Adenosine 5'-phosphosulfate (APS) kinase, the second enzyme in the pathway of inorganic sulfate assimilation, was purified to near homogeneity from mycelium of the filamentous fungus, *Penicillium chrysogenum*. The enzyme has a native molecular weight of 59,000–60,000 and is composed of two 30,000-dalton subunits. At 30 °C, pH 8.0 (0.1 M Tris-chloride buffer), 5.5 μM APS, 5 mM MgATP, 5 mM excess MgCl₂, and "high" salt (70–150 mM (NH₄)₂SO₄), the most highly purified preparation has a specific activity of 24.7 units × mg of protein⁻¹ in the physiological direction of adenosine 3'-phosphate 5'-phosphosulfate (PAPS) formation. This activity is nearly 100-fold higher than that of any previously purified preparation of APS kinase.

APS kinase is subject to potent substrate inhibition by APS. In the absence of added salt, the initial velocity at 5 mM MgATP plus 5 mM Mg²⁺ is maximal at about 1 μM APS and half-maximal at 0.2 and 4.4 μM APS. In the presence of 200 mM NaCl or 70–150 mM (NH₄)₂SO₄, the optimum APS concentration shifts to 4–6 μM APS; the half-maximal values shift to 1–1.3 and 21–27 μM APS.

The steady state kinetics of the reaction were investigated using a continuous spectrophotometric assay. The families of reciprocal plots in the range 0.25–5 mM MgATP and 0.8–5.1 μM APS are linear and intersect on the horizontal axis. Appropriate replots yield $K_{m_{APS}} = 1.5 \text{ mM}$, $K_{m_{ATP}} = 1.4 \text{ μM}$, and $V_{max} = 38.7 \text{ units} \times \text{ mg of protein}^{-1}$. Excess APS is an uncompetitive inhibitor with respect to MgATP ($K_{i_{APS}} = 23 \text{ μM}$). PAPS, the product of the forward reaction, is also uncompetitive with MgATP. PAPS is not competitive with APS.

In the reverse direction, the plots have the characteristics of a rapid equilibrium ordered sequence with MgADP adding before PAPS. The kinetic constants are $K_{m_{PAPS}} = 8 \text{ μM}$, $K_{m_{ADP}} = 560 \text{ μM}$, and $V_{max} = 0.16 \text{ units} \times \text{ mg of protein}^{-1}$. Iso-PAPS (the 2'-phosphate isomer of PAPS) is competitive with PAPS and uncompetitive with respect to MgADP ($K_i = 6 \text{ μM}$).

APS kinase is inactivated by phenylglyoxal, suggesting the involvement of an essential argininyl residue. MgATP or MgADP at 10 K, protect against inactivation. APS or PAPS at 600 and 80 K, respectively, are ineffective alone, but provide nearly complete protection in the presence of 0.1 K, of MgADP or MgATP.

The cumulative results suggest that the kinetic mechanism is ordered in both directions with MgATP adding before APS, and PAPS leaving before MgADP. The kinetics of substrate inhibition by APS are quantitatively consistent with APS (B) binding to the enzyme-MgADP (EQ) complex forming a dead-end EBQ complex. The effectiveness of PAPS plus a suboptimal level of MgATP in protecting against phenylglyoxal-promoted inactivation suggests that an E-MgATP-PAPS can also form.

APS kinase rapidly loses activity at 50 °C. However, a large fraction of the lost activity reappears upon incubating the heated enzyme at 0 °C. MgATP, MgADP, or the free nucleotides at 10 K, accelerate the recovery process, but Mg²⁺, APS, or PAPS ( singly, or in combination) have no effect on the initial loss or subsequent recovery of activity.

Indirect evidence for the action of APS kinase on APSe (the selenium analog of APS) is presented. The rate of PAPSe formation from SeO₂⁻ and MgATP by the combined action of APS sulfurylase and APS kinase is 24% of the rate of PAPS formation at the same substrate concentrations.

The sulfate-activating enzymes, ATP sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4) and APS kinase (ATP:adenylylsulfate-3'-phosphotransferase, EC 2.7.1.25), catalyze the first two steps in the incorporation of inorganic sulfate into biological molecules. The reactions involved in sulfate activation are shown below.

\[
\begin{align*}
\text{SO}_4^{2-} + \text{ATP} & \rightarrow \text{ATP sulfurylase} \rightarrow \text{APS + PP}, \\
\text{APS + ATP} & \rightarrow \text{APS kinase} \rightarrow \text{PAPS + ADP}, \\
\text{PP} + \text{H}_2\text{O} & \rightarrow \text{Pyrophosphatase} \rightarrow 2\text{P}, \\
\text{Sum: } \text{SO}_4^{2-} + 2\text{ATP} & \rightarrow \text{PAPS + ADP + 2P},
\end{align*}
\]

The equilibrium of the ATP sulfurylase reaction lies far to the left (1, 2). Nevertheless, the overall production of PAPS in vivo is promoted by the hydrolysis of inorganic pyrophosphate and the favorable APS kinase reaction.

The sulfate-activating enzymes were discovered in the mid-1950s and have since been shown to occur in most organisms (see Refs. 3–10 for reviews). In spite of the wide distribution of the two enzymes and their obvious biological importance, the abbreviations used are: APS, adenosine 5'-phosphosulfate (5'-adenylylsulfate); PAPS, adenosine 3'-phosphate 5'-phosphosulfate (3'-phospho-5'-adenylylsulfate or 3'-phosphoadenosine-5'-phosphosulfate); APSe, PAPSe, selenium analogs of APS and PAPS; APMo, molybdenum analog of APS.

APS kinase appears to be absent in the anaerobic sulfite-reducing bacteria and in the aerobic sulfide-, sulfite-, and thiosulfate-oxidizing bacteria.

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† Recipient of a Jastro-Shields Research Scholarship.

‡ To whom reprint requests should be sent.

