ATP binding site of mitochondrial creatine kinase

Affinity labelling of Asp-335 with C1RATP

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The ATP binding site of mitochondrial creatine kinase from chicken heart has been studied by modifying the purified enzyme with a ^{14}C-labelled ATP analogue, C1RATP, in which the reactive label was covalently bound to the γ-phosphote group of ATP. The modified enzyme was digested by pepsin, and a single radioactive nonapeptide was isolated by HPLC. Amino acid analysis and direct sequence determination revealed that the isolated peptide corresponds to amino acids 335–343 within the C-terminal region of Mi-CK, this peptide being highly preserved throughout evolution. Asp-335 is very likely the site of modification by C1RATP. The specificity of the ATP analogue for the active site of creatine kinase was demonstrated by the inhibition of the enzymatic activity of Mi-CK by C1RATP and by the prevention of this inhibition bij ADP.

ATP binding site; Creatine kinase; C1RATP analogue

1. INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2) catalyzes the reversible transfer of the γ-phosphate group of ATP to creatine to yield phosphorylcreatine and ADP. CK is found primarily in tissues with high and fluctuating energy demands like brain, heart, skeletal muscle, spermatozoa, and retina where it serves to replenish the high energy phosphate pools (ATP and phosphorylcreatine) and to ensure effective energy transport from the sites of production (mitochondria, glycolysis) to the sites of utilization (kinases, ATPases; for a review see [1]). In higher vertebrates, four different subunit isoforms of CK are known, two of which are localized in the cytoplasm (M- and B-CK) and two in the mitochondrial intermembrane space (Mi_a- and Mi_b-CK) [1,2].

Although the guanidine and nucleotide substrates are known to bind to two adjacent sites on the CK molecule thus enabling a direct 'in line'-transfer of the phosphate group from one substrate to the other [3,4] and although 19 amino acid sequences of creatine kinase isoenzymes from a variety of species (*Schistosoma mansoni*, sea urchin, *Torpedo*, frog, chicken, mouse, rat, rabbit, dog, and man) are known up to date [5–24] (see Table I), little information is currently available on the amino acid residues involved in substrate binding and catalysis. A large number of thiol reagents were shown to modify one very reactive cysteine residue per subunit (Cys-278; numbering according to chicken Mi_b-CK [19]) with a concomitant loss of enzymatic activity (for reviews see [25,26]). Cys-278 is therefore assumed to be essential for the reaction mechanism and is conserved in all 19 sequences. Using 1,5-difluoro-2,4-dinitrophenol as a bifunctional crosslinker, Mahowald [27] demonstrated that in native CK Cys-69 and Lys-191 are in close proximity to Cys-278. Like Cys-278, both Cys-69 (found in 14 of 19 sequences; in the Mi-CK isoenzymes, Cys is replaced by Met) and Lys-191 (found in 18 of 19 sequences) are located in sequence stretches that are highly conserved throughout evolution. NMR experiments and inhibition studies with reagents specific for particular amino acids have revealed that, in addition to the residues mentioned above, a lysine, arginine, histidine, tryptophan as well as an aspartate or glutamate residue are also at or near the active site of creatine kinase [25,26,28,29]. However, the precise location of these amino acids within the primary structure has not been determined.

In this study, the substrate binding site of mitochondrial creatine kinase from chicken heart (Mi_b-CK) was probed with ClRATP, an ATP analogue which had been previously used successfully for the specific labelling of the active site of Na^+/K^+-ATPase [30].

