ATP sulfurylases from *Penicillium chrysogenum*, *Penicillium roqueforti*, *Aspergillus nidulans*, and *Neurospora crassa* are strongly inhibited by 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the product of the second (adenosine-5'-phosphosulfate kinase-catalyzed) reaction in the two-step activation of inorganic sulfate. The *v* versus [PAPS] plots are sigmoidal. At physiological concentrations of MgATP (0.17–3 mM) and SO$_4^{2-}$ (0.4–10 mM), the $K_{0.5}$ for PAPS inhibition of the *P. chrysogenum* enzyme is 35–200 $\mu$M; $K_{0.5}$ is 68–310 $\mu$M. In the presence of PAPS, the $[S]_{0.5}$ values for both substrates are increased and the $v$ versus [MgATP] and $v$ versus [SO$_4^{2-}$] or [MoO$_4^{2-}$] plots are sigmoidal. Fluorosulfonate (FSO$_3^-$) and thiosulfate (S$_2$O$_3^{2-}$) (non-reactive sulfate analogs) inhibit the enzyme at saturating substrate concentrations in the absence of PAPS, but low concentrations of the analogs activate the enzyme when PAPS is present. Thus, PAPS behaves as an allosteric inhibitor of ATP sulfurylase. In contrast, adenosine-5'-phosphosulfate (APS = product $q$), the immediate product of the SO$_4^{2-}$-dependent reaction, is a linear inhibitor of the *P. chrysogenum* enzyme, competitive with both MgATP and MoO$_4^{2-}$ ($K_{iq}$ = 36–73 nM). FSO$_3^-$ or S$_2$O$_3^{2-}$ does not activate the enzyme in the presence of APS. The effect of PAPS on fungal ATP sulfurylase is very similar to that observed when a single highly reactive cysteinyl SH group/subunit (SH-1 or the "regulatory SH") can be chemically modified (Renosto, F., Martin, R. L., and Segel, I. H. (1987) J. Biol. Chem. 262, 16279–16288). The results suggest that in vitro SH-1 modification induces a conformational change in the enzyme that mimics the change induced in vivo by the reversible binding of PAPS. No evidence was obtained to suggest that PAPS covalently modifies SH-1.

ATP sulfurylases from rat liver (Yu, M., Martin, R. L., Jain, S., Chen, L. T., and Segel, I. H. (1989) Arch. Biochem. Biophys. 269, 156–174), spinach leaf, and *Saccharomyces cerevisiae* are not strongly inhibited by PAPS, do not display sigmoidal initial velocity plots in the presence of PAPS, and do not contain a highly reactive cysteinyl residue whose modification induces increased $[S]_{0.5}$ values and sigmoidal velocity curves.

The allosteric effect of PAPS on the fungal ATP sulfurylase may be part of a sequential feedback process unique to a group of organisms that use PAPS for two diverging pathways, reductive assimilation and sulfate ester formation.

---

ATP sulfurylase (MgATP:sulfate adenyllytransferase, EC 2.7.7.4) catalyzes the first reaction in the activation of inorganic sulfate (1, 2):

$$
\text{ATP sulfurylase, Mg}^{2+} + \text{ATP} + \text{SO}_4^{2-} \rightarrow \text{PP}_i + \text{APS}$$

(1)

The action of inorganic pyrophosphatase helps drive the overall synthesis of PAPS:

$$
\text{PP}_i + \text{H}_2\text{O} \rightarrow \text{PP}_\text{ppase, Mg}^{2+} \rightarrow 2 \text{Pi}$$

(3)

PAPS serves as the sulflyl donor for the biosynthesis of sulfate esters (4–6). In bacteria, yeasts, and filamentous fungi, PAPS also serves as the substrate for a reductive sulfate assimilation pathway leading to cysteine (7–11). Higher plants use APS as the form of "activated sulfate" that is reduced (7, 12, 14), but plants also contain APS kinase (15–20).

ATP sulfurylase from *Penicillium chrysogenum* (21–23) is a noncooperative homo-oligomer ($M_r = (61,000 \pm 10\%)$) that contains three free SH groups/subunit ($M_r = 69,000 \pm 5\%$), only one of which (designated SH-1 or the "regulatory SH") can be chemically modified by DTNB or NEM under non-denaturing conditions (24). Modification of SH-1 does not destroy catalytic activity but does cause a marked increase in the $[S]_{0.5}$ values of MgATP and SO$_4^{2-}$ (or MoO$_4^{2-}$, an alternative inorganic substrate). Furthermore, the $v$ versus [S] curves become sigmoidal with Hill coefficients ($n_H$) of 2. Inactivation protection (24), equilibrium binding, and single turnover isotope trapping measurements (25) have confirmed that the sigmoidicity results from true cooperative binding in the formation of the ternary E-MgATP-SO$_4^{2-}$ or binary E-APS.
complex, i.e. positive cooperativity requires occupancy of the adenylyl and sulfate subunits.

The dramatic effect of in vitro modification of SH-1 suggested several possible scenarios including: (a) chemical modification of SH-1 plays a regulatory role, except in vivo, the modifying agent is a natural substance whose level reflects the sulfur or energy sufficiency of the cells and (b) in vitro chemical modification induces a conformational state in the enzyme that is normally induced in vivo by a reversibly-bound allosteric effector.

