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Molecular Characterization and Physiological Role of a Glyoxysome-Bound Ascorbate Peroxidase from Spinach

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cDNAs encoding two cytosolic and two chloroplastic ascorbate peroxidase (AsAP) isozymes from spinach have been cloned recently [Ishikawa et al. (1995) *FEBS Lett.* 367: 28, (1996) *FEBS Lett.* 384: 289]. We herein report the cloning of the fifth cDNA of an AsAP isozyme which localizes in spinach glyoxysomes (gAsAP). The open reading frame of the 858-base pair cDNA encoded 286 amino acid residues with a calculated molecular mass of 31,507 Da. By determination of the latency of AsAP activity in intact glyoxysomes, the enzyme, as well as monodehydroascorbate (MDAsA) reductase, was found to be located on the external side of the organelles. The cDNA was overexpressed in *Escherichia coli (E. coli)*. The enzymatic properties of the partially purified recombinant gAsAP were consistent with those of the native enzyme from intact glyoxysomes. The recombinant enzyme utilized ascorbate (AsA) as its most effective natural electron donor; glutathione (GSH) and NAD(P)H could not substitute for AsA. The substrate-velocity curves with the recombinant enzyme showed Michaelis-Menten type kinetics with AsA and hydrogen peroxide (H_2O_2); the apparent K_m values for AsA and H_2O_2 were 1.89±0.05 mM and 74±4.0 μM, respectively. When the recombinant enzyme was diluted with AsA-depleted medium, the activity was stable over 180 min. We discuss the H_2O_2-scavenging system maintained by AsAP and the regeneration system of AsA in spinach glyoxysome.

Key words: Ascorbate peroxidase (EC 1.11.1.11) — Glyoxysome — Hydrogen peroxide — Isozyme — Spinach (*Spinacia oleracea*).

Abbreviations: AsA, ascorbate; AsAP, ascorbate peroxidase; cAsAP; cytosolic ascorbate peroxidase; gAsAP, glyoxysome-bound ascorbate peroxidase; sAsAP, stromal ascorbate peroxidase; tAsAP, thylakoid-bound ascorbate peroxidase; CCP, cytochrome *c* peroxidase; GSH, glutathione; H_2O_2, hydrogen peroxide; MDAsA, monodehydroascorbate; mAb, monoclonal antibody; SOD, superoxide dismutase.

The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, and GenBank with the accession number D84104.

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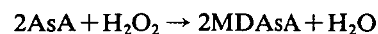
The enzyme AsAP (EC 1.11.1.11) is widely distributed in higher plants and algae, including *Euglena*. AsAP catalyzes the following reaction, which is the first step in the AsA-GSH cycle, a series of reactions which are important in ridding cells of excess H_2O_2 produced under some physiological conditions.



$$2AsA + H_2O_2 \rightarrow 2MDAsA + H_2O$$

In the AsA-GSH cycle, MDAsA is the primary product of the AsAP reaction and spontaneously disproportionates to AsA and dehydroascorbate. MDAsA is also directly reduced to AsA by the action of a NAD(P)H-dependent MDAsA reductase (EC 1.6.5.4). Dehydroascorbate reductase (EC 1.8.5.1) utilizes GSH to reduce dehydroascorbate to AsA. The GSH is then regenerated by GSH reductase (EC 1.6.4.2), utilizing reducing equivalents from NAD(P)H. The regeneration of AsA from the oxidized forms is an absolute necessity for preventing loss of the AsA pool following oxidation and maintaining AsA largely in the reduced form.

In higher plant cells, different types of AsAP isozyme have been clarified with respect to their cellular distribution. One is localized in the cytosol, and the other is found in both stromal soluble and thylakoid-bound forms of chloroplasts. AsAP isozymes have been purified and characterized from many plant sources including spinach (Nakano and Asada 1987, Miyake et al. 1993), tea (Chen and Asada 1989), pea (Mittler and Zilinskas 1991a) and komatsuna (Ishikawa et al. 1996a). Chloroplastic and cytosolic AsAP (cAsAP) isozymes differ in the following aspects: molecular mass, substrate specificity, pH optimum, and stability (Asada 1992). The molecular genetics of AsAP have gained considerable attention in recent years. cDNAs encoding cAsAP isozymes have been isolated, sequenced, and characterized from several plant species such as spinach (Mittler and Zilinskas 1991b, Kubo et al. 1992, Ishikawa et al. 1995). The crystal structure of the recombinant cAsAP has been determined (Patterson et al. 1995). In our preceding report, we have demonstrated the first complete cloning and molecular characterization of stromal (sAsAP) and thylakoid-bound AsAP (tAsAP) isozymes from spinach, differing only in the presence or absence of their C-terminal peptide, which constructs a hydrophobic thylakoid membrane binding domain (Ishikawa et al. 1996b). Yamaguchi et al.

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(1996) have also reported the nucleotide sequence of a cDNA encoding a tAsAP of pumpkin.

More recently, a novel type of AsAP isozyme was found to be localized on the membranes of microbodies in pumpkin (Yamaguchi et al. 1995b) and cotton (Bunkelmann and Trelease 1996). At first, the gAsAP was recognized as a rich glyoxysomal membrane protein with a molecular mass of 31 kDa, which was retained in peroxisomes during the microbody transition from glyoxysome to peroxisome (Yamaguchi et al. 1995a). The partial amino acid sequence of the 31 kDa protein showed a high homology to those of already known cAsAPs, and glyoxysomes retained the activity of AsAP. It thus became clear that a novel AsAP isozyme exists in the microbodies. The detailed properties of the enzyme have not been clarified, but its cDNA has been cloned from cotton cotyledons (Bunkelmann and Trelease 1996).

Microbody is used as a general term which includes both peroxisomes and glyoxysomes (Huang et al. 1983). Peroxisomes in plant cells are known to contain enzymes related to the oxidative photosynthetic carbon metabolic cycle of photorespiration and the fatty acid β-oxidation cycle; glyoxysomes, in addition, contain glyoxylate cycle enzymes and generally occur in germinating fatty seeds. Several H_2O_2-producing flavin oxidases such as glycolate oxidase and acyl CoA oxidase are well know to exist inside microbodies (del Río et al. 1992). Moreover, it has been reported that the superoxide radical is directly generated in the peroxisomal matrix and membranes (Sandalio et al. 1988a, del Río et al. 1989). As scavenging enzymes of these active oxygen species, peroxisomes typically possess catalase and superoxide dismutase (SOD) (Sandalio and del Río 1988b, Droillard and Paulin 1990). These facts reported so far and the occurrence of a new type of the gAsAP raise the question of how catalase and AsAP are functionally utilized as efficient scavengers of H_2O_2. In order to explore the physiological role of the gAsAP isozyme in the active oxygen species metabolism of this organelle, we describe here the identification of a cDNA clone which encodes the gAsAP from spinach cotyledon, the suborganellar distribution of the enzyme in glyoxysomes, and the enzymatic characterization of the recombinant protein overexpressed in *E. coli*.

Materials and Methods

Materials—Spinach seeds (*Spinacia oleracea*) were germinated on moist gauze at 15°C in the dark. The cotyledons from seedlings grown for 10–14 days in the dark were transferred to illumination (140 μE s^{-1} m^{-2}) for 24 h to obtain the greening cotyledons as described previously (Ishikawa et al. 1996b). Restriction enzymes and modifying enzymes were purchased from Takara (Kyoto, Japan). *E. coli* strains Y1090r$^-$ and DH5α F′ were obtained from Amersham (Buckinghamshire, U.K.). The expression vector pET-3a and its companion production strain BL21(DE3)-pLysS were purchased from Novagen (Madison, WI). All other chemicals were of analytical grade and were used without further purification.

