

## The Presence of an Iron-Sulfur Cluster in Adenosine 5'-Phosphosulfate Reductase Separates Organisms Utilizing Adenosine 5'-Phosphosulfate and Phosphoadenosine 5'-Phosphosulfate for Sulfate Assimilation\*

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It was generally accepted that plants, algae, and phototrophic bacteria use adenosine 5'-phosphosulfate (APS) for assimilatory sulfate reduction, whereas bacteria and fungi use phosphoadenosine 5'-phosphosulfate (PAPS). The corresponding enzymes, APS and PAPS reductase, share 25–30% identical amino acids. Phylogenetic analysis of APS and PAPS reductase amino acid sequences from different organisms, which were retrieved from the GenBank<sup>™</sup>, revealed two clusters. The first cluster comprised known PAPS reductases from enteric bacteria, cyanobacteria, and yeast. On the other hand, plant APS reductase sequences were clustered together with many bacterial ones, including those from *Pseudomonas* and *Rhizobium*. The gene for APS reductase cloned from the APS-reducing cyanobacterium *Plectonema* also clustered together with the plant sequences, confirming that the two classes of sequences represent PAPS and APS reductases, respectively. Compared with the PAPS reductase, all sequences of the APS reductase cluster contained two additional cysteine pairs homologous to the cysteine residues involved in binding an iron-sulfur cluster in plants. Mössbauer analysis revealed that the recombinant APS reductase from *Pseudomonas aeruginosa* contains a [4Fe-4S] cluster with the same characteristics as the plant enzyme. We conclude, therefore, that the presence of an iron-sulfur cluster determines the APS specificity of the sulfate-reducing enzymes and thus separates the APS- and PAPS-dependent assimilatory sulfate reduction pathways.

For all living organisms, sulfur is an essential element with many different functions. It is found in reduced form in amino acids, peptides, and proteins and in iron-sulfur clusters, lipoic acid, and other cofactors and in oxidized form as sulfonate group-modifying proteins, polysaccharides, and lipids. Reduced

sulfur compounds, such as hydrogen sulfide, serve as electron donors for chemotrophic or phototrophic growth in a large and diverse group of Archae and bacteria, including purple and green sulfur bacteria (1). On the other hand, oxidized sulfur compounds such as sulfate can function as a terminal electron acceptor in respiration to support the growth of sulfate-reducing bacteria (2).

The majority of sulfur in living organisms is present in the reduced form of organic thiols. For their synthesis, inorganic sulfate is reduced and incorporated into bioorganic compounds in a pathway named assimilatory sulfate reduction. Before reduction, sulfate is activated with ATP to adenosine 5'-phosphosulfate (APS),<sup>1</sup> which can subsequently be converted into phosphoadenosine 5'-phosphosulfate (PAPS) using a second ATP. Either form of activated sulfate can be reduced to sulfite and reduced further to sulfide by sulfite reductase. Sulfide is incorporated into an activated amino acid acceptor, such as *O*-acetylserine, *O*-acetylhomoserine, or *O*-succinylhomoserine, to form cysteine or homocysteine (3–5).

The assimilatory sulfate reduction pathway is present in plants, fungi, and yeast and in a wide range of eubacteria but is missing in metazoa. It was generally accepted that chemotrophic bacteria and fungi utilize PAPS for reduction to sulfite in a reaction catalyzed by a thioredoxin-dependent PAPS reductase, whereas photosynthesizing organisms reduce APS directly (3–9). The boundary line between APS- and PAPS-utilizing organisms was not sharply defined, however, because among phototrophic bacteria and cyanobacteria, both APS- and PAPS-reducing species were described (8, 9). The plant APS reductase (APR), recently cloned from *Arabidopsis thaliana*, is a protein composed of two distinct domains: an N-terminal part is homologous to the *Escherichia coli* PAPS reductase (encoded by the *cysH* gene), and a C-terminal part is similar to thioredoxin with a function modified toward glutaredoxin (10–12). This enzyme is identical to the previously described APS sulfotransferase (13) and contains a [4Fe-4S] iron-sulfur cluster as a cofactor (14). APS reductase is a highly regulated enzyme, and it is considered to have a major control on the flux through assimilatory sulfate reduction in plants (3, 15).

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<sup>1</sup>The abbreviations used are: APS, adenosine 5'-phosphosulfate; APR, adenosine 5'-phosphosulfate reductase; PAPS, phosphoadenosine 5'-phosphosulfate; T, tesla.

