hand, the same technique affords 0% ee with **9b**. None of the methods currently known approaches the desired goal of a substrate-independent asymmetric protonation of enolates. Our IPR method is also not effective with **9b** (11% ee), but it does work with reasonable generality for α-aryl propionamide systems. Thus, both **10** (50% ee) and **11** (60% ee) gave comparable results to those obtained with naproxen amide **1b**. These experiments were carried out by using the procedure optimized for **1a**, without individually optimizing nitrogen substituents or reaction conditions.

The essential role of diamine or triamine ligands in our experiment can be interpreted as evidence for bidentate coordination of lithium, similar to that in Seebach's X-ray structure of an amide enolate (Figure 1).[3,4] Since the optimum stoichiometry for high ee involves a 1:1:1 ratio of enolate **2**:lithium amide **4b**:neutral amine **4a**, the mixed aggregate representation **8** can be used as a starting point for developing predictive models. This structure is based on the Seebach precedent[4] and on an X-ray structure for a mixed aggregate of an enolate with LDA reported by Williard et al.[11] If the BF_3-induced internal proton transfer occurs rapidly relative to structural changes within the mixed aggregate, then a geometry similar to **8** could explain the observed *R* selectivity for IPR. However, important details of the activation process and the identity of the actual proton donor remain to be evaluated. Experiments to address these issues and to explore other substrates are in progress.

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Supplementary Material Available: Experimental details for the preparation of **4a** and the deracemization of **1a** (2 pages). Ordering information is given on any current masthead page.

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Mechanism of Adenylate Kinase. 10. Reversing Phosphorus Stereospecificity by Site-Directed Mutagenesis[1]

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

Department of Chemistry and Ohio State Biochemistry Program The Ohio State University, Columbus, Ohio 43210

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The stereospecificity toward different diastereomers of nucleoside phosphorothioates is a well-established property for many enzymes[2] and has been useful in many research areas of biochemistry and molecular biology.[3] A typical example is shown in Figure 1A for adenylate kinase (AK): when AMP is substituted by AMPS, phosphorylation occurs at the *pro-R* oxygen specifically to give (S_p)-ADPαS;[4,5] the latter can be further converted to (S_p)-ATPαS since the S_p isomer is also preferred over the R_p isomer at the MgATP site.[6-8]

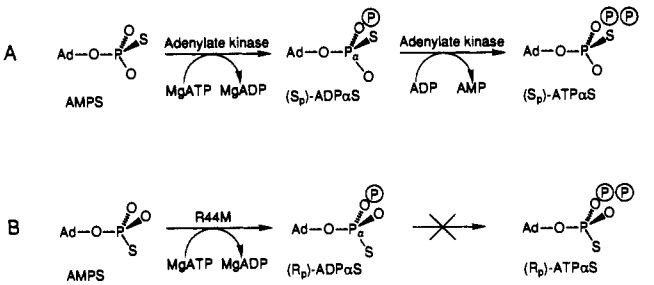

Figure 1. Schemes showing the stereospecificity of wild-type adenylate kinase (A) and R44M (B). As implied by the schemes, the orientation of the acceptor oxygen of AMPS should not change, and the reversal in stereospecificity is likely to be due to a rotation in the *bridging* O–P bond.

Such stereospecificity (not the steric course, which is a different issue) should arise from restricted orientations of the P–O and P–S bonds at the active site, so that the unnatural sulfur atom assumes the position "least painful" to the enzyme. The exact orientations depend on the combined effects of the interactions between the phosphorothioate group and the active-site residues and/or the metal ion. It has been established that changing the metal ion could perturb the stereospecificity.[9] We predict that removal of one of such interactions by site-directed mutagenesis can also change the overall interactions and potentially perturb the stereospecificity.

