

Plant Adenosine 5'-Phosphosulfate Reductase Is a Novel Iron-Sulfur Protein*

Stanislav Kopriva^{‡§}, Thomas Büchert[¶], Günter Fritz[¶], Marianne Suter[‡], Markus Weber[‡], Rüdiger Benda^{**}, Johann Schaller^{‡‡}, Urs Feller[‡], Peter Schürmann^{§§}, Volker Schünemann^{**}, Alfred X. Trautwein^{**}, Peter M. H. Kroneck[¶], and Christian Brunold[‡]

From the [‡]Institute of Plant Sciences, University of Bern, CH-3013 Bern, Switzerland, [¶]Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany, ^{||}Biochemisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland, ^{**}Institut für Physik, Medizinische Universität zu Lübeck, D-23538 Lübeck, Germany, ^{‡‡}Department of Chemistry, University of Bern, CH-3013 Bern, Switzerland, and ^{§§}Laboratoire de Biochimie, University of Neuchâtel, CH-2000 Neuchâtel, Switzerland

Adenosine 5'-phosphosulfate reductase (APR) catalyzes the two-electron reduction of adenosine 5'-phosphosulfate to sulfite and AMP, which represents the key step of sulfate assimilation in higher plants. Recombinant APRs from both *Lemna minor* and *Arabidopsis thaliana* were overexpressed in *Escherichia coli* and isolated as yellow-brown proteins. UV-visible spectra of these recombinant proteins indicated the presence of iron-sulfur centers, whereas flavin was absent. This result was confirmed by quantitative analysis of iron and acid-labile sulfide, suggesting a [4Fe-4S] cluster as the cofactor. EPR spectroscopy of freshly purified enzyme showed, however, only a minor signal at $g = 2.01$. Therefore, Mössbauer spectra of ⁵⁷Fe-enriched APR were obtained at 4.2 K in magnetic fields of up to 7 tesla, which were assigned to a diamagnetic [4Fe-4S]²⁺ cluster. This cluster was unusual because only three of the iron sites exhibited the same Mössbauer parameters. The fourth iron site gave, because of the bistability of the fit, a significantly smaller isomer shift or larger quadrupole splitting than the other three sites. Thus, plant assimilatory APR represents a novel type of adenosine 5'-phosphosulfate reductase with a [4Fe-4S] center as the sole cofactor, which is clearly different from the dissimilatory adenosine 5'-phosphosulfate reductases found in sulfate reducing bacteria.

Sulfur is an essential element, which is found in nature mostly in its oxidized inorganic form of sulfate. In living organisms, however, most sulfur is in the reduced form of organic thiols. Plants, yeast, and most prokaryotes are able to reduce sulfate to sulfide and incorporate it into organic compounds. The sulfate assimilation pathway was first resolved in bacteria, such as *Escherichia coli* and *Salmonella typhimurium* (1, 2). For reduction sulfate must be activated in two steps; ATP sulfurylase forms adenosine 5'-phosphosulfate (APS),¹ which is

further phosphorylated by APS kinase to form adenosine 3'-phosphate 5'-phosphosulfate (PAPS). PAPS is reduced in a thioredoxin-dependent reaction by PAPS reductase (EC 1.8.99) to sulfite. Sulfite is reduced by a NADPH-dependent sulfite reductase (EC 1.8.7.1) to sulfide, which is incorporated into the amino acid skeleton of *O*-acetyl-L-serine, thus forming cysteine (3). Plants and algae were shown to utilize APS rather than PAPS as sulfonyl donor; the corresponding enzyme was originally called APS sulfotransferase because *S*-sulfogluthathione was detected among the reaction products (4, 5). APS sulfotransferase was shown to be highly regulated and to play a key role in controlling sulfate reduction in plants (6). Nevertheless, a PAPS-dependent pathway of sulfate reduction could not be excluded, especially when the purification of PAPS reductase from spinach had been reported (7).

In attempts to clone plant PAPS reductase by complementation of *E. coli CysH* mutants, a small family of three cDNA clones was obtained from *Arabidopsis thaliana* (8, 9). These cDNA clones encoded isoforms of an enzyme with a N-terminal organelle targeting peptide, a central part similar to *E. coli* PAPS reductase, and a C-terminal part similar to thioredoxin. The enzyme produced sulfite from APS, and the cDNAs complemented also *E. coli* mutants deficient in APS kinase. Therefore, the enzyme was named APS reductase (8). When both the PAPS reductase-like and the thioredoxin-like domains of APS reductase were expressed separately in *E. coli*, they were able to reconstitute the APS reductase activity (10, 11). However, the C-terminal thioredoxin-like domain was shown to possess the same enzymatic activity as glutaredoxin but not thioredoxin (10). On the other hand, thioredoxin but not glutaredoxin was able to replace the C-domain in the APS reductase of *Catharanthus roseus* (11), although for the reduction of PAPS in *E. coli* thioredoxin and glutaredoxin are interchangeable (12).

The controversy about the enzyme catalyzing the reduction of APS (13, 14) has been resolved in a recent report by Suter *et al.* (15). The APS sulfotransferase from *Lemna minor* was isolated, the corresponding cDNA was cloned, and the deduced amino acid sequence was shown to be very similar to that of APS reductase from *A. thaliana*. Free sulfite was the only reaction product detected under reducing conditions, and *S*-sulfogluthathione was only formed when oxidized glutathione was present in the enzyme assay (15). From these experiments, the authors concluded that APS sulfotransferase and APS reductase were identical enzymes and should be named APS reductase. The reductase mechanism of APR was corroborated by identification of a reaction intermediate, with sulfite co-

* This work was supported by grants from the Swiss National Science Foundation, the EU project OPTIMISTICK, and the Körber Foundation. 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.

