Adenosine Triphosphate Sulfurylase from Penicillium chrysogenum

II. PHYSICAL, KINETIC, AND REGULATORY PROPERTIES*

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SUMMARY

The molecular weight of Penicillium chrysogenum ATP-sulfurylase (EC 2.7.7.4) has been shown to be between 425,000 and 440,000. Other physical parameters determined were: $s_{20, w} = 13.0$, Stokes' radius $= 72$ A, $D_{20, w} = 2.94 \times 10^{-7}$ cm$^2$·sec$^{-1}$, and $\bar{v} = 0.733$ cm$^3$·g$^{-1}$. The carboxymethylated enzyme breaks down to subunits having a molecular weight of approximately 56,000, in the presence of 0.1% sodium dodecyl sulfate. Titration of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) reveals that there are eight reactive sulfhydryl equivalents per 440,000 g. This, together with the molecular weight of the subunit and amino acid analysis, suggests that the enzyme is an octamer containing one free sulfhydryl and four disulfides per protomer.

Kinetic studies have shown that the actual substrate for the reaction is the ATP-$Mg^{2+}$ complex and that free ATP is a competitive inhibitor with respect to both ATP-$Mg^{2+}$ and $MoO_4^{2-}$ ($K_i = 0.6$ to $1.25$ mM). The $K_i$ for ATP-$Mg^{2+}$ at saturating $MoO_4^{2-}$ is $4.6 \times 10^{-4}$ M. The $K_i$ for $MoO_4^{2-}$ at saturating ATP-$Mg^{2+}$ is $1.5 \times 10^{-4}$ M. In the reverse direction, the $K_i$ values for adenosine 5'-phosphosulfate and $PP_i$ are $7.1 \times 10^{-4}$ and $7.7 \times 10^{-4}$ M, respectively. Initial velocity studies and isotope exchange experiments show that the mechanism of the reaction is of the sequential type. The enzyme is inhibited by adenosine 5'-phosphosulfate ($K_i = 4 \times 10^{-4}$ M) and by sulfide. The inhibition by sulfide is sigmoidal. Sulfide also changes the molbydate concentration dependence from a hyperbolic to a sigmoidal form.

The changes in the level of ATP-sulfurylase when mycelia are grown on different sulfur sources suggest that the synthesis of the enzyme is repressed by methionine, or some close metabolite of methionine.

ATP-sulfurylase (ATP-sulfate adenylyl transferase, EC 2.7.7.4) catalyzes the initial reaction in the metabolism of inorganic sulfate. In this reaction adenosine 5'-phosphosulfate is formed from ATP and sulfate (2). The enzyme is ubiquitous and has been studied from several sources (3-9). However, scant attention has been paid to the physical and kinetic properties of the enzyme and only the yeast enzyme has been purified to homogeneity (10).

The level of ATP-sulfurylase has been shown to be under metabolic control in yeast and in bacteria (5-8). Almost nothing is known about the kinetic and regulatory properties of ATP-sulfurylase of filamentous fungi. For this reason we were prompted to examine ATP-sulfurylase from Penicillium chrysogenum as part of our broader study on the regulation of sulfur metabolism in this organism. The purification and initial characterization of the enzyme have been reported (11).

MATERIALS AND METHODS

Chemicals—Except as noted, all chemicals were of the highest purity obtainable from commercial sources. APS (lithium salt) was synthesized by the procedure of Cherniak and Davidson (12). The identity of the product was established by its participation in the reverse reaction of ATP-sulfurylase. This reaction was also used to determine the concentration of APS in the presence of the AMP which was always a contaminant in the preparation of APS. Other nucleotides were titrated to pH 6.5 and their concentrations determined from their absorbance in the ultraviolet region (13).

Cytochrome c (type VI, equine heart); BSA (Fraction V, crystallized); catalase (bovine liver, twice crystallized); yeast alcohol dehydrogenase (twice crystallized, lyophilized powder); $\beta$-amylase (sweet potato, crystallized), were obtained from Sigma. Myoglobin (equine heart, twice crystallized) and ferritin (equine spleen, twice crystallized) were obtained from Calbiochem. Inorganic pyrophosphatase (yeast, once crystallized) was obtained from Worthington.

Carrier-free $^{35}S$O$_4$ in 0.1 N HCl and carrier-free Na$_2^{32}$P$_2$O$_7$ were obtained from New England Nuclear.

Source of Enzyme—The growth of P. chrysogenum in sub-
merged culture (14) and the purification of ATP-sulfurylase from this organism (11) have been described previously. For the physical studies described in this paper, a homogeneous preparation was used. For kinetic studies the eluate from the second DEAE-Sephadex column described in the purification procedure (11) was used. The concentration of the purified enzyme was determined from its absorbance index (P<sub>280</sub> at 278 nm) of 8.71 determined previously (11).

