The Energetic Linkage of GTP Hydrolysis and the Synthesis of Activated Sulfate†

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ABSTRACT: ATP sulfurylase, from *Escherichia coli* K-12, catalyzes both the hydrolysis of GTP and the synthesis of activated sulfate (APS). This paper describes the energetic linkage of these reactions and the events that couple them. Steady-state and single-turnover experiments suggest that the binding of GTP inhibits APS production and that the hydrolysis of GTP is required to generate the enzyme form(s) that produces APS. It is this progression from the inhibitory, E·GTP, to the productive, E·GDP, complexes in the cycle of APS synthesis that energetically links these two reactions. This model stands in contrast to other GTPase/target systems in which the binding of GTP alone is sufficient to catalyze multiple turnovers of the target reaction. The stoichiometry of GTP hydrolysis to APS synthesis is 1:1, and equilibrium measurements show that −9.1 kcal/mol, produced by the hydrolysis of GTP, is used to thermodynamically drive production of APS and PP_i. These findings establish the mechanism of energy transfer in this novel GTPase/target system, and substantially alter our understanding of the energetics of sulfate activation, an essential step in the metabolic assimilation of sulfur.

ATP sulfurylase (ATP sulfate adenylyltransferase, EC 2.7.7.4), isolated from *Escherichia coli K*-12, catalyzes and chemically links the hydrolysis of GTP and the synthesis of activated sulfate (adenosine 5′-phosphosulfate, APS). These reactions are represented by eq 1 and 2. The native enzyme

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

$$GTP + H_2O \rightleftharpoons GDP + P_i + H^+ \quad (2)$$

is composed of two types of subunits, encoded by *cysD* and *cysN* (Leyh et al., 1987). Sequence similarities between CysN and other GTPases suggest that CysN contains the site for GTP hydrolysis (Leyh et al., 1992). The active site for APS synthesis is not yet mapped to a particular subunit. The initial rate of APS (adenosine 5′-phosphosulfate) formation is stimulated 116-fold at a saturating concentration of GTP; conversely, GTP hydrolysis is stimulated by the APS-forming reactants (Leyh & Suo, 1992). The extent to which this activation results in the transfer of chemical potential between these reactions and the mechanistic basis for this transfer are the topics of this paper.

Activated sulfate is important in a wide variety of metabolic processes. In enteric bacteria, APS is an intermediate in the reduction of sulfate to sulfide, which ultimately is used for the biosynthesis of reduced sulfur metabolites (Siegel, 1975). Certain anaerobic bacteria couple sulfate reduction and electron transport to drive oxidative phosphorylation (Siegel, 1975). In mammalian metabolism, the primary function of activated sulfate is that of the sulfuryl ($-SO_3$) donor. The actual donor, PAPS, is a phosphorylated form of APS. Sulfation is a frequent modification typically occurring at carbohydrate hydroxyl or protein tyrosyl residues (Lindahl & Hook, 1978; Huttner, 1987). Carbohydrate sulfation is important in many cellular processes including cell–cell adhesion (Brauer et al., 1990), the viscoelastic properties of connective tissue (Devered & Whelan, 1986), hemostasis (Atha et al., 1984; Niehrs et al., 1990), detoxication (Jakoby & Ziegler, 1990), and growth factor/receptor recognition (Ishihara et al., 1993). Lowered levels of cartilage sulfation can result in dwarfism (Sugahara & Schwartz, 1979). Protein tyrosyl sulfation regulates the activity of several peptide hormones (Pasqualini et al., 1992) and can be critical in protein–protein recognition (Leyete et al., 1991). Sulfation of factor VIII at tyrosine-1680 is important for its interaction with von Willebrand factor; improper sulfation impedes this interaction, resulting in severe hemophilia (Tuddenham et al., 1991).

