During AMP-dependent sulfite oxidation by some sulfur bacteria, the liberation of sulfate from adenosine-5'-phosphosulfate (APS) is catalyzed by APS:phosphate adenylyltransferase (APAT). Here we report the first biochemical and genetic characterization of APAT. We isolated this enzyme from the chemolithoautotrophic Thiobacillus denitrificans and cloned the corresponding gene. The enzyme is homodimeric with 41,387-Da subunits and exhibits a specific activity of 2100 μmol min⁻¹ mg⁻¹. The Kₘ values are Kₘ(APS) = 300 μm and Kₘ(P₃) = 12 μm. Catalysis occurs by a ping-pong mechanism with a covalently bound AMP as reaction intermediate. The arsenolysis of APS, but not of ADP, CDP, GDP, UDP, or IDP, is also catalyzed, indicating a specific and unidirectional function. The former enzyme name ADP-sulfurylase implies that the reverse reaction is catalyzed; therefore, this name should not be used any longer. Histidine modification of APAT results in complete inactivation that can be suppressed by substrate addition. APAT is highly similar to galactose-1-phosphate uridylyltransferase and also related to Ap₄A phosphorolyase. Active site residues of galactose-1-phosphate uridylyltransferase are conserved in APAT and Ap₄A phosphorolyase, suggesting a histidine as the nucleotide-binding residue in all three enzymes, which together form a new family of nucleotidylyltransferases.

Many bacteria are able to oxidize reduced sulfur compounds such as sulfide or thiosulfate to feed electrons into photosynthetic or respiratory electron transport (1, 2). Two sulfite oxidation pathways may play a role in this dissimilatory oxidative sulfur metabolism as follows: (a) direct oxidation of sulfite to sulfate by sulfite:acceptor oxidoreductase (EC 1.8.2.1), and (b) indirect AMP-dependent oxidation of sulfite to sulfate via the intermediate APS.¹ In the latter pathway APS is formed from sulfite and AMP by the enzyme APS reductase (EC 1.8.9.92).

¹ This work was supported in part by the Deutsche Forschungsgemeinschaft Grant Di 351/1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) 148553.

‡ Supported by a fellowship from the Cusanuswerk.

§ To whom correspondence should be addressed. Tel.: 49 228 732119; E-mail: ChDahl@uni-bonn.de.

The abbreviations used are: APS, adenosine-5'-phosphosulfate; APAT, APS:phosphate adenylyltransferase; Ap₄A, bis(5'-adenosyl)tetraphosphate; DEPC, diethylpyrocarbonate; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ion-acting in reverse. Sulfate is released from APS in a second step either by ATP sulfurylase (EC 2.7.7.4) or by APS:phosphate adenylyltransferase (APAT) (3, 4). The AMP moiety of APS is transferred either to pyrophosphate by ATP sulfurylase or to phosphate by APAT, resulting in the formation of ATP or ADP, respectively. Since ADP can be converted to ATP and AMP by adenylate kinase, both sulfate-liberating enzymes catalyze substrate phosphorylations that may be of energetic importance, especially in chemolithoautotrophic bacteria (5). Significant APAT activity has been reported from various chemotrophic and phototrophic sulfur bacteria (5–10). However, in contrast to ATP-sulfurylase, APAT has never been purified from a bacterium, and a corresponding gene has never been cloned. This led some authors (11) to suggest that APAT does not exist as an enzymatic entity. Observed activities were tentatively explained by the occurrence of side reactions of other enzymes or by incorrect enzyme assays (11, 12). To examine the existence and properties of APAT, we studied the activity from Thiobacillus denitrificans biochemically and genetically.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**Tb. denitrificans strain RT (DSMZ 807) was grown anaerobically on thiosulfate and nitrate as described elsewhere (13). Cells were harvested at late exponential growth phase and kept frozen at —70 °C until use.

