Regulation of the Plant-type 5′-Adenylyl Sulfate Reductase by Oxidative Stress[†,‡]

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ABSTRACT: 5′-Adenylyl sulfate (APS) reductase (EC 1.8.4.9) catalyzes a key reaction in the plant sulfate assimilation pathway leading to the synthesis of cysteine and the antioxidant glutathione. In *Arabidopsis thaliana* APS reductase is encoded by a family of three genes. In vitro biochemical studies revealed that the enzyme product derived from one of them (APR1) is activated by oxidation, probably through the formation of a disulfide bond. The APR1 enzyme is 45-fold more active when expressed in a *trxB* strain of *Escherichia coli* than in a *trxB*$^+$ wild type. The enzyme is inactivated in vitro by treatment with disulfide reductants and is reactivated with thiol oxidants. Redox titrations show that the regulation site has a midpoint potential of −330 mV at pH 8.5 and involves a two-electron redox reaction. Exposure of a variety of plants to ozone induces a rapid increase in APS reductase activity that correlates with the oxidation of the glutathione pool and is followed by an increase in free cysteine and total glutathione. During the response to ozone, the level of immunodetectable APS reductase enzyme does not increase. Treatment of *A. thaliana* seedlings with oxidized glutathione or paraquat induces APS reductase activity even when transcription or translation is blocked with inhibitors. The results suggest that a posttranslational mechanism controls APS reductase. A model is proposed whereby redox regulation of APS reductase provides a rapidly responding, self-regulating mechanism to control the glutathione synthesis necessary to combat oxidative stress.

All aerobic organisms are vulnerable to damage from reactive oxygen species (ROS)[1] (*1*). The life of plants is especially precarious because ROS are generated as a byproduct of oxygenic photosynthesis and carbon dioxide fixation (*2*). In their own defense plants have evolved an antioxidant system in which glutathione plays a pivotal role in the removal of hydrogen peroxide and in maintaining the level of another antioxidant, ascorbic acid (*3, 4*). Glutathione has long been known to accumulate in plants following oxidative stress (*5*−*9*), but the mechanisms regulating this response are far from clear.

Glutathione is an enzymatically synthesized tripeptide composed of the amino acids Glu, Cys, and Gly. It exists in reduced (GSH) and oxidized (GSSG) forms. Together, GSH and GSSG form a biological redox buffer that is maintained predominantly in the reduced state by NADPH-dependent glutathione reductase (*10*). Glutathione is synthesized in two sequential steps catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. First Cys and Glu react to form γ-GluCys. Gly is then added to form glutathione. Regulation of the glutathione synthesis in plants is controlled by γ-glutamylcysteine synthetase (*9, 11*). In addition, the availability of amino acid substrates, in particular Cys, has a significant effect upon the rate of glutathione biosynthesis (*9, 11, 12*).

Cys is produced in four steps (*13, 14*). In the first, inorganic sulfate is activated by use of ATP to form 5′-adenylyl sulfate (APS), a reaction catalyzed by ATP sulfurylase. APS is then reduced to sulfite plus AMP in a two-electron reaction catalyzed by APS reductase, possibly with GSH as a source of electrons (*15*−*17*). A subsequent six-electron reduction of sulfite to sulfide, carried out by sulfite reductase, is followed by incorporation of sulfide into the thiol group of Cys. APS reductase is identical to the enzyme previously known as APS sulfotransferase (*18*). All the enzymes for synthesis of Cys are localized in the chloroplast, and APS reductase is exclusively localized in

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[‡] The EC number for 5′-adenlylysulfate reductase (EC 1.8.4.9) has been newly assigned; see http://www.chem.qmw.ac.uk/iubmb/enzyme/EC1/8/4/9.html. The Protein Identification Resource accession numbers of the enzymes studied in this paper are as follows: APR1, AAC26979.1; APR2, AAC26980.1; APR3, AAC26981.1.

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[1] Abbreviations: Amp, ampicillin; APS, 5′-adenylyl sulfate; Cam, chloramphenicol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; E_m, redox midpoint potential; GSH, reduced glutathione; GSSG, oxidized glutathione; Kan, kanamycin; mBBr, monobromobimane; 2ME, 2-mercaptoethanol; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBST, phosphate-buffered saline with Tween-20; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate, TR, thioredoxin reductase; Trx, thioredoxin; TRXH, thioredoxin h; TRXM, thioredoxin m.

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plastids (*13*). APS reductase is likely a control point of sulfate assimilation in plants. It is the only enzyme activity in the pathway that is significantly regulated by treatments such as sulfur starvation, heavy metal exposure, addition of Cys or other reduced sulfur sources to the irrigation medium (*13*, *14*), or oxidative stress (this study).

In the course of this study, the mechanisms controlling APS reductase activity in response to oxidative stress were explored. The experiments revealed a potential posttranslational mechanism for regulation of APS reductase activity and a role for this enzyme in maintaining glutathione production in response to oxidative stress.

EXPERIMENTAL PROCEDURES

Culture Media, Growth Conditions, and General Methods. Luria−Bertani (LB) medium was used for the growth of *Escherichia coli* cultures. *Arabidopsis thaliana* was grown in Promix irrigated with 0.25× strength Peters 20:20:20 fertilizer (Grace-Sierra Co., Milpitas, CA) in a growth chamber at 24 °C with a diurnal cycle of 14 h light/10 h darkness and a light intensity of approximately 100 μmol of photons m^{-2} s^{-1}. *Nicotiana tabacum* and *Brassica juncea* were grown similarly but in a greenhouse. For some experiments *A. thaliana* seedlings were grown axenically in liquid medium containing 0.5× concentration MS salts (Life Technologies, Gaithersburg, MD, 23118-060), 0.2% (w/v) sucrose, and 25 mM MES (pH 5.8). Approximately 50 surface-sterilized seeds were inoculated into 125-mL Erlenmeyer flasks containing 40 mL of medium and were grown for 7−10 days at 24 °C with constant shaking at 80 rpm and diurnal cycle of 14 h light/10 h darkness and a light intensity of about 100 μmol of photons m^{-2} s^{-1}.

