Mechanism of Adenylate Kinase. Structural and Functional Roles of the
Conserved Arginine-97 and Arginine-132†

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ABSTRACT: The structural and functional roles of two conserved active site residues, Arg-97 and Arg-132, in
chicken muscle adenylate kinase (AK) were evaluated by site-directed mutagenesis in conjunction with one-
and two-dimensional proton nuclear magnetic resonance (NMR), kinetics, and guanidine hydrochloride-induced
renaturation. In addition, 31P NMR analysis was used to evaluate the contribution of Arg-97 to the phosphorus
termspecificity of AK. The results and conclusions are summarized as follows: (i) Kinetic analysis of R97M
reveals 6- and 28-fold increases in the dissociation constant K and Michaelis constant K of AMP, respectively,
and a moderate 30-fold decrease in kcat. The K and V values of MgATP are relatively unaltered. The localized
effect of AMP stabilization was independently confirmed by proton NMR titration, which showed a ca. 20-fold
increase in the dissociation constant of AMP but not of MgATP. (ii) R132M affords a dramatic decrease in kcat
by a factor of 8.0 × 103, with unchanged dissociation and Michaelis constants for either substrate. The lack of
perturbation in the affinities toward substrates was confirmed by proton NMR titration. (iii) Although small chemical shift changes were observed for the free mutants and their complexes with substrates, further analyses by nuclear Overhauser enhanced
spectroscopy with the bisubstrate analogue inhibitor, Pβ,Pβ-bis(5'-adenosyl)pentaphosphate (APβP), indicated
little perturbation in the global conformation. (iv) Contributions to conformational stability by Arg-97 and
Arg-132 are negligible on the basis of the free energy of unfolding, ∆GoH2O. (v) R97M was predicted and
demonstrated to exhibit enhanced stereospecificity at the AMP site by at least 10-fold relative to WT in the
conversion of adenosine 5'-monophosphate to adenidine 5'-1-thiophosphate. This result for R97M was
predicted on the basis of the orientation of Arg-97 relative to Arg-44 and AMP in the active site as observed
in available crystal structures and the stereospecificity results of R44M [Jiang, R.-T., Dahnke, T., & Tsai, M.-D.
conclude that Arg-97 interacts with the phosphoryl group of AMP, beginning at the binary complex (1–2 kcal/mol), continuing through the transition state (3.5 kcal/mol), and that Arg-132 stabilizes the transition state by greater than 5 kcal/mol. (vii) The functional importance of Arg-97 appears to be
similar to that of Arg-44 [Yan, H., Dahnke, T., Zhou, B., Nakazawa, A., & Tsai, M.-D. (1990) Biochemistry
29, 10956–10964]. The results for R97M also clarify conflicting reports from analogous mutants in other
types of AK and support conclusions based upon analysis of the mitochondrial matrix AK-AMP crystal

While significant progresses have been made on the
structure–function relationship of adenylate kinase (AK) as reviewed recently by Tsai and Yan (1991), the functional roles of most active site residues remain to be established. The best characterized region of substrate sites is the phosphate binding region. Crystal structural analyses of the MgAP₆A complexes of yeast AK (AKy) (Egner et al., 1987) and Escherichia coli
AK (AKe) (Müller & Schulz, 1988) suggest that the phosphates are surrounded by several conserved arginine residues. The structure of the AK1-MgAP₆A complex has not been reported; however, Egner et al. (1987) showed an overlay of the structure of free AK1 with that of AKy-MgAP₆A, as shown in Figure 1. The numbering of residues in Figure 1 is according to the "family numbering system" (Schulz et al., 1986); the arginine residues of AK1 addressed in this paper, 44, 97, 132, 138, and 149, correspond to residues 53, 106, 141, 178, and 189, respectively, in the family numbering system.

While the structure of AKy-MgAP₆A best represents the current knowledge on the substrate sites of AK, we consider it as a starting point to probe the quantitative structure–function relationship of AK.