Enzyme activities—The AsAP activity was assayed spectrophotometrically as previously described (Shigeoka et al. 1980). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.4 mM AsA, 0.1 mM H_2O_2, and the enzyme. The oxidation of AsA was followed by a decrease in the absorbance at 290 nm (ε=2.8 mM^{-1} cm^{-1}). The activities with other electron donors were assayed in the same reaction mixture used for AsA, but AsA was replaced with 20 mM pyrogallol (ε_{430} 2.47 mM^{-1} cm^{-1}), 5 mM guaiacol (ε_{470} 26.6 mM^{-1} cm^{-1}), and NAD(P)H (ε_{340} 6.22 mM^{-1} cm^{-1}). MDAsA reductase was assayed using MDAsA generated by AsA and an AsA oxidase system (Shigeoka et al. 1987). The reaction mixture (2 ml) was comprised of 50 mM phosphate buffer (pH 7.0), 1 mM AsA, 1 unit AsA oxidase, 0.1 mM NADH, and the enzyme. Marker enzymes, catalase (Mutsuda et al. 1996) and hydroxypyruvate reductase (Bogin and Wallace 1969) for microbodies and cytochrome *c* oxidase (Hodges and Leonard 1974) and fumarase (Walk and Hock 1977) for mitochondria, were assayed by the methods in the cited references.

Construction of spinach greening cotyledon cDNA library—The construction of the cDNA library was performed as described previously (Ishikawa et al. 1996b). Total RNA was isolated from greening cotyledons of spinach seedlings (5.0 g wet wt.) with guanidine-isothiocyanate, and poly(A)$^+$RNA was then purified using the PolyATtract mRNA Isolation Systems (Promega, WI). A cDNA was synthesized using a cDNA synthesis kit (Amersham, U.K.), and a cDNA library was constructed in λgt11 as described by the supplier (Amersham, U.K.).

Cloning of spinach gAsAP cDNA—The spinach cDNA library in λgt11 was screened with a monoclonal antibody (mAb) raised against *Euglena* AsAP (EAP1) diluted 1 to 1,000 as described previously (Ishikawa et al. 1996c). *E. coli* strain Y1090r$^-$ was infected with recombinant phages; then the plaques formed were imprinted on isopropyl-β-D-thiogalactopyranoside (IPTG)-impregnated nitrocellulose filters (Millipore, MA). The filters were treated with the EAP1 and then with goat anti-mouse IgG-peroxidase conjugate (Cappel, NC), followed by staining with 4-methoxy-1-naphthol (Aldrich, WI). Positive plaques were rescued from the master plate, rescreened, and then amplified in Y1090r$^-$. The insert was excised from the phage and subcloned into the plasmid vector pBluescript SK(+) (Stratagene, CA).

Nucleotide sequence analysis—The nucleotide sequence was determined by the dideoxy method using a 373A DNA sequencer (Applied Biosystems, CA).

Overexpression of recombinant spinach gAsAP in E. coli—For the construction of the plasmid to express the cloned gAsAP cDNA, the following oligonucleotide primers were used. The upstream 24-mer oligonucleotide was derived from the cDNA nucleotide sequence homologous to the non-coding and coding strands corresponding to the nucleotide positions 12 to 35. It included an *Nde*I restriction site (underlined), giving the following sequence: 5′-AAGCTCCAACATATGGCGATGCCG-3′. The downstream 26-mer oligonucleotide was derived from the nucleotide sequence complementary to the non-coding strand of the cDNA nucleotide positions 1,085 to 1,110, which included a *Bam*HI restriction site (underlined), giving the following sequence: 5′-GCTCTTCATAGTTGGATCCATTCTGA-3′.

The PCR reaction was initiated directly with an aliquot of the plasmid containing the full-length cDNA of spinach gAsAP. The plasmid was denatured by heating for 5 min at 94°C. The sample was then subjected to the PCR in 100 μl reaction mixture containing 400 nM of each oligonucleotide, 200 μM dNTPs, 1.5 mM

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$MgCl_2$, and 2.5 units *Taq* DNA polymerase. Thirty cycles (1 min 94°C, 1 min 55°C, 2 min 72°C) were performed, followed by an elongation of 7 min at 72°C. The double strand PCR product with the correct size (1,099 bp) was purified, ligated into pT7Blue T-vector (Novagen, WI), and then transformed into *E. coli* strain DH5*a* F'. The sequence of the insert region of pT7Blue T-vector was verified by DNA sequencing. The plasmid obtained was digested with *Nde*I and *Bam*HI restriction enzymes; then the fragment was ligated into the *Nde*I-*Bam*HI sites of the pET-3a expression vector and introduced into the *E. coli* strain DH5*a* F'. Plasmid DNA was prepared from the ampicillin-resistant transformants and tested by digestion using *Nde*I and *Bam*HI to see if the inserted DNA was indeed present. The resulting construct, named pET-SAP30, was introduced into *E. coli* strain BL21(DE3)pLysS. The recombinant *E. coli* cells were grown at 37°C in Luria-Bertani medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl) supplemented with 50 $\mu g\ ml^{-1}$ ampicillin and 34 $\mu g\ ml^{-1}$ chloramphenicol. IPTG was added to a final concentration of 0.4 mM to midexponential phase cells (OD_{600}~0.7), and the culture was shaken for an additional 3 to 4 h.

Partial purification of recombinant gAsAP—All procedures were carried out at 4°C. The recombinant *E. coli* cells (4.0 g wet wt.) were harvested by centrifugation at 500 × *g* for 10 min, resuspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM AsA, and 20% (w/v) sorbitol, and disrupted by sonication (10 kHz) for 3 min. This lysate was centrifuged at 100,000 × *g* for 30 min. Approximately 10 mg of protein per ml of insoluble suspension was incubated with 0.5% Triton X-100 in the above phosphate buffer for 1 h. The solubilized enzyme was obtained by centrifugation at 105,000 × *g* for 60 min. The supernatant was submitted to a HiLoad 26/10 Q Sepharose column equilibrated with the phosphate buffer. The active fractions were then chromatographed onto a HiLoad 16/60 Superdex 200 column equilibrated with the phosphate buffer.

Isolation of intact glyoxysomes—To avoid contamination by AsAP isozymes from chloroplasts, spinach cotyledons (100 g wet wt.) grown under dark conditions were used for the isolation of glyoxysomes. They were chopped with razor blades in 200 ml of the grinding medium containing 25 mM MOPS (pH 7.8), 0.4 M mannitol, 1 mM EDTA, 0.1% (w/v) BSA, 8 mM cysteine, and 1 mM AsA. The homogenate was squeezed through four layers of cheesecloth. The homogenization of the residue was repeated with a further 200 ml of the grinding medium. The filtrates were combined and centrifuged at 1,000 × *g* for 5 min to remove unbroken cells and cellular debris, followed by centrifuging for 15 min at 14,000 × *g*. The obtained precipitate was suspended in 140 ml of the washing medium containing 5 mM MOPS (pH 7.5), 0.4 M mannitol, 1 mM EDTA, 0.1% BSA, and 1 mM AsA, centrifuged for 5 min at 1,000 × *g*, and then centrifuged for 15 min at 14,000 × *g*. The resultant 1,000–14,000 × *g* pellets were then resuspended in 8 ml of the washing medium and subjected to a linear sucrose density gradient centrifugation (40–70% sucrose concentration) in 10 mM Tricine buffer (pH 7.5) containing 1 mM EDTA, 1 mM AsA described previously (Shigeoka et al. 1980). The tubes were centrifuged at 100,000 × *g* for 3 h at 4°C. Intact glyoxysomes were collected from the gradient by aspiration.

