Plant adenosine 5'-phosphosulfate reductase is a novel iron-sulfur protein*

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ABSTRACT

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 *E. 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 an [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 $^{57}$Fe enriched APR were obtained at 4.2K in magnetic fields of up to 7T which were assigned to a diamagnetic [4Fe-4S]$^{2+}$ cluster. This cluster was unusual since only three of the iron sites exhibited the same Mössbauer parameters. The fourth iron site gave, due to 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 APS reductase with a [4Fe-4S] center as the sole cofactor which is clearly different from the dissimilatory APS reductases found in sulfate reducing bacteria.
INTRODUCTION

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 (APSSTase) since S-sulfoglutathione was detected among the reaction products (4,5). APSSTase 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 by recent report of Suter et al. (15). The APSSTase from *Lemna minor* was isolated, the corresponding cDNA was cloned and the deduced amino acid sequence was showed 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-sulfoglutathione 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 covalently 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 \textit{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 communication 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.

\textbf{EXPERIMENTAL PROCEDURES}

\textit{Materials} – [\textsuperscript{35}S]APS was prepared from [\textsuperscript{35}S]SO\textsubscript{4}\textsuperscript{2-} (Hartmann Analytic) according to Li and Schiff (1991) with recombinant ATP sulfurylase from \textit{A. thaliana} (S. Kopriva, unpublished) 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).

\textit{Overexpression of APR in E. coli} - The APR from \textit{L. minor} (LMAPR) (15), the APR2 isoform of APS reductase from \textit{A. thaliana} (ATAPR2) (9), and the truncated ATAPR2N protein were overexpressed in \textit{E. coli} by the pET14b expression system and were purified to homogeneity by criteria of SDS polyacrylamide electrophoresis with the His\•Tag\textsuperscript{®} System (Novagen) (15, 16).

For the preparation of \textsuperscript{57}Fe labeled \textit{Lemna} APR \textit{E. coli} harboring the expression construct was grown in M9 medium, containing 0.4% glucose, in which \textsuperscript{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 the protein expression.

**Enzyme Assays** - APR activity was either measured as the production of $[^{35}S]$sulfite, assayed as acid volatile radioactivity formed in the presence of $[^{35}S]$APS and DTE (21), or as formation of APS, with K$_3$Fe(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 (24). Protein concentrations were routinely estimated according to Bradford (25) using BSA 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/Vis spectra were recorded on a Lambda 16 Instrument (Perkin-Elmer) equipped with a temperature-controlled cell compartment. For measuring the decrease in OD 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 (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 $\alpha$-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 - In order to identify the putative cofactor of APS reductase the enzymes from L. minor (LMAPR) 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 LMAPR 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, LMAPR was used in most experiments. Freshly purified LMAPR had a specific activity of 30-40 $\mu$mol-min$^{-1}$-mg$^{-1}$ in the reaction with $[^{35}\text{S}]$APS and DTE as electron donor (15) which decreased to approximately 40% after 24 h. Attempts to measure the specific activity of the enzyme with $\text{K}_3[\text{Fe(CN)}_6]$ as electron acceptor and Na-sulfite/AMP as electron donor similar to the dissimilatory APS reductase (22) failed, as the plant enzyme was rapidly denatured by even $\mu$M concentrations of $\text{K}_3[\text{Fe(CN)}_6]$. Substitution of $\text{K}_3[\text{Fe(CN)}_6]$ by other electron acceptors, such as horse heart cytochrome $c$ ($E^{\circ} = +254$ mV), toluidine blue ($E^{\circ} = +115$ mV), or
phenazinemethosulfate (E°' = +80 mV) did not reveal any specific activity of plant APS reductase in the oxidative reaction.

*Electronic spectra* – The UV/Vis spectrum of ATAPR2 exhibited maxima at 280 nm and 390 nm, with shoulders around 324 nm, 475 nm, and 625 nm (Fig. 2A). Upon photochemical reduction with 5-deazaflavin/Na-oxalate, or addition of Na-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/Vis difference spectrum [APS reductase oxidized] – [APS reductase reduced] exhibited maxima at 322 nm and 405 nm as expected for iron-sulfur proteins (Fig. 2B) (28). In the presence of stoichiometric amounts of the oxidant K₃[Fe(CN)₆] the solution turned turbid and the protein started to precipitate, most likely due to destruction of the iron-sulfur centers. Reduction of plant APS reductase was not achieved either by excess of Na-sulfite, or excess of Na-sulfite/2 mM AMP as observed for the bacterial enzyme (22). Incubation with 2 mM dithiothreitol did also not lead to reduction of the enzyme (data not shown).

