α-Thio-APS: A Stereomechanistic Probe of Activated Sulfate Synthesis

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Abstract: Despite their broad application in the phosphoryl-transfer field, thio-nucleotides have not been available for the study of sulfate activation and transfer. There are two known forms of activated sulfate in the cell, APS (adenosine 5′-phosphosulfate) and PAPS (3′-phosphoadenosine 5′-phosphosulfate). PAPS is the only known sulfuryl group donor in metabolism, and sulfuryl transfer is used widely to regulate metabolism. This study presents the first synthesis of a thio-nucleotide analogue of activated sulfate, $APS_{\alpha}S$ ((Sp)- and (Rp)-adenosine 5′-*O*-(1-thiophosphosulfate)). Two syntheses are described, one of which is a novel "one-pot" method that is general for the site-specific delivery of the sulfuryl group. Both epimers of $APS_{\alpha}S$ were purified and their stereochemical configurations were assigned. These compounds were used to address several stereomechanistic issues in the APS-synthesis reaction catalyzed by yeast ATP sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4). The reaction is shown to proceed with inversion of configuration at the α-phosphorus (P_{α}). The enzyme exhibits high selectivity for the (R_p) epimer of $APS_{\alpha}S$ when Mg^{2+} is the activating cation. The "hard" vs "soft" cation dependence of the enzyme's activity indicates that its selectivity is due to cation coordination at P_{α}. The absence of selectivity in the substrate binding reactions indicates that coordination at P_{α} occurs after formation of the $E{\cdot}APS{\cdot}PP_i{\cdot}M^{2+}$ complex.

Since their introduction in 1975,[1] thio-nucleotides have proven extremely useful in the examination of enzymatic and nonenzymatic phosphoryl transfer mechanisms. They have been used to investigate fundamental issues such as determining the degree of transition-state bond formation,[2] altering rate-determining chemical steps,[3] identifying and characterizing enzyme intermediates,[4] determining the stereochemical configuration of metal ion−nucleotide complexes at enzyme-active sites,[5] and determining the stereochemistry and stereospecificity of enzyme-catalyzed reactions.[6] These versatile analogues have not yet been used to explore the enzymatic mechanism of sulfate activation. In the cell, activated sulfate (adenosine 5′-phosphosulfate, or APS) is synthesized by the catalytic action of the enzyme ATP sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4), which transfers the adenylyl moiety of ATP (∼AMP) from PP_i to SO_4 (reaction 1).

$$ATP^{-4} + SO_4^{2-} \leftrightarrows + PP_i^{4-} + APS^{2-} \qquad (1)$$

The extremely high Gibbs potential of the phosphoric−sulfuric acid anhydride bond of activated sulfate ($\Delta G^0_{\text{hydrolysis}}{}' = -19$ kcal/mol) potentiates the sulfuryl group for participation in its subsequent metabolic chemistries, reduction and transfer.[7,8] Sulfuryl-transfer, much like phosphoryl-transfer, regulates the activities of a large, diverse group of metabolites including steroids, peptide hormones, neuropeptides, and selectins.[8,9]

We herein report the first synthesis of the thio-nucleotide analogues of APS, α-thio-APS (adenosine 5′-*O*-[1-thiophosphosulfate] ($APS_{\alpha}S$)). Two synthetic routes to $APS{\alpha}S$ synthesis are presented. One of these is a facile "one-pot" synthesis that can be accomplished exclusively with commercially available reagents. This strategy affords a simple, general method for the site-specific delivery of the sulfuryl group. The Rp and Sp epimers of $APS_{\alpha}S$ were purified, their configurations were assigned, and they were used to investigate stereomechanistic issues in the ATP sulfurylase catalyzed synthesis of APS.

Experimental Section

Materials. Hexokinase, glucose-6-phosphate dehydrogenase, and snake venom phosphodiesterase (*Crotalus durissus*) were purchased from Boehringer Mannheim. ATP sulfurylase, adenosine, glucose, Na_4-PP_i, $NADP^+$, $MgCl_2$, Tris, and Hepes were purchased from Sigma. APS was synthesized as described previously.[10] P_2O_5, $PSCl_3$, triethyl phosphate, tri-*n*-octylammine, and pyridine-*N*-sulfonic acid were purchased from Aldrich. All other reagents were of the highest commercially available grades.

