. [7] at 3 Å resolution with and without ATP. Very recently, the crystal structure of the related arginine kinase (AK) has been solved with a bound transition-state analog [8]. Unlike CK, AK can also function as a monomer [9]. However, most of the biochemical and biological data obtained for this class of enzymes are available only for rabbit MCK. Crystals of rabbit and bovine MCK were grown several years ago. We report here the structure of rabbit MCK, solved by molecular replacement (MR) using the coordinates of the chicken MtCK structure and verified by multiple isomorphous replacement (MIR).

2. Materials and methods

All crystals of rabbit MCK used in this study were grown at neutral pH with a protein concentration of ∼5 mg/ml (type VI-S CK from rabbit heart, purchased from Sigma, St. Louis, MO, USA). Enzyme solutions were initially dialyzed against a 50 mM Na-HEPES buffer at pH 7.2 in a 12-kDa semipermeable membrane, changing the buffer three times. Large crystals (0.2–0.3 mm thick), which were suitable for single crystal X-ray diffraction studies, could reproducibly be grown in standing drops containing equal amounts of the protein solution as well as one containing 55% saturated ammonium sulfate and 2.5% PEG 400. The best crystals diffract to a resolution of ∼2.7 Å at room temperature and to 2.35 Å at 120 K with a laboratory X-ray source. The low-temperature data were collected with crystals that were flash-frozen in mineral oil. The R_{sym} for the data set at 2.35 Å resolution is 0.101 in the space group $I422$, with 89% completeness in the last shell and an overall completeness of 97%, with a 24-fold redundancy. The R_{sym} (0.096) does not decrease significantly when the data were scaled in the space group $I4$.

The crystals of rabbit MCK that were used in this study contain one 43-kDa monomer in the asymmetric part of a tetragonal unit cell with dimensions $a = b = 199.6$ Å, $c = 71.0$ Å in the space group $I422$. The calculated Matthew's volume is 4.3 A³/Da, so the possibility that two CK monomers are present in the asymmetric unit cannot be excluded on this basis. The solvent content in the unit cell is nearly 70% for one CK monomer in the asymmetric unit.

The structure of chicken MtCK was used to initiate the present structure solution by MR. Room temperature data at 3.2 Å resolution were initially used for this purpose. The octameric MtCK contains a tetramer in the asymmetric unit of the tetragonal crystals. A monomer from this model was expected to yield a solution, as there is a 60% amino acid sequence identity (not shown) between chicken MtCK and rabbit MCK. Identical rotation function solutions were obtained from three different programs – MADIRA (J.K.M. Rao, unpublished), X-PLOR [10] and AMoRe [11] – when an MtCK monomer was used as the probe. When a translation search was conducted in the AMoRe suite, there was room only for one CK monomer in the asymmetric part without any overlap of the unit cell contents. At 3.5 Å resolution, the correlation coefficient was ∼0.5 and the R-factor was ∼0.4. Changing the sequence to that of rabbit MCK and refinement of coordinates with individual B-factors by using the X-PLOR program yielded a crystallographic R-factor (R_{work}) of 0.216 ($R_{free} = 0.337$) at 3.2 Å resolution.

When refinement was continued later with the newly collected higher resolution data, certain disconcerting features emerged. With protein atoms alone, the R_{free} was always converging in the 0.32–0.35 range and the R-factor was ∼0.26–0.29 at resolution 2.8–2.5 Å. When the refinement was carried out in the lower symmetry space group $I4$, the R-factor did not drop significantly (only ∼0.01). Even though the MR solutions undoubtedly pointed to the existence of only one CK monomer in the asymmetric unit, we considered it

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worthwhile to confirm this fact in an independent and unbiased manner, using the MIR data.

