Stable Form of Ascorbate Peroxidase from the Red Alga *Galdieria partita* Similar to Both Chloroplastic and Cytosolic Isoforms of Higher Plants

Sakihito KITAJIMA,¹,* Masami UEDA,¹ Satoshi SANO,¹,*** Chikahiro MIYAKE,²,*** Takayuki KOHCHI,² Ken-ichi TOMIZAWA,¹ Shigeru SHIGEOKA,³ and Akiho YOKOTA¹,†

¹Research Institute of Innovative Technology for the Earth (RITE), Soraku-gun, Kyoto, 619-0292, Japan
²Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST), Ikoma, Nara 630-01, Japan
³Department of Food and Nutrition, Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan

Received May 8, 2002; Accepted June 28, 2002

Depletion of the electron donor ascorbate causes rapid inactivation of chloroplastic ascorbate peroxidase (APX) of higher plants, while cytosolic APX is stable under such conditions. Here we report the cloning of cDNA from *Galdieria partita*, a unicellular red alga, encoding a novel type of APX (APX-B). The electrophoretic mobility, $K_m$ values, $k_{cat}$ and absorption spectra of recombinant APX-B produced in *Escherichia coli* were measured. Recombinant APX-B remained active for at least 180 min after depletion of ascorbate. The amino-terminal half of APX-B, which forms the distal pocket of the active site, was richer in amino acid residues conserved in chloroplastic APXs of higher plants rather than cytosolic APXs. In contrast, the sequence of the carboxyl-terminal half, which forms the proximal pocket, was similar to that of the cytosolic isoform. The stability of APX-B might be due to its cytosolic isoform-like structure of the carboxyl-terminal half.

Key words: cDNA; inactivation; reactive oxygen species; recombinant protein

Photosynthetic organisms have a reactive oxygen species (ROS)-scavenging system. In higher plants, one of the most important scavenging systems is the ascorbate-glutathione cycle, composed of ascorbate peroxidase (APX), monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase.¹² APX is a heme enzyme and by its amino acid sequence is a member of the class I family of heme peroxidases, as is cytochrome *c* peroxidase.³ APX first reacts with hydrogen peroxide and is converted to the two-electron-oxidized intermediate, APX(Fe⁴⁺=O)R−, referred to as compound I. In this, the heme moiety is oxidized to the oxyferryl (Fe⁴⁺=O) species and an organic group, R (mainly porphyrin,⁴ see Discussion), is oxidized to its free radical, R·. Compound I is reduced back to the resting ferric (Fe⁴⁺) state in two successive one-electron transfer reactions with ascorbate through APX(Fe⁴⁺ = O)R, referred to as compound II. These reactions produce two molecules of the one-electron-oxidized product, the ascorbate monodehydroascorbic radical.

Crystallographic analysis of recombinant cytosolic APX of pea reveals 12 α-helices.⁵ Domain I, composed of helices A to D, is on the distal side of the heme molecule, and domain II, composed of helices F to J, is on the proximal side. The domains are connected via helix E. On the basis of the results from nuclear magnetic resonance and computer-modeling, the ascorbate-binding site has been proposed to be in a pocket formed by the heme molecule and the domain I.⁶

On the basis of enzyme characteristics and amino acid sequences, APXs of higher plants have been divided into several isoforms;⁷ two soluble isoforms in the cytosol and the stroma, and two membrane-bound isoforms localized in the thylakoids and the microbodies (glyoxysome and peroxisome). The fifth isoform, the subcellular localization of which remains unknown, has also been found. In many plant species, the amino acid sequence of the core region of the stromal isoform is identical to that of the thylakoid-bound isoform.
Different APX isoforms have different stability under ascorbate-depleted conditions. *In vitro* study showed that isoforms in chloroplasts of higher plants are rapidly and irreversibly inactivated when ascorbate is depleted and compound I can not be reduced. This is because autooxidation of remaining ascorbate molecules generates a small amount of hydrogen peroxide, which attacks compound I. Therefore, when excess hydrogen peroxide is generated by various photooxidative stresses, or when the concentration of ascorbate is lowered, APX cannot detoxify ROS that attack proteins, nucleic acids, and lipids in chloroplasts, causing chlorosis and cell death. By contrast, cytosolic and microbody-bound isoforms are relatively stable under low ascorbate conditions.