Preparation of Synthesis Reagents. $PSCl_3$ (thiophosphoryl trichoride) was distilled prior to use. Triethyl phosphate was distilled in

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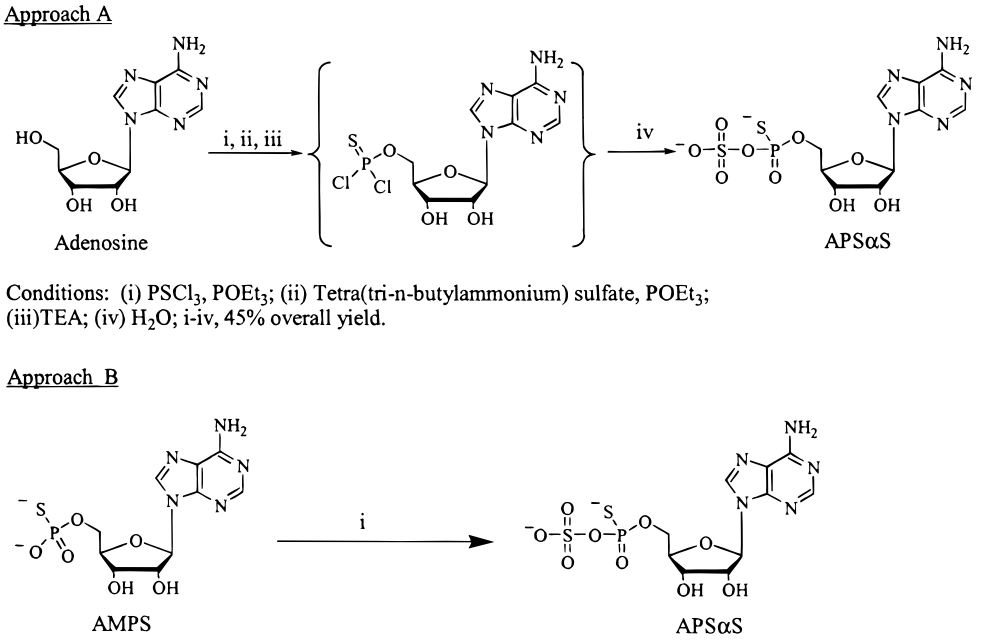
Scheme 1. The Synthesis of APSαS



vacuo over calcium hydride and the compound was stored in a sealed flask over 4 Å molecular sieves in the dark. Tri-*n*-octylamine was distilled in vacuo and stored in a sealed container over P_2O_5. Tri-*n*-butylamine was stirred with calcium hydride overnight, distilled under reduced pressure, and stored under N_2 at 5 °C.

Di(tri-*n*-butylammonium) Sulfate. Na_2SO_4 (0.1 M, 20 mL) was loaded onto a 5 × 5 cm Dowex-50W column in the pyridinium form. $(Py)_2SO_4$ was eluted from the column with water. Column fractions containing $(Py)_2SO_4$ were pooled, and 2.1 equiv of freshly distilled tri-*n*-butylamine were added to the solution. The mixture was evaporated to dryness by rotary evaporation in vacuo. Di(tri-*n*-butylammonium)-sulfate formed a film on the wall of the evaporation flask. The compound was stored in a desiccator over P_2O_5 for 48 h at 25 °C prior to use.

Synthesis of APS$_\alpha$S (Scheme 1). A racemic mixture of APS$_\alpha$S was synthesized using a derivative of the procedure used for the synthesis of other thio-nucleotides (Scheme 1, Approach A).[11] Adenosine (1.3 mmol), dried over P_2O_5 at 110 °C in vacuo for 24 h, was added to a stirred solution of 5.0 mL of anhydrous triethyl phosphate under argon, at 150 °C for 5 min. The adenosine dissolved, but did not decompose, during this interval. The reaction was cooled to 0 °C and 1.1 equiv of anhydrous tri-*n*-octylammine and 1.0 equiv of $PSCl_3$ were then added. The reaction was allowed to proceed under argon at 0 °C for 1 h, at which point >95% of the adenosine was converted to AMPS (adenosine 5′-*O*-[1-thiophosphate]). One equivalent of tetra(tri-*n*-butylammonium) sulfate in 12.5 mL of anhydrous triethyl phosphate was added dropwise to the stirred solution, at 25 °C, over a 30 min interval and the reaction was allowed to proceed for an additional 4 h. Anhydrous triethylamine (30 equiv) was added to the solution to precipitate the compounds containing phosphates, and the resulting white precipitate was filtered. The precipitate was dissolved in 15 mL of water and the solution was stirred for 1 h at 25 °C. The crude product was then loaded onto a DEAE Sephadex A-25 column (3 × 15 cm) and eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05−1.1 M, pH 7.4); APS$_\alpha$S eluted at 0.60 M. This procedure resulted in a 45% yield of an essentially pure racemic mixture of APS$_\alpha$S. The ^{31}P-NMR spectrum of the mixture showed only two peaks with chemical shifts of 47.31 and 47.21 ppm. ^{13}C NMR demonstrated that the thio-phosphosulfate moiety was attached at the 5′-position of the ribose ring. Electrosprary mass spectrometry of the racemic APS$_\alpha$S gave the expected *m*/*z* 442.0.

