Properties of the Cysteine Residues and the Iron−Sulfur Cluster of the Assimilatory 5′-Adenylyl Sulfate Reductase from *Enteromorpha intestinalis*†

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ABSTRACT: The 5′-adenylyl sulfate (APS) reductase from the marine macrophytic green alga *Enteromorpha intestinalis* uses reduced glutathione as the electron donor for the reduction of APS to 5′-AMP and sulfite. The *E. intestinalis* enzyme (EiAPR) is composed of a reductase domain and a glutaredoxin-like C-terminal domain. The enzyme contains a single [4Fe-4S] cluster as its sole prosthetic group. Three of the enzyme's eight cysteine residues (Cys166, Cys257, and Cys260) serve as ligands to the iron−sulfur cluster. Site-directed mutagenesis experiments and resonance Raman spectroscopy are consistent with the presence of a cluster in which only three of the four ligands to the cluster irons contributed by the protein are cysteine residues. Site-directed mutagenesis experiments suggest that the thiol group of Cys250, a residue found only in algal APS reductases, is not an absolute requirement for activity. The other four cysteines that do not serve as cluster ligands, all of which are required for activity, are involved in the formation of two redox-active disulfide/dithiol couples. The couple involving Cys342 and Cys345 has an E_m value at pH 7.0 of −140 mV, and the one involving Cys165 and Cys285 has an E_m value at pH 7.0 of −290 mV. The C-terminal portion of EiAPR, expressed separately, exhibits the cystine reductase activity characteristic of glutaredoxins. It is proposed that the Cys342−Cys345 disulfide provides the site for entry of electrons from reduced glutathione and that the Cys166−Cys285 disulfide may serve as a structural element that is essential for keeping the enzyme in the catalytically active conformation.

Organisms that are capable of reducing sulfur from the +6 to −2 oxidation state are divided into sulfate assimilating and sulfate dissimilating types (*1*). The dissimilators are anaerobic archaea and eubacteria that use sulfate as a respiratory electron acceptor, producing sulfide as an end product. The assimilators, which are typically aerobic microbes and plants, reduce sulfate to sulfide for the synthesis of cysteine and methionine. All sulfate reducers initiate the process by adenylylation of sulfate to produce 5′-adenylyl sulfate (APS), a reaction catalyzed by ATP sulfurylase. Reduction takes place in two steps, a two-electron transfer to yield sulfite, followed by a six-electron transfer to yield sulfide.

An intriguing diversity of mechanisms exists in nature for carrying out the two-electron reduction that generates sulfite. APS reductase exists in dissimilatory and assimilatory forms. Despite their common ability to reduce the sulfur in APS, the two enzyme classes are structurally and mechanistically distinct and are of different evolutionary origins. The dissimilatory APS reductases (EC 1.8.99.2) are heteromeric, contain two iron−sulfur centers, and rely on flavin adenine dinucleotide as a reductant (*2*). The assimilatory APS reductases (EC 1.8.4.9 and EC 1.8.4.10) use dithiol/disulfide redox couples as the reductant. They have primary structures with no similarity to those of the dissimilatory type (*3*, *4*).

Assimilatory APS reductases are further differentiated into two types, one that uses thioredoxin (Trx) or glutaredoxin (Grx) as an electron donor (EC 1.8.4.10) and a second type (EC 1.8.4.9) (*5*) that uses reduced glutathione (GSH).[1] The Trx/Grx-dependent form of APS reductase is found in diverse microbial species, and the GSH-dependent form is found in plants.

Both types of assimilatory APS reductase are related to a Trx/Grx-dependent 3′-phosphoadenylyl sulfate (PAPS) reductase (EC 1.8.4.8), first identified at the level of the corresponding *cysH* gene, which is necessary for cysteine prototrophy in *Escherichia coli* (*6*). A curious difference

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[1] Abbreviations: AtAPR, *A. thaliana* APS reductase; EiAPR, *E. intestinalis* APS reductase; EPR, electron paramagnetic resonance; GSH, reduced glutathione; GSSG, oxidized glutathione; mBBr, monobromobimane; MCD, magnetic circular dichroism; PaAPR, *P. aeruginosa* APS reductase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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between these enzymes is that the APS reductases contain a [4Fe-4S] iron sulfur center and CysH homologues do not. Indeed, the sequence motif delineating the APS reductases from the CysH orthologs includes two cysteine residues that serve as the ligands for the iron−sulfur center (CCXXRKXXPL and SXGCXXCT). It has been proposed, entirely on the basis of phylogenetic evidence, that the conserved cysteines and iron−sulfur center determine the specificity for APS over PAPS (*7*, *8*). However, this idea was recently called into question by the discovery of a FeS cluster-containing APS reductase ortholog from *Bacillus subtilis* that is able to use both APS and PAPS (*9*).

Both CysH and APS reductase share a sequence motif ([KRT]ECG[LI]H) that includes a cysteine residue required for catalytic activity. Mutation of the cysteine residue inactivated one of the GSH-dependent APS reductase isoforms present in the plant *Arabidopsis thaliana* (AtAPR2) and the Trx-dependent enzyme from *Pseudomonas aeruginosa* (PaAPR) (*10*−*12*). Mutation of this cysteine did not disrupt the association of the enzyme with the iron−sulfur center. Mutation of the homologous residue in *E. coli* CysH had a similar inactivating effect (*13*). The same cysteine residue was identified as the ligand for sulfite in AtAPR2 (*10*) and has been proposed, in one of two very different hypotheses about the mechanism of PaAPR (*11*, *12*), to play a similar role in PaAPR (*12*). Previous work from our laboratories (*11*) provided evidence that in PaAPR this cysteine (Cys256), along with Cys140, is part of a redox-active, intramolecular disulfide/dithiol couple. By contrast, an alternative proposal for the role of cysteine residues in PaAPR assigns Cys140 as a ligand to the iron−sulfur cluster and argues that it does not participate in intramolecular disulfide bond formation (*12*). The cysteine corresponding to C256 of PaAPR in *E. coli* CysH was shown to form an intermolecular disulfide bond with the same residue of another CysH monomer (*13*).