Molecular methods were carried out generally as described in Sambrook et al. (*19*). Immunoblotting was carried out as described by Harlow and Lane (*20*). Protein extracts were prepared by grinding freshly prepared plant tissue in extraction buffer. Buffer used to prepare extracts for immunoblotting contained 50 mM Tris-HCl (pH 8.5), and buffer used for enzyme assay contained 50 mM Tris-HCl (pH 8.5) and 500 mM Na_2SO_4. *E. coli* cells were lysed by sonication in the same buffer. The samples were centrifuged at 4 °C for 10 min at 10000*g* and the supernatant was collected. Protein concentration was measured by the Bradford method (*21*) with bovine serum albumin as a standard.

Expression in E. coli and Purification of Recombinant APS Reductase. The coding sequence of APR1 lacking the portion encoding the chloroplast transit peptide was amplified from the cDNA cloned into pBluescript with the following primers: 5′-ACAGAATTCCAAACCTTTAAAC-3′ and 5′-GTAATACGACTCACTATAGGGC-3′. The amplification product was cloned into the *Eco*RI and *Hin*dIII sites of pET30B (Novagen, Inc., Madison, WI). The expression cassette from this plasmid was subcloned into *Xba*I and *Sal*I of pBAD18 (*22*). pBAD18-APR1 was transformed into *E. coli* strain TL3 (*16*), and colonies were selected in LB with 35 μg/mL kanamycin (Kan), 34 μg/mL choramphenicol (Cam), and 100 μg/mL ampicillin (Amp). Overnight cultures grown in liquid LB with the antibiotics were used to inoculate 1 L of the same medium (1:100 inoculum dilution). The cultures were grown to an optical density (600 nm) of 0.6, typically 2−3 h at 30 °C with shaking. APR1 expression was induced by addition of 0.2% (w/v) arabinose followed by overnight incubation.

APR1 protein purification was carried out at 4 °C. The cells were harvested by centrifugation and the cell pellet from 1 L of culture was resuspended in 100 mL of 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl (buffer A). The cells were sonicated on ice and centrifuged, and the supernatant was filtered though a 0.45 μM filter. The supernatant was stirred with 2 mL (bed volume) $Ni2^{+}$-agarose (Talon, New England Biolabs, Beverly, MA) for 2 h, followed by two washes in 20 mL of buffer A and one wash in buffer A containing 10 mM imidazole. The protein was eluted in buffer A containing 125 mM imidazole. The protein was concentrated to approximately 1 mg/mL by use of a Centricon 30 (Amicon, Inc., Beverly, MA) protein concentrator and was stored in aliquots at −70 °C. The protein was diluted to 1 μg/mL in 20 mM Tris-HCl (pH 8.5) just prior to enzyme assays. The purity of the preparations was determined by SDS−polyacrylamide gel electrophoresis followed by staining of the gel with Coomassie blue. The purity was found to be similar to that shown in Figure 2, lane 2, of Bick et al. (*16*). Because of the instability of APS reductase, the affinity tag was not removed, as this would have required proteolytic digestion for extended periods at elevated temperature.

APS Reductase Assay. APS reductase activity was measured at 30 °C in a 100 μL reaction containing 50 mM Tris-HCl (pH 8.5), 500 mM Na_2SO_4, 1 mM EDTA, 10 mM GSH (or DTT, where specified), and 25 μM [^{35}S]APS (specific activity of approximately 500 Bq $nmol^{-1}$) produced from [^{35}S]PAPS (Dupont NEN, Wilmington, DE) as described (*15*). The reactions were started by the addition of tissue extract (5−10 of μg protein) or recombinant enzyme (2 ng of protein or as indicated in the text). In all cases initial velocity conditions were ensured by adjusting incubation time so that no more than 10% of the substrate in the reaction had been consumed. All experiments were performed at least three times.

In Vitro Modulation of APS Reductase Activity and Measurement of the Redox Midpoint Potential of the Regulation Site. Recombinant APR1 APS reductase (2 ng or as indicated in the text) was inactivated by incubation on ice for up to 60 min in a 5 μL reaction with 50 mM Tris-HCl (pH 8.5) and 10 mM DTT, 2-mercaptoethanol (2ME), GSH, or 10 μg of reduced *Spinacea oleracea* thioredoxin *m* (TRXM) or *Chlamydomonas reinhardtii* thioredoxin *h* (TRXH). DTT was removed from an inactivated APR1 APS reductase preparation by buffer replacement with 50 mM Tris-HCl (pH 8.5) by use of a Centricon 3 microconcentrator (Amicon, Inc.). Inactivated protein (2 ng) was reactivated by incubation for up to 120 min on ice in a 5 μL reaction with 50 mM Tris-HCl (pH 8.5) and 100 μM GSSG, 100 μM DTNB, and 0.2 μg oxidized TRXM or TRXH. The progress of inactivation or reactivation was monitored by determining APS reductase activity. The enzyme was diluted 20-fold from the inactivation or reactivation reaction into the APS reductase reaction.