Our earlier efforts were directed toward characterizing the kinetic response of ATP sulfurylase partially purified from cells grown on a high level of L-cysteine or L-methionine, i.e. under conditions where the need for sulfate activation was markedly reduced (25). L-Methionine partially repressed enzyme formation, but the enzyme that was produced displayed normal hyperbolic kinetics. L-Cysteine had no effect on the cabbage leaf (29) were purified as described previously. ATP sulfurylase purified by methods essentially identical to those used for the fungal enzymes. In the latter, leaves were frozen in liquid NP and then ground to a fine powder in a blender. The powder was stirred in an equal volume of 0.3 M NaCl linear gradient. However, the kinetic properties of these enzymes were unchanged when PMSF was omitted as described earlier. ATP sulfurylase from these fungi and from Saccharomyces cerevisiae (baker's yeast cake obtained commercially) were purified by methods essentially identical to those used for the P. chrysogenum enzyme (23). The enzymes from rat liver (28) and cabbages leaf (29) were purified as described previously. ATP sulfurylase from spinach leaf was purified by the method used for the cabbage leaf enzyme (29) and also by a method similar to that used for the fungal enzymes. In the latter, leaves were frozen in liquid N2 and then ground to a fine powder in a blender. The powder was stirred in an equal volume of 0.3 M Tris-Cl, pH 8.0, containing 5 mM EDTA, 5% polyvinylpyrrolidone (suspension), and 0.1 mM PMSF. As soon as the powder was thawed, B-mercaptoethanol was added to a final concentration of 45 mM. The suspension was centrifuged and dialyzed with 40 mM Tris-Cl, pH 8.0, the combined supernatants filtered through glass wool, and the extract fractionated on columns of Bio-Rad Affi-Gel Blue (0–1.5 M NaCl linear gradient), Whatman DEAE-cellulose (0–1.0 M NaCl), Bio-Rad Bio-Gel A-1.5m, and Amicon Matrix gel green A (0–3.5 M NaCl). The standard buffer used for all dialyses and elutions was 40 mM Tris-Cl, pH 8.0, containing 10% (v/v) glycerol. The best yields of the best yields of the enzymes were obtained from Sigma. Choline-O-sulfate was prepared from choline and H2SO4 as described by Bellenger et al. (32). Cysteine-S-sulfate (S-sulfo cysteine) was prepared from L-cysteine and Na2SO3 as described by Segel and Johnson (33). Inorganic compounds were generally Fisher or Mallinkrodt products. Na2S2O3 was obtained from Sigma. Stock Na2S solutions were prepared from crystals that were washed with water until there were 50–75% dissolved and then diluted dry prior to weighing. Stock Na2SO3 solutions were prepared by dissolving the crystals in Tris-Cl buffer (0.05 M, pH 8.0) containing 1 mM sodium azide and 1 mM Na2EDTA.

RESULTS

Properties of ATP Sulfurylases from Various Sources—Table I lists the subunit M, values and specific activities of the ATP sulfurylases used in the present study. Each preparation yielded a single major band on Coomassie Blue-stained sodium dodecyl sulfate gels with less than 5% of the dye-staining material present as minor bands in the fungal and yeast preparations, and less than 25% in the rat liver and two plant preparations (e.g. Fig. 1 of Ref. 28). The kinetic properties of the enzymes from P. chrysogenum (21, 22, 34), P. dupontii (34), and rat liver (28) have been described previously. Rabbit serum antibodies to P. chrysogenum ATP sulfurylase cross-reacted on Western blots with the enzymes from the other filamentous fungi and from yeast but not with the enzymes from plants or rat liver. Antibodies to spinach leaf Arabidopsis thaliana ATP sulfurylase cross-reacted with the cabbage leaf enzyme but not with the others. Antibodies to the rat liver enzyme did not cross-react with any of the other enzymes.

Effect of End Products and Intermediates of the Sulfate Assimilation Pathway on ATP Sulfurylase—The following compounds were tested singly and in combination with one (1 mM each) for their effect on the forward ATP

---

**Inhibition of ATP Sulfurylase by PAPS**

---

Enzymes—ATP sulfurylase and APS kinase from P. chrysogenum were purified to homogeneity (23) from cells grown for 24 h in submerged culture on synthetic medium containing 0.1 × 10−3 L-cysteine acid or 1 × 10−3 Na2SO3 as sole sulfur source. Aspergillus nidulans (26) and Neurospora crassa (27) were grown in media described earlier. ATP sulfurylase from these fungi and from Saccharomyces cerevisiae (baker's yeast cake obtained commercially) were purified by methods essentially identical to those used for the P. chrysogenum enzyme (23). The enzymes from rat liver (28) and cabbage leaf (29) were purified as described previously. ATP sulfurylase from spinach leaf was purified by the method used for the cabbage leaf enzyme (29) and also by a method similar to that used for the fungal enzymes. In the latter, leaves were frozen in liquid N2 and then ground to a fine powder in a blender. The powder was stirred in an equal volume of 0.3 M Tris-Cl, pH 8.0, containing 5 mM EDTA, 5% polyvinylpyrrolidone (suspension), and 0.1 mM PMSF. As soon as the powder was thawed, B-mercaptoethanol was added to a final concentration of 45 mM. The suspension was centrifuged and dialyzed with 40 mM Tris-Cl, pH 8.0, the combined supernatants filtered through glass wool, and the extract fractionated on columns of Bio-Rad Affi-Gel Blue (0–1.5 M NaCl linear gradient), Whatman DEAE-cellulose (0–1.0 M NaCl), Bio-Rad Bio-Gel A-1.5m, and Amicon Matrix gel green A (0–3.5 M NaCl). The standard buffer used for all dialyses and elutions was 40 mM Tris-Cl, pH 8.0, containing 10% (v/v) glycerol. The best yields of the best yields of the enzymes were obtained from Sigma. Choline-O-sulfate was prepared from choline and H2SO4 as described by Bellenger et al. (32). Cysteine-S-sulfate (S-sulfo cysteine) was prepared from L-cysteine and Na2SO3 as described by Segel and Johnson (33). Inorganic compounds were generally Fisher or Mallinkrodt products. Na2S2O3 was obtained from Sigma. Stock Na2S solutions were prepared from crystals that were washed with water until there were 50–75% dissolved and then diluted dry prior to weighing. Stock Na2SO3 solutions were prepared by dissolving the crystals in Tris-Cl buffer (0.05 M, pH 8.0) containing 1 mM sodium azide and 1 mM Na2EDTA.

