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Accelerated Publications

In Vitro Mutagenesis Studies at the Arginine Residues of Adenylate Kinase. A Revised Binding Site for AMP in the X-ray-Deduced Model†

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ABSTRACT: Although X-ray crystallographic and NMR studies have been made on the adenylate kinases, the substrate-binding sites are not unequivocally established. In an attempt to shed light on the binding sites for $MgATP^{2-}$ and for AMP^{2-} in human cytosolic adenylate kinase (EC 2.7.4.3, hAK1), we have investigated the enzymic effects of replacement of the arginine residues (R44, R132, R138, and R149), which had been assumed by Pai et al. [Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37–45] to interact with the phosphoryl groups of AMP^{2-} and $MgATP^{2-}$. With use of the site-directed mutagenesis method, point mutations were made in the artificial gene for hAK1 [Kim, H. J., Nishikawa, S., Tanaka, T., Uesugi, S., Takenaka, H., Hamada, M., & Kuby, S. A. (1989) *Protein Eng. 2*, 379–386] to replace these arginine residues with alanyl residues and yield the mutants R44A hAK1, R132A hAK1, R138A hAK1, and R149A hAK1. The resulting large increases in the $K_{m,app}$ values for AMP^{2-} of the mutant enzymes, the relatively small increases in the $K_{m,app}$ values for $MgATP^{2-}$, and the fact that the R132A, R138A, and R149A mutant enzymes proved to be very poor catalysts are consistent with the idea that the assigned substrate binding sites of Pai et al. (1977) have been reversed and that their ATP-binding site may be assigned as the AMP site.

Adenylate kinase (EC 2.7.4.3, AK), a small and ubiquitous enzyme, catalyzes a phosphoryl-transfer reaction between $MgATP^{2-}$ (or $MgADP^{-}$) and uncomplexed AMP^{2-} (or uncomplexed ADP^{3-}). This enzyme has two distinct nucleotide substrate binding sites: a highly specific one for AMP and a less specific site for MgATP (Noda, 1973). Although three-dimensional X-ray diffraction measurements of AK [from porcine muscle (Sachsenheimer & Schulz, 1977; Dreusicke et al., 1988) and from yeast (Egner et al., 1987)] have been conducted, the substrate-binding sites are still a controversial issue. The substrate-binding sites, suggested by X-ray diffraction studies (Pai et al., 1977; Egner et al., 1987; Dreusicke et al., 1988), by NMR analysis [Fry et al., 1985, 1987; cf. Hamada et al. (1979) and Kuby et al. (1989)], and by molecular mechanics calculations (Caldwell & Kollman, 1988), differ from one another. The proposed binding sites from these studies do not satisfactorily provide a common nucleotide binding sequence motif, especially in the glycine-rich flexible loop region, which has been proposed as a sequence diagnostic of the nucleotide phosphate binding site (Möller & Amons, 1985; Fry et al., 1986). However, AK is often cited as an example of a typical ATP-binding protein when structural topological comparisons or amino acid homology comparisons are made of the nucleotide-binding proteins (Bradley et al., 1987; Garboczi et al., 1988; Taylor & Green, 1989).

Recently, we chemically synthesized the gene for the human cytosolic adenylate kinase (hAK1), expressed it in *Escherichia coli*, and demonstrated that Tyr-95 and Arg-97 were not directly involved in the binding of MgATP, from kinetic analysis of artificial mutant enzymes at these positions (Kim et al., 1989a). In this paper, we tested the X-ray-deduced model of Pai et al. (1977), by point mutation effects on apparent kinetic parameters of replacement of those arginine residues that are inclined toward the so-called "active center cleft" [as designated by Pai et al. (1977)]. These arginine residues appear to be conserved in the AK family [AK1, AK2, AK3, AKy, and AKe, as denoted by Schulz et al. (1986)], and they were replaced with alanyl residues (R44A, R132A, R138A, and R149A). The roles played by these arginine residues, which have been interpreted from their apparent kinetic parameters, made the original model of Pai et al. (1977) untenable, unless their binding site of AMP were interchanged with their ATP site.