E_m was measured for inactivated or reactivated APR1 APS reductase, prepared as described above, with redox buffers containing either DTT, glutathione, or *C. reinhardtii* thioredoxin *h* (TRXH) at defined reduced/oxidized ratios. The measured E_m values were independent of the time used for equilibration (redox equilibration was carried out with

samples kept on ice) from 1 to 2.5 h and independent of the total redox buffer concentration in the range 25−75 mM DTT, 0.8−2.5 mM glutathione, and 25−40 μM TRXH. The calculation of E_m values for the regulatory disulfide of APS reductase is based on E_m values at pH 8.5 of −415 mV for DTT, −400 mV for TRXH, and −325 mV for glutathione. The E_m values used for DTT and glutathione at pH 8.5 were calculated from literature values for E_m at pH 7.0 (*23*, *24*) and the known pK_a values for the thiol groups (*25*) by the method described by Chivers et al. (*26*). In addition, a correction was made to convert literature values from the standard temperature of 25 °C to a temperature of 4 °C because the measurements were carried out on samples incubated in ice. The E_m value for TRXM at pH 8.5 was measured by the monobromobimane (mBBr) fluorescence technique described previously (*27*, *28*). Some experiments were carried out in an anaerobic chamber, but such experiments gave the same E_m values obtained under aerobic conditions. In all experiments, the percentage of inactivation or reactivation was corrected for the redox-independent loss of APS reductase activity when incubated on ice in buffer with 10 mM Tris-HCl (pH 8.5). APR1 APS reductase is unstable, typically losing approximately 12% of its activity over 90 min incubation on ice. Data-fitting to the Nernst equation was carried out as described previously (*27*, *28*).

TRXM and TRXH were completely oxidized as supplied. The proteins were reduced by incubation with an excess of DTT for 1 h on ice. DTT was removed by buffer exchange with 50 mM Tris-HCl (pH 8.5) in a Centricon 3 microconcentrator.

Plant Treatment and Analysis. B. juncea seedlings were grown for 15 days as described above. The plants carried four true leaves in the expansion stage of development. *A. thaliana* was grown for 28 days and the plants carried 8−10 mature leaves in the expansion stage of development. *N. tabacum* was grown for 10 weeks in a greenhouse to just before the flowering stage of development. All plants were exposed to 150−200 ppb ozone for 1−2 h in a fumigation chamber situated within a greenhouse. Ozone treatments were carried out in the early morning within 2 h after sunrise. Ozone was generated by passing oxygen over a UV light source. Ozone concentration was controlled with a UV photometric O_3 analyzer (*29*). Following ozone treatment the plants were removed to the lab bench under ambient laboratory fluorescent light conditions and leaf samples were harvested at various times for analysis. From *B. juncea* the true leaves were sampled. From *A. thaliana*, the entire shoot was harvested, and from *N. tabacum*, leaves at mid-stage of expansion were sampled. APS reductase was assayed immediately. Samples for thiol analysis or immunoblotting were frozen on dry ice and stored at −70 °C prior to analysis.

Treatment and analysis of leaf fragments were carried out as follows. Leaf disks (1 cm diameter) were cut from 14-day-old *B. juncea* seedlings and were placed in buffer T containing 50 mM Tris-HCl (pH 8.5), 200 mM Na_2SO_4, and 0.05% (v/v) Triton X-100 or in buffer T with 10 mM GSH, 10 mM GSSG, or 100 μM paraquat. The leaf disks were vacuum-infiltrated for 5 min and the infiltration buffer was replaced with fresh buffer before the disks were incubated in a growth chamber under light (150 μmol of photons m^{-2} s^{-2}). Samples were periodically harvested and APS reductase activity was measured immediately.

Treatment of axenically grown *A. thaliana* seedlings was carried out as follows. The plants were exposed to chemical treatments as described in the figure legends. The chemicals, including actinomycin D (1 mM), cyclohexamide (10 mM), GSSG (10 mM), and paraquat (100 μM), were added to the growth medium. Samples were harvested for immediate measurement of APS reductase activity; or for later analysis by immunoblotting or RNA blotting, the samples were frozen and stored at −70 °C.

Immunoblot and RNA Blot Analysis. Tissue extracts were separated in a denaturing 12% (w/v) acrylamide gel and transferred onto Immobilon P membrane (Millipore, Inc.). The membranes were blocked overnight in PBST [137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2, and 0.2% (v/v) Tween-20] containing 1% (w/v) BSA. APS reductase was detected with a 1:10 000 dilution of an anti-peptide antibody raised against a conserved region of APS reductase (*30*), followed by incubation with a secondary goat anti-rabbit antibody conjugated with HRP (Sigma, Inc.). Immunocomplexes were detected by use of the Renaissance chemiluminescence detection kit (NEN, Inc.).

RNA was isolated from *A. thaliana* leaf tissue with the Plant RNAeasy Kit (Qiagen, Inc). Total RNA (20 μg) from each sample was separated on a 1.4% (v/v) formamide agarose gel and transferred onto Hybond-N^+ membrane (Amersham) with a vacuum blotter. The membranes were prehybridized for 5 h before overnight hybridization with probe at 65 °C. The membranes were washed in 2× SSC, in 0.5% (w/v) SDS and 0.1× SSC, and in 0.1% (w/v) SDS at 65 °C for 2 h and exposed to film for 3 days. The increase in mRNA was quantitated by cutting out individual bands from the membrane and counting the levels of ^{32}P-label by a scintillation counting with Ready Safe (Beckman-Coulter, Fullerton, CA) scintillation fluid. The APR1, APR2, or APR3 cDNAs (*15*) were labeled by the random primer method with [α-^{32}P]dCTP (111 TBq $mmol^{-1}$).

Thiol Analysis. Thiol compounds were measured from tissue extracts by use of monobromobimane (mBBr) as described by Fahey and Newton (*31*) as modified by Kramer and Grill (*32*). GSSG was measured after blocking GSH with *N*-ethyl maleimide (*31*, *32*).