However, the plant APS reductase is completely unrelated to the dissimilatory APS reductase found in both sulfate-reducing and sulfide-oxidizing bacteria and archaea (16, 17). This dissimilatory APS reductase (EC 1.8.99.2) catalyzes both the reduction of APS to sulfite and the oxidation of sulfite and AMP to APS. This enzyme is a 1:1 heterodimer of a 75-kDa FAD-binding  $\alpha$ -subunit and a 20-kDa  $\beta$ -subunit binding two [4Fe-4S] centers (16). Also, the electron paramagnetic resonance spectral properties of the iron-sulfur clusters from both types of APS reductase are completely different (14, 16).

Very recently, a third type of APS reductase was identified in several sulfate-assimilating bacteria, such as *Pseudomonas*, *Rhizobium*, *Ralstonia*, *Burkholderia*, and *Allochrochromatium vinosum* (18–20). This novel enzyme is homologous to the PAPS reductase from *E. coli* and is even more homologous to the N-terminal part of plant APS reductase. However, the enzyme is missing the C-terminal part of the plant protein and requires thioredoxin as an electron donor. The major difference between these bacterial APS reductases and PAPS reductase from *E. coli* or *Salmonella typhimurium* is the presence of two additional cysteine pairs as in the plant enzyme (Fig. 1). Three of these Cys residues bind the FeS center in the plant APS reductase (14). From this finding, two questions arise: 1) does the bacterial assimilatory APS reductase contain an iron-sulfur center? and 2) are the additional Cys residues a marker for distinguishing APS- and PAPS-dependent sulfate reduction?

#### EXPERIMENTAL PROCEDURES

**Materials**— $[^{35}\text{S}]\text{APS}$  was prepared from  $[^{35}\text{S}]\text{SO}_4^{2-}$  (Hartmann Analytic) according to Li and Schiff (21) with recombinant ATP sulfurylase from *A. thaliana*<sup>2</sup> and inorganic pyrophosphatase (Sigma) (21). Oligonucleotide primers were synthesized at Microsynth GmbH (Balgach, Switzerland).

**Phylogenetic Analysis**—The GenBank<sup>TM</sup> and The Institute for Genomic Research sequence data bases were screened with the BLAST software with the N-terminal portion of *A. thaliana* APR2 as a query sequence. The sequences were aligned by using the CLUSTALW program. The phylogenetic analysis was performed with the Treecon software (22). The tree was constructed by the neighbor-joining method (23) using the Dayhoff matrix. Protein parsimony analysis was performed with the PHYLIP software (24).

**Enzyme Assays**—APS and PAPS reductase activities were measured as production of  $[^{35}\text{S}]\text{sulfite}$ , assayed as acid volatile radioactivity, formed in the presence of 75  $\mu\text{M}$   $[^{35}\text{S}]\text{APS}$  or  $[^{35}\text{S}]\text{PAPS}$ , respectively, and 4 mM dithioerythritol and 4.5  $\mu\text{g}$  of recombinant thioredoxin m from spinach as reductants (25). The protein concentrations were determined with the Bio-Rad kit, with bovine serum albumin as a standard. The measurements were performed in duplicates with two independent protein preparations. The data are presented as means  $\pm$  S.E.

**Protein Overexpression in *E. coli***—The PAPS reductase of *E. coli* (26) and APS reductases of *Pseudomonas aeruginosa* (27) and *Rhizobium meliloti* (18) were overexpressed in *E. coli* BL21(DE3) strain by the pET14b expression system and purified with the His-Tag<sup>®</sup> system (Novagen) according to the manufacturer's instructions. For the preparation of  $^{57}\text{Fe}$ -labeled *P. aeruginosa* APR *E. coli* harboring the expression construct was grown in M9 medium containing 0.4% glucose in which  $^{56}\text{Fe}$  was replaced by  $^{57}\text{Fe}$ . Metal foil consisting of  $^{57}\text{Fe}$  (94.7% enrichment; Glaser, Basel, Switzerland) was dissolved in HCl, neutralized, and added to the culture medium at a final  $^{57}\text{Fe}$  concentration of 20  $\mu\text{M}$ .

**Cloning of APS Reductase from *Plectonema***—DNA was isolated from 0.5 g of *Plectonema* strain 73110 cells according to the standard procedures (28). The major part of the APR coding region was amplified from the DNA by PCR with primers derived from regions conserved in plant APR and bacterial PAPS reductase sequences as described by Suter *et al.* (13). The PCR product was cloned by the TA cloning kit (Invitrogen), and three independent inserts were completely sequenced on both strands. The sequence was deposited in GenBank<sup>TM</sup> under accession number AF214038.

**Determination of Iron**—The iron content of the proteins was estimated by spectrophotometry after reaction with tripyridyl-s-triazine (29). The measurements were performed in duplicates with two inde-

pendent protein preparations. The data are presented as means  $\pm$  S.E.

**Electronic Spectra**—UV-visible spectra were recorded on a Lambda 16 Instrument (PerkinElmer Life Sciences) equipped with a temperature-controlled cell compartment.

