Structural Insights into the NAD(P)H:Quinone Oxidoreductase from Phytophthora capsici

Cancan Yang, Zhenling Huang, Xiuguo Zhang, and Chunyuan Zhu*

ABSTRACT: Soluble quinone oxidoreductases catalyze transfer of electrons from NADPH to quinones. Transfer of electrons is essential for detoxification of synthetic compounds. Here, we present the crystal structure of a NADPH-dependent QOR from Phytophthora capsici (Pc) complexed with NADPH at 2.4 Å resolution. The enzyme exhibits a bi-modular architecture, containing a NADPH-binding groove and a substrate-binding pocket in each subunit. In the crystal, each asymmetric unit of PcQOR contains two molecules stabilized by intermolecular interactions. Gel filtration and ultracentrifugation analyses reveal that it functions as a tetramer in solution. Alignment of homologous structures exhibits a conserved topology. However, the active sites vary among the homologues, indicating differences in substrate specificities. Enzymatic assays indicate that PcQOR tends to catalyze the large substrates, like 9,10-phenanthrenequinone. Computational simulation associated with site-directed mutagenesis and enzymatic activity analysis declares a potential quinone-binding channel. The ability to reduce quinones probably helps P. capsici to detoxify some harmful chemicals encountered during invasion.

INTRODUCTION

Quinone oxidoreductases (QORs) are a class of enzymes that catalyze the reduction of quinones to hydroquinones. Most quinones are classified as cytotoxic molecules widely distributed in all living organisms.1,2 QORs could prevent cells from quinone cytotoxic damage, such as reducing the formation of DNA adducts and lipid peroxides.3,4 QORs are classified into two groups depending on cellular localization: (a) membrane-bound QORs that participate in the respiration chain and generation of energy,5–9 and (b) soluble QORs that reduce quinones to hydroquinones.9 Soluble QORs play a central role in detoxifying quinones by reducing them to corresponding hydroquinones, which may conjugate to either sulfate or glucuronic acid for excretion.10–13 Oxygen can react with hydroquinones and lead to consumption of redundants from cells and lipid peroxidation.14–16 Most QORs exhibit flexible substrate specificities and catalyze various-size quinones.9,10 Significantly, the electron-donating ability of QORs is beneficial to degrade organic contaminants.16

Soluble QORs are categorized into two groups based on their respective reductive mechanism.17 DT-diaphorases (EC 1.6.99.2) belong to two-electron reductive enzymes and require FAD as an additional prosthetic group.15,18 On the other hand, zeta-crystallin-like QORs catalyze one-electron reduction of certain quinones (benzoquinones, naphthoquinones, phenanthrenequinones, and related derivatives) using NADPH as the only cofactor.19–22 Zeta-crystallin-like QORs belong to the medium-chain dehydrogenase/reductase superfamily,23,24 which is divided into two subfamilies: zinc-dependent dehydrogenases25 and non-metallic reductases.26 PcQOR belongs to the latter class, not requiring zinc ions for catalysis. Zeta-crystallin has been found at low levels in the liver and kidney tissues of both humans and guinea pigs,26,27 suggesting a metabolic role in the detoxification of quinones.28 Phytophthora capsici encounters harsh conditions during infection, including oxygen deprivation. Perhaps PcQOR participates in generating energy to accept electrons in the absence of oxygen.

So far, the zeta-crystallin-like QORs have been determined in many organisms, such as Pseudomonas syringae,29 Saccharomyces cerevisiae,30 Thermus thermophilus HB8,31 Escherichia coli,32 Arabidopsis thaliana,33,34 Homo sapiens,35,36 and Streptococcus mutans.37 Notedly, the QORs from humans and yeast have a novel activity to hydrogenate the double-bond α,β-hydrogenation of 2-alkenals and 3-alkenones.35 In addition to the enzymatic activity, they can also bind nucleic acids.36,37 Moreover, these proteins are speculated to bind adenosine–uracil-rich elements to regulate transcription.38 All homologues
of zeta-crystallin-like QORs exist as dimers in the asymmetric unit. Each subunit contains a catalytic pocket and a cofactor-binding pocket. To delineate the structural differences and mechanism of catalysis between PcQOR and its homologues, we perform structural studies of PcQOR.

Here, we determine the crystal structure of the PcQOR complex with NADPH. The complex structure gives noticeable details of the NADPH-binding interaction. We also investigate the substrate-binding site using computational simulation combined with site-directed mutagenesis and enzymatic activity assays. PcQOR could catalyze both benzoquinone and phenanthrenequinone, emphasizing the need for a flexible active site for detoxifying toxic components encountered by P. capsici in the host.