Several heavy atom derivatives were obtained and low temperature data were collected with the soaked and frozen crystals. Interestingly, the soaking times (1–2 h) and concentrations (less than 1 mM) were extremely low. Perhaps this is due to the high solvent content and sparse packing in the unit cell of rabbit MCK that permitted easy entry of the heavy atoms. Mercury (*para*-chloro mercury benzoic acid Na salt) and uranyl (uranyl acetate) derivatives yielded the same heavy atom site with good anomalous dispersion signals. These derivatives, together with three others that yielded sites with relatively lower occupancies (platinum – K_2PtCl_4, lead – methyl lead acetate, and uranyl – $K_3UO_2F_5$) were refined using the program PHASES [12]. The best MIR map with phases from all the derivatives at 3.2 Å resolution was solvent-flattened further and the phases were extended to 2.8 Å resolution. There is no indication of the presence of a second molecule of CK in this map, which is free from any model bias, and the MR model was in good agreement with the MIR map.

The MR model was subjected to further rebuilding and refinement. The resolution was extended in small steps adding less than 1000 reflections at a time. Water molecules were added to the model starting from a resolution of 2.5 Å. All residues except for the first six residues at the N-terminal end and residues 323–331 in the active site (discussed later) were modeled in the final electron density. X-PLOR refinement cycles consisted of a positional refinement (∼400 steps), a simulated annealing slow-cool protocol (starting at 2000 K) and finally an individual *B*-factor refinement. Since the solvent content of the structure is quite high, an unusually large number of water molecules were located in the difference Fourier maps. The final *R*-factor (R_{work}) for 2929 protein atoms, 431 water oxygens and four sulfate groups is 0.195 for 25 023 reflections, with an R_{free} value of 0.288 for 2943 reflections. It is relevant to note that the R_{work} and R_{free} for protein atoms alone are 0.283 and 0.326, respectively. Thus, the solvent molecules contribute significantly to the structure factors, which explains the difficulties encountered in the initial stages of structure refinement. The atomic coordinates of the final model will be deposited in the Protein Data Bank, with the accession number 2crk.

3. Results and discussion

The structure of rabbit MCK obtained in this study is of acceptable quality. Only one residue (Lys[9]) at the N-terminus, where disorder seems to be more prevalent, is outside the allowed region of the Ramachandran plot. The mean *B*-factors for the protein atoms and the water molecules are 26.3 (±9.8) and 36.8 (±10.9) Å^2, respectively. In the region of the active site, the temperature factors near the residues not located in the structure, are indeed high. The rms deviation for the bond lengths is 0.014 Å and the corresponding value for the bond angles is 1.9°.

Among the brain and the muscle CK enzymes, the sequence similarity (not shown) is extensive with ∼80% of the residues identical. The number of identical residues among all the classes is nearly 60%. The identity reaches nearly 90% for each class, brain, muscle, mitochondrial, etc. Fig. 1A and B gives a stereoview and topological diagram, respectively, of the rabbit MCK monomer. The CK monomer consists of two domains: an essentially α-helical N-terminal domain of ∼100 residues and the larger C-terminal domain of ∼250 residues connected by a long linker. The C-terminal domain consists of an antiparallel β-sheet and long α-helices.

A comparison of the chicken MtCK and rabbit MCK dimer structures shows that the N-terminal ends of the two enzymes (about 15 residues) do not superimpose. This may be due to either a different mode of crystal packing for the two enzymes or due to true differences between the two enzymes in this part of the structure. In addition, the sequence similarity

in this region is not very high among the CK enzymes. In fact, Kaldis et al. [13] showed that this region is crucial for the octamerization of the Mt enzyme. The rare occurrence of two parallel α-helices, e.g. in IL-4 [14], may be seen in this domain. There are extensive stretches of identical residues among the CK enzymes, even in random-coil regions. In the linker region between the N- and C-terminal domains, there is an insertion/deletion between the Mt and the other classes of the enzyme.

The major feature of the C-terminal domain is an eight-stranded antiparallel β-sheet flanked by α-helices. The β-sheet itself can best be described as a saddle (or a cradle). The central portion of the sheet is quite hollow and the bend angle is about 120°. There are six helices in this domain and all but one (α8) are on the convex side of the β-sheet. The single helix on the concave side is highly conserved. Out of 50 residues that form the antiparallel β-sheet, ∼40 are identical across the species. Thus, the core of the C-terminal domain (the antiparallel β-sheet) seems to be invariant and this well-conserved structural motif must be common to all CK structures. Among the helices of this domain, the sides that are exposed to the outside solvent seem to show variations in their amino acid sequence, whereas their interior is highly conserved.

