The sulfation activation pathway is essential for the assimilation of sulfate and, in many bacteria, is comprised of three reactions: the synthesis of adenosine 5'phosphosulfate (APS), the hydrolysis of GTP, and the 3'-phosphorylation of APS to produce 3'-phosphoadenosine 5'-phosphosulfate (PAPS), whose sulfuryl group is reduced or transferred to other metabolites. The entire sulfation activation pathway is organized into a single complex in Mycobacterium tuberculosis. Although present in many bacteria, these tripartite complexes have not been studied in detail. Initial rate characterization of the mycobacterial system reveals that it is poised for extremely efficient throughput: at saturating ATP, PAPS synthesis is 5800 times more efficient than APS synthesis. The APS kinase domain of the complex does not appear to form the covalent E-P intermediate observed in the closely related APS kinase from Escherichia coli. The stoichiometry of GTP hydrolysis and APS synthesis is 1:1, and the APS synthesis reaction is driven 1.1×10^10-fold further during GTP hydrolysis; the system harnesses the full chemical potential of the hydrolysis reaction to the synthesis of APS. A key energy-coupling step in the mechanism is a ligand-induced isomerization that enhances the affinity of GTP and commits APS synthesis and GTP hydrolysis to the completion of the catalytic cycle. Ligand-induced increases in guanine nucleotide affinity observed in the mycobacterial system suggest that it too undergoes the energy-coupling isomerization.

The sulfate activation pathway in Mycobacterium tuberculosis is organized into a single complex that consists of three catalytic activities: an adenylyl-transferase (ATP sulfurylase), that catalyzes nucleophilic attack of sulfate at cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain. The organism-dependent fusion of the early cysteine biosynthetic enzymes is particularly interesting, given that the E. coli APS kinase is expressed as a separate polypeptide, rather than a CysN domain.
strains of mycobacterium (11, 12) and can inhibit phagosome-lysosome fusion (13, 14). These tangible links between sulfur-containing metabolites and the viability and antibiotic resistance of M. tuberculosis suggest that sulfur metabolism may prove a fertile area of anti-tubercular research.

**Materials and Methods**

EnzChek phosphate assay kit was obtained from Molecular Probes. mGMPNP was obtained from Jena Biosciences. [32P]SO4/Na2SO4 was purchased from ICN. [γ-33P]ATP was purchased from PerkinElmer Life Sciences. MgCl2, ATP, ADP, AMP, GTP, β-NADH, NADP+, phosphoenol pyruvate (PEP), glucose, KOH, Na2SO4, EDTA, lysozyme, β-mercaptoethanol, and dithiothreitol were the highest grade available from Sigma. Glycerol, KCl, streptomycin sulfate, (NH4)2SO4, and HEPES were purchased from Fisher Scientific. Lactate dehydrogenase (rabbit muscle), glucose-6-phosphate dehydrogenase (yeast), inorganic pyrophosphatase (yeast), phenylmethylsulfonyl fluoride, and pepstatin were purchased from Roche Applied Science. PEI-F TLC sheets were purchased from EM Science. DNA restriction enzymes were acquired from New England Biolabs. Competent E. coli (BL21DE3) were purchased from Novagen, and PhiX Turbo polymerase was purchased from Stratagene. Sephadex G-25, G-10, and DEAE-sepharose fast-flow columns were purchased from Amersham. PAP-agarose was purchase from Sigma. APS was synthesized as described previously (3). PAP-agarose was purchase from Sigma. ATP sulfurylase was expressed and purified as described previously (15, 16). The yeast HAL2 (PAP-2 nucleoside diphosphate kinase) bacterial expression plasmid, PETHAL2, was a generous gift from Prof. John D. York of Duke University Medical Center. Recombinant yeast HAL2 was expressed in BL21(DE3) cells and purified as described previously (17).

**Purification of SAC**—The recombinant mycobacterial sulfate-activating complex was expressed in BL21(DE3) and purified as described previously except for the addition of an anion exchange purification step (Q Sepharose Fast-Flow resin) after size exclusion chromatography (18). SAC eluted in a linear salt gradient (0–0.7 M KCl, 50 mM HEPES, 10 mM MgCl2) in a rate of 0.2 M KCl, 4 °C, and 8% increase in absorbance at 365 nm, by coupling the regeneration of GTP to the oxidation of NADH. The assay conditions were as follows: SAC (0.05 µM), ATP (64, 94, 177, and 1600 µM), (ATP (16, 24, 45, and 400 µM), pyruvate kinase (10 units/ml), lactate dehydrogenase (10 units/ml), HAL2 nucleotidase (0.5 unit/ml), HEPES (50 µM, pH K+ = 8.0), PEP (1.0 µM), NADH (0.25 mM), MgCl2 (2.0 mM), and T = 25 ± 2 °C.

