GTPase Activation of ATP Sulfurylase: The Mechanism†

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ABSTRACT: ATP sulfurylase from *Escherichia coli* K12 catalyzes two, coupled reactions: the hydrolysis of GTP and the formation of activated sulfate (APS). At saturating levels of GTP, the initial rate of APS formation is stimulated 116-fold. The mechanism of this activation has been investigated using isotope trapping, mass spectrometry, and initial velocity kinetic techniques. In the presence of GTP, APS formation proceeds *via* nucleophilic attack of sulfate at the α-phosphoryl group of ATP. Isotope-trapping experiments demonstrate productive, random binding of ATP and GTP. ATP is hydrolyzed to yield AMP and PP_i. AMP production requires GTP and is suppressible by sulfate, suggesting GTP-dependent formation of an E*AMP intermediate in the synthesis of APS. Studies using the hydrolysis-resistant nucleotide analogues AMPCPP and GMPPNP demonstrate that GTP hydrolysis precedes scision of the α–β bond of ATP. Product inhibition studies indicate that PP_i release occurs prior to the addition of sulfate and APS formation. These results are used to construct a proposed mechanism for the GTP-activated synthesis of APS.

The activation of sulfate is required for its metabolic assimilation (De Meio, 1975). Activation is accomplished by transferring the adenosine 5′-phosphoryl moiety of ATP to sulfate. This reaction, catalyzed by ATP sulfurylase (ATP: sulfate adenyltransferase, EC 2.7.7.4), is represented by eq 1(Hiltz & Lipmann, 1955). The sulfuryl moiety of activated

$$ATP + SO_4 \rightleftharpoons APS + PP_i \quad (1)$$

sulfate (APS) poised, by virtue of phosphoric–sulfuric anhydride linkage, for facile, energetically downhill entry into its subsequent metabolic fates of reduction and group transfer (Leyh, 1993).

The metabolic requirements for activated sulfate are diverse and species dependant. Many bacteria reduce APS to provide the sulfide necessary for synthesis of sulfur metabolites (Siegel, 1975). Certain anaerobic microbes couple APS reduction to oxidative phosphorylation (Siegel, 1975). In mammals, the primary function of activated sulfate appears to be that of the sulfuryl group donor. The actual donor is PAPS (3′-phosphoadenosine 5′-phosphosulfate), a phosphorylated form of APS. The sulfuryl recipient is typically a carbohydrate hydroxyl or protein–tyrosyl residue. Sulfated carbohydrates are important in cell adhesion (Brauer et al., 1990), hemostasis (Atha et al., 1984, 1987; Niehrs et al., 1990), and the viscoelastic properties of connective tissue (Devered & Whelan, 1986). Dwarfism results from the undersulfation of cartilage in mice (Sugahara & Schwartz, 1979). Protein–tyrosyl sulfation is a common post-translational modification often found to have profound physiological consequences (Huttner, 1987). It can switch on or off hormonal activity (Brand et al., 1984; Pasqualini et al., 1992) or provide a chemical linchpin anchoring proteins together. Antihemophillic Factor B is tethered to Von Willibrand factor in this way (Leyete et al., 1991). Improper sulfation of Factor B results in severe hemophilia (Tuddenham et al., 1991). Many other examples of the physiological importance of sulfation have been documented (Lindahl & Hook, 1978; Mudd, 1980; Mulder & Meermen, 1982; Jakoby & Ziegler, 1990).

ATP sulfurylase from *Escherichia coli* K12 is composed of two types of subunits, CysN (53 kDa) and CysD (35 kDa) (Leyh et al., 1987). The native (390 kDa) and subunit molecular weights suggest that the enzyme is a tetramer of CysD–CysN heterodimers. Encrypted in the primary sequence of CysN is a GTP-binding motif that is common among members of the GTPase superfamily (Leyh et al., 1992). When the functional implications of the GTP-consensus sequence were pursued, it was found that the enzyme was a GTPase (Leyh & Suo, 1992) and the rate of APS synthesis was stimulated 116-fold at a saturating concentration of GTP. Thus, a novel GTPase/target system had been discovered. In this communication, we present our studies which determine the GTPase-activated mechanism of APS synthesis.

