Adenosine-5'-phosphosulfate kinase (ATP:adenyllylsulfate 3'-phosphotranferase), the second enzyme in the pathway of sulfate activation, has been purified (~300-fold) to homogeneity from an Escherichia coli K12 strain, which overproduces the enzyme activity (~100-fold). The purified enzyme has a specific activity of 153 μmol of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) formed/min/mg of protein at 25 °C. The enzyme is remarkably efficient with a V_{max}/K_{MAPS} of >10^4 M^{-1} s^{-1}, indicating that at physiologically low substrate concentrations the reaction is essentially diffusion limited. Upon incubation with MgATP a phosphorylated enzyme is formed; the isolated phosphorylated enzyme can transfer its phosphoryl group to adenosine 5'-phosphosulfate (APS) to form PAPS or to ADP to form ATP. The phosphorylated enzyme exists as a dimer of identical 21-kilodalton subunits, while a tetrameric form of the enzyme is a minor isolated exists predominantly as a dimer of identical 21-kDa subunits, while a tetrameric form primarily exists as a tetramer. Divalent cations are required for activity with Mg(II), Mn(II), Co(II), and Cd(II) activating. Studies of the divalent metal-dependent stereoselectivity for the α- and β-phosphorothioate derivatives of ATP indicate metal coordination to at least the α-phosphoryl group of the nucleotide.

Steady state kinetic studies of the reverse reaction indicate a sequential mechanism, with a rapid equilibrium ordered binding of MgADP before PAPS. In the forward direction APS is a potent substrate inhibitor, competitive with ATP, complicating kinetic studies. The primary kinetic mechanism in the forward direction is sequential. Product inhibition studies at high concentrations of APS suggest an ordered kinetic mechanism with MgATP binding before APS. At submicromolar concentrations of APS, product inhibition by both MgADP and PAPS is more complex and is not consistent with a solely ordered sequential mechanism. The formation of a phosphorylated enzyme capable of transferring its phosphoryl group to APS or to MgADP suggests that a ping-pong pathway in which the rate of MgADP dissociation is comparable to the rate of APS binding might contribute at very low concentrations of APS. The substrate inhibition by APS is consistent with APS binding to the enzyme, to form a dead-end E*APS complex.

Adenosine-5'-phosphosulfate kinase (ATP:adenyllylsulfate 3'-phosphotransferase, EC 2.7.1.25) catalyzes the phosphorylation of adenosine 5'-phosphosulfate (APS), an activated form of sulfate, yielding 3'-phosphoadenosine 5'-phosphosulfate (PAPS). PAPS is the primary donor of sulfuryl groups in biological systems (1). The net activation of sulfate is the result of the coordinated action of the three enzymes: ATP sulfurylase (EC 2.7.7.4), APS kinase, and inorganic pyrophosphatase.

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

Of the two enzymes involved in activating sulfate, ATP sulfurylase is the better characterized, having been purified from a variety of sources (2-13). In the reaction catalyzed by ATP sulfurylase, inorganic pyrophosphate is displaced from the α-phosphoryl group of ATP by inorganic sulfate. The equilibrium constant of this reaction is approximately 10^{-8} favoring ATP and SO_4^{2-} (14). Therefore, the in vivo concentration of APS is very low, and the formation of APS from ATP and inorganic sulfate occurs to only a very slight extent in the absence of APS kinase and inorganic pyrophosphatase (14).

Very little is known about APS kinase and its reaction mechanism. APS kinase has been partially purified from Escherichia coli (15), yeast (1, 10), Penicillium chrysogenum (16), rat liver (17), and to near homogeneity from rat chondrosarcoma (12).

