Plant Physiology and Biochemistry 120 (2017) 144–155

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Enzymatic characterization and crystal structure analysis of *Chlamydomonas reinhardtii* dehydroascorbate reductase and their implications for oxidative stress

Hsin-Yang Chang [a, b, c, *], Shu-Tseng Lin [a, b], Tzu-Ping Ko [d], Shu-Mei Wu [a, b], Tsen-Hung Lin [a, b], Yu-Ching Chang [a, b], Kai-Fa Huang [d], Tse-Min Lee [a, b, c, **]

[a] Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[b] The Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[c] Doctoral Degree Program in Marine Biotechnology, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[d] Institute of Biological Chemistry, Academia Sinica, Taipei 11529, Taiwan

ARTICLE INFO

Article history:
Received 17 May 2017
Received in revised form
21 September 2017
Accepted 29 September 2017
Available online 3 October 2017

Keywords:
Chlamydomonas
Dehydroascorbate reductase
X-ray crystallography
Enzyme kinetics
Site-directed mutagenesis
Oxidative stress

ABSTRACT

Dehydroascorbate reductase (DHAR) is a key enzyme for glutathione (GSH)-dependent reduction of dehydroascorbate (DHA) to recycled ascorbate (AsA) in plants, and plays a major role against the toxicity of reactive oxygen species (ROS). Previously, we proposed that the increase of AsA regeneration via enhanced DHAR activity modulates the ascorbate-glutathione cycle activity against photooxidative stress in *Chlamydomonas reinhardtii*. In the present work, we use site-directed mutagenesis and crystal structure analysis to elucidate the molecular basis of how *C. reinhardtii* DHAR (CrDHAR1) is involved in the detoxification mechanisms. Mutagenesis data show that the D21A, D21N and C22A mutations result in severe loss of the enzyme's function, suggesting crucial roles of Asp-21 and Cys-22 in substrate binding and catalysis. The mutant K11A also exhibits reduced redox activity (~50%). The crystal structure of apo CrDHAR1 further provides insights into the proposed mechanism centering on the strictly conserved Cys-22, which is suggested to initiate the redox reactions of DHA and GSH. Furthermore, *in vitro* oxidation of the recombinant CrDHAR1 in the presence of 1 mM H_2O_2 has minor effects on the K_m for the substrates but significantly reduces the k_{cat}. The enzyme's activity and its mRNA abundance in the *C. reinhardtii* cells are increased by treatment with 0.2–1 mM H_2O_2 but decreased when H_2O_2 is $\geq$ 1.5 mM. The latter decrease is accompanied by oxidative damage and lower AsA concentrations. These biochemical and physiological data provide new insights into the catalytic mechanism of CrDHAR1, which protects the *C. reinhardtii* cells from oxidative stress-induced toxicity.

© 2017 Elsevier Masson SAS. All rights reserved.

1. Introduction

Plants have developed a number of defense mechanisms against the toxicity of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$) (Halliwell and Gutteridge, 1989). The ascorbate-glutathione cycle is fundamental for plants in the recycling of antioxidants and in detoxifying H_2O_2 using antioxidative enzymes (Halliwell and Gutteridge, 2007; Mittler, 2002). H_2O_2 can be scavenged by ascorbate peroxidase (APX; EC 1.11.1.11) using ascorbate (AsA) as the substrate, and then ascorbate can be oxidized to monodehydroascorbate (MDA). MDA reductase (MDAR; EC 1.6.5.4) can catalytically recycle MDA back to AsA using NADPH as the reductant (Gill and Tuteja, 2010). MDA disproportionates into dehydroascorbate (DHA) and AsA. DHA is then reduced to AsA by DHA reductase (DHAR; EC 1.8.5.1), using glutathione (GSH) as the reducing agent. The regeneration of AsA through MDAR or DHAR is critical to plants for their adaptation to changing environmental conditions (Gallie, 2013a,b). Murik et al. (2014) previously suggested that excess DHA could be the agent responsible for the induction of programmed cell death (PCD) in *C. reinhardtii* under

* Corresponding author. Department of Marine Biotechnology and Resources, National Sun Yat-sen University, No. 70 Lienhai Rd., Kaohsiung 80424, Taiwan.
** Corresponding author. Department of Marine Biotechnology and Resources, National Sun Yat-sen University, No. 70 Lienhai Rd., Kaohsiung 80424, Taiwan.
E-mail addresses: hychang@mail.nsysu.edu.tw (H.-Y. Chang), tmlee@mail.nsysu.edu.tw (T.-M. Lee).

https://doi.org/10.1016/j.plaphy.2017.09.026
0981-9428/© 2017 Elsevier Masson SAS. All rights reserved.