Sulfur dioxide, an air pollutant, inhibits plant growth by generating ROS in chloroplasts. When sulfur dioxide is incorporated into cells and becomes sulfite, it generates hydrogen peroxide in an interaction with photosystem I. Therefore, when excess hydrogen peroxide is generated by various photooxidative stresses, or when the concentration of ascorbate is lowered, APX cannot detoxify ROS that attack proteins, nucleic acids, and lipids in chloroplasts, causing chlorosis and cell death. By contrast, cytosolic and microbody-bound isoforms are relatively stable under low ascorbate conditions.

The enzymes involved in the ascorbate-glutathione cycle have only been studied well in green plants. We therefore isolated cDNA encoding APX-B, a major isoform of two APXs in *G. partita*, and compared its predicted amino acid sequence with APXs of green plants and green algae. Furthermore, we overexpressed the recombinant APX-B in *E. coli*, to examine its enzymatic properties. This is the first report of isolation of the full length of cDNA for APX from non-green photosynthetic organisms. The predicted amino acid sequence and characterization of APX-B provide insight into the cause of different stabilities among APX isoforms.

**Results**

**Cloning and sequence analysis of APX-B cDNA**

The APX-B-specific primer corresponded to the region between the 16th and 22nd amino acid residues. A DNA fragment, amplified from total cDNA using the APX-B-specific and the oligo-dT primers, had a nucleotide sequence similar to those of plant APX genes. Therefore, we screened the cDNA library of *G. partita* by using this DNA fragment as the probe. The APX-B cDNA clones contained an open reading frame of 741 bp within the 1.1-kb insert (Fig. 1).
The EMBL/GenBank/DDBJ accession number is AB037537). Direct sequencing of PCR products of the 5'-end amplified from three independently prepared cDNA libraries confirmed that it encoded the full-length of APX-B cDNA (not shown). One of ten clones isolated had five extra nucleotides, ATTCC, upstream of poly(A) (Fig. 1), indicating that G. partita mRNA had at least two polyadenylation sites.

The first ATG began at base 55, but this reading frame had a stop codon upstream of the first in-frame ATG. Thus, the first in-frame ATG located at bases 198–200 was assigned as the start codon, and the open reading frame encoded a protein of 247 amino acid residues with a predicted molecular mass of 27,835 Da and an isoelectric point of 5.68. Comparison with the amino-terminal amino acid sequence of native APX-B purified from G. partita cells\(^1\) showed that only the first methionine was removed posttranslationally. This indicates that cDNA for APX-B has no sequence for the transit peptide.

**Molecular characteristics of APX-B**

Comparing the amino acid sequence against the PROSITE database showed two motifs conserved in peroxidases. The first is the peroxidases-active-site signature, [SGATV]-x(3)-[LIVMA]-x-[FW]-H-x-[SAC], except the first residue is methionine in APX-B (Fig. 1, double-underlined). The second is the peroxidase-proximal-heme-ligand signature, [DET]-[LIVMTA]-x(2)-[LIVM]-[LIVMFY] (Fig. 1, bold-underlined). Furthermore, APX-B conserved Cys-25, which corresponded to Cys-32 of the cytosolic isoform of pea plants. This residue is conserved in many APXs and has been deduced to be near the ascorbate-binding site and to be important for reduction of the oxidized reaction intermediates compounds I and II.\(^1\)^

**Immunoblotting analysis**

We have reported that the molecular mass of native APX-B was 28 kDa\(^1\)^ which is close to the predicted molecular mass of the cloned sequence. As mentioned above, the first methionine is not present in native APX-B. To examine whether other posttranslational modifications occur, we compared the mobilities of native APXs and recombinant APX-B.

Recombinant APX-B, purified to homogeneity to give a single band at 28 kDa in SDS-PAGE (Fig. 2(a)), was used to immunize rabbits. The antisera were used to probe the APX-B protein in immunoblots. When extracts from G. partita were analyzed, APX-B protein was detected as a band of approximately 28 kDa in the soluble protein fraction (Fig. 2(b)). The recombinant APX-B showed the same mobility as that of the native 28-kDa protein (compare lanes 1 and 2 in Fig. 2(b)). This suggested that there is little if any posttranslational modification, other than the removal of its first methionine. The intensity of staining of 0.06 μg of purified recombinant APX-B was close to that of the 28-kDa native protein in 1.65 μg of total soluble proteins (compare lanes 1 and 2 in Fig. 2(b)). Most of the 28-kDa protein detected by the anti-APX-B antibody in total soluble proteins is presumably APX-B, because the APX-A isoform accounted for only 15% of the total APX activity of G. partita,\(^1\)^ and APX-A was less reactive to the anti-APX-B antibody (Figs. 2(c) and 2(d)).