We have purified APS kinase homogeneity from an overproducing strain of E. coli containing the cloned cysC from E. coli (13), which encodes APS kinase. The enzyme as isolated exists predominantly as a dimer of identical 21-kDa subunits, while a tetrameric form of the enzyme is a minor species. A steady state kinetic mechanism has been determined, and it has been found that a phosphorylated enzyme exists as an intermediate. The preferred divalent metal ion coordination to ATP in the reaction has been investigated using the divalent metal ion-dependent selectivity for phosphorothioate derivatives of ATP.

The abbreviations used are: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PFLC, fast protein liquid chromatography; Hepes, 4-hydroxyethyl-1-piperazineethanesulfonic acid; PEI, polyethylenimine; PMSF, phenylmethylsulfonlfy fluoride; SDS, sodium dodecyl sulfate; TRA, triethylamine; TLC, thin layer chromatography; TMA, tetramethylammonium; ATPγS, adenosine 5'-O-(3-thio)triphosphate; ATPαS, adenosine 5'-O-(1-thio)triphosphate; ATPβS, adenosine 5'-O-(2-thio)triphosphate.

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† Supported by National Institutes of Health Grant CA-09035-13.
Adenosine Phosphosulfate Kinase from Escherichia coli

MATERIALS AND METHODS

RESULTS

Purification of APS Kinase

APS kinase was purified ~300-fold from a strain which overproduces the enzyme activity ~100-fold. The purification scheme is presented in Table I. In many stages of purification a 43-kilodalton polypeptide cochromatographed with APS kinase activity but was separated from APS kinase activity by chromatography on aminohexyl-Sepharose. It is noteworthy that a previous report indicated that a 43-kDa polypeptide was the E. coli APS kinase (see "Discussion") (15). The SDS-polyacrylamide gel profile of the purified enzyme is shown in the Fig. 1 (lane a). The sole observable protein migrated as a 21-kDa polypeptide on denaturing gels. Nondenaturing gel electrophoresis and two-dimensional gel electrophoresis showed that the protein preparation was homogeneous and that APS kinase activity migrated with the only Coomassie-stainable protein band (see "Materials and Methods"). The enzymic activity also comigrated with the purified protein on chromatofocusing. No contaminating polypeptides were detected upon silver staining of SDS-polyacrylamide gel electrophoresis of 2 μg of the purified protein. In order to verify that APS kinase was indeed the product of the cysC gene, the proteins expressed by a plasmid containing cysC were selectively labeled with [35S]methionine. The APS kinase activity in this preparation cochromatographed with the 21-kDa cysC gene product during purification (see "Materials and Methods"). Furthermore, partially purified [35S]methionine-labeled 21-kDa polypeptide comigrated with the purified APS kinase on nondenaturing polyacrylamide gel, Sephadex G-100 column chromatography, and on chromatofocusing. Therefore it appears that cysC is the structural gene for APS kinase.

Physical Characterization of APS kinase

The amino acid composition of APS kinase calculated as residues per 21,000-dalton polypeptide is shown in Table II. Despite its role in sulfur assimilation, the enzyme contains significant amounts of both cysteine and methionine. The purified protein eluted at pH 5.5 from a chromatofocusing column giving an approximate isoelectric point for the native protein. The extinction coefficient at 280 nm of 1.05 (mg/ml)-1 cm-1 was calculated from the absorbance at 280 and 205 nm (30).

Native Molecular Weight Determination

Chromatography on Sephadex G-100 of purified APS kinase showed two forms of the enzyme indistinguishable in specific activity. The molecular masses of these forms were 80 and 40 kDa, corresponding to tetrameric and dimeric forms of the protein. Although the ratio of the two forms varied in different enzyme preparations, the 40-kDa form was generally the predominant species constituting 60-80% of the total enzyme. Incubation of 10 pmol of protein with 1 mM ATP (in 100 μl of 50 mM Tris/HCl 0.5 mM Hepes/KOH, 50 mM KCl, and 5 mM MgCl2, pH 8.0, for 10 min) resulted in all of the enzymic activity migrating as a 40-kDa protein. Nearly 88% of 10 pmol of the 40-kDa protein migrated as a 40-kDa form when 1 mM APS replaced ATP in the incubation. These results indicate a substrate-dependent change in aggregation state of the enzyme.