## MATERIALS AND METHODS

**P. Capsici Strain Culture.** P. capsici LT1534 strain was cultured on oatmeal agar at 25 °C.

**Gene Cloning and Protein Purification.** The open reading frame of PcQOR (GenBank accession no. OK398115) was amplified from the genomic cDNA of P. capsici strain LT1534 using the primer sets described in Supporting Information Table S1 and ligated into the pET28a vector that includes an N-terminal hexahistidine tag. E. coli Rosetta cells were transformed with pET28a-PcQOR plasmids and grown in Luria–Bertani medium under kanamycin selection at 37 °C until the A600nm reached 0.8 absorbance units and then induced with 1 mM isopropyl β-D-thiogalactopyranoside at 16 °C for 24 h. The cells were pelleted by centrifugation at 7000g for 10 min and then resuspended in resuspension buffer containing 30 mM HEPES, pH 7.5, 150 mM NaCl, and 30 mM imidazole. The cells were subjected to sonication on ice using an ultrasonic homogenizer (Ningbo Scientz Biotechnology Co., Ltd.), and the debris was removed by centrifugation (14,000g for 25 min, 4 °C). The supernatant was then loaded onto a Ni-NTA column with an FPLC system (GE Healthcare) equilibrated using lysis buffer and eluted with an elution buffer (30 mM HEPES, pH 7.5, and 150 mM NaCl, followed by a 200–500 mM imidazole gradient), and then washed with 0.5 column volumes of washing buffer. The eluotropic protein was further purified by anion-exchange chromatography with a Resource Q column (GE Healthcare), which was eluted using a linear gradient of NaCl. The protein peak was concentrated to 1 mL and then loaded onto a size exclusion chromatography column (Superdex 200 Increase column, GE Healthcare) pre-equilibrated in 30 mM HEPES, pH 7.5, and 150 mM NaCl buffer. The purified proteins were collected and concentrated to 12 mg/mL. The purity of protein was estimated by SDS-PAGE, and then, the protein was stored at −80 °C for crystallization screening. The mutant proteins were prepared in the same way as the wild-type protein.

**Crystallization, Data Collection, Structure Determination, and Refinement.** The crystals of PcQOR were grown using the sitting drop vapor diffusion technique and a crystal screen kit (HAMPROM RESEARCH). A 1:1 ratio of protein/reservoir solution was equilibrated against 180 µL of reservoir solution at 10 °C. Crystallization of PcQOR was completed within 3 and 5 days under conditions of 28% v/v 2-propanol, 0.1 M Bis-Tris, pH 6.5, and 3% v/v polyethylene glycol 200. The crystals were directly mounted in a cryoloop and soaked in cryoprotectant solution (20% glycerol) and flash-frozen in liquid nitrogen prior to data collection. All X-ray diffraction data sets were collected at a radiation wavelength of 0.97 Å on the beam line BL19U at a temperature of 100 K in the Shanghai Synchrotron Radiation Facility (SSRF), China. The diffraction data from PcQOR were collected with the 360° total rotation range, 1° per image, and with the “auto-correction” option in scaling. The “no merge original index” option was used to generate an alternative, unmerged set of data, intended only to calculate correlation coefficients of anomalous difference for two random half sets (CCano) by phenix.anomalous_signal. The data were scaled separately within the 45, 90, 180, 270, and 360° rotation range to analyze the strength of anomalous signals at different multiplicities. The statistics of diffraction data at maximal redundancy are listed in Table 1. All data were processed using HKL3000 software.

### Table 1. Data Collection and Refinement Statistics of PcQOR Crystals

|                     | PcQOR |
|---------------------|-------|
| data collection     | P3_21 |
| cell dimensions     | 84.28, 84.28, 185.605 |
| a, b, c (Å)         | 90.000 |
| resolution (Å)      | 50–2.40 (2.44–2.40) |
| Rmerge (%)          | 17.9 (42.1) |
| completeness (%)     | 99.9 (100) |
| redundancy           | 90 (9.4) |
| refinement resolution (Å) | 2.39 |
| no. of reflections  | 28,996 |
| Rmerge/Rfree (%)    | 17.28 (19.61) / 22.74 (29.52) |
| no. of atoms        | Protein 5287 |
|                     | ligand/ion 96 |
|                     | Water 244 |
|                     | B-factors 35.9 |
|                     | rms deviations 0.0086 |
|                     | bond lengths (Å) 1.09 |
|                     | bond angles (deg) 100% |
| Ramachandran plot [%] | 96.66 |
|                     | most favored 96.66 |
|                     | additionally allowed 2.62 |

The PcQOR structure using the QOR of *T. thermophilus* HB8 (PDB ID 1HYZ) as a search model was solved by molecular replacement (MR) with the Phaser program from the CCP4 suite. Further refinement was carried out by using the programs Phenix and Coot. Ligand fitting maps were performed using the Phenix.ligand fit. 10% of all data set aside was used to calculate Rfree. Subsequent refinements by incorporating ligands and water molecules were according to the 1.0σ map level. The data collection and refinement statistics are summarized in Table 1. A Ramachandran plot generated with Molprobity indicated that the structure had favorable stereochemistry. High-quality figures of the protein and ligand structures were created with the PyMOL program.

### Gel Filtration Assay

Gel filtration assay was performed for analyzing the solution state of PcQOR. A Superdex 200 Increase column was pre-equilibrated with a buffer containing 30 mM HEPES, pH 7.5, and 150 mM NaCl. 2 mg/mL protein...
of PcQOR was loaded onto the column and eluted under conditions of 30 mM HEPES, pH 7.5, and 150 mM NaCl at a flow rate of 0.5 mL/min. The Bio-Rad’s gel filtration standard was used to estimate the molecular weight of the PcQOR multimer.

