Structural Insight into Bioremediation of Triphenylmethane Dyes by Citrobacter sp. Triphenylmethane Reductase

Myung Hee Kim†1, Yoonjeong Kim‡, Hyo-Jung Park‡, Jong Suk Lee‡, Su-Nam Kwak‡, Woo-Hyuk Jung†, Seung-Goo Lee‡, Dooil Kim‡, Young-Choon Lee‡, and Tae-Kwang Oh†1

From the †Systems Microbiology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806 and the ‡College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea

Triphenylmethane dyes are aromatic xenobiotic compounds that are widely considered to be one of the main culprits of environmental pollution. Triphenylmethane reductase (TMR) from Citrobacter sp. strain KCTC 18061P was initially isolated and biochemically characterized as an enzyme that catalyzes the reduction of triphenylmethane dyes. Information from the primary amino acid sequence suggests that TMR is a dinucleotide-binding motif-containing enzyme; however, no other functional clues can be derived from sequence analysis. We present the crystal structure of TMR in complex with NADP⁺ at 2.0-Å resolution. Despite limited sequence similarity, the enzyme shows remarkable structural similarity to short-chain dehydrogenase/reductase (SDR) family proteins. Functional assignments revealed that TMR has features of both classic and extended SDR family members and does not contain a conserved active site. Thus, it constitutes a novel class of SDR family proteins. On the basis of simulated molecular docking using the substrate malachite green and the TMR/NADP⁺ crystal structure, together with site-directed mutagenesis, we have elucidated a potential molecular mechanism for triphenylmethane dye reduction.

The expanding use of synthetic dyes such as triphenylmethane, azo, and anthraquinone is alarming, given that the release of colored compounds into the environment may cause substantial ecological damage, not only due to their color, which may have an impact on photosynthesis in aquatic plants, but also because many dyes and their breakdown products may be toxic and/or mutagenic to living organisms (1). Biological decolorization and degradation is an environmentally friendly and cost-effective alternative to chemical decomposition (2). One key to efficient dye degradation is to use broad-spectrum and highly efficient dye-decolorizing microorganisms. Several microorganisms, such as bacteria, yeast, and fungi have been investigated for their ability to biodecolorize triphenylmethane dyes (3–9). Partial biochemical studies of the decolorization of triphenylmethane dyes have been carried out in fungi. These studies revealed that the laccase, peroxidase, and lignin peroxidase enzymes from fungi are involved in the decolorization of dyes (10, 11). Recently, a novel dye-decolorizing strain of bacteria, Citrobacter sp., was isolated from effluent-treated plant soil at a textile and dyeing industrial site. The extracellular culture filtrate of this strain showed a broad-spectrum decolorization efficiency for azo and triphenylmethane dyes (12). The enzyme that was responsible for the decolorization of triphenylmethane dyes was purified and biochemically characterized, and the gene encoding the enzyme was cloned (13). Based on biochemical analysis, the enzyme, designated TMR, catalyzes the NAD(P)H-dependent reduction of triphenylmethane dyes as a homodimer in solution, and has a substrate specificity that is dependent on the chemical structures of triphenylmethane dyes. The tmr gene encodes a 287-amino acid protein, and sequence analysis revealed the presence of a dinucleotide-binding motif (GXXGXXG) in the N terminus. Recently, using a transformation-based approach, a 60-kb plasmid, pGNB1, containing a triphenylmethane reductase gene and encoding a protein that is 99.7% identical to TMR, was isolated from a bacterial community in the activated sludge compartment of a wastewater treatment plant. Escherichia coli carrying pGNB1 showed a very similar dye-decolorization spectrum as TMR (14). The tmr gene has also been found in the food-borne pathogens Listeria monocytogenes (15) and Aeromonas hydrophila (7). Although efforts aimed at expanding the bioremediation of dye pollutants have made some progress, research into the development of bioremediation technologies have generally focused on the isolation and characterization of microorganisms able to decolorize synthetic dyes. Key information on the molecular mechanisms underlying the degradation of dye pollutants remains to be elucidated.

In this study, we attempted to extend our knowledge of bioremediation using a structural approach. We crystallized TMR using the introduction of surface entropy reduction method, and solved the TMR structure at a resolution of 2.0 Å. We describe the three-dimensional structure of TMR in complex with the cofactor NADP⁺. Structural inspection of TMR reveals that, although it exhibits a typical SDR fold, the functional characteristics of TMR differ from other members of the SDR family. Triphenylmethane reductase (SDR) family proteins. Functional assignments revealed that TMR has features of both classic and extended SDR family members and does not contain a conserved active site. Thus, it constitutes a novel class of SDR family proteins. On the basis of simulated molecular docking using the substrate malachite green and the TMR/NADP⁺ crystal structure, together with site-directed mutagenesis, we have elucidated a potential molecular mechanism for triphenylmethane dye reduction.

* This work was supported by funding from the 21C Frontier Microbial Genomics and Applications Center Program, MEST, Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence may be addressed. Tel.: 82-42-879-8219; Fax: 82-42-879-8595; E-mail: mhk8n@kribb.re.kr.