**Materials and Methods**

Enzymes—ATP sulfurylase and APS kinase from P. chrysogenum were purified to homogeneity (23) from cells grown for 24 h in submerged culture on synthetic medium containing 0.1 × 10−3 L-cysteine acid or 1 × 10−3 Na2SO3 as sole sulfur source. Aspergillus nidulans (26) and Neurospora crassa (27) were grown in media described earlier. ATP sulfurylase from these fungi and from Saccharomyces cerevisiae (baker's yeast cake obtained commercially) were purified by methods essentially identical to those used for the P. chrysogenum enzyme (23). The enzymes from rat liver (28) and cabbage leaf (29) were purified as described previously. ATP sulfurylase from spinach leaf was purified by the method used for the cabbage leaf enzyme (29) and also by a method similar to that used for the fungal enzymes. In the latter, leaves were frozen in liquid N2 and then ground to a fine powder in a blender. The powder was stirred in an equal volume of 0.3 M Tris-Cl, pH 8.0, containing 5 mM EDTA, 5% polyvinylpyrrolidone (suspension), and 0.1 mM PMSF. As soon as the powder was thawed, B-mercaptoethanol was added to a final concentration of 45 mM. The suspension was centrifuged and dialyzed with 40 mM Tris-Cl, pH 8.0, the combined supernatants filtered through glass wool, and the extract fractionated on columns of Bio-Rad Affi-Gel Blue (0–1.5 M NaCl linear gradient), Whatman DEAE-cellulose (0–1.0 M NaCl), Bio-Rad Bio-Gel A-1.5m, and Amicon Matrix gel green A (0–3.5 M NaCl). The standard buffer used for all dialyses and elutions was 40 mM Tris-Cl, pH 8.0, containing 10% (v/v) glycerol. The best yields of the best yields of the enzymes were obtained from Sigma. Choline-O-sulfate was prepared from choline and H2SO4 as described by Bellenger et al. (32). Cysteine-S-sulfate (S-sulfo cysteine) was prepared from L-cysteine and Na2SO3 as described by Segel and Johnson (33). Inorganic compounds were generally Fisher or Mallinkrodt products. Na2S2O3 was obtained from Sigma. Stock Na2S solutions were prepared from crystals that were washed with water until there were 50–75% dissolved and then diluted dry prior to weighing. Stock Na2SO3 solutions were prepared by dissolving the crystals in Tris-Cl buffer (0.05 M, pH 8.0) containing 1 mM sodium azide and 1 mM Na2EDTA.

**Results**

Properties of ATP Sulfurylases from Various Sources—Table I lists the subunit M, values and specific activities of the ATP sulfurylases used in the present study. Each preparation yielded a single major band on Coomassie Blue-stained sodium dodecyl sulfate gels with less than 5% of the dye-staining material present as minor bands in the fungal and yeast preparations, and less than 25% in the rat liver and two plant preparations (e.g. Fig. 1 of Ref. 28). The kinetic properties of the enzymes from P. chrysogenum (21, 22, 34), P. dupontii (34), and rat liver (28) have been described previously. Rabbit serum antibodies to P. chrysogenum ATP sulfurylase cross-reacted on Western blots with the enzymes from the other filamentous fungi and from yeast but not with the enzymes from plants or rat liver. Antibodies to spinach leaf ATP sulfurylase cross-reacted with the cabbage leaf enzyme but not with the others. Antibodies to the rat liver enzyme did not cross-react with any of the other enzymes.

Effect of End Products and Intermediates of the Sulfate Assimilation Pathway on ATP Sulfurylase—The following compounds were tested singly and in combination with one (1 mM each) for their effect on the forward ATP

---
sulfurylase reaction (P. chrysogenum enzyme) at near \(K_m\) levels of MgATP (0.2 mM) and \(SO_3^-\) (0.5 mM); L-cysteine, L-methionine, L-homocysteine, thiolactone preincubated at pH 11 and then neutralized, L-cystathionine, choline-O-sulfate, L-cysteine-S-sulfate, Na\(_2\)SO\(_3\), Na\(_2\)S\(_2\)O\(_3\), and NaS. S-Adenosylmethionine was tested at 50 µM. Only \(SO_3^-\) exhibited significant inhibition (62%), which was expected given that thiosulfate is a nonreactive sulfate analog (21, 22). The effect of sulfite was difficult to assess because sulfite promoted the reaction in the absence of added sulfate. The rate increased with time suggesting that the effect was caused by contaminating sulfate and sulfate formed by the continuous oxidation of sulfite. The estimated inhibition of sulfate activation by \(1\) mM \(SO_3^-\) was 10-25%. This inhibition was not surprising because a variety of similar anions (FSO\(_3^-,\) ClO\(_3^-,\) CIO\(_3^-,\) NO\(_2^-\) as well as Cl\(^-\)) are inhibitors. Free sulfite may be an intermediate in assimilatory sulfate reduction but is unlikely to be present in vivo at millimolar levels. We conclude that none of the non-nucleotide intermediates or end products of sulfate assimilation acts as a feedback inhibitor of ATP sulfurylase. On the other hand, both APS and PAPS were highly inhibitory (see below).

In another series of experiments, the purified enzyme (6 µg) was incubated in 500 µl of 40 mM Tris-Cl buffer, pH 8.0, containing 25 µl of crude cell-free extract and 1 mM oxidized glutathione, or L-cysteine-S-sulfate, or homocysteine thiolactone, or 100 µM S-adenosylmethionine (potential modifiers). Residual activity in a diluted sample was measured at subsaturating MgATP and \(SO_3^-\) after 30 min. No evidence for SH-1 modification (i.e. enzyme “inactivation”) was obtained.

