Structure and catalytic mechanism of monodehydroascorbate reductase, MDHAR, from Oryza sativa L. japonica

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Ascorbic acid (AsA) maintains redox homeostasis by scavenging reactive oxygen species from prokaryotes to eukaryotes, especially plants. The enzyme monodehydroascorbate reductase (MDHAR) regenerates AsA by catalysing the reduction of monodehydroascorbate, using NADH or NADPH as an electron donor. The detailed recycling mechanism of MDHAR remains unclear due to lack of structural information. Here, we present the crystal structures of MDHAR in the presence of cofactors, nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+), and complexed with AsA as well as its analogue, isoascorbic acid (ISD). The overall structure of MDHAR is similar to other iron-sulphur protein reductases, except for a unique long loop of 63–80 residues, which seems to be essential in forming the active site pocket. From the structural analysis and structure-guided point mutations, we found that the Arg320 residue plays a major substrate binding role, and the Tyr349 residue mediates electron transfer from NAD(P)H to bound substrate via FAD. The enzymatic activity of MDHAR favours NADH as an electron donor over NADPH. Our results show, for the first time, structural insights into this preference. The MDHAR-ISD complex structure revealed an alternative binding conformation of ISD, compared with the MDHAR-AsA complex. This implies a broad substrate (antioxidant) specificity and resulting greater protective ability of MDHAR.

Reactive oxygen species (ROS) are produced as a normal by-product of aerobic metabolism in living organisms. In addition, various environmental stress conditions including the light/dark cycle, nutrient depletion/excess, high salinity, drought, flooding, extreme temperatures, heavy metals, and UV irradiation are also known to accelerate the accumulation of ROS in plant cells, ultimately leading to cell death1,2. Therefore, ROS levels must be tightly regulated in order to prevent oxidative damage of cellular components. In plant cells, ascorbic acid (AsA) is a major antioxidant, which is a part of the AsA-glutathione (GSH) cycle3,4. In order to maintain the antioxidative capacity of AsA, rapid regeneration of AsA is regulated by dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR)1,5,6. Activation of DHAR and MDHAR in these stress responses has been observed in different plants. In transgenic tobacco, overexpression of DHAR protected the plants against drought and ozone stress, whereas high expression of MDHAR increased their tolerance to osmotic stress7,8. In addition, an increase in MDHAR activity resulted in increased tolerance to chilling stress in tomato9,10.

The first reports of the crystal structure of DHAR of Oryza sativa (Os) L. japonica (OsDHAR) suggested that regeneration of AsA by DHAR proceeded via a ping-pong mechanism11. The OsDHAR structure revealed that the Cys20 residue was involved in the reduction of dehydroascorbate (DHA) to AsA. The catalytic cysteine residue is

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Reduced back from sulphenic to sulphonic acid by catalytic cycling via glutaredoxin\(^{11}\). Therefore, regeneration of cysteine residue is required for continued AsA-GSH cycle. However, the catalytic cysteine residue can be irreversibly oxidized to sulphonic acid by hyperoxidation, which inactivates the enzyme. Thus, the role of MDHAR in the AsA regeneration cycle may be more efficient than that of DHAR under conditions of strong oxidative stress. This prediction was supported by the results of an in vivo assay using yeast cells in this study.

We report, for the first time, the crystal structure of MDHAR of *O. sativa* *L.* (*OsMDHAR*) to understand its detailed mechanism. MDHAR reduces the monodehydroascorbate (MDHA) radical to AsA at the expense of an NAD(P)H molecule, with NADH being the preferred donor\(^{5,12}\). The deduced amino acid sequence of MDHAR indicates that it consists of flavin adenine dinucleotide (FAD) and the pyridine nucleotide-binding domain of flavoenzymes. Multiple sequence analysis of MDHAR shows that it shares high sequence similarity with bacterial flavoenzymes, such as iron-sulphur protein reductase, instead of plant flavoenzymes such as ferredoxin-NADP reductase\(^{13}\). Consistently, the overall structure of OsMDHAR is also similar to iron-sulphur protein reductase. However, OsMDHAR has a unique long loop that distinguishes it from the iron-sulphur reductase family.

