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Purification and catalytic mechanism of chloroplast dehydroascorbate reductase from spinach leaves

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Introduction

Ascorbate plays an important role in decomposing hydrogen peroxide in the ascorbate peroxidase (APX) reaction. Ascorbate is oxidized to monodehydroascorbate and dehydroascorbate (DHA) via the APX reaction. The regeneration of ascorbate is essential for the maintenance of function of the active oxygen-scavenging system (Asada, 1999). DHAR, which is one of the key enzymes in the regeneration of ascorbate, has received relatively little attention. Recently, we purified chloroplast DHAR from spinach leaves and examined the enzymatic properties (Shimaoka *et al.*, 2000). The specific activity of spinach chloroplast DHAR was found to be seven times higher than that of DHAR from rice bran (Shimaoka *et al.*, 2000; Kato *et al*., 1997). Moreover, spinach chloroplast DHAR has one hundred times greater specificity for DHA and several-fold higher specific activity than porcine DHAR and other DHA-reducing enzymes (Shimaoka *et al.*, 2000; Wells *et al*., 1990; Washburn *et al*., 1999a; Xu *et al*., 1996; Sha *et al*., 1997).

We also isolated the cDNA for the spinach chloroplast DHAR. Spinach chloroplast DHAR had a C-X-X-C motif, which was commonly included in DHA-reducing proteins. The difference in k_{cat} between spinach chloroplast DHAR and other DHA-reducing enzymes might be due to differences in mechanisms of catalysis. Here, we examined the mechanism of catalysis by spinach chloroplast DHAR. Steady-state kinetic studies showed that catalysis by spinach chloroplast DHAR proceeded by a bi uni uni uni ping-pong mechanism. We obtained evidence that a disulfide bond was formed between the enzyme and GSH to yield a reaction intermediate, when the enzyme had been oxidized by DHA. Site-directed mutagenesis revealed that the amino acid residue Cys-23 of mature DHAR from spinach chloroplasts was essential for the expression of DHAR activity.

Materials and Methods

Purification of recombinant DHAR from Escherichia coli—*E. coli* BL21 (DE3) harboring pET3a-DHAR, in which the cDNA for the mature form of spinach chloroplast DHAR had been ligated (Shimaoka *et al.*, 2000), was grown in 200 ml of LB medium supplemented with 50 μg ml^{-1} ampicillin at 37˚C overnight.

All procedures for purification were performed at 0-4˚C. The cultured cells were resuspended in 50 mM potassium phosphate buffer (pH 7.8) that contained 1 mM EDTA, 40 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 20% (v/v) glycerol, and were disrupted with a French Press apparatus. The recombinant DHAR was purified from the obtained crude extract with hydrophobic, anion-exchange and gel-filtration chromatographies.

Assay of the activity of DHAR—The activity of DHAR was determined by monitoring the glutathione-dependent production of ascorbate at 265 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, various concentrations of DHA and GSH and purified enzyme.

Quantitation of DHAR-bound GSH—The reduced-form enzyme (10 μM) was allowed to react with 1 mM DHA and 10 μM GSH at the room temperature for 30 s in the presence of 50 mM potassium phosphate buffer (pH 7.0) that contained 1 mM EDTA under N_2. Low-molecular-weight compounds were removed from the preparation by passage through a column of PD-10, which had been equilibrated with O_2-free 50 mM borate buffer (pH 8.0) that contained 1 mM EDTA. Then 100 μl of the solution of enzyme were incubated with 50 mM 2-mercaptoethanol at 25˚C for 30 min. The solution was evaporated to dryness and then the residue was dissolved in 50 μl of distilled water. The 2-mercaptoethanol-free solution was mixed with 50 μl of 1 mM ABD-F in 0.1 M borate buffer (pH 8.0) and incubated at 50˚C for 5 min. After incubation, the mixture was cooled on ice and acidified by addition of HCl. The acidified solution was applied to a column (2.3 mm i.d. x 250 mm) of Wacosil-II 5C18 HG, which was part of an HPLC system and which had been equilibrated with a mixture of 50 mM potassium hydrogen phthalate (pH 4.0) and acetonitrile (92:8, v/v). The column was developed with the same solution at a flow rate of 1.0 ml min^{-1}. The GSH that had been modified by ABD-F was detected fluorometrically with an excitation wavelength of 380 nm and an emission wavelength of 510 nm.

Results and Discussion

Characterization of recombinant spinach chloroplast dehydroascorbate reductase—The recombinant enzyme, which was purified to homogeneity, had DHAR activity. The specific activity of the purified recombinant DHAR was 331 μmol mg $protein^{-1}$ min^{-1}, which was almost identical to that of the purified spinach chloroplast DHAR. The K_m values of the recombinant DHAR for DHA and GSH were 60 μM and 1.1 mM, respectively, and were also almost identical those of the DHAR from spinach chloroplasts (Shimaoka *et al.*, 2000).