Aqueous Synthesis (Scheme 1, Approach B). While the above synthesis provides APS$_\alpha$S in good yield, it is somewhat time consuming and is readily accessible to only laboratories familiar with anhydrous organic phase synthesis. What follows is a simple, aqueous phase synthesis of APS$_\alpha$S that can be carried out using commercially available reagents (Scheme 1, Approach B). The reaction between phosphates and anhydrides of carboxylic acids in aqueous pyridine has been used by Avison in the preparation of the corresponding mixed anhydrides.[12] Baddiley, Buchanan, and Letters later reacted pyridine−*N*-sulfonic acid with AMP to produce adenosine 5′-phosphosulfate (APS).[10] We envisioned that this approach might extend to the synthesis of APS$_\alpha$S. This idea was tested by reacting pyridine-*N*-sulfonic acid[13] (0.25 mmol) with AMP$_\alpha$S (0.05 mmol) in 0.50 mL of 0.80 M $NaHCO_3$ buffer at 40 °C. The solution was mixed intermittently by vortexing for 15 min. The reaction mixture was then placed in an ice/water bath, diluted with 2.0 mL of H_2O, and adjusted to pH 5.5 with formic acid (2.0 M). The product of the reaction, obtained in 55% yield, was purified as described above, and was shown by ^{31}P NMR, mass spectroscopy, and C_{18} HPLC to be identical to the APS$_\alpha$S described above.

Intial-Rate Assay. Intial-rate studies were performed using the well-known hexokinase/glucose-6-phosphate dehydrogenase assay for ATP production.[14] This continuous assay links ATP synthesis to the reduction of $NADP^+$, which is monitored spectrophotometrically at OD_{340}. (Sp)-ATP$_\alpha$S, the exclusive product of the ATP sulfurylase reaction when Mg^{2+} is the activating cation, is a good substrate for hexokinase. V_{max} and K_m for (Sp)-ATP$_\alpha$S are 0.20- and 2.7-fold, respectively, those for ATP with Mg^{2+} activation.[15] The kinetic constants associated with (Sp)-ATP$_\alpha$S were used in selecting the assay conditions. The buffer used in the intial-rate experiments was Hepes/K^+ (50 mM, pH 8.0). The experiments were performed at 25(±2) °C. ATP sulfurylase and the coupling enzymes were extensively dialyzed vs Hepes/K^+ (50 mM, pH 8.0); the specific activity of each enzyme was determined in this buffer. The rates were measured within the first 8% of reaction. The

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activities of the primary and coupling enzymes were chosen to allow the reactions to achieve steady state within 20 s.[16]

Mg^{2+}- and Mn^{2+}-Activated V_{max}. The conditions were as follows: APS (90 μM, 160K_m);[17] PP_i (1.0 mM, 142K_m);[17] M^{2+} (1.1. mM); $NADP^+$ (0.20 mM); glucose (10 mM); KCl (10 mM); hexokinase (2.3 μmol/min/mL); glucose-6-phosphate dehydrogenase (0.85 μmol/min/mL); ATP sulfurylase (0.029 μmol/min/mL); Hepes/K^+ (50 mM, pH 8.0); and $T = 25(\pm 2)$ °C.

PP_i Concentration Dependence of (Sp)-$APS_\alpha S$ Inhibition. The conditions were as follows: APS (250 μM, 160K_m); (Sp)-$APS_\alpha S$ (150 μM); PP_i (0.5 mM, 71K_m; 1.0 mM, 142K_m; or, 1.5 mM, 213K_m); M^{2+} ([PP_i] + 0.8 mM); $NADP^+$ (0.20 mM); glucose (10 mM); KCl (10 mM); hexokinase (2.3 μmol/min/mL); glucose-6-phosphate dehydrogenase (0.85 μmol/min/mL); ATP sulfurylase (0.087 μmol/min/mL); Hepes/K^+ (50 mM, pH 8.0); and $T = 25(\pm 2)$ °C.

V_{max} for (Rp)-$APS_\alpha S$. The conditions were as follows: (Rp)-$APS_\alpha S$ (60 μM, 107K_m); PP_i (1.0 mM, 142K_m); Mg^{2+} (1.1 mM); $NADP^+$ (0.20 mM); glucose (10 mM); KCl (10 mM); hexokinase (2.3 μmol/min/mL); glucose-6-phosphate dehydrogenase (0.85 μmol/min/mL); ATP sulfurylase (0.029 μmol/min/mL). An identical experiment was run with APS. The velocities for both APS and (Sp)-$APS_\alpha S$ were 1.1 μmol/min.

K_m for (Rp)-$APS_\alpha S$. The K_m for (Rp)-$APS_\alpha S$ was determined in a competitive rate experiment in which both (Rp)-$APS_\alpha S$ and APS were added at identical and saturating concentrations. Product formation was followed by HPLC using a Vydac semipreparative C_{18} HPLC column with an isocratic mobile phase (4% acetonitrile, 50 mM triethylammonium acetate, pH 6.5, at 4.0 mL/min). Since V_{max} is the same for both compounds (see, V_{max} for (Rp)-$APS_\alpha S$), the relative rates of product synthesis are a direct measure of their relative K_{ms}. Given that $K_{m(APS)} = 560$ nM,[17] and the relative rates, one can calculate $K_{m((Sp)-APS\alpha S)}$. A competitive rate approach was taken, rather than a traditional double-reciprocal initial-rate study, because the affinity of (Rp)-$APS_\alpha S$ is so high, $K_m = 560$ nM (see below), that the quantities of product formed at (Rp)-$APS_\alpha S$ concentrations comparable to K_m (the range used in the traditional study) are too small for accurate quantitation by either the continuous assay, described above, or HPLC. The conditions of the experiment were as follows: (Sp)-$APS_\alpha S$ (1.5 mM); APS (1.5 mM); PP_i (1.0 mM); Mg^{2+} (4.0 mM); and ATP sulfurylase (0.020 μmol/min/mL). The reactions were stopped after 6% of the nucleotide was converted to product. Baseline separation of the (Rp)-$ATP_\alpha S$ and ATP were achieved using a semipreparative C_{18} column. The products were quantitated by integration. The velocities of the two reactions were virtually indistinguishable; hence, $K_{m((Sp)-APS\alpha S)} = K_{m(APS)} = 560$ nM.