MATERIALS AND METHODS

Materials. Nucleotides, NADH, and PEP were of the highest grades available from Sigma Chemical Co. Tricine[1]-stabilized radionucleotides [α-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [γ-^{32}P]GTP (6000 Ci/mmol), and [^{32}P]PP_i (1–60 Ci/mmol) were purchased from DuPont NEN Corp. [^{18}O]H_2O (96 atom % ^{18}O) was obtained from MSD Isotopes. The enzymes inorganic pyrophosphatase (yeast), lactate dehydrogenase (rabbit muscle), and pyruvate kinase (yeast) were purchased from Boehringer Mannheim Corp. ATP sulfurylase was purified according to a published protocol from an *E. coli* K12 strain containing an expression vector that results in production of high levels of the enzyme from *E. coli* K12 (Leyh et al., 1987). The specific activity of the ATP sulfurylase ranged from 0.23 to 0.25 u/mg. The mono Q HR 5/5 and Superose-12 columns were purchased from Pharmacia LKB. The poly(ethyleneimine)–cellulose-F thin-layer chromatography (PEI-F TLC) plates were obtained

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[1] Abbreviations: AMPCPP, 5′-adenyl methylenephosphonate; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GMPPNP, 5′-guanylyl imidodiphosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; TRIS, tris(hydroxymethyl)aminomethane; u, unit, μmol of substrate converted to product/min at V_{max}.

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from E. Merck Co. Other compounds were reagent grade or better.

Synthesis of $[^{18}O_4]SO_4$. $[^{18}O_4]SO_4$ was synthesized by hydrolyzing sulfuryl dichloride (SO_2Cl_2) in $[^{18}O]H_2O$ (96% ^{18}O). Eight microliters of freshly distilled sulfuryl dichloride were added to 100 μL of stirred $[^{18}O]H_2O$ at ambient temperature. Mock reactions were assayed for the extent of completion titrimetrically with a standardized NaOH solution using a phenolphthalein indicator or $Ba(ClO_4)_2$ with a Thorin (2-(2-hydroxy-3,6-disulfo-1-naphthylazo)benzenesulfonic acid) indicator (Fritz & Yamamura, 1955). After 2 h, >95% of the sulfuryl dichloride was converted to sulfate. The $[^{18}O_4]SO_4$ was used for the synthesis of [^{18}O]APS without further purification.

Characterization of Labeled $[^{18}O_4]SO_4$. The mass spectrum of the labeled SO_4 was simulated very well assuming a single chemical species, HSO_4, with an isotopic distribution at a given oxygen position of ^{18}O (90.4%), ^{17}O (2.3%), and ^{16}O (7.3%). The mass to charge ratio of the predominant species in the spectrum was 105, which was 8 mass units higher than that observed in the spectrum of natural abundance HSO_4.

Synthesis of $[^{18}O]APS$. ATP and SO_4 were enzymatically converted to APS and PP_i using ATP sulfurylase. The coupling enzymes inorganic pyrophosphatase and pyruvate kinase were included in the reaction mixture to thermodynamically drive APS formation and to regenerate GTP, respectively. Parallel reactions were run using [^{18}O]-enriched or natural abundance SO_4; the natural abundance APS provided the control for mass spectral analysis. The reaction mixtures contained [^{18}O]-enriched or natural abundance SO_4 (1.0 mM), ATP (1.0 mM), GTP (1.0 mM), PEP (5 mM), $MgCl_2$ (3.0 mM), KCl (10 mM), ATP sulfurylase (0.92 μM), pyruvate kinase (5 u/mL; 1 u = 1 μmol of GDP converted to GTP/min at V_{max}), inorganic pyrophosphate (0.1 u/mL), and 50 mM HEPES/K^+, pH = 8.0; 2.5 mL of each reaction mixture was prepared, and the reactions were run for 2 h at 23 ± 2 °C and quenched by boiling. The reactions yielded 50–55% conversion of the SO_4 to APS. The APS was chromatographically purified using a mono Q HR 5/5 column with a linear, 10 mM–1.0 M gradient of freshly distilled triethylamine/HCO_3, pH = 7.8. The fractions containing APS were pooled and concentrated to dryness by rotary evaporation with gentle heating. To remove residual triethylamine, the APS was suspended and dried three times in methanol followed by three times in H_2O. The APS was then suspended in H_2O to a final concentration of 5–10 mM and the pH adjusted to 8.0 with 1 N KOH.