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only ATP sulfurylase has been studied in significant detail (see Ref. 11 for a summary table). Reports on APS kinase published during the past 25 years have, for the most part, been concerned only with demonstrating the presence of the enzyme in a particular organism or tissue. Consequently, very little is known about the physiological, kinetic, or regulatory properties of the second sulfate-activating enzyme. The major obstacles to quantitative studies of APS kinase have been (a) the marked substrate inhibition exhibited by APS at micromolar concentrations and (b) the lack of a satisfactory assay for the enzyme at subinhibitory APS levels. Attempts to assay APS kinase in impure preparations at subinhibitory levels of APS are frustrated by competing reactions which rapidly remove the substrate or the S-labeled product. APS and PAPS hydrolyses and ATP sulfurylase are the major culprits. ATP sulfurylase will nearly quantitatively convert micromolar APS to ATP and sulfate in the presence of equimolar inorganic pyrophosphate (PPi). As little as 10 μM APS will suffice to reduce 100 μM ATP if both inorganic pyrophosphatase and ATP sulfurylase are present. (The equilibrium constant of the ATP sulfurylase reaction may be as high as 10⁶ in the ATP synthesis direction.)

We have succeeded in purifying APS kinase using a coupled spectrophotometric assay (12) to follow enzyme activity in the final fractionation steps. While the assay is not adequate for characterization studies with crude preparations, it yields clear, reproducible results with the purified enzyme. In this paper, we describe the purification of APS kinase from mycelium of Penicillium chrysogenum and some of the physical and kinetic properties of the enzyme.

MATERIALS AND METHODS

Chemicals and Supplies—APS, PAPS, iso-PAPS, ATP, phospho-
pyruvate (PPP), NADH, pyruvate kinase, lactate dehydro-
genase, nuclease P1, hexokinase, glucose-6-phosphate dehydro-
genase, 70-150 mM ammonium sulfate (provided by the Sigma lactate
enrichment). The solution (16) containing 50 mM Tris(11) and 50 mM MgCl₂, in 0.15
mM sodium pyrophosphate, 2.5 units of glucose-6-phosphate dehydro-
genase, 2.5 units of pyruvate kinase, 2.5 units of pyruvate dehydrogenase/poly
kinase mixture, 25 μg of nuclease P1, and 5 or 10 μl of the APS kinase solution (about 0.2 μg
of the purified enzyme for kinetics studies), all in 0.1
mM Tris-Cl buffer, pH 8, 0.30°C (standard buffer). The reaction was started by adding the APS
after 5 min of equilibration of other components to remove traces of ADP
in the ATP. The decrease in NADH absorbance at 340 nm was followed on a Gilford 250 recording spectrophotometer with the chart
recorder calibrated to give a full scale deflection of 0.02 absorbance units. Absorbance changes were followed by means of the recorder tracing and subtracting the background rate (recorder drift and non-APS kinase-dependent NADH oxidation). At 0.02 absorbance
units, full scale = 100 divisions and the background rate with the purified enzyme was generally four divisions × min⁻¹, or less. The lowest APS kinase-dependent rate used in our kinetic analyses (obtained at the lowest APS and MgATP concentrations used—0.8 μM
and 0.25 mM, respectively) was 10 divisions × min⁻¹ above background. Periodically, the reaction was allowed to go to completion and the overall A₅₄₀ used to verify the initial APS concentration. With crude cell-free extracts and ammonium sulfate fractions, the background rate completely obscured the APS kinase-dependent rate. The inclusion of fluoride and cyanide, as described in the original
recipe for this assay (12), did not help significantly.

The reaction mixture for the reverse reaction contained (in 1-ml total volume) 5 mM MgATP and 5 mM MgCl₂, 0.3 mM NADH, 2.5 units of glucose-6-phosphate dehydro-

4 Nucleoside P1 is much less expensive than commercial rye grass
3'-nucleotidase and works well on PAPS. Alkaline phosphatase
possesses APTPase activity and, thus, is unsuitable for the assay.
4 Contrary to an earlier report (14), pure ATP sulfurylase does not
catalyze APS hydrolysis at a significant rate. This was established by
preincubating the enzyme (0.17 μg/ml) 1.5 h before reaction with
the reaction mixture, containing 0.5 M NaCl, pH 8. The reaction was then stirred for 15 min and 1 μM APS and 6 mM MgCl₂ for 10 min and then measuring the residual APS by adding 10 μM PP, and the other components of the reverse reaction assay.
ADP, PAPS, and Mg\(^{2+}\), all in 0.1 M Tris-Cl buffer, pH 8.0, containing 70 mM (NH\(_4\))\(_2\)SO\(_4\). The reaction was started by adding APS kinase after a brief preincubation period to remove traces of APS in the PAPS. The increase in the absorbance at 340 nm was monitored with the chart recorder set for a full scale deflection of 0.02 absorbance units. In this assay, 2 mol of NADPH are produced for each mole of PAPS. The increase in the absorbance at 340 nm was monitored with 200 \(m\) units.

**TABLE I**

| Fraction          | Total protein mg | Specific activity* units/mg protein | Purification factor | Yield% |
|-------------------|------------------|-------------------------------------|---------------------|--------|
| Crude extract     | \(3.4 \times 10^3\) | ND* \((0.033)^b\)                   | \(1.0\)             | \(100\) |
| Ammonium sulfate, 30-55% saturation | \(5.8 \times 10^3\) | ND* \((0.086)^b\)                   | \(2.9\)             | \(50\)  |
| Affi-Gel blue eluate | 48               | 5.8                                | \(1.0\) \((176)\)   | \(25\)  |
| Matrix-Gel green  | 18.6             | 11.8                               | \(2.0\) \((360)\)   | \(19.6\) |
| A eluate          | 9.2              | 20.7                               | \(3.6\) \((630)\)   | \(17\)  |

1. **Specific activity at 5 mM MgATP, 5.5 \(\mu\)M APS, and 5 mM excess Mg\(^{2+}\) at pH 8.0 and 30 °C in the presence of 70-150 mM (NH\(_4\))\(_2\)SO\(_4\).**
2. **Enzyme activity could not be measured in the crude extract or the 30-55% ammonium sulfate fraction.**
3. **The specific activity of this fraction varied somewhat from preparation to preparation, apparently depending on the sharpness of the cut taken from the A-1.5 column.**
4. **The specific activity of this fraction varied somewhat from preparation to preparation, apparently depending on the sharpness of the cut taken from the A-1.5 column.**
5. **Thus, the enzyme appears to be a dimer composed of two 30,000-dalton subunits.**
6. **Effect of pH and Ionic Strength**—Preliminary experiments showed that APS kinase activity was maximal at pH 8.0 with 90% of maximal activity retained at pH 7.5 and 8.5. All subsequent experiments were performed at pH 8.0 (0.1 M Tris-Cl buffer).