Incidentally, a cursory examination was made of the role played by Asp-119. This residue was postulated by Fry et al. (1985, 1986), through an analogy to homologous systems

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(segment 3), as having a possible role in minimizing hydrolysis or in coordinating Mg^{2+}, even though there is no experimental evidence for a Mg^{2+}-binding site on the enzyme (Kuby et al., 1962). Asp-119 was replaced with an asparagine residue (D119N hAK1).

This suggestion, viz., that of an interchanged assignment for the ATP- and AMP-binding sites, is reconcilable with some other experimental findings on AK [e.g., X-ray studies (Egner et al., 1987; Dreusicke et al., 1988), NMR analyses (Smith & Mildvan, 1982; Fry et al., 1985), chemical modifications (Tagaya et al., 1987), and site-directed mutagenesis (Reinstein et al., 1988, 1989)]. In particular, one should note the suggestions of Smith and Mildvan (1982) and more recently of Egner et al. (1987). Although a model with the interchanged sites of Pai et al. (1977) will be proposed herein, one may carefully note that these data alone will not exclude the model of Fry et al. (1987), since any residues critical to the latter model were not examined. Preliminary reports have been presented (Kim et al., 1989b,c).

Materials and Methods

Materials. The Klenow fragment of *Escherichia coli* DNA polymerase I and DNA sequencing reagents were purchased from Takara Shuzo (Kyoto, Japan). [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from Amersham (Buckinghamshire, U.K.). Other enzymes were from the sources described previously by Kim et al. (1989a). Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems 380A DNA synthesizer (Nishikawa et al., 1987; Kim et al., 1989a).

Site-Directed Mutagenesis. The R132A, R138A, and R149A mutants were obtained by site-directed mutagenesis of the synthetic hAK1 gene (Kim et al., 1989a) essentially as described by Zoller and Smith (1982) with the mutagenic primers as follows: for R132A hAK1, d-TGCTGAAGGCCGGCGAAAC; for R138A hAK1, d-ACTTCGGGAGCCGTGGACG; for R149 hAK1, d-ATTAAGAAAGCTCTGGAAAC (mismatches are underlined). The single-stranded templates used for primer extension were prepared from M13mp19 carrying the *Kpn*I–*Sal*I fragment from the plasmid pAK (Kim et al., 1989a) coding hAK1 lacking the N-terminal 23 amino acid residues. The above synthetic primers were extended in vitro with the Klenow fragment of *E. coli* DNA polymerase I, and the DNA was used to transform *E. coli* JM109. The plaques obtained were screened by DNA sequencing according to the method of Sanger et al. (1977). The *Eco*RI–*Sal*I fragments containing the desired point mutation were recloned into the expression vector. The mutant enzymes R44A hAK1 and D119N hAK1 were obtained by DNA cassette mutagenesis of the hAK1 gene, as described by Kim et al. (1989a), with the oligonucleotide fragments for the mutations MU5A (d-ACTGGTGACCTGCTGGCCTCCGAAGTGAGC) and ML5A (d-AGAGCCGGAGCTCACTTCGGAGGCCAGCAG) for R44A hAK1 and MU13N (d-AACGCGGGCCCGGAGACTATGACTCGTCGC) and ML12N (d-GCCCGCGTTAACGTAGAGAAGCAGAGTCGG) for D119N hAK1 (underlines indicate the changed codons). The entire DNA sequence of the five mutant hAK1 genes was confirmed on the expression vectors by the dideoxy method (Sanger et al., 1977).