SDS-PAGE and immunoblotting—SDS-PAGE was performed in 12.5% slab gels, according to the method of Leammli (Leammli 1970). The gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, gels were transferred to PVDF membranes, using an electroblot apparatus (model 200/2.0, Bio-Rad, CA) at 15 V for 1 h. The membranes were treated with the mAb raised against *Euglena* AsAP and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, as described previously (Ishikawa et al. 1996c). Immunoreactive proteins attached to membranes were visualized after horseradish peroxidase reduction of 4-methoxy-1-naphthol (Aldrich, WI).

N-terminal sequence analysis—The resolved native and recombinant proteins were separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes as described above. The membrane was washed extensively with water, stained with 0.25% Coomassie R-250 in 5% aqueous MeOH and 7.5% acetic acid for 5 min, and destained with 90% aqueous MeOH for 10 min. The portion of the membrane containing the desired protein band was cut out, and the N-terminal sequence was performed by an automated pulse-liquid protein sequencer (model 492, Applied Biosystems, CA) as described previously (Ishikawa et al. 1996c).

Results

cDNA cloning of spinach gAsAP—In the preceding study, we have reported that mAb (EAP1) raised against *Euglena* AsAP cross-reacts with both cAsAP and sAsAP from spinach leaves (Ishikawa et al. 1996c) and becomes a good probe for isolating the four cDNA clones which encode two cAsAP and two chloroplastic AsAP isozymes (Ishikawa et al. 1995, 1996b). Herein, we isolated one positive clone (SAP30) with a nucleotide sequence different from those of the other four AsAP cDNA clones. The cDNA sequence of the SAP30 showed an open reading frame starting from nucleotide 24 up to 881 and coding for a protein containing 286 amino acids with a molecular mass of 31,507 Da (Fig. 1A). The calculated overall identity of the sequence was 83.7% and 80.5% at the protein level with the sequences of cotton (Bunkelmann and Trelease 1996) and *Arabidopsis* gAsAPs (GenBank accession No. X98003), respectively (Fig. 2). A putative membrane-spanning region of the predicted protein was identified near the C-terminal sequence; it possessed a hydrophobic domain, as was the case for the cotton enzyme (Fig. 1B). These data suggested that the SAP30 encodes a spinach gAsAP. The deduced spinach gAsAP showed relatively high homologies to those of cAsAP and chloroplastic AsAPs from spinach with amino acid identities of 64.2% and 47.3%, respectively, indicating that the gAsAP has a higher degree of homology in its amino acid sequence with cAsAP than with chloroplastic AsAP isozymes. However, the C-terminal of gAsAP, which involved the putative transmembrane segment, was approximately 40 amino acids longer than that of cAsAP. AsAP isozymes and yeast cytochrome *c* peroxidase (CCP) have been classified as members of the class I plant peroxidases from their amino acid sequences and have been part of the lineage of prokaryotic peroxidases (Welinder 1992). The deduced spinach gAsAP showed a 34.8% identity over 230 amino acids with yeast CCP and had less homology with the classical plant peroxidases such as horseradish peroxidase. This is also the case for the other previously described AsAP isozymes (Ishikawa et al. 1995, 1996b). A phylogenic tree was

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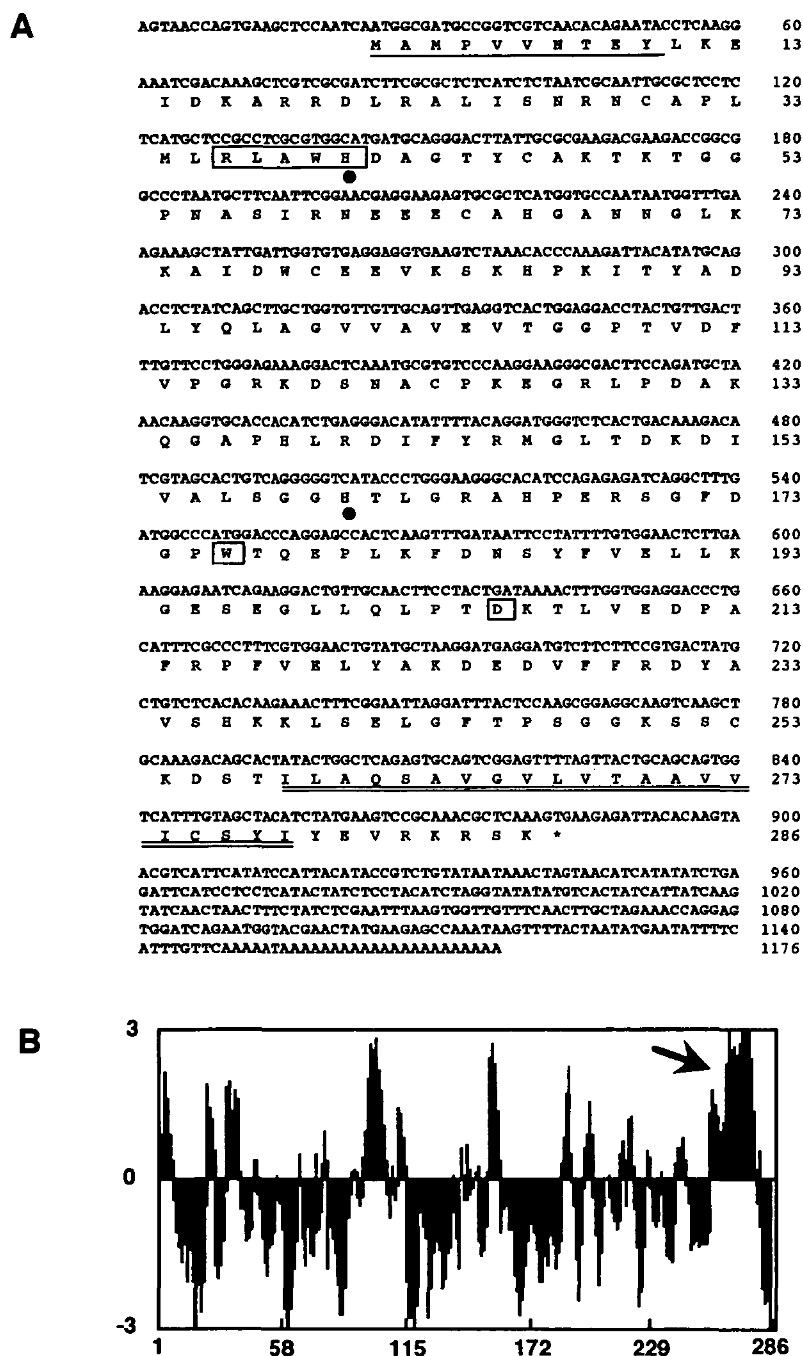

Fig. 1 Nucleotide and predicted amino acid sequence of the cDNA for spinach gAsAP and hydropathy profile of its amino acid sequence. (A) The amino acid sequences deduced from an open reading frame are shown below the nucleotide sequences. The amino acid sequences of the N-terminal of the native gAsAP are single-underlined. Hydrophobic sequences representing the putative membrane binding domain in gAsAP are double-underlined. The distal and proximal His residues are shown by heavy dots. The boxes show amino acid residues that correlate with active sites. (B) Hydrophobicity was analyzed by the GENETYX software program for a window size of nine amino acid residues. Positive hydropathy indicates hydrophobicity. The arrow indicates a putative membrane-binding domain in gAsAP.