§ To whom correspondence should be addressed. Current address: Inst. of Tree Physiology, Georges-Köhler-Allee 053, 79085 Freiburg, Germany. Fax: 49-761-2038302; E-mail: kopriva@uni-freiburg.de.

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

FIG. 1. Comparison of amino acid sequences of mature LMAR, ATAPR2, and PAPS reductase from *E. coli*. Asterisks and dots identify identical residues and homologous substitutions, respectively. Double crosses mark the additional Cys in plant APRs.

LMAR	TSDAVTLVQAAETSTVDVDFEKLASELERASPLEIMDKALEMFGNDIAIAFS-GAEDVALIEYARLTGRPFVSLDTG	79
ATAPR2	ASTLIAPEVEEKGGVEDEFEQLAKKLEDASPLEIMDKALERFGDQIAIAFS-GAEDVALIEYARLTGKPFVSLDTG	77
E. COLI	MSKLDNALNELPKVDRIILAEATNAELKDAEGRVAVALDNLPGYVLSSTFGTQAQVSLHLVNLQIRPDPVILDTG	80
LMAR	RLNPETYRFFDEVEKRYNIRIEMFDDAVEVQALVRSGKGLFSFYEDGHQECCEVRKVRPLRRALRGL--RAWITGQRKDQ	157
ATAPR2	RLNPETYRLDAVEKQYGRIRIEMFDDAVEVQALVRSGKGLFSFYEDGHQECCEVRKVRPLRRALRGL--RAWITGQRKDQ	155
E. COLI	YLFPEYTRFIDELTDKLNKLYRATESAAWQEAARYKGLWEQGVGIEKYLINKEVEMNRALKELNAQTWFAGLRREQ	160
LMAR	SPGTRASVPTVQVDPSEFEGEGTGLIKWNPVANVDGDIWRFLRTMAVPVNSHSQGYVSGICEPCTRPVLPQGHRE	237
ATAPR2	SPGTRSEIPIVQVDPVFEGLDGGVSLVKWNPVANVEGADVWNLRTMDVPVNSHAQGYVSGICEPCTRPVLPQGHRE	235
E. COLI	S-GSRANLPVLAIQR-----GVFKVLPIDWDNRITTYQLKQKGLKYLHFWDEGLYSVGDTHTTRKWPQMAEEE	230
LMAR	GRWWEADAKAKCGLHKGNIKQDESVPPSSNCGNCTAPSDFSVVSLSRFCMENLLRLESRKDWSLVLYAFWCRFCQME	317
ATAPR2	GRWWEADAKAKCGLHKGNIKQDEGAADSKPAAVQEIFESNNVVALSKGGVENLLKLENRKEAWLVLYAFWCPFCQME	315
E. COLI	TRFFG---LKRCEGLHEG	245
LMAR	ESFNEVAGVLGGDSGVKVGKFRADGDARAFA-EEIGLVSEFTILFFPKHASKPVKYSEQRDVSLLAFVNALR	390
ATAPR2	ASYIELAEKLAG-KGVKVAKFRADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYSEHRDVSLSFVNLLR	388

valently bound to a cysteine residue of the enzyme (16). The C-terminal domain was responsible for release of this bound sulfite. Interestingly, the PAPS reductase domain from *A. thaliana* APR2 was able to catalyze sulfite production even without additional thioredoxin. Hereby, only recombinant thioredoxin m but not thioredoxin f enhanced the reaction velocity (16).

APS reductase from both *A. thaliana* and *C. roseus*, which had been overexpressed in *E. coli*, was described as a protein without prosthetic groups or cofactors (9–11). However, the enzyme was purified from *L. minor* as a yellow-brown protein (15), indicating the presence of a cofactor, possibly FAD or/and FeS as in the dissimilatory APS reductases (17). The major difference between the N-terminal part of plant APS reductases and the homologous *E. coli* PAPS reductase (18) was the presence of two additional Cys pairs in the plant enzyme (Fig. 1). From these findings two important questions arise: (i) is there a cofactor in plant APR, and (ii) do the additional Cys residues play any role in the plant enzyme?

In this report we present biochemical and spectroscopic evidence that recombinant plant APS reductase constitutes a new member of the iron-sulfur protein family. On the basis of Mössbauer measurements, we propose that three of the additional Cys residues are involved in binding of the catalytically active [4Fe-4S] center.

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 (19) with recombinant ATP sulfurylase from *A. thaliana*² and inorganic pyrophosphatase (Sigma) (19). Ferredoxin was isolated from spinach leaves according to a standard procedure (20). Oligonucleotide primers were synthesized at Microsynth GmbH (Balgach, Switzerland).

Overexpression of APR in *E. coli*—The APR from *L. minor* (LMAR) (15), the APR2 isoform of APS reductase from *A. thaliana* (ATAPR2) (9), and the truncated ATAPR2N protein were overexpressed in *E. coli* by the pET14b expression system and were purified to homogeneity by criteria of SDS-polyacrylamide electrophoresis with the His-Tag[®] system (Novagen) (15, 16).