The ability of the plant-type APS reductases to use GSH as an electron donor is thought to be a function of the carboxyl-terminal domain that resembles Trx and Grx (*5*). This domain is not present in the Trx-dependent APS reductases. In fact, when synthesized as a separate protein, the C-domain of an *A. thaliana* APS reductase exhibits many of the catalytic activities associated with Grx. It is not yet clear exactly how the C-domain interacts with the reductase domain during catalysis.

Enteromorpha intestinalis is a marine macrophytic green alga. The APS reductase from this species is typical of the plant-type class in that it is composed of reductase and C-terminal domains and it uses GSH as electron donor (*14*). It differs from other plant-type APS reductases in that it contains an eighth cysteine residue in the mature enzyme that is found only in algal APS reductases. It is extremely stable and has proven to be an excellent subject for biochemical analysis (*14*). In this report, the chemical nature of its iron−sulfur cluster and the oxidation−reduction properties of its cysteine residues were studied and site-directed mutagenesis was used to investigate the roles of these cysteine residues. The goals of this study were to gain insight into the function of the cysteine residues and to understand the physical nature of the enzyme and how the N- and C-domains interact during catalysis.

MATERIALS AND METHODS

The pET30b-EiAPR construct (*14*) was used for preparation of the wild-type enzyme and was used for site-directed mutagenesis and preparation of mutant forms of the enzyme. EiAPR contains nine cysteine residues, located at positions 31, 165, 166, 250, 257, 260, 285, 342, and 345 (numbered according to the sequence in GenBank entry AF069951; see Figure 1). The first 54 amino acids of EiAPR do not show significant homology with other APS reductases (Figure 1) and may encode the plastid transit peptide of the pre-enzyme. The HisTAG-EiAPR fusion protein produced from pET30b-EiAPR lacks the first 41 amino acids and so lacks Cys31. It is this form that is termed the wild type enzyme throughout this work. Expression of the C-terminal portion of the enzyme was accomplished using a *Sal*I−*Hin*dIII fragment from the 3′-end of the pET30b-EiAPR construct, subcloned into pET30b. The *Hin*dIII site is from the plasmid polylinker, and the *Sal*I site is located so that the HisTAG at the N-terminus of the expressed protein is fused to Val293 of EiAPR.

All eight cysteine residues present in the wild-type enzyme were individually mutated to serine, with the mutations in the gene produced using the Quickchange kit (Stratagene) according to the manufacturer's instructions. All of the Cys/Ser site-specific variants, as well as the wild-type (WT) enzyme, were expressed in *E. coli* strain BL21(DE3)pLysS. A culture of the *E. coli* host harboring the plasmid pET-EiAPR (*14*) was grown in LB medium containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol at 37 °C to an optical density at 600 nm of 0.6. IPTG (isopropyl β-D-thiogalactopyranoside) was then added, to a final concentration of 1 mM, and growth was continued for 4 h in LB medium at 30 °C. Cells were then harvested by centrifugation, resuspended in 30 mM Tris-HCl buffer (pH 8.0), passed twice through a French press at 18 000 psi, and centrifuged. The supernatant was filtered through a 0.45 μm pore size membrane and applied to a Ni^{2+} affinity column (HiTrap Chelating HP, obtained from Amersham Biosciences) incorporated into a BioCAD perfusion chromatography system (PerSeptive BioSciences). The column was washed with 200 mL of 30 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl (buffer A), supplemented with 25 mM imidazole. The His-tagged EiAPR was then eluted at ∼100 mM imidazole with a gradient from 25 to 250 mM imidazole in 40 mL of buffer A. All solutions used for the Ni^{2+} affinity chromatography were degassed and purged with helium gas. The imidazole was removed by buffer exchange against 30 mM Tris-HCl buffer (pH 8.0) containing 100 mM sodium sulfate, using an Amicon YM10 membrane. The typical yield was ∼5 mg of protein from 1 L of culture. The protein concentration was determined by the Bradford method (*15*) using BSA as a standard. All enzyme samples were stored in 30 mM Tris-HCl (pH 8.0) containing 100 mM Na_2SO_4 [the kosmotropic salt Na_2SO_4 has been shown to enhance the stability and enzymatic activity of the protein (*11*)]. The purity of EiAPR and its molecular mass were estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) on 10% gels. Gels were stained with Coomassie Brilliant Blue, and where necessary, the relative intensities of different bands were estimated using a Molecular Dynamics computing densitometer.