### TABLE I

<table>
<thead>
<tr>
<th>Source</th>
<th>Subunit</th>
<th>Specific activity(^c)</th>
<th>Molybdolysis(^d)</th>
<th>ATP synthesis(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M&lt;sub&gt;o&lt;/sub&gt;</td>
<td>units \times mg protein(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. chrysogenum</td>
<td>67</td>
<td>11</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>P. duponti</td>
<td>66</td>
<td>4</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>65</td>
<td>9</td>
<td>23</td>
<td>73</td>
</tr>
<tr>
<td>N. crassa</td>
<td>63</td>
<td>5</td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>60</td>
<td>7</td>
<td>27</td>
<td>59</td>
</tr>
<tr>
<td>Cabbage leaf</td>
<td>50</td>
<td>4</td>
<td>55</td>
<td>111</td>
</tr>
<tr>
<td>Spinach leaf</td>
<td>48</td>
<td>4</td>
<td>55</td>
<td>104</td>
</tr>
<tr>
<td>Rat liver</td>
<td>62</td>
<td>2</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

\(^a\) Determined by SDS-gel electrophoresis.

\(^b\) 30 °C, pH 8.0 (0.05 M Tris-Cl).

\(^c\) Measured at 10 mM MgATP, 30 mM \(SO_3^-\), and 5 mM excess Mg\(^{2+}\).

\(^d\) Measured at 5 or 10 mM MgATP, 20 mM \(MOO_4^-\), and 5 mM excess Mg\(^{2+}\).

\(^e\) Measured at 400 µM Na\(_4\)PP\(_2\), 12 µM APS, and 5 mM total Mg\(^{2+}\).

Fig. 1. Inhibition of the molybdolysis reaction of P. chrysogenum ATP sulfurylase by APS. a, \(1/0\) versus \(1/[\text{MgATP}]\) at 100 µM \(\sim K_m\) \(MOO_4^-\). b, \(1/0\) versus \(1/[\text{MgATP}]\) at 20 mM \(MOO_4^-\). c, \(1/0\) versus \(1/[\text{MOO}_4^-]\) at 50 µM \(\sim 2 K_m\) MgATP. d, \(1/0\) versus \(1/[\text{MOO}_4^-]\) at 5 mM MgATP. The highest MgATP and \(MOO_4^-\) concentrations used were 10 and 20 mM, respectively. The reaction was started by adding the enzyme. APS (when present) was added immediately before the enzyme. The slope replots yield the following apparent \(K_i\) values: 63 nM (a), 400 nM (b), 80 nM (c), and 400 nM (d).
Fig. 2. Velocity curves of \emph{P. chrysogenum} ATP sulfurylase (APS synthesis reaction) in the presence of varied PAPS and a constant subsaturating cosubstrate level. The reaction was started by adding the enzyme. Velocities were measured during the period 1 to 2 min after starting the reaction. \( a \), varied [MgATP] at subsaturating SO\(^{-2}\); \( b \), Hill plot of data shown in \( a \), \( V_{\text{max,app}} \) was assumed to be 4 units \( \times \) mg protein\(^{-1}\) for all PAPS concentrations. \( c \), varied [SO\(^{-2}\)] at subsaturating MgATP; \( d \), varied [SO\(^{-2}\)] at “high” (15 mM) MgATP. \( e \), Hill plots of data shown in \( d \).

The kinetic constants for the molybdolysis reaction at pH 8.0, 30 °C, 5 mM excess Mg\(^{2+}\) were as follows: \( K_m = 23 \text{ mM} \); \( K_a = 500 \mu\text{M} \); \( K_{mb} = 93 \mu\text{M} \); \( K_i = 2000 \mu\text{M} \); \( V_{\text{max}} = 22 \text{ units} \times \text{mg protein}^{-1} \) where \( A = \text{MgATP and } B = \text{MoO}_4^{2-} \) (data not shown). Except for the lower value of \( K_{mb} \), the constants are nearly identical to those reported earlier (34). If we assume a random addition of MgATP and MoO\(_4^{2-}\) and that APS excludes both substrates, the \( K_{\text{app}} \) values are related to the limiting \( K_i \) for APS binding by \( K_{\text{app}} = K_i [1 + ([S]/K_s)] \) where [S] is the concentration of the non-varied substrate (A or B) and \( K_s \) is the EA or EB dissociation constant (i.e. \( K_a \) or \( K_b \)) (36). The data of Fig. 1 yield, respectively, calculated \( K_{\text{app}} \) values of (a) 60 nM, (b) 37 nM, (c) 73 nM, and (d) 36 nM which are in reasonable agreement with the value of 40 nM obtained from initial velocity measurements of the reverse reaction using a fixed-time radiochemical assay (22).

The spinach leaf enzyme has not been completely characterized, but it appears to be similar to the fungal enzyme with respect to inhibition by APS. At 100 \( \mu\text{M} \) MoO\(_4^{2-}\), \( K_{\text{app}} \) for APS inhibition of the spinach enzyme (competitive with MgATP) was 57 nM. At 50 \( \mu\text{M} \) MgATP, \( K_{\text{app}} \) (competitive with MoO\(_4^{2-}\)) was 70 nM (data not shown).

Feedback Inhibition by PAPS—In earlier initial velocity kinetics measurements of the SO\(^{-2}\)-dependent ATP sulfurylase reaction coupled to excess APS kinase, the level of accumulating PAPS generally did not exceed 5 \( \mu\text{M} \) at any substrate concentration (21, 22). Also, at 1 mM MgATP and 1 mM SO\(^{-2}\) (>\( K_m \) but not saturating), the reaction progress curve was linear up to the accumulation of 20 \( \mu\text{M} \) PAPS (21). Consequently, the inhibition exerted by PAPS was not detected.