**Enzyme Assays—**The assay procedure used in this study was a modification of the molybdolyysis assay of Wilson and Bandurski (15) and has been described previously (11). The standard assay was carried out in a total volume of 0.1 ml. However, in experiments where the ATP concentration was low this resulted in a modification of the molybdolyysis assay of Wilson and Bandurski (278 mp) of 8.7 determined previously (11). The concentration of the purified enzyme was determined from its absorbance index (P<sub>280</sub> at 278 nm) of 8.71 determined previously (11).

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The tube was placed in an ice bath and a suspension of acid-precipitated protein (adjusted to pH 8.0 with HCl); ATP, 0.01 M; MgCl<sub>2</sub>, 0.01 M; K<sub>3</sub>PO<sub>4</sub>, 0.01 M; and inorganic pyrophosphatase, 0.2 pg. The assay was started with ATP-sulfurylase in the forward direction with "<sup>35</sup>S" as the substrate. This assay (described below) depends on the incorporation of radioactive sulfate into APS and (if APS-kinase is present) PAPS. The nucleotides are then adsorbed onto charcoal leaving the unconjugated K<sub>3</sub>PO<sub>4</sub>, in the wash. Inorganic pyrophosphatase is added to the assay mixture in order to remove pyrophosphate from the reaction since the equilibrium is far in the direction of ATP and sulfate. However, even very large excesses of pyrophosphatase could not produce an assay linear with time or protein when purified ATP-sulfurylase was assayed because of inhibition of ATP-sulfurylase by APS. In crude extracts containing APS-kinase the assay is more linear with both time and protein.

The radioactive assay was carried out in a total volume of 0.3 ml containing the following components: Tris base, 0.1 M (adjusted to pH 8.0 with HCl); ATP, 0.01 M; MgCl<sub>2</sub>, 0.01 M; K<sub>3</sub>PO<sub>4</sub>, 10<sup>-3</sup> M; and inorganic pyrophosphatase, 0.2 µg. The assay was started with ATP-sulfurylase and incubated at 30°. At the appropriate time 0.5 ml of cold 1.0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 0.2 M citric acid was added to stop the reaction. The tube was placed in an ice bath and a suspension of acid-washed Norit in water (0.5 ml), 30 mg per ml was added to adsorb the nucleotides. The mixture was centrifuged for 1 min at full speed in a clinical centrifuge. The supernatant was carefully removed by aspiration through a Pasteur pipette. The charcoal pellet was suspended in 3 ml of 0.1 M Na<sub>2</sub>SO<sub>4</sub> (adjusted to pH 4.0) and centrifuged as before. This washing procedure was repeated three times. After the supernatant from the final washing was removed, the charcoal pellet was suspended in 2.0 ml of 50% aqueous ethanol containing 0.4% NaN<sub>3</sub>OIII and 0.1% Tween 80. A 0.5-ml aliquot of this suspension was plated on a 1-inch aluminum planchet and dried under a heat lamp. The radioactivity adsorbed to the Norit was determined with a gas flow counter and compared with the radioactivity in known amounts of standard K<sub>3</sub>PO<sub>4</sub>, plated and dried with an equivalent amount of Norit.

**Ultracentrifuge Studies—**Sedimentation velocities were determined with a Beckman model E analytical ultracentrifuge equipped with Schlieren optics and a temperature control system set to 20°C. Two standard 12-mm single sector cells with quartz windows were used, one being equipped with a 1° wedge window. The enzyme solution was exhaustively dialyzed against 0.01 M Tris (titrated to pH 8.0 with HCl and containing 2 × 10<sup>-4</sup> M EDTA and 0.1 M NaCl) before use. The protein concentration ranged from 0.5 to 6.0 mg per ml. Glass photographic plates were used and the moving boundary distances were measured by the position of maximum ordinate by use of a Bausch and Lomb microcomputor. The slope of the log x versus time curve was determined by the method of least squares with an Olivetti Programma 101 computer. The observed value of x was converted to the s<sub>20</sub> according to Schachman (16). Values for the density and viscosity of the solvent were obtained from standard tables for 0.1 M NaCl (17).

**Discontinuous Gel Electrophoresis—**Discontinuous gel electrophoresis was carried out at several polyacrylamide concentrations according to the procedure described by Hedrick and Smith (18). Several proteins of known molecular weight were used to calibrate the gels. The logarithm of the relative mobility of each protein was plotted against gel concentration and the slope of this line was then plotted against the molecular weight. This calibration curve was used to determine the molecular weight of ATP-sulfurylase.

Disc gel electrophoresis was also performed in the presence of SDS by a modification of procedures described by Shapiro, Vinuela, and Maizel (19) and Weber and Osborn (20). The gel and buffer system used was the same as that used for electrophoresis of the unmodified proteins. ATP-sulfurylase and the standard proteins used were carboxymethylated before electrophoresis (21). The proteins were then diazylated against upper gel buffer to which 0.1% SDS had been added. The samples were applied above the upper gel buffer in 20% sucrose in the normal way. The sample solution and the upper reservoir buffer both contained 0.1% SDS.

At the completion of electrophoresis the dye front was marked with a fine wire and the gels were stained with Coomassie blue according to the procedure of Weber and Osborn (20). The relative mobility of the proteins was then measured and plotted against their molecular weight on a logarithmic scale.