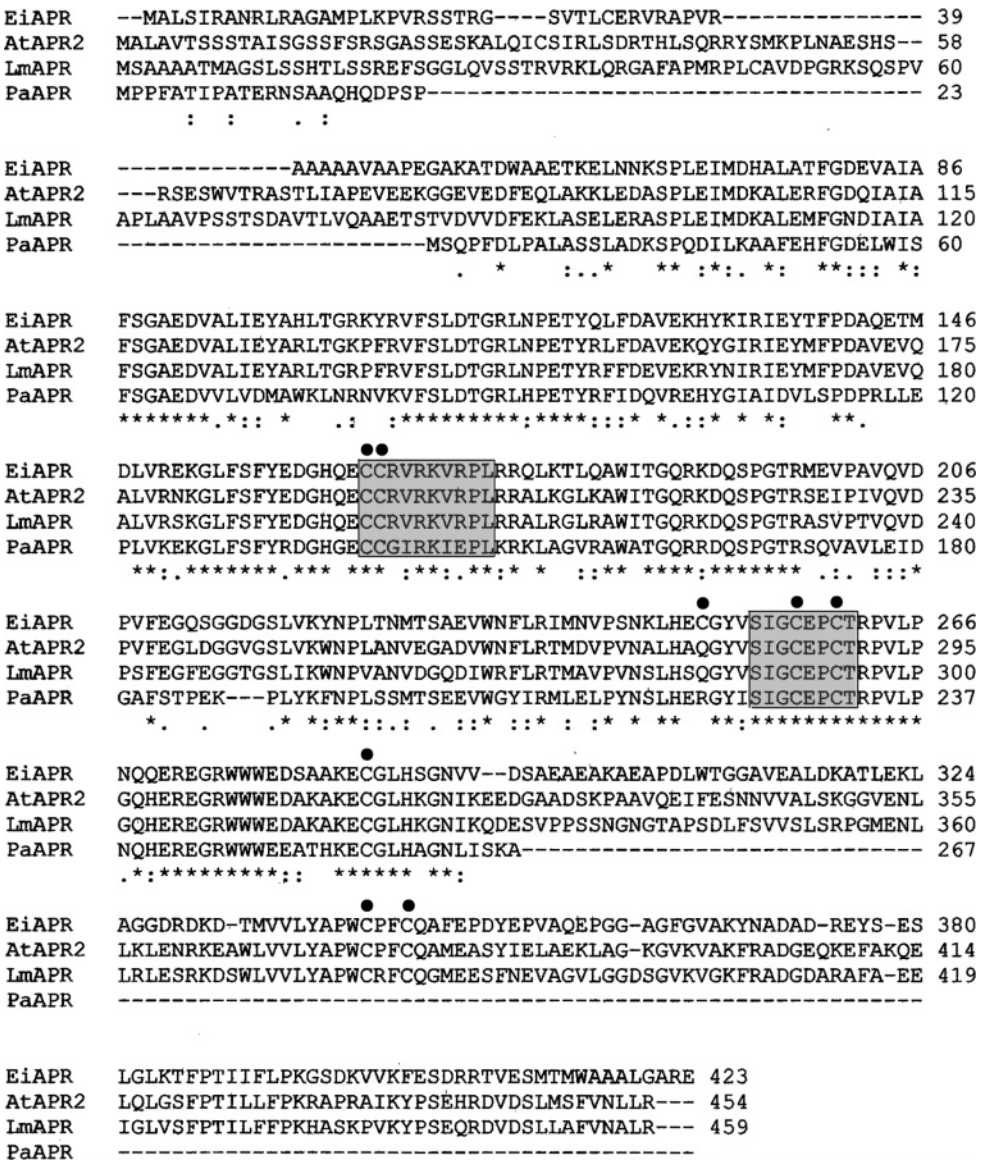

FIGURE 1: Sequence alignment of APRs. EiAPR, AtAPR2, LmAPR, and PaAPR were aligned using ClustalW. The conserved cysteine-containing motifs were shaded using BoxShade. The Cys residues mentioned in the text are indicated with a dot above the EiAPR sequence. Asterisks below the alignment denote amino acid residues that are identical to the consensus amino acid found at that position. Colons denote positions at which the amino acids present show a high degree of similarity, while periods denote positions at which the amino acids present show a significant but lower degree of similarity.

Enzymatic activity, with reduced glutathione as the electron donor for the reduction of APS, was measured as described previously (*14*). Cystine reduction activity was assayed as described previously (*5*). The total iron content was determined according to the procedure of Massey (*16*), using ferric ammonium sulfate as a standard. The amount of acid-labile sulfide was determined using the procedure described by King and Morris (*17*). The absorbance at 670 nm of the preparations was determined after 20 min to allow color development. The iron and sulfide contents of the wild-type enzyme and of its Cys/Ser variants were calculated on the basis of the protein content of the samples, as estimated using the Bradford method, and the known molecular masses of the proteins.

Oxidation−reduction titrations of disulfide/dithiol redox couples in EiAPR were carried out as described previously (*18*, *19*) using thiol labeling with monobromobimane (mBBr), followed by quantitation of the fluorescence arising from the mBBr adducts of the protein (fluorescence was measured using an Aminco-Bowman Series 2 luminescence spectrometer with both excitation and emission monochromators set at 1.0 nm spectral resolution). Oxidation−reduction equilibration was carried out in redox buffers consisting of defined mixtures of either oxidized and reduced dithiothreitol (DTT) or oxidized (GSSG) and reduced (GSH) glutathione, depending on the E_h range being investigated, as described previously (*20*). All the titration data gave excellent fits to the Nernst equation for a two-electron couple. E_m values were independent of redox equilibration time and of the total DTT or total glutathione concentrations present in the redox equilibration buffer. Identical titration curves were observed regardless of whether the titrations were carried out under ambient oxygen concentrations or under an argon atmosphere.

Absorbance spectra were measured using a Shimadzu model UV-2401 PC spectrophotometer at 1.0 nm spectral resolution. Resonance Raman spectra were recorded using the instrumentation and protocols described previously (*11*).

Circular dichroism (CD) spectra were obtained using an OLIS model DSM-10 UV−vis CD spectrophotometer at 1.0 nm spectral resolution. The α-helix and β-sheet contents of the protein were estimated using the algorithm of Sreerama and Woody (*21*).