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1 Abbreviations: ADP, adenosine 5'-diphosphate; ADPβS, adenosine 5'-(1-thiophosphate); AK, adenylate kinase; AMP, adenosine 5'-monophosphate; AMPS, adenosine 5'-monophosphophate; APβP, APβ, Pβ,Pβ-bis(5'-adenosyl)pentaphosphate; ATP, adenosine 5'-triphosphate; APTβS, adenosine 5'-(1-thiothiophosphate); 1D, one-dimensional; 2D, two-dimensional; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; Gdn-HCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl
sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UV, ultraviolet; WT, wild type.

2 The AK from different sources are abbreviated as follows: from muscle, AK1 (followed by the letters c, h, p, and r designating chicken, human, porcine, and rabbit, respectively); from E. coli, AKc; from yeast, AKf; from mammalian mitochondrial intermembrane space, AK2; from mammalian mitochondrial matrix, AK3. Unless otherwise specified, the numbering system used in this paper is the conventional system for AK1. Although cAK has one additional residue near the N-terminus (Kishi et al., 1986), the Met-1 residue is absent in the cAK expressed in E. coli (Tanizawa et al., 1987). This makes numbering of cAK consistent with other AK1.
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R9TM/R140S. Upon this finding, the single mutant at position 97 was reconstructed from the WT gene as described above. R97M was purified and assayed as described by Tien et al. (1988), except for a 0-500 mM linear NaCl gradient rather than the 0-500 mM linear NaCl phosphate column. R132M exhibited weak binding to the phosphocellulose resin and was consequently purified by Blue Sepharose and Sephadex G-100 gel filtration chromatography. At this stage, endogenous E. coli AK was removed by fast performance liquid chromatography (FPLC) using a phenyl-Sepharose CL-4B column pre-equilibrated with a buffer consisting of 30 mM Tris, 1 mM DTT, 1.8 M NaCl, SO4, and 1 mM EDTA, pH 7.7. The pure enzyme was eluted with a gradient of 250-500 mM Tris, 1 mM DTT, and 1 mM EDTA, pH 7.7, and treated as described by Tien et al. (1988).

The purity of each preparation was checked by SDS-PAGE and confirmed to be 95% or greater. The purity of the preparations was determined by scanning the Coomassie blue-stained gel in a Bio-Rad video densitometer and comparing the product bands to the AK standard. No endogenous AK was detected.

Proton NMR Methods. Proton NMR experiments were performed on Bruker AM-500 NMR spectrometers. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4. One- and two-dimensional NMR experiments, sample preparation, and titration procedures were essentially the same as previously described (Yan et al., 1990). Briefer, the buffer was composed of 20 mM potassium glutamate, 15 mM KCl, 2 mM DTT, and 0.5 mM EDTA, pH 7.8 (mM) meter reading without correcting for deuterium isotope contributions. The enzyme concentration was about 1 mM for 1D NMR experiments and 2.5 mM for 2D NMR experiments, and the temperature was 27 °C. For 2D NMR experiments, 90° and phase cycling were used for NOESY experiments (Bodenhausen et al., 1984), with a mixing time of 200 ms. The sweep widths were 11.5 ppm. A total of 256-512 transients were accumulated in a 1000 Hz bin in the time domain in most cases, and were zero-filled to a 4096 x 1024 matrix prior to multiplication by a Gaussian function (L = 3, G = 0.1) and Fourier transforms.

Phosphor NMR Methods. P-31 NMR experiments were performed on Bruker AM-250 and 300 NMR spectrometers at 30 °C. All chemical shifts were referenced to external 85% H3PO4. All spectra were broad-band decoupled with the WALTZ sequence. The spectral width was 75 ppm, and 16-32 data points were recorded for each spectrum in the quadrature detection mode. A 45° pulse and a 0.1-0.2-s delay relaxation were used. Acquisition times ranging from 1.5-2.0 s were used. Repetition times were 2.0-2.5 s, and 1000-2000 transients were obtained for each spectrum. A 2D increase of 2.8 ppm and 2.8 kHz was applied in the time domain prior to Fourier transformation. Unless otherwise noted, only the regions of the P resonances are shown in the spectra.

Assignment and Quantitation of Isomers. The assignment of components within the reaction mixture was accomplished by addition of [1-13C] leucine to the sample, whose relative chemical shifts are in agreement with those reported previously (Shen & Frey, 1977; Jaffe & Cohn, 1978). It should be noted that the chemical shift values of phosphorion resonances are equiproportional to and are independent of the ionic strength (Jaffe & Cohn, 1978). As a result, minor differences in chemical shift for the same species may occur as the sample conditions change under the conditions from reaction to reaction. The intensities of various components described in the text have been measured by cutting and weighing from greatly expanded spectra.