**Electron Paramagnetic Resonance**—Electron paramagnetic resonance spectra (X-band, 9.5 GHz) were recorded on the ESP 300 spectrometer (Bruker) and evaluated as described previously (30). The temperature was maintained with the Helitran system (Air Products).

**Mössbauer Spectroscopy**—Mössbauer spectra were recorded using a conventional spectrometer in the constant acceleration mode. Isomer shifts are given relative to  $\alpha$ -Fe at room temperature. The spectra obtained at 20 mT were measured in a bath cryostat (Oxford MD 306) equipped with a pair of permanent magnets. For the high-field spectra, a cryostat equipped with a superconducting magnet was used (Oxford Instruments). Magnetically split spectra were simulated within the spin Hamiltonian formalism (31); otherwise, spectra were analyzed by least-square fits using Lorentzian line shape.

#### RESULTS

**Evolutionary Relationships of APS and PAPS Reductase**—To characterize the evolutionary relationships among APS- and PAPS-reducing enzymes, we used the sequence of the N-terminal domain of APR2 from *A. thaliana* to retrieve related sequences from the GenBank<sup>TM</sup> and The Institute for Genomic Research databases by the BLAST program. All of these proteins are characterized by a highly conserved (KRT)ECG(LI)H motif (Fig. 1) containing a catalytically active Cys residue (32, 33). In addition, a number of related proteins of unknown function were found in several archaeobacteria, including *Methanococcus jannaschii*, *Pyrococcus horikoshii*, and *Pyrococcus abyssi*. These large proteins contained segments of  $\sim$ 200 amino acids that were 22–27% identical with both plant APS reductases and PAPS reductase of *E. coli* preceded by a 150–200-amino acid domain with no homology to other proteins. Because these proteins did not contain the essential (KRT)ECG(LI)H motif, and because the APR-similar domain showed a 20–25% identity also with the *CysD* subunit of ATP sulfurylase from enteric bacteria, these archaea proteins were not included in the analysis.

Fig. 2 shows a neighbor-joining tree of APS and PAPS reductase-related sequences. The phylogenetic tree is divided into two major branches. The first branch contains a cluster of APS reductases from plants and algae, together with several bacterial enzymes; the other one was subdivided into clusters comprising fungal PAPS reductases and well-characterized PAPS reductases from enteric bacteria and cyanobacteria. However, such a tree topology does not reflect the phylogenetic relationships based on the 16 S rRNA genes. Only the Gram-positive bacteria, fungi, and  $\beta$ -proteobacteria appeared to be monophyletic. In contrast, two species of  $\alpha$ -proteobacteria were found outside of the major  $\alpha$ -proteobacterial group clustered with  $\beta$ -proteobacteria and *A. vinosum*, a  $\gamma$ -proteobacterium. Only  $\gamma$ -proteobacteria could be found in both major branches of the phylogenetic tree. The sequences of the new bacterial assimilatory APS reductases from *Rhizobium*, *Ralstonia*, *Burkholderia*, and *Pseudomonas* were all positioned on the major cluster containing the plant APS reductases. Also, the sequence of CysH from *Acidithiobacillus ferrooxidans*, which most probably encodes an APS reductase (34), was found in this cluster. Although the overall bootstrap support of the neighbor-joining tree topology was only around 50%, phylogenetic analysis using the protein parsimony method resulted in a maximum parsimony tree with an almost identical topology (data not shown). We hypothesized, therefore, that the nod separating the two major branches in this phylogenetic tree represents the border between APS- and PAPS-reducing species, respectively.

To test this hypothesis, we cloned and sequenced part of the gene for putative APS reductase from an APS-reducing cyanobacterium, *Plectonema* strain 73110 (8). We predicted that

<sup>2</sup> S. Kopriva, unpublished observations.