RESULTS

Expression in Escherichia coli trxB Stimulates APS reductase Activity. In the flowering plant *A. thaliana*, APS reductase isoenzymes are encoded by three closely related genes, APR1, -2, and -3 (*15*). The encoding cDNAs were used to produce recombinant proteins for enzymological analysis (*16*). In the course of optimizing the heterologous expression of one of the isoforms, that encoded by APR1, it was found that APS reductase activity in cell lysates is 11−45-fold greater in *E. coli* thioredoxin reductase (TR) (*trxB*) mutants expressing APR1 compared with a *trxB*$^+$ wild-type strain (Table 1). Kinetic analysis of the purified APR1 protein revealed that the enzyme V_{max} is increased by 45-fold and the apparent K_m[APS] is decreased by 3-fold, whereas the apparent K_m[GSH] is unaffected. Similar analysis of the other isoforms encoded by APR2 and APR3 did not reveal marked differences in activity when expressed in the *trxB* and *trxB*$^+$ host backgrounds (Table 1). The effect of the host strain on APR1 APS reductase suggests that the enzyme contains a disulfide bond that is important for activity. Protein disulfide

Table 1: Activity and Kinetic Constants of Recombinant APS Reductase Expressed in Wild-type and *trxB E. coli* Strains

APR isoform	*E. coli* strain[b]	relevant genotype	activity[a] lysate ($\times 10^{-3}$)	activity[a] pure enzyme	K_m[APS] (μM)	K_m[GSH] (mM)
APR1	JM96	*trxB*$^+$	0.22			
APR1	TL3	*trxB*	4.95			
APR1	K1380	*trxB*	5.0			
APR1	AD949	*trxB*	2.54			
APR1	BL21	*trxB*$^+$	0.18	0.18	1.20	0.8
APR1	A326	*trxB*	5.51	8.17	0.38	0.6
APR2	JM96	*trxB*$^+$	2.82			
APR2	A326	*trxB*	2.46			
APR3	JM96	*trxB*$^+$	0.33			
APR3	A326	*trxB*	1.02			

[a] Micromoles per minute per milligram of protein. [b] BL21 and AD494 (Novagen, Inc.) were transformed with pET-APR1 (*15*) and pBAD-APR1, respectively. TL3 (*16*), JM96, A326, and K1380 (Coli Genetic Stock Center, Yale University) were transformed with λYES-APR1, λYES-APR2, or λYES-APR3 (*15*). APS reductase was assayed in lysates of mid-log phase cultures. The kinetic constants were calculated by least-squares nonlinear regression analysis as described in (*16*). *trxB*$^+$ indicates the wild-type allele.

bonds do not normally form in the *E. coli* cytoplasm, but they readily form in *trxB*, probably because oxidized thioredoxin catalyzes the oxidation of protein thiol groups (*33*, *34*).

In Vitro Regulation of APS Reductase by Redox Conditions. Having obtained preliminary evidence that APR1 APS reductase may contain functionally important Cys residues, it seemed useful to investigate the effect of reagents known to alter the redox state of dithiol/disulfide couples on enzyme activity. The recombinant APR1 enzyme expressed in a *trxB* strain was inactivated when it was preincubated with the disulfide reductants DTT, 2ME, reduced TRXM, reduced TRXH, or GSH (Figure 1A). Of these reagents, only GSH and DTT serve as electron donors for catalysis. The low-activity APR1 expressed in a *trxB*$^+$ wild-type *E. coli* was also inactivated with the reductants (not shown), suggesting that the fraction of active enzyme in this preparation may be in the disulfide form. The activity of reduced APS reductase could be restored with the thiol oxidants GSSG, oxidized TRXM, TRXH, or DTNB (Figure 1B). This result further supports the hypothesis that the APR1 enzyme contains a regulatory disulfide bond that, when formed, results in the formation of a catalytically active configuration of the enzyme.

The E_m of this putative regulatory disulfide of APS reductase was measured by monitoring enzyme activity following redox equilibration at pH 8.5 with redox buffers containing either DTT, glutathione, or TRXH at defined reduced/oxidized ratios. It is worth noting that, unlike GSH and DTT, TRXH is not a substrate for the catalytic activity of APS reductase. All of the titrations, the results of which are summarized in Table 2, gave excellent fits to the Nernst equation for a single, two-electron ($n = 2$) redox reaction. Attempts to fit the data to multiple $n = 2$ components did not improve the statistical quality of the fits. The average E_m value, for the six sets of measurements of Table 2, after rounding off to the nearest 10 mV to take into account the ± 10 mV experimental uncertainty, is -330 mV at pH 8.5. The titrations appeared to represent true equilibrium measurements, in that the E_m and n values obtained were

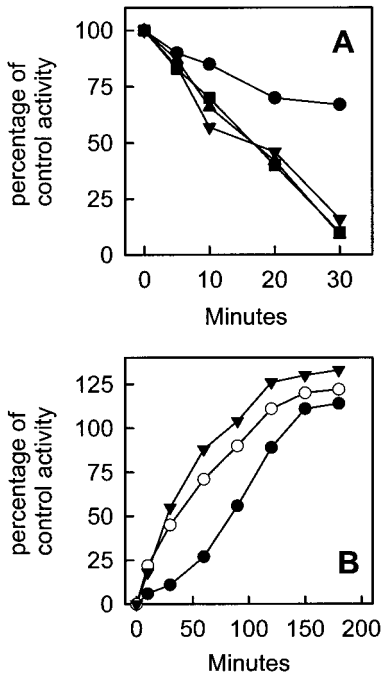

FIGURE 1: Effect of redox buffers on APR1 APS reductase activity. (A) Inactivation with GSH (●), DTT (▲), $TRXM_{RED}$ (▼), and 2ME (■). (B) Reactivation with $TRXM_{OX}$ (○), DTNB (●), or GSSG (▼). Reactivation was carried out on enzyme that had been inactivated with DTT as described under Materials and Methods. All data points represent the mean of three independent experiments. A control activity of 100% reflects the activity of the enzyme before treatment with disulfide reductants or thiol oxidants. The specific activity of the APR1 preparation used in these experiments was ~8.0 μmol min^{-1} mg of protein^{-1}.