**The ATP Sulfurylase Forward Reaction**—The data and experimental conditions associated with this experiment are contained in Fig. 1B and its legend. The progress curves were initiated at a near-saturating concentration of PAPS and containing varying initial ATP concentrations. APS was calculated using the change in absorbance at 365 nm (ε365 = 6.82 mM−1 cm−1) associated with the production of 2 mol of ATP per mole of APS formed. The PAPS concentration was calculated by subtracting the initial ATP concentration from the final concentration of ATP. The initial concentration of PAPS. ATP concentration was determined by adding the concentration of APS formed (which is converted to ADP by the coupling system) to the initial ATP concentration. The initial rates were calculated from the slopes of lines (−dS/dt) taken over intervals that spanned 1.5–2% of the overall reaction. The center points of the slopes were used to calculate the substrate concentrations. Typically, 2.0 mM ATP and 100 units of T. halophaga nucleotidase were used with 20–22% efficiency. The product data points associated with a single progress curve, and kinetic parameters were obtained by statistically fitting the pairs to a model for a sequential reaction mechanism using the SEQUEN program (19).

**The ATP Sulfurylase Reverse Reaction**—The initial rate measurements were performed at a fixed, saturating concentration of ATP (50 µM, 330 × K0.5). GDP formation was monitored continuously at 339 nm using the enzymes pyruvate kinase and lactate dehydrogenase. The assay conditions were as follows: SAC (0.75 µM), ATP (50 µM), GTP (25, 50, 100, 200, and 500 µM; 0.3–6 × K0.5), pyruvate kinase (10 units/ml), lactate dehydrogenase (20 units/ml), HEPES (50 µM, pH K+ = 8.0), PEP (1.0 µM), NADH (0.25 mM), MgCl2 (nucleotide + 1.0 mM), and T = 25 ± 2 °C.

**The Native Molecular Mass of SAC**—The molecular mass of SAC was determined by size exclusion chromatography using a Superdex 200 10/300 GL column (Amersham Biosciences) calibrated with molecular mass standards (ferritin, 440 kDa; catalase, 232 kDa; albumin, 67.0 kDa; ovalbumin, 43.0 kDa; and chymotrypsinogen A, 25.0 kDa). The standards yielded an excellent, linear standard curve when log molecular mass was plotted against Elution volume. The column, sample, and running buffer (50 mM HEPES/K+, 50 mM KCl, pH 7.5) were equilibrated at 25 ± 2 °C. The apparent native molecular mass of SAC is 307 ± 7.0 kDa.

**Results and Discussion**

**The Catalytic Behavior of the M. tuberculosis SAC**—To define and compare the catalytic behavior of the M. tuberculosis SAC domains, their forward and reverse reactions were characterized using initial rate measurements. With the exception of the reverse APS kinase reaction, the experimental designs are classical (see “Materials and Methods”); a representative dataset is shown in Fig. 1A. The best-fit initial rate parameters obtained from these studies are compiled in Table 1. The reverse APS kinase reaction is complicated by the well-established, potent inhibition by APS (20–22). ATP sulfurylase from E. coli, which does not have an APS kinase domain, can be used to remove APS and mitigate inhibition; however, this requires the presence of PP, and produces a second equivalent of ATP for each APS kinase turnover. The ATP is then converted by the coupling enzymes (hexokinase and glucose-6-phosphate dehydrogenase) into ADP, which is a substrate for the APS kinase reaction. Thus, the substrate concentration increases as the system turns over. If the ADP concentration is saturating at t = 0 of reaction, an increasing ADP concentration will not

**Sulfate Activation in M. tuberculosis**

strains of mycobacterium (11, 12) and can inhibit phagosome-lysosome fusion (13, 14). These tangible links between sulfur-containing metabolites and the viability and antibiotic resistance of M. tuberculosis suggest that sulfur metabolism may prove a fertile area of anti-tubercular research.
ence the reaction rate significantly, and linear $1/V$ versus $1/[PAPS]$ data are expected. Alternatively, if the ADP concentration is subsaturating, the velocity will increase until saturation is achieved, predicting a double-reciprocal plot that is non-linear at subsaturation and becomes linear as the enzyme nears saturation with ADP.