**Synthesis of APS—**APS was synthesized from AMP, sulfite, and ferricyanide using APS reductase activity of Tb. denitrificans crude extracts by a method essentially as described elsewhere (14) but modified to obtain salt-free APS after only one chromatographic step. APS synthesis was terminated by boiling for 10 min. Precipitated protein was removed by centrifugation (17,540 × g, 4 °C, 30 min). In 5-ml aliquots the supernatant was loaded onto a 95 × 3-cm G-25 gel filtration column equilibrated with water (flow rate, 1 ml min⁻¹). APS eluted after a yellow ferricyanide peak and before AMP. Fractions were tested by thin layer chromatography for purity (14). AMP-free fractions of APS were pooled. The APS concentration was determined photometrically (ε₂₅₀ = 15.4 mM⁻¹ cm⁻¹) and enzymatically in APAT assays coupled with pyruvate kinase and lactate dehydrogenase (see below).

**Activity Assays—**APAT activity was measured in a coupled photometric assay system. The assay contained 50 mM Tris-HCl, pH 7.6, 50 mM potassium phosphate, pH 7.6, 400 μM APS, 1 mM sodium phophoenolpyruvate, 200 μM NADH, 2 mM MgCl₂, 10 units of pyruvate kinase, 10 units of lactic acid dehydrogenase, and 10 μl of the tested protein solution in a final volume of 0.5 ml. The activity was derived from the velocity of NADH oxidation (ε₃₄₀ = 3.3 mM⁻¹ cm⁻¹). Since no interfering NADH oxidase activity was detectable, the reaction was started by addition of the protein tested. Start by addition of phosphate or APS gave identical velocities. For determination of the optimum pH, the mixture contained 100 mM Tris glycine buffer of the desired pH, Kᵥ values and Vᵥₘₐₓ values for saturating second substrate concentrations were determined from initial velocities at varied pH.

**Printed in U.S.A.**

This paper is available online at http://www.jbc.org
APS-Phosphate Adenylyltransferase from T. denitrificans

graphically according to Cleland (15). APAT was also assayed by thin layer chromatography after incubation of protein with 50 mM Tris-HCl, pH 7.6, 50 mM potassium phosphate, pH 7.6, and 400 μM APS at 30 °C. Chromatograms were developed with isopropyl alcohol:ammonia (33%): water (6:3:1) on F254 silica gel sheets (Merck). This test was especially useful when comparing APAT activities after gel filtration. APAT was eluted in a 400-ml gradient of 0–400 mM NaCl in 20 mM Tris·HCl, pH 7.6, in 150 mM NaCl using a flow rate of 0.5 ml min⁻¹. APAT eluted at 10–20% saturation. Active fractions were pooled, dialyzed against 20 mM Tris·HCl, pH 7.6, and loaded on a Mono Q 5/5 column (calibration standards, Roche Molecular Biochem). The sample (1 ml of purified APAT) was concentrated immediately after the gel filtration step to a final volume of 50 μl (Amicon Centricon system, 10-kDa cut off). This concentration step stabilized enzymatic activity and allowed detection of the enzyme by matrix-assisted laser desorption/ ionization time of flight-mass spectroscopy (MALDI-TOF-MS). MALDI-TOF-MS was carried out using a Voyager RP Workstation (Perkin-Elmer). The sample (~1–3 μl) was 10-fold diluted with 0.5% trifluoroacetic acid, thereafter 1:1 mixed with 110 mM sinapinic acid (3.5-dimethoxy-4-hydroxycinnamic acid) in 0.1% trifluoroacetic acid, 67% acetonitrile, and air-dried. Measurements were carried out in a linear mode. Further parameters are given in the legend of the corresponding figure. For detection of the enzyme-AMP intermediate the sample was incubated with 17 μM APS for 5 min at room temperature and treated as described above.

**RESULTS**

**Purification Results and Some Molecular Properties**

The purification data are summarized in Table I. The gel filtration purification step indicated a native molecular mass of 82 ± 8 kDa. SDS-PAGE analysis of fractions after gel filtration showed a single band at 41 ± 1 kDa which correlated in its intensity exactly with the elution peak absorption and APAT activity and therefore was identified as APAT (Fig. 1). Native and subunit molecular weights strongly suggest a homodimeric structure of APAT. The purified enzyme exhibited a specific activity of 2100 units mg⁻¹ under standard assay conditions. All further kinetic analysis and all experiments on the reactivity of the enzyme under various conditions were done using purified APAT which was stabilized with 5 mg ml⁻¹ bovine serum albumin.

