ATP Sulfurylase from Higher Plants

PURIFICATION AND PRELIMINARY KINETICS STUDIES ON THE CABBAGE LEAF ENZYME

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ABSTRACT

ATP sulfurylase was purified extensively from green cabbage (Brassica capitata L.) leaf. The enzyme appears to be an asymmetric dimer composed of 57,000 dalton subunits. Initial velocity and product inhibition studies of the forward and reverse reactions point to an obligately ordered kinetic mechanism with MgATP adding before MoO₄²⁻ (or SO₄²⁻), and MgPPi leaving before AMP + MoO₄²⁻ (or adenosine-5'-phosphosulfate [APS]). The addition of excess purified fungal APS kinase to assay mixtures increased the rate of ³²SO₄²⁻ incorporation and MgPPi formation and extended the linearity of the forward reaction. This effect can be ascribed to the continual removal of APS, a potent product inhibitor of ATP sulfurylase. The specific activities of the enzyme in the APS synthesis, molybdenylation, MgATP synthesis, and sulfate-dependent [³²P]-MgPPi-MgATP exchange assays were 3.3, 38, 38, and 4.3 micromole product formed per minute per milligram protein, respectively.

ATP sulfurylase (ATP:sulfate adenylyl transferase, EC 2.7.7.4) catalyzes the first reaction in the assimilation of inorganic sulfate:

MgATP + SO₄²⁻ ⇌ MgPPi + APS

Although ATP sulfurylase is widely distributed in nature (for reviews, see 4, 13, 17, 19), only the yeast (5, 9, 15, 16), Penicillium chrysogenum (6-8, 26, 27; for a review, see 22), and spinach leaf (1, 23, 24) enzymes have been studied in any detail. Because of the extremely poor Kₘ of the reaction in the physiological direction (reported to be about 10⁻⁸) and, more importantly, the potent product inhibition by APS (Kᵢₐₐₜ approx. 1 µM), few attempts have been made to study the kinetics of the normal forward reaction. The inclusion of excess inorganic pyrophosphatase in the assay mixture will not pull the reaction to completion because the APS product combines with the free enzyme, not an E-Pi complex (7). For routine assays with relatively pure fractions, the forward reaction has been measured using molybdate (MoO₄²⁻) in place of sulfate (28). This has the advantage of providing a sustained reaction because the putative product, APMo, spontaneously and rapidly decomposes to AMP and MoO₄²⁻. However, there is no reason to assume that the kinetic constants of the molybdenylation reaction are the same as those of the normal forward reaction. The sulfate-dependent [³²P]-MgPPi-MgATP exchange reaction has also been used to assay the enzyme (23). However, the velocity equation of an A-P isotope exchange reaction (Ref. 20, p. 872) is rather complex and does not necessarily yield the kinetic constants of the overall normal forward reaction. In this paper, we describe the purification of ATP sulfurylase from green cabbage leaf and some of the physical and kinetic properties of the enzyme. The normal forward reaction was studied with MoO₄²⁻ as the Group VI anion substrate and also with SO₄²⁻ in the presence of excess APS kinase purified from Penicillium chrysogenum.

MATERIALS AND METHODS

Green cabbage (Brassica capitata L.) was purchased at a local market. Usually, three or four heads (about 3 kilos) were used for each preparation. The heads were washed with deionized H₂O, cooled in an ice-water bath, cut into small portions, and then passed through a household juicer. The juice collector initially held 50 ml of 1 M Tris-Cl buffer (pH 8.0, 0°C), containing 0.1 M EDTA. As the juicing process proceeded, small amounts of solid Tris (free base) were added to the collector to maintain the pH of the extract at 8.0. The collector was packed in ice to maintain the temperature at 0°C. Each cabbage head yielded between 300 and 500 ml of extract.