Under most conditions, sulfate is nonreactive. Virtually all organisms solve this problem by chemically activating sulfate, *via* adenylylation (reaction 1) (De Meio, 1975). Activation poises the sulfuryl moiety for facile, energetically favorable entry into its subsequent metabolic reactions. The chemical potential of the phosphoric/sulfuric acid anhydride bond of APS is quite high; the ΔG associated with the hydrolysis of this bond is estimated at −19 kcal/mol (Robbins & Lipmann, 1958). Consequently, the apparent equilibrium constant for reaction 1 is extremely unfavorable, 1.1×10^{-8} at pH 8.0, T = 37 °C (Robbins & Lipmann, 1958). This formidable impediment to the metabolic acquisition of sulfate has prompted discussions in the literature of how an organisim might circumvent this problem (Leyh, 1993). In this study, we show, for the first time, that the chemical energy released by the hydrolysis of GTP is used to thermodynamically drive APS formation, thereby reducing the energetic barrier to APS synthesis to what is likely a metabolically insignificant level.

MATERIALS AND METHODS

Materials. Nucleotides, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)[1], and PEP were obtained from the Sigma Chemical Co. $[^{35}S]SO_4$ (~4200 Ci/mmol) and $[^{32}P]PP_i$ (1–60 Ci/mmol) were purchased from ICN Biomedicals Inc. and DuPont NEN Corp., respectively.

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[1] Abbreviations: EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GMPPNP, 5′-guanylyl imidodiphosphate; PEP, phosphoenolpyruvate; SE, standard error; U, unit: = micromoles of substrate converted to product per minute at V_{max}.

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Adenylate kinase (rabbit muscle), inorganic pyrophosphatase (yeast), and pyruvate kinase (yeast) were purchased from the Boehringer Mannheim Corp. Poly(ethylenimine)–cellulose-F thin-layer chromatography (PEI-F TLC) plates were obtained from E. Merck Co. Other compounds were reagent grade or better.

ATP Sulfurylase. The enzyme was purified according to a published protocol from an *E. coli K*-12 strain containing an expression vector that causes production of high levels of the *E. coli K*-12 enzyme (Leyh et al., 1987). The specific activity of the purified enzyme ranged from 0.23 to 0.25 U/mg.

Assay for Production of [^{35}S]APS or [^{32}P]ATP. Assays monitoring the formation of [^{35}S]APS, from [^{35}S]SO_4, or [^{32}P]ATP, from [^{32}P]PP_i, were performed as follows: Reactions were stopped by the addition of a stock solution of 100 mM EDTA/HCl, pH 9.5, to a final concentration of 33 mM. Within 1.5 min of the EDTA addition, tubes containing quenched reactions were added to a boiling water bath and heated for 1.0 min to inactivate the enzyme. The tubes were then placed on ice. The boiled reactions were spotted onto PEI-F TLC plates and reactants separated using either a 1.0 M LiCl mobile phase (Randerath & Randerath, 1964), for the separation of APS and SO_4, or a saturated $(NH_4)_2SO_4$/120 mM Na_2EDTA mobile phase (SB buffer; Bochner & Ames, 1982), for the separation of PP_i and ATP. The quantitation of product was accomplished using an AMBIS two-dimensional radioactivity detector (Nye et al., 1988).

Coupling Enzymes. Coupling enzymes were desalted using a Superose-12 size-exclusion 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 activites of pyruvate kinase and adenylate kinase were determined using optical assays (Buchler & Pfleiderer, 1955). The activity of pyruvate kinase was determined using GDP (Davidson, 1959). Inorganic pyrophosphatase was assayed using [^{32}P]PP_i; reactants were separated on PEI-F TLC sheets using a 1.0 M LiCl mobile phase and quantitated using an AMBIS scanner.

Statistical Analysis of Kinetic Data. The data in panels A and B of Figure 4 were statistically fit to competitive and noncompetitive inhibition models using the programs developed by Cleland (1979). The competitive model provided better overall fitting parameters in both cases.

Purification of GMPPNP. Anion-exchange chromatography of the commercially available GMPPNP revealed a ~12%, A_{260} contaminant. Contamination was reduced to <3% by purification using a Mono-Q matrix with a linear salt gradient from 0.0 to 0.60 M, triethylamine (TEA)/HCO_3, pH 7.8 (Porath, 1955). GMPPNP eluted at 0.42 M TEA. The solvent was removed by rotary evaporation. Residual TEA was removed by suspending and drying the sample 3 times in methanol, followed by 3 times in H_2O. The GMPPNP was then suspended in H_2O and the pH adjusted to 7.0 ± 0.2 with KOH.