Fitting the Inhibition Data. Inhibition by (Sp)-$APS_\alpha S$ involves binary and ternary inhibitor complexes (see Results). The literature indicates that the catalytic mechanism is equilibrium ordered, with APS binding first.[17] Assuming the equilibrium condition holds, the algebraic model that describes the initial-rate dependence on substrate and inhibitor concentration is easily derived, and is given by eq 2.

$$1/v = (1 + K_B/[B])/k_{cat} + (k_{cat}[A])^{-1}\{(K_A K_B/[B]) + [I](K_A K_B/K_A'[B] + K_A K_B/K_A' K_B')\} \quad (2)$$

A, B, and I represent APS, $MgPP_i$, and (Sp)-$APS_\alpha S$, respectively. K_A and K_A' are the dissociation constants for the binding of APS and (Sp)-$APS_\alpha S$ to E, respectively. K_B and K_B' are the dissociation constants for the binding of $MgPP_i$ to the E·APS and E·(Sp)-$APS_\alpha S$ complexes, respectively. At an infinite concentration of [B] (i.e, $MgPP_i$), eq 2 reduces to eq 3, which was the equation that was used to fit the data shown in Figure 5 for the unknown, $K_A'K_B'$.

$$1/v = 1/k_{cat} + (k_{cat}[A])^{-1}\{(K_A K_B/[B] + [I]K_A K_B/K_A' K_B')\} \quad (3)$$

The simplification of eq 2 is justified by the fact that the [PP_i] used in the study, 1.0 mM, is 107 times its K_m, and the inhibition is independent of the [PP_i] above 0.50 mM (see Results). The constants used in the model were taken from previous literature.[17] The data were fit using the Sigma Plot program, which uses the Marquardt−Levenberg fitting algorithm.

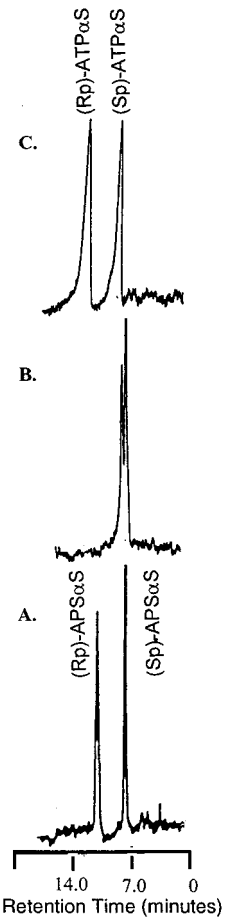

Figure 1. The stereochemistry of the yeast ATP sulfurylase reaction. $APS_\alpha S$ was enzymatically converted to $ATP_\alpha S$ in a reaction that contained $APS_\alpha S$ (~0.5 mM in each epimer), PP_i (2.0 mM), $Mg(OAc)_2$ (3.0 mM), ATP sulfurylase (0.20 units/mL), and Tris/Cl (50 mM, pH 8.0). Panels A and B present A_{260} HPLC profiles of the reaction at 0 and 10 min, respectively. Panel C presents the profile of a control sample containing both epimers of $ATP_\alpha S$ (~0.5 mM in each). The enzyme is stereoselective for the (Rp)-$APS_\alpha S$, which it converts into the (Sp) epimer of $ATP_\alpha S$. The reaction was run at 25 °C and separation of the epimers was achieved using a Vydac semipreparative C_{18} HPLC column with an isocratic mobile phase (4% acetonitrile, 50 mM triethylammonium acetate, pH 6.5, at 4.0 mL/min).