**Kinetic Analysis** — The optimum pH for catalysis was at pH 8.5–9.0. At higher pH some precipitation occurred. To ensure high activity of APAT the coupled enzyme all further kinetic analyses were carried out at pH 8.0. Variation of one substrate concentration at several fixed second substrate concentrations resulted in activities that gave a set of parallel lines in Lineweaver-Burk plots (Fig. 2). This finding strongly indicated a Ping Pong Bi Bi reaction mechanism that involves the formation of a stable enzyme-bound reaction intermediate.
from reaction with the first substrate (APS) before binding of the second substrate (Pi). The $K_m$ values are $K_m(\text{APS}) = 5300$ M and $K_m(\text{Pi}) = 12$ mM at saturated second substrate concentrations. Due to the ping-pong mechanism significantly lower apparent $K_m$ values are observed at unsaturated second substrate concentrations. The theoretical $V_{\text{max}}$ of the reaction can be estimated to be at 3850 units mg$^{-1}$ at optimum pH. Divalent cations in the assay (1 mM MgCl$_2$ or 1 mM MnCl$_2$) did not affect activity of the enzyme. A 1-h preincubation of the enzyme with trace metals had no or slightly negative effects on activity. The following metals (1 mM) were tested (remaining activity): CoCl$_2$ (98%), CuCl$_2$ (100%), FeCl$_2$ (86%), MnCl$_2$ (96%), NiCl$_2$ (95%), or ZnCl$_2$ (89%).

Identification of the Reaction Intermediate—The purified enzyme was subjected to MALDI mass spectrometry. The enzyme molecular mass was determined with this method to be 41,376 ± 20 Da (Fig. 3). After incubation of the enzyme with APS (see “Experimental Procedures”), the main mass peak decreased in parallel to an increase of a new peak of 329 Da at second substrate saturation and, the slope corresponds to $K_m/V_{\text{max}}$.

### Table I

| Method                  | Activity | Total activity | Protein | Specific activity | Relative purity | Yield  |
|-------------------------|----------|----------------|---------|-------------------|-----------------|--------|
| 17,540 × g supernatant | 48.5     | 1940           | 36.8    | 1.32              | 1.00            | 100    |
| Soluble fraction        | 43.6     | 1744           | 20.2    | 2.16              | 1.6             | 90     |
| Membrane fraction       | 13.9     | 556            | 13.5    | 1.03              |                 |        |
| DEAE-Sephacel           | 49.7     | 919            | 7.4     | 6.72              | 5.1             | 47     |
| >40% (NH$_4$)$_2$SO$_4$ | 22.3     | 714            | 1.86    | 12.0              | 9.1             | 37     |
| Phenyl-Sepharose        | 13.9     | 528            | 0.38    | 36.6              | 27              | 27     |
| Mono P                  | 76.8     | 346            | 0.12    | 640               | 485             | 18     |
| Superdex 200            | 21       | 231            | 0.01    | 2100              | 1591            | 12     |

**Fig. 1.** A, elution profile of a Superdex 200 gel filtration (solid line, $A_{280}$ nm; dashed line, activity); B, SDS-PAGE analysis of APAT-containing fractions (maximum activity in fraction 26).