RESULTS AND DISCUSSION

Stoichiometry of GTP Hydrolysis to APS Formation. To assess the extent of chemical coupling between the GTP-hydrolyzing and APS-forming reactions, the stoichiometry of these reactions was determined. This was accomplished by comparing the initial rates of product formation for both reactions. It is important to realize that the stoichiometric coupling of these reactions is a function of the reactant concentrations. The enzyme has an inherent, extremely slow, GTPase activity which is stimulated 13-fold by the addition of ATP, and further by the subsequent addition of sulfate (Leyh & Suo, 1992). In fact, the products, APS and PP_i, also activate GTP hydrolysis. To avoid aberrant stoichiometries, it was necessary to determine the reaction rates under initial velocity conditions for both reactions. In Figure 1, the relative rates of these reactions are plotted as a function of $1/[SO_4]$. The initial concentrations of ATP and GTP used in these experiments were 200 and 100 μM, respectively. At low concentrations of sulfate, the rate of GTP hydrolysis is considerably greater than that for APS synthesis. This is because the preponderance of the hydrolysis under these conditions is due to the ATP-activated hydrolysis of GTP. As the sulfate concentration increases, and the enzyme becomes saturated, the ratio of the initial rates converges on 1:1, indicative of a tightly coupled chemical state in which formation of each molecule of APS and PP_i is associated with the hydrolysis of a single molecule of GTP. The energetic implication of this result is that the full compliment of chemical potential released upon hydrolysis of the β–γ bond of GTP is used to thermodynamically drive APS formation. However, these results do not distinguish between actual energetic linkage of these reactions and some set of coupled and uncoupled chemical processes which adventitiously result in a rate ratio of 1:1.

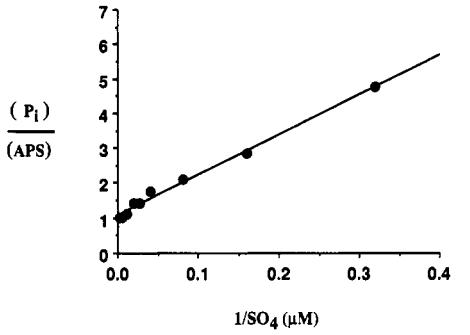

FIGURE 1: Stoichiometry of GTP hydrolysis to APS formation. The ratio of the initial rate of GTP hydrolysis to APS formation is plotted *versus* the reciprocal of the sulfate concentration. The reactions contained ATP (200 μM), [γ-^{32}P]GTP (100 μM, SA ~20 nCi/μL), [^{35}S]SO_4 (at the indicated concentrations, SA ~20 nCi/μL), $MgCl_2$ (1.3 mM), PEP (2.0 mM), ATP sulfurylase (0.30 μM), pyruvate kinase (0.016 U/μL), myokinase (0.007 U/μL), inorganic pyrophosphatase (0.1U/μL), and HEPES (50 mM, pH/K^+ 8.0). Reactions were quenched and reactants separated and quantitated as described under Materials and Methods. Inorganic pyrophosphatase was included to energetically drive APS formation; myokinase and pyruvate kinase were present to regenerate ATP and GTP from the AMP and GDP formed during the reactions. Each point is the average of at least two determinations.

Energetic Linkage of GTP Hydrolysis and APS Formation. To quantitate the chemical potential transferred to the APS-forming reaction by the hydrolysis of GTP, the apparent equilibrium for APS synthesis was determined at a fixed, saturating concentration of GTP. Progress curves for the formation of APS and PP_i from ATP and SO_4 are shown in Figure 2. The initial concentrations of sulfate and GTP were 200 and 500 μM, respectively; the ATP concentration was varied from 100 μM to 1.0 mM. The system demonstrates normal mass action between ATP and SO_4. The equilibrium constants associated with the progress curves were, within experimental error, the same, and were used to obtain the apparent equilibrium constant for APS synthesis in the presence of GTP, which is 0.059 (SD = 0.0055). The Gibbs potential associated with this value is 1.7 kcal/mol.