The crude extract was fractionated with solid ammonium sulfate at 4°C (21). Sufficient salt was added with gentle stirring over a 1-h period to bring the solution to 35% of saturation’ (0.194 g salt/ml original solution). The suspension was stirred for an additional 20 min, then centrifuged (5-10°C) at 16,000 g for 15 min. The pellet thus obtained (usually, dark green in color with a white top layer) was discarded. The supernatant solution was strained through cheesecloth and then brought to 65% of saturation’ with additional solid ammonium sulfate (0.184 g salt/ml solution). After centrifugation as described above, the supernatant solution was carefully removed and the pellet dissolved in a minimum volume of 0.04 M Tris-Cl buffer, (pH 8.0, 4°C) containing 10% (v/v) glycerol and 2mM β-mercaptoethanol. The solution (usually about 200 ml) was then dialyzed for several h at 4°C against 4 L of the above buffer. The buffer was changed three times, and, on the last change, the β-mercaptoethanol was omitted. All subsequent operations were carried out at 4°C.

The dialyzed ammonium sulfate fraction was applied to a 2.5 cm × 40 cm Blue Dextran-Sepharose CL-4B column (18) at a flow rate of 45 to 70 ml/h. The column had been equilibrated with 0.04 M Tris Cl buffer (pH 8.0), containing 10% (v/v) glycerol (hereafter referred to as standard buffer). When the last of the protein solution had been applied, the column was eluted using a linear gradient of NaCl (0–1.5 M) in standard buffer (total volume 800 ml). The total liquid head (including the column) was about 60 cm. Five-ml fractions were collected. The enzyme (detected by means of the molybdenylation reaction) eluted between fractions 75 and 95, with a peak in fraction 85. The pooled fractions were dialyzed against one change of standard buffer and then applied to a second Blue Dextran-Sepharose column (1.5 × 40 cm; 60 cm...
lipid head; flow rate, 25–50 ml/h). The column was eluted with 0–0.15 M NaCl gradient in standard buffer (400 ml total volume). Three-ml fractions were collected. Enzyme activity appeared in fractions 35 to 65 with the peak in fraction 50.

The pooled fraction from the second Blue Dextran column was dialyzed against two changes of standard buffer; 2 ml β-mercaptoethanol was included in the first dialysis buffer. The protein was then loaded onto a 1.5 × 40 cm DEAE-cellulose (Schleicher and Schuell) column equilibrated with standard buffer. Traces of blue dextran (leached from the previous column) adsorbed to the DEAE-cellulose column. The column was eluted with an 0 to 1.0 M NaCl gradient in standard buffer (400 ml total volume) at a 25 to 50 ml/h flow rate. Three-ml fractions were collected. Enzyme activity eluted in fractions 30 to 50 with the peak in fraction 40.

The pooled DEAE fraction was concentrated to a final volume of 1 to 2 ml by ultrafiltration through an Amicon PM-10 membrane, first in a 50-ml, and then in a 10-ml Amicon stirred cell. The concentrate was applied to a 1.5 × 95 cm Bio-Gel A-0.5 m gel filtration column and the column eluted with standard buffer at a flow rate of 6 to 10 ml/h. The total liquid head was maintained at about 80 cm. Small (approx. 1.5 ml) fractions were collected. Enzyme activity was found in fraction 35 to 60 with the peak in fraction 50. The pooled A-0.5 fraction was concentrated to a few ml, made up to contain 30% glycerol, divided into 0.2 ml portions, and stored at −70°C until needed.

The final fraction has a specific activity of about 30 μmol min⁻¹ mg⁻¹ protein in the standard molybdolyase assay (see below) One unit of activity (in all assays) is defined as that amount of enzyme which catalyzes the formation of 1 μmol of product in 1 min under standard assay conditions.