Steady-State Kinetics. The kinetic properties were evaluated by monitoring ADP formation with pyruvate kinase/lactate dehydrogenase as the coupling system (Rhaods & Lowenstein, 1968). The details have been described previously (Tian et al., 1988). The kinetic parameters were obtained by varying both AMP and MgATP concentrations and the data analyzed according to Cleland (1966):
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Figure 2: One-dimensional proton NMR spectra of the aromatic protons of WT (far left), R132M (left), R97M (right), and R97M/R149S (far right) as free (A), AMP complexed (B), and MgATP complexed (C). The spectra of WT are reproduced from Sanders et al. (1989). All spectra were obtained at 7.0 T, C7, and the FIDs were processed with a 1-Hz line broadening. The concentrations of free enzymes were ca. 1 mM for all cases. The millimolar concentrations of AK/AMP are 1.2/14 R132M, 1.0/10.7 R97M, and 0.3/10.5 R97M/R149S; the millimolar concentrations of AK/ATP/Mg\textsuperscript{2+} are 0.9/7.0/8 R132M, 0.8/4.9/11 R97M, and 0.4/5.7/12 R97M/R149S. Due to the low affinity of R97M and R97M/R149S, two mutants may not be saturated by AMP even with a 10-fold excess of AMP.

Table I: Summary of Steady-State Kinetic and Binding Data for WT, R132M, R97M, R97M/R149S, and R97M/R149S

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>WT*</th>
<th>R132M</th>
<th>R97M</th>
<th>R97M/R149S</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_{cat}</td>
<td>(s\textsuperscript{-1})</td>
<td>650</td>
<td>0.003</td>
<td>10 \times 10</td>
<td>0.003</td>
</tr>
<tr>
<td>K_{M,ATP}</td>
<td>(mM)</td>
<td>0.042</td>
<td>0.055</td>
<td>0.083</td>
<td>0.085</td>
</tr>
<tr>
<td>K_{M,AMP}</td>
<td>(mM)</td>
<td>0.009</td>
<td>0.009</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>K_{M,ATP} \textsuperscript{a}</td>
<td>(mM)</td>
<td>1.55 \times 10 \times 10</td>
<td>1.5 \times 10 \times 10</td>
<td>1.0 \times 10 \times 10</td>
<td>0.26 \times 10 \times 10</td>
</tr>
<tr>
<td>K_{M,AMP} \textsuperscript{a}</td>
<td>(mM)</td>
<td>1.66 \times 10 \times 10</td>
<td>1.6 \times 10 \times 10</td>
<td>2.4 \times 10 \times 10</td>
<td>0.24 \times 10 \times 10</td>
</tr>
<tr>
<td>K_{M,ATP} \textsuperscript{b}</td>
<td>(mM)</td>
<td>0.016</td>
<td>0.018</td>
<td>0.066</td>
<td>0.066</td>
</tr>
<tr>
<td>K_{M,AMP} \textsuperscript{b}</td>
<td>(mM)</td>
<td>0.037</td>
<td>0.017</td>
<td>2.22 (5)</td>
<td>2.22 (5)</td>
</tr>
</tbody>
</table>

*Titration Experiments with NMR

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>WT*</th>
<th>R132M</th>
<th>R97M</th>
<th>R97M/R149S</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_{M,ATP} \textsuperscript{a}</td>
<td>(mM)</td>
<td>0.17</td>
<td>0.52</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>K_{M,AMP} \textsuperscript{a}</td>
<td>(mM)</td>
<td>0.50</td>
<td>0.36</td>
<td>0.18</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*The kinetic data were obtained by varying concentrations of both substrates. Numbers in parentheses indicate the ratios of the mutant to WT. *The kinetic data for WT are from Tian et al. (1990); the K_{M} values for WT are from Sanders et al. (1989). *The kinetic data and K_{M} values for R149M are from Yan et al. (1990).