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Abbreviations: CK, creatine kinase; Mi_b-CK, mitochondrial isoenzyme of creatine kinase from chicken heart; ClRATP, γ-[4-(*N*-2-chloroethyl-*N*-methylamino)]benzylamide adenosine triphosphate; BSA, bovine serum albumin

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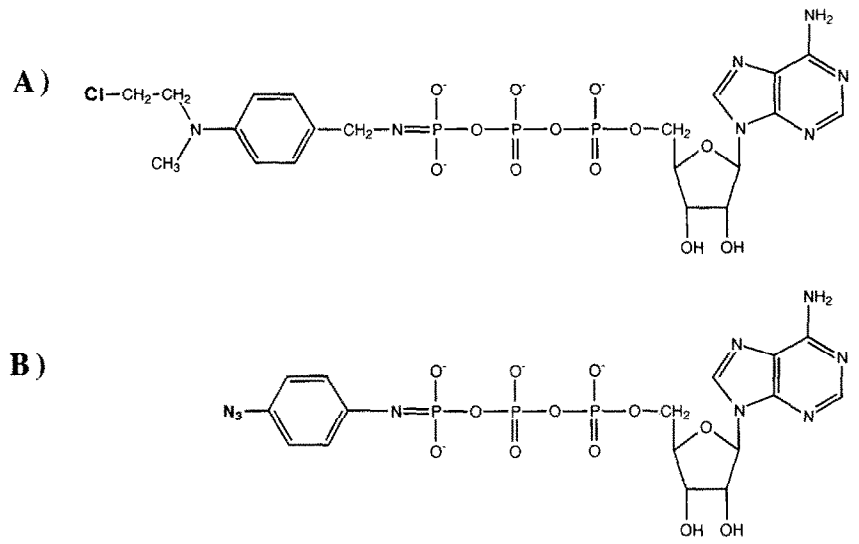

Fig. 1. Structural formula of ClRATP (A) and ATP-γ-*p*-azidoanilide (B) with the reactive label attached to the γ-phosphate group of ATP.

2. MATERIALS AND METHODS

ClRATP was synthesized as described by Dzhandzhugasyan et al. [31]. Pepsin was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA), phosphorylcreatine from Calbiochem (Lucerne, Switzerland), and ADP from Boehringer-Mannheim (FRG). All chemicals used for sequencing and amino acid analysis were from Applied Biosystems (ABI), (Foster City, CA, USA). Mitochondrial creatine kinase (Mi_b-CK) was isolated from chicken cardiac muscle according to [32].

2.1. *Inhibition of Mi-CK activity by ClRATP*

The inhibition of Mi_b-CK from chicken heart by ClRATP was performed essentially as described by Ovchinnikov et al. [30]. Mi_b-CK was dialysed extensively against 20 mM Tris-HCl, pH 7.5 and then incubated at a protein concentration of 1.5 mg/ml and at 37°C with 0.25 mM ClRATP for the indicated periods of time. At the end of the incubation, the CK activity was immediately measured by the pH-stat method at 25°C and pH 7.0 as previously described [33] with 20 mM HCl as the titrating agent, but without BSA in the assay mixture. In control experiments, Mi_b-CK was incubated under identical conditions without ClRATP or, after a preincubation with 4 mM ADP for 1 h on ice, with ClRATP in the presence of 4 mM ADP.

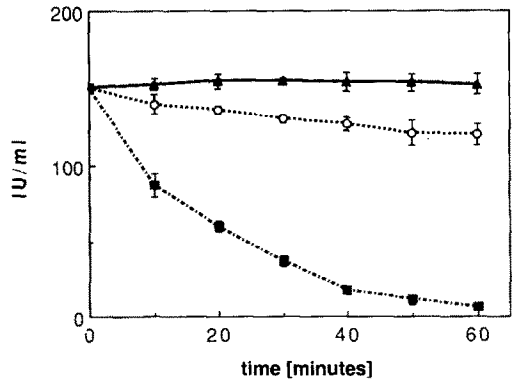

Fig. 2. Inactivation of mitochondrial creatine kinase from chicken heart (Mi_b-CK) by ClRATP. Mi_b-CK was incubated at 25°C and pH 7.5 for the indicated periods of time. (▲) In the absence of ClRATP; (■) in the presence of 0.25 mM ClRATP; (○) in the presence of 0.25 mM ClRATP + 4 mM ADP. CK activity was measured by the pH-stat method. All data points are given as mean ± SD of 3 measurements.