For the preparation of ^{57}Fe labeled *Lemna* APR, *E. coli* harboring the expression construct was grown in M9 medium, containing 0.4% glucose, in which ^{56}Fe was replaced by ^{57}Fe . Metal foil consisting of ^{57}Fe (94.7% enrichment, Glaser, Basel, Switzerland) was dissolved in HCl, neutralized, and added to the culture medium at a final ^{57}Fe concentration of 20 μM .

Site-directed Mutagenesis—The ATAPR2-pET14b construct was used as template for the site-directed mutagenesis. Mutations were produced with the GeneEditor *in vitro* site-directed mutagenesis system (Promega) according to the manufacturer's instructions. The mutations were confirmed by sequencing, and the mutated plasmids were transformed into *E. coli* strain BL21(DE3) for protein expression.

Enzyme Assays—APR activity was either measured as the production of $[^{35}\text{S}]\text{sulfite}$, assayed as acid volatile radioactivity formed in the presence of $[^{35}\text{S}]\text{APS}$ and dithioerythritol (21), or as formation of APS, with $\text{K}_3[\text{Fe}(\text{CN})_6]$ as electron acceptor (22).

Determination of Iron and Sulfur—Iron was quantitated by atomic absorption spectrometry (23). Acid-labile sulfide was determined according to Ref. 24. Protein concentrations were routinely estimated according to Bradford (25) using bovine serum albumin as standard. For the estimation of the iron content of *Lemna* APR, protein measurements were done based on the amino acid determination of hydrolyzed aliquots of APR solutions. The values obtained with this approach were smaller than the values obtained with the Bradford method.

Electronic Spectra—UV-visible spectra were recorded on a Lambda 16 Instrument (PerkinElmer Life Sciences) equipped with a temperature-controlled cell compartment. For measuring the decrease in optical density of *Lemna* APR during storage, a Beckman instrument was used.

Electron Paramagnetic Resonance—EPR spectra (X-band, 9.5 GHz) were recorded on the ESP 300 spectrometer (Bruker) and evaluated as described elsewhere (26). 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 $\alpha\text{-Fe}$ 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 (27); otherwise, spectra were analyzed by least square fits using Lorentzian line shape.

RESULTS

Biochemical Properties of APS Reductase—To identify the putative cofactor of APS reductase, the enzymes from *L. minor* (LMAR) and *A. thaliana* (ATAPR2) were overexpressed in *E. coli*, and the recombinant proteins were purified to homogeneity. In solution, they exhibited a yellow-brown color. Whereas LMAR was losing its activity continuously when kept at 4 °C, ATAPR2 in addition to decrease of activity began to precipitate after 2–3 days under these conditions. Therefore, LMAR was used in most experiments. Freshly purified LMAR had a specific activity of 30–40 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in the reaction with $[^{35}\text{S}]\text{APS}$ and dithioerythritol as electron donor (15), which decreased to ~40% after 24 h. Attempts to measure the specific activity of the enzyme with $\text{K}_3[\text{Fe}(\text{CN})_6]$ as electron acceptor and sodium sulfite/AMP as electron donor similar to the dissimilatory APS reductase (22) failed, as the plant enzyme was rapidly denatured by even micromolar concentrations of $\text{K}_3[\text{Fe}(\text{CN})_6]$. Substitution of $\text{K}_3[\text{Fe}(\text{CN})_6]$ by other electron acceptors, such as horse heart cytochrome *c* ($E'^{\circ} = +254$ mV), toluidine blue ($E'^{\circ} = +115$ mV), or phenazinemetosulfate ($E'^{\circ} = +80$ mV) did not reveal any specific activity of plant APS reductase in the oxidative reaction.

Electronic Spectra—The UV-visible spectrum of ATAPR2 exhibited maxima at 280 and 390 nm, with shoulders around 324, 475, and 625 nm (Fig. 2A). Upon photochemical reduction with 5-deazaflavin/sodium oxalate, or addition of sodium dithionite (data not shown), the absorption decreased in the range 300–750 nm but could be restored after exposure of the solution to dioxygen. The UV-visible difference spectrum, [APS reductase oxidized] – [APS reductase reduced], exhibited maxima at 322 and 405 nm as expected for iron-sulfur proteins (Fig. 2B) (28).

² S. Kopriva, unpublished data.

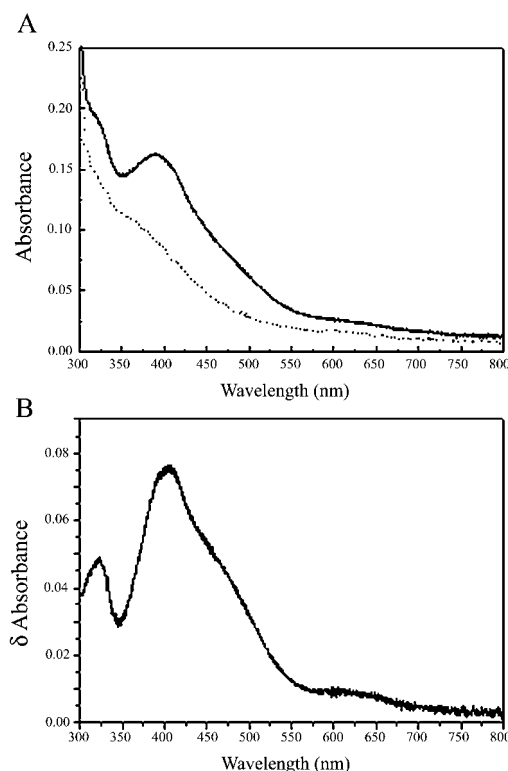


FIG. 2. **UV-visible spectra of APR.** Figure shows electronic spectra of APS reductase from *A. thaliana* (6 μ M) in 20 mM Tris/HCl, pH 8.0, 100 mM imidazole (A) as isolated (solid line); after 60 s of photochemical reduction with 10 mM sodium oxalate and 2 μ M 5-deazaflavin, under exclusion of dioxygen (dotted line). B, difference spectrum: [enzyme as isolated] – [enzyme after 60-s illumination].