Table II: Summary of Conformational Stabilities for WT, R132M, R97M, and R97M/R149S

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>WT</th>
<th>R132M</th>
<th>R97M</th>
<th>R97M/R149S</th>
</tr>
</thead>
<tbody>
<tr>
<td>\Delta G\textsuperscript{a}</td>
<td>kcal/mol</td>
<td>4.5</td>
<td>5.3</td>
<td>7.0</td>
<td>5.3</td>
</tr>
<tr>
<td>\Delta G\textsuperscript{b}</td>
<td>kcal/mol</td>
<td>5.1</td>
<td>5.0</td>
<td>4.5</td>
<td>6.2</td>
</tr>
</tbody>
</table>

*Determined by monitoring guanine nucleotide-induced unfolding with circular dichroism, using the equation \Delta G = \Delta G_{\text{GTP}} - \Delta G_{\text{GDP-4-His}}

Figure 3: One-dimensional proton NMR spectra of the Mg\textsuperscript{2+} complexes of WT (A), R132M (B), and R97M/R149S (C). Peaks a-f are due to bound Mg\textsuperscript{2+} A-H2 (I); H2 (II); H3 (III); H4 (IV); H5 (V); H6 (VI). The spectrum of WT is reproduced from Yan et al. (1990). The millimolar concentrations of AK/AMP/Mg\textsuperscript{2+} are 0.2/3.0/0.3 (B) and 2.0/4.0/4.0 (C). The FIDs were processed with Gaussian multiplication (LB = 5, GB = 0.1). Intermediate exchange rates for spectrum B cause signal broadening and overlap of peaks a-f. It should be noted that the H2 free and H2 (free) resonances in spectrum B overlap H2 (II) and H2 (II) resonances, respectively, whereas H2 (free) resonates at 8.39 ppm. The three sets of adenosine resonances of bound and free forms are exchanged averaged beyond separation in spectrum C.

small, but they are somewhat more extensive than those observed for the binary complex. The peaks arising from H2, H3, and H4 of AP\textsubscript{A} (peaks a-f, assigned from NOEY spectra) differ only slightly between spectra A and B. The enzyme resonances of A and B differ more extensively in the regions of 0.9-0.9 ppm and 6.4-6.9 ppm.

For interpretation of whether differences in enzyme resonances reflect local or global conformational changes, NOEY spectra are compared between WT and R132M as shown in Figure 4 (panels A and B, respectively). A close analysis of the spectra reveals only minimal changes in the pattern of aromatic-aromatic and aromatic-alkaline cross-peaks. The chemical shifts of the aromatic spin systems have been partially assigned from the 1D and 2D NOEY spectra in analogy to the assignment of WT-Mg\textsuperscript{2+}AP\textsubscript{A} (Yan et al., 1990a) and are listed in Table III. There are only four aromatic resonances which differ (>0.02 ppm) in chemical shift: Fa, 0.06 ppm; Fd, 0.03 ppm; Fy, and Hc (His-16), 0.06 ppm. Large differences in Fa have been previously observed (Sanders et al., 1989; Yan et al., 1990a,b) and reflect the sensitivity of this resonance to mutation. The other differences represent minimal changes in the structure. Overall, the differences in the 1D and 2D spectra of the Mg\textsuperscript{2+}AP\textsubscript{A} complexes between R132M and WT, like those of R132M and R149S, are more extensive than those of the binary complexes, yet they are minor and indicate of localized rather than global conformational changes. This result, coupled with the NMR binding studies described in the next section, will allow a quantitative analysis of the kinetic data for R132M.

Also shown in Figure 3 is the 1D spectrum of the complex of Mg\textsuperscript{2+}AP\textsubscript{A} with the double mutant R97M/R149S. There is evidence that Mg\textsuperscript{2+}AP\textsubscript{A} is bound with weaker affinity to the double mutant, since the adenine protons (H2, H3, and H4), free and the two sets of bound, have exchange-averaged to only one set of signals. The 2D NOEY spectrum of the complex (Figure 5C) again indicates that the aromatic-aromatic and aromatic-alkaline cross-peaks have a similar pattern to WT and consequently a conservation in tertiary structure. Comparison of the aromatic-aromatic resonances with WT (Table III) reveals that only two resonances differ significantly with WT: Fa, 0.13 ppm; Hc, 0.09 ppm. The sub-