![Graph A](image1.png)

**Figure 1. Initial rate behavior of the M. tuberculosis SAC.** A. initial rate study of the ATP sulfurylase-catalyzed APS synthesis reaction at a saturating concentration of GTP. The reaction conditions were as follows: SAC (1.5 μM), SO₄²⁻ (75, 120, 250, and 2000 μM), ATP (17, 27, 54, and 500 μM), GTP (1.0 mM; 53 × $K_m$), and Hepes (50 mM, pH 8.0), MgCl₂ ⟨nucleotide⟩ + 1.0 mM, PEP (2.0 mM), NADP (0.30 mM), pyruvate kinase (10 units/ml), lactate dehydrogenase (20 units/ml), inorganic pyrophosphatase (1.0 unit/ml), and $T = 25 \pm 2$°C. Triplicate measurements made at each substrate combination were averaged and fit to a sequential mechanism using the SEQUEN algorithm (19). Rates were measured during the first 7% of reaction. The solid lines represent the behavior predicted by the best-fit initial rate parameters (see Table I).

**B.** double-reciprocal progress curves for the APS kinase-catalyzed synthesis of APS. The reaction conditions were as follows: SAC (8.0 μM, 14 milliunits/ml), PAPS (concentrations are indicated; see “Materials and Methods”), ADP (initial concentrations are indicated and increase during turnover; see “Results and Discussion”), hexokinase (14 units/ml), glucose-6-phosphate dehydrogenase (8.0 units/ml), E. coli ATP sulfurylase (1.0 units/ml), Hepes (50 mM, pH 8.0), glucose (2.0 mM), PP (1.5 mM), NADP ⁻ (0.20 mM), MgCl₂ (1.0 mM + [ADP] + [PAPS] + [PP]), and $T = 25 \pm 2$°C. Initial rates were calculated from progress curve tangents (see “Results and Discussion”). The data were fit to a rapid equilibrium model for a sequential mechanism.

To further characterize the SAC complex and add to what is known about the isoyme-specific formation of the E-P intermediate, an attempt was made to phosphorylate the SAC APS kinase domain under conditions that stoichiometrically label APS kinase from E. coli. To ensure that the labeling conditions were reliable, the E. coli APS kinase was labeled (22) in a positive control experiment. The proteins were incubated with the labeling reagents and then separated from them using size...
exclusion chromatography. The protein concentration and E-32P profiles of the column eluants were constructed and compared (Fig. 2). The profiles associated with the E. coli labeling experiment were coincident, and the active site of the enzyme was 32P-labeled with ~96% efficiency. In contrast, 33P was not detected in the fractions containing SAC. The maximum level of labeled SAC that could have gone undetected in this experiment is <1% of an active site equivalent. The M. tuberculosis and E. coli systems behave quite differently toward E-P formation, and it appears that, like its Penicillium counterpart, the M. tuberculosis system does not form the intermediate; however, E-P formation at very low levels cannot be ruled out by this experiment.

The GTP Hydrolysis/APS Synthesis Stoichiometry—GTP hydrolysis is the only reaction of the SAC complex that produces P and can be monitored using a continuous P detection system (28). The presence of inorganic pyrophosphatase produces two equivalents of P, from the PPy, formed by the APS synthesis reaction. By comparing the initial rate of P formation in the presence and absence of inorganic pyrophosphatase, the stoichiometry of GTP hydrolysis and APS synthesis can be determined. This experiment was performed under the following conditions: SAC (0.44 μM), inorganic pyrophosphatase (0 or 3.2 units/ml), purine nucleoside phosphorylase (40 units/ml), ATP (1.0 mM, 29 × K_m), GTP (1.0 mM, 83 × K_m), Na_2SO_4 (7.6 mM, 9 × K_m), MgCl_2 (3.0 mM), 2-amino-6-mercapto-7-methyl-purine riboside (0.40 mM), Hepes (50 mM), pH/K_ is 8.0, and T = 25 ± 2 °C. The ratio of turnover in the presence of inorganic pyrophosphatase (0.77 ± 0.01 s⁻¹) to that in its absence (0.26 ± 0.01 s⁻¹) is 3.0; hence, the stoichiometry of GTP hydrolysis to APS synthesis is 1:1. Whereas the results suggest that the two reactions are tightly coupled, they do not rule out the possibility of coupled and uncoupled reactions whose net behavior adventitiously produces a 1:1 stoichiometry.