APR2	ASTLIAPEVEEEKGGEVEDFEQLAKKLEDASPLEIMDKALERFGDQIAIAFSGAEDVALIEYARLTG	66
P_AERUG	MPPFATIPATERNSSAAQHQDPSMSPQFDLPALASSLADKSPQDILKAAFEHFHGDDELWISFSGAEDVVLVDMAWKLN	77
E_COLI	MSKLDLNLALNELPKVDRILALAEETNAELEKLDAGRVAWALDNLPGEYVLSSSF-----GIQAAVSLHLVNIQIR	69
	* * * * *	
	↓ ↓	
APR2	KPFRVFLDTGRLNPETYRLFDAVEKQYGI RIEYMPDAVEVQALVRNKGFLSFYEDGHQECCRVKVRPLRRALKGLKA-	146
P_AERUG	RNVKVFSLDTGRLHPETYRFIDQVREHYGIAIDVLS PDRLLLEPLVKEKGLFSFYRDGHGECCGIRKIEPLKRKLAGVRA-	157
E_COLI	PDIPVILTDTGYLFPETYRFIDELTDKLLKLNKLYRATESAAWQEARYGKLEWEGQVEGIEKYNDINKVEPMNRALKELNAQ	150
	* *** ***** *	
	* * * * *	
	↓ ↓	
APR2	-WITGQRKDQSPGTRSEIPIVQVDPVFEGLDGGVGLSVKWNPLANVEGADVWNFLRTMDVPVNALHAQGYVSI GCEPCTR	226
P_AERUG	-WATGQRDQSPGTRSQVAVLEIDGAFSTPEK---PLYKFNPLSSMTSEEVWGYIRMLELPYNSLHERGYISIGCEPCTR	234
E_COLI	TWFAGLRREQS-GSRANLPVLAIQRG-----VFKVLPIDWDNRITYQYLQKHGLKYHPLWDEGYLSVGDTHHTRK	220
	* * * * * * * * * *	
APR2	VLPQGHEREGRWWEDAKAKECGLHGKNIKEEDGAADSKPAAVQEIFESNNVVALSKGGVENLLKLENRKEAWLVVLYAPW	307
P_AERUG	VLPNQHEREGRWWEEATHKECGLHAGNLISKA	267
E_COLI	WEPGMAEEETRFF---GLKRECGLHEG	244
	* * ***** *	
APR2	CPFCQAMEASYIELAEKLAGKGVKAKFRADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYPSEHRDVSLSMFSVNLRLR	453

Fig. 1. Comparison of amino acid sequences of mature APR2 from *A. thaliana*, APS reductase from *P. aeruginosa*, and PAPS reductase from *E. coli*. The sequences were aligned with the program CLUSTAL. Asterisks identify identical residues, and arrows mark the additional Cys in APS reductases. The conserved APS and PAPS reductase signature is underlined.

the position of the *Plectonema* enzyme in the phylogenetic tree would be in the same branch as plant APS reductases and assimilatory APS reductases from *Pseudomonas* and *Rhizobium*, but not as the PAPS reductases from other cyanobacteria, *Synechococcus*, and *Synechocystis*. Indeed, as shown in Fig. 2, the *Plectonema* APS reductase clusters with that of *P. aeruginosa* close to the plant APR proteins. This result indicated that, indeed, solely from the sequence of the *cysH* gene and its position in the phylogenetic tree, one could predict the sulfonucleotide specificity of the corresponding protein.

**Biochemical Characterization of APS Reductase from *P. aeruginosa***—A closer examination of the APS and PAPS reductase sequence alignment used for the phylogenetic analysis revealed that the major difference between sequences in the two branches is the presence of two strictly conserved Cys pairs in the APS-reducing enzymes (compare Fig. 1). These Cys pairs were proposed to coordinate an FeS cluster in plant APS reductases (14). It was thus plausible to expect the same function of these amino acids also in the prokaryotic enzymes. Therefore, the APS reductase from *P. aeruginosa* was overexpressed in *E. coli* by the pET expression system and purified as a yellow-brown protein, similar to the recombinant APR from *A. thaliana* (14). The recombinant protein displayed an APS reductase activity of  $2.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$  with dithioerythritol and thioredoxin m as reductants; the activity decreased to 10% when thioredoxin was omitted from the reaction mixture (Table I). The optical spectrum of the *P. aeruginosa* protein was identical to that of the APR from *Arabidopsis* (Fig. 3), indicating that it possessed the same cofactor as the plant enzyme. Accordingly,  $3.2 \pm 0.4 \text{ nmol Fe/nmol protein}$  were determined in the *P. aeruginosa* APS reductase. On the other hand, similar to previous reports (32), the recombinant PAPS reductase from *E. coli* was isolated as a colorless protein without bound iron (Fig. 3).

To prove indisputably that the *Pseudomonas* enzyme also contains the iron-sulfur cofactor, Mössbauer spectroscopy, which in experiments with the plant APR turned out to be the method of choice (14), was used. The spectra were essentially identical with those obtained with the plant protein (14). The Mössbauer spectrum of *P. aeruginosa* APR obtained at 4.2 K in a small field of 20 mT (perpendicular to the  $\gamma$ -beam; Fig. 4a) exhibited an asymmetric quadrupole doublet. This asymmetry indicates that the iron sites in the cofactor are structurally

different. Applying a strong field of 7 T (perpendicular and parallel to the  $\gamma$ -beam; Fig. 4, b and c) at 4.2 K showed that the iron sites form a diamagnetic cluster. This information, together with the isomer shift  $\delta$  and the quadrupole splitting  $\Delta E_Q$  of the asymmetric doublet, which takes the values  $\delta \sim 0.45 \text{ mm s}^{-1}$  and  $\Delta E_Q \sim 1.2 \text{ mm s}^{-1}$  (see below), strongly points to the presence of  $[4\text{Fe-4S}]^{2+}$  clusters (31). The quantitative analysis of the measured spectra was based on the assumption that, as in the plant enzyme, only three Cys residues were binding to the metal cluster (14). It was assumed that three iron sites exhibit the same  $\delta$  and  $\Delta E_Q$  values, which, however, may differ from the corresponding values of the fourth site. Thus, the fit comprises two doublets with an area ratio of 3:1.