Identifications

When enzymatically determined specific activity of APS kinase was lower than that determined by coupled assays, the enzyme was purified with 1 mM MgCl2, pH 8.0, for 10 min) resulted in all of the enzymic activity migrating as a 40-kDa protein. Nearly 88% of 10 pmol of the 40-kDa protein migrated as a 40-kDa form when 1 mM APS replaced ATP in the incubation. These results indicate a substrate-dependent change in aggregation state of the enzyme.

\[ \text{KDa} \]
\[ a \quad b \quad c \]

\[ \text{FIG. 1. Coomassie Blue stain and autoradiogram of a 15% polyacrylamide gel on which purified APS kinase was used. Lane a, APS kinase, 5 μg of protein purified by step 9 of Table I; lane b, Coomassie Blue-stained gel containing APS kinase followed incubation with [γ32P]ATP (see "Methods"); lane c, autoradiogram of lane b.} \]
reaction. These results indicate that although the γ-phosphoryl group of ATP becomes attached to the enzyme, ADP can dissociate. Phosphorylation of the enzyme was stoichiometric, with incorporation of 1 mol of phosphorus/mole of polypeptide (Fig. 2). Incubation of isolated phosphorylated enzyme with 5 μM APS or 200 μM ADP resulted in the formation of PAPS or ATP, respectively. Both reactions occurred faster than could be measured without a rapid mixing device, consistent with a role of the phosphorylated enzyme as an intermediate in the overall reaction.

Upon incubation of enzyme (1.25 pmol) with 10 pmol of [32P]-PAPS (1 μCi/pmol) in 25 μl of above buffer, no phosphorylated enzyme was detected.

**Effect of Cations on Enzymic Activity**

**The Effect of Divalent Cations**—The effects of metal ions on enzyme activity in the forward direction was determined at two different concentrations of ATP, as illustrated for Mg(II) in Fig. 3A. At both 0.42 and 1.1 mM ATP maximal activation of the enzyme by Mg(II), Mn(II), Co(II), and Cd(II) was at a concentration that equaled the ATP concentration. The trace activity (<1.8% of maximal) observed in the absence of added divalent cation was completely abolished by 250 μM EDTA. Mg(II) in excess of the nucleotide was not inhibitory (Fig. 3A), although excess Mn(II), Co(II), and Cd(II) did inhibit. Free ATP is not a good inhibitor, since a constant rate was obtained when ATP was varied from 1.0 to 5.0 mM with a fixed concentration of Mg(II) (1.15 mM). At 1.1 mM ATP, 0.5 mM Mn(II) and Cd(II) supported enzymic activity as well as Mg(II), while Co(II) at 0.5 mM was ~60% as effective, although at a metal ion concentration of 1.0 mM the enzymic activity was increased by Mn(II) (~10%), inhibited by Cd(II) (~50%), and inhibited by Co(II) (~75%), when compared with Mg(II) activity.

The effect of divalent metal ions on enzyme activity was also determined for the reverse reaction. Maximal activation by Mg(II) was at a concentration that equaled the ATP concentration (0.03 and 0.07 mM). Mg(II) at 0.03 and 0.07 mM. Negligible activity (<0.04% of maximal) was observed in the absence of added divalent cation was completely abolished by 250 μM EDTA. Mg(II) in excess of the nucleotide was not inhibitory (Fig. 3A), although excess Mn(II), Co(II), and Cd(II) did inhibit. Free ATP is not a good inhibitor, since a constant rate was obtained when ATP was varied from 1.0 to 5.0 mM with a fixed concentration of Mg(II) (1.15 mM). At 1.1 mM ATP, 0.5 mM Mn(II) and Cd(II) supported enzymic activity as well as Mg(II), while Co(II) at 0.5 mM was ~60% as effective, although at a metal ion concentration of 1.0 mM the enzymic activity was increased by Mn(II) (~10%), inhibited by Cd(II) (~50%), and inhibited by Co(II) (~75%), when compared with Mg(II) activity.