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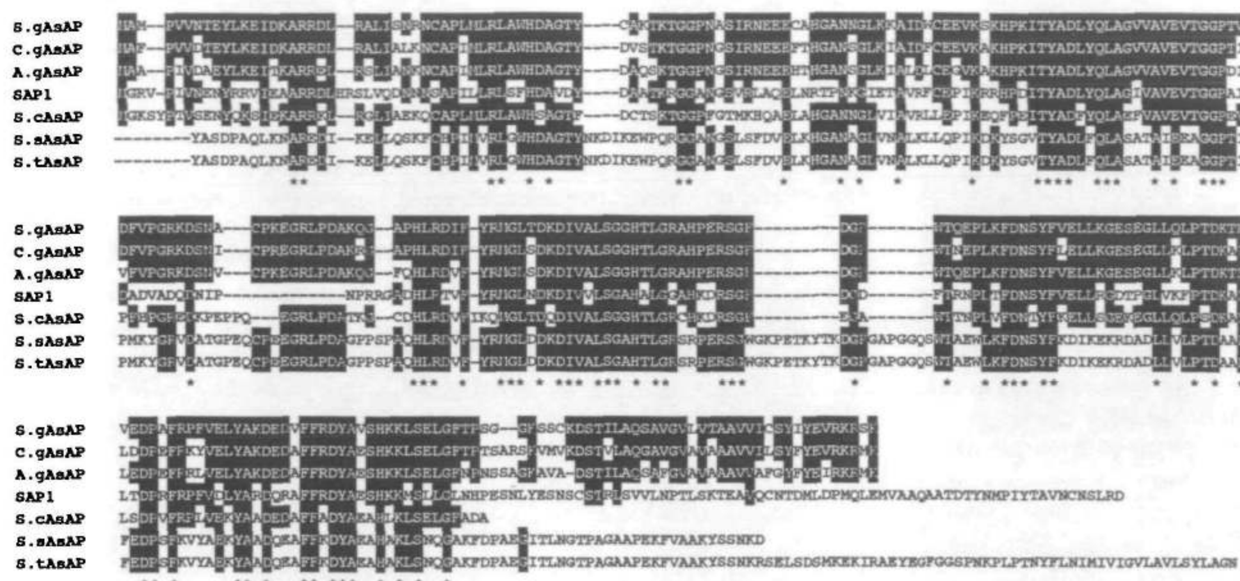

Fig. 2 Comparison of the deduced amino acid sequences of spinach gAsAP with other AsAP isozymes. The deduced sequence of spinach glyoxysome-bound AsAP (S. gAsAP) is aligned with that of cotton glyoxysome-bound AsAP (C. gAsAP) (Bunkelmann and Trelease 1996), *Arabidopsis* glyoxysome-bound AsAP (A. gAsAP) (GenBank accession No. X98003), spinach SAP1 (Ishikawa et al. 1995), spinach cytosolic AsAP (S. cAsAP) (Ishikawa et al. 1995), spinach stromal AsAP (S. sAsAP) (Ishikawa et al. 1996b), and spinach thylakoid-bound AsAP (S. tAsAP) (Ishikawa et al. 1996b) using a single-letter code. The gaps are introduced to optimize the alignment. Residues found at the same position as spinach mAsAP are shown as white letters on black. The asterisks show the consensus amino acids.

constructed according to Hein's alignment algorithm (Hein 1990). As illustrated in Fig. 3, gAsAP was more closely related to the AsAPs and yeast CCP than to other peroxidases. The class I peroxidase shares the common features

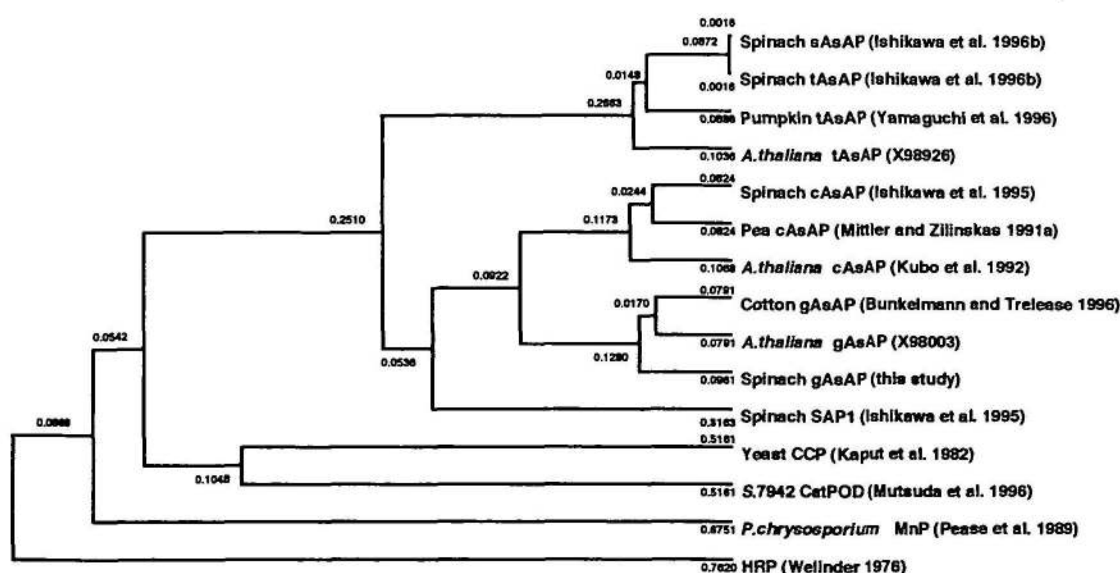

Fig. 3 Phylogenic tree for AsAP isozymes and other peroxidases. The dendrogram is generated by comparison of the known amino acid sequences according to Hein (1990). Numbers indicate branch length as proportional genetic divergence. Numbers in parenthese show the cited references and accession numbers. The enzymes are as follows: sAsAP, stromal AsAP; tAsAP, thylakoid-bound AsAP; cAsAP, cytosolic AsAP; gAsAP, glyoxysome-bound AsAP; SAP1, spinach AsAP; CCP, chytochrome *c* peroxidase; S.7942 CatPOD, *Synechococcus* PCC 7942 catalase-peroxidase; MnP, manganese-dependent peroxidase; HRP, horseradish peroxidase.

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of the distal histidine site (R-L-A-W-H). The spinach gAsAP had a completely conserved residue (His^{40}) in the sequence. The other heme ligand site, the proximal histidine of the gAsAP, was also conserved at the amino acid position of His^{160}. The Asp^{184} residue correlated with the active site of CCP was conserved in the gAsAP as well as AsAP isozymes. The Trp residue, which was correlated with the storage of oxidizing equivalents in Compound I (Fishel et al. 1991), was present at the amino acid position of 176. These results clearly support the argument that gAsAP belongs to the class I peroxidases as do the other AsAP isozymes and that the class I peroxidase genes might have evolved from the same ancestral gene.