‡ To whom correspondence may be addressed. Tel.: 82-42-879-8200; Fax: 82-42-879-8595; E-mail: otk@kribb.re.kr.

3 The abbreviations used are: TMR, triphenylmethane reductase; TEV, tobacco etch virus; rTEV, recombinant TEV protease; SeMet, selenomethionine; PAL, Pohang Accelerator Laboratory; SDR, short-chain dehydrogenase/reductase.
Crystal Structure of Triphenylmethane Reductase

Experimental Procedures

Protein Expression and Purification—The tmr gene encoding TMR from Citrobacter sp. strain KCTC 18061P was amplified from pET-TMR (13) by PCR. The gene was subcloned into the EcoRI and HindIII sites of the expression vector pHis-Parallel1 (17), a hexahistidine (His$_6$) fusion protein expression vector containing a recombinant TEV protease (rTEV) cleavage site, to generate the overexpression plasmid pHisP-TMR. Mutations were introduced into pHisP-TMR using QuikChange (Stratagene), according to the manufacturer’s protocol. E. coli Rosetta-gami (DE3) (Novagen) harboring the overexpression plasmid was grown in LB-ampicillin medium at 37 °C until the cultures reached an A$_{600}$ of 0.6 and 0.8. The temperature was lowered to 25 °C, and protein expression was induced by treatment with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 14 h. The cells were harvested by centrifugation at 5,000 × g for 20 min at 4 °C. The cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, and 300 mM NaCl), and the cell suspensions were ultrasonicated. The crude cell extracts were centrifuged at 11,000 × g for 1 h at 4 °C. Cell lysis containing His$_6$-tagged protein was loaded onto a 10 ml nickel-nitrilotriacetic acid agarose (Qiagen) column that was pre-equilibrated with buffer A at 4 °C. The resin was washed with buffer A, and bound protein was eluted with buffer A containing 200 mM imidazole. The His$_6$-tag was then released from the protein by incubation with rTEV (Amersham Biosciences), followed by nickel-nitrilotriacetic acid agarose and size exclusion chromatography. After purification, the recombinant protein contained a seven-residue cloning artifact (GAMDPEF) in its N terminus. The homogeneity of the protein was assessed by 10% SDS-PAGE and Coomassie Blue staining. Purified protein was dialyzed against 20 mM Tris-HCl, pH 7.5, concentrated to 15 mg/ml using a YM-10 membrane (Amicon), and stored at −80 °C until use. Enzyme assays and kinetic analysis were performed with purified His$_6$-tagged protein in 50 mM Tris-HCl, pH 7.0. Gel-filtration analysis was carried out on a Superdex-75 10/30 column (Amersham Biosciences). The column was calibrated using the following molecular mass standards: ribonuclease (13.7 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), and bovine serum albumin (67 kDa).

Crystalization—A Leu-21 to methionine (L21M)/L235M double mutation was introduced into TMR to facilitate selenomethionine (SeMet) incorporation, because there are no naturally occurring methionines in the protein other than the first and last residues. However, we were unable to successfully crystallize this mutant as an x-ray diffraction quality crystal, despite extensive efforts. Therefore, surface entropy reduction (18) was carried out to improve the crystal quality of the mutant protein. Lys-22 and Lys-23 were replaced with alanine, and the SeMet-labeled L21M/K22A/K23A/L235M mutant protein was expressed in the Escherichia coli axotroph strain E. coli B834(DE3) (Novagen), which was grown in minimal medium supplemented with 50 mg/ml SeMet under the same conditions as the cells containing the native plasmid. The purification procedure for the SeMet-substituted protein was identical to that of the native protein, except for the addition of 5 mM methionine to all of the buffers. All of the crystallization trials were carried out using the sitting-drop technique at 21 °C. The initial trials were performed using the commercially available sparse-matrix screening kits Crystal Screen I and II from Hampton Research, and Wizard I and II from Emerald Biostuctures. Initial crystals of native TMR were produced using 0.05 M KH$_2$PO$_4$ and 20% polyethylene glycol 8000, and x-ray diffraction-quality crystals appeared in the presence of 0.05 M KH$_2$PO$_4$ and 18% polyethylene glycol 8000 in 1 week. Striking results were obtained with the surface entropy reduced L21M/K22A/K23A/L235M mutant protein. The mutant yielded crystal hits under four different screening conditions during the initial screening stage (data not shown). The best crystals of the SeMet-substituted mutant were obtained using 0.28 M Na$_2$HPO$_4$, 1.12 M K$_2$HPO$_4$, 0.2 M NaCl, and 0.1 M imidazole, pH 8.0, in 1 day.

