#95

bered that it has been explicitly assumed that the functional group undergoing positional exchange is free to rotate. This assumption is not always valid since examples have been discovered where the functional group rotation is indeed hindered. For instance, in the reaction catalyzed by argininosuccinate synthetase a PIX reaction was not observed on incubation of ATP and citrulline even though a citrulline–adenylate complex has been identified from rapid quench experiments.[35]

Acknowledgments

The authors are grateful for financial support from the National Institutes of Health (DK30343, GM33894, and GM49706).

[35] L. W. Hilscher, C. D. Hanson, D. H. Russel, and F. M. Rauschel, *Biochemistry* **24**, 5888 (1985).

[16] Manipulating Phosphorus Stereospecificity of Adenylate Kinase by Site-Directed Mutagenesis

By MING-DAW TSAI, RU-TAI JIANG, TERRI DAHNKE, and ZHENGTAO SHI

Introduction

Nucleoside phosphorothioates[1,2] occupy an important niche in the fields of molecular biology, biochemistry, and mechanistic enzymology. The applications in molecular biology, which include DNA sequencing and oligonucleotide-directed mutagenesis, have been reviewed.[3] The applications of nucleoside phosphorothioates to various biochemical problems have also been reviewed by Eckstein.[4] Many applications take advantage of the stereospecificity of enzymes toward specific isomer(s) of nucleoside phosphorothioates, a property uncovered by mechanistic enzy-

[1] ADP, Adenosine 5′-diphosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-monothiophosphate; AP_5A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; ATP, adenosine 5′-triphosphate; ATPαS, adenosine 5-(1-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

[2] D. A. Usher, E. S. Erenrich, and F. Eckstein, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 115 (1972).

[3] F. Eckstein and G. Gish, *Trends Biochem. Sci.* **14**, 97 (1989).

[4] F. Eckstein, *Annu. Rev. Biochem.* **54**, 367 (1985).

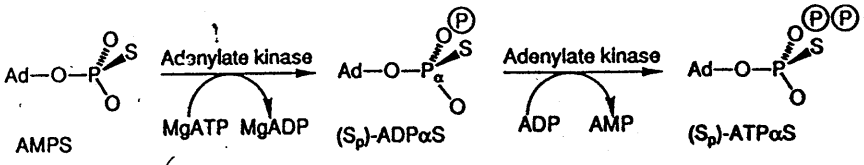

FIG. 1. Scheme showing the stereospecificity of wild-type adenylate kinase at the AMP site (AMPS to ADPαS) and ATP site (ADPαS to ATPαS).

mologists and subsequently exploited to study mechanisms of various enzymatic phosphoryl and nucleotidyl transfer reactions.[5-9]

Adenylate kinase (AK) was one of the first enzymes studied by the use of nucleoside phosphorothioates. Using the methods of coupled enzymatic reactions and ^{31}P nuclear magnetic resonance (NMR) spectroscopy, Sheu and Frey[10] demonstrated that the phosphorylation of AMPS catalyzed by rabbit muscle AK is highly stereospecific and yields predominantly "isomer A" of ADPαS. Later work established the phosphorus configuration of this isomer to be S_p.[6] The specificity toward MgADPαS or MgATPαS at the MgATP site has also been examined in several types of adenylate kinase. Without exception, the S_p isomer is preferred over R_p in AK from rabbit muscle,[11] porcine muscle,[12] and yeast.[13] Figure 1 shows the stereospecificity of both reactions (AMPS to ADPαS and ADPαS to ATPαS).

Although a vast amount of work has been reported on the stereochemical properties 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" (owing 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 in the stereo-

[5] P. A. Frey, J. P. Richard, H.-T. Ho, R. S. Brody, R. D. Sammons, and K.-F. Sheu, this series, Vol. 87, p. 213.
[6] F. Eckstein, P. J. Romaniuk, and B. A. Connolly, this series, Vol. 87, p. 197.
[7] M. Cohn, *Acc. Chem. Res.* **15**, 326 (1982).
[8] M.-D. Tsai, this series, Vol. 87, p. 235.
[9] P. A. Frey, *Adv. Enzymol. Relat. Areas Mol. Biol.* **49**, 119 (1989).
[10] K.-F. R. Sheu and P. A. Frey, *J. Biol. Chem.* **252**, 4445 (1977).
[11] F. Eckstein and R. S. Goody, *Biochemistry* **15**, 1685 (1976).
[12] A. G. Tomasselli and L. H. Noda, *Fed. Proc.* **40**, 1864 (1981).
[13] H. R. Kalbitzer, R. Marquetant, B. A. Connolly, and R. S. Goody, *Eur. J. Biochem.* **133**, 221 (1983).

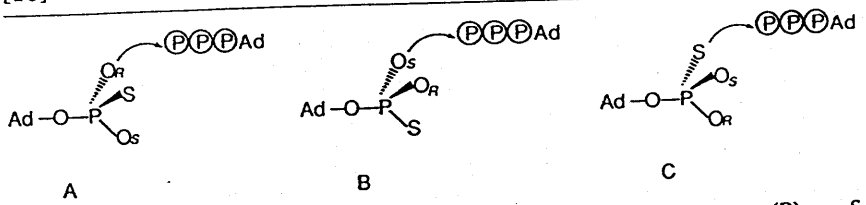

FIG. 2. Possible conformations of the P_α of AMPS with (A) *pro-R* oxygen, (B) *pro-S* oxygen, and (C) sulfur as the attacking atom for the γ-phosphate of ATP.

specificity. As shown by the elegant experiments of Jaffe and Cohn,[14] a reversal in stereospecificity can be achieved for E · metal · ATP complexes by substitution of cadmium, which preferentially coordinates sulfur over oxygen, for magnesium, which favors oxygen over sulfur. A reversal of stereospecificity from such experiments provides strong evidence that the metal ion coordinates directly to the particular phosphorothioate group during catalysis. By a complementary approach, one would predict that site-specific mutants of key residues will perturb the balance of interactions and thus the stereospecificity if these residues interact directly with the phosphoryl moiety. There is always a possibility that a perturbation can also be caused by indirect interactions or by a change in the conformation of the enzyme. However, the indirect interactions are likely to have smaller and nonspecific effects.