Enzyme Kinetics of the Forward Reaction (Hamada & Kuby, 1978). The initial velocity of the forward reaction (see eq 2) was measured by observing the absorbance decrease at 340 nm [for NADH, in the coupled-enzyme reaction with pyruvate kinase (PK) and lactate dehydrogenase (LDH)] to monitor the ADP formation at 25 °C, as previously described (Kim et al., 1989a). The reaction mixture (1 mL) contained 75 mM triethanolamine hydrochloride (pH 7.5), 120 mM KCl, 0.2 mM NADH, 0.3 mM phosphoenolpyruvate, 0.3 mg/mL bovine serum albumin, 10 units of LDH, and 5 units of PK plus varying and calculated amounts of $MgSO_4$, AMP, and ATP. The calculations to correct for Mg^{2+} complexation with AMP and with ATP were made as given by Hamada and Kuby [1978; cf. Kuby (1989)], employing a fixed concentration of free magnesium of 1 mM Mg^{2+}. The concentrations of $MgATP^{2-}$ and AMP^{2-} were as follows: for the R44A, R132A, R138A, and R149A mutant enzymes, 0.1, 0.2, 0.5, 1.0, and 2.0 mM $MgATP^{2-}$ and a single fixed concentration of 5 mM AMP^{2-} were used in the determination of $K_{m,app}^{MgATP^{2-}}$, and 0.5, 1, 2, 3, and 5 mM AMP^{2-} and a single fixed concentration of 2 mM $MgATP^{2-}$ were used in the determination of $K_{m,app}^{AMP^{2-}}$; for D119N hAK1, 0.05, 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mM $MgATP^{2-}$ at a fixed concentration of 1 mM AMP^{2-} were used in the determination of $K_{m,app}^{MgATP^{2-}}$, and 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mM AMP^{2-} at a fixed concentration of 1 mM of $MgATP^{2-}$ were used in the determination of $K_{m,app}^{AMP^{2-}}$. The reaction was initiated by addition of suitable amounts of recombinant hAK1s (wild type and D119N mutant, 5–10 ng; R44A mutant, 68 ng; R132A, R138A, and R149A mutants, 130–160 μg). When the concentration of the paired fixed substrate was not at saturation, the apparent V_{max} was corrected (eq 1; see Table I), because inhibition sets in at a

$$V_{max,cor,app} = V_{max,app}\frac{S_{0,fixed} + K_{m,app}^{fixed\,S}}{S_{0,fixed}} \quad (1)$$

substrate concentration higher than that which could be employed [e.g., Hamada and Kuby (1978)]. The Michaelis constants (K_m) for $MgATP^{2-}$ and AMP^{2-} and the k_{cat} (V_{max}/E_t) values were determined with the use of double-reciprocal plots (Lineweaver & Burk, 1943). However, in this preliminary kinetic analysis, although control was exercised over the metal complex species involved, the analysis was restricted to one fixed high value of the paired substrate and accordingly did *not* permit an evaluation of the intrinsic dissociation constants for the individual substrates in the binary enzyme–substrate complex (K_s) nor in the ternary enzyme–substrate complex (K_s) [see Hamada and Kuby (1978)]. For this reason, the K_m and V_{max} values are designated as $K_{m,app}$ and $V_{max,app}$.

CD spectra were recorded on a JASCO J-500 spectropolarimeter. Spectra were determined at protein concentrations of 0.05–0.5 mg/mL dissolved in 0.05 mM imidazole–0.01 mM EDTA–0.5% glycerol in quartz cells with path lengths ranging from 0.1 to 1 cm for the far- and near-UV regions. Other methods, e.g., the purification of the mutant enzymes on phosphocellulose and on Sephadex G-75 and the determination of the protein concentration (by use of its UV molar absorption coefficient), have been previously described (Kim et al., 1989a).

Results

The mutant enzymes (R44A hAK1, R132A hAK1, R138A hAK1, R149A hAK1, and D119N hAK1) were all purified to homogeneity by a standard procedure (Kim et al., 1989a), since these enzymes possessed basically the same chromatographic elution patterns as the wild-type enzyme. SDS–PAGE (12.5%) analysis (Laemmli, 1970) of the purified enzymes showed single bands with the same mobility as that of wild-type hAK1 (Figure 1). Circular dichroism (CD) spectra in

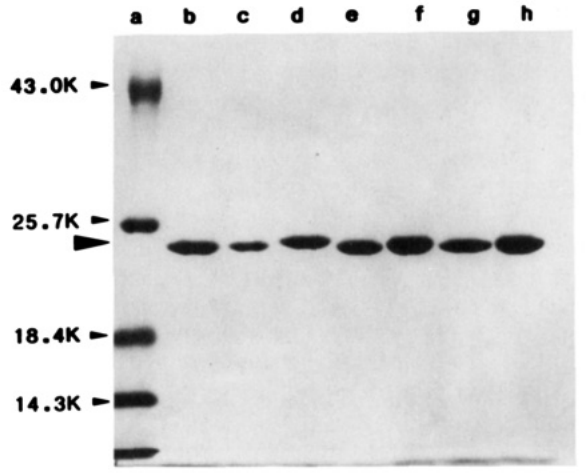

FIGURE 1: Analysis of purified wild-type and mutant hAK1s by SDS–PAGE (12.5%). Lane a, size markers, the molecular masses of which are shown in kDa; lane b, wild-type hAK1; lanes c–h, mutant hAK1s (R44A, R97A, R132A, R138A, R149A, and D119N, respectively). 1 μg of each protein was applied.

the 200–250-nm region of the wild-type and the mutant hAK1s were comparable with that reported for porcine AK1 (Yazawa & Noda, 1976); apparently, the secondary structures of these enzymes were not significantly altered by the mutation (data not shown).