It was important to establish that the APS-forming reaction is in dynamic equilibrium in the plateaus of the progress curves

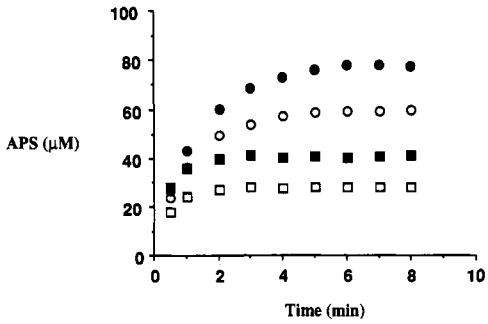

FIGURE 2: Mass action effects of ATP on APS formation in the presence of GTP. The reaction conditions were as follows: $[^{35}S]SO_4$ (200 μM, ~20 nCi/μL), GTP (500 μM), ATP [1.0 mM (●), 500 μM (○), 200 μM (■), 100 μM (□)], $MgCl_2$ ([nucleotide] + 1.0 mM), PEP (2.0 mM), ATP sulfurylase (2.0 μM), pyruvate kinase (0.016 U/mL), HEPES (50 mM, pH/K^+ 8.0), $T = 23 \pm 2$ °C. The reactions were quenched and the radiolabeled products separated and quantitated as described under Materials and Methods. Pyruvate kinase and PEP were present to regenerate GTP from GDP.

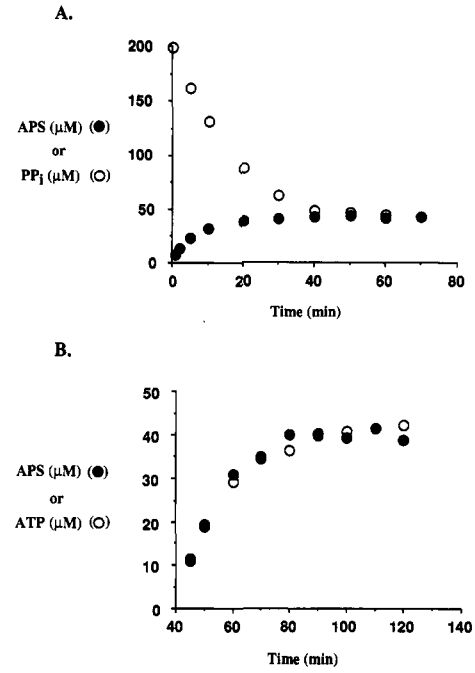

FIGURE 3: APS-forming reaction achieves equilibrium during GTP hydrolysis. (Panel A) Reactions initiated with substrates or products achieve the same end point. Closed circles (●) show the progress curve for the formation of $[^{35}S]$APS in a reaction initiated with ATP and $[^{35}S]SO_4$ (~20 nCi/μl), each at 200 mM. Open circles (○) represent the progress curve for $[^{32}P]PP_i$ consumption in a reaction initiated with $[^{32}P]PP_i$ (~20 nCi/μL) and APS, each at 200 mM. The conditions were as follows: GTP (2.0 mM), $MgCl_2$ ([nucleotide] + $[PP_i]$ + 1.0 mM), PEP (2.0 mM), ATP sulfurylase (0.5 μM), pyruvate kinase (16 mU/μL), HEPES (50 mM, pH/K^+ 8.0), $T = 23$ (±2 °C). The reactions were quenched and the radiolabeled products separated and quantitated as described under Materials and Methods. (Panel B) The rates of the forward and reverse reactions are the same in the steady-state. Conditions are identical to those associated with the open circles in panel A except that 1 μl of carrier-free $[^{35}S]SO_4$ or $[^{32}P]PP_i$, in HEPES (50 mM, pH/K^+ 8.0), was added to 80 μL of the cold reaction mixture 40 min after the reaction was initiated, that is, after the steady-state was established. Closed circles (●), formation of $[^{35}S]$APS from $[^{35}S]SO_4$ and PP_i; open circles (○), formation of $[^{32}P]$ATP from APS and $[^{32}P]PP_i$.