The stereospecificity pertaining to AMPS requires additional explanation. Here the choice is between two diastereotopic oxygens, not between two diastereomers. The preference of wild-type AK in phosphorylating the *pro-R* oxygen can be explained (from the substrate point of view) by a preferred conformation (conformer A in Fig. 2) in which the *pro-R* oxygen is positioned to attack the γ-phosphate of ATP. Such a preferred conformation is again a consequence of the balance of all interactions between the thiophosphoryl group and the active site residues. Perturbations of such interactions could lead to rotation of the O—PSO_2 bond (to find a new conformation least painful to the enzyme) and thus a perturbation in the observed stereospecificity.

The purpose of this chapter is to explain how to use site-directed mutagenesis to manipulate the stereospecificity of adenylate kinase in the conversion of AMPS to ADPαS (at the AMP site) and the conversion of ADPαS to ATPαS (at the MgATP site), by showing examples obtained from our laboratory. The stereospecificity toward R_p and S_p isomers of ATPβS was not examined owing to the very low activity of this substrate.

[14] E. K. Jaffe and M. Cohn, *J. Biol. Chem.* 253, 4823 (1978).

Materials and Methods

Synthesis of Phosphorothioate Analogs

The single isomers (R_p)- and (S_p)-ATPαS, as well as the mixture (R_p + S_p)-ADPαS, are synthesized by the methods of Eckstein and Goody.[11] The single isomers (R_p)- and (S_p)-ADPαS are synthesized as described by Sheu and Frey.[10] The compounds are characterized by the ^{31}P chemical shifts[10,15] and high-performance liquid chromatography (HPLC) retention factors.[16]

Construction and Purification of Enzymes

The *Escherichia coli* expression system for chicken muscle AK was kindly provided by Nakazawa and co-workers.[17] The AK mutants are constructed according to the method of Eckstein and co-workers[18,19] by using the mutagenesis kit from Amersham (Arlington Heights, IL). The full-length AK gene is sequenced by the dideoxy nucleotide method of Sanger to ensure that no undesirable mutations have occurred. The method used to purify wild-type AK as described by Tian *et al.*[20] is modified as required for purifying the mutants. The purity of the enzyme is examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with silver staining on a PhastSystem.

Steady-State Kinetics

The kinetic experiments are carried out at 30° and pH 8.0 by monitoring ADP formation with pyruvate kinase/lactate dehydrogenase as the coupling system.[21] The values of k_{cat}, K (Michaelis constant), and K_i (dissociation constant) are obtained by varying both MgATP and AMP concentrations followed by analyses according to the equation of Cleland[22] for a random Bi Bi system. The details have been described previously.[20]

[15] E. K. Jaffe and M. Cohn, *Biochemistry* **17**, 652 (1978).
[16] R. D. Sammons, Ph.D. Dissertation, The Ohio State University, Columbus (1982).
[17] Y. Tanizawa, F. Kishi, T. Kaneko, and A. Nakazawa, *J. Biochem. (Tokyo)* **101**, 1289 (1987).
[18] J. W. Taylor, W. Schmidt, R. Cosstick, A. Okruszek, and F. Eckstein, *Nucleic Acids Res.* **13**, 8749 (1985).
[19] J. W. Taylor, J. Ott, and F. Eckstein, *Nucleic Acids Res.* **13**, 8765 (1985).
[20] G. Tian, H. Yan, R.-T. Jiang, F. Kishi, A. Nakazawa, and M.-D. Tsai, *Biochemistry* **29**, 4296 (1990).
[21] D. G. Rhoads and J. M. Lowenstein, *J. Biol. Chem.* **243**, 3963 (1968).
[22] W. W. Cleland, *in* "Investigation of Rates and Mechanisms of Reactions, Part I" (C. F. Bernasconi, ed.), p. 791. Wiley, New York, 1986.

Phosphorus-31 Nuclear Magnetic Resonance Methods

All ^{31}P NMR experiments are performed on Bruker AM-250 and AM-300 NMR spectrometers at 30°. All chemical shifts are referenced to external 85% H_3PO_4. All spectra are broadband decoupled with the WALTZ sequence. The spectral width is 75 ppm, and 16–32 K data points are recorded for each spectrum in the quadrature detection mode. A 45° pulse and a 0–1.0 sec relaxation delay are used. Acquisition times range from 1.5 to 2.0 sec, repetition times are 2.0 to 2.5 sec, and 1000–23,000 transients are obtained. A 0.5–2.0 Hz line broadening is applied to the time domain data prior to Fourier transformation. Unless otherwise noted, only the regions of the P_α resonances are shown in the spectra.

Assignment and Quantification of Isomers

The assignment of diastereomers within the reaction mixture is accomplished by addition of known isomers to the sample, whose relative chemical shifts are in agreement with those reported previously.[10,15,23] It should be noted that the absolute values of thiophosphate resonances are extremely sensitive to pH and magnesium ion concentration.[15] As a result, differences in chemical shifts for the same species may occur as the sample conditions differ from reaction to reaction. This fact should be kept in mind in comparing the chemical shifts of the same species in different figures in this chapter. The intensities of various components have been measured by cutting and weighing from greatly expanded spectra.

Manipulation of Stereospecificity toward Adenosine 5′-Monothiophosphate

Confirmation of Wild-Type Adenylate Kinase Stereospecificity

As the first step, the specificity at both binding sites of chicken muscle AK used in our work is established and compared to that of other variants of AK reported previously. The reaction of wild-type AK with MgATP and AMPS is followed by ^{31}P NMR spectroscopy, and a representative spectrum is shown in Fig. 3A. This reaction clearly affords one isomer of ADPαS, which is subsequently assigned as S_p by the addition of known isomers. The result in Fig. 3A also indicates that the newly formed (S_p)-ADPαS is readily converted to (S_p)-ATPαS at the MgATP site, suggesting that the preferred configuration at the P_α position of MgATP is also S_p.