Table 2: E_m Measurements of the APS Reductase Regulation Site[a]

buffer	starting enzyme	E_m (mV) $\pm$ SE
glutathione	active APS reductase	-335 ± 9.5
glutathione	inactive APS reductase	-320 ± 9.9
DTT	active APS reductase	-338 ± 17.5
DTT	inactive APS reductase	-325 ± 15.2
TRXH	active APS reductase	-322 ± 8.2
TRXH	inactive APS reductase	-322 ± 9.6

[a] E_m values of inactivation and reactivation of APR APS reductase with various redox buffers. Each $E_m \pm$ SE was calculated from six titrations carried out with various redox buffer concentrations and equilibration times.

independent of the redox equilibration time and the total concentration of redox buffer used (see Experimental Procedures). The fact that identical E_m values were obtained (see Table 2), within the ± 10 mV experimental uncertainty, for the three chemically different redox buffers indicates that this E_m value is an intrinsic property of APS reductase. Furthermore, independent of which of the three redox buffers was used, or whether fully active enzyme (the oxidized form) or fully inactive enzyme (the reduced form) was titrated, identical E_m values were obtained. This is illustrated, in Figure 2, for titrations in glutathione redox buffer. Taken as a whole, the titration data are consistent with the presence of a single regulatory disulfide, with $E_m = -330$ mV at pH 8.5, in APS reductase. The ability to modulate the activity of APS reductase in vitro with thiol/disulfide redox buffers supports the hypothesis that the enzyme contains two functionally important Cys residues that could be the target of redox regulation in vivo.

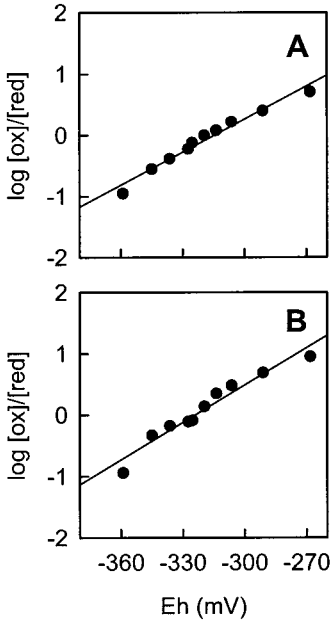

FIGURE 2: Titration of APR1 APS reductase with glutathione redox buffer. (A) Inactivation of APR1. (B) Reactivation of APR1 that had been inactivated with DTT. For each redox titration 25−100 ng of recombinant APR1 was equilibrated on ice with glutathione redox buffer plus 100 mM Na_2SO_4. The *x*-axis is identical in panels A and B.

Induction of APS Reductase in Vivo by Oxidative Stress. The results from in vitro analysis of recombinant APS reductase suggested that the native enzyme might be regulated in vivo by its redox environment. Such a regulatory mechanism could operate during oxidative stress when the redox poise of plant cells is disrupted and the synthesis of glutathione is stimulated (*4*). To test this hypothesis, *B. juncea* seedlings were exposed to ozone in order to invoke an oxidative stress response. *B. juncea* was chosen for this experiment because it is closely related to *A. thaliana*, it contains APS reductase genes with high homology to those in *A. thaliana* (*35*), and it is more easily grown and analyzed than is *A. thaliana*. APS reductase activity increased in the leaves up to 20-fold within 2−3 h after ozone treatment (Figure 3A). Some variation in the magnitude of the response was evident between experiments but not within an experiment. The variability may result from differences in the accessibility of leaf cells to ozone, which is influenced by environmental factors such as relative humidity and temperature that are difficult to control in a greenhouse. However, the kinetics of the response were similar in all of the experiments. APS reductase activity did not change in plants that had not been exposed to ozone (not shown). The increase in activity after exposure to ozone was not accompanied by a change in the level of the APS reductase protein as measured by immunoblotting (Figure 3B). Thus, the response to ozone suggests that APS reductase enzyme is being activated posttranslationally. Similar increases in APS reductase activity were observed in ozone-treated *A. thaliana* and *N. tabacum* (data not shown), indicating that activation of APS reductase activity occurs in several species of flowering plants.

Ozone Stress Induces Synchronous Changes in APS Reductase Activity and Thiol Compounds. Although oxidative stress is well-known to stimulate glutathione synthesis in plants (*4*−*8*), it was of interest to determine whether

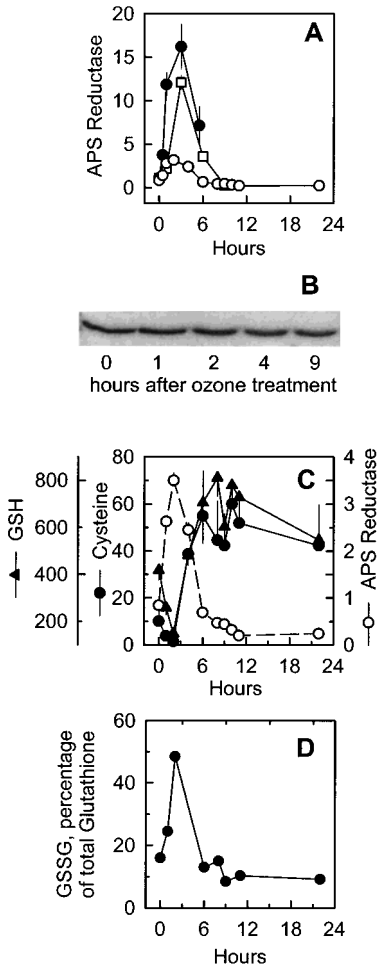

FIGURE 3: Response of *B. juncea* to oxidative stress. (A) APS reductase activity in plants treated for 1 h with 150 ppb ozone (○), 2 h with 150 ppb ozone (□), or 2 h with 200 ppb ozone (●). (B) Level of APR protein measured by immunoblotting of plants treated for 2 h with 200 ppb ozone. The gel was prepared with 12.5% polyacrylamide, and 20 μg of protein from each of the indicated samples was loaded. (C) Temporal changes in cysteine (●) and total glutathione (▲) content and APS reductase activity (○) in plants treated for 1 h with 150 ppb ozone. (D) Temporal change in the redox state of glutathione. All data points are the average ±SD of three individual samples, each sample consisting of the pooled tissue from three plants. Data points with no visible error bars are ones for which the error value is less than the diameter of the line symbol. Zero time represents the moment when ozone fumigation ceased. APS reductase activity is presented in nanomoles per minute per milligram of protein, and metabolites are in nanomoles per gram of fresh weight.