In the presence of stoichiometric amounts of the oxidant $K_3[Fe(CN)_6]$, the solution turned turbid and the protein started to precipitate, most likely the result of destruction of the iron-sulfur centers. Reduction of plant APS reductase was not achieved by either excess of sodium sulfite or excess of sodium sulfite/2 mM AMP, as observed for the bacterial enzyme (22). Incubation with 2 mM dithiothreitol also did not lead to reduction of the enzyme (data not shown).

The more stable LMAPR revealed almost identical UV-visible spectra in the reduction-oxidation experiments (data not shown). In this case, the molar extinction coefficient at 386 nm ϵ_{386} was 14,254 $M^{-1} \cdot cm^{-1}$ per monomer, which accounts rather well for the presence of two [4Fe-4S] clusters per α_2 -homodimer (15).

Iron and Acid-labile Sulfide—In view of its color, the recombinant APS reductases were analyzed for flavin, iron, and acid-labile sulfide. Whereas flavin (FAD or FMN) were absent, freshly isolated LMAPR contained 4.08 ± 0.88 mol of iron and 2.6 ± 0.2 mol of acid-labile sulfide/mol of APR monomer. Note that, parallel to the decrease in LMAPR enzymatic activity, the yellow-brown color of the protein solution faded, as documented by the decay of the absorbance at 386 nm, and the iron content of LMAPR decreased at the same rate (Fig. 3). To characterize the cofactor binding domain of plant APS reductase, a C-terminally truncated ATAPR2 protein (16) was analyzed for iron. The protein, overexpressed in *E. coli* and purified to homogeneity, was again colored and gave UV-visible spectra similar to those for the recombinant ATAPR2 (data not shown). Furthermore, the amount of iron in ATAPR2N was identical to that determined for the native enzyme. Thus, the PAPS reductase-like section of plant APR appears to be the iron-sulfur cluster binding site.

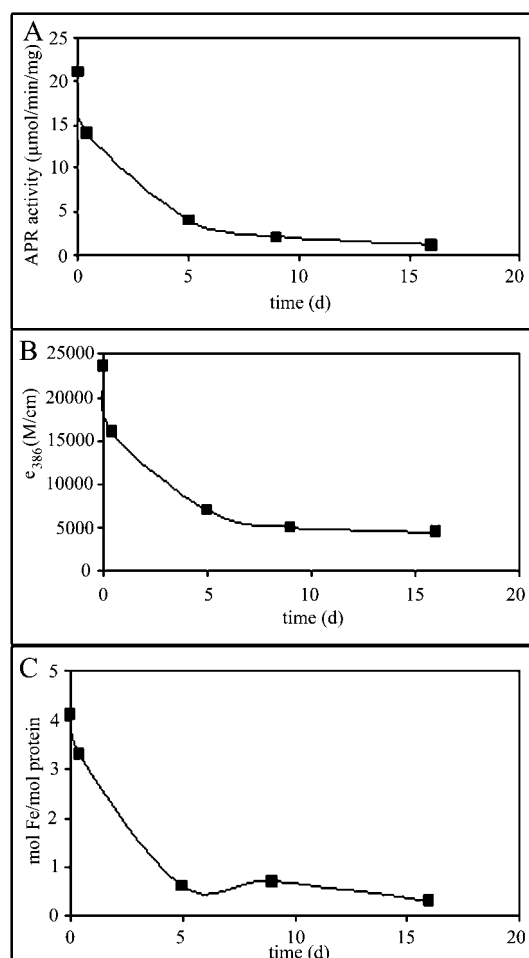


FIG. 3. **Stability of recombinant LMAPR.** Recombinant LMAPR was purified and stored at 4 °C for 16 days. The decrease of enzyme activity (A), molar extinction coefficient (B), and content of iron (C) were monitored during this period.

Site-directed Mutagenesis—The comparison of the PAPS reductase-like section of plant APR with PAPS reductase from *E. coli* revealed the presence of two additional Cys pairs in the plant enzyme, which might potentially bind the iron-sulfur cluster (Fig. 1). Therefore, each of the Cys residues in ATAPR2 was converted into Ser by *in vitro* mutagenesis. Although the ATAPR2 and ATAPR2N remained mostly soluble in the *E. coli* BL21(DE3) strain, the modified proteins C128S, C220S, and C223S were completely excreted into inclusion bodies. The enzymatic activity of the C129S mutant was only very slightly influenced; the V_{max} dropped to 50% of that of the wild type enzyme. The mutation of C248 completely abolished the APR activity (compare with Ref. 16). However, both modified proteins, C129S and C248S, exhibited the same spectral characteristics as the wild type enzyme (data not shown), indicating correct folding, structure, and presence of cofactor. It seems, therefore, likely that Cys-129 and Cys-248 do not contribute to the binding of the iron-sulfur cluster, thus leaving the remaining three Cys, Cys-128, Cys-220, and Cys-223, as only potential Cys ligands.