Kinetic Analysis—The enzyme assay was performed as described previously by Jang et al. (13), with slight modifications. Typical assay mixtures contained 30 mM HEPES, pH 7.5, 20 μM basic fuchsin, 0.1 mM NADH, and a suitable amount of the enzyme in a total volume of 1 ml. The reaction was initiated by the addition of the enzyme at 30 °C and spectrophotometrically monitored at 544 nm. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 μmol of basic fuchsin per minute using a molar absorption coefficient of 116,000 M$^{-1}$ cm$^{-1}$. Kinetic parameters for basic fuchsin were collected at a concentration range of 2 to 80 μM with a constant concentration of NADH (0.1 mM). The kinetic constants for NADPH were determined in the concentration range of 40 to 200 μM in the presence of a fixed concentration of basic fuchsin (40 μM) at 340 nm using a molar absorption coefficient of 6,220 M$^{-1}$ cm$^{-1}$. At least three independent determinations were performed for each kinetic constant. All of the kinetic results were analyzed using SigmaPlot Version 8.0 software (SPSS Inc.).

Data Collection, Structure Determination, and Refinement—The structure of the L21M/K22A/K23A/L235M mutant was determined using the phases obtained from a single-wavelength anomalous diffraction experiment, with data collected at the selenium peak wavelength. Single-wavelength anomalous diffraction data for the SeMet-substituted mutant was collected at Pohang Accelerator Laboratory (PAL) beam line 6C at 2.5-Å resolution at −173 °C using crystals that were frozen in a cryosolution consisting of 0.32 M Na$_2$HPO$_4$, 1.28 M K$_2$HPO$_4$, 0.2 M NaCl, 0.1 M imidazole, pH 8.0, and 35% glycerol. The data for native TMR was collected at beam line 4A at PAL at 2.0-Å resolution, using crystals that were frozen in a cryo-solution consisting of 0.05 M KH$_2$PO$_4$, 25% polyethylene glycol 8000 and 10% glycerol. All of the x-ray diffraction data were processed and scaled using the HKL2000 software package (19). The SeMet-mutant crystal belongs to the space group P2$_1$2$_1$2$_1$. There are four molecules in the asymmetric unit with a packing density of 3.17 Å$^3$/Da, corresponding to an estimated solvent content of 61%. Anomalous differences from the selenium peak data set were used to locate the 12 selenium atoms using the program SOLVE (20). Density modification and subsequent
Crystal Structure of Triphenylmethane Reductase

Overall Structure of TMR—Analysis of the primary amino acid sequence of TMR using a publicly available program failed to reveal any structure-related functional information. Consequently, we generated crystals of a SeMet-incorporated variant of TMR (L21M/K22A/K23A/L235M) and used these crystals to solve the three-dimensional structure of the protein. Similarly, the structure of native TMR was determined, using the structure of SeMet-TMR as a model. The overall TMR structure consists of two domains. The large N-terminal domain is comprised of a Rossmann fold with a central core of parallel β-sheets (β1–β6 and β8) flanked by seven α helices (α1–α5, α7, and α11) and two α helices on both sides. The Rossmann fold generates an NAD(P)H-binding domain with a dinucleotide-binding motif (GXXGXGXG). Typically, the Rossmann fold is composed of three parallel β strands linked by two α helices in the topological order βαβαβ, and binds one nucleotide. Thus, the binding domain for dinucleotides such as NAD(P)H consists of two paired Rossmann folds that each bind one nucleotide moiety of the cofactor molecule. The cofactor-binding domain of TMR has three additional α-helices (α3, α7, and α11) and a β-strand (β8) that disrupt the symmetry of the Rossmann folds. The small C-terminal domain contains a parallel β-sheet (β7 and β9), five α helices (α6, α8–α10, and α12) and a 310 helix. This domain is assumed to function in substrate binding. The α10 and 310 helices, together with the last α helix (α5) of the Rossmann fold domain form a hydrophobic pocket that is immediately adjacent to the nicotinamide ring of the cofactor (Figs. 1a, 3a, and 3c, also see below).

A search conducted using DALI (28) revealed that TMR is structurally related to the SDR fold family of proteins (29), which includes the human NADP signaling protein HSCARG (PDB code 2EXX, Z = 26.8) (30), the Aspergillus nidulans negative transcriptional regulator NmrA (PDB code 1K6l, Z = 24.3) (31), basil eugenol synthase (PDB code 2QZZ, Z = 23.8) (32), Thuja plicata phenylecoumaran benzylc ether reductase (PDB code 1QYC, Z = 23.6) (33) and Arabidopsis thaliana SQD1, which is involved in the biosynthesis of the sulfoquinovosyl headgroup of plant sulfolipids (PDB code 1QRR, Z = 19.9) (34). This structural similarity would not have been readily inferred from sequence data alone, because the pairwise sequence identities of these proteins with TMR are very low (<19%). Key topological features in all of these proteins, however, are conserved, although the secondary structure content in the small C-terminal domain varies among the proteins (Fig. 1b). These differences in the C-terminal regions might lead to different substrate specificities, indicating that the SDR family is a functionally heterogeneous family of proteins. The most closely related structure to TMR appeared to be the NADP+ binding restructured HSCARG protein, with a root mean square deviation of 2.6 Å for 268 equivalent residues.