Fig. 2\( a \) and \( b \) show the effect of 50–125 \( \mu\text{M} \) PAPS on the initial velocity of the APS synthesis reaction catalyzed by the \emph{P. chrysogenum} enzyme. MgATP was varied at subsaturating (0.5 mM) SO\(^{-2}\). The curves are clearly sigmoidal in the presence of PAPS and show a \( V_{\text{max,app}} \) effect at high PAPS.

The velocity curves for varied [SO\(^{-2}\)] at subsaturating (0.25 mM) ATP (Fig. 2\( c \)) are also sigmoidal at low substrate concentrations. Again, there is an effect of PAPS on \( V_{\text{max,app}} \). However, at a much higher fixed MgATP concentration (15 mM), the \( v \) versus [SO\(^{-2}\)] curve approaches the control \( V_{\text{max}} \) (Fig. 2\( d \)).

PAPS also induced sigmoidal velocity curves in the molybdolysis reaction, both in the presence and absence of PP\(_i\)-ase and APS kinase (data not shown). The curves were qualitatively similar to those shown for the APS synthesis reaction. In reaction mixtures lacking APS kinase, the reaction was started by adding the enzyme\(^2\) in order to avoid a preincubation period that would allow APS to accumulate from contaminating SO\(^{-2}\). The induction of sigmoidality by PAPS in the myokinase-coupled molybdolysis reaction eliminates an (hypothetical) interaction of the two sulfate activating enzymes as the site of PAPS action (37).

Fig. 3 shows the effect of PAPS on the APS synthesis reaction of the \emph{P. chrysogenum} enzyme at MgATP and SO\(^{-2}\) concentrations found in vivo. PAPS is a complete inhibitor, \( v \) is driven to zero at all substrate concentrations at the concentration of PAPS approaches infinity. At 170 \( \mu\text{M} \) MgATP and 400 \( \mu\text{M} \) SO\(^{-2}\), the \([I]_{0.5} \) for PAPS is ~35 \( \mu\text{M} \) (Fig. 3\( a \)). Under the same conditions, \([I]_{0.5} \) for PAP was 1.8 mM.

\( ^2 \)APS kinase was originally included in molybdolysis reaction mixtures as a safety measure, i.e. to remove traces of SO\(^{-2}\) that could be converted to inhibitory APS during preincubation with enzyme in the absence of MoO\(_4^{2-}\). (The reactions were started by adding MoO\(_4^{2-}\).) The level of contaminating SO\(^{-2}\) (~1.5 mM) is only about 0.003 \( K_{mb} \). Also, the \( K_m \) for MgATP in the APS synthesis reaction is about seven times that for the molybdolysis reaction. So it was unlikely that contaminating SO\(^{-2}\) or APS derived from this SO\(^{-2}\) had a measurable effect on the MoO\(_4^{2-}\)-dependent initial velocity curves in reactions started by adding the enzyme.
cosubstrate was lower for the SH-l-modified enzyme compared with the native enzyme. This result is consistent with the earlier study of the kinetic consequences of SH-1 modification (24), the experimental velocity curves were obtained at a fixed cosubstrate concentration that was near saturating. Under this condition, SH-1 modification resulted only in a small decrease in $V_{\text{max,app}}$. A decrease in the amount of PAPS originally added. Thus, the primary and coupling enzymes did not contain measurable PAPS sulfohydrolase activity. In the second method, PAPS (27 pM) was incubated with ATP sulfurylase, hexokinase, and glucose-6-phosphate dehydrogenase and the required coupling enzyme substrates, including PP; and 1 mM SO$^-$; c, 1 mM MgATP and 10 mM SO$^-$. The actual $V_0$ values ranged from 1.4 units X mg protein$^{-1}$ (a) to 10.3 units X mg protein$^{-1}$ (d).

Thus, the inhibition is highly specific for the phosphosulfate group and cannot be attributed to contaminating PAP.

**Stability of PAPS in the Reaction Mixture**—The destruction of an inhibitor by contaminating enzymes can lead to non-hyperbolic velocity curves, so it was necessary to assess the stability of PAPS in the complete reaction mixture. This was accomplished by two methods. In the first, 6 or 16 pM [35S]PAPS was measured (22, 38) at zero time and after 20 min. No increase in [35SO$^-$] over the original 11% contamination was observed. Thus, the primary and coupling enzymes did not contain measurable PAPS sulfurylase activity. In the second method, PAPS (27 pM) was incubated with ATP sulfurylase, hexokinase, and glucose-6-phosphate dehydrogenase and the required coupling enzyme substrates, including FF; (23). No reaction was observed. After 20 min, nuclease P-1 was added. The decrease in NADPH was equivalent to the concentration of PAPS originally added. Thus, the ATP sulfurylase and coupling enzymes did not contain measurable 3'-phosphatase activity.

**Velocity Response of the SH-modified Enzyme at Low Cosubstrate Concentration**—In the earlier study of the kinetic consequences of SH-1 modification (24), the experimental velocity curves were obtained at a fixed cosubstrate concentration that was near saturating. Under this condition, SH-1 modification resulted only in a small decrease in $V_{\text{max}}$. The experiments described in Fig. 2, a and c suggest that PAPS has a strong effect on $V_{\text{max,app}}$ at subsaturating cosubstrate levels. In order to further compare the effects of SH-1 modification with PAPS inhibition, the velocity response of the SH-1-modified enzyme was obtained at a fixed cosubstrate concentration. As shown in Fig. 4, saturation with only one cosubstrate did not overcome the inhibited state. The $V_{\text{max,app}}$ attained at the fixed subsaturating concentration of a cosubstrate was lower for the SH-1-modified enzyme compared with the native enzyme. This result is consistent with an effect of SH modification on the [S]$_0$ values for both substrates. That is, at a fixed subsaturating level of B, saturation with A does not yield the control $V_{\text{max,app}}$ because $[B]/[B]_0$ for the modified enzyme is less than $[B]/[B]_0$ for the native enzyme. When both MgATP and SO$^-$ were $\approx K_a$, $V_{\text{max,app}}$ of the SH-modified enzyme approached that of the native enzyme (24), a result similar to that shown in Fig. 2d.