The effect of divalent metal ions on enzyme activity was also determined for the reverse reaction. Maximal activation by Mg(II) was at a concentration that equaled the ADP concentration (0.03 and 0.07 mM). Mg(II) at 0.03 and 0.07 mM. Negligible activity (<0.04% of maximal) was observed in the absence of added divalent cation was completely abolished by 250 μM EDTA. Mg(II) in excess of the nucleotide was not inhibitory (Fig. 3A), although excess Mn(II), Co(II), and Cd(II) did inhibit. Free ATP is not a good inhibitor, since a constant rate was obtained when ATP was varied from 1.0 to 5.0 mM with a fixed concentration of Mg(II) (1.15 mM). At 1.1 mM ATP, 0.5 mM Mn(II) and Cd(II) supported enzymic activity as well as Mg(II), while Co(II) at 0.5 mM was ~60% as effective, although at a metal ion concentration of 1.0 mM the enzymic activity was increased by Mn(II) (~10%), inhibited by Cd(II) (~50%), and inhibited by Co(II) (~75%), when compared with Mg(II) activity.

The requirement for a divalent metal for phosphoryl transfer from chromatographically isolated phosphorylated enzyme (E-P) was assessed. EDTA at 180 μM completely prevented phosphoryl transfer to either: 200 μM ADP or 5 μM APS in a 20-min incubation in 50 μl of 50 mM Tris/HCl, and 100 mM KCl, pH 8.0. Although in control experiments in the absence of added Mg(II) no phosphoryl transfer to ADP (<1.0% of maximal) was observed, phosphoryl transfer to APS was detected (~20% of maximal), under conditions described above.

**Steady State Kinetic Studies**

**Substrate Inhibition**—In our attempts to determine a Km value for APS we observed substantial substrate inhibition. The maximum forward velocity of 153 μmol/min/mg protein was observed at APS and ATP concentrations of 1.1 μM and 3 mM, respectively. Inhibition by APS was alleviated by increasing the concentration of ATP (Fig. 4). At low (“sub-inhibitory”) concentrations of APS, plots of 1/V versus 1/[ATP] at fixed concentrations of APS gave intersecting lines, indicating a sequential mechanism (Fig. 5A). At high (“inhibitory”) concentrations of APS, 1/V versus 1/[ATP] plots at fixed concentrations of APS intersect on 1/V axis.

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Adenosine Phosphosulfate Kinase from Escherichia coli

APS, it was not possible to conduct meaningful inhibition studies with APS as the varied substrate. Addition of 33 concentrations. Due to the potent substrate inhibition by ADP did not alter substrate inhibition by APS. The kinetic constants results are consistent with obligatory ordered steady state and PAPS (Fig. 8A) inhibited the enzyme in a noncompetitive manner. Therefore APS is a competitive inhibitor with respect to MgATP and uncompetitive with respect to PAPS. These results are consistent with an ordered steady state sequence in which MgADP adds before PAPS and APS dissociates before MgATP. APS was not investigated as an inhibitor of reverse reaction because of the requirement for coupling the reaction to ATP sulfurylase. The kinetic constants \( K_a \) and \( K_a \) are given in Table IV.

Substrate Specificity—Neither AMP for the forward reaction, nor 2'-PAPS for the reverse reaction appeared to be substrates for the enzyme. With 1 mM AMP in place of APS, a rate of \(<0.15 \text{ nmol/min/mg protein} \) was obtained. Using 2'-PAPS at 0.7 mM as alternate substrate for 3' PAPS, a rate of \(<0.02 \text{ nmol/min/mg protein} \) was obtained, \(<0.003\% \) of 3' PAPS.