[23] M.-D. Tsai, *in* "Phosphorus-31 NMR: Principles and Applications" (D. G. Gorenstein, ed.), p. 175. Academic Press, New York, 1984.

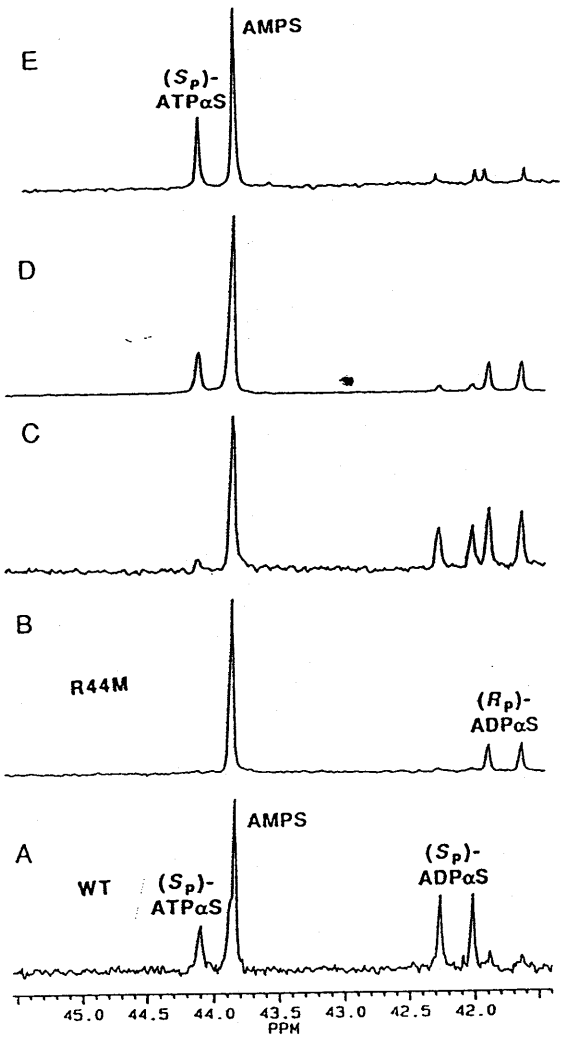

FIG. 3. Comparison by ^{31}P NMR of wild-type and R44M stereospecificity. (A) Wild-type AK, at 4 hr (midpoint of acquisition) after addition of the enzyme; (B) R44M, 2 hr; (C) addition of ADPαS (R_p/S_p = ½) to B, 4 hr; (D) continuation of C, 9 hr; (E) continuation of D, 70 hr. The right half of the doublet of (S_p)-ATPαS overlaps with the singlet of AMPS. The position of (R_p)-ATPαS should be upfield from AMPS. The starting reaction mixture (2 ml) consisted of 22 m*M* AMPS, 75 m*M* ATP, 45 m*M* $Mg(NO_3)_2$, and about 40 μg of wild-type enzyme or 400 μg of R44M, in a 50 m*M* Tris buffer containing 50 m*M* KCl and 2.5 m*M* EDTA, pH 7.8. [Reprinted with permission from R.-T. Jiang, T. Dahnke, and M.-D. Tsai, *J. Am. Chem. Soc.* **113**, 5485 (1991). Copyright 1991 American Chemical Society.]

In conclusion, the stereospecificity of chicken muscle AK at both sites is consistent with previously established stereospecificities of AK from other sources.[10-13]

It is important to note that the degree of stereospecificity revealed by ^{31}P 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 a kinetic event, and the ratio of S_p/R_p isomers should eventually reach the equilibrium value of about 1 on prolonged reaction.

Reversal of AMPS Stereospecificity with R44M Mutant

Our site-specific mutagenesis studies have determined that Arg-44 interacts with AMP starting with the binary complex.[24] This conclusion is based on selective 20- to 30-fold increases in the dissociation and Michaelis constants of AMP for the R44M mutant AK (Table I) and on the observation that the conformation of the mutant is unperturbed. Although the results of kinetic analysis do not specify how Arg-44 interacts AMP, the positively charged arginine side chain most likely interacts with the negatively charged phosphate group of AMP. In the crystal structure of the yeast AK complex with $MgAP_5A$,[25] Arg-53 (family numbering, corresponding to Arg-44 in muscle AK) is in contact with a phosphate group; in the crystal structure of the AMP complex of type 3 AK (from mammalian mitochondrial matrix),[26,27] Arg-41 (also corresponding to Arg-44 in muscle AK) has also been shown to interact with the phosphate group of AMP. This evidence led us to predict that the stereospecificity in the conversion of AMPS to ADPαS could be perturbed in R44M.

The reaction of R44M with AMPS and MgATP is shown in Fig. 3B. The spectrum clearly shows that R44M reverses the stereospecificity of the reaction, since the R_p isomer of ADPαS, instead of S_p as in the case of wild-type AK shown in Fig. 3A, is the predominant product. Furthermore, the fact that no ATPαS is found in Fig. 3B suggests that the stereospecificity at the P_α of the MgATP site has not been altered; that is, the change in stereospecificity appears to be a localized effect as predicted from the kinetic data. Figure 3C confirms the assignment by addition of (R_p + S_p)-ADPαS to the reaction mixture of B, and Fig. 3D confirms that

[24] H. Yan, T. Dahnke, B. Zhou, A. Nakazawa, and M.-D. Tsai, *Biochemistry* **29**, 10956 (1990).
[25] U. Egner, A. G. Tomasselli, and G. E. Schulz, *J. Mol. Biol.* 195, 649 (1987).
[26] K. Diederichs and G. E. Schulz, *Biochemistry* 29, 8138 (1990).
[27] K. Diederichs and G. E. Schulz, *J. Mol. Biol.* 217, 541 (1991).