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed using an Optima AUC analytical ultracentrifuge equipped with two detection systems of ultraviolet light and interference light (Beckman Coulter). 2 mg/mL PcQOR in buffer containing 30 mM HEPES, pH 7.5, and 150 mM NaCl was centrifuged at 4 °C at 42,000 g, and the PcQOR migration was monitored based on absorbance at 280 nm. The sedimentation velocity data were analyzed using SEDFIT.49,50

Computational Docking. The structure of HNQ was acquired from the CYP158A2 complex structure.51 The structures of 1,2-benzoquinone (NQ) (O=C1C=CC2= C(C=CC=C2)C1=O) and PQ (O=C1C(=O)C2= C(C=CC=C2)C3=C1C=C CC=C3) were transformed from the SMILES (simplified molecular input line entry specification) level representations with the program phnix.elbow.52 The three computer-simulated structures were generated using Autodock4.2.6, which is a suite of automated docking tools.53,54 Each molecule could obtain more than 100 initial poses, for each of which conformation was scored. The optimal pose was scored for van der Waals and electrostatic interactions and was rigid-body-minimized.

Site-Directed Mutagenesis. Variants were constructed using a fast site-directed mutagenesis kit (Tiangen Biotech (Beijing) Co Ltd.) with the wild-type PcQOR plasmid as a template. The sequences of the mutagenesis oligonucleotides are listed in Supporting Information Table S2. The PCR products were incubated with DpnI (Tiangen Biotech (Beijing) Co. Ltd.) to digest the original DNA template and then separately transformed into E. coli strain FDM competent cells. Each mutant site change has been confirmed by sequencing.

Activity Assays. The kinetic parameters of enzymes were measured by the previous method.55 Kinase reactions were carried out in a buffer solution containing 100 mM HEPES, pH 7.5, 100 mM NADPH, 0.1 mg/mL PcQOR, and 0.25 mM substrates at 25 °C. The substrates were dissolved in absolute alcohol with final 2% concentration in the assay. The reaction rate was monitored using NanoPhotometer Pearl by recording
the decrease in absorbance of NADPH at 340 nm. The non-enzymatic reduction of NADPH was set as the background control for each assay. The kinetic parameters of mutant enzymes were performed at 25 °C for 15 min.

RESULTS AND DISCUSSION

Overall Structure of PcQOR. We determined the recombinant protein PcQOR crystal structure by MR, which is refined to 2.4 Å resolution (Table 1). In the native structure of PcQOR, ethylene glycol, the smallest component of polyethylene glycol 200, is present in the active site pocket of each monomer. Significantly, judging from the strong difference density peak of definable shape, nicotinamide adenine dinucleotide phosphate (NADPH) is present in the active pocket of each monomer (Figure 1A).

Resembling the other QORs, PcQOR exhibits a bi-modular architecture, which is characterized by two function domains: a catalytic domain (domain1) and a NADPH-binding domain (domain2), which are connected by helix α2. The helix α2 is divided into two segments, labeled α2a and α2b. Domain1 is formed by the N- and C-terminal segments. The N-terminal segment comprises seven β-strands (β1–β7) and the α2a-helix, while the C-terminal segment is composed of two β-strands (β14–β15), an α8-helix, and an α9-helix. These two parts are combined together to form domain1. Significantly, domain1 exhibits a compact bundle, which is formed by strands β3, β4, β5, and β6. Helix α1 is embedded into the bundle. This bundle is in immediate proximity to the NADPH-binding domain; therefore, this motif is very likely to play a pivotal role in substrate binding. Domain2 is formed from G149 to G294 that folds into a classical Rossmann fold, 56 which is constituted by two βαβαβ units flanked by three α-helices on either side. The long helix α2 horizontally inserts into the protein and links the two domains (Figure 1A).

The overall structure of PcQORs is similar to that of QORs from P. syringae pv. tomato DC3000, 9 S. cerevisiae, 10 E. coli, 17 T. thermophilus HB8, 29 and Mycobacterium tuberculosis 57 (Figure 1B). In the Dali server, 58 PcQOR retrieved some alcohol dehydrogenases as top hits, suggesting that PcQOR shares a conserved structure with alcohol dehydrogenases. However, the lack of the zinc-binding motifs probably affects interaction between QORs and hydrogen from alcohols. Importantly, there is a long unique loop (90–108) in PcQOR, compared to other homogeneous QORs, covering the region above the active site in PcQOR (Figure 1B). We speculate that this loop probably plays a guarder role in the substrate entry and product release.

Each asymmetric unit contains one homodimeric molecule in PcQOR. The interface of the dimer is mainly mediated by amino acids from the β13-sheet, α7-helix, and two loops between α7 and α8. The interface area of the dimer is 983.3 Å2, and the ΔG of the interface is −14.5 kcal/mol. Across the dimeric interface, there is a contiguous parallel β-sheet bundle made up of 12 β-strands, which is flanked by helices on either side (Figure 1C). Amino acids from β13, α7, and loops connecting α7 and α8 form intermolecular interactions, holding the monomers within the dimer together (Figure 1D). Within the interactions, L282 from α7 of one monomer interacts with Q292 of the adjacent monomer, and these two residues are in close proximity to the nicotinamide moiety of PcQOR. Sedimentation velocity experiment suggests that PcQOR exists as a tetramer in solution.
NADPH. Therefore, they are likely to play an important role in assisting in the co-factor for catalysis. Further enhancement of the dimer interface is accomplished through interactions of I283, C286, and L288 from one monomer with Q292, G290, and L288 from another monomer, respectively. This interaction is indispensable because these structural elements stabilize the boundary of the active site. Thus, dimerization of PcQOR probably facilitates the formation of the ligand-binding pockets. A similar mode of dimerization of QORs has been observed previously.