The results reinforce the conclusion that SH-1 modification and PAPS binding have a very similar effect on the enzyme.

**Activation by Sulfate Analogs in the Presence of PAPS**—Monovalent oxyanions (FSO$_3^-$, ClO$_2^-$, ClO$_3^-$, NO$_2^-$) and inorganic thiosulfate (S$_2$O$_3^-$) are inhibitors of ATP sulfurylase, competitive with SO$^-$ or MoO$_4^{2-}$. FSO$_3^-$, etc. are uncompetitive with respect to MgATP; S$_2$O$_3^-$ is a noncompetitive inhibitor with respect to MgATP (21, 22). Fig. 5 shows the effect of FSO$_3^-$ on the molybdolysis reaction of the P. chrysogenum enzyme at subsaturating substrate concentrations. In the absence of PAPS, FSO$_3^-$ acted solely as an inhibitor, but in the presence of 150 pM PAPS low levels of FSO$_3^-$ activated the reaction. The activation is not restricted to monovalent oxyanions or to the molybdolysis reaction. In a similar exper-
Aside from disclosing differences in sensitivity to PAPS of ATP sulfurylases from different sources, the experiment depicted in Fig. 6b also served as a further control showing that the sigmoidal response of the fungal enzymes was not an artifact of the assay (e.g., inhibition of a coupling enzyme by PAPS or an effect of minute levels of contaminating APS).

**Correlation Between High Sensitivity to PAPS, Induction of Sigmoidal Velocity Curves by PAPS, and the Presence of a "Regulatory" Sulfhydryl Group—ATP sulfurylases from N. crassa and A. nidulans (3 μm sites based on subunit $M_i^*$ values listed in Table I) were incubated with 250 μM DTNB at pH 8.0, 30 °C, in the presence of 5 mM Mg**$^{2+}$**. The reactions were followed spectrophotometrically at 412 nm. Assuming a molar absorption coefficient of 14,200 M$^{-1}$ cm$^{-1}$ for thionitrobenzoate, each enzyme was found to contain one reactive SH group/native subunit. In the presence of 0.04% (w/v) sodium dodecyl sulfate, a second SH group was exposed. The P. duponti enzyme behaved similarly (34). The results indicate that a single accessible, highly reactive cysteinyl group is present in all fungal ATP sulfurylases although the number of buried SH groups differ among different genera.

In another series of experiments, each of the ATP sulfurylases listed in Table I was preincubated at 40 °C, pH 8.0, with 40 μM DTNB for up to 35 min. (The rat liver and plant leaf enzymes were previously dialyzed to remove residual DTT or β-mercaptoethanol). Remaining activity was measured at 50 μM MgATP and 100 μM Mo**$^{6+}$**, i.e. substrate concentrations in the region of the $K_m$ values so that a chemical modification that affects $K_m$ would be detected. Only the enzymes from the fungi were rapidly "inactivated" by DTNB. For example, $t_{1/2}$ for inactivation of the N. crassa enzyme by DTNB was 5 min; no activity was lost after 35 min in the absence of DTNB. The same enzyme retained 90% of its original activity after 35 min (the same in the presence or absence of DTNB). The spinach enzyme lost activity (70% remaining activity at 30 min), but the same inactivation was observed when activity was measured at 5 mM MgATP and 20 mM Mo**$^{6+}$**. The DTNB-modified ATP sulfurylases from the fungi displayed sigmoidal $v$ versus [MgATP] curves (data not shown). The results confirm the relationship between potent inhibition by PAPS and the presence of a highly reactive SH group whose prevalent modification induces increased [S]$_{50}$ values and sigmoidal velocity curves in the absence of PAPS.

*Does PAPS Modify SH-1?—The similarity between the effect of in vitro SH-1 modification by DTNB, NEM, etc. and the effect of PAPS promoted us to explore the possibility that PAPS acts as an SH-1 modifying agent. In one series of experiments, the P. chrysogenum enzyme (3.4 μm sites) was preincubated at pH 8.0, 30 °C with 200 μM PAPS in the absence and in the presence of MgATP (0.5 mM), or Mg**$^{2+}$** (5 mM), or Mo**$^{6+}$** (0.5 mM), or MgATP (0.5 mM) plus FSO**$^{3-}$** (0.5 mM). Periodically for 30 min, samples were taken and diluted a total of 1300-fold into the molybdolyase assay mixture at subsaturating substrates, (120 μM MgATP, 200 μM Mo**$^{6+}$**, 5 mM Mg**$^{2+}$**, etc.). No loss in activity was observed.

As another approach, ATP sulfurylase (1 nmoL) [35S]PAPS (20 nmoL; 5.2 x 10$^{4}$ cpm x nmoL$^{-1}$). NaPP (2 nmoL), and MgCl$_2$ (1.5 μm) were incubated in 0.3 ml (total vol) of 0.05 M Tris-Cl, pH 8.0, at 30 °C. (The PP, was included to promote the removal of any APS present in the PAPS.) After 10 min, the solution was applied to a Sephadex G-25 column (1.5 x 47 cm) and the column eluted with the Tris buffer. Two-mI fractions were collected. The enzyme eluted in fractions 12-18 (peak at fraction 14), while almost all of the applied [35S]PAPS eluted in fractions 20-32 (peak at fraction 25). The pooled fractions containing the enzyme (0.84 nmoL) contained 0.1