**Gel filtration—**Sephadex G-200, fine (Pharmacia), was allowed to swell for several days at 4° in 0.05 M Tris base adjusted to pH 8.0 with HCl and containing 10<sup>-4</sup> M EDTA. This buffer was used for all experiments. The gel was poured into a column (Pharmacia), 2.5 × 40 cm, and allowed to settle while a slow flow rate was maintained. All operations were performed at 4°. Sample solutions contained 10% sucrose and were applied by layering under the buffer solution just above the gel. The column eluate was monitored at 254 nm with an LKB Uvicord detector. Constant flow rates (8 to 12 ml per hour) were maintained with a Mariotte flask.

The elution volumes of several proteins of known molecular weight were used to calibrate the column according to the method described by Andrews (22). The elution volume of ATP-sulfurylase was then determined and the molecular weight calculated from the calibration curve. In these experiments 0.5-ml fractions were collected and the following standard proteins...
were used: BSA (determined by absorbance at 280 nm); ferritin (determined by absorbance at 415 nm); catalase (determined by its enzymatic activity (23)); yeast alcohol dehydrogenase (determined by its enzymatic activity (24)); myoglobin (determined by absorbance at 430 nm). The position of ATP-sulfurylase was determined by measurement of its enzymatic activity with the molybdobis assay. There was no dependence of elution volume on protein concentration over the range of 0.1 to 1.0 mg per ml. The excluded volume ($V_e$) of the column was determined with blue dextran 2000 and the total volume ($V_t$) with tritiated water.

The data obtained above were also interpreted according to Ackers (25) who has derived an equation relating the measurable column parameters to the Svedberg radius of the effluent molecule and the effective pore radius of the molecular sieve. The free diffusion coefficient ($D$) was then obtained from the Svedberg radius by use of the Stokes-Einstein equation. The molecular weight of the protein was calculated from the free diffusion coefficient and the sedimentation coefficient by use of the Svedberg equation (16).

Sulfhydryl Group Determination—Determination of sulfhydryl groups was performed according to Ellman (26) on both native and SDS-denatured ATP-sulfurylase. The final concentrations used were 0.1 to 0.5 mg per ml of enzyme, $2 \times 10^{-4}$ M DTNB, 0.1 M Tris-HCl buffer, $10^{-3}$ M EDTA (pH 8.0) and (when added) 0.1% SDS. Absorbance measurements were made at 412 nm at 1 min intervals until a constant value was reached.

RESULTS

Physical Characteristics

Sedimentation Velocity—The $s_{20,w}$ of ATP-sulfurylase was determined at several protein concentrations between 0.5 and 6.0 mg per ml. This data was then plotted and extrapolated to zero protein concentration (Fig. 1). A value of 13.0 S was obtained for the $s_{20,w}$ of the enzyme at infinite dilution.

Molecular Weight from Gel Filtration—The molecular weight was determined by gel filtration on a Sephadex G-200 column calibrated with pure proteins of known molecular weight. The results, shown in Fig. 2, indicate a molecular weight of 440,000 for the enzyme. An interesting observation made during the course of these experiments was that if ATP-sulfurylase was applied to the Sephadex G-200 column together with the blue dextran 2000, the enzyme activity peak coincided with the blue dextran peak, i.e. the enzyme was eluted at the excluded volume. Presumably this resulted from the binding of the enzyme to the blue dextran but the phenomenon was not further investigated.

A Sephadex G-200 column was also used to calculate the Stokes' radius of ATP-sulfurylase according to the method of Ackers (25). Table I shows the gel pore radius determined from the elution volumes of ferritin, bovine serum albumin, yeast alcohol dehydrogenase, catalase, and myoglobin. Also shown are the elution volumes for blue dextran 2000 and tritiated water, which were used to determine the column parameters $V_n$, $V_t$, and $V_i$. The gel pore radius calculated from this data showed good agreement for proteins with a wide range of Stokes' radii. The elution volume of ATP-sulfurylase from this column was 75.5 ml which corresponds to a Stokes' radius of 72 Å. The Stokes' radius of ATP-sulfurylase was then used to calculate the free diffusion coefficient for the enzyme according to the

![Fig. 1. Dependence of the sedimentation coefficient of purified ATP-sulfurylase on protein concentration. Sedimentation velocities were determined as described under "Materials and Methods."](image1)

![Fig. 2. Plot of $V_e/V_t$ against log molecular weight of proteins eluted from a Sephadex G-200 column (2.5 X 38 cm). Experimental details are described under "Materials and Methods." The short arrows (I I) indicate the range of $V_e/V_t$ at which ATP-sulfurylase was eluted. The molecular weights of the proteins used were: apoferritin (ferritin) monomer, 450,000 (27); catalase, 240,000 (28); yeast alcohol dehydrogenase, 150,000 (29); bovine serum albumin, 66,000 (30); and myoglobin, 16,900 (31).](image2)

| Compound                  | Elution volume | Stokes' radius | Stokes' radius |
|---------------------------|----------------|----------------|----------------|
| Blue dextran 2000         | 54.5           |                |                |
| Ferritin                  | 72.5           | 79.0           | 205            |
| Catalase                  | 93.0           | 52.2           | 197            |
| Yeast alcohol dehydrogenase| 100.0          | 46.5           | 194            |
| Bovine serum albumin      | 115.0          | 36.1           | 197            |
| Myoglobin                 | 125.5          | 18.7           | 199            |
| Tritiated water           | 210.0          |                |                |

$^a$ Stokes' radii were calculated by means of the Stokes'-Einstein equation from diffusion coefficients reported in the literature.