Mass Spectrometry. Fast atom bombardment mass spectrometry was performed at a resolution of 1500 using a Finnigan MAT-90 spectrometer. APS and sulfate samples were made 50% (v/v) glycerol for spectral analysis. Spectra were obtained at the Laboratory for Macromolecular Analysis located at the Albert Einstein College of Medicine.

Isotope Trapping. (*A*) *Trapping GTP.* The pulse solution contained ATP sulfurylase (241 μM), [γ-^{32}P]GTP (520 μM, 0.2 μCi/μL), and $MgCl_2$ (1.50 mM). The chase solution contained GTP (6.0 mM), $MgCl_2$ (7.0 mM), and SO_4 (1.0 mM), the ATP concentration was varied (see Figure 2). Five seconds following the addition of ATP sulfurylase to the pulse, a 17-fold excess volume of chase was added with vigorous mixing. The reaction was quenched at 2, 3, 4, and 5 s by addition of a stock solution of Na_4EDTA (400 mM, pH = 9.5) to a final concentration of 35 mM. Because these reactions are very slow, mixing was performed with Gilson Pipeteman pipetors. Immediately following the EDTA addition, the tubes containing the reaction mixtures were placed in a boiling water bath for 1 min and then placed on ice. The reaction mixtures were spotted on PEI-F TLC sheets and the reactants separated using a 1.0 M LiCl mobile phase (Randerath & Randerath, 1964). The product, [^{32}P]P_i, was quantitated using an AMBIS 2-D radioactivity counter. A time course was constructed for each reaction and fit using a least-squares linear regression. Each time course was run at least two times. The P_i trapped was calculated by subtracting the back-ground P_i from that obtained by extrapolating the time course of 0 s. Background P_i was determined by reversing the order of addition of the EDTA and chase solution. Background controls were run for each AMP concentration and gave very similar results. The trapping reactions were run at 23 ± 2 °C.

(*B*) *Trapping ATP with GTP ± SO₄.* The manipulations and conditions were the same as those used for the trapping of GTP with the following exceptions. The pulse solution contained ATP sulfurylase (291 μM), [α-^{32}P]ATP (250 μM, 0.2 μCi/μL), and 1.25 mM $MgCl_2$. The chase contained GTP (3.0 mM), SO_4 (1.0 mM or none), and $MgCl_2$ (4.0 mM).

Coupling Enzymes. All coupling enzymes were desalted by size-exclusion chromatography using a Superose-12 column equilibrated with 50 mM HEPES/K^+, pH = 8.0. The specific activity of each enzyme was determined in 50 mM HEPES/K^+, pH = 8.0, the buffer used throughout the current studies. The activities of pyruvate kinase and lactate dehydrogenase were determined using optical assays (Buchler & Pfleiderer, 1955; Dixon & Webb, 1979). Inorganic pyrophosphate was assayed using [^{32}P]PP_i, and reactants were separated on PEI-F TCL sheets and quantitated using an AMBIS scanner.

Optical Assays of GTP Hydrolysis. GTP hydrolysis was monitored at 340 nm by coupling the production of GDP to the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. The rate at which a coupled series of reactions approaches steady state for single- and dual-coupling enzyme systems has been discussed in some detail (McClure, 1969). V/K, the pseudo-first order rate constant for the conversion of substrate to product, for each of the coupled reactions determines the time required to attain steady state. For reactions that recycle product to substrate, V/K also determines the steady-state product/substrate ratio. In the current studies, reactions achieved steady state within 5–10 s; the steady-state GDP levels were 1–5% that of total guanine nucleotides. Assays were performed at 25 ± 2 °C, and they were linear with time and enzyme concentration.

RESULTS AND DISCUSSION

Nucleophilic Character of the APS-Forming Reaction. Sulfate and the α-phosphoryl moiety of ATP are generally considered poor nucleophiles. The pK_a of the α-phosphoryl oxygen is estimated at 0.5 (Jencks & Regenstein, 1976; Dawson et al., 1986), and the most basic pK_a of SO_4 is 2.0 (Weast, 1988). Thus, it was of interest to determine which of these unreactive species acts as the nucleophile in the formation of APS. This was accomplished by comparing the mass of APS synthesized using ^{18}O-enriched SO_4 with that of natural abundance APS. Assuming 100% ^{18}O-enrichment, nucleophilic attack by labeled sulfate will produce APS that is 8 mass units above that of natural abundance APS. Alternatively, a 6-mass-unit increment is expected if the α-phosphoryl oxygen attacks at sulfate.