**Fig. 2.** Ping-Pong kinetics of APAT reaction (Lineweaver-Burk plots). A, plots of $1/v$ against $1/[\text{APS}]$ at several fixed [Pi] concentrations (2, 5, 10, and 50 mM); B, intercept replot of $1/V_{\text{max}}$ at the various fixed [Pi] against $1/[\text{Pi}]$; C, plots of $1/v$ against $1/[\text{Pi}]$ at several fixed APS concentrations (80, 120, 170, 450, and 900 μM); D, intercept replot of $1/V_{\text{max}}$ at the various fixed [APS] against $1/[\text{APS}]$. Data points in A and C correspond to average values of two measurements. All assays were carried out at 30 °C and started by addition of 10–100 ng of APAT after 3 min of preincubation of the assay mixture in the cuvette. The activity was calculated from 20- to 120-s time intervals. The ordinate of the secondary plots (B and D) is crossed at the reciprocal of $V_{\text{max}}$ at second substrate saturation, and the slope corresponds to $K_m/V_{\text{max}}$.
Reversibility of the Catalysis—In order to determine the reactivity of the enzyme with the reaction product ADP and with other NDPs, we carried out arsenolysis experiments. Na₂HAsO₄ is a phosphate analogue that should be able to react with the putative enzyme-AMP intermediate. The resulting phospho-arsenate anhydride is chemically unstable and immediately hydrolyzes to AMP and arsenate. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.

Although an enzyme-AMP intermediate formation with ADP as a substrate was not detectable, ADP inhibited the reaction with APS. By thin layer chromatographic analysis, we found that half-maximum inhibition in assays containing 5 mM Pi and 400 μM APS was reached at 5–10 mM ADP. Traces of AMP were present in the ADP inhibition assays (commercially available ADP had to be used without further purification to reach the concentrations required for inhibition). AMP alone has a comparatively low effect on enzyme activity (21). Sulfate inhibits activity of the enzyme with the reaction product ADP and with AMP. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.

Although an enzyme-AMP intermediate formation with ADP as a substrate was not detectable, ADP inhibited the reaction with APS. By thin layer chromatographic analysis, we found that half-maximum inhibition in assays containing 5 mM Pi and 400 μM APS was reached at 5–10 mM ADP. Traces of AMP were present in the ADP inhibition assays (commercially available ADP had to be used without further purification to reach the concentrations required for inhibition). AMP alone has a comparatively low effect on enzyme activity (21). Sulfate inhibits activity of the enzyme with the reaction product ADP and with AMP. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.

Although an enzyme-AMP intermediate formation with ADP as a substrate was not detectable, ADP inhibited the reaction with APS. By thin layer chromatographic analysis, we found that half-maximum inhibition in assays containing 5 mM Pi and 400 μM APS was reached at 5–10 mM ADP. Traces of AMP were present in the ADP inhibition assays (commercially available ADP had to be used without further purification to reach the concentrations required for inhibition). AMP alone has a comparatively low effect on enzyme activity (21). Sulfate inhibits activity of the enzyme with the reaction product ADP and with AMP. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.

Although an enzyme-AMP intermediate formation with ADP as a substrate was not detectable, ADP inhibited the reaction with APS. By thin layer chromatographic analysis, we found that half-maximum inhibition in assays containing 5 mM Pi and 400 μM APS was reached at 5–10 mM ADP. Traces of AMP were present in the ADP inhibition assays (commercially available ADP had to be used without further purification to reach the concentrations required for inhibition). AMP alone has a comparatively low effect on enzyme activity (21). Sulfate inhibits activity of the enzyme with the reaction product ADP and with AMP. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.