Two different fits have been performed in view of the fact that the asymmetry of the quadrupole doublet (Fig. 4a) could be accounted for by two symmetric doublets with either (I)  $\delta_1 \sim \delta_2$ ,  $\Delta E_{Q,1} \neq \Delta E_{Q,2}$ , or (II)  $\delta_1 \neq \delta_2$ ,  $\Delta E_{Q,1} \sim \Delta E_{Q,2}$ .

With start parameters corresponding to case (I) and (II), respectively, the obtained parameter sets are as follows: (I),  $\delta_1 = 0.46 \text{ mm s}^{-1}$ ,  $\Delta E_{Q,1} = 1.02 \text{ mm s}^{-1}$  (75%),  $\delta_2 = 0.43 \text{ mm s}^{-1}$ , and  $\Delta E_{Q,2} = 1.34 \text{ mm s}^{-1}$  (25%); and (II),  $\delta_1 = 0.49 \text{ mm s}^{-1}$ ,  $\Delta E_{Q,1} = 1.09 \text{ mm s}^{-1}$  (75%),  $\delta_2 = 0.33 \text{ mm s}^{-1}$ , and  $\Delta E_{Q,2} = 1.14 \text{ mm s}^{-1}$  (25%).

Because the two cases yield practically the same goodness of fit, only the results for case (I) have been presented in Fig. 4. Both parameter sets were used to simulate the magnetic pattern of the spectra measured at a magnetic field of 7 T (Fig. 4, b and c). Again, there is no obvious preference for either case.

## DISCUSSION

The properties of APS reductase from *P. aeruginosa* are very similar to those of the C-terminally truncated plant APR, lacking the thioredoxin-like domain (12, 14, 19, 33). The enzyme produces sulfite from APS but not from PAPS, and the activity is stimulated by thioredoxin m but is not absolutely dependent on this compound. The reported  $V_{\text{max}}$  of the *Pseudomonas* APR,  $5.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$  (19), is almost identical to that of the N-domain of APR from *Arabidopsis*,  $5.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ,<sup>2</sup> being  $\sim 8$  times lower than the  $V_{\text{max}}$  of recombinant APR from *Lemna minor* (13). The recombinant proteins are colored yellow-brown and bind 3–4 nmol Fe/nmol protein. Further biochemical analysis of the *P. aeruginosa* APR revealed that the enzyme possessed a diamagnetic  $[4\text{Fe-4S}]^{2+}$  cluster at the ac-