**Molybdolyase Assay.** The molybdate-dependent formation of Ppi was used to follow enzyme activity during purification. The reaction was started by adding 0.10 ml of appropriately diluted enzyme to 0.50 ml of standard reaction mixture. The latter contained 2 mM Na₂ATP, 7 mM MgCl₂, 5 mM Na₂MoO₄, and 0.33 units/ml of inorganic pyrophosphatase (Sigma; sulfate-free) in 0.08 M Tris-Cl buffer (pH 8.0). (Preliminary experiments established that the enzyme was stable and displayed maximal activity at pH 8.0.) After 10 min incubation at 30°C, the reaction was stopped by adding 1.0 ml of 0.5 M sodium acetate buffer (pH 4.0) and 0.20 ml of freshly prepared developer solution. The developer solution contained 100 mg Na₂MoO₄, 200 mg l-tartaric acid (sodium salt), and 20 μl of concentrated H₂SO₄ (added last) in 10 ml of deionized H₂O. After 10 min, the absorbance of the blue color was read at 660 nm against a Tris-Cl buffer blank. Under these conditions, a standard of 0.1 μmol Ppi or 0.2 μmol Pi gave an uncorrected A₆₆₀ of 0.57. Minus-enzyme or zero-time blanks generally had an A of 0.08 (or less) if new or acid-washed reaction tubes were used. The assay is linear with time and enzyme concentration up to an A₆₆₀ of about 0.6. Linearity can be extended by increasing the development time. While the molybdolyase reaction is convenient for scanning column fractions and for kinetics studies with the purified enzyme, it is not suitable for crude extracts and ammonium sulfate fractions. The major obstacles are ATPase activity and, to a greater extent, the turbidity that forms when the acidified developer solution is added. No ATPase (i.e. molybdate-independent activity) was observed in any of the column fractions that contained ATP sulfurylase activity.

**₃⁵SO₄²⁻ Incorporation ([³²P]APS Synthesis) Assay.** The reaction mixture (1.0 ml total volume) contained 0.29 to 2.0 mM MgATP, 5 mM excess (free) Mg⁶⁺ as MgCl₂, 0.20 to 2.0 mM [³⁵SO₄]₂⁻ (2.4 × 10⁻⁴ cm²·μmol⁻¹), or greater), 0.2 units of inorganic pyrophosphatase, 10⁻² units of purified fungal APS kinase, and 0.01 ml of appropriately diluted ATP sulfurylase preparation (approx. 30 μg) in 0.5 ml Tris-Cl buffer (pH 8.0). MgATP was generally prepared as a 50 mM Na₂ATP + 50 mM MgCl₂ stock solution adjusted to pH 8.0 with Tris (free base). The ATP sulfurylase was pre-diluted in the standard buffer. After 5 min incubation at 30°C, the reaction was terminated by adding 3 ml of ice-cold buffer containing 15 mg of acid-washed Norit charcoal (Pfanstiehl). The mixture was centrifuged in a table-top clinical centrifuge and the supernatant fluid carefully removed by suction through a 22-gauge hypodermic needle. The charcoal pellet was vigorously resuspended in 5 ml standard buffer containing 0.1 mM unlabeled Na₂SO₄ and recentrifuged. This washing procedure was repeated 8 to 12 times or until 0.5 ml of the supernatant fluid contained about 200 cpm. Finally, the charcoal pellet was vigorously resuspended in 1.0 ml 50% (v/v) ethanol containing 0.1% (w/v) Tween-80 and 0.4% (w/v) NH₄H₂O. The charcoal was centrifuged, resuspended in the supernatant solution, and centrifuged once more. Then, 0.5 ml of the eluted [³²P]nucleotide solution was carefully removed and counted in 3 ml of PCS scintillation fluid. The specific activity of the [³²SO₄]₂⁻ was determined by counting a suitable dilution of the stock in 0.5 ml of the above eluting solution.

Some batches of carrier-free [³²SO₄]₂⁻ (purchased as H₂³²SO₄ in HCl) contained a nonsulfate impurity that adsorbed to charcoal and produced very high blanks. This impurity (possibly [³²S]-colloidal sulfur or polyphionates) was removed by pretreating the carrier-free [³²SO₄]₂⁻ (diluted in standard buffer) with charcoal. The supernatant solution was passed through a membrane filter to remove all traces of the charcoal.