In a previous kinetic study, Hossain *et al.* suggested that NAD(P)H reduces FAD in MDHAR. The reduced FAD successively donates electrons to MDHA, producing AsA\(^{14}\). The structural information from OsMDHAR, as well as the structures complexed with NAD, NADP, and both NAD and AsA, allows us to elucidate a detailed description of the catalytic mechanism at a molecular level. Structure-guided point mutations indicate that Arg320 and Tyr349 residues are critical for the activity of OsMDHAR. We determined the role of both these residues using structural analysis. Additionally, structural comparison of the OsMDHAR complex with NAD and NADP suggested that the preference for NADH as an electron donor might arise from additional hydrogen bonding near the AMP-ribose of NADH. The quaternary structure of OsMDHAR complexed with isoascorbic acid (ISD), an AsA analogue, suggests that the enzyme can accommodate multiple substrates\(^{15}\).

**Results and Discussion**

**Overall structure of OsMDHAR.** In order to elucidate the electron transfer mechanism employed by OsMDHAR, we determined the crystal structure of OsMDHAR and complexes of the enzyme with NAD and NADP. We also determined quaternary complex structures with FAD, NAD, and AsA or ISD. All four structures contain one molecule of FAD per monomer, which imparts a yellow colour to the crystals. Except for the crystals of apo OsMDHAR and the OsMDHAR-NAD complex, which have one molecule in the asymmetric unit of the crystals, there were two molecules in the asymmetric unit of the crystals. In a size-exclusion chromatography experiment, the purified OsMDHAR protein was eluted as a monomer (Fig. S1), implying that it possibly functions as a monomer.

Structural comparison of OsMDHAR to other proteins, using the DALI server\(^{16}\), indicated that it has the same fold as that of putidaredoxin reductase (Pdr)\(^{17}\), biphenyl dioxygenase (BphA4)\(^{18}\) and rubredoxin reductase (RdxR)\(^{19}\). All these proteins consist of three domains: an NAD(P)-binding domain, an FAD-binding domain, and a C-terminal domain, as shown in Figs 1 and 2. The root mean square deviations (RMSD) after superposition of OsMDHAR with structures of Pdr, BphA4, and RdxR were 1.2–1.4 Å over 285, 255, and 208 C\(_{α}\) atoms, respectively. OsMDHAR also shares 49%, 41%, and 35% sequence similarity with Pdr, BphA4, and RdxR, respectively (Fig. 3). The bacterial reductases, Pdr, BphA4, and RdxR, transfer an electron from NAD(P)H via FAD to low molecular weight, iron-containing proteins: putidaredoxin (Pdx)\(^{20}\), ferredoxin (BphA3)\(^{21}\), and rubredoxin (Rdx)\(^{19}\), respectively. These redox partner proteins bind to the C-terminal domain of their corresponding reductases and mediate the electron transfer in a biological redox reaction. Although OsMDHAR also comprises three domains, similar to the above-mentioned reductases, it transfers an electron directly to its substrate, MDHA, which binds to its active site pocket. Superposition of the OsMDHAR structure with Pdr, BphA4, and RdxR structures complexed with their corresponding partner proteins (Pdx, BphA3, and Rdx) clearly shows the remarkable structural difference in terms of substrate binding site (Fig. 2)\(^{19–21}\). The superposed structures show that the unique long loop of 63–80 residues of OsMDHAR is adjacent to the iron cluster of Pdx, BphA3, and Rdx, and occupies a part of the partner proteins in the complexed structures. It is involved in the formation of a closed and
narrow active site pocket with positively charged residues, which only accommodates small molecules such as MDHA. The amino acid sequence of MDHAR is relatively conserved within the MDHAR homologue proteins, as shown in the multiple sequence analysis in Fig. 3.