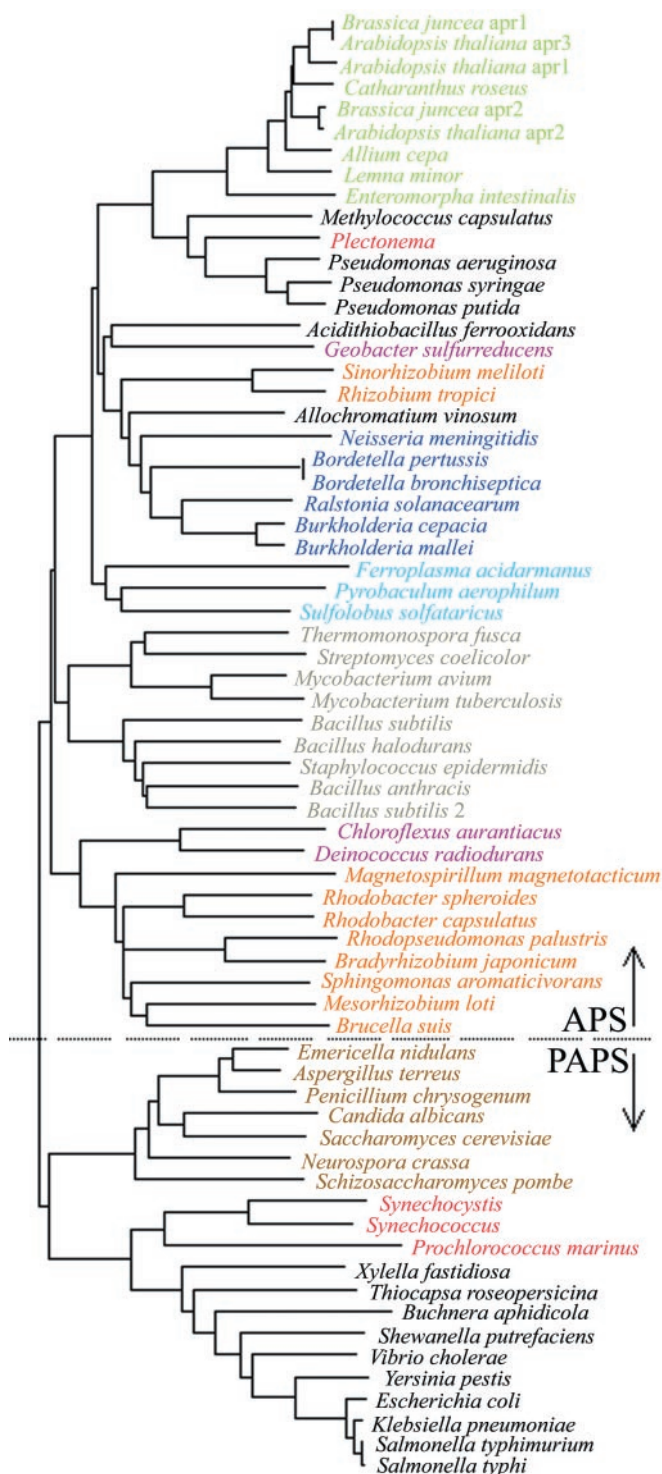


FIG. 2. **Phylogenetic analysis of APS and PAPS reductases.** The protein sequences were retrieved from GenBank™ by the BLAST software and aligned with the program CLUSTAL. The neighbor-joining tree was constructed with the PHYLIP software package. The horizontal dashed line separates the APS and PAPS reductase subclusters. The taxa are color-coded as follows: Viridiplantae, green; fungi, brown; Archaea, light blue; Firmicutes, gray;  $\gamma$ -proteobacteria, black;  $\beta$ -proteobacteria, blue;  $\alpha$ -proteobacteria, orange; cyanobacteria, red;  $\delta$ -proteobacteria, green; non-sulfur bacteria, *Thermus/Deinococcus* group, magenta.

tive site, exactly like the enzyme from higher plants (14). The properties of this cofactor, as interpreted from the Mössbauer spectra, are essentially identical with those of the  $[4\text{Fe-4S}]^{2+}$  cluster of APR from *L. minor*, which was discussed in detail by Kopriva *et al.* (14). Similarly as in the plant protein, one of the

TABLE I  
Comparison of plant and bacterial assimilatory APS reductases

	Activity with thioredoxin m	Activity without thioredoxin	Iron content
	$\mu\text{mol min}^{-1}$ $\text{mg}^{-1}$ protein	$\mu\text{mol min}^{-1}$ $\text{mg}^{-1}$ protein	$\text{mol mol}^{-1}$ protein
<i>P. aeruginosa</i>	$2.8 \pm 0.2$	$0.26 \pm 0.02$	$3.2 \pm 0.1$
APR2 <i>A. thaliana</i>	$22 \pm 1$	$23 \pm 0.8$	$3.1 \pm 0.2$
N-domain APR2	$2.6 \pm 0.3$	$0.6 \pm 0.05$	$2.9 \pm 0.2$

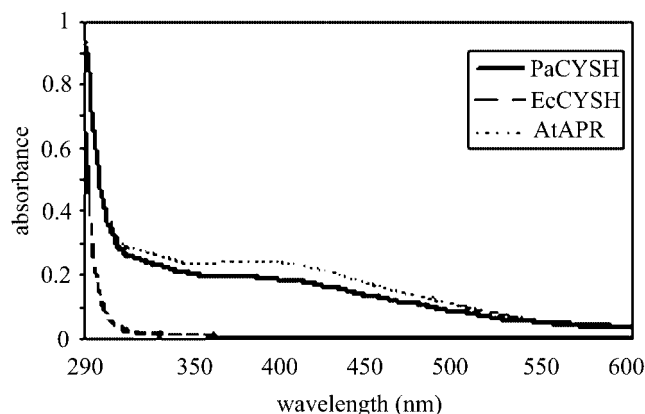


FIG. 3. **Optical spectra of purified recombinant APS- and PAPS-reducing enzymes.** PaCYSH, 20  $\mu\text{M}$  APS reductase from *P. aeruginosa*; EcCYSH, 20  $\mu\text{M}$  PAPS reductase from *E. coli*; AtAPR, 15  $\mu\text{M}$  APS reductase from *A. thaliana*. The recombinant proteins were over-expressed in *E. coli* by the pET14b expression system (Novagen) and purified by the His-Tag® system.