TABLE I
STEADY-STATE KINETIC DATA OF WILD-TYPE ADENYLATE KINASE AND VARIOUS MUTANT FORMS[a]

Parameter	Unit	Wild type[b]	R44M[d]	R97M[c]	R128A[d]	T23A[c]
k_{cat}	sec^{-1}	650	210	22	36	755
$K_{(MgATP)}$	mM	0.042	0.048	0.083	0.65	0.31
$K_{(AMP)}$	mM	0.098	3.53	2.78	1.38	0.60
$k_{cat}/K_{(MgATP)}$	$sec^{-1}\ M^{-1}$	1.55×10^7	0.44×10^6	0.26×10^6	5.5×10^4	0.24×10^7
$k_{cat}/K_{(AMP)}$	$sec^{-1}\ M^{-1}$	0.66×10^7	5.9×10^4	7.9×10^3	2.6×10^4	0.13×10^7
$K_{i(MgATP)}$	mM	0.16	0.11	0.066	1.63	0.76
$k_{i(AMP)}$	mM	0.37	8.25	2.22	3.48	1.47

[a] The kinetic data were obtained by varying concentrations of both substrates.
[b] Data from Tian *et al.*[20]
[c] Data from Dahnke *et al.*[28]
[d] Data from Yan *et al.*[24]
[e] Data from Shi *et al.*[28a]

the S_p isomer of ADPαS is indeed preferentially converted to ATPαS at the MgATP site. Figure 3E demonstrates that on prolonged reaction both isomers can be converted to ATPαS, since the stereospecificity is a kinetic effect. We suggest that this occurs via the back reaction of (R_p)-ADPαS to AMPS, with ephemeral formation of (S_p)-ADPαS, since direct formation of (S_p)-ATPαS by the phosphorylation of (R_p)-ADPαS cannot occur.[28]

Enhancement of AMPS Stereospecificity with R97M Mutant

Like the case with Arg-44, the corresponding mutation to methionine at position 97 yields selective perturbation of AMP binding, beginning at the stage of the binary complex (Table I), with no significant structural aberrations introduced by the site-specific change.[28] The crystal structure of the type 3 AK · AMP complex[26,27] also indicates that the guanidinium group of Arg-92 (Arg-97 in muscle AK) points toward the phosphoryl group. We therefore predicted that the stereospecificity of R97M should also be perturbed; because the effect cannot be identical to that of R44M, it is likely to enhance the stereospecificity of the wild-type reaction.

Because the ratio of R_p/S_p in the product ADPαS depends on the extent of reaction of AMPS as noted earlier, verification of an enhanced stereospecificity requires the demonstration that the ratio R_p/S_p or the percentage of R_p in the total phosphorothioate species in the reaction

[28] T. Dahnke, Z. Shi, H. Yan, and M.-D. Tsai, *Biochemistry* **31**, 6318 (1992).
[28a] Z. Shi, I.-J. L. Byeon, R.-T. Jiang, and M.-D. Tsai, *Biochemistry* **32**, 6450 (1993).

mixture, is smaller at a later stage of the reaction catalyzed by R97M than that at an earlier stage of reaction catalyzed by the wild type. The reaction mixtures of the wild type after 9, 17, and 29%, respectively, of AMPS have been converted to products are shown in Fig. 4A–C. The spectrum of R97M at 27% conversion is seen in Fig. 4D. The percent R_p in the total phosphorothioate species in the reaction mixture present for the wild-type reactions is approximately 0.27, 0.54, and 1.7% for points A–C (Fig. 4), respectively, whereas in spectrum D (R_p)-ADPαS is undetectable. It should be noted that the value of 1.7% for spectrum C could be misleadingly high, since equilibrium has already been established. In the absence of a change in stereospecificity, the theoretical percentage of R_p for R97M at 27% conversion will be considerably larger than 0.54% but less than 1.7%. If we select 1% as a conservative estimate, the amount of (R_p)-ADPαS in spectrum D should be 3–4 times the amount present in spectrum A (0.27%) were there no change in stereospecificity. Because the overall signal-to-noise ratios (*S/N*) of all spectra are similar and the *S/N* for (R_p)-ADPαS in spectrum A is about 3, the actual amount of (R_p)-ADPαS in spectrum D has decreased by at least 10-fold (3 × 3.5). Therefore, the stereospecificity of R97M has been enhanced by at least 10-fold.[28]

Stereospecificity at P_α of MgATP

Unperturbed ATPαS Stereospecificity with R44M and R97M Mutants

The results described above already suggest that the stereospecificity exhibited by the R44M and R97M mutants is not perturbed at the MgATP site. Another experiment using (R_p + S_p)-ADPαS provides additional support for the observed P_α stereospecificity of the MgATP site of wild type, R44M, and R97M.[28] A time course of the reaction of R97M and the ADPαS diastereomers in Fig. 5 illustrates the steady removal of (S_p)-ADPαS for the selective coupling of the S_p component to form (S_p)-ATPαS and AMPS (Fig. 5B–D). By comparison, the R_p isomer remains largely unreacted. Identical results are achieved in the wild-type reaction (Fig. 6A).

An analysis of the reaction of R44M with (R_p + S_p)-ADPαS as the sole substrates in Fig. 6B results in the favorable coupling of (R_p)-ADPαS (at the AMP site) with (S_p)-ADPαS (at the ATP site) to form AMPS and (S_p)-ATPαS, respectively, since both isomers have been consumed. This result not only reaffirms the S_p stereospecificity of the MgATP binding site for R44M, but strengthens the conclusion for a reversal of stereospecificity of the AMP site.