The Equilibrium Constant Determination—The equilibrium constant was determined starting from both directions at several concentrations of enzyme (see “Materials and Methods”). The equilibrium constant determined starting from the forward direction is \( 1.8 \times 10^3 \), and that determined starting with the reverse reaction is \(-2.4 \times 10^2 \).

**DISCUSSION**

APS kinase has been purified to homogeneity from *E. coli*. The purified APS kinase has a specific activity 5-fold higher than previously reported preparations of the enzyme (16). This enzymic activity is diffusion limited in the physiologically relevant direction, \( V_{\text{max}}(K_{\text{Mps}}) \cdot 10^{-4} \text{ M}^{-1} \text{ s}^{-1} \), at the low concentrations of APS present in vivo. The maximal turnover rate of the enzyme is \(-3 \times 10^9 \text{ min}^{-1} \) in the forward direction. Using a sensitive radioactive assay the apparent \( K_{\text{Mps}} \) was estimated to be 0.25 \( \mu \text{M} \), and due to substrate inhibition this may be slightly higher than the actual \( K_{\text{Mps}} \). Determination of the actual \( K_{\text{Mps}} \) may be technically inaccessible due to its small magnitude (requiring the use of carrier-free radioactive compounds) and the potent substrate inhibition.

The isolation of APS kinase has been reported from a strain of *E. coli* C (AN1460) (15). The authors report the preparation to be pure, and the enzyme to be a dimer of 43,000-dalton subunits. The specific activity of the protein at the end of their purification scheme is approximately 200-fold less than the specific activity of APS kinase that we have purified from *E. coli* K12. Although we cannot definitely explain this discrepancy, we would like to point out the strain differences, and that 43- and 14-kDa polypeptides (on denaturing polyacrylamide gels) comigrated with APS kinase activity through most of the steps of purification. The last four steps of purification essentially involve the removal of these contaminating polypeptides. After step 5 of the purification scheme, the 43- and 14-kDa peptides were routinely present at levels approximately 10-fold higher and 10-fold less than that of APS kinase, respectively, despite the 100-fold overproduction of APS kinase. Hydroxylapatite through aminohexyl column chromatographic steps ensure total removal of the 43-kDa
protein, ATP agarose removes the 14-kDa protein and a few other minor contaminating peptides. The three proteins (43-, 21-, and 14-kDa) comigrate upon chromatofocusing of the stage 5 material (Sephacryl S-300 fraction, Table 1). The authors (15) do not mention the use of columns that in our hands separate the 43-kilodalton polypeptide from APS kinase activity. The cloning and mapping of cysC, the radiolabeling of a 21-kDa peptide produced by the cysC gene (13) and copurification of the radiolabeled gene product with APS kinase support the contention that 21-kDa peptide is the monomeric form of APS kinase. We do not think that cysC is truncated in the clone used for purification of APS kinase because clones with 2 kilobases of DNA on either side of cysC produced only the 21-kDa peptide. We anticipate verification of this conclusion by the DNA and protein sequencing studies that are being carried out.