$^b$ The gel pore radius was calculated as described under "Materials and Methods."

$^c$ See Rushen (27).

$^d$ See Sumner and Gralen (28).

$^e$ See Hayes and Velick (32).

$^f$ See Tanford (30).

$^g$ See Theorell (31).
The relative mobilities of proteins on disc gel electrophoresis (in the presence of SDS) as a function of their molecular weights. The proteins were all carboxymethylated prior to electrophoresis. The resolving gel contained 5% acrylamide. Other experimental details are given under “Materials and Methods.” The molecular weights of the proteins used were cytochrome c, 13,400 (34); myoglobin, 16,900 (31); pepsin, 32,777 (35); and bovine serum albumin, 65,000 (30).

Stokes–Einstein relationship. The value obtained for the $D_{20,w}$ was 2.94 x 10^{-5} cm$^2$ sec$^{-1}$.

Calculation of Molecular Weight from $D_{20,w}$ and $s_{20,w}$—The Svedberg equation (10) was used to calculate the molecular weight from the $s_{20,w}$ determined by sedimentation velocity and from the $D_{20,w}$ calculated from the Stokes' radius. The value obtained was 425,000. The value used for the partial specific volume (v) was 0.733 cm$^3$.g$^{-1}$. This was calculated from the previously published amino acid composition (11) by the procedure of Cohn and Edsall (33).

Molecular Weight from Disc Gel Electrophoresis—The method of Hedrick and Smith (18) for the determination of molecular weights by disc gel electrophoresis on gels of varying pore size was applied to ATP-sulfurylase. The standard proteins used were: BSA (monomer and dimer), $\alpha$-amylase, and ferritin (monomer and dimer). This method gave a molecular weight for the enzyme of 440,000.

Subunit Structure

Subunit Size—The subunits of ATP-sulfurylase were investigated by discontinuous gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) as described under “Materials and Methods.” The carboxymethylated ATP-sulfurylase showed a single protein band indicating that the enzyme has subunits of a single molecular size. The relative mobility of this component corresponded to a protein with a molecular weight of 56,000 when compared to the standard proteins shown in Fig. 3. The results suggest that ATP-sulfurylase is composed of eight subunits of molecular weight 56,000. Disc gel electrophoresis of the carboxymethylated enzyme could not be carried out in the absence of either 8 M urea or SDS since the carboxymethylated ATP-sulfurylase precipitated if either of these materials were removed by dialysis.

Sulfhydryl Group Analysis—A typical titration of -SH groups with Ellman's reagent (DTNB) is shown in Fig. 4. DTNB (2 x 10^{-4} M final concentration) was dissolved in 0.1 M Tris-HCl buffer, 10^{-4} M EDTA (pH 8.0). At zero time the enzyme solution was added and the optical density at 412 nm measured at the indicated intervals. The calculations were based on a molar absorbance index of 13,600 at 412 nm (26). Eight sulfhydryl equivalents per 440,000 g were titrated. In the presence of SDS, no additional groups became available. This suggests that there is only one free sulfhydryl equivalent per 56,000 g of subunits. Since amino acid analysis gives a value of 9 half-cystine residues per 56,000 g (11) it is probable that the enzyme contains four disulfide bonds and one free sulfhydryl per subunit. This result also supports the conclusion obtained by gel electrophoresis in the presence of SDS that the enzyme is an octamer.

Kinetic Properties

Interaction with ATP and Magnesium—It has previously been established that a divalent cation (Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$) is necessary for the activity of ATP-sulfurylase (11). The results shown in Fig. 5A show that the substrate for the reaction is the 1:1 complex of ATP and magnesium. Fig. 5A also shows that free ATP is an inhibitor of the enzyme. Fig. 5B shows a Dixon plot of the data from Fig. 5A indicating that the inhibition by free ATP is competitive with respect to molybdate. Double reciprocal plots of these data (not shown) were linear and extrapolated to the same Vmax for all free ATP concentrations, as expected for a competitive inhibitor.