Quaternary Structure of TMR—Most of the SDR enzymes are dimers or tetramers in their active conformation (35). Although size exclusion chromatography revealed that native TMR behaves as a dimer in solution (data not shown), the crystal structure of native TMR has a single molecule in the asymmetric unit with the dimensions 58.8 × 141.7 × 37.8 Å. The crystal packing, however, reveals how the molecule generates a dimer contact with one other protein monomer. In addition, SeMet-TMR (L21M/K22A/K23A/L235M) contains four molecules in the asymmetric unit, which are arranged as a dimer of homodimers. A dimer with a 2-fold pseudosymmetry axis binds to another dimer with a second 2-fold pseudosymmetric axis perpendicular to the first axis. The long helices α4 and α5 (residues 79–97 and 116–132) in the large domain of each monomer pack against one another to form an antiparallel four-helix bundle at the subunit interface (Fig. 1c). Hydrophobic interactions by nonpolar residues are prominent at the interface, and a few hydrogen bonds also appear to play a major role in dimer stability. Residues involved in hydrophobic interactions at the interface are Thr-81, Leu-82, Ile-84, Val-85, Ala-88, Lys-92, His-119, Leu-122, Ala-123, Ala-127, and Thr-130. Hydrogen bonding side chains at the interface include Asp-79, Thr-81, Arg-95, and Thr-130. Asp-79 and Arg-95 also form salt bridges with each other. This dimer arrangement is consistent with the...
Crystal Structure of Triphenylmethane Reductase

FIGURE 1. The crystal structure of TMR in complex with NADP⁺. a, ribbon representation of native TMR. Alpha and 3- helices are shown in purple, β-strands in yellow, and loops in gray. The cofactor NADP⁺ is indicated. b, structural comparison of SDR family proteins. TMR, the human NADP signaling protein HSCARG (PDB code 2EXX) and basil eugenol synthase (PDB code 2QZZ) are displayed in yellow, green, and gray, respectively. The cofactors of each protein are depicted in the corresponding colors of each structure. c, the SeMet-TMR(L21M/K22A/K23A/L235M) molecule in the asymmetric unit is arranged as a dimer of homodimers. Each chain is indicated. d, dimer arrangement of TMR, which may be its active conformation. Unless otherwise noted, figures were prepared using PyMOL (W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA).

The cofactor is bound through a large number of polar and non-polar interactions (Fig. 2c). The adenine ring adopts an anti conformation to its sugar moiety and is stacked between the δ-guanido group of Arg-34 and the phenolic group of Tyr-54. The adenine ring (AN1) is further stabilized by hydrogen bonding with the Tyr-54 backbone amide. The adenine amino group is hydrogen-bonded with the Asp-53 side chain OD1. As is common for dinucleotide-binding proteins, the adenine is selectively recognized by the conserved motifs DHXX and DXXD in classic and extended SDRs, respectively (29). In TMR, this motif consists of DRYNQXX. The adenine-ribose adopts a C2'-endo pucker conformation, and its hydroxyl group is hydrogen-bonded with the backbone amide nitrogen of Gly-10. The 2'-phosphate groups AOP1 and AOP2 are hydrogen-bonded with the side chains OG1 of Thr-9 and NH2 of Arg-34, respectively. It is worthwhile noting that Arg-34 is the determinant basic residue of NADP⁺-preferring enzymes of the extended P1 SDR subfamily (29). The central diphosphate group forms hydrogen bonds with backbone amide nitrogens of Gln-11 and Leu-12, and with the guanidinium group of Arg-175. The nicotinamide ribose has the C3'-endo pucker confor-

results of PISA (36). The monomer surface area buried in the dimer interface is as large as 917 Å², whereas the next largest interface buries only 555 Å² of surface area. This interface has the highest possible PISA complexation significance score (CSS = 0.76), compared with a CSS of 0 for all other possible interfaces. These results suggested that the dimer structure arranged by the antiparallel four-helix bundle represents the active conformation of TMR (Fig. 1d).

Cofactor Binding to TMR—Although no additional cofactor was added during expression, purification, or crystallization, early in the refinement of native TMR, it became evident that the protein contained a molecule of NADP(H). The cofactor groups were well defined by their electron density, with the exception of the nicotinamide moiety, which appeared to have a somewhat poorly defined electron density, particularly at positions C2, C4, and C5, in comparison to the rest of the cofactors (Fig. 2a). The cofactor appeared to be in the oxidized form according to liquid chromatography-tandem mass spectrometry analysis (see supplemental data). We co-crystallized native TMR with 10 mM NADP⁺ under the same crystallization conditions as native TMR to improve the quality of the electron density map of the cofactor. Under these conditions, the crystal diffracted to a 1.96-Å resolution, and the refined model is of the negative transcriptional regulator NmrA (PDB code 1K6I), phenylcoumaran benzylic ether reductase (PDB code 1QYC) and SQD1 (PDB code 1QRK) proteins (data not shown).