individual Fe subsites is different from the other three because it is either tetragonally  $\text{FeS}_3\text{X}$ -coordinated, with X being a non-sulfur ligand (C, N, O) or trigonally sulfur-coordinated. The previously described assimilatory APR from *Sinorhizobium meliloti* (18) also contains the FeS cofactor, as revealed by the dark yellow-brown color of the recombinant protein and the fact that the enzyme binds 4 nmol Fe/nmol protein (data not shown). Remarkably, dissimilatory APS reductases of sulfate-reducing bacteria and archaeobacteria, such as *Desulfovibrio* and *Archaeoglobus*, respectively (35, 36), or sulfur-oxidizing phototrophic bacterium *A. vinosum* (37) also contain  $[4\text{Fe-4S}]$  centers, although these enzymes are not otherwise related to the assimilatory APS or PAPS reductases. The role of the iron-sulfur cluster in the reaction mechanism of assimilatory APRs is not clear, but nevertheless, it seems that the ability to reduce APS is linked to the presence of an iron-sulfur cluster in the enzyme.

These findings have far-reaching implications for understanding the evolution of sulfate assimilation. In contrast to earlier reports (6–9), the results reported previously (18–20, 34) and presented here demonstrate that APS-dependent assimilatory reduction of sulfate is not connected to oxygen-evolving photosynthesis but is present in a large range of eubacterial taxons. From the bacterial species included in the phylogenetic analysis (Fig. 2), preferential reduction of APS over PAPS was confirmed in the  $\gamma$ -proteobacteria *Pseudomonas*, *A. ferrooxidans* (34), and *A. vinosum* (9);  $\beta$ -proteobacteria *Burkholderia* and *Ralstonia* (19); and  $\alpha$ -proteobacteria *S. meliloti* and *R. tropici* (18). Furthermore, because no homologues of APS kinase are present in the completely sequenced genomes of *Neisseria meningitidis* ( $\beta$ -proteobacteria) and *Geobacter sulfurreducens* ( $\delta$ -proteobacteria), one can conclude that these species also possess an APS reductase. In the neighbor-joining tree, the CysH sequences of all these species are found within the large cluster containing the plant APR sequences (Fig. 2). In addition to the species mentioned above, three subclusters are part of

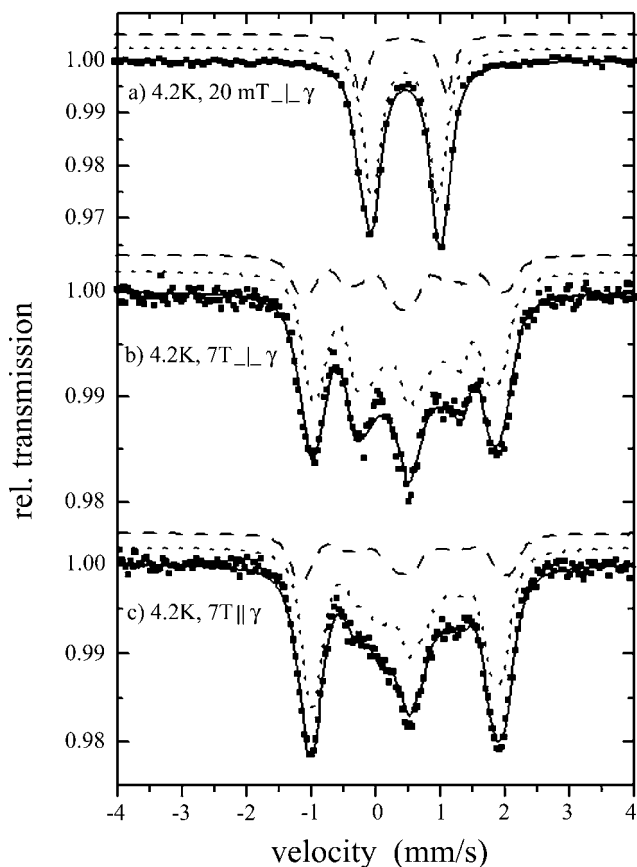


FIG. 4. Mössbauer spectra of APR from *P. aeruginosa*. Mössbauer spectra of APR from *P. aeruginosa* taken (a) at 4.2 K in a field of 20 mT perpendicular to the  $\gamma$ -beam and in a field of 7 T applied (b) perpendicular and (c) parallel to the  $\gamma$ -beam. The solid lines represent (a) a fit and (b and c) simulations with parameters according to case (I) (see the text), and the dashed and dotted lines represent the subspectra according to a subsite ratio of 1:3. The enzyme was dissolved at a concentration of 73.7  $\mu\text{M}$  in 20 mM Tris/HCl, pH 8.0, 100 mM imidazole.