Fig. 5C shows the results of an experiment in which ATP was increased at several fixed concentrations of MgCl$_2$ and at a single molybdate concentration (1.0 mM). Once again the inhibition by free ATP is obvious and a Dixon plot at several ATP-Mg$^{2+}$ concentrations (i.e. Mg$^{2+}$ concentrations) shows that free ATP is a competitive inhibitor with respect to ATP-Mg$^{2+}$ (Fig. 5D). Double reciprocal plots of these data (not shown) were linear and extrapolated to the same Vmax for all free ATP concentrations. Similarly, a replott of the slopes of the Dixon plot (Fig. 5D) against the reciprocal of the ATP-Mg$^{2+}$ concentration was linear with an intercept at the origin. The ATP-Mg$^{2+}$ concentration in these experiments was taken to be equal to either the MgCl$_2$ or the total ATP concentration depending on which was limiting in each experiment (the $K_a$ for ATP-Mg$^{2+}$ is 7.3 x 10^4 M$^{-1}$). The free ATP concentration was calculated from the difference between this value and the total ATP concentration.

Increasing the concentration of Mg$^{2+}$ at a constant concentration of ATP-Mg$^{2+}$ had no effect on the rate of the reaction.
**Initial Velocity Studies**—In order to establish the $K_m$ values of the enzyme for molybdate and ATP-Mg$^{2+}$, a series of initial velocity experiments were performed. The double reciprocal plots of the data obtained (Fig. 6, A and B) show that the $K_m$ for each substrate varies with the concentration of the alternate substrate. The fact that the reciprocal plots are not parallel but intersect to the left of the vertical axis reveals that there is no irreversible step between the addition of the two substrates (36). Since the release of a product before interaction with the second substrate would constitute an irreversible step, this result suggests that the mechanism of the reaction requires the involvement of both substrates prior to the release of any products. Double reciprocal replots of both the slopes (not shown) and the y axis intercepts from Fig. 6, A and B, were linear. The double reciprocal replots of the y axis intercepts from both primary reciprocal replots are shown in Fig. 6C. The intercept on the horizontal axis gives the negative reciprocal of the Michaelis constant for the varied substrate at saturating (infinite) concentration of the alternate substrate (37, 38). The $K_m$ for molybdate at infinite ATP-Mg$^{2+}$ was 0.147 mM while that for ATP-Mg$^{2+}$ at infinite molybdate was 0.0455 mM.

**Isotope Exchange Studies**—Levi and Wolf (4) have obtained data for rat liver ATP-sulfurylase which suggest that the mechanism involves the intermediate formation of an enzyme-AMP complex with pyrophosphate being released. The enzyme-AMP complex would then react with sulfate to form APS. This mechanism is shown in the following reactions.

\[
\text{ATP} + \text{enzyme} \rightleftharpoons \text{enzyme-AMP + PP}_i \quad (1) \\
\text{Enzyme-AMP} + \text{SO}_4^{2-} \rightleftharpoons \text{enzyme} + \text{APS} \quad (2)
\]

This mechanism is of the “ping-pong” type rather than the sequential type and is inconsistent with the initial velocity.
studies described in the previous section. Levi and Wolf (4) base their hypothesis mainly on the fact that there was an exchange of labeled pyrophosphate into ATP in the absence of sulfate and that this exchange was faster in the presence of sulfate. An attempt was made to show ATP $\rightarrow ^{32}$PP$_i$ exchange (Reaction 1) with P. chrysogenum ATP-sulfurylase and the results are shown in Fig. 7. In the absence of added sulfate there was no detectable exchange of $^3$PP$_i$ into ATP. The addition of 2.0 $\mu$moles of ATP-Mg$_2^+$ did enable the exchange to take place but the rate at which $^{32}$PP$_i$ entered ATP could be accounted for by the reaction of the APS produced with $^3$PP$_i$ to give labeled ATP.

The mechanism proposed by Levi and Wolf (4) also predicts an exchange between the sulfur atoms of APS and sulfate in the absence of other substrates (Reaction 2). We were unable to demonstrate this exchange. This result, together with the data obtained from the initial velocity experiments, suggests that the mechanism of the reaction catalyzed by the P. chrysogenum ATP-sulfurylase involves the combination of both ATP-Mg$_2^+$ and sulfate (or molybdate) with the enzyme before the release of any of the products (i.e. sequential type).

Potential Effectors of ATP-sulfurylase—A number of compounds which might be effectors of ATP-sulfurylase were examined. Only sulfate, sulfide, and APS inhibited the enzyme significantly. Sulfate is the physiologically important substrate for the enzyme, but because the rate of the reaction with sulfate is very much slower than the reaction with molybdate, sulfate appears to be a competitive inhibitor with respect to molybdate. Sulfide is the end product of the pathway of sulfate activation and reduction and, as such, would be a likely feedback inhibitor controlling the first step in the sequence. Inhibition by APS is also to be expected since it is a product of the reaction with the physiological substrate. None of the sulfur compounds tested (L-cysteine, L-cystine, L-methionine, L-cysteine sulfonic acid, L-cysteic acid, reduced glutathione, Na$_2$SO$_3$, Na$_2$SO$_4$, and choline-O-sulfate) had any effect on the reaction. Similarly, L-serine, L-homoserine, O-acetyl-L-serine, and O-succinyl-L-homoserine had no effect.

