Reprinted from the Journal of the American Chemical Society, 1991, *113*.


# Mechanism of Adenylate Kinase. 12. Prediction and Demonstration of Enhancement of Phosphorus Stereospecificity by Site-Directed Mutagenesis[1]

Terri Dahnke, Ru-Tai Jiang, and Ming-Daw Tsai*

*Department of Chemistry, The Ohio State University*
*Columbus, Ohio 43210*

*Received August 12, 1991*

Recently we have demonstrated a *reversal* of stereospecificity[2] for the R44M (Arg-44 to Met) mutant enzyme of adenylate kinase (AK, from chicken muscle, overproduced in *Escherichia coli*).[3,4] We now report an *enhancement* of stereospecificity in the conversion of AMPS to ADPαS catalyzed by R97M (Arg-97 to Met) mutant AK, *entirely based upon rational prediction.*

The WT AK is known to convert AMPS to ($S_p$)-ADPαS specifically at the AMP site,[2,5] which is in turn converted to ($S_p$)-ATPαS specifically at the MgATP site.[2,6] However, the stereospecificity is not 100% in either case. Under various conditions, we have detected 5–10% of ($R_p$)-ADPαS and <5% of ($R_p$)-ATPαS in the reaction mixture. As shown in Figure 1, the stereospecificity at the AMP site can be explained by a major conformer A and a minor conformer B at the active site. For R44M,[2] we predicted a possible change in stereospecificity at the AMP site on the basis of the kinetic data (22-fold increase in the $K_d$ and 36-fold increase in the $K_m$ of AMP)[7] and the crystal structures (the yeast AK·MgAP$_5$A complex[8] and the AK3·AMP complex[9]). However, we were unable to predict *how* it would change (i.e., relaxation, reversal, or enhancement). The observed dramatic *reversal* of stereospecificity suggested that Arg-44 plays an important role in orienting the conformation of the phosphorothioate group of bound AMPS. The A to B equilibrium is shifted to B in R44M, as shown in Figure 1.

The crystal structures, however, indicate that another arginine (corresponding to Arg-97 in our system) can also interact with the phosphoryl group of AMP,[8,9] which prompted us to construct the R97M mutant AK. Binding and kinetic analysis yielded 20- and 30-fold increases in the $K_d$ and $K_m$ of AMP, respectively, with no significant perturbation in MgATP binding and a 30-fold decrease in $k_{cat}$. Structural characterization of this mutant by NMR indicated no significant conformational perturbations. These results established that Arg-97 interacts with AMP during the catalysis by AK and led us to predict a change in the stereospecificity of R97M. Since the side chains of Arg-97 and Arg-44 point toward the phosphoryl group of AMP from opposite sides, we also predicted that *R97M and R44M should perturb the stereospecificity in opposite directions*, i.e., *the stereospecificity of R97M should be enhanced relative to WT.*

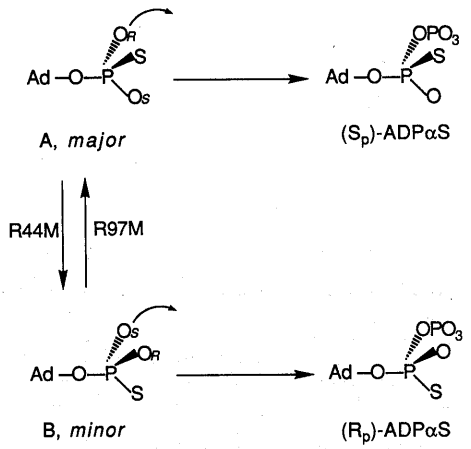


**Figure 1.** Schemes showing the major and minor conformers of AMPS at the active site of WT and the conversion of these conformers to ADPαS. The equilibrium is shifted to conformer B upon R44M mutation and to conformer A upon R97M mutation. It should be noted that a third, nonproductive conformer (with sulfur positioned at the acceptor position) could also be present in WT and both mutants.

To prove that a highly stereospecific reaction has been enhanced, one must demonstrate formation of the minor isomer ($R_p$) at the early stage of reaction for WT, and lack of (or decreased) formation of the $R_p$ isomer at a later stage of reaction for R97M.

(1) Supported by Research Grant DMB-8904727 from the NSF. Paper 11: Reference 4. Abbreviations: ADP, adenosine 5′-diphosphate; ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-monothiophosphate; AP$_5$A, $P^1$,$P^5$-bis(5′-adenosyl)pentaphosphate; ATP, adenosine 5′-triphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); WT, wild type.