Although an enzyme-AMP intermediate formation with ADP as a substrate was not detectable, ADP inhibited the reaction with APS. By thin layer chromatographic analysis, we found that half-maximum inhibition in assays containing 5 mM Pi and 400 μM APS was reached at 5–10 mM ADP. Traces of AMP were present in the ADP inhibition assays (commercially available ADP had to be used without further purification to reach the concentrations required for inhibition). AMP alone has a comparatively low effect on enzyme activity (21). Sulfate inhibits activity of the enzyme with the reaction product ADP and with AMP. This AMP formation can be detected by thin layer chromatography. In a control experiment APS rapidly underwent arsenolysis, indicating the functionality of the assay system (Fig. 4). To our surprise, no reaction could be observed with ADP (Fig. 4). Other NDPs (GDP, UDP, CDP, and IDP) also did not react (data not shown). Even after 24 h of incubation arsenolysis was not detectable, although the enzyme still was active (tested by arsenolysis of APS). Obviously the enzyme-AMP complex cannot be formed from NDP substrates with significant rates. Although a back reaction should be possible in any enzyme-catalyzed reaction, its rate must be infinitely slow in the case of APAT so that the catalysis can be regarded as unidirectional. Therefore the former name “ADP-sulfurylase” is misleading and should not be used any longer.
a protein with very high homology to the E. coli Ras-like protein (Era, BLAST P E value, $2 \times 10^{-79}$, release 2.0.6, see Ref. 22). Era is an essential G-protein that probably is involved in regulation of growth (23, 24). The distance between era and apt (775 base pairs) and the existence of several AT-rich stretches in this region strongly suggest that apt and era transcription are not coupled. Various putative promoters are found upstream of apt. Therefore detailed genetic analyses are necessary to identify the functional promoter region. Downstream of apt a rho-independent termination signal could not be identified in the remaining sequence of the cloned fragment (238 base pairs). It therefore cannot yet be excluded that downstream genes are transcriptionally coupled with apt. Further genetic analysis is in progress. Apt codes for a 41,387-Da protein of 370 residues (without the N-terminal methionine, which is cleaved off). This mass is in agreement with SDS-PAGE and MALDI results (see above). The calculated isoelectric point of the protein is at pH 6.0 (ProtParam, ExPASy home page).

Related Enzymes and Substrate Specificity—Available gene banks were screened for proteins with similarity to APAT (BLAST P, release 2.0.7). The sequence of APAT shows high homology to galactose-1-phosphate uridylyltransferases (GPUT, EC 2.7.7.12) from bacteria, archaea, fungi, plants, and animals (best E value: $3 \times 10^{-27}$ for Thermotoga neapolitana, E value for E. coli-GPUT: $5 \times 10^{-12}$). GPUT catalyzes the UMP transfer from UDP-glucose to galactose 1-phosphate. Lower homology exists to all known Ap4A phosphorylases (EC 2.7.7.-) that catalyze the NMP transfer from bis(5'-nucleosidyl)tetraphosphate dinucleotides to phosphate (see Ref. 25; best E value, 0.32 for Saccharomyces cerevisiae Ap4A phosphorylase 1). All three related enzymes have in common catalysis of NMP transfers to phosphate groups. Catalysis by Ap4A phosphorylase is not very specific; the yeast enzyme reversibly catalyzes reactions also a phosphate exchange on NDPs, and very interestingly the arsenolysis and phosphorolysis of various dinucleotides and in unphysiological reactions also a β-phosphate exchange of NDPs, the arsenolysis of NDPs, and very interestingly the arsenolysis and phosphorolysis of APS (25, 26). For that reason we tested the ability of APAT to catalyze reactions with Ap4A. No catalysis was found with 10 mM Ap4A neither in a phosphorolysis nor in a hydrolysis reaction. Addition of divalent cations (1 mM MgCl2 or CaCl2, 10 mM of CoCl2, CuCl2, FeCl3, MnCl2, NiCl2, or ZnCl2) did not result in any qualitatively detectable reactivity. We also tested the reactivity of GPUT from yeast (Sigma) and found that it neither reacted with Ap4A nor with APS. A summary of the results (see above). Other residues that are strictly conserved in all sequences also may play important roles in nucleotide binding (in analogy to GPUT, Ref. 30). The carbonyl group of His-172 (E. coli His-164) probably interacts with the His-174 (E. coli His-166) imidazole during catalysis, and it can be postulated that Asn-161 and Gln-176 (E. coli Asn-153 and Gln-168) bind to the α-phosphate (Fig. 6B). The structure is probably stabilized

![Figure 5](image-url)
is based on the 15 GPUT sequences and 4 Ap4A phosphorylase sequences available in on-line gene banks. Positions with identical or very similar residues in at least two different enzymes are marked with a broad arrow. Conserved differences between the three enzymes are found at three positions as indicated.