**FAD-binding site.** The FAD-binding domain of OsMDHAR adopts a typical α/β fold consisting of two antiparallel β-sheets and one parallel β-sheet surrounded by four α-helices, as shown in Fig. 1. The FAD is bound to a crevice on the FAD-binding domain of the protein via hydrogen bonds and van der Waals interactions, as shown in Fig. 4a. The residues participating in the van der Waals interactions with FAD are Gly13, Gly15, Ala122, Thr123, Gly297, and Ala319. Most of them are located at the bottom of the crevice and are highly conserved. Lys53, salt-bridged with Glu178, participates in hydrogen bond formation with O4 of FAD, which is also hydrogen bonded with the carbonyl oxygen of Pro49. Both of the residues, Glu178 and Pro49, are highly conserved, and the hydrogen bond network among Lys53, Glu178, and FAD is commonly observed in other enzymes such as glutathione reductase and trypanothione reductase17,18,22,23. The hydrogen bond network indicates that no residues are available for proton transfer to the N5 atom of FAD, thus preventing stabilization of flavin semiquinone by OsMDHAR17,24.

The conserved residue Asp298 forms a hydrogen bond with O3' of the ribitol moiety and the phosphate group is stabilized by hydrogen-bond interactions with Arg48 and Arg147. The Glu41 and Glu148 residues are hydrogen bonded with the O3B and O2B atoms of the adenosine moiety of FAD, respectively. Among those residues, Arg48 helps FAD binding by partially compensating for the negative charge of one of the phosphate groups of FAD22.

**Conformational changes induced by binding of NAD.** The NAD(P) binding site consists of residues 125 to 264. The NAD-binding domain in OsMDHAR consists of antiparallel β-sheets (β8, β12, β13, β14, and β16) and parallel β-sheets (β9, β10, β11, and β15) surrounded by three α-helices (α3, α4, and α5) (Fig. 1b). Reduction of the crystals by binding of NAD quenches the intrinsic yellow colour of the isoalloxazine of FAD, resulting in colourless crystals. The binding of NAD led to a butterfly-like movement of the isoalloxazine ring of FAD as shown in Fig. S2, which was previously observed in the structure of BphA421. This movement is most likely induced by a change in the redox state of FAD21. We observed two different redox states of FAD, namely oxidized and reduced forms, by measuring the UV-visible spectrum of the OsMDHAR protein (Fig. S2). We observed significant conformational changes only in the residues around FAD and NAD binding sites, on comparing the overall structure of OsMDHAR-NAD complex to the NAD-free form. The RMSD between equivalent Cα atoms of the NAD-free and OsMDHAR-NAD complex was 0.61 Å. NAD binding accompanied a large conformational shift in Tyr174, His315, and Phe348 residues. Tyr174, which points toward the NAD binding site in the NAD-free
structure, is moved away from the nicotinamide ring upon NAD binding due to steric hindrance. As a result, the nicotinamide ring is sandwiched between the isoalloxazine ring of FAD and the side chain of Tyr174. Then, the neighbouring Phe348 residue shifts outward to avoid steric conflict with Tyr174. In contrast, His315 is oriented towards the NAD binding site and hydrogen bonded with O7N atom of the bound NAD. The above three residues are only conserved through MDHAR homologues. Thus, this conformational change, induced by NAD binding, has not been observed in other flavoprotein-NAD complexes.

In addition to the van der Waals contacts around the nicotinamide moiety, a conserved Glu178 residue participates in hydrogen bond interaction with the nicotinamide ring, as shown in Fig. 4c. The OE2 atom of Glu178 forms a hydrogen bond with the N7N atom of the nicotinamide ring. The NH2 atom of Arg202 forms hydrogen bond with the O2D of the ribose and the O2A of the phosphate group, respectively. An additional hydrogen bond was formed between the OE1 atom of Glu314 and the O3D atom of ribose. Specifically, in the OsMDHAR structure, an extra hydrogen bond was observed between the O2B of the adenosine ring and Glu196.