The cofactor is bound through a large number of polar and non-polar interactions (Fig. 2c). The adenine ring adopts an anti conformation to its sugar moiety and is stacked between the δ-guanido group of Arg-34 and the phenolic group of Tyr-54. The adenine ring (AN1) is further stabilized by hydrogen bonding with the Tyr-54 backbone amide. The adenine amino group is hydrogen-bonded with the Asp-53 side chain OD1. As is common for dinucleotide-binding proteins, the adenine is selectively recognized by the conserved motifs DHXX and DXXD in classic and extended SDRs, respectively (29). In TMR, this motif consists of DRYNQXX. The adenine-ribose adopts a C2'-endo pucker conformation, and its hydroxyl group is hydrogen-bonded with the backbone amide nitrogen of Gly-10. The 2'-phosphate groups AOP1 and AOP2 are hydrogen-bonded with the side chains OG1 of Thr-9 and NH2 of Arg-34, respectively. It is worthwhile noting that Arg-34 is the determinant basic residue of NADP⁺-preferring enzymes of the extended P1 SDR subfamily (29). The central diphosphate group forms hydrogen bonds with backbone amide nitrogens of Gln-11 and Leu-12, and with the guanidinium group of Arg-175. The nicotinamide ribose has the C3'-endo pucker confor-
mation, and its hydroxyl groups are involved in hydrogen bonds with the carbonyl oxygens of Gly-75 and Ser-74. The nicotinamide ring adopts an anti conformation with its B-face stacked against the side chain of Tyr-143 and the A-face directed toward the substrate binding pocket. This orientation of the nicotinamide ring appears to also be influenced by interactions between the nicotinamide carboxyl and amide groups with the backbone amide of Tyr-143 and the carbonyl of Ala-141, respectively.

Critical residues involved in cofactor binding were assessed using site-directed mutagenesis. The cofactor binding residues Arg-34, Asp-53, Tyr-54, Tyr-143, and Arg-175, were mutated, as indicated in Table 2. Kinetic analysis revealed that replacement of these residues has a relatively small effect on $K_m$, but decreased the turnover number of TMR by 40- to 700-fold ($k_{cat}$). Thus, the catalytic efficiency ($k_{cat}/K_m$) was diminished 20- to 100-fold. These results, together with the crystallographic data, confirmed a key role for Arg-34, Asp-53, Tyr-54, Tyr-143, and Arg-175 side chains in NADP(H) binding.

Modeled Ternary TMR/NADP$^+$/Malachite Green Complex Structure—To gain insight into the catalytic mechanism of TMR, we prepared co-crystals of TMR in complex with cofactor and triphenylmethane substrates, such as malachite green and crystal violet, or their reduced products. However, these efforts were unsuccessful. We then performed a simulated docking of malachite green to the putative substrate binding pocket of TMR (PDB code 2VRB), which is easily identifiable by comparison with homologous structures and is lined by Pro-76, Tyr-78, Tyr-107, Ile-115, Leu-117, His-121, Leu-142, Phe-146, Phe-147, Phe-244, Ile-247, Ala-250, and Ile-251. The calculated volume of the substrate binding pocket was 943.2 Å$^3$ (Fig. 3c) (37). Malachite green docked well into the putative substrate binding site of the TMR/NADP$^+$ binary complex, located in the immediate vicinity of the nicotinamide ring, similar to other SDR family enzymes, making ideal interactions with the cofactor. The volume and hydrophobic environment of the pocket were consistent with the size and structural properties of the substrate. In the model, the distance between NC4 of the nicotinamide ring and C10 of malachite green is $\sim$3.8 Å, which is compatible with a direct hydride transfer from NADPH to the substrate (Fig. 3, a and b). The aliphatic and aromatic residues in the substrate binding site play a crucial role in substrate interaction. The phenylmethyl moiety of malachite green is anchored to a pocket formed by Leu-142, Phe-146, Phe-147,
Crystal Structure of Triphenylmethane Reductase

**TABLE 1**
Crystallographic data collection and refinement statistics
The numbers in parentheses describe the relevant value for the highest resolution shell.

| Dataset | SeMet mutant TMR | Native TMR | NativeTMR · NADP⁺ |
|---------|------------------|------------|------------------|
| Beamline (PAL) | 6C (MXII) | 4A (MXW) | 6C (MXII) |
| Wavelength | 1.97947 | 1.00000 | 1.23985 |
| Space group | P₂₁,₂₁,₂ | C222₁ | C222₁ |
| Cell dimensions (Å) | | | |
| A | 75.96 | 58.80 | 59.26 |
| B | 76.95 | 141.65 | 140.38 |
| C | 272.83 | 75.81 | 76.10 |
| Resolution (Å) | 2.5 (2.59-2.5) | 2.0 (2.07-2.0) | 1.96 (2.02-1.96) |
| No. of total reflections | 348,205 | 122,521 | 261,792 |
| No. of unique reflections | 56,316 | 21,741 | 22,401 |
| Completeness (%) | 100 (99.9) | 99.7 (99.5) | 95.9 (83.3) |
| Redundancy | 6.2 (6.0) | 5.6 (5.4) | 11.7 (7.8) |
| Rcryst (%) | 19.0/23.9 | 17.9/23.2 |
| I/σ(I) | 42.2 (8.45) | 19.33 (4.0) | 8.3 (39.6) |
| Refinement | | | |
| Resolution (Å) | 29.36-2.50 | 30.0-2.00 | 30.0-1.96 |
| Reflections in work/test sets | 51,196/2,747 | 20,569/1,106 | 21,246/1,142 |
| Rcryst/Robs (%) | 19.0/23.9 | 20.0/24.2 | 17.9/23.2 |
| Root mean square deviations | | | |
| Bond lengths (Å) | 0.013 | 0.016 | 0.013 |
| Bond angles (°) | 1.391 | 1.603 | 1.379 |
| Model composition | | | |
| 1,140 residues | 284 residues | 287 residues |
| 242 waters | 136 waters | 167 waters |
| 1 NADP⁺ | 5 glycerols | |
| Geometry | | | |
| Most favored regions (%) | 91.0 | 95.3 | 93.8 |
| Additional allowed regions (%) | 8.9 | 4.7 | 6.2 |
| Generously allowed regions (%) | 0.1 | 4.12 | 5.2 |
| PDB accession code | 2VRC | 2VRB | 2JL1 |