2.2. *Labelling and digestion of mitochondrial creatine kinase*

Mi_b-CK was labelled with [^{14}C]ClRATP (with the radioactivity in the benzyl group; see Fig. 1A) by incubating the enzyme for 30 min at 37°C in 20 mM Tris-HCl buffer, pH 7.5. The concentration of the protein was 1 mg/ml and that of the reagent 0.2 mM. The extent of incorporation of the radioactive label was 66%. Cold reagent was then added to yield a final concentration of 1 mM ClRATP, and the protein/reagent mixture was incubated for a further 30 min to increase the yield of labelled product.

Since the ester bond formed between the label and the protein was known from previous work on the Na^+/K^+-ATPase [30] to be very labile, especially above pH 4.0, a pepsin digest of the modified enzyme was carried out as follows: the protein was precipitated with 10% TCA and washed several times with the same solution to remove unbound label. The final pellet was dissolved in 2% formic acid and subsequently digested with pepsin for 2 h at 37°C, using a ratio of protease:protein of 1:100 (w/w). The digestion was stopped by heating the solution to 100°C for 2 min and then injecting the solution onto the HPLC device. A gradient was run over 30 min from 0–50% *n*-propanol in 10 mM tetraethyl ammonium acetate, pH 5.5 (see Fig. 2). A 300Å C-8 (2.1 × 100 mm) ABI reversed phase column was used. Fractions were collected every 40 s, and a 5% aliquot of each fraction was used for scintillation counting in a Beckman LS180 1 scintillation counter. The peak containing the radioactivity was then repurified on a C-18, 1.0 × 250 mm microbore column using a gradient of acetonitrile with 0.15% TFA to yield a single homogeneous peak. From this material, 20% was used for amino acid analysis (ABI model 420A with on-line PTC detection) and the rest was used directly for amino acid sequencing (ABI model 470A with on-line PTH detection).

3. RESULTS

To ascertain that ClRATP, the structural formula of which is shown in Fig. 1A, binds to the nucleotide binding site of mitochondrial creatine kinase, the effect of 0.25 mM of the ATP analogue on Mi_b-CK activity was investigated (Fig. 2). Within one hour, Mi_b-CK was in-

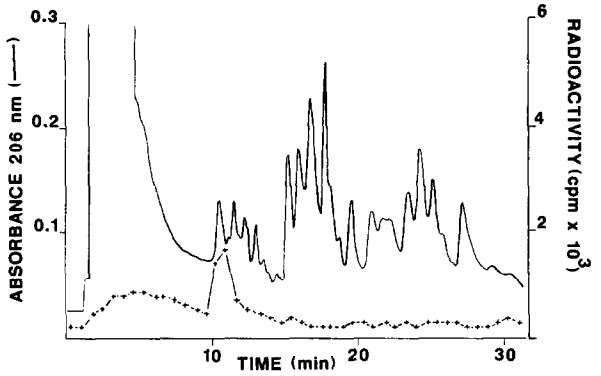

Fig. 3. Reversed phase chromatography profile of the pepsin digest of Mi_b-CK labelled with [^{14}C]ClRATP. The labelling, pepsin digestion, and separation of the peptides obtained were performed as described in section 2. (—) Absorbance at 206 nm; (- + --- + -) radioactivity.

activated to more than 95%. Preincubation with 4 mM ADP, which is expected to compete for the nucleotide binding site, drastically reduced the inactivation rate and thus showed that the covalent modification by ClRATP is not due to unspecific labelling, but to selective interaction of the label with the substrate binding site.