the tentative APR cluster: the Archae, Firmicutes, and  $\alpha$ -proteobacteria. In these organisms, either both APS and PAPS reductase activities were measured or nothing is known about the sulfonucleotide utilized for reduction (9, 19). The hypothesis that this large cluster represents APS reductases was strengthened by the cloning of the *CysH* gene from the APS-reducing cyanobacterium *Plectonema*. Whereas the previous reports and experiments confirmed the APS-dependent activity predicted from the position of the corresponding sequences in the phylogenetic tree, a known enzymatic activity was the starting point in this case (8). Indeed, as expected, the *Plectonema* APR was clustered together with the APS-reducing enzymes of *Pseudomonas* and not with the other cyanobacteria that form a small subcluster in the PAPS reductase cluster of the phylogenetic tree (Fig. 2). Because all enzymes from the tentative APR cluster contain the two additional Cys pairs (Fig. 1), which, in at least four species, bind an iron-sulfur cofactor that seems to be essential for reduction of APS, we conclude that these Cys pairs might serve as a marker for distinguishing between APS- and PAPS-dependent reductases.

The first bacterial species in which sulfate assimilation was investigated were the enterobacteria *E. coli* and *S. typhimurium* (4). The sulfate assimilation in these species requires synthesis of PAPS. PAPS-dependent sulfate reduction was also observed in yeast (5) and several cyanobacteria (8); in contrast, plants, algae, and phototrophic bacteria utilized APS directly (6–9). It was thus

believed that the APS pathway was dependent on oxygen-evolving photosynthesis and that the PAPS pathway was ubiquitous in nonphotosynthetic bacteria (3, 38). However, the results presented here indicate that the PAPS pathway in prokaryotes is restricted to only a few groups of  $\gamma$ -proteobacteria and cyanobacteria. Because APS-reducing species are also found among cyanobacteria and  $\gamma$ -proteobacteria, a horizontal gene transfer must have played an important role in today's distribution of the two enzyme activities (39). Interestingly, the evolution of dissimilatory APS reductase was also affected by frequent horizontal gene transfers (40). An important question arises: was the ancestral sulfate assimilation APS- or PAPS-dependent? The APS reductase pathway seems to be the original one because: 1) APS reductase was present in the evolutionary ancient sulfate-reducing bacteria and Archae, 2) the reduction via APS requires one ATP less than that via PAPS, and 3) the domains similar to APS and PAPS reductase in the archaeobacterial proteins, e.g. the *M. jannaschii* hypothetical protein MJ0973 (GenBank<sup>TM</sup> accession number Q58383) or *P. horikoshii* PH0268, contain the Cys residues required for the coordination of an iron-sulfur cluster (41).

Using protein signature sequences, a linear succession was proposed in which the various phyla evolved from a common ancestor in the following order: Firmicutes, *Deinococcus/Thermus* group, cyanobacteria, Spirochetes, Aquificales + Chlamydiales + green sulfur bacteria,  $\epsilon$ + $\delta$ -proteobacteria,  $\alpha$ -proteobacteria,  $\beta$ -proteobacteria, and  $\gamma$ -proteobacteria (42, 43). If the original gene encoded an APS reductase, the distribution of the reductases can be easily explained by assuming the evolution of PAPS reductase after separation of  $\gamma$ -proteobacteria and a single horizontal gene transfer event into the cyanobacteria. On the other hand, if the original gene encoded PAPS reductase, the evolution of APS reductase would have to occur several times independently, or the horizontal gene transfer would have taken place at least four times. Obviously, the former scenario is more plausible; therefore, we conclude that the APS reductase pathway is the original pathway of sulfate assimilation. Why there are two pathways of sulfate reduction in bacteria still remains an open question. The evolution of a PAPS reductase, which does not need the iron-sulfur cluster, might have been an adaptation to an iron- and/or sulfur-poor environment or to increasing concentrations of oxygen in the atmosphere because the iron-sulfur center is unstable in air. However, an evolutionary advantage of one pathway over the other remains to be shown, as discussed for the dissimilatory APR in *A. vinosum* (44).

Plant APS reductase comprises three domains: a chloroplast-targeting peptide, an APS reductase part, and a C-terminal thioredoxin-like domain. The gene thus most probably originated from a fusion between genes for APS reductase and thioredoxin. Because all APRs isolated or cloned from higher plants, as well as the APR from the green algae *Enteromorpha intestinalis* (45), have the same structure, this fusion must have occurred early in the evolution of plants. The close relation of the *Plectonema* APS reductase to the plant enzymes also implies that plants obtained the gene for APS reductase from the chloroplast ancestor. As discussed above, the ancient sulfate assimilation pathway in cyanobacteria was most probably APS-dependent; therefore, the gene acquired by the original symbiont would be that of APS reductase. The cyanobacterial gene was then allocated to the plant nuclear genome through endosymbiotic gene transfer and supplemented with the sequence encoding the targeting peptide, such as genes coding, e.g. for Calvin cycle enzymes (46).

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