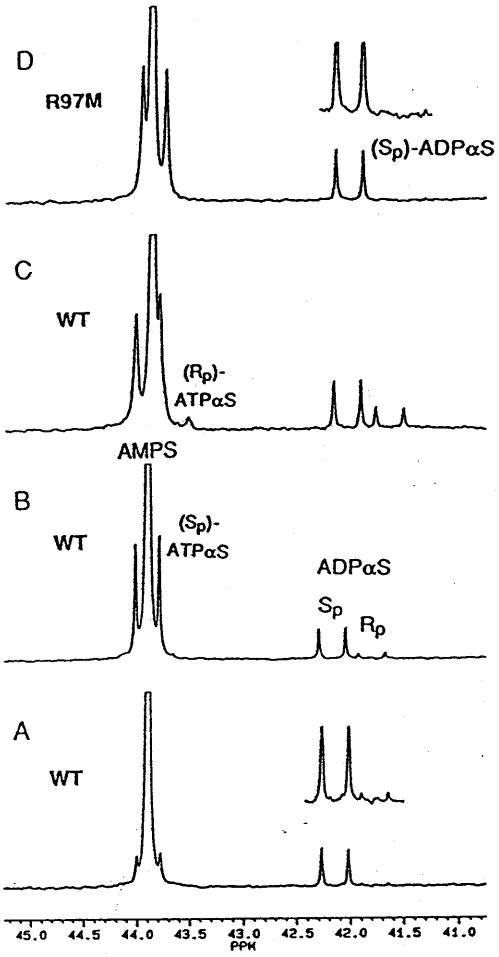

FIG. 4. The ^{31}P NMR spectra showing the conversion of AMPS to ADPαS (and subsequent conversion to ATPαS). (A–C) Wild-type AK, after 9, 17, and 29%, respectively, of AMPS has reacted; (D) R97M, at 27% conversion. Except for sample C, no (R_p)-ATPαS was detectable even under another set of conditions optimized for the detection of ATPαS (spectra not shown). The starting reaction mixture (600 μl) was similar to that of Fig. 3, except for the addition of D_2O (final concentration 15%) to the buffer. Spectra A–C were obtained after the addition of 2, 25, and 10 μg of wild-type AK, whereas 20 μg of R97M was added in D. EDTA and triethylamine were added to optimize conditions for the detection of ADPαS. [Reprinted with permission from T. Dahnke, R.-T. Jiang, and M.-D. Tsai, *J. Am. Chem. Soc.* 113, 9388 (1991). Copyright 1991 American Chemical Society.]

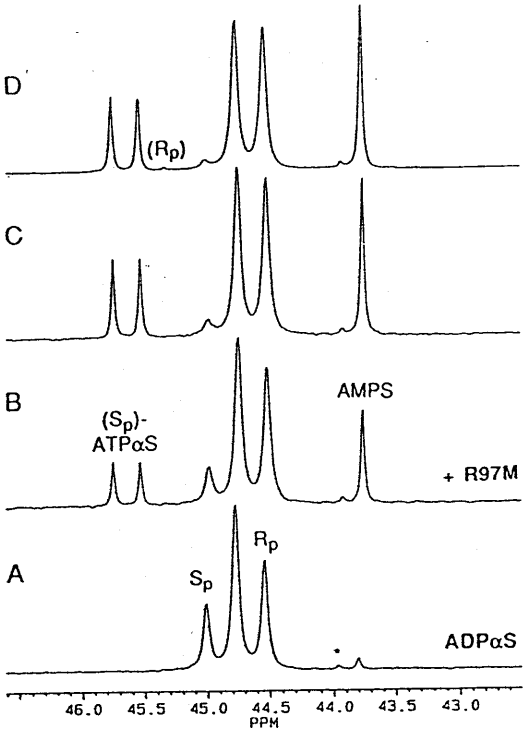

FIG. 5. Time course of the reaction of R97M and (R_p + S_p)-ADPαS. (A) (R_p + S_p)-ADPαS ($R_p/S_p \approx \frac{1}{3}$); (B) 30 min after the addition of R97M; (C) continuation of B, 1.5 hr; (D) continuation of C, 72 hr. A small amount of (R_p)-ATPαS can be detected after prolonged reaction in D. Sample conditions for the reverse reaction were similar to those of Fig. 4, except for the replacement of ATP and AMPS with 22 m*M* ADPαS. Spectra were obtained after the addition of 25 μg of R97M. The asterisk (*) marks a small impurity introduced during the synthesis of the phosphorothioate analogs. (Reprinted with permission from Dahnke *et al.*[28] Copyright 1992 American Chemical Society.)

Relaxation of ATPαS Stereospecificity with R128A Mutant

Arginine-128 does not play a clear structural or functional role since almost every kinetic (Table I) and structural property of the R128A mutant has been perturbed to a small extent.[24] Examination of the stereospecificity of R128A may shed some light on the contribution of this residue to substrate binding.

The two standard experiments were first performed with the R128A mutant. Reaction of AMPS with MgATP gave results (spectrum not shown) essentially the same as that of the wild type shown in Fig. 3A. In

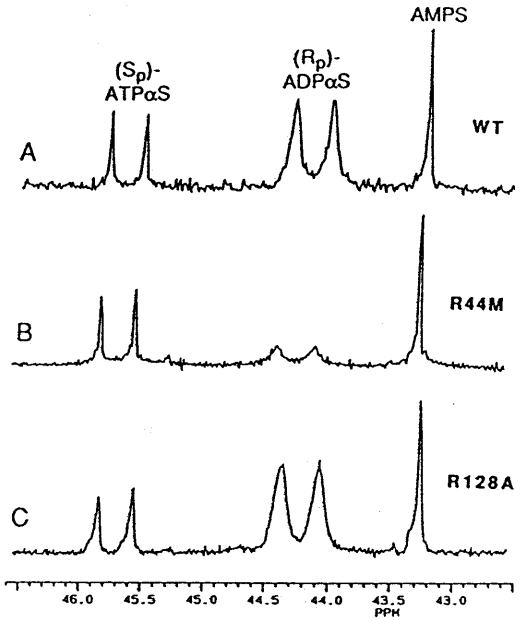

FIG. 6. Reaction of (R_p + S_p)-ADPαS with (A) wild-type AK, (B) R44M, and (C) R128A. Sample conditions for the reactions were similar to those of Fig. 5. The spectra were obtained after the addition of around 20–50 μg of enzyme. (A and B partially reprinted with permission from Dahnke *et al.*[38] Copyright 1992 American Chemical Society.)