Results and Discussion

Assigning the Stereochemical Configuration of the Epimers of $APS_\alpha S$. Exhaustive studies have established the empirical criterion that the Sp thio-nucleotide epimer elutes earlier than its Rp counterpart from a C_{18} HPLC column when an appropriate mobile phase is used (Figure 1, Panel A).[18−20] The epimers of $APS_\alpha S$ are well separated using a semipreparative C_{18} Vydac column (4.0% CH_3CN in 50 mM triethyl ammoniun acetate, pH 6.5) (Figure 1, Panel A). Using this criterion, the $APS_\alpha S$ epimers that elute at 7.5 and 11 min can tentatively be assigned the Sp and Rp configurations, respectively (Figure 1, Panel A). An alternative assignment criterion is provided by the charac-

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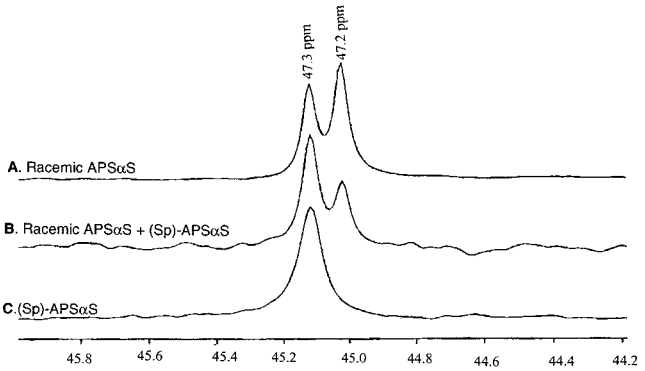

Figure 2. ^{31}P NMR confirmation of the HPLC-based assignment of (Rp)- and (Sp)-APS$_\alpha$S. The Sp epimers of α-thio-nucleotides elute earlier on reverse phase HPLC, and their ^{31}P NMR resonances are shifted ~0.1 ppm downfield from their Rp counterparts. Panels A−C present the ^{31}P NMR spectra of the racemic mixture of APS$_\alpha$S (Panel A), the same racemic mixture to which (Sp)-APS$_\alpha$S (assigned based on HPLC) has been added (Panel B), and (Sp)-APS$_\alpha$S alone (Panel C). The HPLC and ^{31}P NMR assignments are in agreement. The ^{31}P NMR spectra were obtained using a 121.4 MHz Bruker DRX 300 spectrometer. The samples were ^{1}H-broad-band decoupled and the data were acquired at 25 °C. The chemical shifts in ppm are referenced to an external H_3PO_3 standard.

teristic differences between the ^{31}P NMR spectra of thio-nucleotide epimers.[18,19] Chemical shift differences are typically ~0.10 ppm, and the literature suggests that the downfield peak invariably corresponds to the Sp species. ^{31}P NMR spectra of the epimers of APS$_\alpha$S (Figure 2) corroborate the HPLC assignment: the compounds that elute at 7.5 and 11 min correspond to the Sp and Rp configurations, respectively. The third criterion used to assign the configuration of the epimers was their ability to serve as substrates for snake venom phosphodiesterase (E.C. 3.1.4.1). Phosphodiesterase, which hydrolyzes nucleotide-5′-phosphodiesters to the corresponding monoesters, demonstrates broad substrate specificity, and a high degree of stereoselectivity for the (Rp)-P$_\alpha$ configuration of the diester. When a racemic mixture of APS$_\alpha$S is treated with phosphodiesterase, only the isomer predicted by the NMR and HPLC data to be the Rp isomer was hydrolyzed (Figure 3). The agreement of these three independent criteria strongly suggests that the compounds that elute at 7.5 and 11 min are, in fact, the Sp and Rp epimers of APS$_\alpha$S, respectively.

Stereochemistry and Stereoselectivity of the ATP Sulfurylase Catalyzed Reaction. The stereochemisty and stereoselectivity of the ATP sulfurylase catalyzed reaction were evaluated by monitoring the progress of a reaction in which a racemic mixture of the epimers of APS$_\alpha$S (~0.5 mM in each) was incubated with ATP sulfurylase (0.20 units/mL), $Na_4P_2O_7$ (2.0 mM), $Mg(CH_3CO_2)_2$ (3.0 mM), and Tris/Cl (50 mM, pH 8.0). The progress of the reaction was monitored by C_{18} HPLC (isocratic, 4% CH_3CN in triethylammonium acetate, pH 6.5). (Rp)-APS$_\alpha$S was completely converted to (Sp)-ATP$_\alpha$S within 10 min (the epimers of ATPαS were synthesized and purified according to an established protocol[21] and their relative retention times on C_{18} HPLC are well characterized[18]). We could not detect any formation of the (Rp)-ATP$_\alpha$S or consumption of (Sp)-APS$_\alpha$S, indicating that the enzyme exhibits a very high degree of stereoselectivity (Figure 1). These results demonstrate that the transfer of the adenylyl moiety between pyrophosphate and sulfate occurs with stereochemical inversion at the α-phosphorus. It is generally accepted that inversion of stereochemistry implies a single displacement reaction in which the enzyme catalyzes an "in-line" nucleophilic attack leading directly to the formation of product.[21] This finding rules out both an adjacent attack, followed by a pseudorotation, and a double-displacement mechanism with an adenylyl enzyme intermediate, as was postulated by Segel and co-workers,[22] because these mechanisms predict stereochemical retention. Our result agrees with an early study by Lowe et al. that used the isotopomers of adenosine-5′-[(*S*)-^{16}O,^{17}O,^{18}O]-phosphosulfate to determine the stereochemical course of the yeast ATP sulfurylase catalyzed reaction.[23] The agreement between Lowe's and our results demonstrates that the stereochemical course of the reaction is not altered by sulfur substitution, and that in addition to providing the stereochemistry of the overall reaction, the thio analogues provide an assessment of the stereoselectivity of the enzyme.[24]

