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Cytosolic isoform-like structure of domain II is important for the stability of ascorbate peroxidase of *Galdieria partita* under ascorbate-depleted conditions.

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Introduction

Ascorbate peroxidase (APX) is an enzyme involved in the reactive oxygen species (ROS)-scavenging system. In the catalytic cycle the ferric resting state heme is first oxidized by hydrogen peroxide to the intermediate Compound I, which is characterized by an oxyferryl iron and a radical cation. Two sequentical single-electron reduction by ascorbate then restore the resting state enzyme via the second intermediate Compound II which is one redox equivalent above the resting state and which has an oxyferryl heme iron. Higher plants have several isoforms of APX. When isoforms functioning in chloroplasts of higher plants are incubated under ascorbate-depleted media, they are rapidly and irreversively inactivated. This is because small amounts of hydrogen peroxide generated by autooxidation of oxygen molecule attack the Compound I (Miyake and Asada 1996). In contrast, cytosolic and glyoxysome-bound isoforms are highly stable. It is interesting to know the molecular mechanisms of inactivation process of APX and of different stability among APX isoforms. In the present study, we show

the domain II is important for the stability of the stable APX of *G. partita*, red algae.

Materials and Methods

A partial cDNA fragment of APX-B was obtained by RT-PCR with oligo-dT and APX-B-specific primer. The latter primer was designed according to the *N*-terminal amino acid sequence of native APX-B purified from *G. partita*. By using this fragment as the probe, clones encoding the full length of APX-B cDNA were isolated from the cDNA library of *G. partita*. To create a plasmid for expression of reconbinant APXs in *E. coli*, coding regions of cDNAs were inserted into a pET vector. DNA cassette for chimeric APX was produced by PCR amplification of the *N*-terminal half of APX-B cDNA and the *C*-terminal half of stromal APX cDNA that were joind by T4 DNA ligase. DNA was reamplified with two of the original primers and inserted into the vector. Recombinant APX proteins expressed in *E. coli* were purified with a butyl toyopearl column (Tosoh, Tokyo, Japan) and a Hiload 26/60 superdex 75 prep grade column (Amerscham Pharmacia Biotech).

The APX activity was assayed at 25℃ in a reaction mixture (1 ml) that contained 50 mM MES-KOH buffer (pH7.0), 0.5 mM ascorbate and 0.1 mM hydrogen peroxide.

Results and Discussion

The APX-B cDNA of *G. partita* contained an open reading frame which encodes a protein of 247 amino acid residues with the predicted molecular mass of 28 kDa. Comparison of its deduced amino acid sequence with the *N*-terminal amino acid sequence of native APX-B purified from *G. partita* cells (Sano et al. 2001) showed that only the first Met was removed posttranslationally. APX-B had no *C*-terminal extension found in stromal, thylakoid-bound or glyoxysome-bound isoforms of higher plants. Immunoblotting analysis showed that APX-B was present as a soluble protein in *G. partita* cells. These findings strongly suggest that APX-B is localized in the cytosol. Recombinant APX-B was not inactivated by decreasing the concentration of ascorbate to 10 μM for at least 3hr at 25℃ (Fig. 1), indicating the recombinant APX-B is highly stable under ascorbate-depleted conditions.

Multiple sequence alignment showed that APX-B is likely to have a hybrid-type structure of chloroplastic and cytosolic isoforms of higher plants (Fig. 2). The sequence

of domain I (helixes A to D) of APX-B, especially, helixes B and D, is relatively similar to that of chloroplastic isoforms. In contrast, the domain II (helixes F to J) had a primary structure similar to that of the cytosolic isoform.

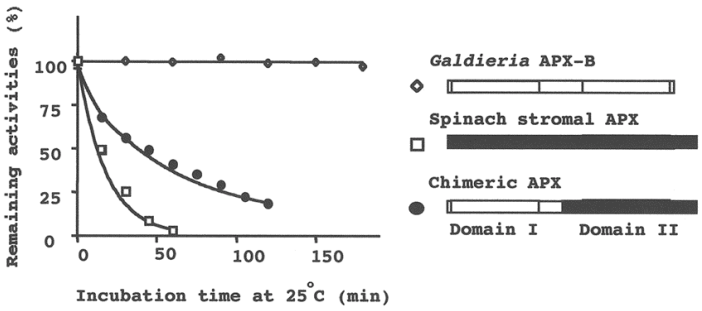

Fig. 1 Change in activity of recombinant APXs after incubation under ascorbate-depleted condition. **(**Left) Highly purified APX-B (diamond) and partially purified stromal APX (square) of spinach and chimeric APX (circle) were analyzed. After indicated times of incubation in 50 mM MES-KOH, 0.01 mM ascorbate and 0.1 mg/ml BSA (pH 7.0) at 25ºC, enzymes were mixed with 0.5 mM of ascorbate and the activities were measured. (Right) Illustration of recombinant APX proteins used in this experiment.

We created a chimeric APX protein having the domain I of APX-B fused to the domain II of stromal APX of spinach. The chimeric APX retained its activity for at least 2 hr if 0.5 mM of ascorbate was supplemented in the solution, but lost its activity ($T_{1/2}$ = 50 min) by decreasing concentration of ascorbate to 10 μM, as recombinant stromal APX of spinach did ($T_{1/2}$ = 13 min) (Fig. 1). This result indicates that the domain II of APX-B is important for its stability under ascorbate-depleted conditions.

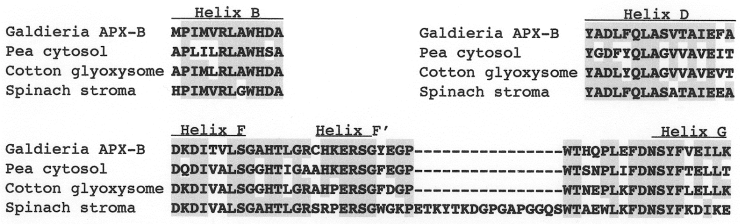

Fig. 2 Comparison of amino acid sequence of APX-B with those of APXs of higher plants. Helixes B and D of domain I and regions between helixes F and G of domain II are aligned. Lines show regions of α-helixes assigned according to the crystal structure of cytosolic APX of pea (Patterson and Poulos 1995). Conserved amino acids are shaded.

Domain II of APX contains several regions or amino acids, which may be involved

in the enzyme activity; amino acid residues forming the proximal pocket, cation-binding site, and Arg which may be involved in substrate-binding. In cytochrome *c* peroxidase of yeast, electron transfer is occurred from iron of heme to tryptophan of proximal pocket, Trp-191, then the radical in the Compound I resides on Trp-191 (Sivaraja et al. 1989). In cytosolic APX, however, a cation near the proximal Trp residue prevents this electron transfer by increasing the electrostatic potential in proximal pocket, and therefore cytosolic APX forms preferentially a porphyrin π cation radical (Patterson et al. 1995). The four amino acid residues of pea cytosolic APX binding to the cation are located between helixes F and G of domain II (Patterson et al. 1995 and Cheek et al. 1999). Interestingly, the amino acid sequences of this region are quite different between stable and unstable APX isoforms of higher plants (Figure 2). Another important residue within domain II is Arg-172 of cytosolic APX of pea. Mandelman et al. (1998) suggested that, in cytosolic APX of pea, ascorbate binds to APX near the heme edge containing the propionates by possibly interacting with Arg-172 (Arg-168 in APX-B) from helix F' of domain II.

More detailed analysis using various chimeric APX proteins will clarify relationships between these regions and the inactivation process of APX or different stability among APX isoforms.

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