**TABLE 2**
Kinetic properties for NADPH of the TMR and cofactor-binding mutants

| TMR | Kₘ | kₗ | kₗ/Kₘ |
|-----|----|----|-------|
| WT | 370.56 ± 6.17 | 185.00 ± 7.07 | 4.99 ± 10⁻³ |
| R34V | 166.67 ± 3.18 | 3.45 ± 0.07 | 0.21 |
| D53L | 279.03 ± 4.70 | 1.50 ± 0.03 | 0.05 |
| Y54A | 538.64 ± 4.12 | 3.71 ± 0.03 | 0.07 |
| Y143F | 26.16 ± 0.89 | 0.26 ± 0.01 | 0.10 |
| R175L | 193.43 ± 2.77 | 4.62 ± 0.07 | 0.24 |

and Ile-251. The phenyl ring of the substrate is stacked with the phenyl of Phe-146 through a π-π interaction and stabilizes enzyme-substrate interactions that facilitate a proper distance for the hydride transfer from NADPH to malachite green. The dimethylaminophenyl moiety of malachite green makes hydrophobic contacts with Tyr-107, Ile-115, Leu-117, and His-121. The other dimethylaminophenyl moiety forms hydrophobic contacts with Pro-76, Tyr-78, and Ile-247. The pseudo-phenyl ring of the moiety is stacked against the cofactor nicotinamide ring, which is a common feature of the substrate binding modes of SDR family enzymes. The hydroxyl group of Tyr-78 interacts with the amino group of the dimethylaminophenyl moiety and allows the dissipation of the delocalized positive charge of malachite green. Tyr-78 located in the loop between β4 and α4 is highly flexible in the SeMet-substituted mutant TMR structure and swings in toward the catalytic center and out into the solvent active site, exhibiting remarkably different side-chain conformations in each chain (Fig. 3d). It is also highly flexible in the structure of native TMR. Thus, it is likely that Tyr-78 plays a role in substrate recruitment into the catalytic site.

To further explore the roles of potential key residues involved in substrate recognition, we performed site-directed mutagenesis of the targeting residues (Table 3). The mutations Y78A, I115A, L142D, and I247A resulted in a 2- to 50-fold decrease in kₗ and a 4- to 7-fold decrease in catalytic efficiency (kₗ/Kₘ). The mutations L117A, H121L, and I251A did not result in significant differences in catalytic efficiency as compared with wild-type TMR, whereas L117D, H121D, and I251S resulted in substantial decreases in catalytic efficiency. These results support the hypothesis that hydrophobic interactions between TMR and its substrates are key for catalysis. It is worthwhile noting that the positions of Leu-117 and His-121 correspond to the positions of the strictly conserved catalytic residues Tyr and Lys, respectively, in other SDR enzymes (see “Discussion”).

**DISCUSSION**

**TMR Comprises a Novel Class of SDR Family Proteins**—The SDR enzyme family has great functional diversity, with amino acid sequence identities that are only 15–30%. Their substrate spectrum ranges from alcohols, sugars, steroids, and aromatic compounds to xenobiotics (35). Despite low sequence identity, the three-dimensional structural folds can be superimposed on specific conserved sequence motifs that exhibit only small deviations. Variations in these motifs have been used to define SDR subfamilies. As expected, TMR contains an NAD(P)H-binding-
associated sequence motif ($^6$TGXXGXXG$^{13}$) in the N-terminal domain, which indicates that TMR belongs to the extended SDR family (29). The presence of the NADP(H)-preferring sig-

nature basic residue Arg-34 located at the first loop position after the second β-strand suggests that TMR can be further classified into the extended P1 SDR subfamily (29). On the other hand, the adenine amino group recognition motif 53DXXX56 in TMR indicates that the enzyme might belong to the classic SDRs. Surprisingly, functional assignments revealed that the TMR active site is unique. In general, SDR family enzymes have two invariant catalytic residues, Tyr and Lys, which are present in a conserved YXXXK (or YXXXMXXK) motif located in α5 (29). In addition, a Ser residue in a conserved GXXXXSS or S$\text{S}$$\text{S}$XXXXG motif located in β5 is also partly involved catalysis. These conserved catalytic residues are missing in TMR. The $^{117}$LXXXH$^{121}$ motif is present in the corresponding position, and although there is no equivalent Ser residue, Tyr-107 is present. These results suggest that TMR does not belong to any of the existing SDR subfamilies and may represent the first member of a new class of SDR family.