Inhibition by APS—The effect of APS on the reaction is shown in Fig. 8. When this data was plotted in double reciprocal form (inset, Fig. 8) the pattern obtained was characteristic of noncompetitive inhibition. The replot of the slopes of the double reciprocal plot versus the inhibitor concentration (not shown) was linear. A Dixon plot of the original data (not shown) yielded a series of straight lines, intersecting on the horizontal axis. This is also characteristic of noncompetitive inhibition. A $K_i$ of 0.04 $\text{mM}$ for APS was obtained from the Dixon plot. APS does not appear to have any allosteric effect on the enzyme since all of the reciprocal plots were linear (36). Further evidence that APS is not an allosteric effector was obtained when the reverse reaction was studied (Fig. 10). APS did not exhibit substrate inhibition of the reaction even at quite high concentration. It therefore appears to be a simple product inhibitor of ATP-sulfurylase. The inhibition of ATP-sulfurylase by APS was also evident when K$_2$SO$_4$ was used as the substrate and the APS produced was adsorbed to Norit. This reaction was never linear either with time or protein concentration even in the presence of large amounts of inorganic pyrophosphatase. The inhibition must have been caused by APS produced during the reaction since pyrophosphate is continually

![Fig. 7. Exchange of $^{32}$P-labeled pyrophosphate into ATP in the presence and absence of sulfate. ATP-Mg$_2^+$ mixture was prepared containing the following components in a total of 1.0 ml: Tris-HCl (pH 7.5), 50 $\mu$moles; MgCl$_2$, 10 $\mu$moles; ATP, 2.0 $\mu$moles; $^{32}$PP$_i$, 0.99 $\mu$ mole. When K$_2$SO$_4$ was added the concentration was 2.0 $\mu$moles per ml. At zero time 10 $\mu$g of homogenous ATP-sulfurylase were added to all reaction mixtures except those used as minus enzyme blanks. At the times indicated 100-ml aliquots were taken and the reaction was stopped with 0.1 ml of ice-cold 0.1 M sodium pyrophosphate in 0.05 M citric acid. One-tenth milliliter of a 30 mg per ml suspension of Norit was added to adsorb the nucleotides. After 5 min the Norit suspension was centrifuged in a clinical centrifuge and the clear supernatant was removed by aspiration with a filter pump. The Norit was then washed four times with ice-cold 0.1 M sodium pyrophosphate which had been adjusted to pH 4.0 with acetic acid. After removal of the final wash the Norit was suspended in 2.0 ml of ethanol-water-NH$_2$OH (50:50:1) and a 0.5-ml aliquot was plated onto an aluminum planchet and dried before being counted in a gas flow counter. PP$_i$ standards were mixed with an equivalent amount of Norit before plating to correct for any absorption of radioactivity by the Norit.](https://www.jbc.org/issue/249/14/2443/a1/fig7.png)

![Fig. 8. Dependence of the rate of reaction of ATP-sulfurylase on molybdate concentration at several concentrations of APS. The enzyme velocity was determined by molybdoanalyses in a total volume of 1.0 ml. The ATP concentration was 2.0 $\text{mM}$ while the Mg$^{2+}$ was 10 $\text{mM}$. The APS concentrations used were (millimolar): •, 0.0; ○, 0.01; △, 0.02; □, 0.03; □, 0.05. The inset shows the double reciprocal plot of these data.](https://www.jbc.org/issue/249/14/2443/a1/fig8.png)
The inset shows the double reciprocal plot of these data. The enzyme activity was determined by molybdolysis in a total volume of 1.0 ml. The ATP concentration was 5.0 mM and the Mg²⁺ concentration was 10.0 mM. The NanS sodium sulfide. The enzyme activity was determined by molybdolysis in a total volume of 1.0 ml. The ATP concentration was 5.0 mM and the Mg²⁺ concentration was 10.0 mM. The sodium molybdate concentrations were (millimolar): ●, 0.0; △, 0.5; ▲, 1.0; ○, 1.5. The inset shows the double reciprocal plot of these data. The same symbols are used for Na₂S concentrations. B, Hill plots of the data shown in A. C, dependence of the rate of reaction of ATP-sulfurylase on sulfide concentration at several molybdate concentrations. The ATP concentration was 5.0 mM and the Mg²⁺ concentration was 10.0 mM. The sodium molybdate concentrations were (millimolar): ○, 5.0; ▲, 2.0; ●, 1.0;△, 0.5. The Hill plots are shown in D. D, Hill plots of the data shown in C.

FIG. 9. A, dependence of the rate of reaction of ATP-sulfurylase on molybdate concentration at several concentrations of sodium sulfide. The enzyme activity was determined by molybdolysis in a total volume of 1.0 ml. The ATP concentration was 5.0 mM and the Mg²⁺ concentration was 10.0 mM. The Na₂S concentrations were (millimolar): ●, 0.0; △, 0.5; ▲, 1.0; ○, 1.5. The inset shows the double reciprocal plot of these data. The saturation curve for each substrate was obtained at saturating concentrations of the other substrate. The enzyme activity was determined as described previously (11). The APS concentration used when PPi was the varied substrate was 0.5 mM, while the PPi concentration used when APS was the varied substrate was 5.0 mM. The inset shows the double reciprocal plot of these data.

removed. The concentration of APS produced in these experiments was between 0.02 and 0.05 mM, i.e. in the same range as the Ki for APS determined with molybdate as substrate.