**APS Kinase Preparation.** The APS kinase used in the [³²SO₄]₂⁻ incorporation assay was purified from *Penicillium chrysogenum*. The purification steps included ammonium sulfate fractionation, affinity chromatography on Blue Dextran Sepharose and Affi-gel Blue agarose, followed by DEAE cellulose chromatography, then gel filtration through Bio-Gel A 1.5 m and A-0.5 m. The final preparation had a specific activity of 2 units mg⁻¹ protein at 5 mM MgATP, 5 mM excess Mg⁶⁺, and 3 μM APS as measured by the coupled assay of Burnell and Whately (2). The details of the purification and properties of this enzyme will be published elsewhere.

**³²P** Incorporation ([³²P]MgATP Synthesis) Assay. The reaction mixture (1.0 ml total volume) contained 0.5 to 5 μM APS, 2 to 8 μM total [³²P]Pi (10⁴ cpm μmol⁻¹), or greater) 10 mM total MgCl₂, and 0.05 ml of appropriately diluted enzyme (approx. 5 × 10⁻⁴ units of reverse reaction activity) all in 0.05 M Tris Cl buffer (pH 8.0). Under these conditions, the MgPi concentration was 0.156 × total [Pi] (29). ATP, when present as a product inhibitor, was added as MgATP (i.e. a 1:1 mixture of Na₂ATP and MgCl₂). After 3 min incubation at 30°C, the reaction was terminated by adding 3 ml ice-cold buffer (0.05 M imidazole [pH 7.0]) containing 0.01 M unlabeled Na₂P₂O₇ and 10 mg Norit charcoal. The Norit pellet was washed with the imidazole buffer as described for the [³²SO₄]₂⁻ incorporation assay. Finally, the [³²P]ATP was eluted in 1.0 ml of ethanol-Tween 80-NH₄Cl solution and 0.5 ml of the eluate counted in 3 ml of PCS scintillation fluid.

The concentration of APS in the stock solution was determined by (a) running the pyruvate kinase and lactate dehydrogenase coupled APS kinase reaction (2) to completion in the presence of excess MgATP, PEP, and NADH; and (b) running the reverse ATP sulfurylase reaction to completion in the presence of [³²P]MgPi of known specific radio-activity. The commercial APS (Sigma) was determined to be 90% pure based on A₂₅₀ of 15.400 M⁻¹ cm⁻¹.

**P-MgPi-MgATP Isotope Exchange Assay.** The reaction mixture (1.0 ml total volume) contained 0.65 to 5.2 mM MgATP and 0.39 to 3.12 μM [³²P]MgPi (varied together at about a 165:1 ratio) by pre-mixing MgATP and total Na₂P₂O₇ in a 25:1 ratio), 10 mM excess Mg⁶⁺ as MgCl₂, 0.2 to 10 mM Na₂SO₄, and 0.05 ml of appropriately diluted enzyme (approx. 0.2 μg of enzyme) all in 0.05 M Tris-Cl buffer, pH 8.0. The samples were processed as described for the [³²P]MgATP synthesis assay.
Fig. 1. Molybdoysis reaction; initial velocity in the absence of product. A, reciprocal of forward velocity versus 1/[MgATP] at a fixed 5 mM excess Mg²⁺ and the following fixed concentrations of MoO₄²⁻: (●), 5 mM; (△), 2.0 mM; (■), 1.0 mM; (○), 0.667 mM. B, slope and intercept replots. C, reciprocal of forward velocity versus 1/[MgO₄²⁻] at a fixed 5.0 mM excess Mg²⁺ and the following fixed concentrations of MgATP: (●), 2.0 mM; (△), 1.0 mM; (■), 0.53 mM; (□), 0.35 mM; (◆), 0.22 mM. D, slope and intercept replots. Each assay tube contained 0.2 µg purified enzyme (per 0.6 ml total volume).

Gel Filtration. Estimates of the mol wt and Stokes radius were obtained by gel filtration at 5°C through calibrated columns of Bio-Gel A-1.5 m (93 × 1.5 cm) and Sephadex G-200 (110 × 1.5 cm). All samples (0.5-ml volumes) were applied and run in 0.04 M Tris-Cl buffer (pH 8.0) containing 0.1 M KCl. The flow rates were approximately 10 ml h⁻¹. Two-ml fractions were collected. The apparent mol wt of the enzyme was obtained from a standard plot of log mol wt versus elution volume. The Stokes radius of the enzyme was obtained from a standard plot of (−log Kᵅ)¹/² versus Stokes radius (25).