shown in Figure 2, rather than a static state resulting from product inhibition. The criteria used to assess that equilibrium was achieved were that the end point of the reaction be independent of whether it was initiated using substrates or products and that the rates of the forward and reverse reactions in the plateau be equal. The results of experiments testing conformity with these criteria are depicted in panels A and B of Figure 3. Panel A shows that the same reaction end point is achieved whether the reaction is initiated using substrates, ATP and SO_4 (each at 200 μM), or products, APS and PP_i (each at 200 μM). In each case, GTP was maintained at 2.0 mM throughout the reaction using PEP and pyruvate kinase. Control experiments, performed under identical conditions with α-GTP, verified that the GTP concentration did not vary significantly during these measurements. The results shown in panel B demonstrate that the rates of the forward and reverse reactions in the steady-state are the same. The conditions of these experiments were identical to those for the reaction initiated with APS and PP_i shown in panel A except that instead of adding the carrier-free $[^{35}S]SO_4$ or $[^{32}P]PP_i$ at the time the reaction was initiated, $t = 0$ min, it was added 40 min later, that is, after the system had reached the putative equilibrium condition. These results clearly indicate that the APS-forming reaction has reached equilibrium in these experiments.

To sustain the steady-state attained in the plateau of each progress curve, it was necessary to convert the GDP produced to GTP; this was accomplished with pyruvate kinase and PEP. Increasing the concentration of GTP to as high as 5.0 mM, 10 times the concentration used in these experiments, had no measurable effect on the position of the plateau. In this sense, then, the system is saturated with GTP. If one defines the system to include both reactions, then chemical equilibrium is not achieved in the plateau, since GTP must be continually replenished. Nevertheless, the APS-forming reaction has achieved a steady-state or quasi-equilibrum condition, and the energetics of this reaction can be treated as though the chemical potential of APS and PP_i has been lowered, relative to ATP and SO_4, at the expense of the chemical energy afforded by the hydrolysis of GTP. An equilibrium constant of 1.1×10^{-8} has been measured for this reaction in the absence of GTP under conditions similar to those used in the current studies. Given these equilibrium constants, one can calculate the chemical potential transferred from the hydrolytic to the APS-forming reaction as follows: $\Delta\Delta G = \Delta G_{(+GTP)} - \Delta G_{(-GTP)} = 1.7 - 10.8 = -9.1$ kcal/mol. Thus, it appears that most, if not all, of the chemical potential available from the hydrolysis of the β–γ bond of GTP is used to drive APS formation.

This finding is of considerable metabolic significance. To overcome the energetic problems associated with APS synthesis, it is often suggested that the reactions catalyzed by APS kinase (which phosphorylates APS at the 3′ position of the ribose using ATP) and inorganic pyrophosphatase could be used to draw the APS-forming reaction forward *in vivo*. While this is certainly possible, the extent to which the chemical potential of these reactions can be utilized for this purpose is determined by how near equilibrium these coupling reactions are *in vivo*. Measurements of the *in vivo* concentration of PP_i, ~0.5 mM, seem too high for the pyrophosphatase reaction to be near equilibrium; however, these measurements do not address the possibility of unique conditions within microenvironments of the cell (Kukko-Kalske et al., 1989). An alternative suggestion is that ATP sulfurylase and APS kinase might physically associate to allow the channeling of APS from one active site to the other. In this case, the protein surfaces become the solvents for the products, and the energetic profile for the ATP sulfurylase reaction could change dramatically (Renosto et al., 1989; Leyh, 1993). While these mechanisms might, in fact, be operational *in vivo*, there is clearly no need to invoke them to overcome the energetics associated with APS formation in *E. coli*. The *in vivo*