Preference for NAD over NADP. Previous reports have demonstrated that reduction of Pdr by NADPH was three orders of magnitude slower than with NADH. This is possibly due to hydrogen bonding of an additional Glu186 with the ADP-ribose of NAD. In addition, the crystal structure of Pdr indicated that the relatively long and flexible Glu186 of Pdr might disrupt the binding of the 2′-phosphate group of NADP, thereby increasing selectivity of the enzyme for NAD. However, because the structure of Pdr complexed with NAD or NADP is not available, the above speculation is not confirmed. The biochemical data shown in Fig. 5a indicates that OsMDHAR also prefers NADH to NADPH. We postulated that, similar to Pdr, the preference for NAD in the OsMDHAR structure may arise from an additional hydrogen bond with the ADP-ribose of NAD. Our speculation was confirmed from the structure of the OsMDHAR-NAD complex, where we observed hydrogen bonding between Glu196 and the O2B molecule in the adenosine ring of NAD (Fig. 4c). Abolishing the hydrogen-bonding interaction by introducing an Ala196 mutation (E196A) reduced the enzyme activity, as shown in Fig. 5b.
NADP was docked into the NAD binding pocket of OsMDHAR, the Glu196 residue occupied the site of the 2’-phosphate group of NADP (Fig. 4c). Figure 3 shows the sequence alignment of OsMDHAR that revealed Glu196 to be a highly conserved residue throughout MDHAR homologues. For more detailed information, we attempted to determine the structure of the enzyme in complex with NADP. We prepared an E196A mutant protein, which facilitates the binding of NADP by eliminating the carboxylic acid group. Unexpectedly, the structure of the E196A mutant-NADP complex revealed that the 2’-phosphate group of NADP was not located in the position of the carboxylic acid group of Glu196.

To test whether the long and flexible Glu196 interferes with the binding of NADPH, we measured the binding affinity of wild type (WT) and E196A for NADH and NADPH using isothermal titration calorimetry (ITC). ITC experiments clearly showed an increase in the affinity of OsMDHAR toward NADP on removing the carboxylate group of Glu196 (Fig. S3). ITC data showed that the binding affinity of WT for NAD was 20-fold higher than that of WT for NADP. On the other hand, E196A had only about 3 times higher affinity for NAD than that for NADP. Although there is no difference between WT and E196A in NAD binding affinity, E196A showed a 16-fold increase in affinity for NADP compared to the WT. Therefore, together from the structural studies and ITC experiments, we hypothesized that Glu196 has an important role in the NAD selectivity of the OsMDHAR.

Substrate binding site. Some of the bacterial reductases like Pdr, BphA4, and RdxR, which have a high structural similarity with OsMDHAR, interact with their redox partner proteins to form redox-protein complexes. In the electron transfer pathway from structures of the redox-protein complexes, the conserved Trp residues in Pdr and BphA4 are able to mediate electron transfer from FAD to the [2Fe-2S] cluster, whereas RdxR has a bridging catalytic water molecule instead of a catalytic residue for the same. In OsMDHAR, Tyr349 is a completely conserved residue in the homologues of MDHAR, which structurally corresponds to the catalytic residues in similar enzymes (Figs 2 and 3). Mutation of this Tyr349 residue to Ala, Phe, and Trp abolishes the enzyme activity, as shown in Fig. 5b. Based on these results, we speculate that Tyr349 is a catalytic residue of OsMDHAR.