The mass spectra of heavy leveled (black rectangles) and natural abundance (open rectangles) APS are shown in Figure 1. The predominant signal in the natural abundance spectrum

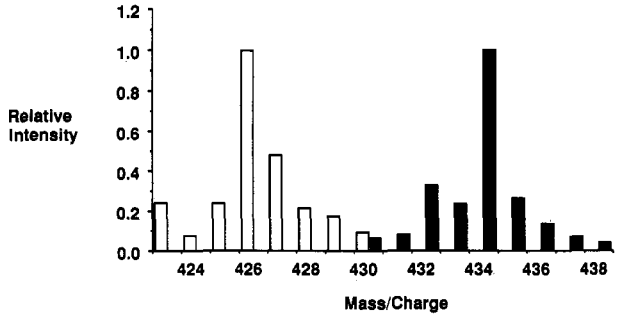

FIGURE 1: Mass spectra of APS synthesized enzymatically using ^{18}O-labeled or natural abundance SO_4. The natural abundance and ^{18}O-enriched spectra are displayed with open and solid rectangles, respectively. The APS synthesis and purification are described in Materials and Methods; GTP was used in the synthesis. The signal intensities were normalized such that the maximum intensity in each spectrum was assigned an arbitrary value of 1.0.

occurs at a mass/charge of 426, the value predicted for the APS^- species. The corresponding signal in the ^{18}O-enriched APS spectrum is 8 mass units higher, at a mass/charge of 434. The distribution of signals in the enriched spectrum arises from the nonuniform isotopic composition of the water used for the synthesis of ^{18}O-enriched SO_4 estimated at: ^{18}O (90.4%), ^{17}O (2.3%), and ^{16}O (7.3%) (see Materials and Methods). This distribution is very similar to that seen in the spectrum of the labeled sulfate used for the APS synthesis. Thus, the reaction proceeds *via* nucleophilic attack by sulfate at the α-phosphoryl position resulting in cleavage between the α-phosphorus and its briding oxygen.

Order of Substrate Binding. To determine the order of substrate binding, isotope-trapping experiments were performed using either [γ-^{32}P]GTP or [α-^{32}P]ATP. These experiments involve dilution of a pulse solution—a mixture of enzyme (E), labeled nucleotide (A*), and the putative E·A* complex—with a chase solution containing a large excess of unlabeled A and a fixed variable concentration of the complementary reactant(s) (B) required for the conversion of the labeled species to product (Rose, 1980). Dissociation of A* after addition of the chase is essentially irreversible due to very large dilution with unlabeled A. If product formation requires that B binds the enzyme before A, as in the case of an ordered mechanism, no labeled product will form. If, however, B can bind to E·A* and cause product formation, it is possible that some fraction of the E·A* in the pulse will be converted directly to product without dissociating into the chase. This "trapping" of the labeled substrate as product requires that the desorption of A* from the binary (E·A*) and ternary (E·A*·B) complexes be sufficiently slow to allow a measurable quantity of the labeled GTP to proceed through the first irreversible step toward product formation.

The results of three such experiments are shown in panels A–C of Figure 2. The data in panel A demonstrate ATP-dependent trapping of GTP; 1.0 mM sulfate was included in the chase. Thus, GTP binds productively to the enzyme, and activation of GTP hydrolysis occurs as a consequence of the binding of the ATP and/or sulfate to the E·GTP complex. Panel B demonstrates that ATP also forms a productive complex with the enzyme. In this case, the chase contained a fixed variable concentration of GTP and 1.0 mM sulfate. To trap ATP as APS, both GTP and sulfate must add to the enzyme subsequent to the addition of ATP. No trapping of ATP was observed using only sulfate (1.0 mM) in the chase; however, a small fraction of ATP could be trapped with 1.0 mM GTP alone in the chase (see panel C). AMP is the product