A faint band of 28 kDa was visible in the insoluble fraction (lane 3 in Fig. 2(b)) and was probably contamination by APX-A and/or B. No other band was detected, suggesting that G. partita does not have a membrane-bound APX isoform having a primary structure similar to that of APX-B. This is consistent with the observation that the molecular mass of native APX-B was 28 kDa, which is close to the predicted molecular mass of the cloned sequence.
Table 1. Enzyme Properties of Recombinant and Native APX-B

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>K_m(AsA) (µM)</th>
<th>K_m(H_2O_2) (µM)</th>
<th>k_cat (sec^{-1} heme^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant APX-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>216</td>
<td>82</td>
<td>3460</td>
</tr>
<tr>
<td>25</td>
<td>119</td>
<td>42</td>
<td>2190</td>
</tr>
<tr>
<td>Native APX-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>174</td>
<td>28</td>
<td>ND</td>
</tr>
</tbody>
</table>

AsA, ascorbate; ND, not done.

with the previous result that APX enzyme activity was not detected in the membranous fraction.13)

**Reaction kinetics**

The specific heme content of recombinant APX-B was measured by the pyridine hemochromogen method. The reduced pyridine hemochromogen for APX-B showed an absorbance maximum at 556 nm and a minimum at 540 nm (not shown), which is characteristic of protoporphyrin IX.15) One molecule of recombinant APX-B contained on average 0.56 molecule of heme, indicating that 44% of recombinant APX-B was the apoenzyme.

Recombinant APX-B showed a specific activity of 2900 ± 300 units min^{-1} mg protein^{-1} at 37°C. The activity at 25°C was approximately 60% of that at 37°C. The K_m value for ascorbate was 216 µM in the presence of 100 µM hydrogen peroxide, and that for hydrogen peroxide was 82 µM in the presence of 500 µM ascorbate. The k_cat calculated from the maximum activity and the heme content was 3460 sec^{-1} site^{-1}. When assayed at 25°C, K_m values for ascorbate and hydrogen peroxide and k_cat were 119 and 42 µM, and 2190 sec^{-1} site^{-1}, respectively (Table 1).

**Absorption spectra of recombinant APX-B**

Absorption spectra were measured using 0.15 mg/ml of recombinant APX-B in buffer B containing 0.15 M of KCl. A Soret peak was found at 407 nm with an absorption coefficient of 125 mM^{-1} cm^{-1} per heme, and the peak was shifted to 435 nm (119 mM^{-1} cm^{-1} per heme) upon reduction by dithionite, with the α-peak at 556 nm (15.3 mM^{-1} cm^{-1} per heme). A cyanide complex with recombinant APX-B gave peaks at 421 nm (124 mM^{-1} cm^{-1} per heme) and 545 nm (13 mM^{-1} cm^{-1} per heme).

**Stability of recombinant APX-B under ascorbate-depleted conditions**

In higher plants, chloroplastic APX isoforms are rapidly and irreversibly inactivated when ascorbate is depleted. This is because a small amount of hydrogen peroxide is produced via autooxidation of the remaining ascorbate, which attacks compound I.8) In contrast, cytosolic16) and microbody-bound17) isoforms of higher plants are relatively stable under these conditions.

We therefore examined the stability of recombinant APX-B (Fig. 3). Recombinant APX-B in buffer B containing 0.15 M of KCl (0.34 units min^{-1} µl^{-1} at 25°C) was diluted 100-fold with 50 mM MES-KOH (pH 7.0) with or without 0.5 mM ascorbic acid, to give final concentrations of 0.5 mM (squares) or 10 µM ascorbate (circles), respectively. After incubation at 25°C for indicated times, the activity remaining was assayed.

**Discussion**

APX-B lacked a transit peptide as found in the chloroplastic isoforms, and also lacked the carboxyl-terminal extension found in stromal, thylakoid-bound, or microbody-bound isoforms of higher plants. APX-B has been detected as a soluble protein in G. partita cells (Fig. 2 and Sano et al.13)). These findings suggest that APX-B is localized in the cytosol. Since the site where sulfur dioxide generates ROS is the chloroplast, APX-B is thought to scavenge hydrogen peroxide that diffuses out from the organelle. Similarly in Euglena gracilis, APX, dehydroascorbate reductase,18) and glutathione reductase19) are found only in the cytosol. Hydrogen peroxide generated in chloroplasts is presumably decomposed in a similar way.20)

The K_m for substrates and the absorption spectra of recombinant APX-B were similar to those of native APX-B partially purified from G. partita (Table 1). Comparison of electrophoretic mobilities
Fig. 4. Comparison of the Amino Acid Sequence of APX-B with Other APXs.