![Fig. 6 A, ClustalW alignment of Tb. denitrificans APAT and E. coli GPUT](image)

**DISCUSSION**

High APAT activity was present in crude extracts of *Tb. denitrificans* strain RT, and the responsible enzyme could be purified to homogeneity to allow further characterization and cloning of the corresponding gene. Results obtained from kinetic analysis, arslenoanalysis, molybdolysis, and MALDI experiments suggest the following overall reaction scheme.

\[
E + APS \rightarrow E.APS \rightarrow E.AMP \rightarrow E.ADP \rightarrow E + ADP
\]

where \(S\) indicates sulfate. Phosphorylase of the enzyme-bound intermediate is irreversible since the intermediate cannot be formed from ADP.

APAT exhibits high sequence similarity to GPUT, which is also a homodimer of a very similar size (28). GPUT introduces galactose-phosphate into the sugar metabolism by transferring an UMP to its phosphate group. The product UDP-galactose can be epimerized to UDP-glucose which can be further metabolized. The reaction mechanism of both enzymes involves the formation of a covalent bond between a conserved active center histidine and a nucleotide. For GPUT this has been shown by x-ray analysis (30). We propose the same mechanism for APAT based on several lines of evidence. 1) The ping-pong kinetics of APAT suggests a tightly enzyme bound reaction intermediate (Fig. 2). 2) MALDI measurements are in agreement with the formation of a covalent enzyme-AMP intermediate (Fig. 3). 3) Histidine modification resulted in a complete loss of activity that was prevented by substrate addition (Fig. 5). 4) Sequence comparisons strongly suggest that the active site of APAT resembles that of GPUT, especially in a highly conserved region that contains the active histidine and some other intermediate stabilizing residues (Fig. 6).

In former modification studies with crude APAT preparations from other sources, only cysteine-modifying agents were tested (7). Complete inactivation was not achieved by those agents, and it has to be considered that N-ethylmaleimide, which resulted in highest inhibition (80%), also modifies histidines with a slower rate (34). However, it cannot be excluded that different types of APAT exist in organisms other than *Tb. denitrificans* and that cysteines may play more important roles in these enzymes.

**SCHEME 1**

Based on sequence similarities, the reaction mechanism of APAT and GPUT also has to be postulated for Ap4A phosphorylase. In agreement with this proposal, ping-pong kinetics have been observed for AP4A phosphorylase of *Ap4A phosphorylase* (35). Ap4A phosphorylase from yeast is the only other purified enzyme with APAT activity (26) and catalyzes this reaction as an unphysiological side reaction which probably enzymes that p-chloromercuribenzoic acid modification can lead to steric inhibition even when nonessential residues are modified (33). The Cys-160 of the active center from *E. coli* GPUT is not conserved among GPUT sequences and does not occur in the known sequences of Ap4A phosphorylases and APAT.

Beside the homologies mentioned above, a conspicuous stretch of eight identical residues exists near the C terminus of APAT (Glu-360 to Arg-367) and *E. coli* GPUT (Glu-329 to Arg-336). These residues form an α-helix in GPUT which is neither close to the active center nor to the subunit interface (Rutgers University Protein Data Bank code 1HXQ, Ref. 30). The function of this conserved structure is not clear yet.
occurs because of the similarity of Ap₄A phosphorylase to authentic APAT. In yeast there are two isoforms of Ap₄A phosphorylase. It was shown by studies on mutants that Ap₄A phosphorylase 1 is responsible for 85% of the APAT activity in this organism (36). Ap₄A phosphorylase 2 is suggested to cause the remaining 15% activity (36). This is supported by the higher similarity of bacterial APAT to type 1 Ap₄A phosphorylase (Blast P E value, 0.35) compared with Ap₄A phosphorylase 2 (Blast P E value 8.1). Ap₄A phosphorylase probably does not need to be more specific, because in yeast APS is formed only under sulfate assimilation conditions by a highly regulated ATP-sulfurylase (37). In addition, a substrate shuttle mechanism may transfer APS from ATP-sulfurylase to APS kinase, similar to the case of bifunctional sulfate-activating enzymes in higher eukaryotes (38). It is therefore unlikely that APS serves as a substrate for Ap₄A phosphorylase in yeast. Interestingly, Ap₄A phosphorylase catalyzes the arsenolysis of both ADP and ATP (39). For that reason the reversibility of the APAT reaction by Ap₄A phosphorylase has to be considered as possible, although energetically unfavorable. However, the enzyme-AMP complex of Ap₄A phosphorylase might not react with sulfate, and in this case the APAT reaction of Ap₄A phosphorylase may also be irreversible. Very interesting is the observation that Ap₄A phosphorylase, similar to APAT, is sensitive against arginine-modifying agents (39). In both cases substrate binding has some effect on inactivation kinetics. However, it seems possible that the inactivation by arginine modification may not be due to a functional role of arginine in the catalytic center.