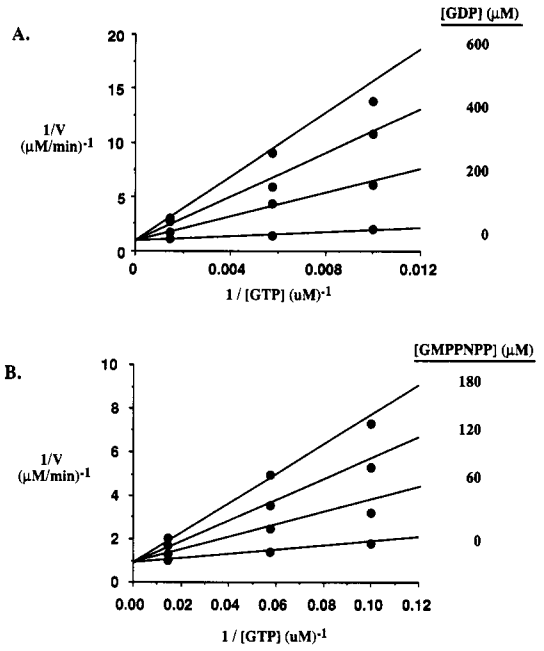

FIGURE 4: Inhibition of APS formation by GMPPNP and GDP. GMPPNP and GDP are competitive inhibitors, *versus* GTP, for APS formation. The assay used in these experiments monitors the production of radiolabeled APS from $[^{35}S]SO_4$. The reaction conditions were as follows: ATP sulfurylase (0.20 μM), ATP (1.0 mM), $[^{35}S]SO_4$ (25 μM, SA ~20 nCi/μL), $MgCl_2$ ([total nucleotide + 1.0 mM), HEPES (50 mM, pH/K^+ 8.0), 3 mU/mL inorganic pyrophosphatase, $T = 23 \pm 2$ °C. The GMPPNP assay mixtures also contained adenylate kinase (2.1 U/mL), pyruvate kinase (6.0 U/mL), and 0.70 mM PEP, which regenerate GTP and ATP from the AMP and GDP produced during the reaction. Each reaction rate was determined 2 or more times from a nonweighted least-squares fit of progress curves composed of four successive time points. Less than 10% of substrate was converted to product in all cases. The lines represent the statistically optimized fit of the data to a competitive inhibition model (see Materials and Methods). The reactions were quenched and the reactants separated and quantitated as described under Materials and Methods.

concentration of GTP in *E. coli* is estimated at 0.9 mM (Neuhard & Nygaard, 1987). This is sufficient to shift the chemical potential of the APS reaction to 1.7 kcal/mol, which, generally speaking, is a metabolically insignificant barrier. Thus, ATP sulfurylase is functioning as a conduit for the transfer of chemical potential from GTP, a reservoir of metabolic high energy, to the production of activated sulfate, the extremely unfavorable first step in the metabolic acquisition of sulfate.

Activated Turnover Requires GTP Hydrolysis. To determine which guanine nucleotide form of the enzyme, E·GDP or E·GTP, is active in stimulating APS synthesis, GMPPNP (Eckstein et al., 1971; Yount, 1975) and GDP were tested as activators and inhibitors, *versus* GTP, of APS synthesis. Both compounds were inhibitors. The inhibition patterns, shown in Figure 4, are competitive *versus* GTP; the slope replots are linear in each case with K_i(GMPPNP) = 29 μM (SE 4.7 μM) and K_i(GDP) = 39 μM (SE 5.0 μM). These experiments do not indicate whether, at saturating concentration, these inhibitors suppress the rate of APS synthesis to zero or to the nonactivated rate, or whether they are simply relatively poor activators of the reaction. These results do demonstrate that the energy associated with the binding of GDP or GMPPNP (and presumably GTP) is insufficient to fully activate the catalytic cycle of APS formation and strongly suggest that GTP hydrolysis is required to generate forms of the enzyme that are essential for activated catalysis.