Inhibition by Sulphide—Fig. 9A shows the effect of sulfide on the molybdate dependence of the reaction rate. In the absence of sulfide a normal hyperbolic saturation curve is obtained. Sulfide inhibits the reaction and changes the saturation curve to a sigmoidal form. The double reciprocal plots of velocity versus substrate concentration (inset, Fig. 9A) are concave upward but all extrapolate to the same V₅₀. This is characteristic of an allosteric inhibitor of the K type as described by Monod, Wyman, and Changeux (39). The Hill plots (40) from the data in Fig. 9A are shown in Fig. 9B. Sulfide at 1.5 mM changes the interaction coefficient (n) for molybdate from 1 (in the absence of inhibitor) to 1.54. The change in the S₅₀ for molybdate is even more marked, increasing from 0.65 mM in the absence of inhibitor to 4.5 mM at 1.5 mM sulfide.

The dependence of the reaction rate on sulfide concentration at several molybdate concentrations is shown in Fig. 9C. Fig. 9D shows the Hill plots of this data. At all the molybdate concentrations tested the inhibition curve for sulfide was sigmoidal with an interaction coefficient between 1.30 and 1.55. The greatest effect of molybdate was on the I₅₀ for sulfide. At high molybdate (5.0 mM) the I₅₀ for sulfide was 4.02 mM but at a lower molybdate concentration (0.5 mM) the I₅₀ for the inhibitor was 0.46 mM. These results suggest that sulfide may play a significant role in the control of sulfate activation in P. chrysogenum.

Reaction in Direction of ATP Synthesis—Preliminary studies were carried out on the reaction of ATP-sulfurylase in the nonphysiological direction (ATP synthesis) with the assay procedure described previously (11). The concentration dependence for each substrate in the presence of saturating amounts of the alternate substrate was examined and the results are shown in Fig. 10. The K₅₀ for APS was 0.0071 mM and the K₅₀ for PPi was 0.077 mM. The reciprocal plots for PPi concentration dependence (inset, Fig. 10) are linear even at high substrate concentrations (up to 2.0 mM) showing that PPi does not inhibit ATP-sulfurylase.

Control of Enzyme Synthesis

ATP-sulfurylase Levels in Mycelia Grown on Various Sulfur Sources—P. chrysogenum was grown with several different sulfur compounds as the sole sulfur source and the enzyme activity of the mycelia determined with the molybdolysis assay. The initial concentration of the sulfur source in the medium was 10 μmoles of sulfur per ml. Sulfur sources at this concentration are not depleted in a 24-hour period.³ The results of this experiment are shown in Table II. The most dramatic difference in enzyme level was shown by the mycelia grown on methionine. The level of the enzyme was about 15% of that present in mycelia grown on thiosulfate (the sulfur source yielding the highest level of enzyme). If methionine-grown mycelium were washed and resuspended in media without a sulfur source, the level of ATP-sulfurylase rose to 30 to 40 enzyme units per g, dry weight, after 10 to 12 hours. The combined activating enzyme levels were also determined in crude extracts with the radioactive ⁸⁵S⁰₄⁻ assay described under “Materials and Methods.” The relative activities on different sulfur sources were the same as shown with the molybdolysis assay (Table II). Although the actual values were much lower because of the slower rate of reaction with sulfate as substrate. These results are consistent with a control mechanism involving the repression and

³ J. W. Tweedie and I. H. Segel, unpublished results.
**Table II**

*Activity of ATP-sulfurylase in mycelia grown on various sulfur sources*

| Sulfur source | Enzyme activity<sup>a</sup> |
|---------------|-----------------------------|
|               | Units/mg mycelium, dry weight | Units/mg extracted protein |
| L-Methionine  | 7.4                         | 0.011                       |
| Na<sub>2</sub>SO<sub>4</sub> | 20.6                        | 0.034                       |
| Choline-O-sulfate | 31.0                       | 0.047                       |
| Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> | 30.0                        | 0.075                       |
| L-Cysteine acid | 40.5                        | 0.064                       |
| L-Cysteine sulfonic acid | 39.2                        | 0.057                       |
| L-Djenkolic acid | 35.6                        | 0.090                       |
| L-Cystine   | 40.1                        | 0.062                       |
| L-Methionine for 24 hrs, then sulfur-starved for 12 hrs | 35.0 | 0.048 |

<sup>a</sup> One unit of enzymatic activity = 1 amole of Pi released per min in the molybdolyase assay.