Kinetic Analysis of Mutant hAK1. Adenylate kinase catalyzes the following reversible reaction:

$$MgATP^{2-} + AMP^{2-} \rightleftarrows MgADP^{-} + ADP^{3-} \qquad (2)$$

(Noda, 1973; Kuby, 1989). In this study, kinetic analysis was performed only on the forward reaction, but under conditions where metal (Mg^{2+}) complexation by the nucleotide substrates was quantitatively considered [Hamada & Kuby, 1978; cf. Kuby (1989)]. The apparent kinetic parameters of the steady-state enzyme kinetics for the forward reaction are summarized in Table I. For comparison, the previously reported kinetic data for the wild-type hAK1 and R97A hAK1 (Kim et al., 1989a) were reevaluated by this slightly more sophisticated analysis and are also included in Table I. However, as mentioned above, in this preliminary method of data analysis, the intrinsic dissociation constants for each substrate in the binary ($\bar{K}_s$) and in the ternary enzyme complexes (K_s) were not evaluated. The method of data processing that had been employed earlier for the rabbit muscle enzyme (Hamada & Kuby, 1978) did permit such an evaluation of both $\bar{K}_s$ and K_s values, which, as they pointed out, would differ in value if substrate-induced conformational changes took place in the ternary complexes. If warranted, a more detailed kinetic analysis will be carried out at a later date.

The $K_{m,app}$ values for AMP^{2-} (Table IA) of R44A hAK1 and R138A hAK1 markedly increased ca. 44- and 21-fold, respectively, while those for $MgATP^{2-}$ (Table IB) in the case of both mutant enzymes show much smaller changes (4- and 1.3-fold, respectively) compared to that of wild-type hAK1. On the other hand, in the case of R132A hAK1, the $K_{m,app}$ values increase ca. 13-fold for AMP^{2-} and 18-fold for $MgATP^{2-}$ and, in the case of R149A hAK1, 18-fold for AMP^{2-} and 8-fold for $MgATP^{2-}$. However, in the case of mutant D119N, the $K_{m,app}$ values for both AMP^{2-} and $MgATP^{2-}$ are nearly the same as that of the wild-type hAK1 (2.1- and 0.6-fold, respectively). These results strongly suggest that in the transition state Arg-44 and Arg-138 make closer contact with the substrate AMP^{2-} than with $MgATP^{2-}$; Arg-132 makes a similar degree of contact with both $MgATP^{2-}$ and AMP^{2-}; Arg-149, by contrast, makes closer contact with AMP^{2-} than with $MgATP^{2-}$, whereas Asp-119 may only appear to show little direct interaction with either AMP^{2-} or $MgATP^{2-}$.

Table I: Summary of Kinetic Parameters of Wild-Type hAK1 and Mutant hAK1s

enzyme	$K^{AMP^{2-}}_{m,app}$ (mM)	k'_{cat} (s^{-1})[a]	$k'_{cat}/K^{AMP^{2-}}_{m,app}$ (s^{-1} M^{-1})	relative value (%)[b]
	(A) AMP^{2-} as Variable Substrate			
wild type	0.16 (1.0)[c]	7.51×10^{2}	4.7×10^{6}	100.0
R44A	6.99 (43.7)	1.67×10^{2}	2.4×10^{4}	0.5
R97A	0.34 (2.1)	1.43×10	4.2×10^{4}	0.9
R132A	2.09 (13.1)	5.57×10^{-2}[d]	2.7×10	5.7×10^{-4}
R138A	3.28 (20.5)	1.57×10^{-2}	4.8	1.0×10^{-4}
R149A	2.92 (18.3)	1.08×10^{-1}	3.7×10	7.8×10^{-4}
D119N	0.34 (2.1)	8.37×10^{2}	2.5×10^{6}	53.0
	(B) $MgATP^{2-}$ as Variable Substrate			
wild type	0.10 (1.0)[c]	1.10×10^{3}	1.1×10^{7}	100.0
R44A	0.41 (4.1)	1.64×10^{2}[d]	4.0×10^{5}	3.6
R97A	0.02 (0.2)	1.28×10	6.4×10^{5}	5.8
R132A	1.84 (18.4)	3.87×10^{-2}	2.1×10	1.9×10^{-4}
R138A	0.13 (1.3)	1.16×10^{-2}	8.9×10	8.1×10^{-4}
R149A	0.79 (7.9)	9.57×10^{-2}[d]	1.2×10^{2}	1.1×10^{-3}
D119N	0.06 (0.6)	6.46×10^{2}	1.1×10^{7}	100.0