The results of gel filtration and ultracentrifugation analysis reveal that PcQOR exists as a tetramer in solution. Gel filtration results show that PcQOR eluted as a wide peak close to molecular weight marker 158 kDa (Figure 2C). Ultra-centrifugation demonstrates that the macromolecule has a molecular weight of 146 kDa in solution, which approximately quadruples the weight of PcQOR (Figure 2D). Two dimers screwing together form a tetramer (Figure 2A). Amino acids from the α5-helix, α8-helix, and β8-sheet, combined with residues from the loop that connects α2 with β7 and the loop that connects α3 with β9, form intermolecular interactions that hold the dimers within the tetramer together (Figure 2B). The interface area between molecule 1 and molecule 2 is 643.3 Å, and the ΔG of the interface is −0.5 kcal/mol. Within the tetramer, R308 and R309 from α8 of molecule 1 interact with T167, A189, and F229 of the adjacent molecule 4. Further consolidation of the tetramer interface is achieved via interaction of D134 from one monomer with N231 of another monomer. Thus, the intermolecular interactions of these residues guide the protein tetramerization in the solution.

PcQOR functions in the tetramer conformation, a more compact conformation compared to the dimeric topology, which probably contributes to the formation of catalysis-competent ligand-binding pockets.

**NADPH-Binding Site.** NADPH binds to the cleft between domain1 and domain2 and embeds in a positively charged cavity (Figure 3A,B). Residues from both domain1 and domain2 participate in tethering the co-factor. The adenine ring of NADPH in PcQOR is wrapped in a groove formed by Q171, Y216, I241, G242, T246, L266, and A267 via van der Waals contacts (Figure 3C). The side chain of Y216 stacks against the adenine ring of NADPH. The phosphate group attached to the adenosine ribose is encompassed by positively charged residues (T173, S195, S196, K200, and Y216). The pyrophosphate moiety establishes three hydrogen bonds with G175-N, V176-N, and A44-N. The interaction of the ribose sugar with the protein is perceived by the C2 hydroxyl oxygen with the side chain of Q48 and by the C3 hydroxyl oxygen with the main chain of Y265 through a water atom. The nicotinamide moiety fits into a more open cavity and forms strong interactions with I263-O, V291-O, and A293-N. These interactional amino acids enhance and orient the co-factor optimally for catalysis and provide an applicable binding environment for NADPH.

Superposition of the PcQOR with other homologues reveals that the adenine ring of NADPH in PcQOR exhibits a mirrored orientation compared to the dimeric topology, which probably contributes to the formation of catalysis-competent ligand-binding pockets.
substrates such as phenanthrenequinone cannot be reduced. residues L50, A51, and W243, which explains why the large or Ser in the NADPH pocket (Figure S1). Side chain of “Glu” Tyr around the NADPH pocket, while Arg is replaced with Gly and Tyr plays an important role in stacking against the adenine (Figure 3D). Perhaps it is the difference in the nucleotide-binding residues causing the conformational change of NADPH in PcQOR. The unique orientation of NADPH in PcQOR may be relevant to the species specificity. BLAST analysis of the MtbQOR against other oomycetes revealed some orthologues from Phytophthora palmivora, Saprolegnia parasitica, and Aphanomyces astaci. Sequence alignment shows that these three QORs also contain Tyr around the NADPH pocket, while Arg is replaced with Gly or Ser in the NADPH pocket (Figure S1). Side chain of “Glu” or “Ser” residue may be too short to stabilize the adenine ring. Therefore, we surmise that unique orientation of NADPH could be found in QORs of some oomycetes species.

Enzymatic Assays. In T. thermophilus HB8 QOR, the entrance to the substrate-binding pocket is blocked by nearby residues L50, A51, and W243, which explains why the large substrates such as phenanthrenequinone cannot be reduced. In contrast, the access to the substrate-binding pocket in PcQOR, which is guarded by nearby residues A57, A56, and Q292, exhibits greater opening for substrates compared to T. thermophilus HB8 QOR. A57, A56, and Q292 locate above the nicotinamide moiety and establish a large opening through which NADPH may accomplish the hydride transfer (Figure 4A).

To characterize the enzymatic activity of PcQOR, we determined the enzymatic activities against substrates such as NQ and 9,10-phenanthrenequinone (PQ) kinetics. PcQOR catalyze transfer of electrons from NADPH to substrates. The C4 atom in the nicotinamide ring of NADPH is the key site where electron transfer occurs. Thus, the substrate-binding site is speculated to be located in the groove between the nicotinamide ring and domain1. We examine the region around the C4 atom of the nicotinamide moiety to acquire information about the substrate-binding site. The electrostatic potential surface of PcQOR—NADPH indicates that the substrate-binding site is located in a region surrounded by polar residues, which construct two sidewalls. The hydrophilic sidewall is composed of N43, R45, D46, Q48, Y54, S67, D68, C147, T148, and K341 from domain1, while the hydrophobic sidewall consists of V176, A293, and NADPH. Importantly, C147, T148, and V176 are within 5 Å of the C4 atom of the NADPH nicotinamide moiety. In addition, ethylene glycol is found in the substrate-binding pocket, held tightly by hydrogen bonds to the protein and NADPH (Figure 5A).