Suborganellar distribution of gAsAP—Fig. 4A shows the subcellular distribution of the AsAP activity together with marker enzymes (catalase, hydroxy pyruvate reductase, cytochrome *c* oxidase, fumarase) in a linear sucrose density gradient centrifugation. The AsAP activity was present only in the glyoxysome fraction; no activity was detected in the other fractions. Furthermore, in order to explore the occurrence of AsAP in spinach glyoxysomes, each fraction in a linear sucrose density gradient centrifugation was subjected to SDS-PAGE for immunoblotting using the mAb (EAP1) raised against *Euglena* AsAP. The EAP1 cross-reacted with a 31 kDa protein band in glyoxysomal fractions, which correlated well with the calculated molecular mass of SAP30; no cross-reactivity was found in any other fraction such as mitochondria (Fig. 4B). The N-terminal amino acid sequence of the 31 kDa protein was determined to be M-A-M-P-V-V-N-T-E-Y-, which was completely identical with that of the deduced amino acid sequence of the SAP30 clone. These findings clearly in-

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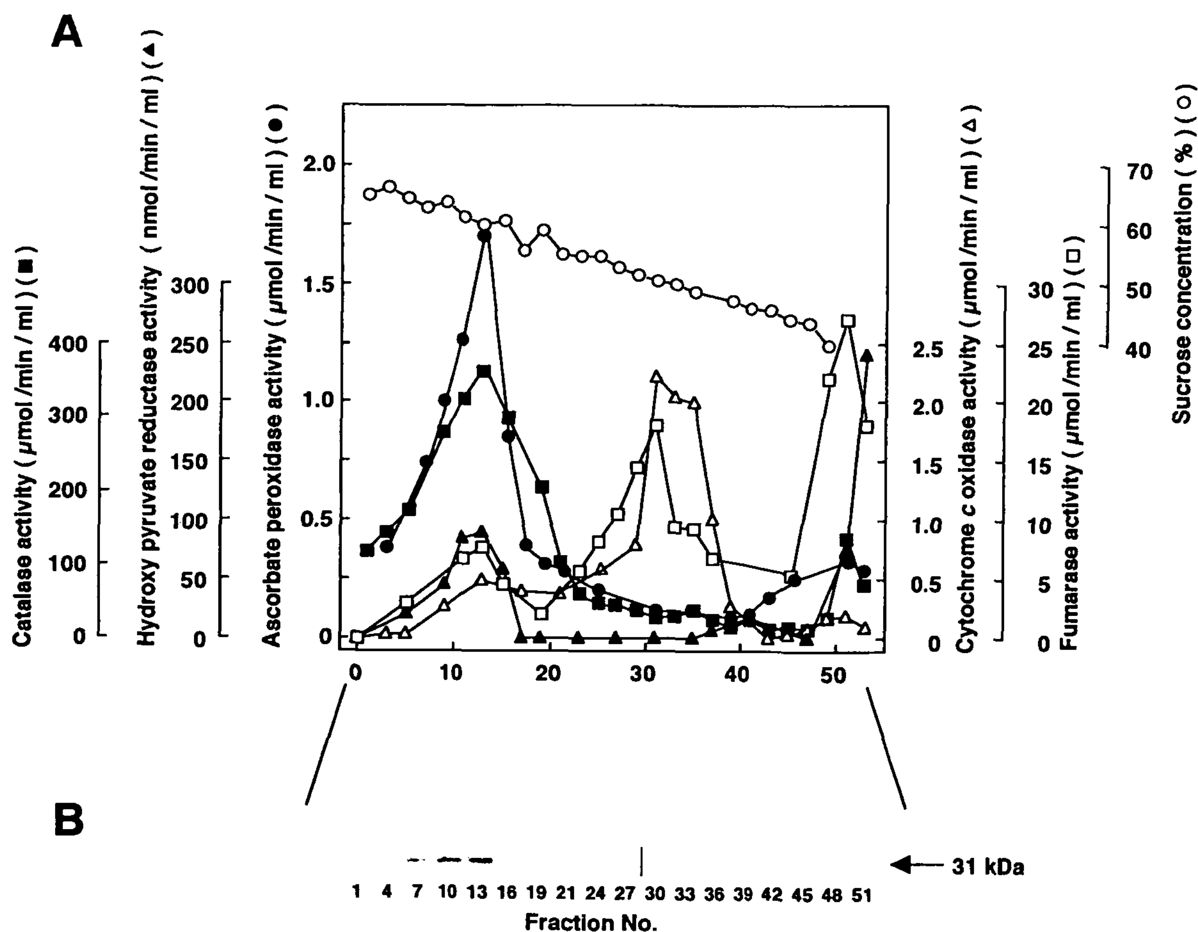

Fig. 4 Subcellular distribution of AsAP and marker enzymes following linear sucrose density gradient centrifugation (A) and immunoblot analysis using the mAb (EAP1) raised against *Euglena* AsAP (B). (A) The 1,000–14,000 × *g* pellets obtained by differential centrifugation from spinach cotyledon homogenate were resuspended in 8 ml of the washing medium containing 5 mM MOPS (pH 7.5), 0.4 M mannitol, 1 mM EDTA, 0.1% BSA, and 1 mM AsA and were then subjected to linear sucrose density gradient centrifugation (sucrose concentration from 40% to 70%). (B) Aliquots of the fractions (20 μl of each fraction) were treated with 1% SDS at 100°C for 3 min and subjected to SDS-PAGE and immunoblotting. The arrow indicates the position of the gAsAP (31 kDa). Detailed procedures are described in the Materials and Methods section.

Table 1 Suborganellar distribution of activities of AsAP, monodehydroascorbate reductase (MDAsAR), and catalase (Cat) in spinach glyoxysomes

	Enzyme activity		
	AsAP (nmol min^{-1})	MDAsAR (nmol min^{-1})	Cat (μmol min^{-1})
Crude extract	915±33	108 ±7.5	174 ±12
KCl-soluble fraction	nil	nil	177 ±15
KCl-insoluble fraction	638±22	99.4±4.2	1.5± 0.1

The intact glyoxysomes (0.6 mg of protein) were treated with 50 mM MES buffer (pH 6.0) containing 0.2 M KCl in a final volume of 500 μl. Preparations were incubated on ice and vortexed every 10 min. After 30 min of incubation, each preparation was centrifuged at 100,000 × *g* for 30 min at 4°C. The resuspended pellet and the soluble fraction were assayed for enzyme activity as described in the Materials and Methods section. Data are mean values ± SD from three assays.

dicated that SAP30 is the cDNA clone encoding AsAP isozyme which localizes in glyoxysomes of spinach. The presence of the C-terminal hydrophobic membrane-spanning domain of gAsAP suggested that the gAsAP exists as a glyoxysome membrane-bound form. The suborganellar distribution of the AsAP in glyoxysomes was confirmed. Most of the AsAP activity was detected in the KCl-insoluble membrane fraction of osmotically disrupted glyoxysomes unlike catalase, a typical soluble matrix enzyme of glyoxysomes (Table 1). Furthermore, the latency experiment using Triton X-100 showed a high latent catalase activity in glyoxysomes. However, intact glyoxysomes showed no latent AsAP activity (Table 2). The treatment of intact glyoxysomes with trypsin resulted in an irreversible inactivation of the AsAP; it had no effect on the catalase activity (Table 3). The MDAsA reductase activity was also observed in the membrane fraction (Table 1). The latent activity and tryptic treatment of the MDAsA reductase resembled those of gAsAP (Table 3). The MDAsA reductase activity was present only in the glyoxysome fraction; no activity was detected in any other fraction in a linear sucrose density gradient centrifugation (data not shown). The MDAsA reductase activity detected in glyoxysome fraction does not seem to be due to contamination by other organelles. These results clearly imply that both gAsAP and MDAsA reductase are bound to the external side of the membrane of spinach glyoxysomes.