The commercial pyruvate kinase and lactate dehydrogenase which were used as coupling enzymes contain a high level of ammonium sulfate. At one point, we had occasion to use salt-free coupling enzymes (obtained commercially from the same sources, or prepared by extensive dialysis or gel filtration of the sulfate-containing enzymes). With the salt-free coupling enzymes, the APS kinase activity under standard assay conditions (5 \(\mu\)M APS, 5 mM MgATP, 5 mM Mg\(^{2+}\)) was markedly depressed. A further investigation of the salt effect yielded the \(v\) versus [APS] profiles shown in Fig. 1. In the presence of added salt (or using the sulfate-containing coupling enzymes), the maximal velocity occurs between 4 and 6 \(\mu\)M APS.

Thus, the enzyme appears to be a dimer composed of two 30,000-dalton subunits.

**RESULTS AND CONCLUSIONS**

**Purification of the Enzyme—**Preliminary experiments showed that APS kinase passes through blue dextran but is retained on Affi-Gel blue. On the other hand, ATP sulfurylase is retained on blue dextran. Thus, the two blue dye columns arranged in series separate the two sulfate-activating enzymes early in the purification process and permit both to be purified in parallel. The blue dextran column very likely also removes other nucleotide-binding enzymes from the APS kinase preparation. Although we have not experimented with variations in the purification scheme, it seems likely that the blue dextran column could be omitted and the ammonium sulfate fraction (or perhaps, the dialyzed crude extract) loaded directly onto the Affi-Gel blue column. Any ATP sulfurylase present in the dye column eluates would be removed in the final gel filtration step. Another potential variation would be the inclusion of a heat step early in the purification process and permit both to be purified.

**Native and Subunit Molecular Weight—**The enzyme eluted from a calibrated Sephadex G-200 gel filtration column at a position corresponding to a Stokes radius of 3.3 nm (data not shown). Density gradient centrifugation in a 10 to 30% (v/v) glycerol gradient in 40 mM Tris-Cl buffer, pH 8, at 4 °C. The samples were run in 9.53 × 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 and assayed for the standards and APS kinase activity.

**Protein Determination—**Protein concentrations were determined from the difference in absorbance at 280 and 260 nm (p. 334 of Ref. 17). [protein] \(\text{mg} = 1.55 \times \text{Abs}_{280} \text{mg} = 0.76 \times \text{Abs}_{260} \text{mg} \)

**Enzyme Units—**One unit of APS kinase is the amount of enzyme which catalyzes the formation of 1 umol of primary product in 1 min at pH 8 (0.1 M Tris-Cl buffer) and 30 °C. The "units" for the coupling enzymes are as defined by the supplier (phosphatase activity for nuclease P1).

**Density Gradient Centrifugation—**The \(s_{20,w}\) of the enzyme was obtained by sedimentation in a preformed 10 to 30% (v/v) glycerol at pH 8.0 and 30 °C. The samples were run in 9.53 × 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 and assayed for the standards and APS kinase activity.

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produced the widest spread between the [APS] values and yielded the highest maximal velocity. All subsequent kinetics experiments on the forward reaction were performed using the ammonium sulfate-containing coupling enzymes which we estimate provided about 70–150 mM (NH₄)₂SO₄. The advantages of the higher ionic strength are that (a) MgATP can be varied in the region of 0.25–5 mM (0.25 to 5.0 K⁺) along with a 5 mM excess of Mg⁺ without introducing secondary ionic effects and (b) APS concentrations can be varied over a reasonably broad range before substrate inhibition complicates the diagnostic initial velocity plots. It could also be argued that the higher ionic strength is closer to the in vivo situation.

Metal Ion Specificity— Manganese could replace Mg²⁺ (pyruvate kinase will accept Mn⁺⁺). Under standard assay conditions (5 mM ATP, 5 µM APS), 5 mM total Mn⁺⁺ yielded the same reaction velocity as 5 mM total Mg²⁺. But as shown in Fig. 2, excess (free) Mn⁺⁺ inhibited slightly. The inhibition may be an artifact because unchelated Mn⁺⁺ at pH 8 slowly generates a turbidity (probably Mn(OH)₂), increasing the A₃₄₀ in opposition to the APS kinase-dependent decrease in A₃₄₀. The greater stimulation afforded by Mn⁺⁺ over Mg⁺⁺ at total cation concentrations below [ATP], very likely reflects the greater affinity of ATP for Mn⁺⁺ (19). Co⁺⁺ at 5 mM total concentration was 58% as effective as Mg⁺⁺ or Mn⁺⁺.

Other experiments showed that free ATP (i.e. ATP in excess of total Mg⁺⁺) was inhibitory. This inhibition is responsible for the slight sigmoidicity of the μ versus [Mg⁺⁺] plot (Fig. 2). Increasing the Mg⁺⁺ concentration simultaneously deinhibits the enzyme (by reducing the concentration of free ATP) and increases the concentration of the true substrate (MgATP).

Initial Velocity Studies of the Forward Reaction—Fig. 3 shows an initial velocity reciprocal plot for the APS kinase reaction in a noninhibitory range of APS concentrations. The family of 1/μ versus 1/[MgATP] plots also intersected on the horizontal axis (data not shown). The patterns indicate either an ordered or a random mechanism (20). Replots of the 1/μ axis intercepts yield a theoretical Vₘₐₓₙ at 30 units × mg of protein⁻¹ for the preparation used (a value which can never be attained experimentally because of the substrate inhibition by APS). The primary plots and replots also yield Kₘₐₜₐₚₜ = 1.5 mM and Kₐₜₚ = 1.4 µM.