The analysis of steady state kinetic data for the forward reaction is complicated by the pronounced substrate inhibition by APS. The observation that APS is a competitive inhibitor with respect to MgATP suggests that E:APS is a dead-end complex. The uncompetitive inhibition by PAPS with respect to MgATP and competitive inhibition by MgADP with respect to MgATP at high APS concentration (~5 K,) indicate that the reaction sequence is ordered sequential (35) with MgATP binding first, as illustrated in Fig. 10. The binding of the enzyme to an ATP agarose matrix and the studies of the reverse reaction are consistent with this pathway. Also consistent are the data for the forward reaction at subinhibitory concentrations of APS (Fig. 5A). However, at a subinhibitory (~K,;) concentration of APS both MgADP and PAPS exhibited noncompetitive inhibition with respect to MgATP, which is uncharacteristic of an ordered sequential mechanism. Since during gel filtration, MgADP dissociates from the E-P-MgADP complex formed from MgATP in the absence of APS, and since E-P transfers the phosphoryl group to APS or MgADP, a ping-pong pathway might also be operative, as illustrated in Fig. 10. Clearly the steady state ordered sequence can degenerate to a ping-pong type of mechanism, if the rate of dissociation of MgADP from E-P is greater than the (second order) rate of APS binding. Although diffusion limited, the rate of APS binding would be quite slow at the submicromolar concentrations needed to avoid substrate inhibition, concentrations which are also physiologically relevant. Therefore, it may be concluded that the steady state ordered sequential mechanism reflects the preferred rather than the exclusive pathway. The mechanism proposed here differs from the mechanism for APS kinase from P. chrysogenum proposed by Renosto et al. (16). They propose an ordered sequential mechanism in which E-APS-MgADP is the dead-end complex and suggest the possible formation of E-MgATP-PAPS also. In our experiments the addition of ADP did not potentiate the substrate inhibition by APS, consistent with the lack of potent inhibition by an E-APS-MgADP complex.

The phosphorylated enzyme exists as a dimer, while the dephosphorylated enzyme exists mainly as a tetramer. The interconversion of these forms of the enzyme is not likely to be part of the normal steady state reaction sequence, because the rate of tetramerization, even if diffusion-controlled (36), would be at least 3 orders of magnitude slower than the rate of enzyme reaction at the low concentration of enzyme used in our work (50–100 pm). Schriek and Schwenn (15) report the formation of a 40,000–45,000-dalton APS kinase from a previously 80-kDa species upon treatment of the enzyme with dithiothreitol. We observed similar changes in the molecular weight forms of our enzyme preparations upon incubation of the enzyme with reductants followed by chromatography in the presence of reducing agents. No tetrameric form of the enzyme was detected when dithiothreitol or 2-mercaptoethanol was included in the elution buffer, for either the phosphorylated or the dephosphorylated enzymes. No loss of phosphoryl group was detected when phosphorylated enzyme was chromatographed with reductants. We do not know if or how these two phenomena are related. However, this appears to be an in vitro phenomenon, since the reduced cellular environment might preclude the formation of the 80-kDa enzyme. Changes in subunit aggregation appear to be common to APS kinase from various sources. The 68-kDa tetrameric rat liver enzyme (15-kDa identical subunits) shows properties of aggregation and disaggregation in the absence and in the presence of reducing agents (17). The P. chrysogenum enzyme is a dimer of 60 kDa with identical 30-kDa subunits, and shows temperature-sensitive monomerization and loss of activity; upon incubation with MgATP the enzymic activity is restored and the protein dimerizes (37).

APS kinase has an absolute requirement for a divalent cation, but no requirement for monovalent cations. It appears that a M(II)-nucleotide complex is the true substrate. Mg(II), Mn(II), Co(II), and Cd(II) activated the enzyme to comparable extents. Since Mg(II) is required for phosphorylation of APS by E-P, metal ion(s) other than those coordinated to ATP or ADP appear to be involved. Studies of divalent cation activation using diastereoisomers of phosphorothioate derivatives of ATP reveal the probable metal ion-ATP coordination scheme preferred by APS kinase. The 36-fold stereoselectivity of the R over the S isomer of ATPoS in the presence of Mg(II) and reversed stereoselectivity (~4-fold) in the presence of Cd(II) suggests that metal coordination to the pro-R oxygen of P, is preferred. The absence of pronounced stereoselectivity of ATPoS (R versus S) in the presence of Mg(II) or Cd(II)