RESULTS

The construct used to express the wild-type form of EiAPR in *E. coli* was designed with a six-histidine extension at the N-terminus to facilitate purification of the enzyme from soluble *E. coli* extracts using a Ni^{2+} affinity column. Nonreducing SDS−PAGE analysis of freshly prepared samples of the His-tagged, recombinant EiAPR showed a single major Coomassie-staining band with an apparent molecular mass of 48 kDa and a much fainter band with an apparent molecular mass of 96 kDa, consistent with the presence of a small amount of a disulfide-linked homodimer (the M_r = 96 kDa band represents no more than 5% of the total protein present, as quantitated by densitometer analysis of Coomassie Blue-stained SDS−PAGE gels prepared under nonreducing conditions, and is absent in reducing SDS−PAGE gels). No other Coomassie Blue-staining bands were detected on these gels. It should be mentioned that SDS−PAGE analysis of His-tagged, recombinant EiAPR that was purified by conventional Ni^{2+} affinity chromatography under aerobic conditions, instead of being purified rapidly under a He atmosphere (as described in Materials and Methods), showed considerably larger amounts of EiAPR homodimer and also contained Coomassie-staining bands that are likely to arise from higher-molecular mass homo-oligomers (densitometer quantitation of nonreducing, Coomassie-stained SDS−PAGE gels indicated that ca. 16% of the protein was present as a homodimer and another 16% was present as higher-order homo-oligomers when the protein was purified under aerobic conditions). Reducing SDS−PAGE analysis (i.e., experiments in which the samples were incubated with 2-mercaptoethanol prior to electrophoresis) of aerobically prepared samples showed only the M_r = 48 kDa EiAPR monomer and neither the M_r = 96 kDa dimer nor any higher-molecular mass components, indicating that the dimer and oligomers likely resulted from formation of disulfide bonds. All of the experiments described below were carried out with EiAPR samples prepared under anaerobic conditions.

Figure 2 shows the absorbance spectrum of EiAPR in the visible and near-ultraviolet regions of the spectrum. The spectrum, which contained a peak at 280 nm and a broad absorbance feature centered at 386 nm, was similar to that reported previously for PaAPR (*7*, *11*) and for two other APS reductases known to contain a single [4Fe-4S] cluster (*22*), the *Lemna minor* APS reductase and one of the three APS reductases (AtAPR2) found in chloroplasts of *A. thaliana*. Non-heme iron and acid-labile sulfide analyses of freshly prepared EiAPR indicated the presence of 3.72 ± 0.63 mol of iron and 3.06 ± 0.52 mol of sulfide per mole of enzyme (Table 1). As acid-labile sulfide determinations often underestimate the actual sulfide contents, these results are consistent with the presence of one [4Fe-4S] cluster per enzyme monomer in EiAPR.

Mössbauer spectroscopy of PaAPR (*7*) and of the *L. minor* enzyme (*22*) has provided evidence consistent with the presence of a [4Fe-4S] cluster in both the bacterial and higher-plant APS reductase that is ligated to the protein by only three cysteine ligands, instead of the four cysteine ligands found in most [4Fe-4S] cluster-containing proteins. Resonance Raman spectroscopy and site-directed mutagenesis studies of PaAPR, carried out in our laboratories (*11*), provided additional evidence for a [4Fe-4S] cluster ligated by only three cysteine residues and allowed identification of the three cysteines that serve as cluster ligands [it should be pointed out that an alternative model for a [4Fe-4S] cluster in PaAPR with four rather than three cysteinyl ligands has recently been proposed (*12*)]. Figure 3 shows a comparison of the resonance Raman spectrum of EiAPR and PaAPR in the Fe−S stretching region. The spectra are very similar, and the features in the spectrum of EiAPR could be assigned by direct analogy with published band assignments for PaAPR (*11*), indicating that the [4Fe-4S] centers in the bacterial and algal enzymes are structurally similar.

Site-directed mutagenesis was carried out to obtain additional evidence for the number of cysteine ligands to the [4Fe-4S] cluster and to identify these cluster ligands. Figure 1 shows the location of the eight cysteine residues present in EiAPR and compares their locations to the cysteine residues present in three other APS reductases known to contain a [4Fe-4S] cluster. It should be mentioned that all eight variants showed the contents of iron/sulfide were lower than that of WT EiAPR, indicating that the variants are unstable to some extent. Five of the cysteines in EiAPR, i.e., Cys165, Cys166, Cys257, Cys260, and Cys285, lie in two conserved cysteine-containing motifs. Two other cysteines present in EiAPR, Cys342 and Cys345, lie in a

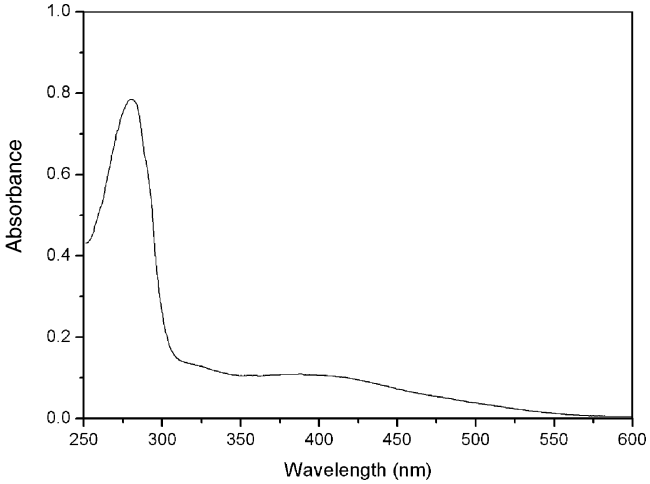

FIGURE 2: Absorbance spectrum of purified wild-type EiAPR. The EiAPR was at 7 μM in 30 mM Tris-HCl (pH 8.0) with 100 mM sodium sulfate.