H_2O_2-induced oxidative stress. It has been reported that a change in DHAR activity and/or gene expression results in a change in AsA-recycling ability and oxidative tolerance (Chen and Gallie, 2005; Wang et al., 2010). Our recent study further showed that DHAR overexpression in *Chlamydomonas reinhardtii* not only increases the AsA regeneration via enhanced DHAR activity but also improves the organism's tolerance against photooxidative stress (1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and oxidative stress (3 and 6 mM H_2O_2) (Lin et al., 2016). These findings suggest that the DHAR responsible for the recycling of DHA back to AsA is critical for *C. reinhardtii* cells to cope with oxidative stress. Until now, however, the basic enzymatic characteristics of *C. reinhardtii* DHAR, such as its catalytic mechanism and substrate specificity, have not been well studied.

It is generally believed that the DHA-reducing enzymes possess a CxxC/S motif located in their catalytic domain. Site-directed mutagenesis analysis indicates that the first cysteine residue of this motif in DHAR from spinach, rice and poplar is essential for the reduction of the DHA substrate (Shimaoka et al., 2003; Tang and Yang, 2013; Do et al., 2016). Previous work on the identification and enzymatic characterization of DHARs also showed that the enzymes from different species exhibit quite different catalytic activities although they all share high sequence identity. The peroxisome-localized DHAR from *Arabidopsis* (AtDHAR1) shows a high enzymatic activity of 936 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (Rahantaniaina et al., 2013), but its cytosol-localized DHAR (AtDHAR3) and the enzyme from rice have much lower activity, 120 and 49 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively (Rahantaniaina et al., 2013; Kato et al., 1997). Such differences in the biochemical characteristics among various DHA-reducing enzymes may be due to their functional divergence in catalytic mechanism during the redox cycling of each substrate from different plant cell organelles and species. Despite their physiological importance, the structural information on DHARs was not clear until several breakthrough studies on the three-dimensional (3D) structure of DHARs were recently reported (Do et al., 2016; Lallement et al., 2016; Krishna Das et al., 2016; Bodra et al., 2017). Do et al. (2016) and Bodra et al. (2017) have demonstrated the location of the DHA- and GSH-binding sites as well as the surrounding catalytic residues, which might serve as a prototype for studying other eukaryotic DHARs.

Although the induction of CrDHAR1 activity, the mRNA abundance of *CrDHAR1* (Cre10.g456750, *Chlamydomonas* v5.5, Phytozome) in response to oxidative stress, and the improvement of oxidative tolerance in CrDHAR1-overexpressing *C. reinhardtii* cells have been reported (Murik et al., 2014; Urzica et al., 2012), to the best of our knowledge, the structure-based catalytic mechanism and its relation to oxidative stress have not yet been examined in the green algae. In our previous study, we cloned *CrDHAR1* from the model green alga *C. reinhardtii* for the preparation of recombinant CrDHAR1 protein, which was overexpressed using *E. coli* as a host. We found that the recombinant CrDHAR1 possessed activities of 965 and 940 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein against DHA and GSH (DHA k_{cat} = 400.43 s^{-1} and GSH k_{cat} = 390.06 s^{-1}), respectively, and its affinity (K_m) for DHA and GSH was 0.09 and 0.27 mM, respectively (Lin et al., 2016). In the present study, we determined the enzymatic characteristics of the recombinant CrDHAR1 and its crystal structure at 2.46 Å resolution. A structural model of the enzyme-substrate complex of CrDHAR1 is also proposed to rationally explain the changes in enzyme kinetics produced by wild-type and mutant enzymes. The role of the catalytic amino acids in the regulation of enzyme activity during substrate redox processes and the susceptibility of these amino acids to H_2O_2 are also discussed. Finally, H_2O_2 was exogenously applied to the recombinant CrDHAR1 to investigate the modification of the characteristics of CrDHAR1 using *in vitro* experiments. The *in vivo* effect of H_2O_2 on CrDHAR1 activity in relation to its mRNA abundance, on AsA recycling (AsA/DHA ratio), and on oxidative stress in *C. reinhardtii* (at various H_2O_2 concentrations) was also determined and compared to the results of the *in vitro* experiments.