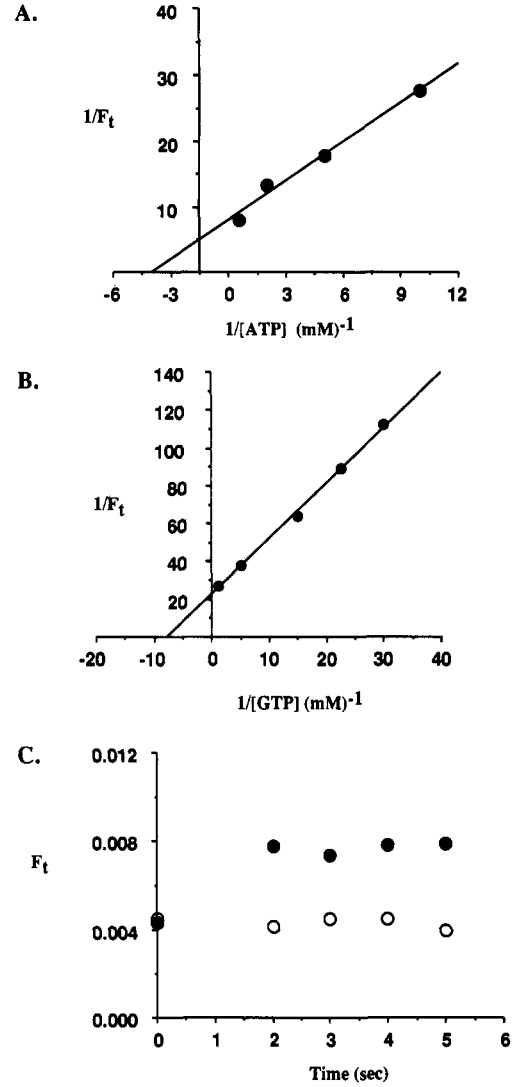

FIGURE 2: Isotope trapping of ATP and GTP. The ordinate label, F_t, represents the fraction of enzyme active sites that yielded product. Panel A shows the titration of [^{32}P]P_i formation from E·[γ-^{32}P]GTP with ATP; SO_4 was 1.0 mM in the chase. Panel B presents the titration of [α-^{32}P]APS formation from E·[α-^{32}P]ATP with GTP; SO_4 was 1.0 mM in the chase. Panel C shows a progress curve for the trapping of [α-^{32}P]ATP with either 1.0 mM GTP (●) or 1.0 mM SO_4 (○). Trapping with GTP yields [α-^{32}P]AMP and PP_i (see text). The F_t value at $t = 0$ s was obtained by adding the quenching agent, EDTA, prior to the chase solution and quantitating the CPM at the AMP position on the TLC sheets. See Materials and Methods for a more detailed protocol.

trapped using GTP—ATP is hydrolyzed to AMP and PP_i in the presence of GTP (see below). This confirms that GTP can add directly to the E·ATP complex without an intervening sulfate-binding step. We were unable to trap ATP at sulfate concentrations as high as 8.0 mM; higher concentrations of sulfate inhibit the enzyme. Failure to trap with sulfate does not rule out the possibility that sulfate can add to the E·ATP complex. These isotope-trapping experiments demonstrate random, productive binding of ATP and GTP.

Isotope-trapping experiments provide values for the maximum trappable fraction of enzyme, $F_{t_{max}}$, and the concentration of the fixed variable ligand required to trap one half of this maximum value, $K_{1/2}$. The parameters associated with trapping GTP and ATP respectively are $F_{t_{max}} = 0.12$, $K_{1/2} = 250\ \mu M$, and $F_{t_{max}} = 0.043$, $K_{1/2} = 130\ \mu M$. Once equilibrium constants for the binding of nucleotides are established, it will be possible to use these $F_{t_{max}}$ and $K_{1/2}$ values to estimate the