---

3 F. Renosto and I. H. Segel, unpublished results.
Moreover, in the presence of PAPS, the $v$ versus [MgATP] and $v$ versus [SO$_3^2$] or [MoO$_4^{2-}$] curves are sigmoidal (Fig. 2). In the absence of PAPS, nonreactive sulfate analogs inhibit the enzyme, but in the presence of high PAPS low analog concentrations activate the enzyme (Fig. 5). Also, at a fixed saturating cosubstrate concentration, saturation with the other cosubstrate does not yield the control $V_{	ext{max,app}}$. Saturation with both substrates overcomes the inhibition. The effects of PAPS on $P$. chrysogenum ATP sulfurylase are essentially identical to those observed when a single, highly reactive cysteiny1 residue/subunit is covalently modified in vitro (24, 25). ATP sulfuryrases from several other fungi ($P$. duponti, $A$. nidulans, $N$. crassa) are also highly sensitive to PAPS and display sigmoidal velocity curves in the presence of PAPS (Fig. 6). The ATP sulfuryrases from these fungi also possess a reactive cysteiny1 group whose modification results in increased $K_{	ext{m},S}$ values and sigmoidal velocity curves in the absence of PAPS. The results suggest that in vitro modification of fungal ATP sulfurylase triggers a conformational change that is very similar to that produced by the reversible binding of PAPS. In contrast, ATP sulfuryrases from baker’s yeast, rat liver, cabbage leaf, and spinach leaf do not possess a regulatory cysteiny1 group, are not strongly inhibited by PAPS, and do not display sigmoidal velocity curves in the presence of PAPS. The results strongly suggest that ATP sulfurylase of filamentous fungi is regulated by feedback inhibition by PAPS. As shown in Fig. 3, 35 to 200 $\mu$M PAPS causes a 50% inhibition of enzyme activity at physiological concentrations of MgATP (0.17–3 mM) and SO$_3^2$ (0.4–10 mM); 90% inhibition is obtained at 68 to 310 $\mu$M PAPS. The intracellular PAPS concentration is unknown, but as shown with the plant ATP sulfurylase coupled to fungal APS kinase (Fig. 7), there is no thermodynamic barrier to the accumulation of PAPS to inhibitory levels.

Several lines of evidence showed that the sigmoidal velocity curves induced by PAPS were not artifacts of the assay. (a) Sigmoidal curves were obtained with MoO$_4^{2-}$ as the inorganic substrate (myokinase-coupled reaction) as well as with SO$_3^2$ (APS kinase-coupled reaction). PAPS caused sigmoidal curves for the molybdolysis reaction in the presence or absence of APS kinase and PP$tase$. Thus, the sigmoidicity cannot be attributed to PAPS inhibition of a primary coupling enzyme. (b) $APS$ (the only likely contaminating effector) inhibits the forward ATP sulfurylase reaction but does not induce sigmoidal velocity curves. Furthermore, if the inhibition exerted by PAPS was actually caused by contaminating APS, the spinach and rat liver (28) enzymes (which have $K_{\text{app}}$ values for APS nearly the same as those of the $P$. chrysogenum ATP sulfurylase (22) at identical cosubstrate levels) would have been just as sensitive as the fungal enzyme to “PAPS.” (c) ATP sulfuryrases from plants, yeast, and animal sources do not display sigmoidal curves in the presence of PAPS when all conditions are identical to those used to assay the fungal enzymes.

Why should strong inhibition of ATP sulfurylase by PAPS be restricted to the fungal enzymes? The answer probably lies in the unique sulfate metabolism of fungi. Eucosmcytes and Fungi Imperfecti use PAPS not only as the substrate for reductive sulfate assimilation (forming cysteine, methionine, etc.), but also as the sulfurylating agent (39) for the synthesis of choline-O-sulfate (sulfurylcholine) (40–42). This sulfate ester is presumed to be a sulfur storage compound and can account for 1.5% of the dry weight in fungal spores (43) and up to 0.26% of the mycelial dry weight (44, 45). This amounts to 70 and 13 $\mu$mol/g dry weight, respectively. Mycelium growing on excess sulfate contains about 120 $\mu$mol of total organic

**FIG. 7. Effect of the source of ATP sulfurylase on the accumulation of PAPS. $P$. chrysogenum APS kinase (1.2 units x ml$^{-1}$) was incubated with either $P$. chrysogenum or spinach leaf ATP sulfurylase (0.1 APS synthesis units x ml$^{-1}$) in the presence of 1 mM $^{35}$SO$_3^2$ (1.2 x 10$^6$ cpm x $\mu$mol$^{-1}$), 2 mM MgATP, and 5 mM excess Mg$^{2+}$ (total initial volume 1.5 ml). Conversion was monitored by periodically measuring the residual charcoal non-adsorbable $^{35}$SO$_3^2$ in a 0.1-ml aliquot of the incubation mixture (38).**

nmol of $^{35}$S. After dialysis for 3 h, the $^{35}$S content was reduced to <0.02 nmol.

In another series of experiments, 1 mM PAP or 1 mM DTT was added to a molybdolysis assay mixture containing saturating substrates and 200 $\mu$M PAPS. The rationale was that excess PAP would drive the putative modification reaction (ESH + PAPS $\Rightarrow$ ESSO$_3^2$ + PAP) in the demodification direction, while a high DTT level would promote the reduction of the modified enzyme (ESSO$_3^2$ + R(SH)$_2$ $\Rightarrow$ ESH + RS$_2$ + HSO$_3$). Neither addition reversed the inhibition by PAPS. (PAP caused additional inhibition.)

The above results suggest that PAPS does not covalently modify SH-1 of fungal ATP sulfurylase. However, we cannot exclude the possibility that PAPS reversibly modifies SH-1, but the coproduct, PAP, remains trapped at the site blocking adsorbable material (presumably, $[^{35}$S]PAPS) after 2 h. But 90% of the $^{35}$S content after 2 h. But 90% of the $^{35}$SO$_3^2$ was converted to the nucleotide product in 2 h.