In all the CK enzymes, the functional unit is at least a dimer. In this study, the crystallographic asymmetric unit is a monomer. Thus, the CK dimer has an exact two-fold symmetry. The dimer created by using the diagonal two-fold axis in the (001) plane could be superimposed on any dimer of chicken MtCK. Therefore, this dimer is functional in all CK enzymes. A dimer created in this way superimposes quite well with four such dimers in the chicken MtCK octamer. The rms deviations (in Å) for superimposed Cα pairs are 0.64 (576 pairs), 0.61 (582 pairs), 0.69 (519 pairs) and 0.69 (525 pairs). The difference in the number of superimposable atoms seems to be due to the presence of a dimer with perfect two-fold symmetry in rabbit MCK and to the presence of dimers related by pseudo dyads in chicken MtCK.

The monomer itself makes a limited number of contacts with residues of other monomers. The hydrogen-bonded interactions in a proper dimer of rabbit MCK are shown in Fig. 2. There are only eight hydrogen bonds that involve the protein atoms of the constituent monomers. One of these hydrogen-bonded interactions is a salt bridge between Asp[53] and the symmetry-related Arg[147]. In addition to these, there are a few other water-mediated interactions at the dimer interface. We are surprised to observe that such a small number of interactions is enough to form and hold such a large dimer.

The crystals of rabbit MCK used in this work were grown with ammonium sulfate as the precipitant (see Section 2). We were able to locate four sulfate groups in the structure, with three in the putative active site. Fig. 3 gives a stereoview of the active site region in rabbit MCK. For comparison, the ATP bound to chicken MtCK is also shown. Clearly two sulfates occupy the α- and γ-phosphate positions of ATP. Efforts to displace the sulfates and replace them by ATP have been unsuccessful so far under the present conditions of crystal growth. Interestingly the administration of magnesium sulfate to cardiac patients with unstable angina lowers the release of MBCK [15]. It is not clear whether sulfate binding to the active site of CK is responsible for this effect.

In addition to the two sulfates that occupy the ATP phosphate positions, there is another sulfate group located 5.2 Å
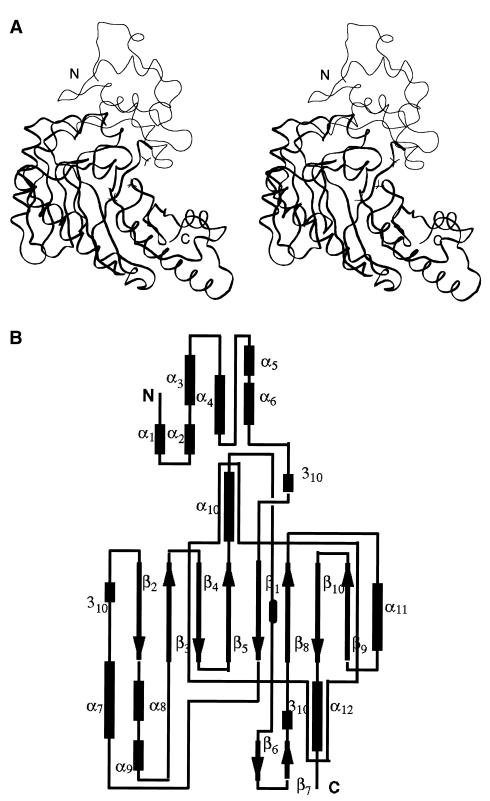
Fig. 1. A: A stereoview of the rabbit MCK monomer. The thin lines represent the N-terminal domain and the thick lines the C-terminal domain. Three sulfate groups occupy the active site. B: Topological diagram of the rabbit MCK monomer. Helices are represented by cylinders and strands by arrows. The residue numbers for the α helices are: α1, 16–19; α2, 29–33; α3, 36–42; α4, 53–62; α5, 82–85; α6, 87–96; α7, 148–162; α8, 181–188; α9, 200–203; α10, 246–266; α11, 308–315; and α12, 346–367. The residue stretches for the 310 helices are 112–115, 167–169 and 284–286. The residues for the β strands are: β1, 126–135; β2, 171–175; β3, 216–220; β4, 225–229; β5, 235–242; β6, 273–274; β7, 278–279; β8, 292–298; β9, 317–320; and β10, 333–338. The boxed area is the domain that has a pseudo two-fold symmetry.
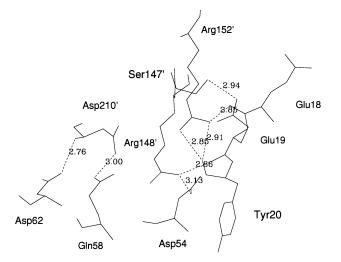
Fig. 2. The dimer interface. The hydrogen bonds with their distances in Å are shown as dotted lines. The prime symbol for residue numbers (e.g., Asp210′) indicates that the residues belong to the symmetry-related molecule.