(2) Jiang, R.-T.; Dahnke, T.; Tsai, M.-D. *J. Am. Chem. Soc.* **1991**, *113*, 5485–5486.
(3) Tanizawa, Y.; Kishi, F.; Kaneko, T.; Nakazawa, A. *J. Biochem.* (*Tokyo*) **1987**, *101*, 1289–1296.
(4) Tsai, M.-D.; Yan, H. *Biochemistry* **1991**, *30*, 6806–6818.
(5) Sheu, K.-F. R.; Frey, P. A. *J. Biol. Chem.* **1977**, *252*, 4445–4448.
(6) (a) Eckstein, F.; Goody, R. S. *Biochemistry* **1976**, *15*, 1685–1691. (b) Eckstein, F.; Romaniuk, P. J.; Connolly, B. A. *Methods Enzymol.* **1982**, *87*, 197–212. (c) Tomasselli, A. G.; Noda, L. H. *Fed. Proc.* **1981**, *40*, 1864. (d) Kalbitzer, H. R.; Marquetant, R.; Connolly, B. A.; Goody, R. S. *Eur. J. Biochem.* **1983**, *133*, 221–227. (e) Tomasselli, A. G.; Noda, L. H. *Eur. J. Biochem.* **1983**, *132*, 109–115.
(7) Yan, H.; Dahnke, T.; Zhou, B.; Nakazawa, A.; Tsai, M.-D. *Biochemistry* **1990**, *29*, 10956–10964.
(8) Egner, U.; Tomasselli, A. G.; Schulz, G. E. *J. Mol. Biol.* **1987**, *195*, 649–658.
(9) Diederichs, K.; Schulz, G. E. *Biochemistry* **1990**, *29*, 8138–8144.
(10) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652–657.

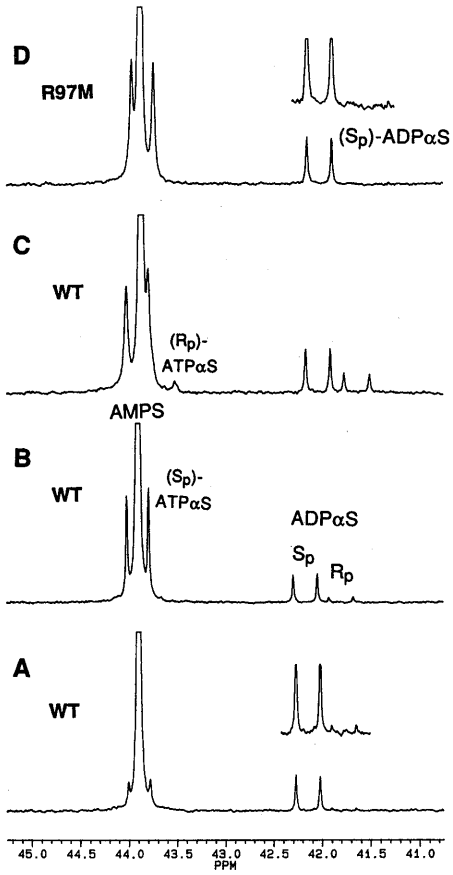


**Figure 2.** $^{31}P$ NMR spectra showing the conversion of AMPS to ADPαS (and subsequent conversion to ATPαS). Only the $P_\alpha$ resonances are shown. (A–C) WT AK, after 9%, 17%, and 29%, respectively, of AMPS has reacted. (D) R97M, at 27% conversion. Spectra were obtained after addition of 100 mM EDTA and 150 μL of triethylamine to optimize for the detection of ADPαS. Except for sample C, no ($R_p$)-ATPαS was detectable even under other conditions, optimized for the detection of ATPαS (spectra not shown). The intensities of various components described in the text were measured by cutting and weighing from greatly expanded spectra. The assignments of different species have been confirmed by mixing with known compounds and agree with previous reports.[5,10] The starting reaction mixture (600 μL) consisted of 22 mM AMPS, 75 mM ATP, and 80 mM $MgCl_2$, in a 50 mM Tris buffer containing 50 mM KCl, 2.5 mM EDTA, and 15% $D_2O$, pH = 7.8. The broadband decoupled spectra were obtained at 121.5 MHz with a pulse width of 45°. The acquisition time was 1.5 s, the repetition time was 2 s, and ca. 23 000 transients were accumulated for each spectrum. The FID was processed with 2.0-Hz exponential multiplication.

These were accomplished by $^{31}P$ NMR analysis as shown in Figure 2. Spectra A, B, and C are the reaction mixtures of WT after 9%, 17%, and 29%, respectively, of AMPS has been converted to products. The minor isomer, ($R_p$)-ADPαS, is clearly detectable in these spectra and constitutes 5%, 15%, and 28%, respectively, of the total ADPαS, or 0.27%, 0.54%, and 1.7%, respectively, of the starting AMPS. In D, 27% of AMPS has been converted to products by R97M, but *no* ($R_p$)-ADPαS can be detected. If there was no change in stereospecificity, sample D should have consisted of more than 0.54% but less than 1.7% of the $R_p$ isomer relative to the starting AMPS (the 1.7% of spectrum C could be an overestimation since the reaction has passed equilibrium). If we take a conservative value of 1%, the $R_p$ isomer in D should have been 3–4 times that in A. Since the signal/noise ratios in D and A are comparable and the signal/noise ratio of ($R_p$)-ADPαS in A is ca. 3, *formation of the $R_p$ isomer has decreased at least 10-fold in D.* These results and analysis indicate *an enhancement of stereospecificity in R97M relative to WT*, i.e., the A to B equilibrium has been shifted to A in R97M, as also indicated in Figure 1.

In terms of molecular events at the active site, the two arginine side chains appear to "compete" for the nonbridging sulfur and/or oxygen. Such competing interactions should start at the AK·AMPS binary complex and persist through the transition state. The balance between the two competing interactions results in the observed stereospecificity in WT, which is shifted one way or the other upon removal of one of the two interactions. Since the major conformer has been perturbed upon R44M mutation, Arg-44 appears to "win" over Arg-97 in orienting the phosphorothioate. The molecular detail of such interactions, however, remains to be established.