### Table 3

| TMR     | $K_m$ (μM)       | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$s$^{-1}$) |
|---------|------------------|----------------------|----------------------------------|
| WT      | 15.83 ± 0.63     | 0.49 ± 0.02          | 3.10                             |
| Y78A    | 29.29 ± 1.40     | 0.24 ± 0.01          | 0.82                             |
| Y107A   | 7.25 ± 0.60      | 0.11 ± 0.01          | 1.52                             |
| I115A   | 12.64 ± 0.63     | 0.11 ± 0.01          | 0.87                             |
| L117A   | 11.95 ± 0.66     | 0.39 ± 0.02          | 3.26                             |
| L117D   | 14.28 ± 0.68     | 0.01 ± 0.00          | 0.07                             |
| H121L   | 5.26 ± 0.27      | 0.16 ± 0.01          | 3.04                             |
| H121D   | 5.93 ± 1.04      | 0.03 ± 0.00          | 0.51                             |
| L142D   | 7.53 ± 0.17      | 0.02 ± 0.00          | 0.66                             |
| I247A   | 5.80 ± 0.40      | 0.03 ± 0.00          | 0.52                             |
| I251A   | 16.55 ± 1.02     | 0.79 ± 0.05          | 4.77                             |
| I251S   | 13.54 ± 5.54     | 0.05 ± 0.03          | 0.37                             |

### FIGURE 3

Model of the ternary TMR/NADP$^+$/malachite green complex structure. (a) the substrate malachite green was docked into the putative substrate binding site of the TMR-NADP$^+$ complex structure (PDB code 2VRB). Malachite green is shown in green. Residues involved in substrate binding are displayed in yellow. The cofactor NADP(H) is indicated. (b) schematic diagram depicting interactions between malachite green and its amino acid binding partners, identified with the program LIGPLOT (16). (c) surface representation, colored according to electrostatic potential. The cofactor, malachite green, and residues involved in substrate binding, as described for a are exhibited. (d) superimposition of TMR(L21M/K22A/K23A/L235M) in the asymmetric unit and native TMR (PDB code 2VRB). The location of Tyr-78 is illustrated by the red circle. The close-up view shows the side-chain conformation of Tyr-78 for each molecule.
enzymes. These differences might also explain why we were unable to extract structural or functional information from the primary amino acid sequence of TMR using the public data base. Sequence alignment using BLAST (38) revealed that the positions of the Leu and His residues of TMR are well conserved among a number of bacterial proteins of unknown function, showing similarity levels of 53–99% (Fig. 4). The putative substrate-binding residues in these proteins are similar to those of TMR, with a few exceptions. The NADPH determinant Arg and the adenine recognition Asp are also strictly conserved in this group of proteins. However, it is not clear whether these proteins possess similar functions to TMR. Nonetheless, the distinct active site and the presence of highly conserved sequence motifs that are a mix of classic and extended SDR family members suggest that these proteins define a novel class of SDR family enzymes.

Implications for Substrate Binding and Electron Transfer—The proposed catalytic mechanism of the typical SDRs is governed by a catalytic triad of Ser(Thr)-Tyr-Lys that is involved in oxidoreductase activity. Previous studies support the concept that, in general, the Tyr residue functions as the catalytic base, whereas the Ser (or Thr) residue stabilizes the substrate and the lysine forms hydrogen bonds with the nicotinamide ribose hydroxyl group and assists in the reprotonation of the tyrosine residue during catalysis (39). The standard reduction of SDRs is considered to take place through two steps: first, the cofactor
Hydride is transferred onto a polarized double bond in the substrate molecule, and second, the emerging negative charge on the substrate is neutralized in a subsequent protonation, probably using the catalytic triad. When TMR is superimposed with SQD1 (PDB code 1QRR) (34), one of the most structurally closed enzymes, the catalytic residues Thr, Tyr, and Lys are replaced with Tyr-107, Leu-117, and His-121, respectively, in TMR, suggesting that the catalytic chemistry of TMR is different than other SDRs (Fig. 5). In our mutagenesis analysis, L117D and H121D resulted in a 52- and 7-fold, respectively, decrease in catalytic efficiency, whereas L117A and H121L mutants had a similar catalytic efficiency as wild-type TMR (Table 3). These results imply that Leu-117 and His-121 function as catalytic residues in TMR through a hydrophobic interaction system with substrates, rather than the proton-relay reaction system that occurs in typical SDRs via hydrogen-bond and hydrophobic interactions, as described in the docking model. In this case, His-121 and Tyr-107 might be involved in a proton-relay catalytic reaction, and Leu-117 probably contributes to hydrophobic interactions with the substrate. A detailed understanding of the catalytic mechanism of TMR with real substrates in Citrobacter sp. remains to be elucidated.