Table 1: Analysis of Iron and Acid-Labile Sulfide Contents and Specific Activity for Wild-Type EiAPR and Its Variants

	sulfide content (nmol/nmol of protein)	iron content (nmol/nmol of protein)	visible-region absorbance	specific activity (nmol min^{-1} mg^{-1})
wild type	3.06 ± 0.52	3.72 ± 0.63	yes	7248
C165S	0.69 ± 0.16	0.59 ± 0.13	yes	0
C166S	0.23 ± 0.02	0.35 ± 0.03	no	0
C250S	1.13 ± 0.17	1.23 ± 0.18	yes	3500
C257S	none	0.71 ± 0.20	no	0
C260S	none	0.24 ± 0.04	no	0
C285S	1.42 ± 0.48	1.31 ± 0.44	yes	0
C342S	2.79 ± 0.26	3.96 ± 0.38	yes	11
C345S	2.44 ± 0.33	3.92 ± 0.53	yes	150

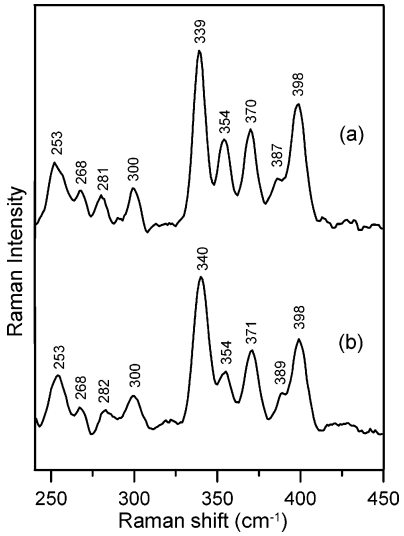

FIGURE 3: Comparison of the resonance Raman spectra of wild-type PaAPR (a) and EiAPR (b). Spectra were recorded at 17 K using 488.0 nm excitation (200 mW of laser power at the sample), with samples that contained ~2 mM APR and ~2 mM DTT. Each spectrum is the sum of 100 scans, with each scan involving photon counting for 1 s at 1 cm^{-1} increments using 8 cm^{-1} spectral resolution. Bands originating from the frozen buffer solution were subtracted after the intensity of the dominant ice-band at 231 cm^{-1} had been normalized.

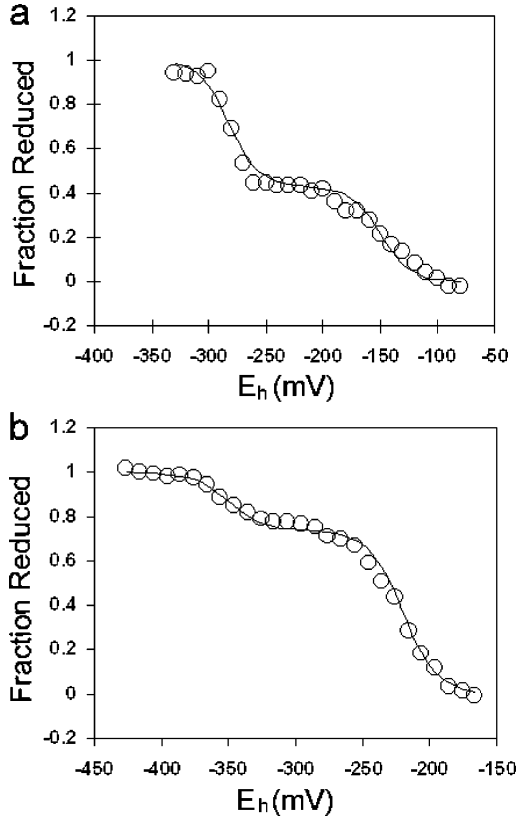

FIGURE 4: Oxidation−reduction titrations of wild-type EiAPR. EiAPR, at a concentration of 50 μg/mL, was titrated using redox buffers that contained either a total DTT concentration of 2.0 mM or a total glutathione concentration of 2.0 mM. Redox equilibration was carried out for 2.5 h. (a) Titration at pH 7.0 in which 100 mM Hepes buffer was used. (b) Titration at pH 8.5 in which 100 mM Tricine buffer was used.

conserved region in the C-terminal portion also found in higher-plant APS reductases that shows homology to thioredoxin and glutaredoxin. However, one EiAPR cysteine residue, Cys250, is found in algal APRs, EiAPR, and also the APS reductase from *Chlamydomonas reinhardtii* (*23*).

Each of the eight EiAPR cysteine residues was changed to serine, one at a time. The visible-region spectra of the C165S, C250S, C285S, C342S, and C345S variants of EiAPR retained the features seen in the spectrum of the wild-type enzyme that arise from the [4Fe-4S] cluster, although in some cases the amplitudes of the visible-region feature were smaller for a C-to-S variant than for the wild-type enzyme. On the basis of the assumption that elimination of any cysteine residue that serves as a cluster ligand would result in a complete loss of the visible-region spectral feature that arises from the cluster, these data suggest that Cys165, Cys250, Cys285, Cys342, and Cys345 do not serve as ligands to the [4Fe-4S] cluster in EiAPR. In contrast to the results obtained with the C165S, C250S, C285S, C342S, and C345S variants, changing any of the remaining EiAPR cysteines (i.e., Cys166, Cys257, or Cys260) to serine produced enzymes with almost no detectable absorbance in the visible region. Furthermore, unlike the C165S, C250S, C285S, C342S, and C345S variants, the C166S, C257S, and C260S mutants contained little or no iron or sulfide (Table 1). On the basis of this evidence, we concluded that the [4Fe-4S] cluster of EiAPR is ligated by three cysteine residues, Cys166, Cys275, and Cys260.

Table 1 also summarizes the enzymatic activities of all eight C-to-S variants and of wild-type EiAPR. As we have shown earlier for PaAPR, all three of the C-to-S variants that are missing the [4Fe-4S] cluster exhibit no detectable activity, suggesting a crucial role for the cluster in the mechanism of the enzyme. Four of the C-to-S EiAPR variants in which one of the conserved cysteine residues that does not serve as a ligand to the [4Fe-4S] cluster has been replaced with a serine either are completely inactive (C165S and C285S) or exhibit extremely low activity (0.1% of wild-type activity for C342S and 2% of wild-type activity for C345S). Replacement of the unique cysteine in EiAPR, Cys250, with serine produces a C-to-S variant that exhibits ca. 50% of the wild-type activity (although in other side-by-side purifications with wild-type EiAPR, C250S exhibited activity as high as 80% of that of the wild type).