Figure 4: Partial NOEY spectra of the complexes of Mg\textsuperscript{2+}AP\textsubscript{A} and WT (A), R132M (B), and R97M/R149S (C) (same samples as used for Figure 3). The cross-peak f arises from exchange between the two H2 protons of bound Mg\textsuperscript{2+}AP\textsubscript{A}, while cross-peaks a-f arise from interresidue NOEYs, as described previously (Yan et al., 1990a,b); cross-peaks a-f are not detectable for (B) or (C).
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TABLE III: Chemical Shifts of the Aromatic Residues of the MgA/P Complex (pH 7.4)*

<table>
<thead>
<tr>
<th>spin axis</th>
<th>R133M-MgA/P</th>
<th>R197M/R140S-MgA/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>6.28 (±0.06)</td>
<td>6.05 (±0.03)</td>
</tr>
<tr>
<td>Fe</td>
<td>6.42</td>
<td>7.00</td>
</tr>
<tr>
<td>Fe</td>
<td>6.69</td>
<td>6.88</td>
</tr>
<tr>
<td>Fe</td>
<td>5.54 (±0.03)</td>
<td>7.20</td>
</tr>
<tr>
<td>Fe</td>
<td>4.41</td>
<td>7.15</td>
</tr>
<tr>
<td>Fe</td>
<td>5.61</td>
<td>6.62</td>
</tr>
<tr>
<td>Fe</td>
<td>6.75</td>
<td>7.03</td>
</tr>
<tr>
<td>Fe</td>
<td>6.65 (±0.04)</td>
<td>6.88 (±0.03)</td>
</tr>
<tr>
<td>Fe</td>
<td>6.92</td>
<td>7.76</td>
</tr>
<tr>
<td>Fe</td>
<td>6.96</td>
<td>7.35</td>
</tr>
<tr>
<td>Fe</td>
<td>7.69</td>
<td>7.69</td>
</tr>
<tr>
<td>Fe</td>
<td>7.92</td>
<td>7.75</td>
</tr>
<tr>
<td>Fe</td>
<td>8.00 (±0.04)</td>
<td>7.95 (±0.05)</td>
</tr>
<tr>
<td>Fe</td>
<td>7.54</td>
<td>7.94</td>
</tr>
</tbody>
</table>

*The underlined are the resonances which differ by >0.02 ppm from the corresponding resonances of WT-MgA/P, and the magnitudes of the differences are shown in parentheses. Fe and Fe correspond to Xa and Xb, respectively, of the WT spin systems in previous reports (Yan et al., 1999a,b).

stantial shift of Fa can be expected for this double mutant on the basis of the previous demonstrations of the sensitivity of this aromatic residue. We have also detected differences of Fe (H336) with R128A, R138K, and R140M (Yan et al., 1999a,b). It is quite remarkable that a double mutant, in which two conserved residues have been changed, remains conformationally intact. These results suggest that the kinetic data of R132M and R97M can be interpreted with confidence, as described in the Discussion.

Analysis of Substrate Binding affinity by NMR Titration. Another technique used to evaluate the impact of structural perturbations introduced by mutagenesis, as well as to confirm dissociation constants determined by kinetic analysis, is NMR substrate titration. The dissociation constants of MgATP and AMP for R132M (Table I) determined from such experiments are 0.52 and 1.36 mM, respectively, which are comparable to the corresponding Kd values obtained from kinetics and the corresponding Kd values from the NMR experiments of WT AK, as is listed in Table I. These data support the lack of changes in the Kd values of R132M determined from kinetics.

For R97M, the kinetic data for AMP binding show a relatively small (6-foil) increase in Kd although the Michaelis constant increases nearly 30-fold. However, NMR titrations with AMP indicate that binding affinity for the binary complex is quite weak for this mutant. As illustrated in Figure 5, the characteristic upfield shift of the aromatic resonance Fa and downfield shift of the adenine H1 for the binary complex, clearly observed for WT, which has pronounced in the corresponding experiment with R97M. Nonlinear least-squares fitting of the data to obtain binding constants is quite difficult in this case since the affinity of AMP for the AMP site could be comparable to or even weaker than the affinity of AMP for the MgATP site (ca. 4.3 mM; Sanders et al., 1989).