**Discussion**

ATP sulfurylase from $P$. chrysogenum is strongly inhibited by PAPS, the product of the APS kinase-catalyzed reaction.
sulfur/g dry weight (52). Thus, choline-O-sulfate formation represents a substantial fraction of the total sulfate metabolized by fungi. Yeast and bacteria also use PAPS for reductive sulfate assimilation, but with a few exceptions (42, 46), these organisms do not normally accumulate high levels of sulfate esters. Higher plants (47) and red algae (48) have been reported to synthesize choline-O-sulfate. Marine algae also synthesize large amounts of carbohydrate sulfate esters. But higher plants and algae use APS rather than PAPS for sulfate reduction. Animals form many types of sulfate esters but do not reduce APS or PAPS. Thus, PAPS is a definite branch point metabolite in fungi but not in any of the other organisms examined. We can envision a sequential feedback process occurring in fungi whereby excess cysteine (or another sulfate assimilation end product) feedback inhibits the PAPS reductase system. PAPS, in turn, inhibits ATP sulfurylase reducing the expensive sulfate activation sequence (3 → P/SO₄⁻) to a level commensurate with the need for choline-O-sulfate. Over the long term, excess methionine represses ATP sulfurylase to about 30% of the fully derepressed level (25, 49).

Aside from the feedback inhibition of the fungal enzyme by PAPS, there is no other well-documented evidence for regulation of ATP sulfurylase activity. Previous reports of inhibition of the fungal (49) and yeast (50) ATP sulfurylase by inorganic sulfide need to be reassessed for several reasons. (a) Reagent grade Na₂S crystals contain small amounts of SO₄⁻ (probably on their surface). This contamination can generate APS in prolonged, fixed-time molybdenylation assays conducted in the absence of APS kinase. As shown in Fig. 1, APS is an extremely strong inhibitor of the molybdenylation reaction. Thus, it is not surprising that an APS-insensitive ATP sulfurylase from a strain of wine yeast also appeared to be insensitive to sulfide (50). (b) Contaminating polysulfides in Na₂S may have covalently modified SH-1 of the fungal enzyme causing decreased activity at subsaturating substrate levels (49). Finally, (c) Na₂S at millimolar levels may interfere with some types of colorimetric molybdenylation assays. ATP sulfurylase has a very high affinity for APS, probably a result of evolutionary pressure to maximize kcat/km, for an enzyme that catalyzes a reaction with a very small km in the physiological direction (35). There is no doubt that product inhibition by APS (and PP) limits the net forward reaction under steady-state conditions in vivo (37), but it is doubtful that this inhibition plays a regulatory role in the usual physiological sense. One reason is that MgATP and SO₄⁻ are both competitive with APS. At in vivo levels of the substrates, the apparent km for APS is about 1 μM. But the maximum intracellular level of APS cannot exceed 1.5 μM (37). Another reason is that APS kinase is not subject to feedback inhibition by sulfate assimilation end products (53) and, consequently, the cellular level of APS would not vary in direct response to the sulfur sufficiency of the cells.

The exact mechanism of PAPS inhibition remains to be established although the kinetic behavior of the system provides some clues. The activation produced by non-reactive sulfate analogs in the presence of PAPS (Fig. 5) indicates that PAPS binds to an allosteric site distinct from the active site (51; p. 450 of Ref. 36). A degree of normal competitive inhibition may be superimposed because of the structural similarity between PAPS and APS and the extremely high affinity of the active site for the phosphosulfate group. Within the context of a general concerted transition model, the cumulative data are compatible with the following: (a) the native enzyme exists almost entirely in the R state. That is, L, the allosteric constant is very small so that the T state is a very small fraction of the enzyme in the absence of PAPS. (Thus, control velocity curves are essentially hyperbolic.) (b) PAPS binds predominantly to an allosteric site on the T state. PAPS may also compete with MgATP and SO₄⁻ at the active site on the R state, but the net effect of PAPS binding is to promote formation of the T state. (c) MgATP and SO₄⁻ individually bind to both states; the affinity of the T state for MgATP is lower than that of the R state, while a T state E-MgATP-SO₄⁻ complex may not form at all. Thus, in the presence of PAPS, the velocity curves are sigmoidal (mainly a result of ternary EAB complex formation on the R state). The nonexclusive binding of MgATP and SO₄⁻ is responsible for the Hill coefficients being smaller than the actual number of subunits, the highly unfavorable formation of a T state EAB is responsible for Vmax,app effects at subsaturating, but not at saturating cosubstrate concentrations. (d) Chemical modification of SH-1 is assumed to stabilize a T state-like conformation of the enzyme in the absence of PAPS. The modified enzyme binds MgATP or SO₄⁻ but reverts to an R state conformation when both substrate subsites are occupied (24, 25).

Systematic kinetics studies on the reverse reaction combined with equilibrium binding measurements may further elucidate the mechanism of PAPS action. Structure-function studies on ATP sulfurylases from filamentous fungi that have been reported to not synthesize choline O-sulfate (e.g. Phycomyces, Mucor, some Rhizopus species) (40–42) may also prove informative.

Acknowledgments—We thank Sharon Lafferty and Gregory W. Hunt for their assistance in purifying the rat liver enzyme, and Jonathan S. Mazer for his assistance in purifying the plant leaf ATP sulfurylases.

REFERENCES
17. Acknowledgments—We thank Sharon Lafferty and Gregory W. Hunt for their assistance in purifying the rat liver enzyme, and Jonathan S. Mazer for his assistance in purifying the plant leaf ATP sulfurylases.
Inhibition of ATP Sulfurylase by PAPS

Regulation of inorganic sulfate activation in filamentous fungi. Allosteric inhibition of ATP sulfurylase by 3'- phosphoadenosine-5'- phosphosulfate.

F Renosto, R L Martin, L M Wailes, L A Daley and I H Segel


Access the most updated version of this article at [http://www.jbc.org/content/265/18/10300](http://www.jbc.org/content/265/18/10300)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/18/10300.full.html#ref-list-1](http://www.jbc.org/content/265/18/10300.full.html#ref-list-1)