Because the product of the ATP-sulfurylase reaction is inorganic pyrophosphate, the true specific activities (in international units) are half the indicated values. *P. chrysogenum* was grown on citrate No. 3 medium containing the indicated sulfur source at a concentration of 10 amoles of sulfur per ml. After 24 hours, the mycelia were filtered through a sintered glass funnel and washed once with deionized water. The wet mycelial mat was divided into two parts and weighed. One of these was used to determine the dry weight after 8 hours at 100°. ATP-sulfurylase activity was determined in the second piece after extraction by shaking for 2 min in a Brown-Wall cell homogenizer. The ratio of wet mycelia-1 mm glass beads-0.05 x Tris buffer, pH 8.0, was 1:2:1 (w/w/v). The volume of the extract was determined after decanting from the glass beads. ATP-sulfurylase activity was determined in the extract by the molybdolyase assay after dialysis against 0.05 x Tris buffer, pH 8.0. Protein was determined by the biuret procedure.

**Discussion**

The molecular weight of ATP-sulfurylase was shown by several methods to be between 425,000 and 440,000. This is greater than the molecular weight of 100,000 calculated for the yeast enzyme from the data of Robbins and Lipmann (10). Levi and Wolf (4) have shown that rat liver ATP-sulfurylase is very large, with an approximate molecular weight of 900,000. These results suggest that the ATP-sulfurylases from the three sources are unrelated proteins.

*P. chrysogenum* ATP-sulfurylase appears to be composed of eight subunits of molecular weight 56,000. Other data suggest that there are 4 cysteine and 4 cysteine residues in each subunit. The fact that the actual substrate for the enzyme is the ATP-Mg<sup>2+</sup> complex is not unexpected since most other enzymes for which ATP is a substrate require magnesium. It has invariably been shown that the substrate is actually the ATP-Mg<sup>2+</sup> complex (43, 44). The data obtained in the present study are consistent with a mechanism in which ATP-Mg<sup>2+</sup> is the substrate and free ATP is an inhibitor of the enzyme.

The inhibition of ATP-sulfurylases by APS has been noted by other workers (3, 4, 9). Levi and Wolf (4) observed substrate inhibition of the rat liver enzyme when the reaction was measured in the direction of ATP synthesis. We could find no evidence of substrate inhibition by APS when the reaction was studied in this direction (Fig. 10). It is possible that the actual substrate in this direction is the APS-Mg complex and that free APS is inhibitory as was shown for free ATP in the forward direction. In the experiment shown in Fig. 10 the Mg<sup>2+</sup> concentration was 10 mm which was much higher than the highest APS concentration (1.0 mm). In any case, the inhibition observed by Levi and Wolf (4) was very slight since the reciprocal plot of their data showed only a leveling off at high APS concentrations and there appeared to be no change in the sign of the slope of the curve. It is difficult to state whether the inhibition by APS is physiologically significant. The concentration of APS in the cell is likely to be much lower than the value of the K<sub>i</sub> for APS (0.04 mm). Robbins and Lipmann (45) have reported that the K<sub>i</sub> for APS-kinase for APS is less than 0.1 μm. This fact, together with the fact that the equilibrium of both the ATP-sulfurylase and APS-kinase reactions are in the direction of APS breakdown (10), would indicate that the steady state concentration of this metabolite is very low. Consequently, product inhibition by APS probably has no physiological significance, but this conclusion can only be confirmed by a knowledge of the concentrations in vivo of APS under various sulfur nutritional conditions.

The inhibition of enzyme activity by sulfide shows sigmoidal kinetics and the I<sub>50</sub> is strongly dependent on the concentration of substrate. This suggests that an interaction between the substrate and inhibitor plays a part in regulating the activity of the enzyme in vivo. Sulfide is the end product of the pathway of sulfate activation and reduction and as such would be a logical candidate for a feedback inhibitor of ATP-sulfurylase, the initial enzyme in this pathway. DeVito and Dreyfuss (3) have also noted that ATP-sulfurylase from yeast is inhibited by sulfide. These authors did not observe a sigmoidal dependence on substrate concentration; however, closer examination of Fig. 3 from their paper suggests that the double reciprocal plot of velocity and substrate concentration could be redrawn to give curves which are concave upward, similar to those shown in the inset to Fig. 9A.

The experiments described in this report suggest that the amount of ATP-sulfurylase present in the mycelia is controlled by the intracellular concentration of some sulfur metabolite related to methionine. When the organism is grown on sulfur sources which provide high levels of methionine, or a close metabolite of methionine, the synthesis of ATP-sulfurylase is repressed. Sulfur starvation of a culture previously grown on methionine results in a 6- to 7-fold increase in the level of ATP-sulfurylase activity. This type of control is similar to that previously shown for the sulfate transport system of *P. chrysogenum* (46, 47). Spencer and Hussey (48) have suggested that the rate of synthesis of PAPS in *Aspergillus nidulans* is inversely proportional to the intracellular concentration of cysteine. This was not supported by our results which showed that the level of ATP-sulfurylase and of the combined activating enzymes was increased during sulfur starvation when the cysteine concentration would be expected to fall. DeVito and Dreyfuss (3) found that ATP-
sulfurylase of yeast was repressed during growth on methionine. These authors found that the highest levels of ATP-sulfurylase were present in cells grown on cysteine, while growth on sulfate gave intermediate values. This result is similar to that obtained in the present work and suggests that similar mechanisms are involved in the control of ATP-sulfurylase synthesis in yeast and filamentous fungi.

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