glutathione and Cys, both end-products of sulfate assimilation, accumulate in synchrony with APS reductase activity. After ozone treatment, the level of Cys and total glutathione decreased to a low point at 2 h (Figure 3C). The decrease in total glutathione occurred at the expense of GSH in that the level of GSSG as a percentage of total glutathione increased from 12% to 50% after 2 h (Figure 3D). The decrease in Cys and glutathione, and the large change in the redox state of the glutathione pool, correlated precisely with the increase in APS reductase activity. After the peak in activity, total glutathione and Cys increased sharply and reached a plateau after 6 h. Over the same period, GSSG declined to 10% of total glutathione, and APS reductase activity declined to the level in untreated plants. γ-GluCys, an intermediate in glutathione synthesis, remained unchanged over the experimental

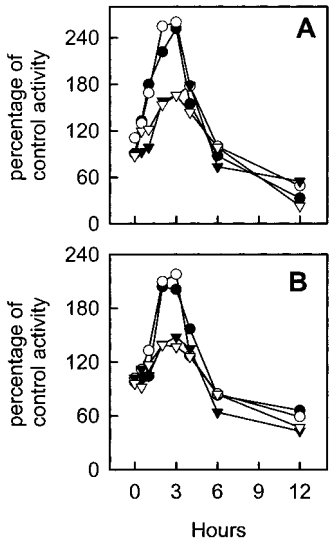

FIGURE 4: Analysis of APS reductase expression in *A. thaliana* seedlings exposed to oxidative stress and inhibitors of transcription or translation. (A) Treatment with GSSG (○) or paraquat (●); pretreatment with actinomycin D followed by treatment with GSSG (▼) or paraquat (▽). (B) Treatment with GSSG (○) or paraquat (●); pretreatment with cycloheximide followed by treatment with GSSG (▼) or paraquat (▽). The *x*-axis is identical in panels A and B.

period (not shown). Thus, APS reductase activity correlated directly with the oxidized state of the glutathione pool, and the peak in activity preceded the increase in Cys and total glutathione. The rapidity and synchrony of the responses to ozone resemble a homeostatic regulatory mechanism.

APS Reductase Activity Is Induced by Oxidative Stress in Vivo in the Absence of Transcription or Translation. The in vitro and in vivo results suggest that a posttranslational mechanism plays a role in regulation of APS reductase. This hypothesis was explored further by examining whether inhibition of transcription or translation affects the oxidative stress response of APS reductase. In *A. thaliana* seedlings grown in liquid medium, APS reductase activity increased 2.5-fold approximately 3 h after treatment with GSSG or paraquat (Figure 4). After the peak was reached, the activity rapidly declined to the level in untreated control plants. The kinetics of induction are similar to those observed for plants treated with ozone, but the magnitude of the response was lower. Similar results were obtained when *B. juncea* leaf fragments were vacuum-infiltrated with either paraquat or GSSG (data not shown). Paraquat is an herbicide that induces oxidative stress by inhibiting photosynthetic electron transport (*36*). In contrast, GSSG treatment may cause a transient shift in the redox state of the glutathione pool (*37*), thereby mimicking the effects of oxidative stress.

Because seedlings grown in liquid medium are small and in uniform contact with the culture fluid, they are more likely to take up actinomycin D or cycloheximide than are soil-grown plants, affording the possibility of testing whether transcription or translation is required for activation of APS reductase. Treatment with the inhibitors alone resulted in a steady decline in APS reductase activity, reaching 15% of the initial activity over 36 h (not shown), presumably due to turnover of APS reductase mRNA and protein. The rate of decay indicates that the half-life of APS reductase is approximately 10 h, similar to that previously reported for APS reductase of *Lemna minor* (*38*).

A pretreatment resulting in approximately 50% decline in APS reductase activity (12 h with 1 mM actinomycin D or 10 mM cycloheximide) was selected as the starting point for induction of APS reductase activity by oxidative stress. At this point it is certain that transcription and translation of APS reductase are inhibited. The results in Figure 4 show that even though synthesis of APS reductase is blocked, its activity increases after treatment with paraquat or GSSG. Although the response is greater if transcription or translation are not blocked, it should be noted that the decay of APS reductase continues throughout the experiment, accounting in part for attenuation of the response to GSSG or paraquat. Blot analyses revealed that the level of APS reductase protein did not change markedly during the period of rapid change in activity (not shown). This was confirmed by use of three independently raised anti-APS reductase antibodies (not shown). Thus, the GSSG- or paraquat-induced increase in APS reductase activity is principally due to activation of the enzyme rather than de novo synthesis. The steady state level of mRNA for the three APS reductase genes also did not change significantly (not shown). From these results it is possible to conclude that a posttranslational mechanism plays a major role in regulation of APS reductase activity in response to oxidative stress.

DISCUSSION

In this report, both in vitro and in vivo evidence is presented in support of the hypothesis that APS reductase is posttranslationally activated by oxidative stress. Such a regulation of the sulfur assimilation pathway could play a role in maintaining or increasing the pool of glutathione, a key component in the metabolic cycles that operate to mitigate oxidative stress in plants. The data suggest that the posttranslational mechanism for regulation of APS reductase may involve a redox-responsive Cys pair in the enzyme that, when oxidized to a disulfide bond, results in the formation of an active enzyme configuration. The trigger for disulfide bond formation may be GSSG, which accumulates when the redox poise of plant cells is disturbed by oxidative stress. Each of these points will be individually addressed in the following discussion.