We tested our prediction using AK from chicken muscle overproduced in *Escherichia coli*.[10] Recently we found that substitution of Arg-44 by Met (to give the R44M AK) resulted in 36-fold and 22-fold increases in the Michaelis and the dissociation constants, respectively, of AMP but not MgATP, and only a 3-fold decrease in k_{cat}.[11] After detailed structural analysis to ensure that the conformation of the mutant AK had not been perturbed, we concluded that Arg-44 interacts specifically with AMP starting from the binary complex. Although the kinetic results do not reveal *how* Arg-44 interacts with AMP, the positively charged arginine is likely to interact with the negatively charged phosphate, as also suggested by the crystal structure of a yeast AK–inhibitor complex.[12]

Using ^{31}P NMR, we monitored the conversion of AMPS to ADPαS (at the AMP site) and its subsequent conversion to ATPαS (at the MgATP site). As shown in Figure 2A, the products from wild-type (WT) AK are mainly the S_p isomers of both ADPαS and ATPαS. Separate experiments using (R_p+S_p)-ADPαS confirmed that the S_p isomer is indeed preferred over the R_p isomer at the MgATP site. Thus chicken muscle AK behaves like other types of AK in the stereospecificity at the P_α of both AMP and MgATP.[4-8]

As shown in Figure 2B, *the stereospecificity at the AMP site is completely reversed in the reaction catalyzed by R44M AK* since the product is mainly (R_p)-ADPαS. The fact that no ATPαS formed in Figure 2B suggests that *the stereospecificity at the P_α of the MgATP site has not changed.* This was further confirmed by addition of (R_p+S_p)-ADPαS to the sample, which resulted in specific conversion of the S_p isomer to (S_p)-ATPαS (Figure 2, C and D). The stereospecificity of the reactions catalyzed by R44M is shown in Figure 1B. Although we have predicted a change in stereospecificity, it was surprising that the change was not to relax the stereospecificity, and fortuitous that it was not to enhance the existing stereospecificity.

* To whom correspondence should be addressed at Department of Chemistry, The Ohio State University, 120 W. 18th Ave., Columbus, OH 43210.
(1) Supported by Research Grant DMB-8904727 from the NSF. Paper 9 in the series: Yan, H.; Tsai, M.-D. *Biochemistry* **1991**, *30*, 5539–5546. Abbreviations: ADP, adenosine 5′-diphosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine, 5′-monophosphate; AMPS, adenosine 5′-monothiophosphate; ATP, adenosine 5′-triphosphate; ATPαS, adenosine 5′-(1-thiotriphosphate); WT, wild-type.
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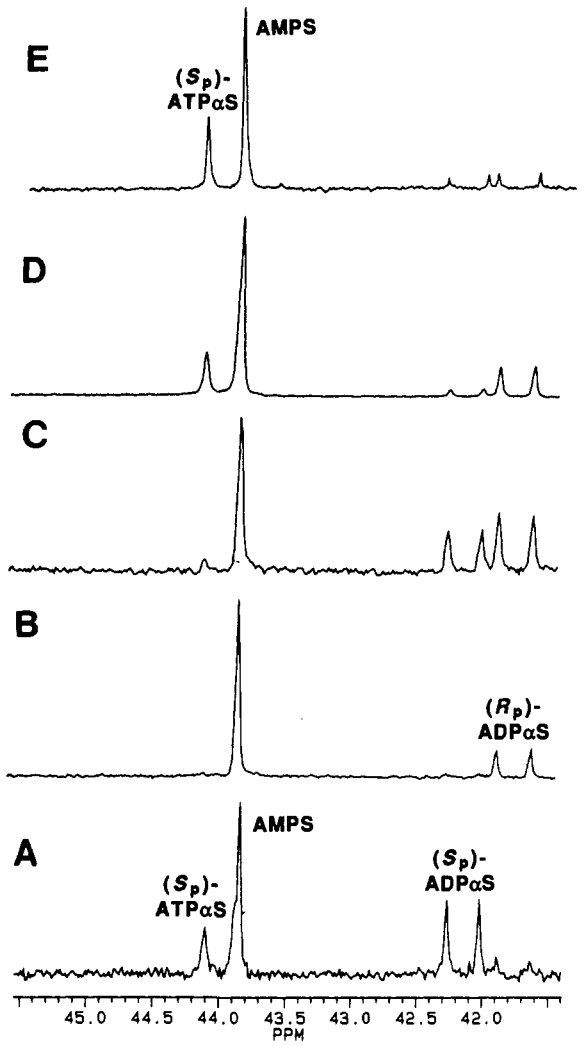