Regions between helices A and J are aligned. Helices were assigned according to the structure of cytosolic APX of pea. Identical and similar amino acid residues are marked by asterisks and dots, respectively. Green letters, residues conserved in the chloroplastic but not in the cytosolic isoforms of higher plants. Red letters, residues conserved in the cytosolic but not in the chloroplastic isoforms. The proteins shown are cytosolic APXs from pea (DNA or protein database P48534), Arabidopsis (Q05341), spinach (D85864), tobacco (U15933), maize (Z34934) and rice (D45364), microbody-bound APXs from cotton (U37060) and Arabidopsis (X98276), APXs locations of which are unknown from spinach (D49679) and iceplant (AF079512 and AF079513), chloroplastic APXs from cucurbit (D88420), spinach (AB002467), iceplant (AF069316), Arabidopsis (X98925 and X98926), APX from Chlamydomonas W80 (AB009084), and APX from C. reinhardtii (A3223352). APX of Chlamydomonas W80 strain is in chloroplasts.
of recombinant and native APX-B proteins suggested that there is no significant posttranslational modification, other than the first methionine being removed.

Recombinant APX-B remained active for at least 180 min at 25°C in 10 μM ascorbate (Fig. 3), as do recombinant microbody-bound and cytosolic APXs of spinach. (17)

The deduced amino acid sequence of APX-B was compared with those of other APXs. The first residue of the peroxidases-active-site signature [SGATV]-x(3)-[LIVMA]-R-[LIVMA]-x-[FW]-H-x-[SAC] is not conserved in APX-B, as described above. The first residue of the signature is also replaced by other amino acids in APXs of chloroplasts of higher plants and in an isoform of *Chlamydomonas reinhardtii* (Fig. 4). Thus, it seems unlikely that this residue is important to peroxidase activity in APXs.

In this report, helixes of other APXs were assigned according to the structure of cytosolic APX of pea. (5) Overall, the core region (helixes A to J) of APX-B was only 46–60% identical to those of APXs of higher plants and *Chlamydomonas* and APX-B could not unambiguously be classified as a member of any isoform group.

However, we found, by a multiple sequence alignment, that some parts of APX-B shared sequence similarity with chloroplastic isoforms of higher plants, whereas other parts shared similarity with cytosolic isoforms (see Fig. 4).

In this study, amino acid sequences of nine cytosolic APXs including those of soybean (accession number AAD20022), cucumber (BAA13671), and barley (CAA06996) and five chloroplastic APXs were compared (Fig. 4). Sequences of soybean, cucumber, and barley enzymes are not shown in the figure to save space. Residues conserved in at least eight cytosolic APXs but not found in chloroplastic APXs were defined as cytosolic residues. The residues conserved in at least four chloroplastic APXs but not found in cytosolic APXs were defined as chloroplastic residues. APX-B domain I (helixes A to D) was richer in chloroplastic residues than domain II (helixes F to J); of 24 chloroplastic residues found in helixes A to J, thirteen were located in domain I and only four in domain II. In particular, helixes B and D were similar to those of the chloroplastic isoforms. Helix B of APX-B has three chloroplastic and one cytosolic residues as well as six residues common to both isoforms. Helix D of APX has five chloroplastic and one cytosolic residues as well as six residues common in both isoforms.

Helix B contains a peroxidase-active-site signature, and in pea cytosolic APX, this region forms the distal pocket of the active site. (7) The native APX-B partially purified from *G. partita* showed a substrate specificity for electron donors similar to those of the chloroplastic APXs, rather than that of the cytosolic APX of higher plants. (13) The specificity of APX-B for electron donors might be due to the chloroplastic-like structure of helices B and D.