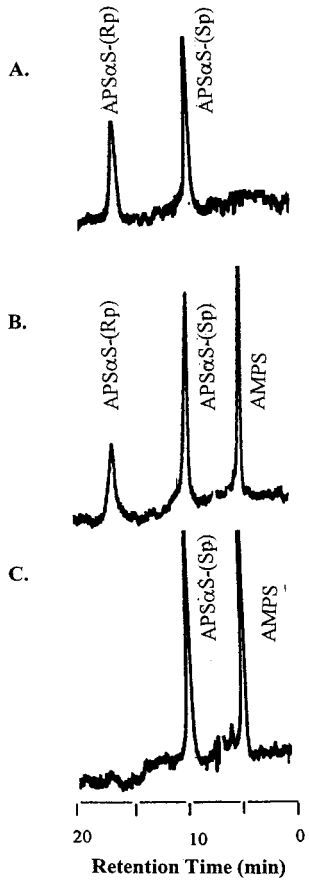

Figure 3. The enzymatic assignment of the stereochemical configuration of (Rp)- and (Sp)-APS$_\alpha$S. A racemic mixture of APS$_\alpha$S was treated with snake venom phosphodiesterase from *Crotalus durissus*. Panels A−C present the C_{18} HPLC chromatograms of the reaction mixture at 0, 10, and 30 min, respectively. The late-eluting isomer is virtually completely hydrolyzed to AMP$_\alpha$S during this time interval, while the early-eluting species is essentially unaffected. The high degree of selectivity of the phosphodiesterase for the late-eluting isomer strongly supports that it is the (Rp)-APS$_\alpha$S epimer. The reaction conditions were as follows: APS$_\alpha$S (~0.50 mM in each epimer); $MgCl_2$ (2.0 mM); Tris/acetate (100 mM, pH 8.0); phosphodiesterase (0.033 u/ml); and $T = 25 \pm 2$ °C. The HPLC conditions are described in the Figure 1 legend.

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To assess how (Rp)-APS$_\alpha$S compares with APS as substrate, K_m and V_{max} were determined for both substrates using Mg^{2+} as the activating cofactor (see Experimental Section). Remarkably, the constants are virtually indistinguishable (K_m = 560 and 560 nM and V_{max} = 1.1 and 1.1 μmol/min/OD$_{280}$, for (Rp)-APS$_\alpha$S and APS, respectively). Given the known effects of sulfur substitution on bond order,[25] electronic distribution,[25] and the rates of enzymatic and nonenzymatic chemistry,[26,17b] it is reasonable to expect that if bond breaking were rate determining, thio substitution would influence V_{max}. The absence of such an effect suggests, but does not prove, that a step other than chemistry is rate determining.

Divalent Cation Coordinates P$_\alpha$. It is well established that the hardness or softness of a divalent cation correlates with its propensity to coordinate oxygen or sulfur, respectively, of a nucleotide thiophosphoryl group.[27] This hard−hard, soft−soft complementarity can be used to assess whether a divalent cation coordinates a particular phosphoryl group during the catalytic cycle, and which prochiral oxygen is coordinated to the cation. If, for example, Mg^{2+} (a hard metal ion) coordinates a particular prochiral oxygen, substituting sulfur for that oxygen would result in anticomplementarity and a poor substrate compared to the epimer in which oxygen coordinates Mg^{2+}, and complementarity is satisfied. To verify that such stereoselective effects are associated with cation coordination rather than other topological constraints of the active site, the specificity can be studied as a function of metal ion hardness. The following rank ordering of the affinities of divalent cations for oxygen over sulfur has been deduced from solution studies using thionucleotides: $Mg^{2+} \gg Mn^{2+} \sim Co^{2+} \gg Cd^{2+}$.[27b,c]

The high degree of selectivity for (Rp)-APS$_\alpha$S when Mg^{2+} is the activating cation is consistent with Mg^{2+} coordination to the P$_\alpha$ proS oxygen at some point during the catalyic cycle. The results obtained with Mn^{2+} confirm that this is the case. Mn^{2+} is an excellent surrogate for Mg^{2+} in the native reaction. The Mn^{2+}-activated V_{max}, obtained at saturating concentrations of APS and PP$_i$, is 0.59-fold less than that obtained with Mg^{2+} (see Experimental Section). Like Mg^{2+}, Mn^{2+} is highly stereoselective for (Rp)-APS$_\alpha$S; however, unlike Mg^{2+}, which does not discriminate between APS and (Rp)-APS$_\alpha$S on a V_{max} basis, the V_{max} with Mn^{2+} decreases 14-fold when (Rp)-APS$_\alpha$S is substituted for APS. This cation-dependent selectivity demonstrates that P$_\alpha$ and the cation interact, suggesting that P$_\alpha$ is coordinated to the cation. The results of studies using Co^{2+} and Cd^{2+} provide further credence for P$_\alpha$−cation interaction and reveal an intriguing cation-dependent change in the enzyme's mechanism. Co^{2+}, which demonstrates a slight preference for oxygen over sulfur,[27b] is stereoselective for (Rp)-APS$_\alpha$S; however, the complete consumption of (Rp)-APS$_\alpha$S produces only 15% of the expected (Sp)-ATP$_\alpha$S, the remainder is the hydrolysis product, AMP$_\alpha$S (Figure 4). Cd^{2+}, which has a very strong preference for sulfur, results in the hydrolysis of both isomers of APS$_\alpha$S, and does not produce detectable levels of ATP$_\alpha$S (Figure 4). Thus, softening the cation fosters an alternative catalytic path (i.e., hydrolysis) and a partial reversal of the hydrolysis stereoselectivity seen with Co^{2+}.