To elucidate the detailed mechanism of the electron pathway adopted by OsMDHAR, we co-crystallized OsMDHAR with AsA, which is the final product of the enzyme, in the presence of NAD. We determined the structure of the catalytically compromised Y349F mutant in complex with AsA. Superposition of the Tyr349 and Phe349 of apo and mutant structure indicates that there is no conformational change of Phe349 (Fig. S4a).
both apo and NADH complex structure, Tyr349 formed hydrogen bonds with two water molecules, whose position was corresponded to the bound substrate ligand in the AsA complex structure (Fig. S4b). This suggests that hydrogen bonding with water molecules might limit the flexibility of Tyr349, and ligand binding is attained by the replacement of the hydrogen-bonded water molecules. In case of other enzyme-protein complexes, such as the BphA4-BphA3 complex, binding of the redox partner protein induced rotational movement of the NAD-binding domain and C-terminal domain21. However, we did not observe any large conformational changes in the structure of the OsMDHAR-NAD-AsA complex. The active site of OsMDHAR consists of highly positively charged electrostatic potential pockets, which easily bind to the negatively charged AsA, as shown in Fig. 6a. Structural studies revealed three highly conserved residues, G72, R320, and R351, positioned close to the AsA molecule (Figs 3 and 5c). Of these residues, Arg320 forms a hydrogen bond with AsA bound to the structure (Fig. 5d). In addition, in the mutational analysis, only R320A had significantly reduced activity (Fig. 5b). Therefore, we suggest that R320 is important for substrate binding.

**Electron transfer mechanism.** The structural and mutagenesis studies suggest that the electron is transferred from the flavin N atom to the FAD flanking Tyr349, which subdivides the distance between FAD and AsA into two shorter segments of 5.7 and 3.8 Å, and then directly to the substrate, MDHA (Fig. 6b). As shown in Fig. 6c, initially, two electrons are transferred from NAD(P)H in the hydride transfer to FAD in OsMDHAR. During the oxidative reaction, two electrons are sequentially transferred from FADH₂ to Tyr349, resulting in a flavin semiquinone reaction intermediate. The electrons are then transferred from the Tyr349 to the MDHA radical, generating two molecules of AsA.

MDHAR uses a catalytic tyrosine residue to regenerate AsA unlike DHAR, which uses a catalytic cysteine residue that is inactivated by hyperoxidation. Mutation of Cys70, which is the only Cys residue in OsMDHAR, had no significant effect on enzyme activity (Fig. 5b). Based on this observation, we postulated that MDHAR plays a major role in AsA recycling. To verify our hypothesis, we cultured three types of yeast cells harbouring different genes: the OsDHAR gene, the OsMDHAR gene, or an empty vector. Cells expressing either OsMDHAR or OsDHAR were more resistant under H₂O₂-induced oxidative stress compared to WT cells that harbour an empty vector (Fig. 6d). However, yeast cells overexpressing MDHAR were more tolerant to oxidative stress than those expressing DHAR, verifying the predominant role of MDHAR in oxidative stress (Fig. 6d).

During data interpretation, we noticed that although most eukaryotic organisms, including plants, produce L-ascorbic acid, yeast cells instead produce D-erythroascorbic acid, a five-carbon analogue of L-ascorbic acid26,27. The fact that both enzymes are functional in our in vivo assay implies that MDHAR and DHAR can use an alternate substrate analogue such as D-erythroascorbic acid. For structural confirmation of binding of the enzyme to an AsA analogue, we co-crystallized OsMDHAR bound to NADH and isoascorbic acid (ISD), as D-erythroascorbic acid is not commercially available. We observed an interpretable electron density map, suitable for structural confirmation.
for ISD fitting. Superimposed structures of OsMDHAR-AsA and OsMDHAR-ISD complexes showed distinct binding conformations for both substrate ligands (Fig. 5c,d). In detail, ISD did not fit perfectly into the binding site of AsA. The binding site of ISD had more exposed surface area. In addition, we observed clear electron density for the di-hydroxyfuranone part of ISD, but not its di-hydroxyethyl part. This suggests that ISD may bind more loosely to the enzyme than AsA. Nevertheless, ISD is hydrogen bonded to the conserved Arg320, which is within an accessible distance for electron transfer from the Tyr349 residue. Kinetic analysis of OsMDHAR for AsA and ISD revealed that the catalytic Vmax values were 37.5 ± 2.5 and 32.6 ± 2.7, respectively, whereas the Km values were almost identical (Fig. S5). The Arg320 residue forms hydrogen bonds with both AsA and ISD in the complex structures. This is evidence that Arg320 plays an important role for AsA as well as ISD despite their dissimilar binding modes. We confirmed this through an enzyme activity assay using an R320A mutant which retained only 20% activity compared to WT. The catalytic hydroxyl group of the active site Y349 residue was positioned within reasonable distance to be able to transfer electrons to O3 of AsA or ISD. The Y349X mutants also displayed almost no activity compared to WT enzyme (Fig. 5). Arg320 and Tyr349 are highly conserved in other MDHAR homologues (Fig. 3). Therefore, we concluded that Arg320 actually has a substrate-binding role for both substrates and that the Tyr349 residue mediates electron transfer toward AsA or ISD.