the presence of (R_p + S_p)-ADPαS as sole substrates, the products shown in Fig. 6C are also essentially the same as those for the wild type (Fig. 6A). Based on the results of the two experiments, one might prematurely conclude that the stereospecificity of wild-type AK and R128A is identical. However, this was disproved on the basis of a third experiment. Figure 7 shows the products of the reactions of ADP and the single diastereomer (R_p)-ADPαS catalyzed by the wild type and R128A. The reaction mixture of wild-type AK consists of (R_p)-ADPαS, AMPS, and (S_p)-ATPαS, whereas that of R128A consists of (R_p)-ADPαS, AMPS, and (R_p)-ATPαS. The formation of (S_p)-ATPαS with the wild type can be explained by the (R_p)-ADPαS being dephosphorylated to form AMPS at the AMP site (allowed since the AMP site stereospecificity is not absolute), and further combined with ATP to form (S_p)-ADPαS (not detectable in Fig. 7) and subsequently (S_p)-ATPαS. The formation of (R_p)-ATPαS with R128A, however, can only come from direct phosphorylation of (R_p)-ADPαS at the MgATP site. This behavior indicates a relaxation of stereospecificity at the ATP site. The degree of this relaxation may not be large judging

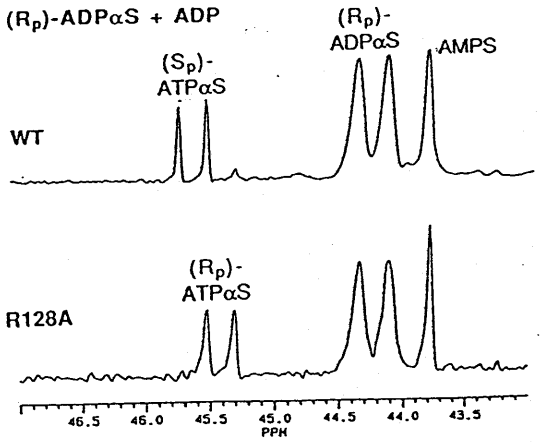

FIG. 7. Reaction of (R_p)-ADPαS and ADP with wild-type AK and R128A. Sample conditions for the reactions were similar to those of Fig. 5, except for the replacement of (R_p + S_p)-ADPαS with 22 m*M* (R_p)-ADPαS and 22 m*M* ADP. The spectra were obtained after the addition of around 20–50 μg of enzyme.

from the lack of perturbations (relative to wild type) in the two reactions starting with AMPS plus ATP and with (R_p + S_p)-ADPαS.

Because there is a relaxation of stereospecificity at the P_α site of ATP, a tempting interpretation is that the guanidinium group of Arg-128 interacts with the α-phosphate of ATP. However, because the degree of change in stereospecificity with the mutant is quite small, we can at best say that the results are consistent with such an interpretation. Further evidence needs to be sought to confirm the functional roles of this residue.

ATPαS Stereospecificity of T23A Mutant is Significantly Perturbed

The T23A mutant presents a case where kinetic data of the mutants are only minorly perturbed, but structural results suggest that the residue is likely to interact with the substrate.[29] The ^{31}P NMR analysis of the reaction between AMPS and MgATP catalyzed by T23A is illustrated in Fig. 8, which shows a noticeable difference from the result of wild-type AK in that there is a considerable amount of the R_p isomer of ATPαS (20% relative to S_p) in the product of T23A. This indicates a perturbation in the stereospecificity at the α-phosphate site of MgATP. The result, however, gives no indication as to the extent of perturbation in stereospecificity since the formation of (R_p)-ATPαS is limited by the availability of

[29] Z. Shi, I.-J. L. Byeon, R.-T. Jiang, and M.-D. Tsai, *Biochemistry* **32**, 6450 (1993).

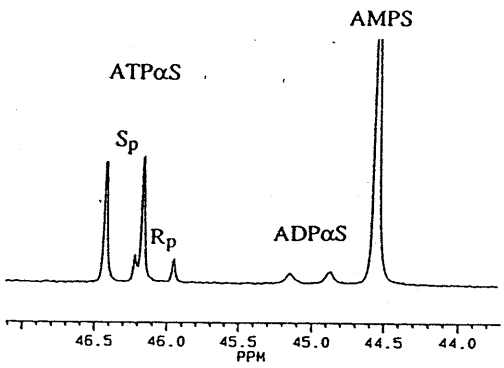

FIG. 8. Analysis by ^{31}P NMR of the reaction of AMPS and MgATP catalyzed by T23A showing the presence of (R_p)-ATPαS. The starting reaction mixture (0.6 ml) consisted of 22 m*M* AMPS, 75 m*M* ATP, 45 m*M* $Mg(NO_3)_2$, and around 100 μg of T23A in 50 m*M* Tris buffer containing 50 m*M* KCl and 2.5 m*M* EDTA, pH 7.8. The spectrum was obtained in the absence of the deuterium lock. Data acquisition started right after the addition of T23A. For better resolution, samples were adjusted to pH 14 before the spectra were taken. Notice that the peak of AMPS is shifted upfield under this condition. (Reprinted with permission from Shi *et al.*[29] Copyright 1993 American Chemical Society.)

(R_p)-ADPαS, which is controlled by the stereospecificity of the AMP site. The result also gives no indication of the stereospecificity of the AMP site since (R_p)-ADPαS does not accumulate.

