Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic analysis of monodehydroascorbate radical reductase from cucumber

Satoshi Sano,[a] You-Na Kang,[b] Hiroko Shigemizu,[a] Nobuhiko Morishita,[a] Hye-Jin Yoon,[c] Kazumi Saito,[a] Kozi Asada[d] and Bunzo Mikami[b]*

[a]Laboratory of Chemistry of Biological Function, Graduate School of Agriculture, Kyoto Prefectural University, Shimogamo, Sakyo, Kyoto 606-8522, Japan, [b]Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan, [c]Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea, and [d]Department of Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University, Fukuyama, Hiroshima 702-0292, Japan

Correspondence e-mail: mikami@kais.kyoto-u.ac.jp

Received 10 May 2004
Accepted 17 June 2004

Monodehydroascorbate (MDA) radical reductase (EC 1.6.5.4) is an FAD enzyme that catalyzes the univalent reduction of MDA radical to ascorbate using NAD(P)H as an electron donor. The recombinant MDA reductase from cucumber was crystallized using polyethylene glycol 6000 as a precipitant. The crystals belong to space group $P2_1$, with unit-cell parameters a = 60.8, b = 138.6, c = 61.7 Å, β = 114.5°, and contained two molecules per asymmetric unit. The Matthews coefficient (V_M) and the solvent content are 2.46 Å^3 Da^{-1} and 50.0%, respectively. Diffraction data were collected to a resolution of 2.4 Å at 100 K using Cu $K\alpha$ radiation with a multi-wire area detector and gave a data set with an overall R_{sym} of 10.0% and a completeness of 92.5%.

1. Introduction

Ascorbate (AsA) plays a central function in the protection of eukaryotes from oxidative stress. When AsA acts as an antioxidant in cells, in most cases monodehydroascorbate (MDA) radical is produced as the primary oxidation product. In plants, the reaction catalyzed by AsA peroxidase is a major source of MDA radicals, which scavenges hydrogen peroxide (Hossain *et al.*, 1984). AsA oxidase catalyzes the univalent oxidation of AsA to MDA by dioxygen, especially in plants of the Cucurbitaceae family. Superoxide and hydroxyl radicals directly oxidize AsA to MDA; other radicals such as tocopherol chromanoxy, carbon-centred, aminoxy, peroxy, phenoxy and thiyl radicals also generate MDA (Bielski, 1982; Forni *et al.*, 1983). Thus, MDA is a 'sink' for radicals generated in eukaryotic cells. To maintain the antioxidant activity of AsA, the regeneration of AsA from MDA radical is indispensable.

MDA radical reductase (EC 1.6.5.4) is an FAD enzyme that catalyzes the reduction of MDA radical to AsA using NAD(P)H as an electron donor (Hossain *et al.*, 1984). It is the only enzyme known to use an organic radical as an enzyme substrate. The molecular size of cytosolic MDA reductase from cucumber is approximately 47.3 kDa and the enzyme is composed of 433 amino-acid residues, as deduced from its cDNA (Sano & Asada, 1994). From its amino-acid composition, the pI is calculated to be 5.6. Its amino-acid sequence contains the putative FAD and pyridine nucleotide-binding motifs of flavoenzymes. However, MDA reductase exhibits a high sequence identity with bacterial flavoenzymes such as iron–sulfur protein reductase rather than plant flavoenzymes such as ferredoxin–NADP reductase (Sano & Asada, 1994). A homology search against the PDB indicated that putidaredoxin reductase from *Pseudomonas putida* (PDB codes 1q1r and 1q1w) and ferredoxin reductase from *Pseudomonas* sp. (PDB code 1f3p) give the highest homology score in a BLAST search, exhibiting sequence identities of 27.2 and 27.3% with E values of 1×10^{-28} and 6×10^{-24}, respectively.