[a] For calculation of k'_{cat} a molecular weight of 21 700 was employed. [b] The relative values of $k'_{cat}/K_{m,app}$ to the wild-type hAK1 value, which is taken as 100%. [c] Figures in parentheses indicate the relative change compared to the wild-type hAK1 value, which is taken as 1.0. [d] To correct to "saturation" of the fixed substrate, V_{max} was extrapolated according to eq 1, which is defined under Materials and Methods; these corrected values were used to calculate the k_{cat} values.

As shown in Table I, the k_{cat} values for AMP^{2-} and for $MgATP^{2-}$ of R132A, R138A, and R149A hAK1s are drastically reduced to below 0.01%, whereas, in the case of the other mutants, R44A hAK1 and R97A hAK1, these values are reduced to 20 or 2% for AMP^{2-} and 15 or 1% for $MgATP^{2-}$ as the substrate. In the case of D119N hAK1, k_{cat} values are 110% for AMP^{2-} and 58% for $MgATP^{2-}$ compared with that of wild-type hAK1. Arginines 132, 138, and 149, therefore, appear to be important residues for the catalytic activity of the human adenylate kinase. The role played by Arg-44 can be more clearly understood by a comparison of R44A hAK1 with R97A hAK1, which have nearly the same, i.e., within a factor of 2, catalytic efficiencies, $k_{cat}/K_{m,app}$. The change in the $k_{cat}/K_{m,app}$ value for R44A hAK1 compared to wild type is predominantly caused by an increase in the $K_{m,app}$ term, whereas for R97A hAK1 it is the result of a decrease in the k_{cat} term. Moreover, the observed small reduction in k_{cat} of R44A hAK1 (4–7-fold) (Table I) suggests that Arg-44 is *not* directly involved in catalysis. Therefore, from the 40-fold increase in $K_{m,app}$ for AMP^{2-}, Arg-44 may possibly be involved in AMP binding and not in catalysis. Since Arg-138 appears to be involved in both catalysis and in AMP binding, in the transition state, one may tentatively assign Arg-138 and Arg-44 to the AMP substrate binding site of Pai et al. (1977).

From the results obtained in this study and from consideration only of the model originally proposed by Pai et al. (1977) for the X-ray crystal structure of porcine muscle adenylate kinase (Sachsenheimer & Schulz, 1977), one may propose the following change in the model of Pai et al. (1977), for the substrate-binding sites of AK. Thus, when viewed from the direction as illustrated in Figure 2, MgATP would bind at the left side of the enzyme and span to Arg-132, and AMP would bind at the right side of the enzyme, with the hydrophobic pocket containing Arg-44 and Arg-138. Arg-138 might act as a lid for this pocket by hydrogen bonding with the phosphoryl group of AMP.