In order to identify the position of the amino acid residue(s) modified by the ATP analogue, Mi_b-CK labelled with [^{14}C]ClRATP was digested by pepsin. Separation of the peptide mixture by HPLC revealed a single peak of radioactivity (Fig. 3) which upon rechromatography proved to contain only one peptide species (not shown). The yield of radioactively labelled peptide, based on the amount of radioactivity recovered from the TCA-washed Mi-CK pellet before digestion, was 18%. The rest of the radioactive label was lost during purification of the peptide due to instability of the ester linkage between the ATP analogue and Mi_b-CK. Sequence analysis revealed the radioactively labelled peptide to correspond to amino acid residues 335–343 of Mi_b-CK [19]: Asp-Arg-Met-Gly-Arg-Ser-Glu-Val-Glu. Most of the radioactivity was recovered in the first cycle of sequencing, indicating either that the ester bond was completely unstable under sequencing conditions, or more likely that Asp-335 was the site of labelling. This is supported by the fact that the yield of Asp was 600 pmol, whilst the other 8 amino acids were detected at the nmol level. Since the modified aspartate would not be detected under the HPLC conditions used, the labelling therefore very probably occurred at Asp-335. Amino acid analysis of the radioactive peptide revealed the same composition as that derived from sequencing. As can be seen from Table I, the amino acid sequence around Asp-335 is highly conserved throughout evolution confirming its structural and/or functional importance for the CK molecule.

Table I

Comparison of amino acid residues 332–346 from all known CK sequences (numbering of amino acids according to chicken Mi_b-CK)

	332	*		*	*	346
(1)	S	NL	**D**	R M G	R S	E V E L V Q
(2)	S	NI	**D**	R I G	R S	E V E L V Q
(3)	S	NL	**D**	R L G	K S	E V E L V Q
(4)	S	NA	**D**	R L G	F S	E V E L V Q
(5)	S	NA	**D**	R L G	F S	E V E Q V Q
(6)	S	NA	**D**	R L G	S S	E V E Q V Q
(7)	S	NA	**D**	R L G	F S	E V E Q V Q
(8)a	S	NA	**D**	R L G	F S	E V E Q V Q
b	S	NK	**R**	R L G	L T	E L D A V T
(9)a	S	NL	**D**	R L G	T S	E V Q Q V Q
b	S	NL	**D**	R L G	S S	E V Q Q V Q
c	S	NS	**D**	R I G	F S	E V H L V Q

Amino acid residue 335, which was labelled by ClRATP in Mi_b-CK, is written in bold letters. The three acidic residues (Asp and Glu) conserved throughout evolution are marked by stars. The ClRATP-labelled Mi_b-CK nonapeptide obtained by pepsin digestion is underlined.

(1) chicken leg muscle Mi-CK (Mi_b-CK) [19]; (2) human heart Mi-CK [22]; (3) human placenta Mi-CK [20]; (4) chicken [14], rabbit [12], rat [10], human [17,18], and dog B-CK [15], *Xenopus* CK-IV [21]; (5) chicken M-CK [7]; (6) rabbit [5], rat [6], mouse [11], human [16], and dog M-CK [13]; (7) *Torpedo marmorata* CK [9] and *Torpedo californica* CK [8]; (8) two corresponding regions (a,b) within the amino acid sequence of the ATP-guanidino phosphotransferase (showing CK activity) of *Schistosoma mansoni*, where a duplicated gene is expressed as a contiguous dimer [23]; (9) three corresponding regions (a,b,c) within the amino acid sequence of the creatine kinase from sea urchin spermatozoa tails, where a triplicated gene is expressed as contiguous CK trimer [24].

4. DISCUSSION

Inspection of all 19 CK sequences known up to date reveals that none of the criteria used for identifying nucleotide binding domains in proteins is applicable to CK. There is no glycine-rich bend (GxggxG) which forms the phosphate binding loop in a variety of enzymes [34], and there is no β-sheet ending in an α-helix which could be a candidate for a specific ATP binding site [34,35]. By using ClRATP as a label for the active site of Mi_b-CK, more information was sought about the amino acid residues involved in substrate binding in the hope of finding homologies to other nucleotide or creatine binding proteins.