However, the binding affinity observed in this titration (as judged by the concentration of substrate required to affect the AMP-sensitive resonances) is comparable to that of R44M, another mutant AK with a selective and dramatic effect on the MgATP site (20-30-fold increases in binding constants; Yan et al., 1990b). These data indicate 3-4-fold differences between the estimate Kd (from NMR) and Kd (from kinetics). The specific reason behind such differences is unknown, but it could be due to slight deviation of the mutant from the rapid equilibrium random model (Dahneke et al., 1993). The perturbation in the AMP binding is not carried over into the MgATP site, since titrations with the latter substrate were quite similar to those of the WT and afforded comparable dissociation constants (Table 1)

While the binding affinities of R97M/R140S toward substrates were not measurable from kinetics due to the very undetectable activity, the NMR titration experiments (Figure 6) showed that this double mutant has lost its capability for ATP binding almost entirely (>50-fold increase in Kd, to the best of our estimation) while the MgATP binding capability remains nearly intact. This is perhaps the most interesting of all results reported in this paper, and it strongly supports of stereospecificity at the AMP site (formation of [R97S]-ADP-PoS exclusively) but not at the MgATP site ([R97S]-ADP-PoS was not converted to [R97S]-ATP-PoS) (Jiang et al., 1991).

In Figure 7a, ca. 5% of the R97 isomer is observed for ADP-PoS and no R57 isomer is observable for ATP-PoS. Thus, both sites show a high degree of stereospecificity. However, it is important to note that the degree of stereospecificity revealed by 3P NMR is only qualitative and depends on the extent of reaction. Although the observed stereospecificity arises from differences in the thermodynamics of the interactions between active site residues and the two isomers (or conformers), there is little difference in the free energies of the isomers outside of the active site. Thus the observed stereospecificity is not a kinetic event, and the ratio of S2/R2 should eventually reach the equilibrium value of ca. 1 upon prolonged reaction.

Endonuclease AK Site Stereospecificity with R97M. Since the ratio of S2/R2 in the product ADP-PoS depends on the extent of reaction of AMPS as noted above, verification of an enhanced stereospecificity requires the demonstration that the ratio of S2/R2 or the percentage of S2 in the total phosphorothioate species in the reaction mixture is smaller at a later stage of the reaction catalyzed by R97M than that at an earlier stage of reaction catalyzed by WT. The reaction mixtures of WT after 98%, 17%, and 9%, respectively, AMPS has been converted to products are shown in panels A-C of Figure 8, respectively. The spectrum of R97M at 27% conversion is shown in Figure 8D. The percentage of R97, in the total phosphorothioate species in the reaction mixture present for the WT reactions is approximately 0.27%, 0.52%, and 1.7% for points A-C, respectively, whereas in spectrum D, (R97)-ADP-PoS is undetectable. It should be noted that the value of 1.7% for the WT reaction is misleadingly high, since equilibrium has already been previously established. In the absence of a change in stereospecificity, the theoretical percentage of R97, for WT at 27% conversion is considered larger than 0.5%. If we select 1% as a conservative estimate, the amount of (R97)-ADP-PoS in spectrum D should be considered smaller than 0.02% (0.27%) if there was no change in stereospecificity. Since the overall signal to noise ratio of all of these spots are similar and the signal to noise ratio for (R97)-ADP-PoS in spectrum A
basis of our best estimation), our techniques are not sensitive enough to detect significant enhancement at the MgATP site.

Discussion

Arginine-97 Interacts with AMP Starting with the Binary Complex.

The absence of structural perturbation in the double mutant MgATP and AMP shows that Arg-97, like the other conserved arginines we have examined, does not contribute significantly to the structural integrity of AK. Free energy calculations indicate that Arg-97 stabilizes AMP by ca. 1–2 kcal/mol starting with the binary complex. Stabilization continues to the ternary complex (K_M effect) and further intensifies at the transition state (K_M, transition state effect). The latter interpretation is justified since we have previously shown that the chemical step is nearly rate-limiting (Tian et al., 1994). The energy contribution at the transition state should be 3.5 kcal/mol according to the relationship ΔG* = RT ln \( \frac{[E-K_M]}{[E-K_M]_{transition}} \), where A and B represent the two substrates.