The Chemical Potentials of GTP Hydrolysis and APS Synthesis Are Coupled—In the absence of GTP, APS synthesis is extremely unfavorable, K_ is ~10⁻⁷ to 10⁻⁸ at near-physiological conditions (29). The SAC APS kinase domain couples APS synthesis to PAPS formation, and PAPS synthesis is sufficiently favorable (K_ is = 2 × 10³, 50 mM Hepes, pH/K_ is = 8.0, T = 25 °C) (22) to produce detectable, albeit low, quantities of [32P]PAPS from ATP and 35SO_4 in the absence of GTP; reactions initiated at 4.0 mM ATP and 0.50 mM 35SO_4 produce a maximum of ~2% conversion of SO_4 to PAPS, and APS levels under these conditions are too low to detect (see Fig. 2A). In the absence of GTP, the SAC concentrations needed for the PAPS synthesis reactions to reach completion within several hours are higher than the quantities of product formed. The 35SO_4

assays measure the total product formed (i.e. enzyme-bound solution-phase product); thus, it is possible that a significant fraction of the product is bound to SAC, a situation that prevents using the reaction end points to calculate solution-phase equilibrium constants. To assess whether the majority of the PAPS formed in the reaction is in solution, the progress curve was determined as a function of SAC concentration. SAC-bound PAPS will titrate with SAC concentration. If the SAC-PAPS complexes represent a significant fraction of the total PAPS formed at the reaction end point, the progress curve plateau will increase with increasing SAC concentration, which is not observed (Fig. 3A). Thus, the 9.5 μM PAPS that forms at the end point of the reactions is predominantly in solution, and one can calculate that the net equilibrium constant for conversion of ATP and SO_4 to PAPS, ADP, and PP_ is 4.4 × 10⁻⁸. Given K_ for the overall and PAPS-forming reactions, K_ for the APS synthesis reaction is calculated at 2.2 × 10⁻⁷, which corresponds to ~9.0 kcal/mol, a number that agrees well with previously published data (30, 31). It is important to realize that APS formation is extremely unfavorable despite the fact that the α-β-bond of ATP is cleaved to produce it. ΔG_ for the hydrolysis of the α-β-bond, ~10.4 kcal/mol, can be used in conjunction with the ΔG_ for APS synthesis to estimate that ΔG_ for the hydrolysis of the phosphoric-sulfuric acid anhydride bond of APS is a remarkably favorable ~19.4 kcal/mol.

The addition of GTP has a profound effect on the extent of

**Table I**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Substrate</th>
<th>K^a_μM</th>
<th>K^b_μM</th>
<th>k_{cat} min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP sulfurylase</td>
<td>Forward</td>
<td>ATP</td>
<td>84 ± 2</td>
<td>35 ± 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>SO₄</td>
<td>940 ± 30</td>
<td>390 ± 5</td>
</tr>
<tr>
<td>APS kinase</td>
<td>Forward</td>
<td>APS</td>
<td>51 ± 3</td>
<td>11 ± 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>PP</td>
<td>1500 ± 200</td>
<td>350 ± 20</td>
</tr>
</tbody>
</table>

* a Steady-state affinity of substrate for the unliganded form of the enzyme, E.
* b Steady-state affinity of substrate for E+S, where S is the complementary substrate.
* c Determined at a fixed, near-saturating concentration of GTP (1.0 mM, 83 × K_m).
* d Determined at a fixed, near-saturating concentration of ATP (1.0 mM, 29 × K_m) and SO₄ (4.0 mM, 10 × K_m).
* e ND, not determined.
SAC-catalyzed PAPS synthesis. A comparison of PAPS synthesis progress curves performed with and without GTP (at a saturating concentration) reveals that the PAPS formed at the end point of the (+)GTP reaction (470 µM) is 50-fold greater than that formed in its absence (see Fig. 3B). The apparent equilibrium constant for the APS-forming reaction in the presence of GTP is 0.23, which is comparable with the value of 0.059 determined using the enzyme from \( \text{E. coli} \) (16, 34). The chemical potential of the hydrolysis reaction that is coupled to APS synthesis can be calculated from the difference in the potential of the APS-forming reaction in the presence and absence of GTP. This difference, \(-8.1 \text{ kcal/mol}\), agrees well with that expected for the hydrolysis of GTP (33). The thermodynamic and stoichiometry data both support the hypothesis that GTP hydrolysis and APS synthesis are tightly coupled and that the full complement of chemical potential available in the GTP hydrolysis reaction is used to drive the APS synthesis reaction forward.