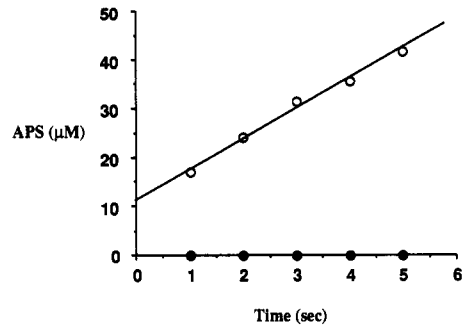

FIGURE 5: GTP hydrolysis is required for formation of E·APS. Single-turnover production of $[^{35}S]$APS in the presence of GTP or GMPPNP. Reaction conditions: ATP sulfurylase (25 μM), SO_4 (100 μM, SA ~20 nCi/μL), ATP (2.0 mM), GTP or GMPPNP (1.5 mM), inorganic pyrophosphatase (12.5 mU/mL), $MgCl_2$ (4.5 mM), HEPES (50 mM, pH/K^+ 8.0), $T = 23 \pm 2$ °C. Reactions were quenched and reactants separated and quantitated as described under Materials and Methods. Inorganic pyrophosphatase was included to prevent the back-reaction and energetically draw APS formation forward. Each point is the average of two determinations.

GTP Hydrolysis Is Required for and Precedes, or Is Concomitant with, Formation of E·APS. The steady-state inhibition studies demonstrate that GTP hydrolysis is required for activated production of APS but do not rigorously address the stringency of this requirement, nor do they indicate where in the reaction coordinate for GTP hydrolysis APS is formed. Knowledge of the interdigitation of the steps of these reactions is fundamental to understanding how energy is transferred between them. It is possible, for example, that binding of GTP drives formation of E·APS and that the subsequent hydrolysis is required for product release. Single-turnover experiments using radiolabeled substrate provide a sensitive assay with which to approach these issues since they can detect product formation in situations where the enzyme cannot complete a full catalytic cycle.

Figure 5 shows the results of single-turnover experiments that monitored the production of radiolabeled APS from $[^{35}S]$-SO_4 at a saturating concentration of either GTP or GMPPNP. The GTP reaction produced 1.7 active-site equiv of APS in 5 s. During this same time interval, no APS formation was observed in the reaction containing GMPPNP. The lower limit of detection of APS was estimated at 0.4% of an enzyme active-site equivalent. Thus, to the extent that GMPPNP successfully mimics GTP, we can conclude that E·APS formation requires the hydrolysis of GTP. These results support the conclusion that GTP hydrolysis either precedes or occurs spontaneously with the production of APS, ruling out a mechanism in which the energy of binding of GTP is used to drive formation of E·APS.

GDP Activates, P_i Does Not. When GDP was tested as an activator in the absence of GTP, it was found to enhance the rate of APS formation 2.9-fold. The apparent k_{cat} for this activation, 0.0074 s^{-1}, is ~1/40 that for activation by GTP. The k_{cat} was determined at a theoretically infinite concentration of ATP at near-saturating, (8–10)K_m, concentrations of SO_4 and GDP. The conditions were the following: ATP sulfurylase (2.0 μM), SO_4 (1.5 mM), GDP (4.0 mM), $MgCl_2$ ([nucleotide] + 1.0 mM), ATP (10.0, 14.7, 27.8, 250 μM), HEPES/K^+ (50 mM, pH 8.0), $T = 23 \pm 2$ °C. In contrast to the inhibition caused by the binding of GMPPNP, some fraction of the energy associated with the binding of GDP is used to lower the energy of activation for APS. This suggests that product or product-like enzyme forms might be the activating species.

To test whether the product complexes E·GDP·P_i or E·P_i could activate the enzyme, P_i was tested singly and in combination with GDP as an activator. No increase in the rate of APS formation was detected in the presence of P_i, or P_i and GDP under the following conditions: P_i (2.0 mM), GDP (0 or 200 μM), ATP (1.0 mM), [^{35}S]SO_4 (25 μM, SA ~20 nCi/μL), $MgCl_2$ ([nucleotide] + 1.0 mM), ATP sulfurylase (0.20 μM), HEPES (50 mM, pH 8.0), $T = 23 \pm 2$ °C. The concentration of P_i was chosen on the basis of inhibition of the GTP-activated reaction. AT 300 μM GTP, using the conditions outlined above, P_i inhibited APS formation by 55%. The lack of detectable activation under these conditions suggests that these product complexes cannot activate APS synthesis; however, since we are not certain that the appropriate complexes form under these conditions, the role of P_i as an activator remains ambiguous.

