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Crystallization and preliminary X-ray diffraction analysis of glutathione-dependent dehydroascorbate reductase from spinach chloroplasts

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Glutathione-dependent dehydroascorbate reductase (GSH-DHAR) catalyzes the reduction of dehydroascorbate to ascorbate using reduced glutathione as the electron donor. GSH-DHAR from spinach chloroplasts produced in *Escherichia coli* was crystallized by the hanging-drop vapour-diffusion method. The crystals were monoclinic, space group $C2$, with unit-cell parameters a = 98.25, b = 39.96, c = 106.86 Å, β = 110.46°. The asymmetric unit contained two molecules, giving a crystal volume per enzyme mass (V_M) of 2.06 Å^3 Da^{-1} and a solvent content of 40.3%. A full set of X-ray diffraction data were collected to 2.2 Å Bragg spacing from three native crystals with an overall R_{merge} of 6.5% and a completeness of 93.4%.

1. Introduction

Ascorbate is a vital compound in both animal and plant cells. It functions as an electron donor in a variety of physiological processes. For example, ascorbate influences many enzyme activities as a cofactor, often by keeping metal ions associated with 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, a 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 direct chemical reduction of DHA by reduced glutathione (GSH) or through an enzymatic reduction (Rose & Bode, 1992).

Glutathione-dependent dehydroascorbate reductase (GSH-DHAR: E.C. 1.8.5.1) catalyzes the reduction of DHA to ascorbate using GSH as the electron donor. Although enzymatic reduction of DHA was detected 70 y ago (Szent-Györgyi, 1928), extensive kinetic studies of purified enzymes were not performed until the 1990s (Table 1). These studies revealed the following two things. Firstly, intracellular GSH-DHAR activities were derived not only from a reaction of authentic GSH-DHAR, but also from the side reactions of several other enzymes, *e.g.* thioltransferase (glutaredoxin), protein disulfide isomerase (PDI), trypsin inhibitor, glutathione transferase (GST) and glutathione peroxidase (GPX). 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 been reported. Secondly, the enzymes possessing GSH-DHAR activity, with the exception of selenoenzyme GPX, 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 in the regeneration of ascorbate, which is oxidized in large quantities to scavenge active oxygen species generated in the process of photosynthesis (Asada, 1999). Very recently, we isolated a highly specific GSH-DHAR from spinach chloroplasts (Shimaoka *et al.*, 2000). The specificity constants for DHA and GSH of spinach chloroplast GSH-DHAR (V_{max}/K_m^{DHA} of 5.1 × 10^3 and V_{max}/K_m^{GSH} of 3.3 × 10^2 U mg^{-1} mM^{-1}) were approximately 40- and 35-fold higher than those of pig liver thioltransferase (V_{max}/K_m^{DHA} of 1.2 × 10^2 and V_{max}/K_m^{GSH} of 9.4 U mg^{-1} mM^{-1}), 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 this question *via* the comparison of its active-site structure with that of pig liver thioltransferase. In this paper, we report the first crystallization and preliminary crystallographic study of recombinant spinach chloroplast GSH-DHAR produced in *E. coli*.

2. Purification

Recombinant spinach chloroplast GSH-DHAR was purified as described previously

Table 1
Enzymes possessing GSH-DHAR activity and their characteristics.

GSH-DHAR activity (V_{max}) was determined spectrophotometrically from the increase in ascorbate absorbance around 265 nm except for 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^{-1}. K_m^{DHA} and K_m^{GSH} are Michaelis constants for DHA and GSH, respectively.