We failed to acquire crystals of the PcQOR tertiary complex with NADPH and ortho-quinones. Therefore, we docked two classical ortho-quinone substrates, PQ and NQ, and one typical ortho-quinone product, HNQ to the PcQOR—NADPH complex using the program Autodock4.2.6. Docking produced 10 clusters for PQ, NQ, and HNQ, respectively. For every docking substrate, we picked out the lowest-energy restraint, which is packed parallel to the nicotinamide ring. The carbonyl groups of quinone of HNQ and NQ point outward the N43, R45, Q48, C147, T148, and K341, while the carbonyl groups of quinone of PQ pointed inward the N43, R45, Q48, C147, T148, and K341. Importantly, all three molecules stack between NADPH and Y54. One carbonyl oxygen atom of HNQ and NQ interacts with the side chain of Y54 through a hydrogen bond, and the other carbonyl oxygen is stabilized by the C2 hydroxyl oxygen atom of the nicotinamide ribose and the side chain of Q48 (Figure 5B–D). Thus, we deduce that the polar residues in the active pocket create an ideal environment for catalysis.

Site-Directed Mutagenesis of PcQOR. To confirm the docking results, we detect the catalytic activities of mutants of R45, Q48, Y54, C147, and T148, which exhibit a stronger affinity to substrates predicted from the above docking pattern. The result discriminates that the five amino acids are essential for substrate binding (Figure 6). R45, Q48, and Y54 are predicted to stabilize the carbonyl group of the substrates. As
shown in Figure 6, the enzyme activities of the three mutants are very low, indicating that residues R45, Q48, and Y54 are essential for catalytic activity. C147 and T148 are predicted to form hydrogen bonds with the phenyl rings of ortho-quinones. Variants of these two amino acids show an almost complete loss of enzyme activity. All these imply that these five residues play an important role in shaping a suitable environment for catalysis of substrates. Although N43, D46, S67, D68, V176, and K341 are predicted to surround the substrate, mutations of these residues result in inapparent loss of activity. All these show that simulation of complex structures could provide important information of active sites.

We also measure the activity of some key residues interacting with NADPH. Mutation of Y216, which stacks against the adenine ring of NADPH, shows a great loss of enzyme activity, indicating the critical role of the hydrophilic tyrosine for NADPH stability. Similarly, mutagenesis of I263 and V291, which form strong interactions with the nicotinamide moiety of NADPH, results in a significant loss of activity. Therefore, we infer that most residues in the NADPH-binding groove strengthen the protein−cofactor interaction.

**Sequence Alignment.** Multiple sequence alignment shows that some active site residues, like N43, Y54, T151, V176, and K341, are conserved in homologous QORs, while differences are also found in some other active site residues. For instance, in the structure of PcQOR, the polar residue R45 replaces the corresponding hydrophobic residue Ile from other homologues and the polar residue C147 replaces the nonpolar residues Leu. In addition, some QORs like Zta1 from S.cerevisiae have been reported to be able to reduce the αβ double bond of alkenals/alkenones using two adjacent tyrosine residues as catalytic residues. However, in PcQOR, the glutamine replaces one tyrosine in Zta1, implying that PcQOR might not reduce the αβ double bond of alkenals/alkenones (Figure 7).

### CONCLUSIONS

This work reports the structure of the PcQOR−NADPH complex. Computational simulation combined with site-directed mutagenesis and enzymatic activity assays enable us to define the potential quinone-binding site of PcQOR. Based on these, we propose a catalytic mechanism of PcQOR. When quinone enters the active pocket, the substrate will be redistributed by the side chains of R45, Q48, Y54, C147, and T148 and the NADPH nicotinamide ring. Electron transfer proceeds once the phenyl ring of quinone stacks against the nicotinamide ring. The increased hydrophobicity around the positively charged nicotinamide cavity stimulates electron transfer from NADPH to the substrate in the ternary

---

**Figure 5.** Docking of HNQ, NQ, and PQ to (A) PcQOR−NADPH complex. Ethylene glycol and active residues of the interaction network are shown as sticks. Docking pattern of (B) HNQ, (C) NQ, and (D) PQ to PcQOR. Ligands and active residues are shown as sticks. NADPH colored blue is shown as a line. Distances <4.0 Å are indicated with dashed lines.

**Figure 6.** Enzymatic activity of site-directed mutants of PcQOR. Enzyme activity rates were tested by monitoring the decrease in absorbance of NADPH at 340 nm. Mutation of residues that were estimated to interact with substrates exhibited lower enzyme activity than other mutants. Each assay was conducted in triplicate, from which the average ± s.d. was calculated.
After reduction of the quinone carbonyl group, the hydrogen bonds between quinone and the side chain of R45, Q48, and Y54 are broken. As the reduction reaction is complete, the substrate-binding pocket opens to release the product.

**DATA AVAILABILITY STATEMENT**

The atomic coordinates and structure factors of the reported structure have been deposited in the Protein Data Bank under accession codes 7VEM. All other relevant data are available from the corresponding author upon request.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02954.