The reaction kinetics of MDA reductase show that the reaction proceeds *via* a ping-pong mechanism (Hossain & Asada, 1985) as follows:

$$E\text{-FAD} + \text{NAD(P)H} + \text{H}^+ \rightarrow E\text{-FADH}_2\text{-NAD(P)}^+, \quad (1)$$

$$E\text{-FADH}_2\text{-NAD(P)}^+ + \text{MDA} \rightarrow E\text{-FAD}^{\cdot}\text{-NAD(P)}^+ + \text{AsA}, \quad (2)$$

$$E\text{-FAD}^{\cdot}\text{-NAD(P)}^+ + \text{MDA} \rightarrow E\text{-FAD} + \text{AsA} + \text{NAD(P)}^+, \quad (3)$$

where, E-FAD, E-FADH$_2$-NAD(P)$^+$ and E-FAD$^{\cdot}$-NAD(P)$^+$ represent the oxidized form, the reduced charge-transfer complex and the semiquinone form of MDA reductase, respectively.

MDA reductase can also reduce phenoxyl radicals to their respective parent phenols *via* a mechanism similar to the reduction of MDA (Sakihama *et al.*, 2000).

The activity of MDA reductase is inhibited by thiol-modifying reagents at the step of the reduction of FAD by NADH, producing a charge-transfer complex (equation 1). The rate constant of this step ($1.2 \times 10^8\ M^{-1}\ s^{-1}$) is larger than that of a diffusion-controlled reaction assuming a simple collision of the enzyme-bound FAD with MDA ($2.5 \times$

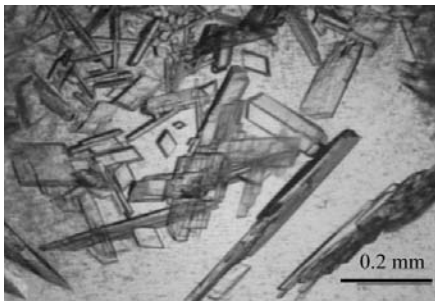

Figure 1
Crystals of MDA radical reductase. The crystal has approximate dimensions of 0.23 × 0.08 × 0.01 mm.

$10^6\ M^{-1}\ s^{-1}$; Sano *et al.*, 1995). The reduced FAD also donates electrons to MDA with a similar rate constant, $3 \times 10^8\ M^{-1}\ s^{-1}$ (Kobayashi *et al.*, 1995). The rate of the whole reaction decreases with an increase in ionic strength, suggesting that the rapid reaction is facilitated by electrostatic interaction between the enzyme and substrates. To understand the reaction mechanism of this characteristic flavoenzyme, its three-dimensional structure is indispensable, and we report here the crystallization and preliminary crystallographic analysis of the recombinant MDA reductase of cucumber produced in *Escherichia coli*.

2. Crystallization

The cDNA of the MDA reductase from cucumber was cloned (Sano & Asada, 1994) and the recombinant protein was over-expressed and purified as described previously (Sano & Asada, 1994). The MDA reductase was further purified on a Mono Q ion-exchange column (Amersham). The purified enzyme exhibited a single band on SDS–PAGE and a single peak on gel filtration that corresponded to the peak position of the monomer, suggesting that MDA reductase exists mainly as a monomer in solution. The purified enzyme was crystallized immediately after purification by using the hanging-drop vapour-diffusion method. O_2 was not excluded from the crystallization trials. All drops were prepared by mixing 5 µl protein solution at 10 mg ml^{-1} with 5 µl reservoir solution containing 20–25% polyethylene glycol (PEG) 6000, 0.1 *M* $CaCl_2$ in 0.05 *M* Tris–HCl buffer pH 8.0. Droplets were placed on siliconized cover slips and equilibrated against 1 ml reservoir solution at 293 K. Yellow-coloured crystals with a prismatic shape appeared within a week and reached maximum dimensions of 0.23 × 0.08 × 0.01 mm (Fig. 1).

Table 1
Data-collection statistics for MDA reductase.