2. Material and methods

2.1. Crystallization and X-ray data collection

For the expression and purification of recombinant CrDHAR1 enzymes, we followed our previously reported protocol of using nickel-nitrilotriacetic acid resin and size exclusion chromatography (Lin et al., 2016). For the crystallization of the recombinant enzyme, we failed to obtain crystals using the full-length CrDHAR1 (amino acids 1–226), CrDHAR1ΔN (amino acids 5-226) or CrDHAR1ΔC (amino acids 1-218) in Tris-NaCl buffer, so we tried to grow crystals of a variant with both the N- and C-termini truncated, denoted CrDHAR1ΔNΔC (amino acids 5-218) (Fig. 3B). The initial crystallization screening produced sitting drops with ~1000 different conditions. The crystals obtained from initial conditions were further refined manually. Finally, one crystallization condition was selected, 0.1 M Tris-HCl, pH 8.0, with 2 M ammonium sulfate. The crystals were grown at 20 °C by mixing the CrDHAR1ΔNΔC solution with an equal volume of crystallization buffer via the sitting-drop vapor-diffusion method. The rod-shaped crystals appeared after one day and grew to approximate dimensions of 0.3 × 0.05 × 0.05 mm within five days (Fig. 3C). The X-ray high-resolution data were collected in the Core Facilities for Protein Structural Analysis (CFPSA) with the protein X-ray diffraction system FR-E+ & R-AXIS HTC at the Academia Sinica (Taipei, Taiwan) and at beamlines 13C or TPS-05A of the National Synchrotron Radiation Research Center (Hsinchu, Taiwan). Before being mounted on the goniometer, the crystals were cryoprotected by the addition of 25% (v/v) glycerol. All diffraction data were processed and scaled with the HKL-2000 package (Otwinowski and Minor, 1997). The data collection statistics are listed in Table 1. The space group of the crystals is P 4_1 2_1 2, with the typical unit cell dimensions of a = b = 104.6 Å, c = 47.7 Å, $\alpha = 90.0°$, $\beta = 90.0°$, and $\gamma = 90.0°$. The asymmetric unit comprises a CrDHAR1ΔNΔC monomer with an estimated solvent content of 54.53%.

2.2. Structure determination and refinement

The crystal structure of CrDHAR1 was solved using the molecular-replacement method with the program MOLREP (Vagin and Teplyakov, 2010) using the structure of OsDHAR (reduced wild-type OsDHAR, 5D9T) as the search model. The initial model building and further iterative structural modifications were carried out in COOT (Emsley and Cowtan, 2004). The resulting model was subjected to computational refinement with the program REFMAC5 (Murshudov et al., 2011). A randomly selected 5% of the data was set aside as a free dataset, and the model was refined against the remaining data with F > 0 as a working dataset throughout refinement. Subsequently, several rounds of model adjustment with COOT and refinement with PHENIX were performed using a 2.46 Å resolution dataset to improve the quality and completeness of the structure (Adams et al., 2010). The well-ordered water molecules were located with COOT. Finally, the refinement converged at a final R factor and R_{free} of 0.189 and 0.222, respectively, in the 29.2–2.46 Å resolution range. The final refinement statistics are listed in Table 1. The molecular figures were produced with PyMOL (Schrödinger, New York, USA).

Download English Version:

https://daneshyari.com/en/article/5515275

Download Persian Version:

https://daneshyari.com/article/5515275

Daneshyari.com