Expression and partial purification of recombinant gAsAP—The expression systems using *E. coli* for the two cAsAP and one sAsAP isozymes reported in the literature yielded soluble proteins (Patterson and Poulos 1994, Dalton et al. 1996, Ishikawa et al. 1996b). These AsAPs exist as a soluble form in vivo but not as a membrane-bound form. We examined the optimum conditions for the expression of our recombinant gAsAP which was subcloned into the pET-3a expression vector. The initiation of induction after growth saturation (optical density of 0.7 at 600 nm) and the continuation of induction for 3 h resulted in a high yield of recombinant gAsAP. The recombinant enzyme protein prepared from the insoluble fraction correlated with the deduced molecular mass from SAP30 (Fig. 5A). The immunoblots of the soluble and insoluble fractions using the EAP1 raised against *Euglena* AsAP also revealed a predominant band at 31 kDa (Fig. 5B). More than 95% of the

Table 2 Effect of Triton X-100 on enzyme activities of AsAP, monodehydroascorbate reductase (MDAsAR), and catalase (Cat) in whole glyoxysomes

Treatment	Enzyme activity		
	AsAP (nmol min^{-1})	MDAsAR (nmol min^{-1})	Cat (μmol min^{-1})
None	1,760±146	380±31	5.0± 0.2
+0.2% Triton X-100	1,470±125	325±27	165 ±11

The intact glyoxysomes were treated with Triton X-100 in 50 mM MES buffer (pH 6.0) containing 0.3 M sucrose at detergent-to-protein ratios (w/w) of 10 to 1. The glyoxysome protein (1.21 mg) was used in a final volume of 0.5 ml. The total enzyme activities were measured in the presence of 0.3 M sucrose as described in Materials and Methods. Each value represents the mean of three assays ± SD.

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Table 3 Effect of trypsin on enzyme activities of AsAP, monodehydroascorbate reductase (MDAsAR), and catalase (Cat) in whole glyoxysomes

Treatment	Enzyme activity		
	AsAP (nmol min^{-1})	MDAsAR (nmol min^{-1})	Cat (μmol min^{-1})
None	1,493±87	403 ±33	100 ±8.2
+5 μg Trypsin	176±14	29.3± 1.0	85.1±5.0
+5 μg Trypsin and Trypsin inhibitor	1,365±95	390 ±25	95 ±7.5

The intact glyoxysomes (1.2 mg of protein) were treated with trypsin in 50 mM MES buffer (pH 6.0) containing 0.3 M sucrose at a trypsin-to-protein ratio (w/w) of 1 to 250. After 15 min incubation at room temperature, the reaction was stopped by trypsin inhibitor in an eight-fold excess relative to the weight of trypsin. As a control the reaction was incubated for the same time with trypsin and trypsin inhibitor mixed prior to addition to the organelles. Both preparations were immediately used for the enzyme assays. The enzyme activities were measured in the absence of 0.3 M sucrose as described in Materials and Methods. Each value represents the mean of three assays±SD.

recombinant gAsAP was accumulated in the insoluble membrane fraction as active enzyme. We therefore developed the solubilization of the enzyme using some detergents and partial purification. Sodium deoxycholate (6 mM), Triton X-100 (0.5%) and CHAPS (0.2%) in 50 mM potassium phosphate (pH 7.0) containing 1 mM AsA solubilized about 76, 78, and 69%, respectively, of the recombinant gAsAP from the bacterial membrane. Triton X-100 was thus a useful detergent for the membrane-bound recombinant gAsAP because of the high yield and enzymatic stability. When using CHAPS or octyl-glucoside, the solubilized enzyme lost its activity within one day (data not shown).

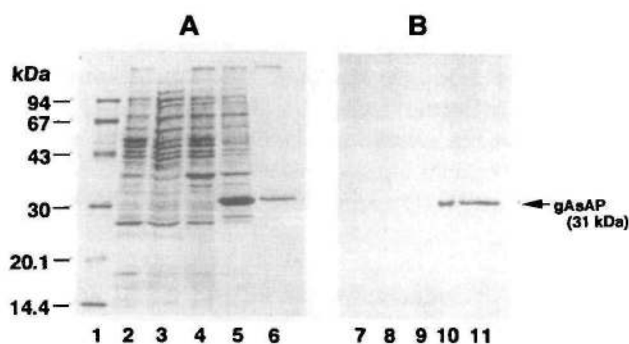

Fig. 5 Overexpression and partial purification of recombinant gAsAP in *E. coli*. The overexpression of pET-SAP30 in *E. coli* and partial purification of the recombinant gAsAP were carried out as described in the Materials and Methods section. (A) Coomassie Brilliant Blue staining of SDS-PAGE. (B) Immunoblot analysis using the *Euglena* AsAP mAb, EAP1. Positions and sizes in kilodaltons of marker proteins are shown on the left side of the panel. Lane 1, molecular mass markers; lanes 2 and 7, a soluble fraction of the cells without pET-SAP30; lanes 3 and 8, a soluble fraction of the cells harboring pET-SAP30; lanes 4 and 9, an insoluble fraction of the cells without pET-SAP30; lanes 5 and 10, an insoluble fraction of the cells harboring pET-SAP30; lanes 6 and 11, partially purified recombinant gAsAP.

The procedure yielded a recombinant enzyme preparation purified approximately 2.2-fold over the crude enzyme, giving a final 32.0% recovery of the activity. The SDS-PAGE of the partially purified recombinant gAsAP showed one major and a few faint minor protein bands; the major band contained approximately 95% of the total protein (Fig. 5A). The specific activity of the recombinant gAsAP was 81.0±2.5 μmol of AsA/min per mg of protein. N-terminal amino acid sequence analysis of the recombinant gAsAP revealed the sequence M-A-M-P-V-V-N-T-E-Y-L-K-E-I-D-K-, identical to that deduced from the cDNA.

Molecular mass analysis—In cotton oilseed glyoxysomes, gAsAP was found to be a homodimer, because the antiserum raised from 67-kDa peroxisomal membrane protein recognized a 31 kDa protein (Bunkelmann and Trelease 1996). The authors, however, did not identify the native gAsAP activity. Judging from SDS-PAGE and immunoblotting (Fig. 5), the subunit molecular mass of both the native and recombinant gAsAPs was found to be 31 kDa, which correlated with the deduced molecular mass from SAP30 open reading frame. The *E. coli* soluble fraction corresponding to a few % of the total recombinant gAsAP activity was subjected to a HiLoad 16/60 Superdex 200 column equilibrated with a 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM AsA, and 20% (w/v) sorbitol. The soluble recombinant enzyme had a molecular mass of 31 kDa, indicating that the glyoxysome enzyme exists as a monomeric form in its native state. We failed to determine the exact molecular mass of the Triton X-100-solubilized recombinant enzyme because it aggregated during solubilization; this might have been caused by its C-terminal hydrophobic region.

