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Crystallographic study of glutathione-dependent dehydroascorbate reductase from spinach chloroplasts

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Introduction

Ascorbate is the vital compound in both animal and plant cells. It functions as an electron donor in a variety of physiological processes. For example, ascorbate influences on many enzyme activities as a cofactor often by keeping metal ions associated with such enzymes in the reduced form. Ascorbate also reduces and scavenges many types of active oxygen species directly or enzymatically through peroxidase. Two-electron oxidation of ascorbate produces dehydroascorbate (DHA). Because DHA spontaneously decays through hydration, the net loss of ascorbate is anticipated in the absence of a mechanism to maintain high concentrations of ascorbate in the cell. This could be accomplished either through a direct chemical reduction of DHA by reduced glutathione (GSH) or through an enzymatic reduction (Rose & Bode, 1992).

Glutathione-dependent dehydroascorbate reductase (GSH-DHAR: EC 1.8.5.1) catalyzes the reduction of DHA to ascorbate using GSH as the electron donor. Although enzymatic reduction of DHA was detected seventy years ago (Szent-Györgyi, 1928), the extensive kinetic studies of purified enzymes were performed mainly from ’90s (Table 1). These studies revealed the following two things. First, intracellular GSH-DHAR activities were derived not only from a reaction of authentic GSH-DHAR but also from the side reactions of several the other enzymes; e.g., thioltransferase (glutaredoxin), protein disulfide isomerase (PDI), trypsin inhibitor, glutathione transferase (GST), and glutathione peroxidase (GPX). In fact the most studied GSH-DHAR activity is that of pig liver thioltransferase (Wells *et al.*, 1995). Its crystal structure (Katti, *et al.*, 1995) and reaction mechanisms (Washburn & Wells, 1999*b*) have also been reported. Secondly, the enzymes possessing GSH-DHAR activity, excluding selenoenzyme GPX, would commonly contain at least one reactive cysteine residue participating in the DHA reduction at the active site.

GSH-DHAR existing in chloroplasts has been considered to play a pivotal role to regenerate ascorbate which was oxidized in large quantities to scavenge active oxygen species generated in the process of photosynthesis (Asada, 1999). Very recently, we

isolated the most specific GSH-DHAR from spinach chloroplasts (Shimaoka, *et al.*, 2000) in the history of GSH-DHAR studies. For instance, the specificity constants for DHA and GSH of spinach chloroplast GSH-DHAR (V_{max} / K_m^{DHA} of 5.1 x 10^3 and V_{max} / K_m^{GSH} of 3.3 x 10^2 U / mg / m*M*) were approximately 40 and 35-fold higher than those of pig liver thioltransferase (V_{max} / K_m^{DHA} of 1.2 x 10^2 and V_{max} / K_m^{GSH} of 9.4 U / mg / m*M*), respectively. Primary structures and molecular masses were also very different between the two enzymes.

It is very intriguing how spinach chloroplast GSH-DHAR establishes its high specificities. The three-dimensional structure of spinach chloroplast GSH-DHAR will answer the question through comparing its active site structure with pig liver thioltransferase's one. In this paper, we report the first crystallization and preliminary crystallographic study of recombinant spinach chloroplast GSH-DHAR produced in *Escherichia coli*

Table 1. Enzymes possessing GSH-DHAR activity and their kinetic parameters. GSH-DHAR activity (V_{max}) was determined spectrophotometrically from the increase in ascorbate absorbance around 265 nm except values designated by †. It was measured indirectly following the oxidation of NADPH at 340 nm coupled to glutathione reductase. 1 unit (U) is 1 μmol / min. K_m^{DHA} and K_m^{GSH} are Michaelis constants for DHA and GSH, respectively.

Enzyme name	Origin	Preparation	Mass (kDa)	V_{max} (U / mg)	K_m^{DHA} (m*M*)	K_m^{GSH} (m*M*)	V_{max} / K_m^{DHA} (U / mg / m*M*)	V_{max} / K_m^{GSH} (U / mg / m*M*)	Reference
GSH-DHAR	spinach chloroplast	native	26	360	0.070	1.1	5.1×10^3	3.3×10^2	Shimaoka *et al.*, 2000
	spinach leaf	native	25	400	0.080	2.5	5.0×10^3	1.6×10^2	Shimaoka *et al.*, 2000
	spinach leaf	native	23	370	0.07	2.5	5×10^3	1.5×10^2	Hossain & Asada, 1984
	spinach leaf	native	25	5.60	0.34	4.43	1.6×10	1.26	Foyer & Halliwell, 1977
	rice bran cytosol	native	26	49.1	0.35	0.84	1.4×10^2	5.8×10	Kato *et al.*, 1997
	potato tuber	native	23	9.57	0.39	4.35	2.5×10	2.20	Dipierro & Boraccino, 1991
	rat liver	native	31	4.5	0.245	2.8	1.8×10	1.6	Maellaro *et al.*, 1994
	human erythrocyte	native	32	9.88	0.21	3.5	4.7×10	2.8	Xu *et al.*, 1996
thioltransferase	*Escherichia coli*	—	—	7.4	—	—	—	—	Trümper *et al.*, 1994
(glutaredoxin)	rice bran	native	11	91.7	—	—	—	—	Sha *et al.*, 1997
	pig liver	recombinant	11.7	32.0	0.26	3.4	1.2×10^2	9.4	Wells *et al.*, 1995
	human placenta (B)	native	12	103†	—	—	—	—	Padilla *et al.*, 1995
	human placenta	commercial	12	3.4	0.27	—	1.3×10	—	May *et al.*, 1997
	phage T4 (NrdC)	recombinant	11	0.176†	—	—	—	—	Gvakharia *et al.*, 1996
	phage T4 (Y55.7)	recombinant	12	0.121†	—	—	—	—	Gvakharia *et al.*, 1996
PDI	bovine liver	native	12	1.3	2.8	2.9	4.6×10^{-1}	4.5×10^{-1}	Wells *et al.*, 1995
	bovine liver	commercial	57	1.6	1.8	—	8.9×10^{-1}	—	May *et al.*, 1997
trypsin inhibitor	spinach chloroplast	native	38	1.3	1	7	1	2×10^{-1}	Trümper *et al.*, 1994
	soybean	commercial	21	0.25	—	—	—	—	Trümper *et al.*, 1994
GST	human	recombinant	56	0.16	—	—	—	—	Board *et al.*, 2000
GPX	bovine erythrocyte	commercial	22.6	6.19	4.1	2.0	1.5	3.1	Washburn & Wells, 1999*a*