The three enzymes Ap₄A phosphorylase, GPUT, and APAT belong to a new family of nucleotidyltransferases. For evaluation of former and future studies, it has to be considered that low specific activities of APAT in crude extracts (below ~100 milliunits mg⁻¹) can be due to Ap₄A phosphorylase or other enzymes of this family and therefore should not be overinterpreted. Higher activities (above ~100 milliunits mg⁻¹), which have up to now only been detected in Thiocapsa roseopersicina (4), Thiobacillus thioparus (5), and Tb. denitrificans (this study), are likely to be due to a specific APAT.

Within the last years the arrangement of APAT into a cytoplasmic sulfite oxidation pathway was generally doubted (11, 12). Tb. denitrificans is an organism for which it has been postulated that APAT functions in a pathway by which sulfite produced by a cytoplasmic reverse siroheme sulfite reductase is further oxidized to sulfate (40). This pathway involves APS reductase, which produces APS from sulfite and AMP (41). The formation of APS allows coupling of sulfate liberation to a substrate phosphorylation step. We are convinced that Thiobacillus APAT is involved in this sulfate liberation for various reasons as follows: 1) no other substrate than APS is known for Thiobacillus APAT; 2) the reverse reaction is not catalyzed; 3) the enzyme is present with high activities; 4) the enzyme is present in the same compartment as APS and therefore the reaction must take place if APS is not protected somehow (which is very unlikely at high turnover rates).

Interestingly, ATP-sulfurylase exists in the same organism during growth on reduced sulfur compounds. All other known bacteria with high APAT activity also simultaneously contain high ATP-sulfurylase activity (Te. roseopersicina see Ref. 10, Tb. thioparus see Ref. 5). We suggest the following model which may explain this finding at least for chemotrophs: ATP-sulfurylase activity is limited by pyrophosphate availability, since pyrophosphate is hydrolyzed by pyrophosphatase and used up by ATP-sulfurylase. APAT activity may allow a higher APS turnover and could thereby prevent the accumulation of toxic sulfite in the cytoplasm. Since ATP-sulfurylase is known to have a very high affinity for APS (Kₘ(APS) = 6.6 µM, see Ref. 11) and pyrophosphate (Kₘ(PP) = 14 µM, see Ref. 11), this enzyme is efficiently employed even in the presence of APAT as long as pyrophosphate is available. Note that for APAT the apparent Kₘ values are higher (the Kₘ(APS) is ~55 µM with 2 mM P₄ and the Kₘ(PP) is ~1.7 mM with 80 µM APS). APAT therefore could prevent accumulation of APS without interfering with ATP-sulfurylase. By this mechanism a maximum energy conservation by both enzymes, ATP-sulfurylase and APAT, is guaranteed which could be of importance for chemolithoautotrophic growth of Tb. denitrificans.