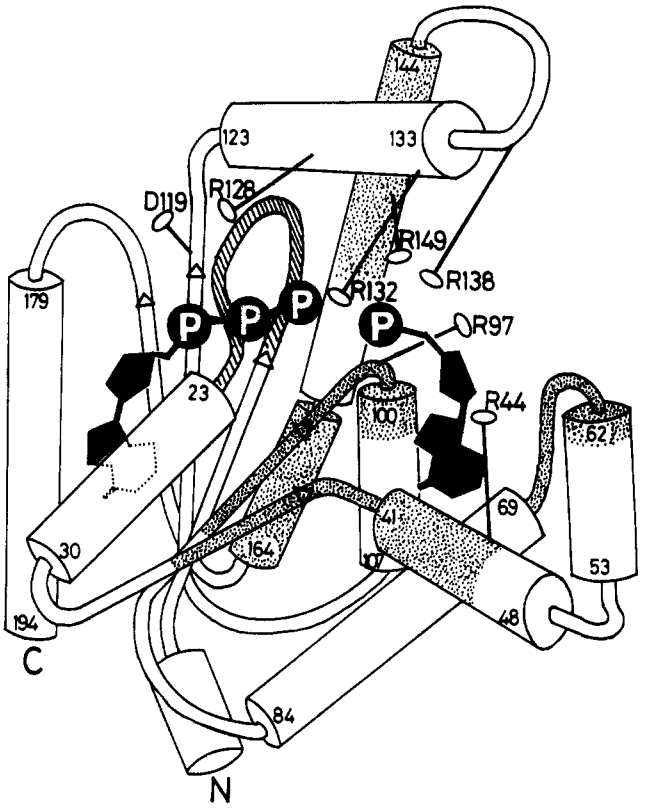

FIGURE 2: A proposed model of the binding sites for AMP and MgATP in adenylate kinase which has been constructed by interchanging the substrate-binding sites originally proposed by Pai et al. (1977) with some modifications (see text). The model is presented in a schematic form of crystal form A (the substrate-bound form of AK1) of crystalline porcine AK1, which, in turn, is taken in modified form from the X-ray studies of Sachsenheimer and Schulz (1977) [see also Pai et al. (1977)]. The side chains of Arg-44, -97, -128, -132, -138, and -149 and Asp-119 are depicted, and the direction of the arginine residues, which have been replaced by alanyl residues in the mutant enzymes, is based on the refined X-ray structure of porcine AK1 at 2.1-Å resolution (Dreusicke et al., 1988; Dreusicke & Schulz, 1988). The dotted areas indicate the postulated regions of a high degree of homology among the AK families, which may constitute the substrate-binding site for AMP; the glycine-rich flexible loop, which is also a region of a high degree of homology, is shown as hatched in the figure.

Discussion

In the AK family [AK1, AK2, AK3, AKe, and AKy, where the designations are given in Schulz et al. (1986) and e and y stand for the *E. coli* and the yeast enzyme, respectively], the following arginine residues are conserved: Arg-44, Arg-128, Arg-132, Arg-138, and Arg-149 (Haase et al., 1989). These residues are *presumed*, from the X-ray crystal studies, to participate in phosphoryl binding and to form a solvent-protected phosphoryl-transfer region [for yeast AKy, see Egner et al. (1987); for porcine AK1, see Dreusicke et al. (1989) and Pai et al. (1977)]. The catalytic region proposed by NMR analysis is a distal solvent-exposed one located on the opposite side of the arginine-lined cleft with the glycine-rich loop acting as the boundary between them (Fry et al., 1985, 1987; Mildvan & Fry, 1987).

As a test of the Pai et al. (1977) model, however, a replacement of the critical residues, Arg-44, -132, -138, and -149, with an alanyl residue in their so-called "catalytic cleft" would permit some ideas as to the correct assignments of the substrate-binding sites.

Kinetic studies on the forward reaction with R44A, R132A, R138A, R149A, and D119N hAK1s (see Results and Table I) have led to the following conclusions. (1) Arg-44 would more likely be located at the AMP-binding pocket in the Pai et al. (1977) model to maintain the proper geometry of the binding site, but it would not appear to be a catalytically required residue. (2) Arg-132 would appear to be an essential residue for catalysis, interacting largely with $MgATP^{2-}$ and only partially with AMP^{2-}. (3) Arg-138 would also appear to be an important residue for catalysis and would apparently interact with only AMP^{2-}. (4) Arg-149 would appear to interact largely with AMP^{2-} and only partially with $MgATP^{2-}$ and would also appear to be important for the catalytic action of the enzyme. (5) By the preliminary simplified kinetic analysis employed here, Asp-119 does not appear to be involved in substrate binding or in catalysis.

After taking into consideration the loci of these arginine residues in the X-ray crystal structure of porcine AK1 [Sachsenheimer & Schulz, 1977; see Pai et al. (1977)], the model as originally proposed by Pai et al. (1977) appears to be inconsistent with points 1 through 4. If, however, one interchanges the assigned positions of ATP and AMP (as shown in Figure 2), the revised model becomes compatible with the data presented.