Electron Paramagnetic Resonance Spectroscopy—Compared with the bacterial APS reductases, which carry two [4Fe-4S] centers with intense resonances in the reduced state (22), EPR spectroscopy proved not to be the method of choice to investigate the iron-sulfur centers of the plant enzymes. EPR spectra of ATAPR2 exhibited a minor nearly isotropic signal around $g = 2.01$ with the typical features of a [3Fe-4S] center (22) (data

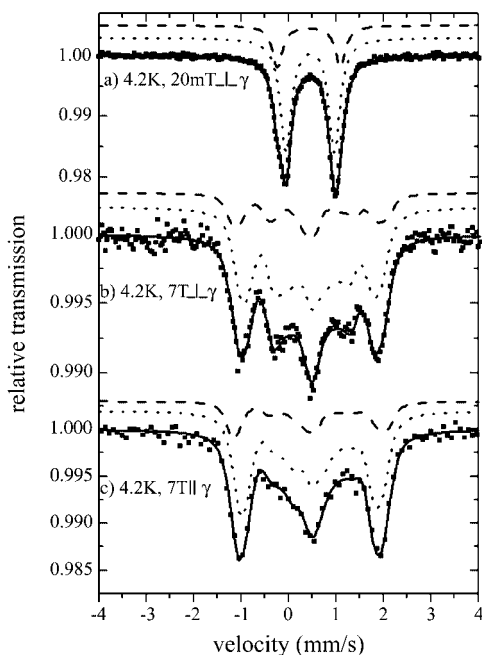


FIG. 4. **Mössbauer spectra of APR from *L. minor*.** Mössbauer spectra of LMAPR taken (a) at 4.2 K in a field of 20 mT perpendicular to the γ -beam and in a field of 7 T applied perpendicular (b) and parallel (c) to the γ -beam. The solid lines represent a fit (a) and simulations (b and c) according to case 1 with parameters summarized in Table I, and the dashed and dotted lines represent the subspectra according to subsite ratio 1:3. The enzyme was dissolved at concentration of 73.7 μ M in 20 mM Tris/HCl, pH 8.0, 100 mM imidazole.

not shown). Similarly, the LMAPR revealed the nearly isotropic signal at $g = 2.01$. In both cases spin quantitation gave $\leq 0.1\%$ of the chemically determined iron. In the recombinant LMAPR isolated from *E. coli*, which had been cultivated on ^{57}Fe , the weak signal at $g = 2.01$ was again present, and its linewidth was slightly increased compared with the signal of the corresponding ^{56}Fe enzyme. In all preparations, there was a signal at $g = 4.3$, which was assigned to nonspecifically bound Fe^{III} . All attempts to generate new EPR signals under reducing conditions, either by titrating the enzyme as isolated with varying amounts of dithionite or Ti(III) citrate or by photochemical reduction with the deazaflavin/oxalate system, failed. Similarly, titration of the enzyme with the oxidant $\text{K}_3[\text{Fe}(\text{CN})_6]$ did not yield any significant resonances.

Mössbauer Spectroscopy—The Mössbauer spectrum of LMAPR obtained at 4.2 K in a small field of 20 mT (perpendicular to the γ -beam; Fig. 4a) exhibited an asymmetric quadrupole doublet. This asymmetry indicates that APR comprises structurally different iron sites. Applying a strong field of 7 T (parallel and perpendicular to the γ -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 δ and the quadrupole splitting ΔE_Q of the asymmetric doublet, which take 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 toward the presence of $[\text{4Fe-4S}]^{2+}$ clusters (27). The quantitative analysis of the measured spectra was based on the assumption that only three (and not four) Cys residues were binding to the metal cluster (see above). It was assumed that three iron sites exhibit the same δ and ΔE_Q values, which, however, may differ from the corresponding values of the fourth site. Thus, the fit comprises two doublets with area ratio 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 1) $\delta_1 \sim \delta_2$, $\Delta E_{Q,1} \neq \Delta E_{Q,2}$, or 2) $\delta_1 \neq \delta_2$, $\Delta E_{Q,1} \sim \Delta E_{Q,2}$.

With start parameters corresponding to cases 1 and 2, respectively, the obtained parameter sets are: 1) $\delta_1 = 0.46 \text{ mm s}^{-1}$, $\Delta E_{Q,1} = 1.02 \text{ mm s}^{-1}$ (75%), $\delta_2 = 0.43 \text{ mm s}^{-1}$, $\Delta E_{Q,2} = 1.33 \text{ mm s}^{-1}$ (25%); and 2) $\delta_1 = 0.49 \text{ mm s}^{-1}$, $\Delta E_{Q,1} = 1.08 \text{ mm s}^{-1}$ (75%), $\delta_2 = 0.35 \text{ mm s}^{-1}$, $\Delta E_{Q,2} = 1.15 \text{ mm s}^{-1}$ (25%).