In Vitro Evidence for Redox Regulation of APS Reductase and Its Physiological Implications. The initial evidence for redox regulation of APS reductase was obtained by serendipity during functional complementation analysis of *E. coli* strains when it was observed that the activity of APR1 APS reductase is greatly increased when expressed in a *trxB* genetic background. It is now firmly established that the *trxB* mutation causes the *E. coli* cytoplasm to become oxidizing, promoting the formation of protein disulfide bonds that would not normally form in the cytoplasm of wild-type *E. coli* (*33*, *34*, *39*). The concept of a redox-regulated APS reductase was further supported by studies demonstrating that the activity of the purified recombinant enzyme could be modulated in vitro by incubation in thiol/disulfide buffers of the appropriate redox potential and by in vivo studies showing that a posttranslational mechanism operates to regulate APS reductase in vivo. It has been known for some time that native APS reductase is readily inactivated by disulfide reducing agents (*40*). Reactivation with dithiol oxidants was never observed for the native enzyme, perhaps because of its extreme lability in protein extracts from plant

cells (*18*, *40*). Inactivation by disulfide reductants and enzyme lability were shown to act through different mechanisms (*40*).

The ability to modify APS reductase activity by chemical agents and proteins that reduce disulfide bonds or that cause the oxidative formation of disulfide bonds provides strong indirect evidence for a redox-sensitive Cys pair in APS reductase. Moreover, it was possible to determine the E_m of the Cys pair, which behaved, in accordance with the Nernst equation, as predicted for a two-electron-transfer reaction. The E_m of the regulation site was measured at −330 mV (at pH 8.5), close to that of glutathione at this pH. This E_m value is also consistent with the observation that oxidized DTT is a poor activator and reduced DTT is a strong inactivator, since the E_m of DTT (pH 8.5) is much more negative (−415 mV) than the regulation site of APS reductase.

The physiological implication of a regulatory disulfide with an E_m so close to that of glutathione is that only relatively large changes in the GSH:GSSG ratio, which is normally highly reduced, would shift the steady state of APS reductase activity. Thus, the activation state of the enzyme would only change significantly when GSSG accumulates during oxidative stress. The effect might not be limited to glutathione since thiol−disulfide oxidoreductases such as TRX also are potent modulators of APR1 (Figure 1). Analysis of *cad2* (*41*), a γ-glutamylcysteine synthetase mutant of *A. thaliana*, revealed that the APS reductase activity is similar to that of wild-type plants even though the total glutathione content of the mutant is only ∼20% that of the wild type. However, despite lower overall glutathione levels, the GSH:GSSG ratio of the *cad2* plants is identical to that of the wild type (data not presented). It may therefore be the redox status of the glutathione pool, rather than the absolute level of glutathione that influences APS reductase activity. The importance of the GSH:GSSG ratio has previously been implicated in the signaling of oxidative stress and the subsequent initiation of the appropriate antioxidant responses (*3*, *37*). Furthermore, the chloroplast GSH:GSSG ratio has been associated with the transcriptional regulation of the rubisco large subunit in *C. reinhardtii*, providing a mechanism for linking the stress response with the potential for photoacclimation (*42*).

Structure of APS Reductase Supports the Hypothesis of a Redox Regulation Site. All the plant APS reductases described to date, including those from *A. thaliana*, have the same general structure and contain seven conserved Cys residues (*30*). These enzymes are composed of two domains. At the amino terminus lies a region termed the "reductase domain" showing amino acid homology with CysH, a cysteine biosynthetic enzyme from bacteria (*43*). At the carboxyl terminus is a domain with sequence homology to thioredoxins (*15*). Despite the homology to thioredoxins, the C-domain functions like glutaredoxin, a redox cofactor related to thioredoxin that accepts electrons only from GSH. Plant APS reductase efficiently uses GSH as an electron source for catalysis and the C-domain is necessary for this activity, suggesting that it functions in catalysis (*16*). Two of the APS reductase Cys residues are found in the C-domain in the sequence CPFC (C385 and C388 of APR1, PIR accession number AAC26979.1), comprising the glutaredoxin-like active site.

The other five Cys residues are located in the reductase domain at residues C202 and C203 (context ECCR), C294 and C297 (context CEPC), and C322 (context ECGLH). The three-dimensional structure of *E. coli* CysH, the orthologue of the reductase domain of plant APS reductases, has been determined (PDB filename SUL1). By carrying out a simple modeling exercise whereby the APR1 reductase domain is fitted to the structure of *E. coli* CysH, it became evident that C202/C203 and C385/C388, which lie distant from each other in the primary sequence, may lie near each other within the context of the folded protein, suggesting that they could potentially form disulfide bridges.

Any effort to predict the APR1 regulatory site must explain why its closely related paralogues, APR2 and APR3, appear to be insensitive to redox control since all the forms contain all the conserved Cys residues. It is possible that an activating disulfide bond exists in the paralogues, but due to differences in primary sequence, the disulfide is more stable and less sensitive to disulfide reductants in APR2 and APR3 than in APR1. In this regard, future examination of the oxidation state of the Cys in the three APR paralogues of *A. thaliana* would be informative.

In Vivo Response of APS Reductase to Oxidative Stress. An increase in APS reductase activity was observed in vivo in a number of experimental systems in response to various treatments that induce oxidative stress. In all of the systems studied there was a transient increase in activity, which returned to the initial level as the stress was mitigated. The increase in activity did not correlate with a change in the immunodetectable level of enzyme, and the response was still observed in *A. thaliana* seedlings blocked in mRNA synthesis or translation. This is indicative of the activation, rather than increased de novo synthesis, of APS reductase. In the example of plants treated with ozone, a transient change in the redox state of the glutathione pool was observed that correlated precisely with the change in APS reductase activity. These results are consistent with the hypothesis that APS reductase is regulated by the redox state of the plant cell. Although all the results of this study are correlative, taken together they provide a group of results from widely varying systems that all point toward the hypothesis that APS reductase is subject to posttranslational regulation.