Density Gradient Centrifugation. The S₂₀,W of the enzyme was obtained by the sedimentation velocity method in a preformed 10 to 30% (v/v) glycerol gradient in 0.04 M Tris-Cl buffer (pH 8.0, 4°C). The samples were run in 9.53 cm × 1.45 cm cellulose nitrate tubes containing 13 ml of gradient solution. A Beckman SW40 rotor operating at 25,000 rpm for 40 h was used. At the end of the run, 0.27-ml fractions were collected from each tube.

Protein Determination. Protein was usually determined by the method of Kalb and Bernlohr (10):

\[ \text{[Protein]µg/ml} = 183 A_{280\text{ nm}} - 7.5 A_{280\text{ nm}} \]

Gel Electrophoresis. Discontinuous polyacrylamide slab gel electrophoresis of the native enzyme was performed by the method of Davis (3) using a 3% stacking gel and 5.5% to 10.5% running gels. Gels were stained with a solution containing 12% (w/v) TCA, 12% (v/v) isopropanol, and 0.4% Coomassie Blue R. The gels were destained with successive changes of 10% (w/v) TCA. The enzyme was detected by cutting an unstained strip of the slab into 3 mm segments and soaking the pieces in 1.0 ml of 0.04 M Tris-Cl buffer (pH 8.0). The eluates were assayed by the molybdoysis reaction. Enzyme activity was located at a position corresponding to the most intensely stained protein band.

Discontinuous SDS-PAGE was carried out by the method of Laemmli (11) using a 3% stacking gel (pH 6.8) and a 9% running gel (pH 8.8). Between 1 and 10 µg of protein was applied. Bio-Rad SDS-PAGE standards (mol wt, 14,700–200,000) were run simultaneously. The gels were stained and destained as described above.

RESULTS AND CONCLUSIONS

Purification and Some Physical Properties of the Enzyme. Table I summarizes the purification of the cabbage leaf enzyme. The crude extract and 35 to 65% ammonium sulfate fraction were not assayed. However, negligible enzyme activity was recovered when the 0 to 35% and 65 to 100% ammonium sulfate fractions were applied to and eluted from the Blue Dextran column.

On a Bio-Gel A-1.5m column calibrated according to mol wt, the cabbage ATP sulfurylase eluted at a position corresponding to a mol wt of 172,000 ± 8,000. On a calibrated Sephadex G-200 column, the enzyme eluted at a position corresponding to a mol wt of 145,000 ± 15,000. The standard proteins used were thyroglobulin (660,000), β-galactosidase (595,000 and 1,190,000), ferritin (495,000), catalase (237,000), aldolase (158,000), BSA (67,000 and 134,000), hemoglobin (64,000), and chymotrypsinogen A (25,000).

When the A-1.5 and G-200 columns were calibrated according to the Stokes radii of the standards, ATP sulfurylase eluted at a position corresponding to a Stokes radius of 4.6 ± 0.1 nm. The standards were thyroglobulin (8.5 nm), ferritin (6.9 nm), catalase (5.2 nm), aldolase (4.8 nm), BSA (3.6 nm), hemoglobin (2.4 nm), and chymotrypsinogen A (2.1 nm).

Density gradient centrifugation in a 10 to 30% (v/v) glycerol gradient yielded an S₂₀,W of 5.5 ± 0.1 relative to standards of β-galactosidase (15.93 S), catalase (11.30 S), and aldolase (7.35 S). Combining the Stokes radius and S₂₀,W, and assuming a partial
specific volume \((\bar{v})\) of 0.725 cm\(^3\) g\(^{-1}\), a mol wt of 108,000 ± 4,000 was calculated (25).

PAGE of the native enzyme yielded a major dye-staining band and four relatively minor bands, two with a slightly greater mobility and two with a slightly lower mobility than the major band. The major band (which corresponded to the peak of enzyme activity eluted from the gel) was estimated to account for more than 75% of the total dye-staining protein in the applied sample.