![Fig. 10. The steady state reaction scheme for APS kinase. The primary pathway is shown in bold. The steady state reaction scheme is shown under increasing APS concentrations from the bottom up. At very high APS concentrations the binding of APS leads either to a E-APS (shown in italics) complex formation which is either a dead-end complex, or the route of E-MgATP. APS complex is slow. At very low APS concentrations ADP may dissociate before APS binds (lower arm of the pathway). It is not known whether phosphorylation of the enzyme always precedes productive APS binding.](attachment:image.png)
was unexpected since in all characterized cases kinases do coordinate to oxygens on the $\beta$-phosphoryl group (38). However, the small stereoselectivity did invert when Cd(II) replaced Mg(II). This smaller change in the stereoselectivity of the ATPS with Mg(II) and Cd(II) is a less definitive result, reminiscent of studies with phosphoglycerate kinase (39). The $V_{\text{max}}$ with ATP$\gamma$S was nearly 500 times less than that obtained for ATP. This reduction in $V_{\text{max}}$ when PO$_2$S rather than a PO$_3$ group is transferred suggests that phosphoryl transfer is a rate limiting step (38). Rapid reaction kinetic studies will reveal whether enzyme phosphorylation or dephosphorylation is predominantly rate determining.

We have found no evidence for allosteric activation of APS kinase by MgATP as reported for the rat liver APS kinase (17). ATP sulfurylase is readily separated from APS kinase by ammonium sulfate precipitation (13), or by ATP agarose chromatography. Therefore the E. coli sulfate activating enzymes do not cochromatograph as reported for rat chondrosarcoma enzymes (12). Given the equilibrium constants for ATP sulfurylase and APS kinase, and the rate of APS kinase catalysis, channeling of products and substrates seems appropriate for the sulfate activating pathway. However, we have not found any evidence for channeling either in crude extracts that contain overproduced ATP sulfurylase and APS kinase or in mixtures of purified APS kinase and ATP sulfurylase.

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REFERENCES


Continued on next page.
Adenosine Phosphosulfate Kinase from Escherichia coli

MATERIALS AND METHODS

Centrifugation and Supernatant Fractionation: The 

Centrifugation of Proteins and Enzymes: The proteins and enzymes were subjected to centrifugation at 100,000 g for 1 h in a Beckman L-85B ultracentrifuge, and the supernatant fraction was collected.

Supernatants and Centrifugates Fractionated by Polyacrylamide Gel Electrophoresis: The proteins and enzymes were separated by polyacrylamide gel electrophoresis (PAGE) using 7.5% acrylamide gels and 0.1% SDS. The gels were stained with Coomassie blue R-250 and destained in 7.5% acetic acid and 40% methanol.

Supernatants and Centrifugates Fractionated by Silver Staining: The proteins and enzymes were separated by 2-D PAGE using 10% acrylamide gels and 0.1% SDS. The gels were stained with silver nitrate and destained in 7.5% acetic acid.

Supernatants and Centrifugates Fractionated by Immunoblotting: The proteins and enzymes were separated by 1-D PAGE using 7.5% acrylamide gels and 0.1% SDS. The gels were transferred to nitrocellulose membranes and probed with specific antibodies.

Supernatants and Centrifugates Fractionated by Western Blotting: The proteins and enzymes were separated by 2-D PAGE using 10% acrylamide gels and 0.1% SDS. The gels were transferred to nitrocellulose membranes and probed with specific antibodies.

Supernatants and Centrifugates Fractionated by Mass Spectrometry: The proteins and enzymes were separated by 1-D PAGE using 7.5% acrylamide gels and 0.1% SDS. The gels were transferred to nitrocellulose membranes and probed with specific antibodies.

Supernatants and Centrifugates Fractionated by Nuclear Magnetic Resonance: The proteins and enzymes were separated by 1-D PAGE using 7.5% acrylamide gels and 0.1% SDS. The gels were transferred to nitrocellulose membranes and probed with specific antibodies.