Figure 4a shows an oxidation−reduction titration of EiAPR, at pH 7.0, using the mBBr modification of thiols described in Materials and Methods to monitor the thiol content of the enzyme. The data gave a good fit to the Nernst equation for two separate two-electron redox couples with E_m values of −292 and −141 mV, respectively. The E_m values and the $n = 2$ character of the titration curve, as well as the known specificity of mBBr for chemical modification of thiols (*18*, *19*), strongly suggested that the two components seen in the redox titration can be attributed to two dithiol/disulfide redox couples. The average E_m values (from four determinations) for the two redox couples were -292 ± 7 and -142 ± 10 mV at pH 7.0. Redox titrations of EiAPR carried out at pH 8.5 (Figure 4b), the pH optimum for enzymatic activity (*14*), gave E_m values of -352 ± 10 and -223 ± 10 mV for the two components (Figure 4b). A process in which the reduction of a disulfide is accompanied by the uptake of two protons per disulfide reduced would be expected to have an E_m value at pH 8.5 that is ca. 90 mV more negative than the E_m value observed at pH 7.0.

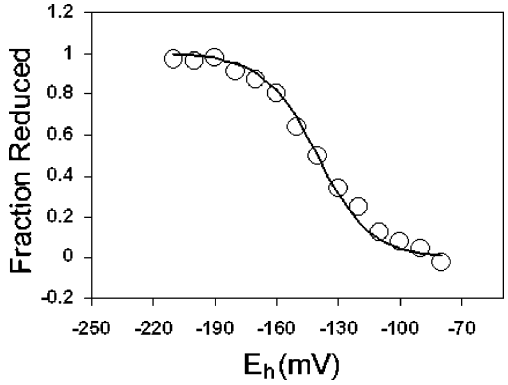

FIGURE 5: Oxidation−reduction titration of the C-terminal domain of EiAPR. The C-terminal domain of EiAPR was titrated at a concentration of 50 μg/mL in 100 mM Hepes (pH 7.0) buffer with a redox equilibration time of 2.5 h. The redox buffers contained either a total DTT concentration of 2.0 mM or a total glutathione concentration of 2.0 mM.

Although a detailed study of the pH dependence of the E_m values of the two dithiol/disulfide couples found in EiAPR was not carried out, the pH dependence of E_m for the more positive of the two components is consistent, within the combined uncertainties of the two sets of measurements, with the uptake of two protons per disulfide reduced over the pH range from 7.0 to 8.5. In contrast, the fact that the observed difference in the E_m values of the more negative EiAPR redox component at these two pH values is only 60 mV indicates a transition from the uptake of two protons per disulfide reduced to one proton taken up per disulfide reduced over this pH range (i.e., to the presence of a pK_a for a group that couples disulfide reduction to proton uptake somewhere between pH 7.0 and 8.5).

It was of interest to identify which specific pairs of cysteines are involved in formation of the two redox-active disulfides found in EiAPR. Figure 5 shows the results of a redox titration, carried out at pH 7.0, of the C-terminal domain of the enzyme expressed separately. The data give a good fit to the Nernst equation for a single two-electron redox couple with an E_m value of −140 mV. The average E_m value (three determinations) for the C-terminal domain was −143 ± 3 mV, identical to the value measured for the more positive component seen in titrations of the intact enzyme (see above). As this separately expressed C-terminal domain contains only two cysteine residues, Cys342 and Cys345, we can assign the E_m = −140 mV component to the Cys342/Cys345 pair. To further characterize this domain of EiAPR and to assess the functional integrity of the separately expressed recombinant C-domain, its ability to catalyze GSH-dependent reduction of cystine was assayed and compared to that of the intact EiAPR protein [this activity is typical of glutaredoxins and has been demonstrated for the AtAPR1 isoform of *A. thaliana* APS reductase and its separately expressed C-domain (*5*)]. The C-domain of EiAPR exhibited cystine reduction activity of 180.0 μmol min^{-1} (mg of protein)$^{-1}$, and the intact enzyme exhibited an activity of 121.5 μmol min^{-1} (mg of protein)$^{-1}$, consistent with sequence comparisons that were used to assign a glutaredoxin-like character to this domain of EiAPR.

As Cys166, Cys275, and Cys260 each serve as a ligand to an iron in the [4Fe-4S] cluster, the more negative component seen in titrations of EiAPR must involve two of the remaining three cysteine residues, Cys165, Cys250, and Cys285. Cysteine residues corresponding to Cys165 and Cys285 in EiAPR are also found in PaAPR, while Cys250 appears to be unique to EiAPR (see Figure 1). PaAPR has a single redox-active disulfide with an E_m value of −300 mV at pH 7.0 (*11*), quite similar to that measured for the more negative component observed in titrations of intact EiAPR. Thus, it seemed reasonable to assume that the E_m = −290 mV component present in EiAPR involves the Cys165/Cys285 pair. To test this hypothesis further, oxidation−reduction titrations were carried out on C-to-S variants involving these three cysteine residues. Titrations (not shown) of the C250S variant resembled those of the wild-type enzyme, with average E_m values (from two independent titrations) for the two n = 2 components of −148 ± 3 and −273 ± 16 mV at pH 7.0. The fact that replacement of Cys250 with serine did not appreciably affect the redox properties of the enzyme is most readily interpreted in terms of a model in which this residue is not involved in disulfide bond formation and that the E_m = −290 mV disulfide/dithiol couple involves Cys165 and Cys285. To test this hypothesis further, titrations of C165S and C285S were carried out (one would expect that, if Cys165 and Cys285 are indeed the residues involved in the E_m = −290 mV disulfide bond, titrations would show only the E_m = −140 mV component). Unfortunately, these titrations were complicated by the fact that both variants contained large amounts of disulfide-linked homodimers that could not be distinguished from intramolecular disulfides in mBBr-based dithiol/disulfide redox titrations. (Quantitation of Coomassie-stained, nonreducing SDS−PAGE gels indicated that ca. 30% of the C285S variant was present as a homodimer and that ca. 42% of the C165S variant was present as a homodimer. Preparations of both variants also contained ca. 4−5% of higher-molecular mass, disulfide-linked oligomers.) Titrations of both the C165S and C285S variants exhibited two components, one of which had an E_m value of −155 ± 10 mV, equal to that measured for the Cys342/Cys345 pair, within the combined uncertainties of the two sets of measurements. The simplest interpretation of these data is to assign this component to the intramolecular C342/Cys345 pair. The second component seen in the titrations (at pH 7.0) of the C165S variant had an E_m value of −270 ± 15 mV, and the second component seen in titrations (also at pH 7.0) of the C285S variant had an E_m value of −295 ± 10 mV. On the basis of the presence of large amounts of disulfide-linked homodimers in the two variants, it seems appropriate to tentatively assign the more negative E_m components in both cases to intermolecular disulfide bonds.