SDS gel electrophoresis of the purified enzyme yielded two very closely-migrating bands. The more intensely-stained band migrated with a mobility corresponding to a mol wt of 57,000. The second band (perhaps a partially proteolyzed monomer) migrated with a mobility corresponding to a mol wt of 56,000. The cumulative results suggest that the native enzyme is a non-spherical dimer.

**Kinetics of the Molybdolysis Reaction.** Figure 1 shows the initial velocity reciprocal plots for the molybdolysis reaction. The
intersecting pattern indicates 'sequential' (i.e. ordered or random) mechanism. APS was competitive with MgATP and a mixed type inhibitor with respect to MoO$_4^{2-}$ (data not shown). The pattern is consistent with either an ordered or random mechanism.

Because of the nature of the molybodysis assay, the kinetics of the PPI product inhibition could not be conveniently investigated. However, a preliminary experiment suggested that MgPPI does not significantly inhibit the reaction: ATP sulfurylase was incubated with all the assay components except pyrophosphatase. After 10 min, the ATP sulfurylase was inactivated by heating the assay tubes for 1 min in a boiling water bath. The solution was cooled to 30°C, pyrophosphatase was added, and the mixture incubated for another 10 min. The color was developed in the usual way. The two-stage assay yielded the same A$_{600}$ as the usual one-stage assay. The results showed that up to 200 µM PPI could accumulate during the assay without significantly affecting the initial molybodysis reaction velocity.

In another experiment, 0.1 mM (0.05 µmol/0.5 ml) PPI was added to the standard reaction mixture at zero-time. The reaction mixture was run in two stages as described above with sufficient enzyme to produce 0.1 mM PPI in 10 min. The total PPI found was, within experimental error, 0.2 mM. These results show that MgPPI at concentrations in the neighborhood of 0.1 mM (approx. 100 times the $K_m$ for MgPPI in the normal reverse reaction) does not significantly inhibit the molybodysis reaction. From this, we can conclude that MgPPI does not bind to the free enzyme, which, in turn, means that product release must be obligately ordered with MgPPI leaving first. The results also suggest that E-APMo complex does not account for a significant fraction of the total enzyme in the steady state.

Chlorate and nitrate are dead end inhibitors of ATP sulfurylase (7,24). These ions may resemble the transition state of the sulfuryl group. As shown in Figure 2, ClO$_3^-$ is competitive with MoO$_4^{2-}$ and uncompetitive with respect to MgATP. The results are consistent with an ordered kinetic mechanism in which MgATP adds before MoO$_4^{2-}$ (Ref. 20, pp 777–779).

**Kinetics of the Normal Forward (APS Synthesis) Reaction.** In the presence of excess pyrophosphatase and the absence of APS kinase, ATP sulfurylase catalyzes the formation of charcoal-adsonorbeable [35S]APS (Fig. 3). Under the specific assay conditions (1.5 mM MgATP, 5 mM free Mg$^{2+}$, and 2 mM $^{35}$SO$_4^{2-}$), the reaction appears to be linear with time up until 0.3 to 0.4 µM [35S]APS accumulates. Since substrate depletion is negligible, the sloping off of the reaction progress curve must result from product inhibition by APS. Indeed, at lower MgATP concentrations, the reaction progress curve departs from linearity at lower levels of accumulated APS. The linearity of the reaction during the first 3 or 4 min is probably illusory because if excess APS kinase is included in the assay, the initial rate increases by 60% (and the reaction remains linear for a much longer time). It is highly

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4 Renosto and Segel (14) and Farley et al. (7) have pointed out that a dead end inhibitor can be uncompetitive with respect to substrate A in a rapid equilibrium random system if the inhibitor competes with substrate B for the EA complex, but does not combine with the free enzyme. This limiting case cannot be excluded for the cabbage leaf ATP sulfurylase. However, direct equilibrium binding experiments with the *P. chrysogenum* enzyme (8) have shown that $^{35}$SO$_4^{2-}$ at concentrations up to 10 $K_m$ does not bind to the free enzyme, a result consistent with the ordered mechanism deduced from kinetics studies.