Substrate Inhibition Kinetics—APS at concentrations of >5 µM is a competitive inhibitor with respect to MgATP (Fig. 4). Thus, substrate inhibition models in which APS binds to free E or to E–APS in direct or indirect competition with MgATP can be excluded (p. 819 of Ref. 20). These include a pseudo-competitive random model where both nucleotide subunits are accessible on free E, but the binding of APS first to its own subsite blocks the entry of MgATP. The linear competitive pattern also eliminates a steady state random sequence in which the route E → E–APS → E–APS·MgATP is much slower than the route E → E–MgATP → E–APS·MgATP. In this model, saturating MgATP would yield the same Vₘₐₓₙ at all inhibitory levels of APS. Also, the μ versus [MgATP] curve at ~20 µM APS would be sigmoidal. No sigmoidicity was observed.

The most likely explanation is that APS binds to E–MgADP forming an "abortive" E–APS·MgADP complex. Of such models, the simplest would involve an obligately ordered steady state sequence in which MgATP (A) adds before APS (B), and PAPS (P) dissociates before MgADP (Q), as shown in Scheme 1. Although a random sequence with MgADP
Equation agreement with the value of \([\text{APS}]_{\text{eq}}\), was 5.5

dissociation constant of APS from \(k_2\), release being partially rate-limiting cannot be eliminated on the basis of the initial velocity and substrate-inhibition pattern.

Evidence described later suggests that an also forms in the presence of added PAPS and MgATP.

uncompetitive substrate inhibition by APS with respect to MgATP.

yielding the maximal velocity at a given \([\text{MgATP}]\); and

At 5 mM MgATP

FIG. 3 (left). Initial velocity at subinhibitory APS concentrations. \(1/v\) versus 1/[APS] at different fixed concentrations of MgATP. INT, \(1/v\) axis intercept. The preparation used for this and the following kinetics experiments had a standard assay specific activity of 20.7 units \(\times\) mg of protein\(^{-1}\).

FIG. 4 (right). Substrate inhibition. \(1/v\) versus 1/[MgATP] at different fixed inhibitory concentrations of APS. INT, \(1/v\) axis intercept.

As described below, \(V_{\text{max,}}/V_{\text{max}}\) is about 200. But, because \(K_m/K_a\) is much less than \(K_B\) (see later).

Multiple Inhibition Analysis—Plots of \(1/v\) versus [PAPS] at several different fixed inhibitory concentrations of APS were parallel (data not shown), indicating that PAPS and APS cannot occupy the enzyme simultaneously ( Chap. 8 of Ref. 20). The result is consistent with the suggestion that APS inhibits by promoting the formation of a dead-end \(E\cdot\text{APS}\cdot\text{MgADP}\) complex, i.e. that APS binds in place of PAPS at the catalytic site, rather than at a regulatory site.

Initial Velocity Studies of the Reverse Reaction—The reverse reaction could be studied by taking advantage of the extremely high equilibrium constant of the ATP sulfurylase reaction in the direction of MgATP and \(\text{SO}_4^{2-}\) formation. If APS was not removed by the sulfurylase, the reverse reaction could not be demonstrated, even in the presence of hexokinase which removes MgATP.

Figs. 5 and 6 show the initial velocity patterns. The intersection of the \(1/v\) versus 1/[PAPS] plots on the \(1/v\) axis and the corresponding zero intercept of the slope replot in Fig. 5 are characteristic of a rapid equilibrium ordered sequence with MgADP adding before PAPS (pp. 320-326 of Ref. 20). The replots yield \(V_{\text{max,}} = 0.13\) units \(\times\) mg protein\(^{-1}\), \(K_m = 560\) \(\mu\)M and \(K_{\text{eq}} = 8\) \(\mu\)M. \(K_{\text{eq}}\) is much less than \(K_a\) and cannot be experimentally determined.

\(V_{\text{max,}}/\left[E\right]\), which is equivalent to \(k_+, k_-/(k_- + k_-)\) (p. 591 of Ref. 20), is only 0.08 s\(^{-1}\) for the most highly purified preparation. The rate constant \(k_+\), calculated from \(V_{\text{max,}} K_m/\left[E\right]\), for the same preparation (p. 588 of Ref. 20), is about 19 s\(^{-1}\). Thus, catalysis or APS release (i.e. \(k_-\)) must be the slowest and rate-determining step of the reverse reaction (\(k_- = 0.08\) s\(^{-1}\)). Furthermore, \(k_+\) must be \(\gg V_{\text{max,}}/\left[E\right]\), to yield the rapid equilibrium pattern (p. 591 of Ref. 20).
Evidence presented later suggests that PAPS can form a dead-end complex with E-MgATP. But, in assays of the reverse reaction, no substrate inhibition by PAPS was observed. This was not surprising because if \( k_{-2} \ll k_{-1} \), there would be virtually no E-MgATP in the steady state to combine with PAPS in the absence of added MgATP.

Another potential explanation for the reciprocal plot patterns shown in Figs. 5 and 6 is that the mechanism is rapid equilibrium random with strongly cooperative binding of MgADP and PAPS. This possibility can be experimentally tested. If substrate binding is indeed random with an interaction factor \( \alpha \ll 1 \) (pp. 274-281 of Ref. 20), then a pair of reciprocal plots symmetrical to those shown in Figs. 5 and 6 would be obtained when MgADP is varied in the region of \( K_{m2} \) and PAPS is varied in the region of \( K_{m1} \). We do not know either \( K_{m1} \) or \( K_{m2} \), but, allowing for an extreme case of \( \alpha = 0.01 \) (i.e. the binding of one substrate increases the affinity of the enzyme for the other by a factor of 100), we would have observed the characteristic plots when MgADP was varied in the region of 5.6 \( \mu \)M and PAPS was varied in the region of 800 \( \mu \)M. Experimentally however, patterns symmetrical to Figs. 5 and 6 were not observed. Thus, the original diagnosis of a rapid equilibrium ordered reverse reaction seems to be correct.