The more stable LMAPR revealed almost identical UV/Vis spectra in the reduction-oxidation experiments (data not shown). In this case, the molar extinction coefficient at 386 nm ε₃86 was 14,254 M⁻¹·cm⁻¹ per monomer which accounts rather well for the presence of two [4Fe-4S] clusters per α₂-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 Fe and 2.6±0.2 mol acid labile sulfide per mol 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 Fe content of LMAPR decreased at the same rate (Fig. 3). In order 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 similar UV/visible spectra as the recombinant ATAPR2 (data not shown). Furthermore, the amount of Fe 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.

**Site Directed Mutagenesis** - The comparison of the PAPS reductase-like section of plant APR with PAPS reductase from *E. coli* revealed presence of two additional Cys pairs in the plant enzyme, that 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 $V_{\text{max}}$ dropped to 50% of that of the wild type enzyme. The mutation of C248 completely abolished the APR activity (compare 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 C129 and C248 do not contribute to the binding of the iron-sulfur cluster leaving thus the remaining three Cys, C128, C220, and C223, as only potential Cys ligands.
Electron Paramagnetic resonance spectroscopy – Compared to 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 not shown). Similarly, the LMAPR revealed the nearly isotropic signal at $g = 2.01$. In both cases spin quantitation gave $= 0.1\%$ 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 to the signal of the corresponding $^{56}$Fe enzyme. In all preparations, there was a signal at $g = 4.3$ which was assigned to non-specifically bound Fe$^{\text{III}}$. All attempts to generate new EPR signals under reducing conditions, either by titrating the as isolated enzyme 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 $K_3[Fe(CN)_6]$ 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 $\gamma$-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 $\gamma$-beam; Fig. 4b 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 take the values $\delta \sim 0.45$ mms$^{-1}$ and $\Delta E_Q \sim 1.2$ mms$^{-1}$ (vide infra), strongly points towards the presence of [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 $\delta$ and $\Delta E_Q$ values which, however, may differ from the corresponding values of the fourth site. Thus, the fit comprises two doublets with 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 (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:

(I) $\delta_1 = 0.46$ mms$^{-1}$, $\Delta E_{Q,1} = 1.02$ mms$^{-1}$ (75 %), $\delta_2 = 0.43$ mms$^{-1}$, $\Delta E_{Q,2} = 1.33$ mms$^{-1}$ (25 %) and

(II) $\delta_1 = 0.49$ mms$^{-1}$, $\Delta E_{Q,1} = 1.08$ mms$^{-1}$ (75 %), $\delta_2 = 0.35$ mms$^{-1}$, $\Delta E_{Q,2} = 1.15$ mms$^{-1}$ (25 %).

As 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. 4b 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 [4Fe-4S]$^{2+}$ cluster at the active site. UV/Vis 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 [4Fe-4S] clusters as prosthetic group. However, EPR spectroscopy only
yielded a minor signal of a [3Fe-4S] cluster. No EPR signals were observed for the oxidized and reduced enzymes which could be assigned to a [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 [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 which show that the isomer shifts $\delta$ 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 $\delta = 0.24 \text{ mms}^{-1}$ $\delta = 0.69 \text{ mms}^{-1}$ (with variation $\pm 0.03 \text{ mms}^{-1}$) case (I) represents the situation that the four iron sites in APR form a [4Fe-4S]$^{2+}$ cluster and have the same intermediate oxidation state $\approx 2.5+$ (corresponding to $\delta_1 = 0.46 \text{ mms}^{-1}$ and $\delta_2 = 0.43 \text{ mms}^{-1}$) but slightly different individual coordination geometry (different quadrupole splittings) as, e. g., in aconitase or CO-dehydrogenase (Table 1). Along this line of discussion case (II) represents a [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 which deviate from the classical FeS₄ 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 [4Fe-4S]²⁺ cluster from 0.46 mms⁻¹ to 0.89 mms⁻¹, while that of the other three iron sites remains at 0.46 mms⁻¹. This increase has been attributed to an increase of iron-ligand coordination larger than four (29). A corresponding increase of isomer shift by increasing Fe coordination has been observed also in biomimetic [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, (I) or (II), the isomer shift is not larger than 0.49 mms⁻¹.