The ClRATP derivative is such that the reactive label is attached to the γ-phosphate group of ATP. When the nucleotide substrate is bound to the CK molecule, this is the position usually occupied by a Mg^{2+} ion coordinating the oxygen atoms of the α-, β- and γ-phosphate groups of ATP [26]. Because the label attached to the γ-phosphate group in ClRATP is rather large and bulky, two possibilities for the attachment site of the ATP analogue must be considered.

(i) ClRATP binds to an amino acid residue involved in the binding of the Mg^{2+} ion. Support for this interpretation comes from structural comparisons with adenylate kinase, the crystal structure of which is known [36]. NMR studies with Cr^{3+}-ATP as the

nucleotide substrate suggested that in adenylate kinase the metal ion coordinated to the ATP comes into close contact with His-189 [37]. This histidine is part of an α-helix between residues 179 and 194 which in addition contains an acidic region. It seems very reasonable to assume that the aspartate and glutamate residues in this acidic region are involved in the binding of the metal ion. The region of Mi_b-CK labelled by ClRATP would be analogous to the acidic region of adenylate kinase and could therefore be the coordination site for the Mg^{2+} ion bound to the ATP. This interpretation is further corroborated by the high degree of conservation throughout evolution of the three aspartate and glutamate residues within the Mi_b-CK peptide labelled by ClRATP (Table I, marked by stars).

(ii) ClRATP binds to an amino acid residue involved in the binding of the guanidinium substrate. Since pH studies showed that an aspartate or glutamate residue of CK is involved in the binding of creatine [29] and since affinity labelling of CK with epoxycreatine led to the modification of an aspartate or glutamate residue [28], Asp-335 might be involved in the binding and proper orientation of the creatine molecule for catalysis. Asp-335 might bind to one of the two nitrogen atoms of the guanidinium group, attract the positive charge of this group and therefore render the other nitrogen atom more nucleophilic (for a discussion see [29]). Unfortunately, comparison of the labelled peptide around Asp-335 of Mi_b-CK with creatine amidinohydrolase, the only creatine binding protein besides CK for which the primary (and tertiary) structure is known [38], revealed no significant homology. The additional fact that the two enzymes have no secondary structure pattern in common indicates that the creatine binding sites of CK and creatine amidinohydrolase are likely to be markedly different.

Although both interpretations must be viewed as tentative, they can serve as working models for future mutagenesis studies which, together with work now in progress on the analysis of recently obtained Mi_b-CK crystals [39,40], are expected to bring about conclusive evidence for or against these two possibilities.

Using ATP-γ-*p*-azidoanilide, a photoactivatable ATP analogue structurally very similar to ClRATP (Fig. 1B), Vandest et al. [41] were able to specifically label one cysteine residue per subunit of rabbit muscle M-CK, seemingly Cys-278. Both Cys-278 and Asp-335 are therefore very likely to be at or near the substrate binding site, probably in close proximity to each other. This interpretation is in accordance with the NMR and EPR experiments of McLaughlin et al. [4] who demonstrated that the distance between a nitroxide label attached to Cys-278 and the nucleotide and guanidine substrates is only 7–8 Å.

The findings that Cys-278 [25,26], Cys-69, Lys-191 [27], and Asp-335 (this study) all are near the active site of creatine kinase indicates that the substrate binding site of CK is formed by a complex refolding of the polypeptide chain. The structural and/or functional importance of the four amino acid residues for CK activity is further corroborated by the high degree of sequence conservation around each of these residues (for Asp-335 see Table I). Interestingly, digestion of M-CK [42,43] as well as Mi_b-CK (M. Wyss, unpublished results) with proteinase K, which selectively cleaves the polypeptide chain between Ala-328 and Ala-329 in M-CK and between Ala-324 and Val-325 in Mi_b-CK, respectively, leads to complete inactivation of the CK isoenzymes, although the two parts of the molecule remain associated with each other. This further indicates that the C-terminal 50 amino acid residues of the CK molecule in general and Asp-335 in particular are essential for the enzymatic mechanism.

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