If the forward and reverse reactions are identical in form (which is usually the case), then the ordered reverse reaction—MgADP adding before PAPS—suggests that the forward reaction is also obligately ordered—MgATP adding before APS.

**Dead-end Inhibition of the Reverse Reaction by Iso-PAPS**—Iso-PAPS did not serve as a substrate for the reverse APS kinase reaction but did act as an inhibitor. The 2'-phosphate isomer was competitive with normal PAPS (data not shown) and uncompetitive with MgADP (Fig. 7), confirming the ordered sequence deduced from initial velocity studies. The limiting \( K_{i} \) for iso-PAPS (6 \( \mu \)M) is nearly identical to the \( K_{m} \) for PAPS (\( K_{m} \) = 8 \( \mu \)M). \( K_{i} \) is equivalent to \( k_{-1}(k_{-2} + k_{-1})/ k_{-2}(k_{-1} + k_{-2}) \) (p. 563 of Ref. 20). Because \( k_{i} \) cannot be less than \( V_{max}/[E] \) (i.e. 19 s\(^{-1}\)) and \( k_{-2} \) is much smaller than \( k_{i} \) (see above), \( K_{m} \) reduces to a simple dissociation constant, \( k_{-2}/k_{-1} \). It appears then, that PAPS and iso-PAPS have about the same affinity for E-MgADP.

**Product and Dead-end Inhibition Studies of the Forward Reaction**—At 4 \( \mu \)M APS, PAPS (within experimental error) an uncompetitive inhibitor with respect to MgATP (instead of a noncompetitive or mixed type inhibitor, as expected for a sequential reaction; data not shown). The intercept replot yielded a \( K_{m2} \) of 192 \( \mu \)M. Because the APS concentration was only about 3 \( K_{m} \), the competitive pattern could not have been caused by saturation with a co-substrate which adds between the points of addition of PAPS and MgATP. The pattern must then result from some unusual
ratios of kinetic constants. If we assume that PAPS combines with $E\cdot$MgATP as well as with $E\cdot$MgADP and the reaction is ordered in both directions with MgATP = $A$ and PAPS = $P$, the slope and intercept are given by

$$\text{slope} = \frac{K_{mA}}{V_{max}} \left[ \frac{1 + K_m[P]}{K_{mA}(B)} \left( 1 + \frac{K_m[P]}{K_{mA}[P]} \right) \right]$$

$$\frac{1}{V_{max}} = \frac{1}{V_{max}} \left[ \frac{1 + K_m[P]}{K_{mA}(B)} \left( 1 + \frac{K_m[P]}{K_{mA}[P]} \right) \right]$$

where $K_m$ is the $P$ dissociation constant from EAP. We can see that PAPS would have a relatively large intercept effect compared to the slope effect if the $K_{mA}[P]/K_{mA}K_m$ term is very small compared to $[P]/K_m$ and/or $[P]/K_{mA}$, that is if $K_m$ and/or $K_{mA}$ is much greater than the slope and intercept of the experimental $K_m$, of 8 $\mu$M, a feature consistent with $k_{-2} \ll k_{-1}$ and $k_{-2} \ll k_{1}$, (i.e., with a rapid equilibrium ordered reverse reaction where catalysis or APS release is rate-limiting). Note that $K_m$, unlike $K_{mA}$ or the $K$ for iso-PAPS, is not a simple dissociation constant. Rather, $K_m = (k_3 + k_{-3})/k_{-3}$ (p. 563 of Ref. 20). If $k_{-3}/k_{-2} = 8$ $\mu$M, a $K_m$ of at least 126 $\mu$M requires that $K_m$ be at least 15 $K_m$, or $\geq 285$ s$^{-1}$. Substituting $K_m/K_m$, $\geq 15$ into Equation 1, we can calculate that $K_{mA}$ is $\geq 1.4$ $\mu$M. Equation 3 yields $K_{mA} \geq 0.15$ $\mu$M.

Iso-PAPS was uncompetitive with respect to MgATP (data not shown). At 4 $\mu$M APS, $K_{mA}$ was 200 $\mu$M, or about the same as the $K_{mA}$ of normal PAPS.

In the absence of nuclease P1, initial rates are difficult to measure accurately at subinhibitory APS concentrations. Consequently, the nature of the inhibition exerted by PAPS with respect to APS could not be established by conventional means. Also, the nature of the assay (i.e. coupled to pyruvate kinase) precluded any studies of the MgADP product inhibition patterns.

In an attempt to determine the PAPS/APS inhibition pattern, APS was varied in the subinhibitory range of 2-5 $\mu$M along with 200-500 $\mu$M PAPS in a constant ratio ($[PAPS] = 78[APS]$). The reciprocal plot of $1/v$ versus $1/[APS]$ at 1.0 $\mu$M MgATP was nonlinear with a minimum occurring at a point corresponding to 2.5 $\mu$M APS (data not shown). This result shows that PAPS is not simply a competitive inhibitor with respect to APS. (Varying a substrate and a competitive inhibitor at a constant ratio yields a linear reciprocal plot. Uncompetitive, noncompetitive, and mixed type inhibitors yield an apparent substrate inhibition. The results are similar to those described on pp. 147-150 of Ref. 20.)

Table II summarizes the kinetic and physical properties of the fungal APS kinase.