As mentioned above, EiAPR uses reduced glutathione (GSH) as its electron donor. In contrast, PaAPR utilizes the thermodynamically stronger reductant, reduced thioredoxin, as its electron-donating substrate. The E_m value, at pH 7.0, for the GSH/GSSG couple at pH 7.0 is −245 mV (*24*), sufficiently negative so that reduction of the E_m = −140 mV Cys342/Cys345 disulfide in EiAPR is thermodynamically quite favorable. In contrast, reduction of the E_m = −290 mV Cys165/Cys285 disulfide by GSH would be thermodynamically much less favored. Although the ambient redox potential in the chloroplast stromal space of *E. intestinalis* (the likely location of EiAPR) is not known, if it is significantly more positive than −290 mV, the Cys165 and

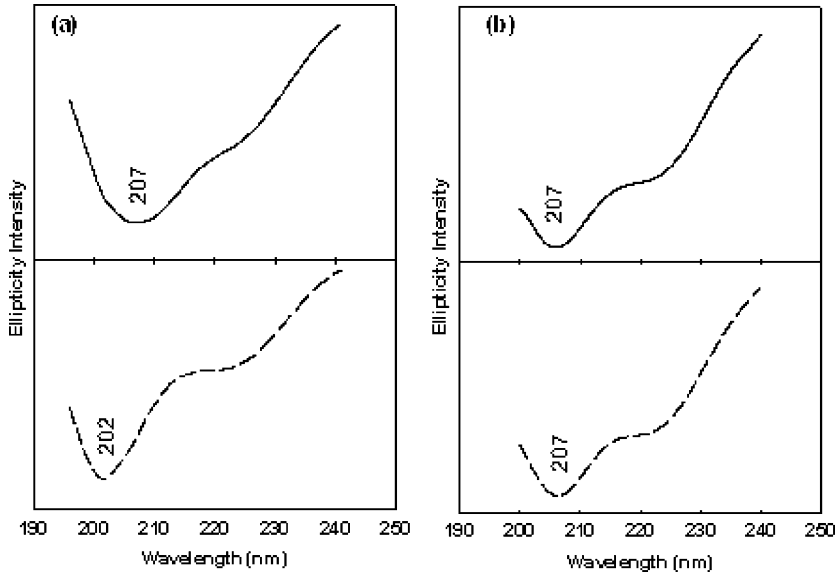

FIGURE 6: CD spectra of wild-type (a) and C285S EiAPR (b) poised at defined redox potentials. Solid lines represent data for the fully reduced form of the enzymes; dashed lines represent data for the half-reduced form of the enzymes. The enzymes are incubated in 5 mM reduced DTT for the fully reduced form and incubated at −220 mV for the half-reduced form. All spectra were obtained at ambient temperature in 10 mM potassium phosphate buffer (pH 7.5) at an enzyme concentration of 1−2 μM.

Cys285 residues would remain permanently in the oxidized, disulfide form under all physiological conditions. If this were indeed the case, one must reconcile the fact that these two cysteine residues do not participate in redox reactions, yet the site-directed mutagenesis data of Table 1 show both thiol groups are essential for activity. One possible explanation is that the disulfide formed between these two cysteines is an essential structural element that stabilizes a catalytically active conformation of the enzyme. Figure 6a shows circular dichroism (CD) spectra of wild-type EiAPR, in the ultraviolet region where α-helices and β-sheets contribute to the CD spectra of proteins, poised at two different ambient redox potentials chosen so that either only the Cys342/Cys345 disulfide has been reduced (the "half-reduced form" of the enzyme) or both disulfides are reduced (the "fully reduced form"). As can be seen, the two spectra differed significantly, indicating that reduction of the $E_m = -290$ mV Cys165/Cys285 disulfide produced a change in the conformation of the enzyme. Using an algorithm that allows one to estimate secondary structure contents from CD spectra (*21*), one can estimate that reduction of the Cys165/Cys285 disulfide increases the α-helix content from 13.5 to 18.7% and decreases the β-sheet content from 29.9 to 25.8% (the "random coil" content increases from 53.7 to 55.8%). If the hypothesis that reduction of a Cys165/Cys285 disulfide bond alters the secondary structure of EiAPR is correct, then the CD spectra of either a C165S variant or a C285S variant, each of which is incapable of forming a Cys165/Cys285 disulfide, should not depend on the ambient redox potential over the E_h range where this disulfide becomes reduced in the wild-type enzyme. Figure 6b shows CD spectra of C285S EiAPR under the same conditions used to generate the half-reduced and fully reduced forms of the wild-type enzyme for the CD spectra shown in Figure 6a. As predicted, there was little effect of ambient potential on the CD spectrum of this C285S EiAPR variant. Furthermore, the CD spectrum of C285S EiAPR poised at a E_h value where the wild-type enzyme would be half-reduced showed a resemblance to the CD spectrum of the fully reduced WT enzyme (the wavelength positions for the major features of these spectra show that the CD spectrum of C285S is more like the spectrum of the fully reduced, wild-type enzyme rather than that of the half-reduced protein), as would be expected if neither contained the putative conformation-stabilizing, low-potential disulfide.