Our results and interpretations regarding the role of Arg-97 in AK1 are thus different from those of the same residue in AK1 reported by Kim et al. (1989, 1990); the latter failed to recognize the specific role of this residue toward binding of AMP. While it is possible that the residue plays different roles in the two muscle enzymes, we view this as highly unlikely since (1) these two enzymes should function quite similarly, on the basis of a high average sequence homology among muscle AK of 89% (Schulz, 1987) and (2) the specific K_M effect on AMP has also been reported for a more closely related (ca. 31% homology) AK, AK6 (Reinstein et al., 1989).

On the basis of qualitatively similar kinetic data, however, Reinstein et al. interpreted that the arginine residue "is involved in binding of AMP or that the two nucleotide sites are tightly coupled" (for the K_M effect) and that "it possibly stabilizes the structure of the γ-phosphate group from AMP to AMP in the transition state" (for the K_M, transition state effect). We disagree with the role of Arg-97 in interacting with the transferring phosphophosphate group and suggest that the decreased K_M for R97M merely reflects the further enhancement of the interaction of Arg-97 with AMP at the transition state. These functional results support the structural observations that Arg-88 of AK1 (corresponding to Arg-97 of AK1 or Arg-106 in the systematic numbering system) is in proximity to a phosphate group in the AK-MgAP complex (Eigner et al., 1993) and that Arg-92 of AK1 (corresponding to Arg-97 of AK1) is in contact with the phosphate group of AMP in the AK1-MAMP complex (Diederen & Schlembach, 1991).

It is an interesting exercise to compare the kinetic and structural differences between R97M and R97M/R149S. We already know from previous work that Arg-149 is critical for transesterification (as shown in Table I) (Yan et al., 1999b), so the virtually undetectable activity of this double mutant is not at all surprising. Furthermore, structural perturbations observed by 1D NMR analysis in free R97M/R149S is nearly identical to those in free R97M, whereas free R149M is structurally identical to WT (Yan et al., 1999b). This indicates that no additional conformational changes have occurred for the double mutant that are not inherently part of either of the single mutants. Finally, the conformational stability (free energy of unfolding) of this double mutant, within experimental error, is the same as that of WT and indicates that a relatively "unsatable" enzyme (small ΔG* value) after AMP perturbation is stable in the absence of both AMPs.

Manipulation of AMP Phosphoryl Stereospecificity.

While a vast amount of work has been reported on the stereospecificity of the enzyme at the substrate level, little is known about the structural basis of the observed stereospecificity at the enzyme level. In general terms, the specific isomer preferred by the enzyme is the isomer that can fit into the active site and cause the least "pain" (due to replacing an O with S) to the enzyme during the catalytic process. The stereospecificity, therefore, is a consequence of the balance of the enzyme–substrate interactions at the active site. A perturbation of such interactions can then be expected to lead to a perturbation of the stereospecificity. The prediction and demonstration of changes in stereospecificity for R794M and R794M (Jiang et al., 1991) have provided strong, irrefutable evidence for the interaction of the arginine and the phosphate during catalysis. In addition, the results have provided further insight into the enzyme–substrate interactions at the active site. As shown in Figure 11, the stereospecificity of WT at the AMP site can be explained by an equilibrium between a major conformer A and a minor conformer B. The equilibrium is shifted toward conformer B in R794M, and toward conformer A in R797M, as indicated by the arrows. We speculate that Arg-44 is more important than Arg-97 in positioning the catalytic group during catalysis, since the major conformer is perturbed in the R794M mutant.

Arginine-122 is Critical for Transition-State Stabilization.

Although many changes have been observed for this mutant in the AMP and the MgAMP complexes, as well as 1D and 2D spectra of the MgAP/A complex, these changes do not affect the basis of the NOESY spectra and the chemical shifts of aromatic spin systems. As shown in Table I, the dissociation constants for AMP and MgATP determined from NMR titration experiments are also comparable to the WT values, and to those derived kinetically.