Supernatants and Centrifugates Fractionated by X-ray Crystallography: The proteins and enzymes were separated by 1-D PAGE using 7.5% acrylamide gels and 0.1% SDS. The gels were transferred to nitrocellulose membranes and probed with specific antibodies.
Adenosine Phosphosulfate Kinase from Escherichia coli

Table II

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*The kinetic and steady-state parameters are described in Ref.*

a) The reaction and steady-state parameters are described in Ref. (1).
### TABLE III

Effect of Mg(II) and Cr(III) on V_{max} and K_{cat} using phosphate derivatives of ATP

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<td>0.32</td>
</tr>
<tr>
<td>ATP+G+D</td>
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<td>0.52</td>
</tr>
<tr>
<td>ATP+G+I+D</td>
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<td>0.28</td>
</tr>
</tbody>
</table>

### TABLE IV

Kinetic constants and inhibition studies

<table>
<thead>
<tr>
<th></th>
<th>vs ATP</th>
<th>noncomplexed with ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.15</td>
<td>0.32</td>
</tr>
<tr>
<td>PAPS</td>
<td>0.32</td>
<td>0.34</td>
</tr>
<tr>
<td>ADP</td>
<td>0.34</td>
<td>0.34</td>
</tr>
</tbody>
</table>

### FIG. 3

Effect of Cations on the rate of PAPS formation.

A. Activation by Mg(II) at 0.43 mM ATP (O), and 1.1 mM ATP (■). The reaction mixture contained 1.5 μM ADP, 100 mM KCl and 50 mM Hepes/NaOH, pH 6.0. 
B. The effect of KCi. The reaction mixture contained 50 mM Tris/HCl, 5 mM MgCl_2, 1 mM ATP/Tris, and 1.2 mM PAPS/NaOH, pH 8.0. V is velocity expressed as pmol/min/mg protein.

### FIG. 6

Variation of V_{max} and K_{cat} with PAPS concentration.

A. Effect of increasing PAPS concentration on V_{max} of the forward reaction. B. Effect of PAPS on K_{cat}. The kinetic constants were estimated from the data presented in Figures 5A and 5B using the Michaelis-Menten curve equation, as described in Methods. V_{max} is expressed as pmol/min/mg protein.

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The values presented in the table are derived from the data.
Adenosine Phosphosulfate Kinase from Escherichia coli

**FIG. 1.** Product inhibition of the forward reaction by ADP.

A. Competitive inhibition by ADP with respect to ATP at a high (11 μM) concentration of APS. The APS concentration in the assay was: none added (●), 0.1 (○), 0.3 (△), and 0.5 (■) μM. B. Non-competitive inhibition by ADP with respect to ATP at a low (0.25 μM) concentration of APS. The ADP concentration in the assay was: none added (●), 0.1 (○), 0.3 (△), and 0.5 (■) μM. V is velocity expressed as μmol/mg protein.

**FIG. 2.** Double reciprocal plots of the reverse reaction.

A. Substrate saturation by ADP. The concentrations of pAPS in mM were: 0.05 (●), 0.08 (○), 0.16 (△), 0.3 (■), 0.5 (●), and 0.7 (○). B. Substrate saturation by PAPS. The concentrations of ADP in mM were: 0.95 (●), 0.90 (○), 0.85 (△), 0.80 (■), and 0.75 (●). The maximal velocities were similar to the conditions described for the forward reactions in the legend to Figure 1. V is velocity expressed as μmol/mg protein.

**FIG. 3.** Product inhibition of the forward reaction by PAPS.

A. Non-competitive inhibition by PAPS with respect to ATP at a low (0.25 μM) concentration of APS. PAPS concentrations in mM were: none added (●), 0.1 (○), 0.3 (△), and 1.0 (■). B. Non-competitive inhibition by PAPS with respect to ATP at a high (11 μM) concentration of APS. PAPS concentrations in mM were: none added (●), 0.1 (○), 0.3 (△), and 0.5 (■) μM. The reaction conditions were identical to those described in the legend to Figure 1. V is velocity expressed as μmol/mg protein.