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**Fig. 4.** APS synthesis reaction: initial velocity in the absence of products. A, reciprocal of forward velocity versus 1/[MgATP] at 5.0 mM excess Mg$^{2+}$ and the following concentrations of $^{35}$SO$_4^{2-}$: (●), 2.0 mM; (△), 1.0 mM; (■), 0.50 mM; (□), 0.33 mM; (●), 0.25 mM; (○), 0.20 mM. B, slope and intercept replots. C, reciprocal of forward velocity versus 1/[MgATP] at 5.0 mM excess Mg$^{2+}$ and the following concentrations of MgATP: (●), 2.0 mM; (△), 0.68 mM; ( ■ ), 0.40 mM; (□), 0.29 mM. D, slope and intercept replots. Each assay tube contained 0.1 µg purified enzyme (per 1.0 ml total volume).
unlikely that the stimulation of ATP sulfurylase activity by APS kinase results from a direct interaction of the two enzymes to form a 'PAPS synthetase' complex ('substrate channeling') because the two enzymes are derived from completely unrelated organisms. The stimulation most likely results from the continual removal of the inhibitory product, APS. The kinetics studies on the normal forward ATP sulfurylase-catalyzed reaction (described below) were performed with an excess of fungal APS kinase in the assay mixtures.

Figure 4 shows the initial velocity reciprocal plots for the APS synthesis reaction measured by the \(^{32}\)P incorporation assay. Essentially the same results are obtained using the Pi colorimetric assay with sulfate and excess APS kinase replacing molybdate in the incubation mixture. The patterns and the \(K_m\), \(K_a\), and \(K_m\) values (Table II) are very similar to those of the molybdoysis reaction, although the \(V_{max}\) of APS synthesis (3.3 units mg\(^{-1}\) protein) is considerably less than the \(V_{max}\) of molybdoysis (38 units mg\(^{-1}\) protein).

<table>
<thead>
<tr>
<th>Kinetic mechanism</th>
<th>(K_m) (MgATP with SO(_4^{2-}) as substrate)</th>
<th>(K_m) (MgATP with MoO(_4^{2-}) as substrate)</th>
<th>(K_m) (MgATP with APS)</th>
<th>(K_m) (for APS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH optimum</td>
<td>0.31 mM</td>
<td>0.33 mM</td>
<td>&lt;1 (\mu)M</td>
<td></td>
</tr>
<tr>
<td>(V_{max}/V_{max}) (with natural substrates)</td>
<td>0.75-1.3 mM</td>
<td>0.3 mM</td>
<td>0.99(^b)</td>
<td>0.99(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Based on one active site per subunit of mol wt 57,000.  
\(^b\) Forward reaction rates were measured in the presence of excess APS kinase from \(P.\) chrysogenum.

\(^c\) Corresponds to 4.6 \(\mu\)M total PPI at 10 mM free Mg\(^{2+}\).

activity of that enzyme was not reported.

**[32P]MgPPi-MgATP Exchange Reaction.** The cabbage leaf enzyme catalyzes the sulfate-dependent isotope exchange between [\(^{32}\)P]MgPPi and MgATP with a \(V_{max}\) of 4.3 units mg\(^{-1}\) protein. Substrate inhibition by sulfate (substrate 'B'), common for A-P isotope exchange reactions in ordered mechanisms (Ref. 20, p 872), was not observed. In the absence of sulfate, the exchange rate at saturating [\(^{32}\)P]MgPPi and MgATP was only about 0.02 units mg\(^{-1}\) protein. The absolute value of the exchange \(V_{max}\) is similar to that reported by Shaw and Anderson (23) for the spinach leaf enzyme (3 to 4 units mg\(^{-1}\) protein). However, the ratio, \(V_{max}(\text{exchange})/V_{max}(\text{reverse})\), is 0.11 for our cabbage leaf preparation compared to 0.01 reported for the spinach enzyme. The difference stems from the 10-fold higher \(V_{max}\) (reverse) of the latter.

Table II summarized some of the physical and kinetic properties of the cabbage leaf enzyme.