DISCUSSION

Seven of the eight cysteine residues present in what is likely to be the mature form of the EiAPR have been shown to be essential for activity. Only Cys250, which is unique to algal APS reductases (*14*, *23*), is not absolutely essential for activity. The possibility that this residue may be important for the stability of the enzyme is currently under investigation. Three of the essential cysteine residues, Cys166, Cys257, and Cys260, appear to serve as ligands to the [4Fe-4S] cluster present in the enzyme. The cluster in EiAPR is spectroscopically similar (and thus likely to be structurally similar) to that in PaAPR, LmAPR, and AtAPR2, which also have been proposed to contain only three cysteinyl ligands (*11*, *22*). By contrast, Carroll et al. (*12*) inferred that the [4Fe-4S] cluster of *Mycobacterium tuberculosis* APR and PaAPR is tetragonally coordinated on the basis of their detection of only a single free thiol (the residue analogous to Cys285 in EiAPR) by alkylation with 4-vinylpyridine followed by mass analysis. They reasoned deductively that if the thiol group analogous to Cys165 or Cys166 (in EiAPR) is not free or involved in a disulfide bond it must be coordinated to the [4Fe-4S] cluster. However, Mossbauer and resonance Raman spectroscopy of PaAPR, LmAPR, and AtAPR2 are clearly consistent with a [4Fe-4S] cluster in which only three of the ligands to Fe are cysteine residues in these proteins (*11*, *22*). Moreover, site-directed mutagenesis studies of PaAPR and AtAPR2 (*11*, *22*) and DTNB titration of free thiol groups in PaAPR (*11*) are also consistent with the presence of a [4Fe-4S] cluster in which only three of the ligands to Fe are cysteine residues. This discrepancy could be resolved when the three-dimensional structure of an assimilatory APS reductase becomes available. It is of interest to note that our

previous PaAPR mutagenesis experiments identified the cysteine residue of the adjacent Cys139/Cys140 pair closer to the N-terminus of the protein, i.e., Cys139, as a [4Fe-4S] cluster ligand and the other cysteine of the pair, i.e., Cys140, as a member of a redox-active Cys140/Cys256 disulfide. In contrast, the mutagenesis experiments reported above identify the cysteine residue of the adjacent Cy165/Cys166 pair that is closer to the C-terminus of EiAPR, i.e., Cys166, as a [4Fe-4S] cluster ligand, with the other cysteine of the pair, i.e., Cys165, serving as a member of a redox-active disulfide. Considering the conserved alignment of five of the eight cysteine residues of EiAPR with the five cysteines present in PaAPR (see Figure 1), and the fact that Cys285 in EiAPR, the residue that forms a disulfide with Cys165, corresponds to Cys256, the disulfide bond partner for Cys140 in PaAPR, this observation was somewhat surprising. It should be mentioned that, unlike the case for our previous investigation of PaAPR, where C-to-S variants of cysteines thought not to function as cluster ligands had iron and sulfide contents similar to that of the wild-type enzyme, in the case of the C165S variant, the low iron and sulfide contents may arise from the fact that this cysteine is so close to a cysteine that actually serves as a cluster ligand (i.e., Cys166) that altering this amino acid (even with the quite conservative replacement of it with a serine) alters the local environment in a manner that partially destabilizes the cluster.

As can be seen in Figure 1, the two cysteine residues present in the C-terminal region of EiAPR have no counterparts in PaAPR. Our experiments indicate that these two residues, Cys342 and Cys345, form a redox-active disulfide/dithiol couple with an E_m value of −140 mV at pH 7.0. This E_m value would make the Cys342/Cys345 disulfide present in the "as isolated" form of EiAPR able to be reduced by GSH, the known physiological electron donor for the enzyme, in a reaction that is thermodynamically quite favorable, and for this reason, we propose that this C-terminal disulfide is the point for entry of electrons from GSH. A similar conclusion for the C-terminal domain of one of the chloroplastic isoforms of *A. thaliana* APS reductase was reached by Bick et al. (*5*), based on the finding that the C-terminal domain of this enzyme is active in several glutathione-dependent reductase assays that are typical of glutaredoxins, and we have demonstrated that the C-terminal domain of EiAPR exhibits at least one glutaredoxin-like activity, i.e., cystine reduction. We have also demonstrated the presence of a second disulfide in EiAPR, which we assign to a Cys165/Cys285 linkage. Although this $E_m = -290$ mV disulfide has an E_m value very similar to that of the Cys140/Cys256 disulfide found in PaAPR, we propose quite different functions for this low-potential disulfide in the two enzymes. While we have suggested that the Cys140/Cys256 bond is the redox-active point of entry for electrons from reduced thioredoxin in the case of PaAPR, we propose that, in contrast, the Cys165/Cys285 disulfide remains oxidized throughout the catalytic cycle of EiAPR (which uses the thermodynamically weaker reduced glutathione as its electron donor) and serves to lock the enzyme in a catalytically efficient conformation.

Our assignment of a purely structural role to a disulfide is not the only possible interpretation of our data. For example, it is possible that the putative Cys165/Cys285 disulfide plays a regulatory role, even though at present there is no evidence from the studies for dithiol/disulfide-linked redox activation of an APS reductase as the ambient potential becomes more negative. It should also be mentioned that our model proposes a purely structural role for EiAPR cysteine residues when the corresponding cysteines have a catalytic role in related bacterial APS reductases. Experiments designed to further document these possible differences between plant and bacterial APS reductase (including attempts to crystallize EiAPR to confirm the proposed disulfide assignments and the ligation pattern for the [4Fe-4S] cluster in EiAPR) are currently underway in our laboratories.

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