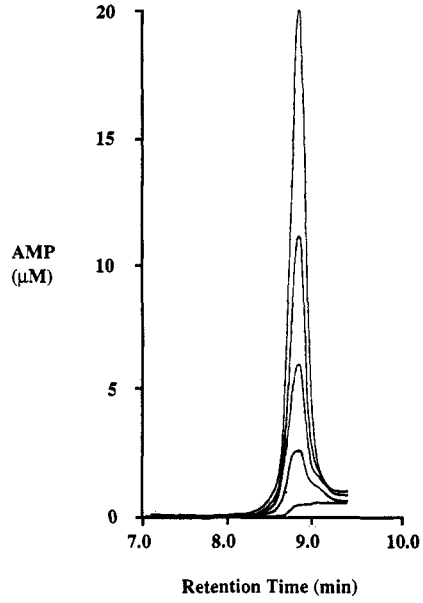

FIGURE 3: Suppression of AMP formation by sulfate. The plot is a superposition of the chromatographic profiles of the AMP produced in reactions containing ATP sulfurylase, ATP, GTP, and varying concentrations of sulfate. The sulfate concentrations, in order of descending peak intensity, were 0, 125, 250, and 500 μM. The lowest curve is the chromatographic background obtained from the 500 μM SO_4 reaction quenched immediately upon addition of substrates. AMP was detected at λ = 254 nm. The reaction conditions were ATP (0.30 mM), GTP (3.0 mM), ATP sulfurylase (2.0 μM), $MgCl_2$ (4.3 mM), inorganic pyrophosphatase (0.01 u/mL), HEPES/K^+ (50 mM, pH = 8.0), and T = 23 ± 2 °C. Reactions were run for 45 min and quenched by placing the tubes containing the reaction mixtures in a boiling water bath for 1.0 min. Products were separated on a mono Q HR 5/5 column using an FPLC gradient system. The gradient was linear in NaCl, and the buffer was TRIS (25 mM, pH/HCl = 7.5); AMP eluted at 95–105 mM NaCl.

net rate constants for nucleotide dissociation from the binary and ternary complexes.

ATP Is Cleaved at the α,β-Position in a Reaction Requiring Hydrolysis of GTP. ATP sulfurylase hydrolyzes GTP at an extremely low rate and does not significantly hydrolyze ATP. However, when both nucleotides are present, GTP hydrolysis is stimulated and ATP is hydrolyzed at the α,β-position. The GTP hydrolysis produces GDP and P_i and has been previously characterized (Leyh & Suo, 1992). The ATP hydrolysis products were identified on the basis of their chromatographic behavior on poly(ethyleneimine) TLC plates using a 1.0 M LiCl mobile phase. The products obtained using [γ-^{32}P]- or [α-^{32}P]ATP behaved like authentic PP_i or AMP and could be converted into compounds with the chromatographic properties of P_i and ATP using inorganic pyrophosphatase or a combination of myokinase and pyruvate kinase. At a saturating concentration of GTP (1.0 mM), the k_{cat} for ATP hydrolysis is 0.46 min^{-1} which is 4.5% of the rate of GTP hydrolysis under these conditions.

To establish that ATP hydrolysis and APS synthesis are chemically linked, AMP formation was studied as a function of sulfate concentration at a fixed concentration of ATP and GTP. The results of this experiment, shown in Figure 3, demonstrate that the production of AMP is suppressed by the addition of sulfate and that the extent of suppression is titratable with sulfate. Thus, it appears that there is a GTP-dependent formation of an E·AMP intermediate, perhaps an enzyme adenylate, that can react with sulfate to produce APS.

GTP Hydrolysis Precedes ATP Hydrolysis. The sequence of the hydrolytic reactions was determined using the hydrolysis-

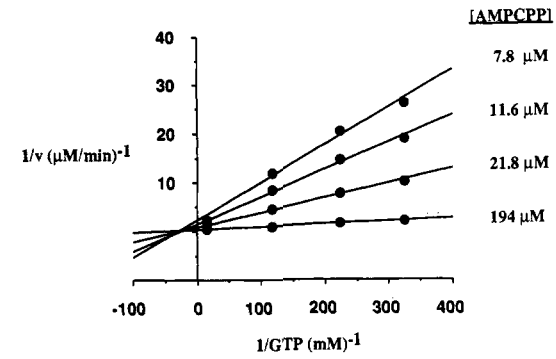

FIGURE 4: AMPCPP activation of GTP hydrolysis. GTP hydrolysis was monitored using the coupled enzyme assay described in Materials and Methods. Assay mixtures contained ATP sulfurylase (0.32 μM), $MgCl_2$ ([nucleotides] + 1.0 mM), PEP (0.67 mM), NADH (110 μM), lactate dehydrogenase (3.4 u/mL), pyruvate kinase (5.9 u/mL), HEPES/K^+ (50 mM, pH = 8.0), and GTP (77.1, 8.6, 4.5, 3.1 μM); the AMPCPP concentrations are shown. Assays were run at 25 ± 2 °C. The data are the average of two determinations.