We also confirmed the physiological role of AsA and ISD against oxidative stress in yeast mutants. The enzymatic activity of OsMDHAR, produced from yeast cells, was consistent with both AsA and ISD (Fig. S6a). The antioxidant effect and regeneration of AsA and ISD in yeast cells was confirmed using yeast mutant cells (ara2Δ), which lacked the ARA2 gene coding D-erythroascorbic acid biosynthesis. Exogenous AsA and ISD in the yeast mutant cells recovered rapidly, when challenged with H2O2 compared to the cells lacking antioxidant molecule supplementation. As shown in Fig. S6b, there is a subtle difference between the adaptative abilities of AsA and ISD. The yeast mutant cells expressing OsMDHAR were more tolerant to oxidative stress, compared to the control cells without OsMDHAR. These results suggest that both antioxidants, AsA and ISD, are able to substitute D-erythroascorbic acid, and their reduced forms are regenerated by MDHAR activity in yeast cells. Given the structural and functional analysis, we conclude that the binding site of OsMDHAR may be able to accommodate variable substrate compositions including MDHA.

Methods
Cloning, purification, and preparation of mutants. The cloning of the OsMDHAR gene was described in the previous report24. In brief, OsMDHAR was amplified from cDNA of O. sativa L. japonica by PCR and PCR products were integrated into the pKM260 vector (Euroscarf, Germany). The recombinant plasmid was transformed into Escherichia coli strain NiCo21 (DE3) cells. For purification, cells were resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 200 mM NaCl, and 5 mM imidazole) and lysed by sonication. The lysate was
MDHAR activity was assayed spectrophotometrically (UV-1650PC; Shimadzu Corp., Kyoto, Japan). The assay was performed at 25 °C with a reaction mixture (1 mL) containing 50 mM potassium phosphate, pH 7.2, 0.25 mM NADH or NADPH, 2 mM AsA, 1 unit AsA oxidase (Sigma-Aldrich, USA), and purified protein (10 μg). The absorbance was measured at 340 nm by monitoring NAD(P)H oxidation, and the activity was calculated using an absorbance coefficient of 6.2 mM−1 cm−1. One unit is the amount of enzyme that oxidizes 1 nmol of NADH per min at 25 °C. Enzyme activity was represented relative to WT proteins, which was defined as 100%. The enzymatic assay was independently performed at least 3 times and results are expressed as the mean ± standard deviation (SD).