In contrast, domain II was similar to the cytosolic isoform. Only eight of 23 cytosolic residues were in domain I, while 14 residues were in domain II. The hybrid-type structure of APX-B suggests that its stability under ascorbate-depleted conditions might be due to its cytosolic-like domain II. Domain II of pea cytosolic APX contains several regions or amino acid residues that may be involved in enzyme activity. Domain II forms a pocket at the proximal face of the heme. In cytochrome *c* peroxidase of yeast, electrons are transferred from the proximal pocket tryptophan to the iron of heme, forming the tryptophan radical in compound I. (21) In cytosolic APX, a porphyrin π cation radical is preferentially formed because a cation (possibly a potassium ion) near the tryptophan (Trp-179) prevents electron transfer by increasing the electrostatic potential. (5) In pea cytosolic APX, the four cation-binding residues, Thr-164, Thr-180, Asn-182, and Asp-187, are in domain II, in the region containing helixes F to G. (5,22) It should be noted that, under certain conditions, cytosolic APX can form the tryptophan radical. Hiner *et al.* (21) reported that the reaction of cytosolic APX of pea with hydrogen peroxide generates the tryptophan radical in the absence of ascorbate.

The other important residue within domain II is Arg-172, which may interact with ascorbate during its binding near the heme edge containing the propionate residues. (14)

The proximal tryptophan, the cation-binding residues apart from Asn-182, and Arg-172, that are seen in pea cytosolic APX, are all well conserved among cytosolic, microbody-bound and chloroplastic APXs of higher plants and APX-B. In contrast, residues near these amino acids are quite different among the isoforms. Interestingly, chloroplastic APXs have 16 amino acid residues inserted between Pro-178 and Trp-179 of the pea cytosolic APX. These neighboring amino acid residues might influence the function of the proximal tryptophan, the cation-binding site, and/or the Arg-172, changing the stability of APXs under ascorbate-depleted conditions.

APX-A had enzyme characteristics similar to those of APX-B and is also highly stable under ascorbate-depleted conditions. (13) Nevertheless, immunological studies (Fig. 2 and Sano *et al.* (13)) indicated that its primary structure was different from that of APX-B. We have not obtained a cDNA clone encoding APX-A, but comparison between the amino acid sequences of APXs-A and B would give us further information on the significance of each amino acid residue of domain II.
Materials and Methods

Culture of Galdieria partita. G. partita Tokara was cultured as described by Uemura et al.\textsuperscript{20} Cells were harvested by centrifugation at 10,000 \( \times \) g for 20 min at 4\textdegree{}C, flash-frozen in liquid N\(_2\) and stored at \(-80\textdegree{}C\).

Isolation of cDNA clones of APX-B. DNA and RNA were manipulated by the procedures described by Ausubel et al.\textsuperscript{29} and Sambrook et al.\textsuperscript{26} Total RNA of G. partita was reverse-transcribed using an oligo-dT primer and Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and amplified by PCR with the oligo-dT and an APX-B-specific primers. The APX-B-specific primer (5\textprime{}-GTIAARYTITYGARCARAC-3\textprime{}) was designed according to the amino-terminal sequence of APX-B, determined by Edman degradation.\textsuperscript{13} The PCR program consisted of 32 cycles: 95\textdegree{}C for 30 sec, 42\textdegree{}C for 30 sec, and 72\textdegree{}C for 1.5 min. PCR products were separated by agarose gel electrophoresis and the 0.8-kb product was ligated into the pT7Blue vector (Novagen, Madison, WI).

Polyadenylated RNA of G. partita was used to construct a cDNA library in the \( \lambda \)ZAPII vector (Stratagene, La Jolla, CA). A total of 1 \( \times \) 10\textsuperscript{5} clones were screened by plaque hybridization using a \( ^{32} \)P-labeled cDNA fragment of APX-B obtained by reverse transcription-PCR as the probe. Positive clones were identified by autoradiography and converted into phagemids for sequencing.

Sequence analysis. Nucleotide sequences were analyzed by the dye-deoxy-chain-termination method using a Thermosequenase II DNA sequencing kit (Amersham Pharmacia Biotech) with an automated DNA sequencer (model 377, PE Applied Biosystems, Foster City, CA). DNA sequence of both strands was sequenced. DNA sequences were analyzed using the Gene Works (IntelliGenetics, Campbell, CA) and MacVector (Oxford Molecular Group, San Diego, CA). The PROSITE database\textsuperscript{27} (http://motif. genome.ad.jp) was used to find motifs in the amino acid sequence. Multiple sequence alignment was done by Clustal X.\textsuperscript{28} Identities between amino acid sequences were calculated with FASTA3\textsuperscript{29} (http://www2.ebi.ac.uk/fasta3/).