Sequence alignment of OQRs from some oomycetes, primers used in the study, and mutagenesis oligonucleotides (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**
Chunyuan Zhu — College of Life Sciences, Shandong Agricultural University, Taian 271018, China;
Email: zhuchunyuan@sdau.edu.cn
Authors
Cancan Yang — Shandong Provincial Key Laboratory for Biology of Vegetable Diseases and Insect Pests, College of Plant Protection, Shandong Agricultural University, Taian 271018, China; orcid.org/0000-0002-9841-3545
Zhenling Huang — Shandong Provincial Key Laboratory for Biology of Vegetable Diseases and Insect Pests, College of Plant Protection, Shandong Agricultural University, Taian 271018, China
Xiuguo Zhang — Shandong Provincial Key Laboratory for Biology of Vegetable Diseases and Insect Pests, College of Plant Protection, Shandong Agricultural University, Taian 271018, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c02954

Funding
This work was supported by the Major Science and Technology Innovation Project of Shandong Province (2019JZZY010714).

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We acknowledge the Shanghai Synchrotron Radiation Facility (SSRF) for providing resources for this research.

REFERENCES
(1) Bolton, J. L.; Trush, M. A.; Penning, T. M.; Monks, T. J. Role of quinones in toxicoology. Chem. Res. Toxicol. 2000, 13, 135–160.

(2) Qian, L.; Yang, F.; Lin, X.; Jiang, S.; Zhang, Y.; Tang, Y. Pyrroloquinoline quinone ameliorates liver injury in mice induced by cyclophosphamide. Environ. Sci. Pollut. Res. Int. 2022, 29, 30383–30393.

(3) Dinkova-Kostava, A. T.; Talalay, P. Persuasive evidence that quinone reductase type 1 (DT diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. Free Radiic. Biol. Med. 2000, 29, 231–240.

(4) Nioi, P.; Hayes, J. D. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nu2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. Mutat. Res. 2004, 555, 149–171.

(5) Qiu, D.; Song, S.; Wang, Y.; Bian, Y.; Wu, M.; Wu, H.; Shi, Y.; Duan, H. NAD(P)H: quinone oxidoreductase 1 attenuates oxidative stress and apoptosis by regulating Sirt1 in diabetic nephropathy. J. Transl. Med. 2022, 20, 44.

(6) Kim, I. K.; Yim, H. S.; Kim, M. K.; Kim, D. W.; Kim, Y. M.; Cha, S. S.; Kang, S. O. Crystal structure of a new type of NADPH-dependent quinone oxidoreductase (QOR2) from Escherichia coli. J. Mol. Biol. 2008, 379, 372–384.

(7) Feng, Y.; Li, W.; Li, L.; Wang, J.; Ge, J.; Xu, D.; Liu, Y.; Wu, K.; Zeng, Q.; Wu, J. W.; Tian, C.; Zhou, B.; Yang, M. Structural insight into the type-II mitochondrial NADH dehydrogenases. Nature 2012, 491, 478–482.

(8) Yagi, T. Bacterial NADH-quinone oxidoreductases. J. Bioenerg. Biomembr. 1991, 23, 211–225.

(9) Pan, X.; Zhang, H.; Gao, Y.; Li, M.; Chang, W. Crystal structures of Pseudomonas syringae pv. tomato DC3000 quinone oxidoreductase and its complex with NADPH. Biochem. Biophys. Res. Commun. 2009, 390, 597–602.

(10) Guo, P. C.; Ma, X. X.; Bao, Z. Z.; Ma, J. D.; Chen, Y.; Zhou, C. Z. Structural insights into the cofactor-assisted substrate recognition of yeast quinone oxidoreductase Zta1. J. Struct. Biol. 2011, 176, 112–118.

(11) Kiew, J. J.; Haystead, T.; Rudolph, J. Kinetic Mechanism of Quinone Oxidoreductase 2 and Its Inhibition by the Antimalarial Quinolines. Biochemistry 2004, 43, 4538–4547.

(12) Tedeschi, G.; Chen, S.; Massey, V. DT-diaphorase. J. Biol. Chem. 1995, 270, 1198–1204.

(13) Prestera, T.; Holtzclaw, W. D.; Zhang, Z. P.; Talalay, P. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc. Natl. Acad. Sci. U.S.A. 1993, 90, 2965–2969.

(14) Beyer, R. E.; Segura-Aguilar, J.; Di Bernardo, S. D.; Cavazzoni, M.; Fato, R.; Fiorentini, D.; Galli, M. C.; Setti, M.; Landi, L.; Lenaz, G. The role of DT-diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems. Proc. Natl. Acad. Sci. U.S.A. 1996, 93, 2528–2532.

(15) Li, R. B.; Blanchet, P.; Talalay, L. M.; Amzel, M. A. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprevention and chemotherapy: mechanism of the two-electron reduction. Proc. Natl. Acad. Sci. U.S.A. 1995, 92, 8846–8850.

(16) Porté, S.; Moeini, A.; Reche, I.; Shafqat, N.; Oppermann, U.; Farrés, J.; Parés, X. Kinetic and structural evidence of the alkenal/one reductase specificity of human ζ-crystallin. Cell. Mol. Life Sci. 2011, 68, 1065.