from the nearest of these sulfates, which is linked through a water-mediated hydrogen bond. This sulfate group is quite close to the reactive Cys283. Although the role of Cys283 is not clearly understood at present, it has been shown that this residue, which is strictly conserved in all CK enzymes, is not essential for catalysis, but is required for shaping and maintaining the conformation between the substrate-binding sites [16]. The proximity of this sulfate to both the ATP γ-phosphate (see Fig. 3) and Cys283 suggests that this could be the location of creatine, the moiety to be transphosphorylated. Furthermore, ATP is known to transphosphorylate as well as autophosphorylate CK [17]. The residues which are candidates for autophosphorylation are Thr282, Thr289 and Ser285. In the rabbit MCK structure, Thr282 and Thr289 are quite far from this sulfate group whereas Ser285 is within hydrogen-bonding distance (-CH-CH$_2$-O-H···O-SO$_3$ is 3.04 Å). Near the reactive Cys283 are several water molecules that form a hydrogen-bonded network with the sulfate group. This observation leads to the hypothesis that Ser285 and Cys283 are both involved in auto- and transphosphorylation.

Olcott et al. [18] concluded that residues 237–241 and 280–291 are localized near the adenine moiety of ATP. This conclusion has been verified from the present structure. James et al. [19] suggested that Asp340 might be involved in the ATP binding site. However, this residue is far from the active site.

Residues 323–331 are disordered and could not be modeled in this study, which was conducted in the absence of ATP or ADP. However, even in the chicken MtCK structure [7], where the corresponding residues could be located, the temperature factors for atoms in this region are extremely high. Furthermore, the conformation in this region is not the same in all monomers of MtCK. Still, several conclusions can be drawn from the present structure of rabbit MCK, even without ADP or ATP. The active site cavity, occupied by sulfates, is surrounded by a cluster of positive charges consisting mainly of arginines (see Fig. 3). There are five arginines (Arg130, Arg132, Arg236, Arg292 and Arg341) in the immediate vicinity of the ATP moiety and one more (Arg96, belonging to the N-terminal domain) near the third sulfate. The residue Trp228 [20] is crucial for the activity of the enzymes as its replacement through site-directed mutagenesis inactivates CK. This residue which is at ∼5 Å from the sulfate group occupying the α-phosphate of ATP could interact with the ATP through Arg236 and a channel of water molecules. There are two histidine residues near ATP (His191 and His296), with His296 directly stacking with the adenine ring and His191 interacting through a water-mediated hydrogen bond. In rabbit MCK with the mutation H296N, the rate of inactivation by diethylpyrocarbonate was shown to be dramatic [21]. There are two more histidines (His29 and His96) near the third sulfate. Interestingly, some of the hydrogen-bonding distances are extremely short (2.5–2.6 Å). It is suggested that residues involved in such short hydrogen bonds play a major role in enzymatic mechanisms [22].