Coupling Events. In many cases, it is the GTPase·GTP complex which is competent to activate a target enzyme (Bourne et al., 1991). Activation ceases upon hydrolysis of GTP, and the duration of the activation is regulated by effectors that influence GTP turnover. In these systems, the binding of GTP is sufficient to activate the target which often turns over many times before GTP is hydrolyzed. Hydrolysis-resistant GTP analogues sustain the active form of these GTPases, prolonging target activation.

Another class of GTPase directly couples the energetics of GTP hydrolysis to the reaction catalyzed by the target enzyme. Functional hallmarks of this class are that GTP hydrolysis is tightly stoichiometrically coupled (1:1) to the target reaction and activation is inhibited by nonhydrolyzable analogues of GTP. These GTPases can be subclassified on a mechanistic basis. One subgroup conformationally couples the energetics of GTP hydrolysis to the target reaction (Kaziro, 1978; Jencks, 1980); in the other, GTP directly participates in the chemistry of the target reaction (Bridger, 1974; Arnelle & O'Leary, 1992; Frey, 1992).

To understand the mechanistic basis of the linkage in the ATP sulfurylase/GTPase system, it is necessary to understand the interdependence of the steps of the two reactions. The transfer of chemical energy requires that there be certain events that oblige the two reactions to occur in concert, either simultaneously or sequentially, in order to proceed. The results of the single-turnover experiments using GMPPNP suggest that upon binding of GTP, progress of the APS-forming reaction "ceases" until hydrolysis occurs. This progression from inhibition, upon binding of GTP, to activation, upon hydrolysis, energetically weds these two reactions.

While it is not possible to determine the exact point at which the events that result in the production of E·APS occur, they can be mapped to a particular region of the reaction coordinate for GTP hydrolysis. The section of the coordinate up to and including the formation of E·GTP can be excluded, since E·APS forms after these steps. If hydrolysis precedes such events, one expects the product complexes E·GDP, E·P_i, or E·GDP·P_i to activate APS synthesis. Our studies indicate that the E·GDP complex is weakly activating and that P_i has no demonstrable influence on the rate of APS formation. The ~40-fold increase in activation by GTP, compared to GDP, suggests that other complexes or steps preceding E·GDP formation are far more competent in activating APS synthesis. Thus, it is plausible, if not likely, that the events that are directly linked to the production of E·APS occur during the transition from the ground-state E·GTP to E·GDP·P_i complexes. However, the ambiguity in the role of P_i as an activator extends the relevant region of the reaction coordinate to include all points between the incipient attack of water and the release of P_i.

Studies of the kinetic mechanism of the GTP-activated reaction have shown that GTP hydrolysis is required for formation of a high-energy E*AMP intermediate, possibly an enzyme adenylate. This species undergoes nucleophilic attack by sulfate, producing APS (Liu et al., 1994). This intermediate, which forms either during or immediately following GTP hydrolysis, is the earliest detected product of the APS-forming reaction. Hence, GTP hydrolysis and formation of the intermediate appear to provide the chemical linkage in this system.

CONCLUSIONS

Under fully coupled conditions, virtually all of the chemical potential of the β–γ bond of GTP is given-over, upon hydrolysis, to the production of APS and PP_i. This energetic linkage occurs because the reactions proceed through an inhibitory E·GTP complex(es), to an activating complex(es) or event(s) which occur(s) during, or following, GTP hydrolysis. The chemical link between these reactions appears to be the coupling of the hydrolysis of GTP to the formation of an E*AMP intermediate. These findings substantially alter our understanding of the energetics of sulfur assimilation, and provide an in-depth view of the events that link GTP hydrolysis to formation of product in this novel GTPase/target system.

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