Enzymatic properties of gAsAP—The enzymatic properties of the almost purified recombinant gAsAP were consistent with those of the native enzyme from intact glyoxysomes. Both recombinant and native gAsAPs utilized AsA

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as the most effective natural electron donor; GSH and NAD(P)H could not substitute for AsA. They catalyzed the oxidation of pyrogallol at a rate one third lower than that with AsA. With respect to its donor specificity, gAsAP resembled the chloroplastic AsAP isozymes more than the cAsAP isozymes (Nakano and Asada 1987, Chen and Asada 1989, Mittler and Zilinskas 1991a, Miyake et al. 1993, Ishikawa et al. 1996a). K_m values for AsA and H_2O_2 were determined using Lineweaver-Burk plots with substrate concentrations of 0.1–0.5 mM for AsA and 20–350 μM for H_2O_2. The substrate-velocity curves with recombinant and native gAsAPs showed Michaelis-Menten type kinetics with AsA and H_2O_2. The recombinant cAsAPs from pea and soybean failed to obey Michaelis-Menten kinetics because of the complication of the monomer/dimer equilibrium (Dalton et al. 1996, Patterson and Poulos 1994). From Lineweaver-Burk plots, the apparent K_m values for AsA of the native and recombinant gAsAP at 0.1 mM H_2O_2 were determined to be 1.82 ± 0.05 mM and 1.89 ± 0.05 mM, respectively. These values were approximately 3.5–7-fold higher than those of other AsAP isozymes (Chen and Asada 1989, Mittler and Zilinskas 1991a, Ishikawa et al. 1996a). The K_m values for H_2O_2 of the native and recombinant gAsAP were 80 ± 5.3 μM and 74 ± 4.0 μM, respectively, when the concentration of AsA was 0.4 mM. The partially purified recombinant gAsAP was completely inhibited by 0.6 mM azide and 50 μM cyanide, indicating that the spinach gAsAP, like other AsAP isozymes, is a typical hemeprotein. The enzyme was also sensitive to the thiol-modifying agent *p*-chloromercuric benzoate at 0.1 mM.

The optimum pH and temperature of the recombinant gAsAP were 7.0 and 37°C, respectively. Previous reports with respect to cAsAP and chloroplastic AsAP isozymes from various sources indicate optimum pH ranges of 6.0–8.0 (Chen and Asada 1989, Mittler and Zilinskas 1991a, Ishikawa et al. 1996a). The enzyme retained its full activity up to 45°C between pH 6.3 and 8.0 and lost its activity completely at 53°C. One of the specific properties of AsAPs is rapid inactivation in an AsA-depleted medium. This is especially true for chloroplastic AsAP, whose half-inactivation time was only 15 s (Miyake and Asada 1996). cAsAP isozymes are more stable than the chloroplastic AsAP isozymes (Asada 1992). When the partially purified recombinant gAsAP was diluted with the AsA-depleted medium, the activity was stable over 180 min (Fig. 6), which was in agreement with pea cAsAP (Mittler and Zilinskas 1991a). Miyake and Asada (1996) have reported that the inactivation of sAsAP isozyme in an AsA-depleted medium is caused by the instability of Compound I to H_2O_2 when AsA is not available for Compound I. When spinach recombinant gAsAP is incubated with 2 μM H_2O_2 under anaerobic conditions, no inactivation is observed (data not shown). Accordingly, one of the reasons for the stability

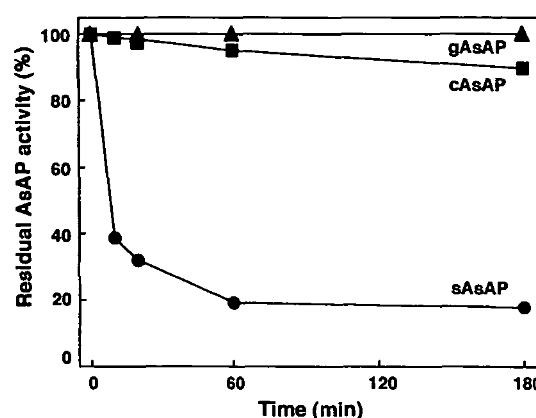

Fig. 6 Effect of the AsA-depletion on AsAP activities. The recombinant cytosolic AsAP (cAsAP) and recombinant stromal AsAP (sAsAP) from spinach were prepared as described previously (Ishikawa et al. 1995, 1996b) and partially purified. Each AsAP protein (1.2 mg ml^{-1}) suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM AsA and 20% (w/v) sorbitol was diluted with 10 mM potassium phosphate buffer (pH 7.0) to a final AsA concentration of less than 10 μM. After incubation for the indicated time, 1 mM AsA was added, and the enzyme activity was assayed.

mechanism in spinach gAsAP may be the insusceptibility of Compound I to H_2O_2. The exact reason for the difference in stability among AsAP isozymes is not clear at present.

Discussion

In spinach, we have demonstrated the molecular characteristics of many AsAP isozymes, including the one in this study (Ishikawa et al. 1995, 1996b). This novel AsAP isozyme, gAsAP, has some characteristic properties distinguishing it from other AsAP isozymes. The hydropathy analysis of its primary structures indicated that a putative significant hydrophobic segment is present in the C-terminal region (Fig. 1B). A similar membrane-spanning region at the C-terminal region is present in the tAsAP isozyme, although no other similarity of the C-terminal region is observed (Ishikawa et al. 1996b). As far as we know, no other reports exist of such a unique membrane spanning system. It is an interesting problem as to whether or not the C-terminal hydrophobic region of the gAsAP serves as a novel targeting motif of the microbody outer-membrane and the anchor of the protein.

Based on the latency of gAsAP activity by solubilization assay with Triton X-100 and the tryptic digestion in intact glyoxysomes (Table 2), the catalytic domain of AsAP on glyoxysome membranes was found to be exposed to the cytosol. This was also true in an AsAP from pumpkin microbodies (Yamaguchi et al. 1995b). In cotton oilseed, how-