The Isomerization of SAC—The mechanism of ATP sulfurylase from \( \text{E. coli} \) includes an isomerization that is driven by allostERIC interactions between ligands at the GTPase and adenylyltransferase active sites (16, 34). The isomerization, which precedes and partially rate-limits both GTP hydrolysis and APS synthesis, is a central energy-coupling step in the mechanism (34, 35). The isomerization commits the chemistries to forward reaction and appears to bring residues at both active sites into their catalytic positions, from which chemistry occurs quickly (35). Together, the substrate analogues AMP and PP\(_1\) can substitute for ATP and SO\(_4^\text{2-}\) in driving the isomerization and activating GTP hydrolysis (36).

The fluorescent, \(3'-\text{O-}-(\text{N-methylanthraniloyl})-2'-\text{deoxyguanosine} \) nucleotide derivatives (m-nucleotides) have proven valuable tools for understanding the allosteric interactions in the \( \text{E. coli} \) system and other GTPase-catalyzed reactions (37). These analogues are excellent functional mimics of their native counterparts and can be used to monitor nucleotide binding and hydrolysis. The quaternary GMPPNP-AMP-PP\(_1\) complex of the \( \text{E. coli} \) system (and presumably the \( \text{M. tuberculosis} \) system) resembles a point in the native reaction in which the \( a, \beta \)-bond of ATP has been broken, and the \( \beta, \gamma \)-bond of GTP has not. The system has isomerized and is stalled because it cannot cleave the \( \beta, \gamma \)-imido bond of GMPPNP. Cleavage of the \( \beta, \gamma \)-bond of GTP produces the GDP-AMP-PP\(_1\) complex, which is not isomerized (34, 35). AMP and PP\(_1\) are needed to experimentally observe the isomerization, and these activators increase the equilibrium of affinity of mGMPPNP for the \( \text{E. coli} \) enzyme 4700-fold (27 µM to 5.8 mM) (34).

To assess whether the \( \text{M. tuberculosis} \) complex undergoes an activator-dependent isomerization, the binding of mGMPPNP was studied in the presence and absence of saturating concentrations of AMP and PP\(_1\). The equilibrium binding of mGMPP-
PNP to SAC is shown in Fig. 4A. The data are described well by a simple non-allosteric binding model with a $K_a$ of 19 μM. The addition of AMP and PP$_i$ at saturating concentrations causes the affinity of mGMPPNP to increase to 120-fold, to 160 μM (Fig. 4B). Thus, like its E. coli counterpart, SAC isomerizes in a ligand-dependent fashion in what is likely an energy-coupling step in the mechanism.

The Stoichiometry of mGMPPNP Binding to SAC—The stoichiometry of mGMPPNP-SAC complex was determined in equilibrium titrations in which the mGMPPNP concentration was held fixed at 11×$K_a$ mGMPPNP. Under these conditions, the majority of SAC remains bound to nucleotide until the concentration of SAC GMPPNP-binding sites begins to exceed that of the nucleotide, at which point the titration transitions into the plateau. The transition break point yields the stoichiometry.

The ATP present in the stoichiometry titrations also prevents, by competitive binding, complications caused by mGMPPNP binding at the active site of the APS kinase domain. As is the case with APS kinase from other organisms, the SAC APS kinase domain is capable of using GTP as a substrate to phosphorylate APS (38–41). The kinetic constants associated with the ATP-dependent phosphorylation of APS are as follows: $K_m$ GTP $= 90 \pm 10$ μM, and $k_{cat} = 17 \pm 1$ min$^{-1}$ (see "Materials and Methods"). It appears that the metabolic links between sulfate activation and guanine nucleotides may extend beyond the coupling of GTP hydrolysis and APS synthesis.

**Conclusion**—The *M. tuberculosis* SAC has been physically and mechanistically characterized. The catalytic efficiency of the PAPS synthesis reaction is far greater than that of either of the reactions catalyzed by the ATP sulfurylase domain, a design that helps ensure that the system will produce PAPS efficiently in the presence of a metabolically demanding demand. The complex appears to be organized as a trimer of heterodimers, (αβ)$_3$, which is distinctly different from the (αβ)$_3$ organization of the *E. coli* enzyme, which lacks the APS kinase domain. The stoichiometries of the APS synthesis and GTP hydrolysis reactions are, within error, identical, and the apparent equilibrium constant for APS synthesis increases $1.2 \times 10^{6}$-fold ($8.1$ kcal/mol) during GTP hydrolysis; these facts reveal that the full chemical potential of the GTP hydrolysis reaction is harnessed to drive the synthesis of APS and that the energy-coupling efficiency of the enzyme is quite high.

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The Trifunctional Sulfate-activating Complex (SAC) of \textit{Mycobacterium tuberculosis}
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doi: 10.1074/jbc.M409613200 originally published online December 22, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409613200

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