Statistics for the highest resolution shell are given in parentheses.

X-ray source	Cu $K\alpha$
Detector	Bruker Hi-star
Wavelength (Å)	1.5418
Crystal-to-detector distance (cm)	15.0
$\Delta\varphi$ per frame (°)	0.25
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 60.8, b = 138.6, c = 61.7, β = 114.5
Resolution range (Å)	46.3–2.4 (2.5–2.4)
Measured reflections	104718 (3821)
Unique reflections	33585 (2729)
Multiplicity	3.1
Completeness (%)	92.5 (75.5)
R_{sym} (%)	10.0 (28.0)
$I/\sigma(I)$	8.7 (2.8)

3. Data collection

A preliminary diffraction data set was collected with Cu $K\alpha$ radiation (λ = 1.5418 Å) using a Bruker Hi-Star area detector coupled to a MAC Science M18XHF rotating-anode generator operating at 45 kV and 90 mA. The crystal diffracted to 2.4 Å resolution. After cryoprotection had been achieved by soaking the crystal in a buffer consisting of 0.05 *M* Tris–HCl pH 8.0, 0.1 *M* $CaCl_2$, 25%(*w*/*v*) PEG 6000 and 30%(*v*/*v*) glycerol, data were collected at 100 K by the standard oscillation method using a crystal-to-detector distance of 150 mm. Images were collected in 0.25° increments with an exposure time of 1 min per image over a total of 360°. The collected diffraction data were processed with the *SADIE* and *SAINT* software packages (Bruker). Data-collection statistics are summarized in Table 1.

4. Results

The crystals of MDA reductase belonged to space group $P2_1$, with unit-cell parameters a = 60.8, b = 138.6, c = 61.7 Å, β = 114.5°. The asymmetric unit presumably contains two molecules, each with a molecular weight of 47.3 kDa. This corresponds to a solvent content of 50.02% and a V_M value of 2.46 Å^3 Da^{-1}, which is within the range usually observed in protein crystals (Matthews, 1968). A total of 104 718 reflections (33 585 unique reflections) were collected with 92.5% completeness and an R_{sym} of 10.0% to 2.4 Å resolution. Structure determination of MDA reductase using heavy-atom derivatives and molecular-replacement methods using the coordinates of putidaredoxin reductase from *P. putida* (PDB code 1q1r) and the ferredoxin reductase (BphA4)–NADH complex from *Pseudomonas* sp. strain KKS102 (PDB code 1f3p; Senda *et al.*, 2000) as search models is currently under way.

References

Bielski, B. (1982). *Ascorbic Acid: Chemistry, Metabolism and Uses*, edited by P. A. Seib & B. M. Tolbert, pp. 81–100. Washington, DC: American Chemical Society.

Forni, L. G., Mönig, J., Mora-Arellano, V. O. & Wilson, R. L. (1983). *J. Chem. Soc. Perkin Trans. 2*, pp. 961–965.

Hossain, M. A. & Asada, K. (1985). *J. Biol. Chem.* **260**, 12920–12926.

Hossain, M. A., Nakano, Y. & Asada, K. (1984). *Plant Cell Physiol.* **25**, 385–395.

Kobayashi, K., Tagawa, S., Sano, S. & Asada, K. (1995). *J. Biol. Chem.* **270**, 27551–27554.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Sakihama, Y., Mano, J., Sano, S., Asada, K. & Yamasaki, H. (2000). *Biochem. Biophys. Res. Commun.* **279**, 949–954.

Sano, S. & Asada, K. (1994). *Plant Cell Physiol.* **35**, 425–437.

Sano, S., Miyake, C., Mikami, B. & Asada, K. (1995). *J. Biol. Chem.* **270**, 21354–21361.

Senda, T., Yamada, T., Sakurai, N., Kubota, M., Nishizaki, T., Masai, E., Fukuda, M. & Mitsui, Y. (2000). *J. Mol. Biol.* **304**, 397–410.