As the two cases yield practically the same goodness-of-fit, only the results for case 1 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

Both biochemical and spectroscopic techniques were used to demonstrate that plant-type APS reductase carries a diamagnetic $[\text{4Fe-4S}]^{2+}$ cluster at the active site. UV-visible spectra of the enzymes from *A. thaliana* (ATAPR2) and *L. minor* (LMAPR) together with the results from iron and acid-labile sulfur analysis indicated the presence of $[\text{4Fe-4S}]$ clusters as prosthetic group. However, EPR spectroscopy only yielded a minor signal of a $[\text{3Fe-4S}]$ cluster. No EPR signals were observed for the oxidized and reduced enzymes, which could be assigned to a $[\text{4Fe-4S}]$ cluster. A comparison of the homologous amino acid sequences of both enzymes and site-directed mutagenesis revealed the possibility that one iron center of the $[\text{4Fe-4S}]$ cluster might be coordinated by a non-cysteine ligand. This interpretation would agree with the high instability of plant-type APS reductases observed even under exclusion of dioxygen and might explain their unusual EPR properties.

Clearly, Mössbauer spectroscopy was decisive in unraveling the structural and electronic features of the iron-sulfur center. The interpretation of the obtained parameters is based on data that show the isomer shifts δ of Fe-S clusters are closely correlated to oxidation states and iron-ligand coordination numbers of the individual iron sites (29–37). According to typical values for the average isomer shifts at 4.2 K of tetrahedrally sulfur-coordinated ferric and ferrous high spin iron, Fe^{3+} ($\delta = 0.24 \text{ mm s}^{-1}$) $\rightarrow$ Fe^{2+} ($\delta = 0.69 \text{ mm s}^{-1}$) (with variation $\pm 0.03 \text{ mm s}^{-1}$), case 1 represents the situation that the four iron sites in APR form a $[\text{4Fe-4S}]^{2+}$ cluster and have the same intermediate oxidation state $\sim 2.5+$ (corresponding to $\delta_1 = 0.46 \text{ mm s}^{-1}$ and $\delta_2 = 0.43 \text{ mm s}^{-1}$) but slightly different individual coordination geometry (different quadrupole splittings) as, e.g., in aconitase or CO-dehydrogenase (Table I). Along this line of discussion, case 2 represents a $[\text{4Fe-4S}]^{2+}$ cluster with one iron site having slightly larger ($2.75+$) and the other three slightly smaller ($2.42+$) oxidation state than the average of $2.5+$. The assumption that only three (and not four) Cys residues are binding to the 4Fe cluster is, so far, implied from our analysis only by considering a 3:1 area ratio of subspectra. We therefore extend our discussion to cases that deviate from the classical FeS_4 coordination of each individual iron site.

Substrate binding to aconitase results in an increase of isomer shift of one of the four iron sites in the $[\text{4Fe-4S}]^{2+}$ cluster from 0.46 mm s^{-1} to 0.89 mm s^{-1} , whereas that of the other three iron sites remains at 0.46 mm s^{-1} . This increase has been attributed to an increase of iron-ligand coordination larger than 4 (29). A corresponding increase of isomer shift by increasing iron coordination has been observed also in biomimetic $[\text{4Fe-4S}]^{2+}$ models (37). In the present case, an iron-ligand coordination larger than 4 is very unlikely, because APR provides only three Cys residues for cluster formation and in either case, 1 or 2, the isomer shift is not larger than 0.49 mm s^{-1} .

Another factor that is responsible for an increase of isomer shift of one iron site within a $[\text{4Fe-4S}]^{2+}$ cluster is the replacement of one Cys residue by a non-sulfur ligand. Examples are the 4Fe clusters in the ferredoxin from the hyperthermophilic

TABLE I
Isomer shifts δ and quadrupole splittings ΔE_Q of $[4\text{Fe-4S}]^{2+}$
cluster-containing proteins

	Relative area	δ	ΔE_Q	Reference
	%	mm s^{-1}	mm s^{-1}	
APR case 1	75	0.46	1.02	This work
	25	0.43	1.33	
APR case 2	75	0.49	1.08	This work
	25	0.35	1.15	
Aconitase	75	0.46	1.30	29
	25	0.46	0.83	
CO-dehydrogenase (nonlabile A_{ox} -state)	50	0.45	1.26	30
	50	0.45	0.96	
Anaerobic ribonucleotide reductase	75	0.43	1.00	31
	25	0.50	1.18	
Pyruvate formate-lyase activating enzyme	Site 1	0.45	1.15	32
	Site 2	0.45	1.00	
3S-coordinated iron in iron- molybdenum cofactor		0.40 (0.41)	0.76	33 (34)
3S-coordinated iron in iron- iron cofactor	Site 1	0.37	0.74	35
	Site 2	0.42	1.00	
3S-coordinated iron in iron- vanadium cofactor		0.39	0.94	36

archeon *Pyrococcus furiosus* (with one of the four sites yielding $\delta = 0.55 \text{ mm s}^{-1}$, ΔE_Q not reported, Ref. 38) and in the anaerobic ribonucleotide reductase from *E. coli* (with one site yielding $\delta = 0.50 \text{ mm s}^{-1}$, $\Delta E_Q = 1.18 \text{ mm s}^{-1}$; Ref. 39). It is important to note that the increase of isomer shift caused by the replacement of a Cys residue may be counterbalanced by a corresponding decrease of isomer shift caused by a redistribution of electron charge within $[4\text{Fe-4S}]^{2+}$. Such redistribution of electron charge in 4Fe clusters is readily accessible by minor changes of cluster symmetry (40). An example for the compensation of counter effects upon the isomer shift is provided by the $[4\text{Fe-4S}]^{2+}$ cluster in the native pyruvate formate lyase-activating enzyme. This enzyme provides only three Cys residues for 4Fe cluster formation; the fourth terminal ligand has not yet been identified (32). In this example the isomer shift is 0.45 mm s^{-1} for all four iron sites, and only the quadrupole splittings are slightly different, i.e. 1.15 mm s^{-1} and 1.00 mm s^{-1} . The parameters obtained for case 1 from APR are compatible with this situation.