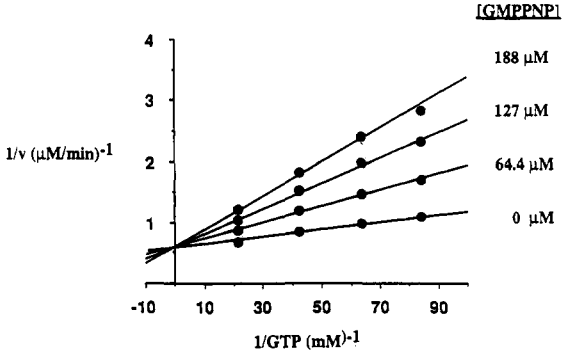

FIGURE 5: GMPPNP inhibition of GTP hydrolysis. The rate of GTP hydrolysis was monitored by 340 nM by coupling GDP formation to NADH oxidation using pyruvate kinase and lactate dehydrogenase. Assay mixtures contained ATP sulfurylase (0.31 μM), ATP (1.4 mM), GTP (1.0 mM), $MgCl_2$ ([nucleotides] + 1.0 mM), PEP (0.63 mM), NADH (110 μM), lactate dehydrogenase (3.9 u/mL), pyruvate kinase (5.4 u/mL), inorganic pyrophosphatase (0.014 u/mL), HEPES/K^+ (50 mM, pH = 8.0), and GTP (47.4, 23.8, 15.8, 11.9 μM); the GMPPNP concentrations are shown. Assays were run at 25 ± 2 °C. Inorganic pyrophosphatase was included to remove PP_i generated by the hydrolysis of ATP, which was less than 10% in all cases. The data are the average of two determinations.

resistant analogues of ATP and GTP, AMPCPP and GMP-PNP (Larsen et al., 1969; Eckstein et al., 1971; Yount, 1975). AMPCPP, like ATP, activates GTP hydrolysis (see Figure 4). The activation parameters are, for AMPCPP, K_a = 31 μM, K_{ia} = 232 μM, and, for GTP, K_m = 31 μM, K_i = 232 μM; k_{cat} = 0.17 s^{-1}. K_a and K_m are the Michaelis constants obtained at a theoretically infinite concentration of the complimentary nucleotide; K_{ia} and K_i are the affinity constants at the complementary nucleotide concentration extrapolated to zero. This allosteric activation of GTP hydrolysis will be the subject of a subsequent communication. Clearly, hydrolysis of ATP is not required for hydrolysis of GTP. Thus, it is the energy of binding, not hydrolysis, of ATP that is used to lower the kinetically significant barrier(s) to GTP hydrolysis.

GMPPNP is a linear competitive inhibitor of GTP hydrolysis with a K_i of 83 μM (see Figure 5). Within experimental error, the inhibition is purely competitive, demonstrating that GMPPNP can bind to all, or virtually all, of the enzyme forms that can bind GTP. At concentrations at or above its K_i, GMPPNP does not promote ATP hydrolysis (data not shown). Thus, it appears that GTP hydrolysis is required for, and precedes or is concomitant with, ATP hydrolysis. This interpretation of the data does not withstand the possibility