(17) Thorn, J. M.; Barton, J. D.; Dixon, N. E.; Ollis, D. L.; Edwards, K. J. Crystal Structure of Escherichia coliQR Quinone Oxidoreductase Complexed with NADPH. J. Mol. Biol. 1995, 249, 785–799.

(18) Jaiswal, A. K. Characterization and Partial Purification of Microsomal NAD(P)H:Quinone Oxidoreductases. Arch. Biochem. Biophys. 2000, 375, 62–68.

(19) Oppermann, U. Carboxyl reductases: the complex relationships of mammalian carboxyl- and quinone-reducing enzymes and their role in physiology. Annu. Rev. Pharmacol. Toxicol. 2007, 47, 295–322.

(20) Porté, S.; Crossas, E.; Yakovtseva, E.; Biosca, J. A.; Farrés, J.; Fernández, M.; Parés, X. MDR quinone oxidoreductases: the human and yeast zeta-crystallins. Chem. Biol. Interact. 2009, 178, 288.

(21) Huang, Q. L.; Russell, P.; Stone, S. H.; Zigler, J. S. ζ-Crystallin, a novel lens protein from the guinea pig. Curr. Eye Res. 1987, 6, 725–732.

(22) Garland, D.; Rao, P. V.; Corso, A. D.; Mura, U.; Zigler, J. S., Jr. ζ-Crystallin is a major protein in the lens of Camels dromedarius. Arch. Biochem. Biophys. 1991, 285, 134–136.

(23) Persson, B.; Zigler, J.; Jornvall, H. A Super-Family of Medium-Chain Dehydrogenases/Reductases (MDR). Sub-Lines including zeta-Crystallin, Alcohol and Polyol Dehydrogenases, Quinone Oxidoreductases, Enolyl Reductases, VAT-I and other Proteins. Eur. J. Biochem. 1994, 226, 15–22.

(24) Nordling, E.; Jornvall, H.; Persson, B. Medium-chain dehydrogenases/reductases (MDR). Eur. J. Biochem. 2002, 269, 4267–4276.

(25) Cedergren-Zeppezauer, E.; Samama, J. P.; Eklund, H. Crystal-structure determination of reduced nicotinamide adenine dinucleotide complex with horse liver alcohol dehydrogenase maintained in its apo conformation by zinc-bound imidazole. Biochemistry 1983, 22, 5761–5772.

(26) Rao, P. V.; Zigler, J. S., Jr. Purification and characterization of ζ-crystallin/quinone reductase from guinea pig liver. Biochim. Biophys. Acta 1992, 1117, 315.

(27) Gonzalez, P.; Rao, P. V.; Zigler, J. S. Molecular Cloning and Sequencing of ζ-Crystallin/Quinone Reductase cDNA from Human Liver. Biochem. Biophys. Res. Commun. 1993, 191, 902–907.

(28) Tumminia, S. J.; Vasanth Rao, P. V.; Zigler, J. S.; Russell, P. Xenobiotic induction of quinone oxidoreductase activity in lens epithelial cells. Biochim. Biophys. Acta 1993, 1203, 251–259.

(29) Shimomura, Y.; Kakuta, Y.; Fukuyama, K. Crystal Structures of the Quinone Oxidoreductase from Thermus thermophilus HB8 and Its Complex with NADPH: Implication for NADPH and Substrate Recognition. J. Bacteriol. 2003, 185, 4211–4218.

(30) Mano, J.; Yoon, H. J.; Asada, K.; Babychuk, E.; Inzé, D.; Mikami, B. Crystallization and preliminary X-ray crystallographic...
analysis of NADPH: azodicarboxyl/quinone oxidoreductase, a plant ς-crystallin. Biochim. Biophys. Acta 2000, 1480, 374−376.

(31) Mano, J.; Babiychuk, E.; Belles-Boix, E. B.; Hiratake, J.; Kimura, A.; Inzé, D.; Kushnir, S.; Asada, K. A novel NADPH:diamide oxidoreductase activity in Arabidopsis thaliana P1 ς-crystallin. Eur. J. Biochem. 2000, 267, 3661−3671.

(32) Mano, J.; Torii, Y.; Hayashi, S.; Takimoto, K.; Matsui, K.; Nakamura, K.; Inzé, D.; Babiychuk, E.; Kushnir, S.; Asada, K. The NADPH:Quinone Oxidoreductase P1-ς-crystallin in Arabidopsis Catalyzes the αβ-Hydrogenation of 2-Alkenals: Detoxication of the Lipid Peroxide-Derived Reactive Aldehydes. Plant Cell Physiol. 2002, 43, 1445−1455.

(33) Kim, S.-Y.; Mori, T.; Min, F. C.; Furuya, S.; Hakoshima, T. Structural insights into vesicle amine transport-1 (VAT-1) as a member of the NADPH-dependent quinone oxidoreductase family. Sci. Rep. 2021, 11, 2120.

(34) Wang, Z.; Li, L.; Dong, Y. H.; Su, X. D. Structural and biochemical characterization of MdDa from cariogenic Streptococcus mutans reveals an NADPH-specific quinone oxidoreductase. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2014, 70, 912−921.