As mentioned above, Egner et al. (1987), from an X-ray study on yeast AK_y with its inhibitor P^1,P^2-bis(5′-adenosyl)-pentaphosphate (Ap_5A), proposed interchanging the substrate-binding locations. Moreover, Dreusicke et al. (1988), by X-ray crystallographic analysis at 2.1-Å resolution on porcine AK1, determined that the guanidinium groups of Arg-138 and Arg-149 contact a sulfate ion designated as 2 and that of Arg-132 contacts a sulfate ion designated as 1, but only weakly with sulfate ion 2. The bound sulfate ions were assumed to mimic the phosphoryl groups of the substrates. Therefore, the binding sites for MgATP and AMP have been depicted (Figure 2) by taking into consideration our kinetic data and the suggestions from the above X-ray crystallographic studies. In Figure 2, the binding site for the base and the sugar of AMP is located at the "adenosine B" site of Ap_5A (Egner et al., 1987) and that of MgATP is placed at the AMP-binding site proposed by Pai et al. (1977). The α-phosphate of AMP and γ-phosphate of ATP are set in the positions of the bound sulfate ions 2 and 1, respectively (Dreusicke et al., 1988). In this model, the α- and β-phosphate groups of ATP are likely to go around the glycine-rich loop in a fashion similar to that of Ap_5A in yeast AKy (Egner et al., 1988) and not through it.

Moreover, in this binding mode, a similar topology exists at the glycine-rich loop as in those of other GTP/GDP-binding proteins such as EF-Tu (La Cour et al., 1985) and ras p21 (Tong et al., 1989).

Earlier NMR studies by Smith and Mildvan (1982) had pointed out that the substrate-binding sites proposed by the X-ray study of Pai et al. (1977) were incorrectly assigned, and they suggested an alternative arrangement of ATP and AMP which would provide a better fit to their data. Fry et al. (1985, 1987) proposed a model based on NMR data, where the conformations of both of the substrates and the loci of both binding sites were, in turn, based on measurements of intra- and interproton distances with the paramagnetic probe–T_1 method and by time-dependent nuclear Overhauser effects in the native enzyme and in the synthetic peptide I_{1-45}. At present, their proposed MgATP-binding site is regarded as the most appropriate structure. If, however, in the model of Fry et al. (1985), the metal triphosphate group were rotated so as to be located in the so-called catalytic region of Pai et al. (1977), then an alternate orientation is possible. In this alternate proposal, the binding sites of MgATP and AMP as

proposed by Pai et al. (1987) would be interchanged (Figure 2), and we have an arrangement which would also satisfactorily explain the high degree of homology among the AK family. It has long been known that the AK family shows a higher degree of substrate specificity for AMP than for ATP (Noda, 1973), and as shown in Figure 2, there would be many highly conserved homologous regions (Haase et al., 1989; Schulz et al., 1986), which might cooperatively constitute the substrate-binding site for AMP (dotted region in Figure 2). Moreover, this pocket had been postulated as occupied by an adenosine moiety in all the X-ray studies reported to date. In this model, the binding site for ATP can now satisfy the homology requirements and also the topology requirements in the glycine-rich flexible loop, i.e., as a phosphate-binding region, in common with the other nucleotide-binding proteins. This glycine-rich loop is ubiquitous among the ATP- and GTP-binding proteins and is considered to be a sequence diagnostic of the nucleotide phosphate binding site (Möller & Amons, 1985; Fry et al., 1986). Our results would also be in agreement with some other chemical modification results and some site-directed mutagenesis observations (Crivellone et al., 1985; Tagaya et al., 1987; Reinstein et al., 1988, 1989).

In conclusion, by the data presented here, one may propose that the substrate-binding sites of adenylate kinase are *not* consistent with that originally proposed by Pai et al. (1977); however, with an interchange in the assignments for the ATP- and AMP-binding sites, the model of Pai et al. (1977) can be made to be consistent with the data presented herein. One should take note, however, that these data, by themselves, may not rule out the model proposed by Fry et al. (1987).

Other mutants with altered AMP-binding properties are currently under investigation, and future work should be directed toward a more detailed examination of the model of Fry et al. (1987).

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