Not only in Tb. denitrificans, but also in other sulfur compound oxidizing chemotrophic and phototrophic bacteria, sulfite may be formed in the cytoplasm. In those cases two scenarios for sulfite oxidation are possible (Fig. 7) as follows: sulfite can be either oxidized in the cytoplasm via the APS pathway or extruded into the periplasmic space where it is oxidized by sulfite:acceptor oxidoreductase (Fig. 7, A–C). Sulfite:acceptor oxidoreductases from various thiobacilli are known to be c-type cytochromes and therefore a periplasmic localization can be assumed (42). When the cytoplasmic APS pathway is employed, toxic sulfite accumulation in the cytoplasm may be prevented by APAT as rationalized above. We propose a mixture of both scenarios for those sulfur compound

---

2 T. Brüser, unpublished results.
oxidizing organisms that contain APS reductase and ATP sulfurylase but no APAT; in those cases accumulating sulfite could be extruded to the periplasm when ATP-sulfurylase is limited by low pyrophosphate availability (Fig. 7B).

After the herein reported biochemical and genetic analyses of Thiobacillus APAT, future studies will have to concentrate on the relation to other APATs and on the physiological role of APATs. The proposal of the various sulfite oxidation pathways will hopefully support such studies.

Acknowledgments—We thank R. Deutzmann (Regensburg) for automated Edman degradation of APAT. We are grateful to R. K. Thauer and W. Buckel for supporting MALDI measurements at the Max Planck Institute for Terrestrial Microbiology (Marburg). We particularly thank H. G. Trüper for discussions and encouragement throughout the work.

REFERENCES

1. Brune, D. C. (1995) in Anoxygenic Photosynthetic Bacteria (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., eds) pp. 847–870, Kluwer Academic Publishers Group, Dordrecht, Netherlands
2. Kelly, D. P., Shergill, J. K., Lu, W.-P., and Wood, A. P. (1997) in Variations in Autotrophic Life (Skirely, J. M., and Barton, L. L., eds) pp. 121–146, Academic Press, London
3. Smith, D. W., and Strohl, W. R. (1991) in Enrichment and Characterization of the ADP Sulfurylase from Thiobacillus denitrificans RT. Diploma thesis, University of Bonn, Germany
4. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
5. March, P. E., Lerner, C. G., Ahn, J., Cui, X., and Inouye, M. (1988) Oncogene 2, 539–544
6. Lin, Y. P., Sharer, J. D., and March, P. E. (1994) J. Bacteriol. 176, 44–49
7. Plateau, P., Fromant, M., Schmitter, J.-M., and Blanquet, S. (1998) J. Bacteriol. 172, 6892–6899
8. Ruzicka, F. J., Wedekind, J. E., Kim, J., Rayment, I., and Frey, P. A. (1995) Biochemistry 34, 5610–5617
9. Wedekind, J. E., Frey, P. A., and Rayment, I. (1995) Biochemistry 34, 11049–11061
10. Higgins, D. G., and Sharp, P. M. (1989) Gene (Amst.) 73, 237–244
11. Wedekind, J. E., Frey, P. A., and Rayment, I. (1996) Biochemistry 35, 11560–11569
12. Thoden, J. B., Ruzicka, F. J., Frey, P. A., Rayment, I., and Holden, H. M. (1997) Biochemistry 36, 1212–1222
13. Adams, C. A., and Nicholas, D. J. D. (1972) Biochem. J. 128, 647–654
14. Caligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. (1995) Current Protocols in Protein Science, John Wiley & Sons, New York
15. Nicholls, R. G. (1977) Biochem. J. 165, 149–155
16. Plateau, P., Fromant, M., Schmitter, J.-M., Buhler, J.-M., and Blanquet, S. (1989) J. Bacteriol. 171, 6437–6445
17. Marzluf, G. A. (1997) Annu. Rev. Microbiol. 51, 73–96
18. Schwartz, N. B., Lyle, S., Oxer, J. D., Li, H., Dayrup, A., Ng, K., and Westley, J. (1998) Chem. Biol. Interact. 109, 143–51
19. Robinson, A. K., and Barnes, L. D. (1991) Biochem. J. 270, 135–139
20. Schneider, M., and Trupper, H. G. (1979) Biochim. Biophys. Acta 568, 454–467
21. Bowen, T. J., Huppol, F. C., and Taylor, B. F. (1966) Biochim. Biophys. Acta 118, 566–576
22. Hooper, A. B., and DiSpirito, A. A. (1985) Microbiol. Rev. 49, 140–157