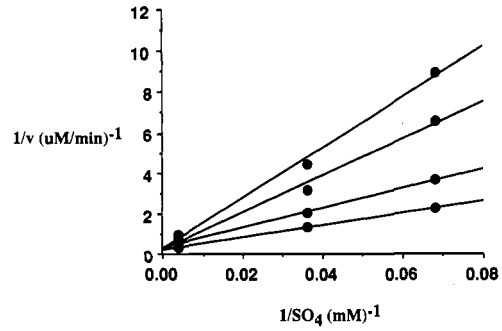

FIGURE 6: PP_i inhibition of APS formation. The reaction mixtures contained ATP sulfurylase (0.20 μM), ATP (10.0 mM), GTP (1.0 mM), $MgCl_2$ ([nucleotides + PP_i] + 1.0 mM), PEP (4.0 mM), pyruvate kinase (4.0 u/mL), HEPES/K^+ (50 mM, pH = 8.0), SO_4 (14.7, 27.8, 250 μM, 0.3 Ci/μL; concentration indicated), and PP_i (0, 100, 200, or 300 μM). The reactions, run at 23 ± 2 °C, were quenched by addition of a stock EDTA solution (100 mM, pH = 9.5) to a final concentration of 33 mM. After addition of EDTA, the tubes containing the reaction mixtures were immediately placed in a boiling water bath for 1 min and then placed on ice. A time course was run for each reaction; the velocity was determined by a least-squares fit to the time course. Each time course was run in triplicate, and the averaged data are shown. Product formation was less than 10% in all cases. The radiolabeled reactants were separated on PEI-F TLC sheets using a 1.0 M LiCl mobile phase and quantitated using an AMBIS scanner.

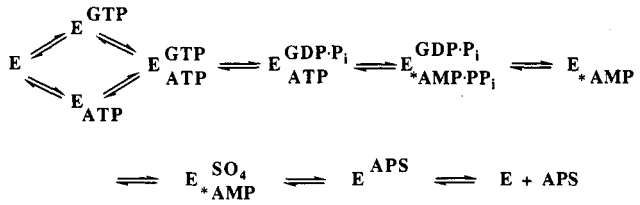

FIGURE 7: Proposed kinetic mechanism for the GTP-activated synthesis of APS.

that GMPPNP is an insufficiently exact analogue of GTP to elicit the E·GTP-like species necessary for ATP hydrolysis.

Dissociation of PP_i Precedes SO_4 Addition and APS Formation. Formation of an E·AMP intermediate suggests that PP_i, released by scission of the α,β-bond of ATP, might need to dissociate prior to the binding of SO_4 and APS formation. This model predicts that PP_i will be a competitive inhibitor, versus sulfate, of APS formation, that is, PP_i and SO_4 will compete for the E·AMP form of the enzyme. The results of this inhibition study are shown in Figure 6. PP_i does, indeed, competitively inhibit the addition of sulfate and APS synthesis, consistent with overlapping binding sites and departure of PP_i prior to sulfate addition. To observe competitive inhibition, it was necessary to perform this experiment at a high, 10 mM, concentration of ATP. Lower ATP concentrations resulted in mixed-type inhibition. The dependence of inhibition type on ATP concentration is indicative of a form of the enzyme, other than that associated with competitive binding of sulfate and PP_i, to which both PP_i and ATP can bind. This is anticipated, since ATP and PP_i are likely to compete for the same binding site.

Proposed Mechanism. A minimal kinetic scheme embodying the findings of this work is shown in Figure 7. The binding of GTP and ATP to E is demonstrated by the isotope-trapping experiments, as is binding to GTP to E·ATP. Binding of ATP to E·GTP is inferred from the trapping of E·GTP with ATP and SO_4 and the fact that SO_4 addition follows PP_i release. The intermediate, written E*AMP, is suggested by the GTP-dependent hydrolysis of ATP. The suppression of AMP formation by sulfate indicates that the intermediate is on the catalytic path leading to APS synthesis. The sequence of hydrolytic steps was determined using the hydrolysis-resistant nucleotides AMPCPP and GMPPNP. Ordered release of PP_i prior to sulfate binding is suggested by the competitive inhibition of PP_i versus sulfate for APS formation. Mass spectral analysis of [^{18}O]APS synthesized from [$^{18}O_4$]SO_4 of known isotopic enrichment was used to show that APS formation proceeds *via* nucleophilic attack of SO_4.

While this mechanism includes what appears to be the key steps in the GTP-activated synthesis of APS, there are other reactions, not on the central path to APS synthesis, which are not included. For example, we now know that each of the reactants in eq 1 can, to varying degrees, activate GTP hydrolysis. Each of these hydrolysis reactions has associated with it a minimum of six enzyme complexes. This allosteric activation of GTP hydrolysis, currently under study, allows one to investigate the ability of various substrates and analogues to elicit the conformational changes which alter the affinity of the enzyme for the ground-state and rate-determining, transition-state(s) complex(es) associated with GTP hydrolysis.

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