Trigonally sulfur-coordinated iron sites have the tendency to exhibit isomer shifts that are smaller than those from corresponding tetragonally sulfur-coordinated iron sites. For the ferrous high spin complex $[\text{Fe}(\text{SC}_6\text{H}_2-2,4,6-t\text{-Bu}_3)_3]$, for which a trigonal thiolate coordination is enforced by use of sterically encumbered ligands, an isomer shift of $\delta = 0.57 \text{ mm s}^{-1}$ at 4.2 K was reported (41). This value is significantly smaller than the corresponding average value $\delta = 0.69 \text{ mm s}^{-1}$ for tetrahedral Fe^{2+}S_4 compounds. The same trend was observed for polynuclear Fe-S clusters, which comprise individual subsites with oxidation state 2.5+ and μ_3 -sulfido coordination. Examples of this type ($\text{Fe}^{2.5+}\text{S}_3$) belong to the family of cofactors of nitrogenase, i.e. iron-vanadium cofactor, iron-iron cofactor, and iron-molybdenum cofactor, for which isomer shifts were reported ($\delta = 0.40\text{--}0.37 \text{ mm s}^{-1}$; Refs. 33–37), which are well below the value $\delta = 0.46 \text{ mm s}^{-1}$ for $\text{Fe}^{2.5+}\text{S}_4$ coordination. Within the frame of conditions discussed here, the parameters obtained for case 2 from APR are compatible with a $[4\text{Fe-4S}]^{2+}$ cluster with one iron site being trigonally and three iron sites being tetragonally sulfur-coordinated.

In summary we conclude that APR comprises four paramagnetic iron sites, which form a diamagnetic $[4\text{Fe-4S}]^{2+}$ cluster.

One of the individual subsites is different from the other three because it is either tetragonally FeS_3X -coordinated, with X being a non-sulfur ligand (C, N, O), or it is trigonally sulfur-coordinated.

The experimental finding of such an iron-sulfur cluster in APR, however, does not agree with data recently published by Prior *et al.* (11), who described APR from *C. roseus* as a protein devoid of chromophores. The *C. roseus* APR is 74.4% identical to ATAPR2 and 70.7% to LMAPR, and possesses the same 7 Cys residues as the other plant APR proteins. However, the V_{max} of the *C. roseus* enzyme obtained with APS and DTT ($4.2 \mu\text{mol/min/mg}$) was 10 times lower than the V_{max} determined for the recombinant LMAPR or with the isolated *L. minor* enzyme (15). Possibly, the expression system used by Prior *et al.* (11) yielded only partially active protein because of incomplete incorporation of the iron-sulfur center.

The presence of an iron-sulfur cluster in plant APS reductase raises new questions about the evolution of this enzyme. Obviously, APR does not represent a simple fusion protein of PAPS reductase and thioredoxin, although the original gene might have been formed this way. The C-terminal, thioredoxin-like, part of the APR has clearly changed its function into a glutaredoxin despite the sequence homology with thioredoxin (10). Furthermore, the N-terminal part of APR did not just modulate the active site to react preferentially with APS and not PAPS, but acquired a new cofactor and a new reaction mechanism. Note that dissimilatory APS reductase, an enzyme catalyzing reduction of APS in anaerobic, sulfate-reducing bacteria such as *Desulfovibrio sp.*, carries two $[4\text{Fe-4S}]$ centers in addition to FAD. The structure of dissimilatory APR is completely different from that of plant APR. The protein is a dimer of a large FAD containing subunit with a small one possessing two $[4\text{Fe-4S}]$ clusters (17), and there is no sequence homology among these proteins. Whether there really is a link between presence of an iron-sulfur cluster and reduction of APS rather than PAPS will be a subject of additional experiments.

Acknowledgments—We thank Dr. T. Brunold, Dr. M. Droux, and Dr. B. Buchanan for helpful discussions and Dr. C. Schäublin for help in administrative matters.