Materials and methods

Recombinant spinach chloroplast GSH-DHAR was purified as described previously (Shimaoka *et al.*, 2000) with some modifications. *E. coli* cells overexpressing the enzyme were harvested, suspended in the extraction buffer (50 m*M* HEPES-KOH pH 7.6 at 4 ˚C, 20% (*v*/*v*) glycerol, 1 m*M* EDTA, 25 m*M* dithiothreitol, 1m*M* phenylmethylsulfonyl fluoride, and 10 μ*M* leupeptin), and sonicated. The sample was brought to 40% $(NH_4)_2SO_4$ and centrifuged at 20,000 x *g* for 20 min. The supernatant was applied to a column (2.6 cm i.d. x 40 cm) of butyl-Toyopearl (Tosoh, Tokyo, Japan) and eluted with a 40-0% $(NH_4)_2SO_4$ gradient in the elution buffer (the extraction buffer without leupeptin). The fractions containing GSH-DHAR activity were pooled and concentrated with a PM-10 membrane (Amicon, MA, USA). The sample was applied to a HiLoad™ 26/60 Superdex™ 75 prep grade (Amersham Pharmacia Biotech, Tokyo, Japan) and eluted with the elution buffer. The purified enzyme was frozen with liquid N_2 and stored at –80 ˚C until crystallization.

Frozen spinach chloroplast GSH-DHAR was thawed, buffer-exchanged, and concentrated to 50 mg/ml in 80 m*M* HEPES-KOH (pH 8.08 at 20 ˚C) containing 1 m*M* EDTA and 120 m*M* dithiothreitol using Centriprep-10 (Amicon). The amount of the enzyme was determined spectrophotometrically by using an extinction coefficient of 1.278 absorbance units for 1 mg/ml at 280 nm. A large number of crystallization conditions were analyzed using the preparation.

X-ray diffraction data were measured at room temperature using an R-AXIS IV imaging-plate detector with Cu $K\alpha$ radiation (λ = 1.5418 Å) produced by a Rigaku ultraX18 rotating-anode generator operated at 45 kV and 100 mA. The distance between a crystal and the detector and the crystal oscillation angle per image were set to 100 mm and 1˚, respectively. The data were processed with *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997).

Results and Discussion

The best crystals were grown at 4 ˚C by hanging-drop vapour-diffusion method. Drops consisted of 2 μl of 50 mg/ml enzyme solution with an equal volume of the well liquor (30% PEG4000, 80 m*M* sodium acetate pH 5.25 at 20 ˚C). Crystals took 3 – 6 days to appear and up to 7-10 days to grow to a suitable size for diffraction analysis (Fig. 1). Usually, the grown crystals were overlapped with each other. The crystals were separated with Micro-Tools (Hampton Research, CA, USA) and were mounted in glass capillary tubes. Then X-ray diffraction data collection and data reduction were performed. A summary of the data statistics is shown in Table 2.

Assuming two molecules of the GSH-DHAR in the asymmetric unit, the crystal volume per enzyme mass (V_m) and the solvent content were calculated to be 2.06 $Å^3$ Da^{-1} and 40.3%, respectively. These values are within the frequently observed ranges for protein crystals (Matthews, 1968). Preparation of heavy-atom derivatives for

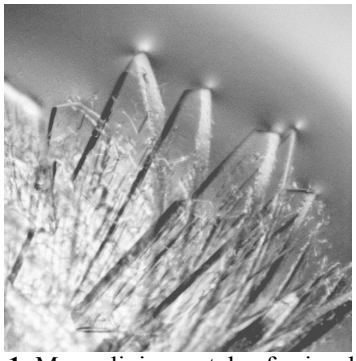

Fig.1. Monoclinic crystals of spinach chloroplast GSH-DHAR. The dimensions of the largest crystals are approximately 0.4 x 0.25 x 0.03 mm.

Table 2. Data collection statistics for spinach chloroplast GSH-DHAR.

Beam source and detector	Rigaku ultraX18 / R-AXIS IV
Wavelength (Å)	1.5418
Number of crystals and images	3 / 158
Space group	*C*2
Cell dimensions (Å, ˚)	*a* = 98.25, *b* = 39.96, *c* = 106.86, ß = 110.46
Resolution range (outer shell) (Å)	40 – 2.2 (2.28 – 2.2)
Measured reflections	154,384
Unique reflections	18,608
$I/\sigma(I)$	8.3
Completeness (outer shell) (%)	93.4 (92.3)
R_{merge} (outer shell) (%)	6.5 (24.0)

phase determination is in progress.

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