Recently, it was reported that *A. thaliana* APS reductase is diurnally regulated by a transcriptional or posttranscriptional mechanism (*44*). Diurnal regulation of APS reductase is unlikely to explain the results reported here. First, the kinetics and magnitude of diurnal regulation are very different from regulation by oxidative stress. Moreover, regulation by oxidative stress was not accompanied by changes in steady-state mRNA or protein level, whereas both mRNA and protein change in response to diurnal regulation.

Mechanisms for Regulation of Glutathione and Cys Synthesis. The mechanisms regulating glutathione synthesis in plants are currently not well understood. γ-Glutamylcysteine synthetase (*9*, *11*) controls glutathione synthesis, but it is ultimately dependent upon the level of its substrates Glu and Cys. If Cys availability is a limitation, as has been hypothesized (*9*, *11*, *12*), it raises the question of whether the regulation of Cys synthesis may play a role in regulating glutathione level. APS reductase is the only enzyme of the sulfate assimilation pathway that is strongly regulated in plants by various treatments that alter the requirement for Cys. These include the exposure of plant roots to heavy

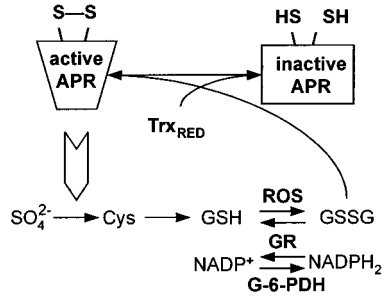

FIGURE 5: Working model for regulation of sulfate assimilation by oxidative stress. The diagram depicts the activation state of APR being a function of the ability of reduced TRX to inactivate APR and the ability of GSSG to activate APR. Glutathione is normally maintained in a reduced state by glutathione reductase (GR), which depends on NADPH produced by the pentose phosphate pathway under control by glucose-6-phosphate dehydrogenase (G-6-PDH). But GSSG accumulates in plant cells when ROS are produced during oxidative stress. The activation of APR stimulates the production of GSH needed to mitigate ROS.

metals (*35*, *45*) and sulfur starvation (*13*, *46*). Thus, the finding that oxidative stress induces APS reductase activity is consistent with previous reports. However, the response to ozone appears to be fundamentally different from the others in that the oxidative stress induces APS reductase activity in leaves and a component of the regulation appears to be at a posttranslational level. By contrast, heavy metal stress and sulfur starvation induce APS reductase primarily in roots and regulation is mediated through increases in the steady-state level of mRNA for APS reductase.

In plants, the level of glutathione and its reduction state are metabolically controlled. The glutathione concentration has been reported as between 3 and 10 mM, and it is maintained in the reduced state by NADPH-dependent glutathione reductase. During oxidative stress, glutathione is used for reduction of ROS, resulting in a transient increase in the level of GSSG. In addition, the level of glutathione also increases after oxidative stress (*5*−*9*), presumably to provide increased capacity to neutralize ROS. On the basis of the results presented here, the activation of APS reductase can be placed within a coordinated system for maintenance of the glutathione pool and glutathione reduction state (Figure 5). Glutathione reductase is another enzyme that has been proposed to have redox-regulated isoforms that are activated by GSSG or oxidants (*47*). The chloroplast form of glucose-6-phosphate dehydrogenase, the pentose phosphate pathway enzyme needed for generation of NADPH, is another enzyme that is activated by oxidation (*48*). Other posttranscriptional, redox-dependent mechanisms have been described for the regulation of APX, SOD, and γ-glutamylcysteine (γ-EC) synthetase (*49*). The availability of reduced glutathione is important in the restoration of the redox status of the cell, particularly in the chloroplasts, and plays a central role as an antioxidant and in the recycling of other antioxidant systems. However, a recent study demonstrating that overexpression of γ-EC synthetase, and hence increased GSH biosynthesis in chloroplasts, paradoxically resulted in an increase in oxidative stress levels. This probably reflects the complexity of the redox-sensing mechanism in plants and our limited understanding of the metabolic responses to oxidative stress.

Concerted Regulation of the Cycles for Mitigation of Oxidative Stress. In this report we propose that APS reductase activity is controlled by the equilibrium between thioredoxin and glutathione, two independent redox systems operating in the chloroplast (Figure 5). The role of thioredoxin in coupling the light reactions of photosynthesis to the reductive activation of Calvin cycle enzymes is well-known (*50*). In the absence of oxidative stress, thioredoxin would serve to inactivate the redox-regulated form of APS reductase. However, a side effect of photosynthesis is the production of ROS whose removal produces GSSG. When ROS production exceeds the capacity to maintain the GSH pool, the accumulating GSSG would activate APS reductase, thereby inducing the synthesis of additional glutathione. As glutathione accumulates and the GSH pool is restored, thioredoxin inactivates APS reductase. This hypothesis is compelling because direct activation of a key enzyme in glutathione biosynthesis would serve as a dynamic mechanism for controlling the rate of synthesis in direct response to the requirement for glutathione needed to combat oxidative stress.

This study is the first to demonstrate redox regulation of sulfur assimilation in plants through enzyme regulation. The precise mechanism of APS reductase regulation and the Cys residues involved have yet to be elucidated. Nevertheless, in identifying a correlation between the activity of a key enzyme in Cys biosynthesis and the redox status of the glutathione pool, we are closer to understanding the mechanisms involved in overcoming oxidative stress in the chloroplasts.

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