Construction of a plasmid for expression of recombinant APX-B in E. coli. An NcoI site (CCATGG) was created at the translation-start site by PCR-mediated site-directed mutagenesis using a mutagenic primer (5\textprime{}-GGTCCACACCATGGTTGC-TTCTAAG-3\textprime{}). The cDNA fragment between the introduced NcoI and XhoI sites of the vector plasmid downstream of poly(A) sequence of the cDNA was inserted into NcoI-XhoI site downstream of the T7 promoter of pET16b (Novagen) to generate pET16b::APX-B. The fragment amplified by PCR was sequenced to check the valid incorporation of nucleotides in PCR.

Purification of recombinant APXs. E. coli strain BL21(DE3) harboring pET16b::APX-B was grown at 37\textdegree{}C in 100 ml of Circlegrow medium (Bio101, Carlsbad, CA) containing 50 mg l\(^{-1}\) ampicillin. At OD\textsubscript{600} = 0.5, isopropyl-\( \beta \)-D-thiogalactoside was added to a final concentration of 1 mM. When OD\textsubscript{600} reached 1.5, the cells were harvested by centrifugation and stored at \(-80\textdegree{}C\).

The E. coli cells (2.2 g wet weight) were suspended in 20 ml of buffer A [50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM ascorbate, 1 mM PMSF], and disrupted by sonication. The lysate was centrifuged at 5,000 \( \times \) g for 20 min. Recombinant APX-B was purified from the supernatant with a Butyl Toyopearl column (Tosoh, Tokyo, Japan) and a HiLoad 26/60 Superdex 75 prep grade column (Amersham Pharmacia Biotech) as previously described.\textsuperscript{33} Recombinant stromal APX of spinach was purified by the same procedure as for APX-B, except that ammonium sulfate precipitation was omitted. Purified recombinant APXs was stored at \(-80\textdegree{}C\) in buffer B [10 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM ascorbate] with 0.15 M KCl. The obtained recombinant stromal APX was still contaminated by a few proteins, as seen in SDS-PAGE (not shown).

Protein was assayed by the procedure of Bradford\textsuperscript{31} with BSA as the standard.

The heme content of purified recombinant APX-B was assayed by the pyridine hemochromogen method\textsuperscript{32} with myoglobin from dog heart as the standard.

Enzyme assay. APX activity was assayed as described by Nakano and Asada\textsuperscript{33} in a reaction mixture (1 ml) that contained 50 mM MES-KOH buffer (pH 7.0), 0.5 mM ascorbate, and 0.1 mM hydrogen peroxide. The hydrogen peroxide-dependent oxidation of ascorbate was followed at 37\textdegree{}C or 25\textdegree{}C by monitoring of the decrease in absorbance of ascorbate at 290 nm, assuming an absorption coefficient of 2.8 mM\(^{-1}\) cm\(^{-1}\). One unit of APX is defined as the amount of enzyme that oxidizes 1 \( \mu \)mol of ascorbate per minute under these above assay conditions.

SDS-PAGE and Immunoblotting. G. partita cells (0.15 g wet weight) were homogenized in 0.6 ml of buffer A with a Mini-beadbeater (Biospec products, Bartlesville, OK). Soluble proteins were separated by ultracentrifugation at 70,000 \( \times \) g for 60 min from the membranous pellets, which were suspended by sonication in buffer B containing 300 mM NaCl, and cen-
trifuged at 70,000 × g for 30 min. After washing two times by the same procedure, the pellets were suspended in 0.6 ml of buffer B and used as the insoluble membranous fraction. All procedures were done at 4°C.

To compare the reactivities of APXs-A and B to anti-APX-B antibody, native APXs-A and B were partially purified with a Butyl toyopearl column as previously described. These proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels and detected by silver staining. For immunoblot analysis, proteins in gels were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) with a TransBlot apparatus (BioRad). After transfer, blots were treated with rabbit anti-APX-B antibody, then treated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Jackson Immuno Research, West Grove, PA), and stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega, Madison, WI, U.S.A.).

Acknowledgments

We thank Dr. Hiroshi Yamamoto for helpful discussion and advice with FPLC. We also thank Ms. Yuki Shinzaki, Mr. Yukihisa Yamauchi, and Ms. Satoko Sugahara for technical assistance. This study was partly supported by the Petroleum Energy Center and the Research Association for Biotechnology, subsidized by the Ministry of Economy, Trade, and Industry, Japan.

References

22) Cheek, J., Mandelman, D., Poulos, T. L., and Dawson, J. H., A study of the K⁺-site mutant of as-