Quantitative results were obtained by using (R_p)-ADPαS and (S_p)-ADPαS as substrates. In the case of the wild type, this experiment produces exclusively (S_p)-ATPαS (and AMPS) at the expense of exclusively (S_p)-ADPαS (at both sites), leaving (R_p)-ADPαS unreacted (Fig. 6A). The results with T23A, as shown in Fig. 9, indicate that a mixture of ATPαS with $R_p/S_p = 0.37:1$ is formed (spectrum B). This ratio should represent the stereochemical preference at the α-phosphorus of the MgATP site since the enzyme has ample supply of either isomer of ADPαS (spectrum A; an excess of S_p isomer was used). Because the ratio (R_p)-ATPαS/(S_p)-ATPαS is less than 0.02 (to the limit of detection) for the wild type,[28] the ratio R_p/S_p has been enhanced by more than 20-fold at the P_α of the MgATP site for T23A.

Before the above results can be used to suggest that the side chain of Thr-23 interacts directly with the α-phosphate of ATP during the catalysis by wild-type AK, it is necessary to address two issues. The first issue is whether the perturbation in stereospecificity is specific to the MgATP site. If the stereospecificity at the AMP site is also relaxed, it may be argued that the stereospecificity is relaxed nonspecifically owing to a

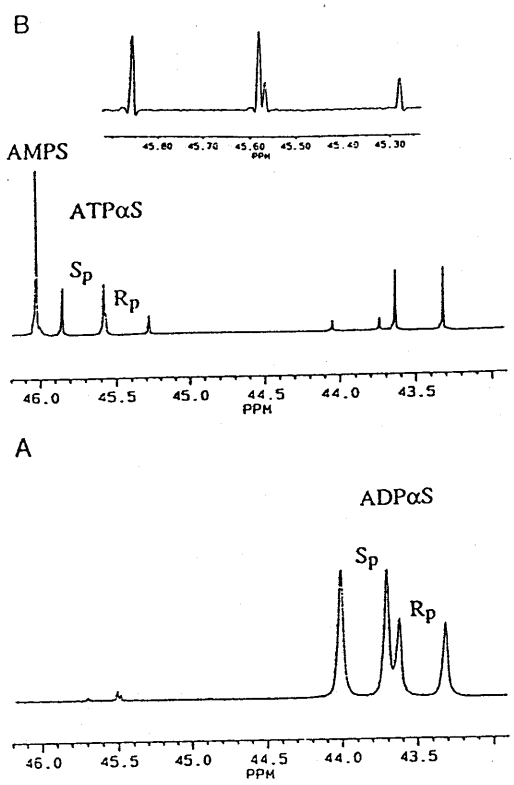

FIG. 9. Analysis by ^{31}P NMR of the reaction of (R_p)-ADPαS and (S_p)-ADPαS catalyzed by T23A. (A) Starting substrates: (R_p + S_p)-ADPαS (S_p/R_p = 1.6). (B) Reaction mixture, obtained at 30 min after the addition of T23A; the inset is a resolution-enhanced analysis of the product (R_p + S_p)-ATPαS. Sample conditions were similar to those of Fig. 6, except for the replacement of ATP and AMPS with 22 m*M* ADPαS. For better resolution, samples were adjusted to pH 5.6 before the spectra were taken. Notice that the peak of AMPS is shifted downfield under this condition.

perturbation in conformation or mechanism. This issue should be reflected in the R_p/S_p ratio of ADPαS consumed, and thus can be addressed by quantitative analyses of the results of Fig. 9. If the stereospecificity at the AMP site is strictly S_p, then the amount of R_p isomer of ADPαS consumed should be equal to the amount of R_p isomer of ATPαS formed, and AMPS should come exclusively from (S_p)-ADPαS. In other words, the ratio of [AMPS] + [(S_p)-ATPαS] + [(S_p)-ADPαS remaining] to [(R_p)-ATPαS] + [(R_p)-ADPαS remaining], defined as R, should be equal to the S_p/R_p ratio of the starting ADPαS (1.6). On the other hand, a relaxation

in the stereospecificity of the AMP site should result in a larger R value since some of the (R_p)-ADPαS will be converted to AMPS at the AMP site, in addition to being converted to ATPαS at the MgATP site. The observed R value, on the basis of both peak height as well as integration of Fig. 9B, is 1.8; after correcting for the fact that the stereospecificity at the AMP site is around 95% instead of 100%, the observed R value becomes 1.65, which agrees with the predicted value of 1.6 within experimental errors. Thus, the stereospecificity at the AMP site of T23A is the same as that of the wild type.

The second issue is to ensure that the stereospecificity (at the MgATP site) is a "preequilibrium effect," that is, a kinetic effect. Because equilibration between R_p and S_p isomers must occur via AMPS as an intermediate, it should occur for ADPαS before ATPαS. This is not the case as shown in Fig. 9B. In addition, such equilibration should also make the observed R value deviate from the predicted value, which is not the case. Most importantly, the result is similar at an earlier stage of reaction (not shown).

The results of analysis of the T23A mutant allow us to interpret the stereochemical results: the side chain of Thr-23 interacts directly with the α-phosphate of ATP during the catalysis by wild-type AK, most likely via hydrogen bonding.[29]

Interpretation of Stereochemical Results

In Terms of Microscopic Rates

A scheme summarizing the differences in stereospecificities for wild-type AK and mutants is shown in Fig. 10. For the wild-type enzyme, the small arrows between AMPS and (R_p)-ADPαS indicate the unfavorable formation of the R_p isomer relative to the S_p isomer at the AMP site. The conversion of (R_p)-ADPαS to (R_p)-ATPαS is indicated as "unallowed" by a cross; however, it only means the rate is very slow. For R44M, the stereospecificity at the AMP site has been reversed, as indicated by the relative lengths of the arrows. For R97M, the stereospecificity at the AMP site has been enhanced; the conversion of AMPS to (R_p)-ADPαS is crossed out to indicate the low rate of this path. For R128A, the cross between (R_p)-ADPαS and (R_p)-ATPαS is replaced by small arrows to indicate relaxation of this path. In a scenario where the enzyme is given a "choice" of diastereomeric substrates, R128A still favors the S_p isomer at the ATP site (and the AMP site), as evidenced by the result of Fig. 6C. However, in the absence of (S_p)-ADPαS, the acceptance of the R_p isomer at the ATP site can be manifested as shown in Fig. 7B. For T23A, the R_p isomer