Gross et al. [23] established that in MtCK the C-terminal domain starting from residue 173 (the numbering corresponds to the sequence of rabbit MCK) could fold into a compact globule exhibiting enzymatic activity. In the present structure,
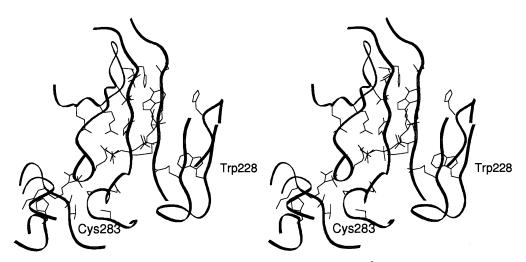

Fig. 3. A stereoview of the active site region of rabbit MCK. All residues at a distance of 5.5 Å or less around the sulfate groups are shown in the diagram. ATP from chicken MtCK is also displayed. Note the cluster of arginines around the sulfates. For clarity, no water molecule is shown, even though there are 25 water molecules satisfying the distance criterion. The sulfate near Cys283 is hypothesized to be the locale for creatine.

we also see that the C-terminal domain is quite compact in comparison to the N-terminal domain, with fewer loops and with better defined secondary structures. However, residues from the N-terminal domain do approach the active site. In fact, His^{29} (in helix α1 29–33) and Arg^{96} (in helix α6 87–96) in the N-terminal domain are quite close to the third sulfate (the probable locale for creatine) in the active site region. Thus although the N- and the C-terminal domains are quite distinct in the CK monomer, there seems to be sufficient cross-talk between them, particularly in the active site region.

Though CK functions as a dimer, the active sites of the two molecules, marked by the positions of the α-phosphates (sulfates in this work), are separated by more than 40 Å. Therefore, it is unclear how such distant active sites communicate with each other, if they do at all. It is also unclear whether the two active sites act independently or interact through the dimer interface. However, kinetic studies on denatured rabbit MCK suggest that proper refolding of the enzyme involves a rapid association of two folded monomers followed by a slow rearrangement of the dimer [24].

The saddle-shaped motif of the antiparallel β-sheet of the C-terminal domain, which is the location of the active site of the enzyme, is not unique to CK. The topological relationship between the C-terminal domain of CK and other proteins was examined by the use of DALI [25]. Fragments that have topological equivalence with the C-terminal domain of CK were found in glutamine synthetase complexed with AMP [26], in adenylyl cyclase complexed with forskolin [27] and in a TATA-box binding protein [28]. In the first two cases, AMP and forskolin moieties occupy positions very similar to ATP in chicken MtCK, whereas in the third, DNA is found at this site. In the TATA-box binding protein, the sheet structure has a pseudo two-fold symmetry, with 30% amino acid residue identity. In view of the pseudo two-fold symmetry found in TATA-box binding protein, a search was made to locate a pseudo dyad and, interestingly, such a pseudo dyad was found in the C-terminal domain of CK (see the boxed region in Fig. 1B). For 49 pairs of Cα atoms, the rms deviation is 1.8 Å. The two long helices (α10 and α12) superimpose with an average difference of about 1.0 Å. It is therefore possible that the C-terminal domain evolved through gene duplication and fusion, with subsequent mutations that eliminated sequence identities but preserved the topological similarities.

In order to understand why octamers are formed only by MtCK enzymes, but not by their cytosolic counterparts, a hypothetical octamer was created with a rabbit MCK dimer. For such an octamer, we found that there are extensive short contacts at the dimer interfaces of the octamer. This is partly due to the fact that the hypothetical octamer is made up of perfect dimers of rabbit MCK, whereas the chicken MtCK dimers constituting the octamer have only approximate two-fold symmetry. More importantly, the residues that make contacts in the octamer show sequence differences in these two classes of enzymes. It would be interesting to mutate such residues in MtCK to those in the cytosolic ones and demonstrate that octamers are indeed prevented from being formed. In the future, we plan to study CK structures with bound ATP and ADP, as well as creatine and other inhibitors, in order to investigate the mechanism of the enzymatic reaction.

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