### Table II

**Limiting kinetic constants and other properties of APS kinase from *P. chrysogenum***

The values are given for the most highly purified preparation at pH 8.0 (0.1 M Tris-Cl), 30 °C, in the presence of 70-150 $\mu$M (NH$_4$)$_2$SO$_4$.

| Constant or property | Description | Value |
|----------------------|-------------|-------|
| $K_{mA}$ | $K_m$ for MgATP at saturating APS, assuming no substrate inhibition by APS* | 1.5 $\mu$M |
| $K_m$ | Dissociation constant of the $E\cdot$MgATP complex | 1.5 $\mu$M |
| $K_{mA}$ | $K_m$ for APS at saturating MgATP | 1.4 $\mu$M |
| $K_a$ | Apparent inhibition constant ($K_{mA}$) of APS at saturating MgATP | 23 $\mu$M |
| $K_{mA}$ | $K_m$ for MgADP at saturating APS | $\geq 560$ $\mu$M |
| $K_{mA}$ | $K_m$ for MgADP at saturating PAPS | 560 $\mu$M |
| $K_{mA}$ | $K_m$ for PAPS at saturating MgADP | 8 $\mu$M |
| $K_{iso-PAPS}$ | Dissociation constant for iso-PAPS release from $E\cdot$MgADP iso-PAPS | 6 $\mu$M |
| $K_p$ | Inhibition constant for PAPS (estimated from $K_{sea}$) | $> 126$ $\mu$M |
| $V_{max}$ | Forward (theoretical) maximum velocity | 38.7 units $\times$ mg of protein$^{-1}$ |
| $k_{cat}$ | Active site catalytic rate constant for forward reaction | 19 s$^{-1}$ |
| $V_{max}$ | Maximal forward velocity at saturating MgATP and [APS]$_{sat}$ | 24.7 units $\times$ mg of protein$^{-1}$ |
| [APS]$_{sat}$ | APS concentration yielding maximal forward velocity at saturating MgATP | 5.5 $\mu$M |
| [APS]$_{iso}$ | APS concentration yielding half-maximal velocity before substrate inhibition | 1.3 $\mu$M |
| $V_{max}$ | Reverse maximum velocity | 27 $\mu$M |
| $K_{mA}$ | $V_{max}$, $K_m$ for PAPS | 0.16 units $\times$ mg of protein$^{-1}$ |
| $K_m$ | Equilibrium constant calculated from Haldane equation | 492 |
| $R$ | Stokes radius of native enzyme | 3.3 nm |
| $\xi_{max}$ | Sedimentation coefficient | 4.4 S |
| Native molecular weight | Calculated from $R$, $\xi_{max}$ and assumed $\phi$ | 59,000 |
| Subunit molecular weight | From SDS-gel electrophoresis | 30,000 |
| $A_{280}/A_{400}$ | Absorbance ratio: $A_{280}/A_{400}$ | 1.92 |

* Fortuitously, the $K_{sea}$ of one substrate does not depend on the concentration of the cosubstrate at subinhibitory levels of APS. Consequently, the $K_m$ for MgATP is 1.5 $\mu$M for all APS concentrations of $\leq 5$ $\mu$M. Above 5 $\mu$M APS, the $K_m$ for MgATP decreases with increasing [APS].
Chemical Modification with Phenylglyoxal and the Effect of Substrates and Products—Preincubation of APS kinase with phenylglyoxal resulted in a first order decay of enzyme activity, suggesting the presence of an essential arginine residue (21). MgATP, free ATP, MgADP, or free ADP at ~10 K, provided significant protection against inactivation. APS at ~600 K,, PAPS at ~80 K,, or free MgATP at 5 mm were each ineffective by themselves (Table III). However, in the presence of 0.1 K, of MgADP (not sufficient by itself for measurable protection), APS or PAPS provided nearly complete protection against the phenylglyoxal-dependent inactivation. Low MgATP together with high APS or PAPS also provided protection. The latter result suggests the formation of a dead-end E·MgATP-PAPS complex (and perhaps explains why enzymatic preparations of PAPS give low yields). These results confirm that the substrates bind synergistically, but do not distinguish between an obligately ordered sequence and a highly cooperative random sequence for the binding of MgADP and PAPS. (The fact that APS alone provided no protection is not diagnostic of an ordered forward reaction because the phenylglyoxal-sensitive residue could reside at the MgATP/MgADP subsite.)

Effect of 3'-AMP and Other Compounds—Burnell and Anderson (22) reported that PAPS accumulation by a spinach chloroplast extract increased markedly if 3'-AMP was included in the reaction mixture. They concluded that either (a) 3'-AMP is an activator of the chloroplast APS kinase or (b) 3'-AMP inhibited the utilization of PAPS by other enzymes in the extract. The nature of the putative PAPS-utilizing system was not explored, although 3'-nucleotidase (phosphatase) activity was deemed to be unlikely. We examined the effects of 3'-AMP and several other nucleotides on the activity of the purified fungal APS kinase. At 1 mm MgATP, 5 mm excess MgATP, and 5 μm APS (minus nuclease P1), none of the following compounds at 1 mm had any significant effect: 3'-AMP, 5'-AMP, 3',5'-ADP, 2',5'-ADP, 3',5'-cyclic AMP, adenosine-5'-sulfate, or adenosine-5'-phosphoamidate.

| Substrates or products added to preincubation mixture | Activity remaining after 60 min |
|------------------------------------------------------|---------------------------------|
|                                                      | -MgATP                         |
|                                                      | +5 mm excess MgATP              |
|                                                      | % zero time activity            |
| None                                                 | 22                             |
| ATP (150 μM)                                         | 28                             |
| ATP (15 mM)                                          | 80                             |
| ADP (50 μM)                                          | 24                             |
| ADP (5 mM)                                           | 62                             |
| APS (5 μM)                                           | 22                             |
| APS (840 μM)                                         | 25                             |
| PAPS (10 μM)                                         | 24                             |
| PAPS (640 μM)                                        | 24                             |
| ATP (150 μM) + APS (840 μM)                          | 87                             |
| ATP (150 μM) + PAPS (640 μM)                         | 37                             |
| ADP (50 μM) + APS (840 μM)                           | 71                             |
| ADP (50 μM) + PAPS (640 μM)                          | 48                             |

TABLE III

Effect of substrates and products on the inactivation of APS kinase by phenylglyoxal

APS kinase (32 μg x ml⁻¹) was incubated at 30 °C with 27 mm phenylglyoxal in 0.05 M NaHCO₃ (pH 8.1) containing the indicated additions. After 60 min, a 5-μl sample was removed, added to a standard 1.0-ml reaction mixture, and the residual activity determined. In the absence of added substrates or products, the enzyme lost activity in a first order fashion with a half-life of about 26 min. Residual activity is expressed as the percentage of the activity remaining compared to the zero time activity of the same mixture. Because of the large dilution factor, the zero time activities of the different preincubation mixtures were essentially the same and the same as that of a control incubated for 60 min in the absence of phenylglyoxal or other additions.