(35) Crosas, E.; Porté, S.; Moeini, A.; Farrés, J.; Biosca, M.; Parés, X.; Fernández, M. R. Novel alkenal/one reductase activity of yeast NADPH:quinone reductase Zta1p. Prospect of the functional role for the ς-crystallin family. Chem. Biol. Interact. 2011, 191, 32−37.

(36) Kranthi, B. V.; Natarajan, B.; Rangarajan, P. N. Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, Pichia pastoris and its identification as zeta crystallin. Nucleic Acids Res. 2006, 34, 4060−4068.

(37) Lulli, M.; Nencioni, D.; Papucci, L.; Schiavone, N. Zeta-crystallin: a moonlighting player in cancer. Cell. Mol. Life Sci. 2020, 77, 965−976.

(38) Fernández, M. R.; Porté, S.; Crosas, E.; Barberí, N.; Farrés, J.; Biosca, J. A.; Parés, X. Human and yeast ς-crystallins bind AU-rich elements in RNA. Cell. Mol. Life Sci. 2007, 64, 1419−1427.

(39) LaVallie, E. R.; DiBlasio, E. A.; Kovacic, S.; Grant, K. L.; Sauter, N. K.; Terwilliger, T. C. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2002, 58, 1948−1954.

(40) Minor, W.; Cymborowski, M.; Otwinowski, Z.; Chruszcz, M. HKL-3000: the integration of data reduction and structure solution – from diffraction images to an initial model in minutes. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2006, 62, 859−866.

(41) Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 1997, 282, 307−326.

(42) McCoy, A. J.; Grosse-Kunstleve, R.; Adams, M., B.; Winn, B.; Storoni, C.; Read, A. Phasercrystallographic software. J. Appl. Crystallogr. 2007, 40, 658−674.

(43) Winn, M.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, K. S.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; Nichol, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S. Overview of the CCP4 suite and current developments. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2011, 67, 235−242.

(44) Adams, P. D.; Afonine, P. V.; Bunkóczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2010, 66, 213−221.

(45) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2004, 60, 2126−2132.

(46) Terwilliger, T. C.; Klei, H.; Adams, P. D.; Cohn, J. D.; Moriarty, N. W. Automated ligand fitting by core-fragment fitting and extension into density. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2010, 66, 915−922.

(47) Chen, V. B.; Arendall, W. B.; Headd, J. J.; Keedy, D. A.; Immormino, D. C.; Kapral, G. J.; Murray, L. W.; Richardson, J. S.; Richardson, D. C. MolProbity: All-Atom Structure Validation for Macromolecular Crystallography. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2010, 66, 12−21.

(48) Delano, W. L. The PyMol Molecular Graphics System. Proteins 2002, 30, 442−454.

(49) Schuck, P. Size-Distribution Analysis of Macromolecules by Sedimentation Velocity Ultracentrifugation and Lamm Equation Modeling. Biophys. J. 2000, 78, 1606−1619.

(50) Schuck, P. On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. Anal. Biochem. 2003, 320, 104−124.

(51) Zhao, B.; Guengerich, F. P.; Voehler, M.; Waterman, M. R. Role of Active Site Water Molecules and Substrate Hydroxyl Groups in Oxygen Activation by Cytochrome P450 15A2. J. Biol. Chem. 2005, 280, 42188−42197.

(52) Adams, P. D.; Grosse-Kunstleve, R. W.; Hung, W. H.; Joeger, T. R.; McCoy, T. C.; Moriarty, N. W.; Read, R. J.; Saschettini, J. C.; Sauter, N. K.; Terwilliger, T. C. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2002, 58, 1948−1954.

(53) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 2009, 30, 2785−2791.

(54) Huey, R.; Morris, G. M.; Olson, A. J.; Goodsell, D. S. A semiempirical free energy force field with charge-based desolvation. J. Comput. Chem. 2007, 28, 1145−1152.

(55) Rao, P. V.; Krishna, C. M.; Zigler, Z. J. Identification and characterization of the enzymatic activity of zeta-crystallin from guinea pig lens. A novel NADPH:quinone oxidoreductase. J. Biol. Chem. 1992, 267, 96−102.

(56) Rao, S. T.; Rossmann, M. G. Comparison of super-secondary structures in proteins. J. Mol. Biol. 1973, 76, 241.

(57) Zheng, Q.; Song, Y.; Zhang, W.; Shaw, N.; Zhou, W.; Rao, Z. Structural views of quinone oxidoreductase from Mycobacterium tuberculosis reveal large conformational changes induced by the co-factor. FEBS J. 2015, 282, 2697−2707.

(58) Holm, L.; Rosenström, P. Dali server: conservation mapping in 3D. Nucleic Acids Res. 2010, 38, W545−W549.

(59) Larkin, M. A.; Blackshields, G.; Brown, N. P.; Chenna, R.; McGettigan, P. A.; McWilliam, H.; Valentin, F.; Wallace, I. M.; Wilm, A.; Lopez, R.; Thompson, J. D.; Gibson, T. J.; Higgins, D. G. Clustal W and Clustal X version 2.0. Bioinformatics 2007, 23, 2947−2948.

(60) Robert, X.; Gouet, P. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res. 2014, 42, W320−W324.

(61) Eklund, H.; Samama, J. P.; Wallen, L.; Brandén, C.; Åkeson, A.; Jones, T. A. Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase with 2.9 Å resolution. J. Mol. Biol. 1981, 146, 561−587.