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ever, the majority of gAsAP, including the active site, was predicted to be on the matrix side of the glyoxysomes (Bunkelmann and Trelease 1996). SODs in peroxisomes from many plant species were detected as isozymes differing in their suborganellar localization. In watermelon cotyledons, a Cu/Zn-SOD has been detected in a soluble matrix and a Mn-SOD on the external side of the peroxisomal membrane as a binding form. On the other hand, in pea leaves, a Mn-SOD has been located only in the peroxisomal matrix (Sandalio and del Río 1988). Similarly, the suborganellar localization of AsAP in glyoxysomes may also depend on the tissue origin. In pumpkin, the gAsAP had been recognized as a rich glyoxysomal membrane protein with a molecular mass of 31 kDa, which was retained in peroxisomes during the microbody transition from glyoxysomes to peroxisomes (Yamaguchi et al. 1995a). The fact that the SDS-PAGE analysis detected a main band which corresponds to the 31 kDa gAsAP protein in intact glyoxysomes from spinach cotyledons also suggests that the AsAP commonly plays an essential role in these organelles (data not shown). In recent years, it has become clear that microbodies carry out essential functions in almost all eukaryotic cells and have an essentially oxidative type of metabolism (van den Bosch et al. 1992). The acyl CoA oxidase in β-oxidation and the glycolate oxidase in photorespiration directly produce H_2O_2. Furthermore, there are two sites of O_2^- production in peroxisomes; one is the generating system of xanthine oxidase in the matrix and the other is the NADH-dependent electron transport system in the membrane. Some xenobiotics, such as clofibrate, stimulate the production of O_2^- and H_2O_2 inside microbodies (del Río et al. 1992). Taking into account these data, it seemd important to determine the extent to which AsAP and catalase are involved in the detoxification of H_2O_2 generated in microbodies. The catalase localized in their matrix appears to be rather unsuited to the natural state of leaves because it suffers from a light-induced inhibition of function accompanied by a loss of the enzyme protein (Feierabend et al. 1992). In addition, catalase is very inefficient at removing low concentrations of H_2O_2, because the K_m value for H_2O_2 is high (~1 M) (Huang et al. 1983). The K_m value for H_2O_2 of spinach gAsAP was 74±4.0 μM. The K_m value for AsA of spinach gAsAP was 1.89±0.05 mM, which is approximately 3.5-7-fold higher than those of other AsAP isozymes (Chen and Asada 1989, Mittler and Zilinskas 1991a, Ishikawa et al. 1996a). It has been reported that the concen-

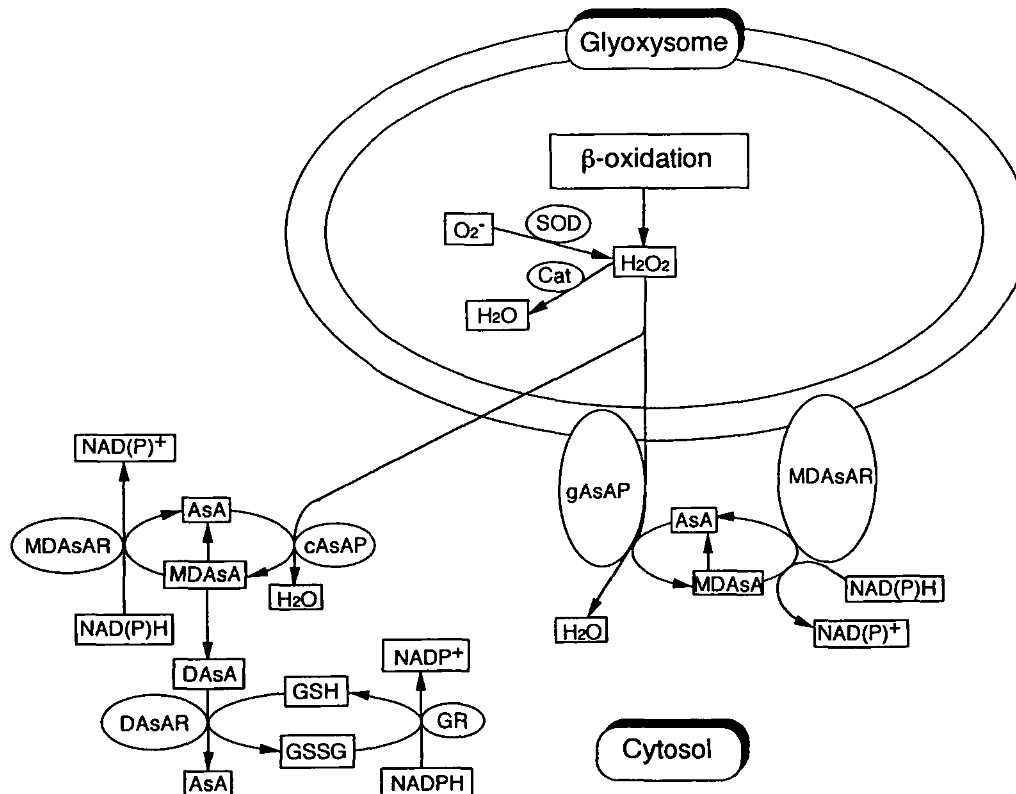

Fig. 7 Possible model of the functional organization of the H_2O_2-scavenging system in glyoxysomes. AsA, ascorbate; cAsAP, cytosolic ascorbate peroxidase; gAsAP, glyoxysome-bound ascorbate peroxidase; Cat, catalase; SOD, superoxide dismutase; MDAsA, monodehydroascorbate; MDAsAR, monodehydroascorbate reductase; DAsA, dehydroascorbate; DAsAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase.

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tration of AsA in apoplasts of spinach leaves is estimated to be between 0.1 and 0.5 mM and that the average intracellular AsA concentration is far higher than the apoplastic AsA concentration (Takahama and Oniki 1992). Accordingly, the spinach cytosolic AsA concentration seems to be at least several mM, allowing gAsAP to remove H_2O_2 in situ.

Based on our present data and other data so far, we propose the H_2O_2-scavenging system involving spinach glyoxysomes shown in Fig. 7. The MDAsA is the primary product of the AsAP reaction, and rapid regeneration of AsA is an absolute necessity for the scavenging of H_2O_2 by AsAP (Asada 1992). In chloroplasts, AsA is regenerated from the oxidation products in the sAsAP and tAsAP reaction by MDAsA reductase using NAD(P)H as the electron donor and direct reduction by ferredoxin photoreduced in PSI (Miyake and Asada 1994). In caster bean and cotton glyoxysomes, MDAsA reductase has been found to be membrane-associated and appears to be bound to the matrix side of the organelles (Bowditch and Donaldson 1990, Bunkelmann and Trelease 1996). In contrast, the MDAsA reductase of spinach glyoxysomes was bound to the external side of the membrane of glyoxysomes, in analogy with AsAP. It seems conceivable that the MDAsA which is generated by the peroxidase reaction is effectively regenerated to AsA by utilizing cytosolic NAD(P)H via glyoxysome outer-membrane-bound MDAsA reductase. Since the microbody membrane is sparsely permeable to NAD(P)H (Liang et al. 1984), the reduction of MDAsA radical by the enzyme seems not to be affected by permeation of the NAD(P)H from the cytosol to the glyoxysome matrix site. Furthermore, in caster bean, Bowditch and Donaldson (1990) assessed the suitability of MDAsA as a physiological acceptor for the glyoxysomal membrane-bound NADH dehydrogenase.

H_2O_2 diffuses readily through biological membranes (Takahashi and Asada 1983). In NaCl-sensitive pea cultivars, the catalase activity was inhibited, and glycolate oxidase was stimulated by NaCl (Corpas et al. 1993). The authors thus have suggested that H_2O_2 can diffuse out of peroxisomes into the cytosol as a result of NaCl-induced leakage of the peroxisomal membranes. We have demonstrated that H_2O_2 formed in *Euglena* organelles, including chloroplasts and mitochondria diffuses from them into the cytosol (Ishikawa et al. 1993). The excretion of H_2O_2 has been reported from many photosynthetic organisms, including green microalgae (Zepp et al. 1987). It seems likely that some of the H_2O_2 generated in the microbody matrix diffuses from the organelle toward the cytosol and then is decomposed by the mAsAP binding to the outside of the membrane. A set of the enzymes responsible for the AsA-GSH cycle located in the cytosol may serve as a partialy H_2O_2-scavenging system. Thus, the AsAP isozymes and AsA regeneration systems may function to protect microbodies from intoxication by H_2O_2 produced during some physiological activites.

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