As shown in Fig. 8, MgATP (5 mm = 3.3 K,) accelerated the recovery process (and may have also protected against the initial loss of activity). APS (840 μM) had no effect. Other experiments (Table IV) established that free ATP, free ADP, or MgADP at 10 K, levels were also quite effective in accelerating the recovery from thermal inactivation, but MgCl₂ (5 mm) or PAPS (640 μM), alone or in combination, were ineffective. Suboptimal concentrations (0.1 K,) of MgATP or MgADP plus APS or PAPS were no more effective than the suboptimal MgATP or MgADP alone.

The physical basis of the reversible thermal inactivation and the possibility of exploiting the unusual heat property as a purification step are under investigation.

**Evidence for the Activation of Selenate by ATP Sulfurylase and APS Kinase**—Selenium is an essential nutrient for animals and some plant and microbial species. The sulfur analog

![Fig. 8. Reversibility of heat inactivation.](https://example.com/fig8.png)
is an essential constituent of several bacterial enzymes including glycine reductase (23), formate dehydrogenase (24), fatty acyl-S-CoA thiolase (25), and, possibly, xanthine dehydrogenase (26) and nicotinate hydroxylase (27). Glutathione peroxidase, the major selenium-containing protein in animal tissues (28), has been shown to contain selenocysteine at its active site (29). Selenocysteine is also present in formate dehydrogenase (30) and hydrogenase (31). Selenomethionine has not been shown to form seleno amino acids. However, selenomethionine has been isolated from the thiolase of Penicillium and other fungi and other fungi have not been shown to form seleno amino acids. However, inorganic selenate is taken up by mycelium and reduced to volatile dimethylselenide (33). Selenate uptake is mediated by the sulfate-transport system, which also accepts molybdate and thiocysteine (34). It seems likely that selenate is activated by the sulfate-transport system, which also accepts molybdate and thiosulfate (34). It seems likely that selenate is activated by the sulfate-transport system, which also accepts molybdate and thiosulfate (34). It seems likely that selenate is activated by the sulfate-transport system, which also accepts molybdate and thiosulfate (34). It seems likely that selenate is activated by the sulfate-transport system, which also accepts molybdate and thiosulfate (34). It seems likely that selenate is activated by the sulfate-transport system, which also accepts molybdate and thiosulfate (34).

Evidence for the action of the sulfate-activating enzymes on selenate

| Reaction mixture | Rate with the following inorganic substrates* |
|------------------|----------------------------------------------|
|                  | SO₄⁻ | SeO₄²⁻ | MoO₄²⁻ |
| Complete³       | 6.7  | 1.6   | 0     |
| Minus APS kinase| 0    | 0     | 0     |
| Plus adenylyl kinase⁴ | 6.6² | 1.6⁵  | 17.0⁶ |
| Minus APS kinase, plus adenylyl kinase⁴ | 0    | 1.7   | 17.8  |

*Activity based on an ATP sulfurylase content of 0.33 µg x ml⁻¹. The complete reaction mixture contained 5 mM MgATP, 5 mM excess MgCl₂, 5 mM Na₂SO₄ (or Na₂SeO₄), or Na₂MoO₄, 0.4 mM PEP, 0.3 mM NaH₂O, 1 ml KCL.6.5 x 10⁻³ units x ml⁻¹ of ATP sulfurylase (molybdoysis activity), 0.58 units x ml⁻¹ of APS kinase, 2.5 units x ml⁻¹ of pyrophosphatase, 13 units x ml⁻¹ of pyruvate kinase, and 18 units x ml⁻¹ of lactate dehydrogenase (the latter two were desalted by gel filtration prior to their use). This assay measures ADP formation. None of the inorganic substrates produced a rate above background in the absence of ATP sulfurylase. 9 µ units x ml⁻¹. The observed rates of sulfate-promoted and selenate-promoted NADH oxidation were the same as in the absence of added adenylyl kinase. The values shown assume a 1:1 relationship between NADH oxidized and MgATP used. This assay measures AMP formation. The rate of AMP formation is taken as half the rate of NADH oxidation. Essentially the same rate is obtained when PP; formation is measured.

Table IV

Effect of substrates and products on the overall heat inactivation and reactivation of APS kinase

| Substrates or products added to 50 °C preincubation mixture | Activity remaining after 0.5 min at 50 °C followed by incubation at 0 °C | % unheated control |
|------------------------------------------------------------|-----------------------------------------------------------------------------|-------------------|
| None                                                       | 44                                                                          | 85                |
| MgCl₂ (5 mM)                                                | 44                                                                          | 85                |
| ATP (15 mM)                                                 | 88                                                                          | 100               |
| MgATP (150 µM)                                              | 63                                                                          | 96                |
| MgATP (15 mM)                                               | 98                                                                          | 100               |
| ADP (5 mM)                                                  | 95                                                                          | 100               |
| MgADP (500 µM)                                              | 60                                                                          | 98                |
| MgADP (50 µM)                                               | 96                                                                          | 100               |
| APS (840 µM)                                                | 41 (39)*                                                                    | 85 (77)*          |
| PAPS (640 µM)                                               | 38 (39)*                                                                    | 78 (82)*          |
| MgATP (150 µM) + APS (540 µM)                               | 56                                                                          | 96                |
| MgATP (150 µM) + PAPS (640 µM)                              | 61                                                                          | 100               |
| MgADP (50 µM) + APS (840 µM)                                | 62                                                                          | 87                |
| MgADP (50 µM) + PAPS (640 µM)                               | 54                                                                          | 93                |
| APS (420 µM) + PAPS (320 µM)                                | 42                                                                          | 86                |

*The preincubation mixture contained 5 mM MgCl₂.
Acknowledgements—We thank Linh Hoang and Paul Knudson for their assistance in the purification of the enzyme and the SDS gel electrophoresis.