Initial velocity and product inhibition of the reaction catalyzed by spinach

chloroplast DHAR—DHAR reduces one mole of dehydroascorbate (DHA) to one mole of ascorbate with two moles of GSH as the electron donor. Double-reciprocal plots for activity *versus* various concentrations of one substrate yielded straight lines at the various fixed concentrations of the other substrate. Replot for the slopes and the intercepts with the *y*-axis in the above double-reciprocal plots against the reciprocals of the concentrations of the substrates yielded straight lines. These results show that the reaction catalyzed by DHAR proceeds via a bi uni uni uni ping-pong mechanism and that substrate that binds last is GSH.

The double-reciprocal plots of reaction velocity against the concentration of DHA in the presence of GSH and GSSG gave straight lines that pivoted counterclockwise on the point at which the lines. Double-reciprocal plots of velocity *versus* the concentration of GSH in the presence of DHA and GSSG yielded parabolic curves. These results indicated that GSSG acted as a competitive inhibitor with respect to DHA. The observed competitive inhibition by GSSG with respect to DHA is consistent with a mechanism in which DHA binds first to the enzyme.

The most plausible reaction mechanism that incorporates the results of our steady-state kinetic studies seems to be the bi uni uni uni ping-pong mechanism (Fig. 1). Our observations are inconsistent with the mechanism proposed for the other DHA-reducing proteins, such as pig liver thioltransferase and *Trypanosoma* p52 (Moutiez *et al*., 1997; Washburn *et al*., 1999b).

Detection of the reaction intermediate—Fig. 1 suggests the occurrence of an intermediate complex, in which DHAR and GSH are linked by a disulfide bond. To identify the intermediate complex, we performed the experiment as described in Materials and Methods. When DHAR was reacted with excess DHA prior to GSH, we detected GSH with a retention time of 7.4 min (Fig. 2). No material was detected with this retention time when the enzyme was reacted with GSH only (Fig. 2). The results indicate that the reduced form of DHAR reacted first with DHA and then the intermediate, namely, the cysteinyl-thiohemiketal complex, reacted with GSH to generate the oxidized form of the enzyme, in which DHAR and GSH were linked by a disulfide bond.

Identification of the cysteine residue involved in the reaction catalyzed by chloroplast DHAR—Our results (Fig. 1 and Shimaoka *et al.*, 2000) indicated that a cysteine residue was involved in the reaction catalyzed by DHAR. Spinach chloroplast DHAR contains three cysteine residues, namely, Cys-9, Cys-23 and Cys-26. We identified the cysteine residue that was involved in the DHAR-catalyzed reaction by measuring the activities of recombinant DHARs with variously mutated cysteine residues. C23S DHAR had almost no activity (Table 1). The k_{cat} of C26S was half of that of wild-type DHAR. The k_{cat} of C9S was slightly lower than that of wild-type DHAR. The respective K_m values for the two substrates of wild-type, C9S, C26S and C9S/C26S DHARs were almost identical. These results indicated that Cys-23 was essential for the DHAR-catalyzed reaction and suggested that this residue might form the disulfide bond with GSH in the cysteinyl-thiohemiketal complex and

the oxidized form of the enzyme during the DHAR-catalyzed reaction.

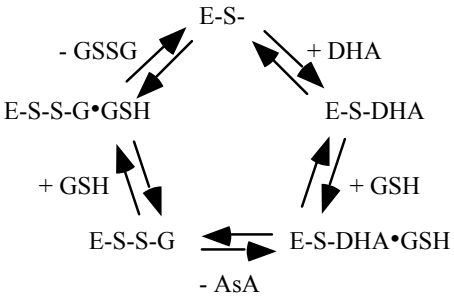

Fig. 1 Plausible reaction mechanism of catalysis by chloroplast DHAR.

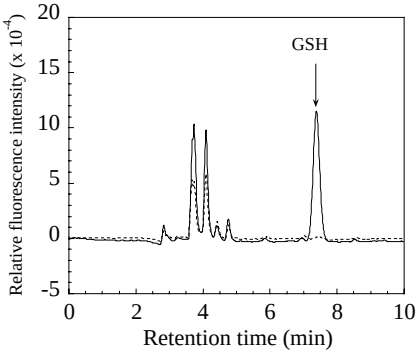

Fig. 2 Detection of DHAR bound GSH by HPLC. The reduce form of the enzyme was reacted with 1 mM DHA and 10 μM GSH (solid line) or 10 μM GSH (dashed line).

Table 1: Kinetic Parameters of wild-type and mutant forms of DHAR

	k_{cat} (s^{-1})	K_m for DHA (μM)	K_m for GSH (mM)
Wild-type	490 ± 40[*a]	53 ± 12	1.1 ± 0.5
C9S	420 ± 30	19 ± 14	0.95 ± 0.07
C23S	< 1	-	-
C26S	280 ± 30	26 ± 6	0.69 ± 0.2
C9/26S	210 ± 10	58 ± 7	1.1 ± 0.3

[*a] Standard deviation (N = 3-5).

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