### Data collection and refinement statistics

| Entry       | Space group | PDB ID | Resolution (Å) | Completeness (%) | %Reflections | %I/σ | %Merging | Rmerge (%) | Rfree (%) | Rwork (%) |
|-------------|-------------|--------|----------------|------------------|--------------|------|----------|------------|-----------|------------|
| E196A_NAD  | P4_122      | 5JCI   | 50.0–1.7       | 99.2 (100)       | 85440        | 6.3 (29.5) | 6.3 (29.5) | 17.3       | 20.4      |
| E196A_NADP | P4_122      | 5JCK   | 50.0–2.0       | 99.7 (100)       | 85440        | 14.5 (35.5) | 14.5 (35.5) | 20.2       | 24.1      |
| E349F_NAD  | P2_121      | 5JCL   | 50.0–1.8       | 99.7 (100)       | 85440        | 7.2 (69.7)  | 7.2 (69.7)  | 15.9       | 19.7      |
| E349F_NADP | P2_121      | 5JCN   | 50.0–2.3       | 99.5 (100)       | 85440        | 7.3 (50.2)  | 7.3 (50.2)  | 18.1       | 21.0      |
| E349F_Asa  | P2_121      | 5JCM   | 50.0–1.9       | 99.3 (98.8)      | 85440        | 8.2 (65.6)  | 8.2 (65.6)  | 20.6       | 26.4      |

### Crystallization and structure refinement

The crystallization of apo OsMDHAR was described in the previous report28. Briefly, apo OsMDHAR crystals were obtained under conditions consisting of 100 mM Tris–HCl, pH 8.0, 200 mM lithium sulphate, 30% (w/v) PEG 5000 MME by the hanging-drop vapour-diffusion method. The initial crystal screening of OsMDHAR mutant crystals (E196A and Y349F) was also performed with subsequent purification of mutants OsMDHAR protein was identical as described above for the wild-type protein28.

In a previous paper, the data collection and structural refinement of apo OsMDHAR was described28. In the case of complex crystals, Perfluoropolyether Cryo Oil (Hampton Research, USA) was introduced as a cryoprotectant. X-ray diffraction data for complex crystals with NAD were collected at a resolution of 2.0 Å using an
were exposed to 20 mM H2O2 for 1 h at 28 °C and serially diluted (100 to 10−4) in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and cultured for 8 h. The mid-log phase cells (OD600 = 0.5−1) were inoculated in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and cultured for 8 h. Mid-log phase cells (OD600 ≈ 3.0) were exposed to 20 mM H2O2 for 1 h at 28 °C and serially diluted (100 to 10−4) in YPD medium. Five microliters of diluted solutions were spotted onto YPD agar plates and incubated for 2−3 days at 28 °C. The results of the spotting assay are representative of at least 2 independent experiments performed under identical conditions.

**Protein data bank accession number.** The coordinates of the structures together with the structure factors have been deposited in the Protein Data Bank [http://www.rcsb.org/pdb] with accession codes of 5ICL, 5CK, 5ICI, 5CM, and 5CN.

**Stress response in OsMDHAR- and OsDHAR-expressing yeast cells.** CDNA containing the open reading frame (ORF) of OsMDHAR and OsDHAR was subcloned into the yeast expression vector p426GPD (yeast 2μ expression vector with URA3 marker; GenBank Accession No. DQ019861), which allows constitutive expression of a target gene under the control of the yeast GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter. To measure cell viability, cells (4 × 104 cells/mL) were inoculated in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) and cultured for 8 h. Mid-log phase cells (OD600 ≈ 3.0) were exposed to 20 mM H2O2 for 1 h at 28 °C and serially diluted (100 to 10−4) with YPD medium. Five microliters of diluted solutions were spotted onto YPD agar plates and incubated for 2−3 days at 28 °C. The results of the spotting assay are representative of at least 2 independent experiments performed under identical conditions.

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Author Contributions
H.-S.Y., J.H.L. and H.-W.K. designed and supervised the project. A.K.P., I.-S.K. and H.D. performed the cloning, expression, and protein purification. A.K.P., H.D. and B.W.J. crystallized, collected X-ray data, and solved the protein structures. I.-S.K. C.W.L., A.K.P. and Y.-S.K. performed the enzyme activity experiment. I.-S.K., S.J.R., S.C.S. and H.P. performed stress response experiment in yeast cell. A.K.P., I.-S.K., J.H.L. and H.-W.K. wrote the initial manuscript and H.D. and H.-S.Y. contributed to revisions. All authors discussed the results, commented on the manuscript, and approved the manuscript.

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