Enzyme name	Origin	Preparation	Identity (%)	Mass (kDa)	V_{max} (U mg^{-1})	K_m^{DHA} (mM)	K_m^{GSH} (mM)	V_{max}/K_m^{DHA} (U mg^{-1} mM^{-1})	V_{max}/K_m^{GSH} (U mg^{-1} mM^{-1})	Reference
GSH-DHAR	Spinach chloroplast	Native	100	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	56.5	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	37.9	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	*E. coli*	—	—	—	7.4	—	—	—	—	Trümper *et al.* (1994)
(glutaredoxin)	Rice bran	Native	27.1	11	91.7	—	—	—	—	Sha *et al.* (1997)
	Pig liver	Recombinant	24.8	11.7	32.0	0.26	3.4	1.2×10^2	9.4	Wells *et al.* (1995)
	Human placenta (B)	Native	25.2	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	22.0	11	0.176*	—	—	—	—	Gvakharia *et al.* (1996)
	Phage T4 (Y55.7)	Recombinant	27.1	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	31.8	21	0.25	—	—	—	—	Trümper *et al.* (1994)
GST	Human	Recombinant	37.3	56	0.16	—	—	—	—	Board *et al.* (2000)
GPX	Bovine erythrocyte	Commercial	35.0	22.6	6.19	4.1	2.0	1.5	3.1	Washburn & Wells (1999*a*)

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

Values in parentheses are for the outer resolution shell.

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, β = 110.46
Resolution range (Å)	40–2.2 (2.28–2.2)
Measured reflections	154384
Unique reflections	18608
$I/\sigma(I)$	8.3
Completeness (%)	93.4 (92.3)
R_{merge} (%)	6.5 (24.0)

(Shimaoka *et al.*, 2000) with some modifications. *E. coli* cells overexpressing the enzyme were harvested, suspended in the extraction buffer [50 mM HEPES–KOH pH 7.6 at 277 K, 20%(*v*/*v*) glycerol, 1 mM EDTA, 25 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 10 µM leupeptin] and sonicated. The sample was brought to 40% $(NH_4)_2SO_4$ and centrifuged at 20 000*g* for 20 min. The supernatant was applied to a column (2.6 × 40 cm) of butyl-Toyopearl (Tosoh, Tokyo, Japan) and eluted with a 40–0% $(NH_4)_2SO_4$ gradient in 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) column and eluted with the elution buffer. The purified enzyme was frozen with liquid N_2 and stored at 193 K until crystallization.

3. Crystallization

Frozen spinach chloroplast GSH-DHAR was thawed, buffer-exchanged and concentrated to 50 mg ml^{-1} in 80 mM HEPES–KOH (pH 8.08 at 293 K) containing 1 mM EDTA and 120 mM dithiothreitol using a Centriprep-10 (Amicon). The amount of the enzyme was determined spectrophotometrically using an extinction coefficient of 1.278 absorbance units for 1 mg ml^{-1} at 280 nm. Crystals were grown at 277 K by the hanging-drop vapour-diffusion method. Drops consisted of 2 µl of 50 mg ml^{-1} enzyme solution with an equal volume of the well liquor (30% PEG 4000, 80 mM sodium acetate pH 5.25 at 293 K). Crystals took 3–6 d to appear and up to 7–10 d to grow to a suitable size for diffraction analysis (Fig. 1). Usually, crystals grew overlapping with each other.

4. Data collection and analysis

The overlapping crystals were separated with Micro-Tools (Hampton Research, CA, USA) and were mounted in glass capillary tubes. 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 crystal-to-detector distance 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). 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 phase determination is in progress.

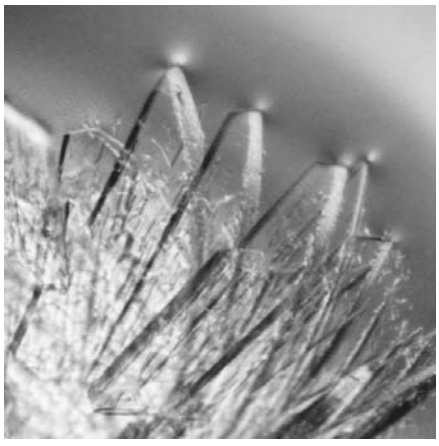

Figure 1
Monoclinic crystals of spinach chloroplast GSH-DHAR. The dimensions of the largest crystals are approximately 0.4 × 0.25 × 0.03 mm.

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