Stereoselective Events Occur after the E·APS·PP$_i$·M^{2+} Complex Has Formed. The data described in the preceding section strongly support coordination at P$_\alpha$; however, they do not address the issue of where in the catalytic cycle the P$_\alpha$ proS oxygen of APS inserts into the first coordination sphere of the divalent cation. If the cation interacts with P$_\alpha$ in the binary, E·APS, and/or ternary, E·APS·PP$_i$, complex, the reactions that form these complexes should be stereoselective. An absence of stereoselectivity in these binding reactions would indicate that the cation−P$_\alpha$ interactions occur in other regions of the catalytic cycle. With Mg^{2+} as the activating cation, the system is highly selective on a turnover basis for (Rp)-APS$_\alpha$. If the binding reactions are stereoselective, the affinity of (Sp)-APS$_\alpha$S, the epimer that is selected against during turnover, will be weak compared to that of the native substrate, APS.

The affinity of (Sp)-APS$_\alpha$S was assessed in a classical initial-rate inhibition study in which (Sp)-APS$_\alpha$S was used as an inhibitor vs APS, with Mg^{2+} as the activator (Figure 5). Given that the native binding mechanism is ordered (APS adds prior to PP$_i$),[17] the observed linear-competitive inhibition pattern is consistent with mechanisms in which PP$_i$ either can or cannot add to the E·(Sp)−APS$_\alpha$S complex. This is an important issue because it addresses whether the ternary, E·(Sp)−APS$_\alpha$S·PP$_i$·M^{2+} complex can form. If substrate binding is ordered and at equilibrium during turnover (as is believed to be the case)[17] the affinity of the first substrate, APS, is linked by mass action to the concentration of the second substrate, PP$_i$, such that the

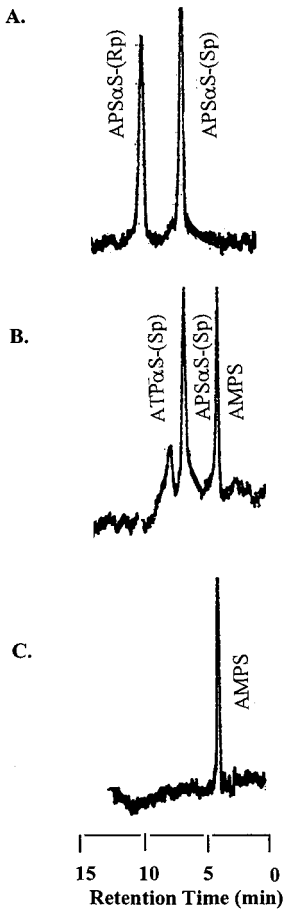

Figure 4. The Co^{2+}- and Cd^{2+}-dependent reactions catalyzed by ATP sulfurylase. The reaction conditions were as follows: APS$_\alpha$S (~0.5 mM in each epimer); PP$_i$ (2.0 mM); $CoCl_2$ or $CdCl_2$ (3.0 mM); ATP sulfurylase (0.2 units/ml); Tris/Cl (50 mM, pH 8.0); and T = 25(±2) °C. A control, A_{260} HPLC profile of the (R_p)- and (S_p) epimers of APS$_\alpha$S, is shown in Panel A. Panels B and C present the profiles of the completed Co^{2+} and Cd^{2+} activated reactions, respectively. The HPLC conditions are described in the Figure 1 legend.

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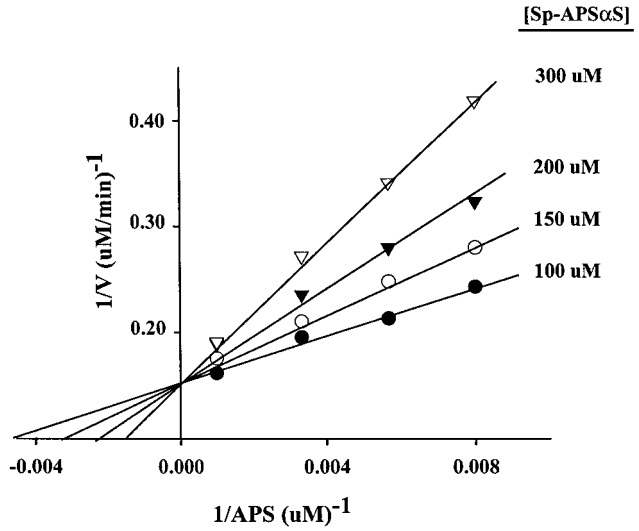