REFERENCES

- Jones-Mortimer, M. C. (1968) *Biochem. J.* **110**, 589–595
- Kredich, N. M. (1971) *J. Biol. Chem.* **246**, 3474–3484
- Brunold, C. (1990) in *Sulfur Nutrition and Sulfur Assimilation in Higher Plants* (Rennenberg, H., Brunold, C., De Kok, L. J., and Stulen, I., eds) pp. 13–31, SPB Academic Publishing, The Hague, The Netherlands
- Schmidt, A. (1972) *Arch. Mikrobiol.* **84**, 77–86
- Schmidt, A. (1975) *Plant Sci. Lett.* **5**, 407–415
- Brunold, C. (1993) in *Sulfur Nutrition and Sulfur Assimilation in Higher Plants* (De Kok, L. J., Stulen, I., Rennenberg, H., Brunold, C., and Rauser, W. E., eds) pp. 61–75, SPB Academic Publishing, The Hague, The Netherlands
- Schwenn, J. D. (1989) *Z. Naturforsch.* **44c**, 504–508
- Gutierrez-Marcos, J. F., Roberts, M. A., Campbell, E. I., and Wray, J. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13377–13382
- Setya, A., Murillo, M., and Leustek, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13383–13388
- Bick, J.-A., Åslund, F., Chen, Y., and Leustek, T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8404–8409
- Prior, A., Uhrig, J. F., Heins, L., Wiesmann, A., Lillig, C. H., Stoltze, C., Soll, J., and Schwenn, J. D. (1999) *Biochim. Biophys. Acta* **1430**, 25–38
- Tsang, M. L.-S. (1981) *J. Bacteriol.* **146**, 1059–1066
- Leustek T., and Saito K. (1999) *Plant Physiol.* **120**, 637–644
- Hawkesford, M. J., and Wray, J. L. (2000) *Adv. Bot. Res.* **33**, 159–223
- Suter, M., von Ballmoos, P., Kopriva, S., Op den Camp, R., Schaller, J., Kuhlmeier, C., Schürmann, P., and Brunold, C. (2000) *J. Biol. Chem.* **275**, 930–936
- Weber, M., Suter, M., Brunold, C., and Kopriva, S. (2000) *Eur. J. Biochem.* **267**, 3647–3653
- Verhagen, M. F., Kooter, I. M., Wolbert, R. B., and Hagen, W. R. (1994) *Eur. J. Biochem.* **221**, 831–837
- Krone, F. A., Westphal, G., and Schwenn, J. D. (1991) *Mol. Gen. Genet.* **225**, 314–319
- Li, J., and Schiff, J. A. (1991) *Biochem. J.* **274**, 355–360
- Buchanan, B. B., and Arnon, D. I. (1971) *Methods Enzymol.* **23**, 413–440
- Brunold, C., and Suter, M. (1990) in *Methods in Plant Biochemistry* (Lea, P., ed) Vol. 3, pp. 339–343, Academic Press, London

22. Fritz, G., Buchert, T., Huber, H., Stetter, K. O., and Kroneck, P. M. (2000) *FEBS Lett.* **473**, 63–66
23. Stieger, P. A., and Feller, U. (1994) *Plant Soil* **166**, 173–179
24. Fischer, D. S., and Price, D. C. (1964) *Clin. Chem.* **10**, 21–31
25. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
26. Riester, J., Zumft, W. G., and Kroneck, P. M. H. (1989) *Eur. J. Biochem.* **178**, 751–762
27. Trautwein, A. X., Bill, E., Bominaar, E. L., and Winkler, H. (1991) *Struct. Bond.* **78**, 1–95
28. Cammack, R. (1992) *Adv. Inorg. Chem.* **38**, 281–322
29. Emptage, M. H., Kent, T. A., Kennedy, M. C., Beinert, H., and Münck, E. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 4674–4678
30. Xia, J., Hu, Z., Popescu, C. V., Lindahl, P. A., and Münck, E. (1997) *J. Am. Chem. Soc.* **119**, 8301–8312
31. Tamarit, J., Gerez, C., Meier, C., Mulliez, E., Trautwein, A. X., and Fontecave, M. (2000) *J. Biol. Inorg. Chem.* **275**, 15669–15675
32. Krebs, C., Henshaw, T. F., Cheek, J., Huynh, B. H., and Broderick, J. B. (2000) *J. Am. Chem. Soc.* **122**, 12497–12506
33. Ravi, N., Moore, V., Lloyd, S. G., Hales, B. J., and Huynh, B. H. (1994) *J. Biol. Chem.* **269**, 20920–20924
34. Yoo, S. J., Angove, H. C., Papaefthymiou, V., Burgess, B. K., and Münck, E. (2000) *J. Am. Chem. Soc.* **122**, 4926–4936
35. Krahn, E., Schneider, K., Müller, A., Weis, B. J. R., Cramer, S. P., Kröckel, M., Trautwein, A. X., Groppe, J., and Henkel, G. (2001) *J. Biol. Inorg. Chem.*, in press
36. Huynh, B. H., Henzl, M. T., Christner, J. A., Zimmermann, R., Orme-Johnson, W. H., and Münck, E. (1980) *Biochim. Biophys. Acta* **623**, 124–138
37. Holm, R. H., Ciurli, S., and Weigel, J. A. (1990) *Prog. Inorg. Chem.* **38**, 2–64
38. Bates, D. M., Popescu, C. V., Khoroshilova, N., Vogt, K., Beinert, H., Münck, E., and Kiley, P. J. (2000) *J. Biol. Chem.* **275**, 6234–6240
39. Ollagnier, S., Meier, C., Mulliez, E., Gaillard, J., Schuenemann, V., Trautwein, A. X., Mattioli, T., Lutz, M., and Fontecave, M. (1999) *J. Am. Chem. Soc.* **121**, 6344–6350
40. Coropceanu, V., Meier, C., and Trautwein, A. X. (2000) *Chem. Phys. Lett.* **323**, 14–20
41. MacDonnel, F. M., Rohlandt-Senge, K., Ellison, J. J., Holm, R. H., and Power, P. P. (1995) *Inorg. Chem.* **34**, 1815–1822