Figure 2. ^{31}P NMR analysis (at 121.5 MHz) of stereospecificity at neutral pH. Only the regions of the P_α resonances are shown. The starting reaction mixture (2 mL) consisted of 22 mM AMPS, 75 mM ATP, 45 mM $Mg(NO_3)_2$, and ca. 0.04 mg of WT or ca. 0.4 mg of R44M, in a 50 mM Tris buffer containing 50 mM KCl and 2.5 mM EDTA, pH 7.8. The temperature in the NMR probe was 30 °C (A) WT AK, at 4 h (midpoint of acquisition) after addition of the enzyme; (B) R44M, 2 h; (C) addition of ADPαS (R_p/S_p = 1:2) to B, 4 h; (D) continuation of C, 9 h; (E) continuation of D, 70 h. The free induction decay was acquired with broad-band decoupling and processed with 0.5- or 1-Hz exponential multiplication. The chemical shifts are referenced to external 85% H_3PO_4. 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 relative chemical shifts agree with the values reported previously[4,13] (the absolute values are sensitive to pH and buffer conditions).

Since the stereospecificity in the present case is a kinetic phenomenon, it is important to follow the time course of the reaction. Otherwise complication could arise from equilibrium effects. For example, further incubation of the sample of Figure 2D resulted in the conversion of the R_p isomer of ADPαS to the S_p isomer of ATPαS (Figure 2E), apparently via back reaction to AMPS and transient formation of (S_p)-ADPαS. Also, the stereospecificity should not be considered to be 100%. In Figure 2A, for example, a small amount of (R_p)-ADPαS (ca. 15% of total ADPαS, or ca. 8% of total products) can also be detected.

Overall, our results are significant in several aspects: (a) Site-directed mutagenesis can be used to manipulate the P-stereospecificity of enzymes and produce useful diastereomers of phosphorothioates. In the present case, R44M provides a direct way to synthesize pure (R_p)-ADPαS from AMPS, which is simpler than the previously available procedure of chemically synthesizing (R_p+S_p)-ADPαS followed by enzymatic removal of the S_p isomer.[6] (b) A reversal, or a significant perturbation in the stereospecificity, is strong evidence that the mutated residue interacts with the phosphorothioate group. The results in this work unequivocally establish that Arg-44 interacts with the phosphoryl group of AMP during the catalytic reaction. Such delineation of the functional role of an active-site residue is a step forward from direct interpretation of the static crystal structure or the kinetic data of mutant enzymes. (c) In-depth investigation of various systems can enhance our understanding of the chemical basis of enzymatic catalysis and the biological effects of phosphorothioates.

Nitrogen-15-Labeled Oligodeoxynucleotides. 3. Protonation of the Adenine N1 in the A·C and A·G Mispairs of the Duplexes $\{d[CG(^{15}N^1)AGAATTCCCG]\}_2$ and $\{d[CGGGAATTC(^{15}N^1)ACG]\}_2$

Chuan Wang, Hetian Gao, Barbara L. Gaffney, and Roger A. Jones*

Department of Chemistry
Rutgers, The State University of New Jersey
Piscataway, New Jersey 08855
Received February 25, 1991

Nitrogen NMR of specifically labeled molecules has the potential to provide unique information about structure and interactions.[1,2] The chemical shift of an sp^2 nitrogen, for example, is strongly affected by protonation. In the case of the adenine N1, protonation is known to cause an upfield shift of ~70 ppm.[2] Thus, nitrogen NMR should allow unambiguous determination of when or if a specific (labeled) adenine N1 is protonated, regardless of the size of the molecule, within the limit of τ_c, the molecular correlation time. We synthesized the labeled molecules d[CG($^{15}N^1$)AGAATTCCCG] (**1**) and d[CGGGAATTC-($^{15}N^1$)ACG] (**2**), using an H-phosphonated method, and monitored the ^{15}N chemical shift of each as a function of pD over a range of ~5 to ~8.[3] The ^{15}N chemical shifts were detected indirectly through the corresponding H2 atom by using a ^1H-detected heteronuclear 2D NMR experiment.[4] This technique reduces dramatically the NMR time required, relative to direct ^{15}N detection, to achieve a given level of sensitivity. In the ^1H–^{15}N 2D NMR spectrum obtained, the observed signals provide both ^1H and ^{15}N chemical shifts for the individual heteronuclear J-coupled spin systems.

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