Another factor which is responsible for an increase of isomer shift of one iron site within a [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 archeon Pyrococcus furiosus (with one of the four sites yielding δ = 0.55 mms⁻¹, ΔE_Q not reported, 38) and in the anaerobic ribonucleotide reductase from E. coli (with one site yielding δ = 0.50 mms⁻¹, ΔE_Q = 1.18 mms⁻¹, 32). It is important to note that the increase of isomer shift due to the replacement of a Cys residue may be counter-balanced by a corresponding decrease of isomer shift due to a redistribution of electron charge within [4Fe-4S]²⁺. Such redistribution of electron charge in 4Fe clusters is readily accessible by minor changes of cluster symmetry (39). An example for the compensation of counter effects upon the isomer shift is provided by the [4Fe-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 mms⁻¹ for all four iron sites, and only the quadrupole splittings are slightly different, i.e. 1.15 mms⁻¹
and 1.00 mms$^{-1}$. The parameters obtained for case (I) from APR are compatible with this situation.

Trigonally sulfur-coordinated iron sites have the tendency to exhibit isomer shifts which are smaller than those from corresponding tetragonally sulfur-coordinated iron sites. For the ferrous high-spin complex $[\text{Fe(SC}_{6}\text{H}_{2}-2,4,6\text{-t-Bu}_{3})_{3}]$, for which a trigonal thiolate coordination is enforced by use of sterically encumbered ligands, an isomer shift of $\delta = 0.57$ mms$^{-1}$ at 4.2 K was reported (40). This value is significantly smaller than the corresponding average value $\delta = 0.69$ mms$^{-1}$ 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 $\mu_{3}$-sulfido coordination. Examples of this type (Fe$^{2.5+}$S$_{3}$) belong to the family of cofactors of nitrogenase, i.e. FeVco, FeFeco and FeMoco, for which isomer shifts were reported ($\delta = 0.40$–0.37 mms$^{-1}$, 33-37), which are well below the value $\delta = 0.46$ mms$^{-1}$ for Fe$^{2.5+}$S$_{4}$ coordination. Within the frame of conditions discussed here the parameters obtained for case (II) from APR are compatible with a $[4\text{Fe-4S}]^{2+}$ cluster with one Fe site being trigonally and three Fe 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$_{3}$X 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 the recently published data of Prior et al. (11), who described APR from $C.\text{roseus}$ as a protein devoid of chromophores. The $C.\text{roseus}$ 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 $V_{\text{max}}$ of the $C.\text{roseus}$ enzyme
obtained with APS and DTT (4.2 µmol/min/mg) was ten times lower than the $V_{\text{max}}$ determined for the recombinant LMAPR or with the isolated $L. \text{minor}$ 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 [4Fe-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 [4Fe-4S] clusters (17) and there is no sequence homology among these proteins. If 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.

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FOOTNOTES

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1 The abbreviations used are: APR, adenosine 5’-phosphosulfate reductase; APS, adenosine 5’-phosphosulfate; DTE, dithioerythritol; GSH, glutathione; PAPS, phosphoadenosine 5’-phosphosulfate.
FIGURE LEGENDS

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

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

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

Fig. 4. **Mössbauer spectra of APR from *L. minor*.** Mössbauer spectra of LMAPR taken (a) at 4.2K in a field of 20mT perpendicular to the γ-beam and in a field of 7T applied (b) perpendicular and (c) parallel to the γ-beam. The solid lines represent (a) a fit and (b,c) simulations according to case I with parameters summarized in Table 1, and the dashed and dotted lines represent the subspectra according to subsite ratio 1:3. The enzyme was dissolved at concentration of 73.7 µM in 20 mM Tris/HCl, pH 8.0, 100 mM imidazole.
Table 1  Isomer shifts $\delta$ and quadrupole splittings $\Delta E_Q$ of [4Fe-4S]$^{2+}$ cluster containing proteins

|                              | rel. area | $\delta$ [mms$^{-1}$] | $\Delta E_Q$ [mms$^{-1}$] | Reference |
|------------------------------|-----------|------------------------|----------------------------|-----------|
| APR case I                   | 75        | 0.46                   | 1.02                       | this work |
|                              | 25        | 0.43                   | 1.33                       |           |
| APR case II                  | 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       | site 1    | 0.45                   | 1.15                       | 32        |
|                              | site 2    | 0.45                   | 1.00                       |           |
| 3S-coordinated Fe in FeMoco  |           | 0.40 (0.41)            | 0.76                       | 33 (34)   |
| 3S-coordinated Fe in FeFeco  | site 1    | 0.37                   | 0.74                       | 35        |
|                              | site 2    | 0.42                   | 1.00                       |           |
| 3S-coordinated Fe in FeVco   |           | 0.39                   | 0.94                       | 36        |
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