APPENDIX

For a steady state ordered Bi Bi sequence with dead-end EBQ and EAP as the only “abortive” complexes in the concentration ranges studied (Equation 7),

\[
\begin{align*}
A & \xrightarrow{k_{-1}} B \\
& \xrightarrow{k_1} (EAB \rightleftharpoons EPQ) \\
& \xrightarrow{k_2} EQ \\
& \xrightarrow{k_3} EAP \\
& \xrightarrow{k_4} EBQ
\end{align*}
\]

the King-Altman figure is as shown in Equation 8.

\[
\begin{align*}
E & \xrightarrow{k_1} [A] \\
& \xrightarrow{k_{-4}} [B] \\
& \xrightarrow{k_2} [Q] \\
& \xrightarrow{k_3} [P] \\
& \xrightarrow{k_4} [EAP] \\
& \xrightarrow{k_5} [EAP] \\
& \xrightarrow{k_6} [P] \\
& \xrightarrow{k_7} [B] \\
& \xrightarrow{k_8} [A] \\
& \xrightarrow{k_9} [Q] \\
& \xrightarrow{k_{10}} [P] \\
& \xrightarrow{k_{11}} [EAP] \\
& \xrightarrow{k_{12}} [EBQ]
\end{align*}
\]

Letting \( u = k_1[A][E] - k_{-1}[EA] \) and defining groups of rate constants in the usual way, we obtain the following equation for the initial forward velocity.

\[
\frac{v}{V_{max}} = \frac{[A][B]}{K_aK_m + K_m[A] + K_m[B] + [A][B] + [A][P] + [A][P]^2}{K_{IP}}
\]

All the kinetic constants are defined (and have the same rate constant composition) as for a normal steady state Bi Bi reaction with the exception of the following.

\[
K_n = \frac{\text{coeff}_{IP} - \text{coeff}_{AP}}{\text{coeff}_{AP}}
\]

Equation 9 was derived using the King-Altman figure with an EBQ corner, but without the EAP corner. The formation of EAP was then taken into account by multiplying the resulting \([A]\) and \([A][P]\) terms by \((1 + [P]/K_m)\), as described on p. 789 of Ref. 20. If the EAP corner is included from the start and the groups of rate constants defined in the usual manner, the two \([A][P]\) terms of Equation 9 will automatically be combined into a single term which can be written as

\[
\frac{K_n[A][P]}{K_{IP}} \quad \text{or} \quad \frac{K_nK_mK_{IP}[A][P]}{K_{IP}K_mK_n}
\]

where

\[
K_n = \frac{\text{coeff}_{IP} - \text{coeff}_{AP}}{\text{coeff}_{AP}} = \frac{K_{IP}}{1 + \frac{K_mK_{IP}}{K_nK_m}}
\]

and

\[
K_n = \frac{\text{coeff}_{IP} - \text{coeff}_{AP}}{\text{coeff}_{AP}} = K_{IP} \left( 1 + \frac{K_mK_{IP}}{K_nK_m} \right)
\]

Also, the \([A][P]^2\) term can be written as

\[
\frac{K_n[A][P]^2}{K_{IP}K_n}
\]

where

\[
K_{IP} = \frac{\text{coeff}_{IP} - \text{coeff}_{AP}}{\text{coeff}_{AP}} = K_{IP} \left( 1 + \frac{K_mK_{IP}}{K_nK_m} \right)
\]

When \([A]\) is varied at different fixed levels of \([B]\) (or a constant \([B]\) and different fixed levels of \([P]\)), the equation is as follows.

\[
\frac{v}{V_{max}} = \frac{1 + \frac{K_mK_{IP}}{K_nK_m[B]} + [B]}{1 + \frac{K_mK_{IP}}{K_nK_m[B]} + [B] + \frac{[A][B]}{K_{IP}}}
\]

The factor multiplying \(K_m\) is the factor by which the slope of the reciprocal plot changes in response to changing \([B]\) or \([P]\). The factor multiplying \([A]\) is the factor by which the \(1/v\) axis intercept of the reciprocal plot changes in response to changing \([B]\) and \([P]\). Reciprocal plots of \(1/v\) versus \(1/[A]\) are linear at all \([B]\) and \([P]\). The \(1/v\) axis intercept versus \(1/[B]\) replot (treating \([B]\) as a substrate) and the \(1/v\) axis intercept versus \(1/[P]\) replot (treating \([B]\) as an inhibitor) are nonlinear, but will appear linear over the appropriately restricted \([B]\) range if \(K_{IP}\) is significantly greater than \(K_m\).

The reciprocal plot for \(1/[B]\) is nonlinear but can appear so in the region of \(K_m\) if \(K_{IP}\) is sufficiently greater than \(K_m\). The equation for the \(1/v\) versus \(1/[B]\) plot can be written as follows.

\[
\frac{1}{v} = \frac{1 - K_m[1/[A]] + [A][B]}{V_{max} + [A][B] + 1 + \frac{K_mK_{IP}}{K_nK_m[B]} + 1 + \frac{K_mK_{IP}}{K_nK_m[B]} + \frac{1}{[B]}}
\]

At the minimum of the reciprocal plot, \(1/[B] = 1/[B]_opt\), and \(d(1/v)/d(1/[B]) = 0\), or

\[
\frac{1}{[B]_opt} = K_{IP}K_m \left( 1 + \frac{K_m}{[A]} \right)
\]

or

\[
[1/[B]_opt] = \sqrt{K_{IP}K_m \left( 1 + \frac{K_m}{[A]} \right)}
\]

In the \(v\) versus \([B]\) plot, the maximum occurs at \([B]_opt\).
APS Kinase from P. chrysogenum

\[
[B]_{tot} = \sqrt{K_{mB}K_{mE} \left(1 + \frac{K_m}{[A]}\right)}
\]

Other properties of an ordered Bi Bi sequence with a dead-end EBQ complex (but without a dead-end EAP complex) are described by Pennings and Van Kempen (36).

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