### DISCUSSION

The most striking feature of ATP sulfurylase is its extremely high affinities for the products of the normal reaction, APS and MgPPi. The \(K_m\) values and \(K_a\) values of the products are in the micromolar range, while the \(K_m\) values and \(K_a\) values of the substrates, MgATP and SO\(_4^{2-}\), are in the mM range. The remarkably low \(K_{product}/K_{substrate}\) ratio very likely reflects evolutionary pressure to maximize \(V_{max}\) for the forward reaction in the face of an extremely unfavorable \(K_{eq}\). This can be best appreciated by referring to the Haldane relationship between \(K_{eq}\) and the kinetic constants. For an ordered Bi Bi reaction, this relationship is (Ref. 20, p 574):

\[ K_{eq} = \frac{V_{max}K_{eq}K_{m}}{V_{max}K_{m}K_{a}} \]
The $K_a$ of the APS synthesis reaction is reported to be about $10^{-8}$ (16, 28). This value is probably incorrect (too low), but it can be used to illustrate the point. We see that if $K_a = 10^{-8}$ and the products had $K_a$ values equal to (or greater than) the $K_a$ values of the substrates (a usual expectation), the forward $V_{\text{max}}$ would be fixed at no greater than one hundred millionth of the reverse $V_{\text{max}}$. But because $K_a$ and $K_{\text{eq}}$ are each three orders of magnitude lower than $K_a$ and $K_{\text{eq}}$, $V_{\text{max}}$ can be $1\%$ of $V_{\text{max}}$. Experimentally, we find a $V_{\text{max}}/V_{\text{max}}$ ratio close to 0.1 (Table II). Combining this ratio with the other relevant experimental constants, the calculated value of $K_{\text{eq}}$ is close to $10^{-8}$.

The only other ATP sulfurylase that has been characterized extensively is the enzyme from Penicillium chrysogenum (6–8, 22, 26, 27). The cabbage leaf enzyme differs from the fungal enzyme in several respects. First of all, the plant enzyme has a native mol wt of at least 104,000, and at most, 180,000 (the experimental value depends on the method of determination), while the native fungal enzyme has a mol wt of at least 400,000 (26) and at most, 545,000 (I. H. Segel et al., unpublished results). SDS-gel electrophoresis yields a subunit mol wt of 57,000 for the plant enzyme and 68,000 for the fungal enzyme (I. H. Segel et al., unpublished results). Thus, the plant enzyme appears to be an asymmetric dimer, while the fungal enzyme is a relatively spherical hexamer or octomer. Rabbit serum antibodies raised against purified P. chrysogenum ATP sulfurylase did not cross-react with the purified cabbage leaf enzyme. (Crossreaction was observed against the enzymes purified from P. duponti and Aspergillus nidulans, two other fungi.) The plant and fungal enzymes also display different $V_{\text{max}}$ ratios for the various activities that can be measured. For example, in the presence of fungal APS kinase, the fungal ATP sulfurylase has a specific APS synthesis activity of 8 units mg\(^{-1}\) protein, which corresponds to an active site turnover number of 554 min\(^{-1}\) (I. H. Segel et al., unpublished results). The specific APS synthesis activity of the plant enzyme in the presence of APS kinase is 3.3 units mg\(^{-1}\) protein, corresponding to an active site turnover number of only 188 min\(^{-1}\). Thus, the $\frac{[32P]MgPP\text{I}}{MgATP}$ exchange activity of the fungal enzyme is also higher than that of the plant enzyme—816 min\(^{-1}\) (8) compared to 245 min\(^{-1}\). However, the molybdoysis and MgATP synthesis activity of the plant enzyme (2,162 min\(^{-1}\)) is higher than that of the fungal enzyme (approx. 1,000 min\(^{-1}\)) (8). It is too early to comment on the physiological or physical significance of these differences. But it is worth noting that fungal APS kinase was used as the coupling enzyme in the APS synthesis assays. It would be of interest to compare the effects of fungal and cabbage leaf APS kinase on the APS synthesis activities of the fungal and plant sulfurylases.

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