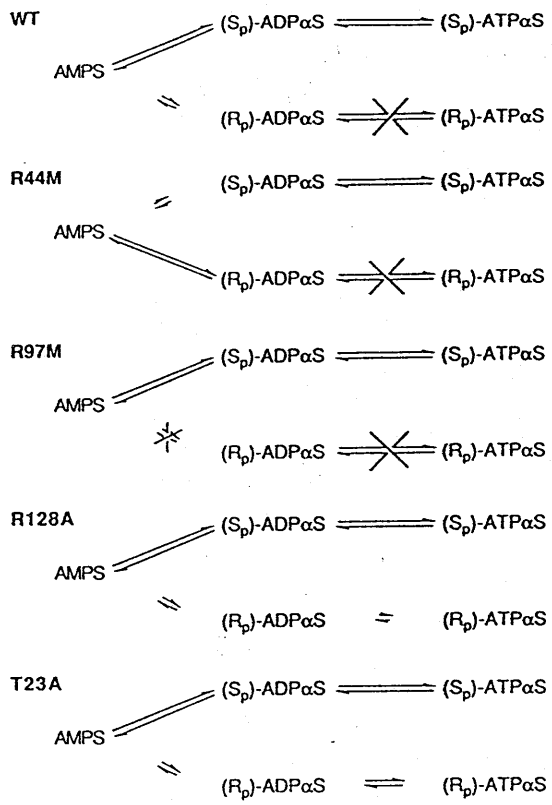

FIG. 10. Schemes summarizing the differences in stereospecificities for wild-type AK and R44M, R97M, R128A, and T23A mutants.

at the ATP site is acceptable to an extent nearly comparable to that for the S_p isomer, as indicated by the relative lengths of the arrows.

In Terms of Active Site Conformations

As shown schematically in Fig. 11, the stereospecificity of wild-type AK 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 R44M, and toward conformer A in R97M, as indicated by the arrows. We speculate that Arg-44 is more important than Arg-97 in positioning the phosphoryl group during catalysis, since the major conformer is perturbed in the R44M mutant.

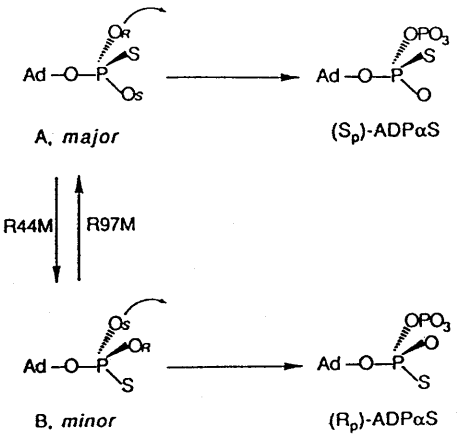

FIG. 11. Schemes showing the major and minor conformers of AMPS at the active site of wild-type AK and the conversion of these conformers to ADPαS. The equilibrium is shifted to conformer B on R44M mutation and to conformer A on R97M mutation. [Reprinted with permission from T. Dahnke, R.-T. Jiang, and M.-D. Tsai, *J. Am. Chem. Soc.* **113**, 9388 (1991). Copyright 1991 American Chemical Society.]

The role of conformer C in Fig. 2 is unclear and thus is not represented in Fig. 11. This conformer could lead to the formation of ADP with a bridging sulfur between P_β and P_α. Because such a product has never been detected, it may be tempting to consider this as a minor or nonproductive conformer. However, the finding of the high chemical reactivity of the bridging-monothio analog of pyrophosphate[30] suggests that even if a product is formed from conformer C it could be hydrolyzed back to AMPS (chemically) immediately and thus escape detection.

Potential Applications to Other Systems

The implications and possible applications of our work are many. First of all, a significant perturbation in stereospecificity is strong evidence that the mutated residue interacts with the phosphorothioate group. Our results unequivocally establish that both Arg-44 and Arg-97 interact with the phosphoryl group of AMP, and Thr-23 interacts with the P_α group of ATP, during the catalytic reaction of adenylate kinase. This method of assessing the functional role of residues is a step forward from direct interpretation of the static crystal structure or the kinetic data of mutant enzymes and

[30] C. J. Halkides and P. A. Frey, *J. Am. Chem. Soc.* **113**, 9843 (1991).

can enhance our understanding of the chemical basis of enzymatic catalysis and the biological effects of phosphorothioates. Furthermore, a combination of site-directed mutagenesis and changes in metal ions could be used to manipulate phosphorus stereospecificity so that enzymes may either accept or produce alternative diastereomers of phosphorothioate analogs. Potential candidates for manipulation of substrate stereospecificity include DNA and RNA polymerases which, without exception, all prefer the S_p isomer of dNTPαS or NTPαS.[3] With respect to immediate application, R44M provides a more direct route to the synthesis of pure (R_p)-ADPαS from AMPS, which has been previously achieved by the chemical synthesis of (R_p + S_p)-ADPαS followed by enzymatic removal of the S_p isomer.[11]

Acknowledgments

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[17] Equilibrium Isotope Exchange in Enzyme Catalysis

By FREDERICK C. WEDLER

Background and General Properties

Theoretical Basis

Isotope exchange kinetics at chemical equilibrium has been recognized as an important, if underdeveloped, subcategory of steady-state kinetics for over three decades. The fundamental concepts were pioneered and delineated by Boyer,[1] Boyer and Silverstein,[2] and Alberty *et al.*[3] Later contributions to theoretical concepts were made by Yagil and Hoberman,[4] Yagil,[5] Britton,[6,7] Britton and Dann,[8] Morales *et al.*,[9] Flossdorf and Kula,[10]

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