Figure 5. (Sp)-$APS_\alpha S$ is a competitive inhibitor vs APS. Intial rates were determined at each of the 16 conditions obtained from a 4 × 4 matrix of APS and (Sp)-APS_α concentrations. The substrate and inhibitor concentrations were as follows: APS (125, 176, 300, 1000 μM); (Sp)-$APS_\alpha S$ (100, 150, 200, 300 μM). The assays also contained the following: PP_i (1.0 mM, 107K_m); Mg^{2+} (1.1. mM + [nucleotide]); $NADP^+$ (0.20 mM); glucose (10 mM); KCl (10 mM); hexokinase (13 μmol/min/mL); glucose-6-phosphate dehydrogenase (0.85 μmol/min/mL); and ATP sulfurylase (0.30 μmol/min/mL). The rates were determined at 25(±2) °C. Each rate was determined at least two times, the rates at each condition were averaged, and the data set was then statistically fit as described in the Experimental Section.

affinity of APS will become infinite at an infinite concentration of PP_i. Thus, if PP_i were incapable of binding to the binary inhibitor complex (i.e., E·(Sp)−$APS_\alpha S$), increasing the concentration of PP_i would remove the inhibition by selectively increasing the affinity of APS over that of the inhibitor. Alternatively, if PP_i binds to both the native and inhibitor binary complexes, inhibition is *not* removed with increasing PP_i concentration, rather, the percent inhibition becomes independent of PP_i concentration as the binary complexes become saturated with PP_i. To address this issue, initial-rate experiments were performed at three, saturating concentrations of PP_i (71 to 284 × K_m). The reactions contained both APS (250 μM) and (Sp)-$APS_\alpha S$ (150 μM) (see Experimental Section). The initial rates were independent of pyrophosphate concentration (25(±1)% inhibition was observed at each PP_i concentration). Hence, PP_i can add to the binary complex and form the ternary inhibitor complex, E·(Sp)−$APS_\alpha S$·PP_i·M^{2+}.

The algebraic model describing the inhibition is sufficiently complex that the individual kinetic constants governing the binding of (Sp)-$APS_\alpha S$ to E (i.e., K_A') and PP_i to the E·(Sp)−$APS_\alpha S$ (i.e., K_B') complex cannot be obtained from the inhibition study, Figure 5. However, the product of these two constants, $K_A'K_B'$, which represents the dissociation of the ternary complex, E·(Sp)−$APS_\alpha S$·PP_i, into its three components, can be obtained from the data (see the Experimental Section). $K_A'K_B' = 0.90\ \mu M^2$ and is extremely similar to that for the native reaction, $K_AK_B = 0.63\ \mu M^2$. This similarity suggests that the steady-state affinities of (Sp)-$APS_\alpha S$ and APS for E, and of PP_i for the E·(Sp)−$APS_\alpha S$ and E·APS complexes, are virtually identical. It is possible, while seemingly unlikely, that the kinetic constants for the native and analogue binding reactions are actually quite different, and that these differences compensate such that their products are virtually identical.

(Sp)-$APS_\alpha S$ appears to be an excellent mimic of APS throughout the substrate binding region of the reaction coordinate. It forms both the binary and ternary complexes, and its affinity is similar to that of APS. The apparent lack of discrimination against (Sp)-$APS_\alpha S$ in the binding reactions strongly suggests that the event(s) responsible for the stereoselectivity (i.e., the ligand exchange reaction that results in P_α coordination) occurs in the catalytic cycle after the E·APS·PP_i·M^{2+} complex has formed. The data demonstrate that either epimer of $APS_\alpha S$ is capable of proceeding into the catalytic cycle to the point at which the coordination attempt occurs. If oxygen is presented to Mg^{2+}, the substrate proceeds unabated through the catalytic cycle (i.e., (Rp)-$APS_\alpha S$ is as good a substrate as APS). However, if sulfur is presented to Mg^{2+} the substrate is rejected, and the enzyme is left to oscillate among its nondiscriminatory forms, up to and *including* the point of rejection.

Conclusions

$APS_\alpha S$ has been synthesized for the first time. A novel, facile synthesis that may be extended to other nucleotides has been developed. The epimers of $APS_\alpha S$ were purified and their configurations were assigned. $APS_\alpha S$ was used to investigate stereomechanistic aspects of the reaction catalyzed by ATP sulfurylase. The reaction is highly stereoselective and proceeds with inversion of configuration at P_α. The cation dependence of the enzyme's activity strongly supports metal ion coordination at P_α (Mg^{2+} coordinates the pro-S oxygen of APS). Initial-rate inhibition studies indicate that coordination at P_α occurs in the catalytic cycle after formation of the ternary E·APS·PP_i·M^{2+} complex. Further applications of these compounds are underway in this laboratory. The results of these studies will be published in due course.

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