Having established that Arg-122 does not play a structural role in AK, a quantitative evaluation of kinetic indicators that Arg-122 does play an important role in function as it stabilizes the transition state by 5.1 kcal/mol. This finding places Arg-122 in a group with two other critical transition-state residues, Arg-138 and Arg-149. These three residues have in common large transition-state stabilizations (5.1, 7.0, and 7.3 kcal/mol for Arg-132, Arg-138, and Arg-149, respectively) and no correlation on the complex alkyl group at either substrate. The effect of stabilization of the ternary complex increases in the order Arg-132 < Arg-138 < Arg-149. Interestingly, except for the correlation of the two deuterium H resonances, both 1D and 2D NMR spectra of R132M-MgAP/A are very similar to those of the MgAMP complexes of R138K and R149M (Yan et al., 1999b).
spectral differences in the R138K and R149M complexes with MgAP(A) are attributed to the $K_m$ effect, i.e., minor conformational changes in the ternary complex as a result of increased $K_m$; no such conclusion can be made with R132M due to the distinct absence of this $K_m$ effect for either substrate.

Current Status on the Structure-Function Relationship of AK. The interactions of Arg-44 and Arg-97 with the phosphoryl group of AMP have been well established on the basis of the results presented in this work and pertinent structural and functional studies reviewed by Tsai and Yan (1991). On the basis of the position of the three arginine residues 132, 138, and 149, as well as their functional roles, we have proposed that the side chains of these three arginine residues, possibly in concert with that of Lys-21, surround the transferring phosphate and stabilize the enzymatic metaphosphate in the case of a dissociative mechanism or the pentasaccharide transition state in a case of an associative mechanism (Tsai & Yan, 1991). However, such a proposal remains to be verified by further structural studies. Since no crystal structure of a MgAP(A) complex of AKI is available, we constructed Figure 2 by manually placing MgAP(A) into the structure of free AKI, with side chains of the pertinent residues highlighted. In this model, the "fit" between the four side chains (132, 138, 149, and 21) and the phosphates is not optimal. There are two possible reasons for the discrepancy: (i) The AKI structure in Figure 12 is that of the free form and does not represent the actual conformation of the complex. A conformational change in AKI will definitely bring the arginine side chains closer to the phosphates. However, even in the co-crystal AKy-MgAP(A) the side chain of Arg-149 is not within 3.5 Å of the bound inhibitor (Egner et al., 1987). (ii) The results of AKy upon which the position of Arg-149 in Figure 1 is based cannot be directly translated into AKI. This is likely since the adenosine moiety at the ATP site in the AKy-MgAP(A) complex is surrounded by a 30-residue "intron segment" which is absent in AKI (Egner et al., 1987). In addition, there is some uncertainty regarding which residue in AKy corresponds to the Arg-132 of AKI. Eckstein & Goody (1979) have suggested that in the scissile alignment (Egner et al., 1987), the Arg-132 of AKI is given a systematic number of 141; in the alignment proposed by Haase et al. (1989), it is in Arg-97 (also an arginine). In the former alignment, Arg-128 of AK would correspond to Arg-137 in systematic numbering (a leucine in AKy and AKs) while in the latter it would correspond to residue 141 (conserved).

Conclusions. In light of the results described above, the following important points can be made: (i) Neither Arg-97 nor Arg-132 is required for maintaining the native tertiary structure of AK, as judged by 1D and 2D proton NMR spectra; (ii) the conformational stability of the mutants of both arginines as well as those of the remaining arginines in the catalytic cleft, see Tsai and Yan (1991) is identical to WT, and it provides evidence, in conjunction with the NMR data, that the role of these arginines is strictly functional in cytoskeletal muscle AK; (iii) Arg-132 is critical to stabilization of the transition state, as demonstrated by the dramatic decrease of the $k_{cat}/K_m$ effect of 0.5 kcal/mol in the methionine mutant; (iv) Arg-97 affords a selective stabilization of the phosphoryl group of AMP (1.2 kcal/mol), beginning at the stage of the binary complex and continuing into the ternary complex and the transition state (3.5 kcal/mol); and (v) we have been able to manipulate the phosphorescent stereospecificity of AK by site-directed mutagenesis.

ADDED IN PROOF

The interpretations in this paper and related earlier papers from our laboratory are in full agreement with the refined structure of the E. coli AK-PAP-A complex which has just appeared (Müller & Schulz, 1992).

REFERENCES