Previous results showed that TMR decolorization activity is dependent on the chemical structure of the dyes (13). Our model, in combination with a structural inspection of the substrate binding pocket, might help explain the substrate specificity of TMR. The most efficient TMR substrate appeared to be malachite green, because it exhibited favorable structural features when modeled with the ternary complex. Crystal violet was a less favorable TMR substrate than malachite green, perhaps because of the additional dimethylamino group. The corresponding phenylmethyl moiety of malachite green is anchored to a pocket formed by Leu-142, Phe-146, Phe-147, and Ile-251, and stabilizes the enzyme-substrate interaction through a π-π stacking system with the phenyl of Phe-146. The magenta dye basic fuchsin, which has a positively charged amino group in each phenylmethyl moiety, may be a less favorable substrate in the hydrophobic environment of the substrate binding pocket as compared with malachite green. The structures of other dyes, such as brilliant green, bromphenol blue, methyl red, and Congo red, suggest that they would be incompatible with the size and hydrophobic restrictions of the pocket environment, resulting in no TMR activity in the presence of these dyes.

In summary, structural analysis of TMR enabled us to distinguish it as a unique member of the SDR family of proteins, in terms of functional motifs and its potential catalytic chemistry. Structure-based mechanisms of decolorization of triphenylmethane dyes will provide essential clues for the development of bioremediation systems.

Acknowledgments—We thank Drs. Kyung-Jin Kim and Ghyung-Hwa Kim at PAL (MXII 6C and MXW 4A) for help with data collection.

REFERENCES
1. Weisburger, J. H. (2002) Mutat. Res. 506–507, 9–20
2. Robinson, T., McMullan, G., Marchant, R., and Nigam, P. (2001) Biore- souer. Technol. 77, 247–255
3. Azmi, W., Sani, R. K., and Banerjee, U. C. (1998) Enzyme Microb. Technol. 22, 185–191
4. Cha, C. J., Doerge, D. R., and Cerniglia, C. E. (2001) Appl. Environ. Microbiol. 67, 4358–4360
5. Liu, W., Chao, Y., Yang, X., Bao, H., and Qian, S. (2004) J. Ind. Microbiol. Biotechnol. 31, 127–132
6. Jadhav, J. P., and Govindwar, S. P. (2006) Yeast 23, 315–323
7. Ren, S., Guo, J., Zeng, G., and Sun, G. (2006) Appl. Microbiol. Biotechnol.
Crystal Structure of Triphenylmethane Reductase

72, 1316–1321
8. Guerra-Lopez, D., Daniels, L., and Rawat, M. (2007) Microbiology 153, 2724–2732
9. Shedbalkar, U., Dhanve, R., and Jadhav, I. (2008) J. Hazard. Mater. 15, 472–479
10. Tekere, M., Mswaka, A. Y., Zvauya, R., and Read, J. S. (2001) Enzyme Microb. Technol. 28, 420–426
11. Shin, K. S., and Kim, C. J. (1998) Biotechnol. Lett. 20, 569–572
12. An, S. Y., Min, S. K., Cha, I. H., Choi, Y. L., Cho, Y. S., Kim, C. H., and Lee, Y. C. (2002) Biotechnol. Lett. 24, 1037–1040
13. Jang, M. S., Lee, Y. M., Kim, C. H., Lee, J. H., Kang, D. W., Kim, S. J., and Lee, Y. C. (2005) Appl. Environ. Microbiol. 71, 7955–7960
14. Schlu¨ter, A., Krahn, I., Kollin, F., Bo¨nemann, G., Stiens, M., Szcz-epanowski, R., Schneiker, S., and Pu¨hler, A. (2007) Appl. Environ. Microbiol. 73, 6345–6350
15. Nelson, K. E., Fouts, D. E., Mongodin, E. F., Ravel, J., DeBoy, R. T., Kolonay, J. F., Dodson, R. J., Madupu, R., Haft, D. H., Selengut, J., Van Aken, S., Kouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L. D., Uhlich, G. A., Bayles, D. O., Luchan-sky, J. B., and Fraser, C. M. (2004) Nucleic Acids Res. 32, 2386–2395
16. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) Protein Eng. 8, 127–134
17. Sheffield, P., Garrard, S., and Derewenda, Z. (1999) Protein Expr. Purif. 15, 34–39
18. Cooper, D. R., Boczek, T., Grelewksa, K., Pinkowska, M., Sikorska, M., Zawadzki, M., and Derewenda, Z. (2007) Acta Crystallogr. D Biol. Crystallogr. 63, 636–645
19. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
20. Terwilliger, T. C., and Berendzen, J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 571–579
21. Terwilliger, T. C. (2001) Acta Crystallogr. D Biol. Crystallogr. 57, 1755–1762
22. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
23. Jones, A. T., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110–119
24. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
25. Vagin, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
26. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
27. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) Nucleic Acids Res. 28, 235–242
28. Holm, L., and Sander, C. (1996) Science 273, 595–603
29. Kallberg, Y., Oppermann, U., Jörnvall, H., and Persson, B. (2002) Eur. J. Biochem. 269, 4409–4417
30. Zheng, X., Dai, X., Zhao, Y., Chen, Q., Lu, F., Yao, D., Yu, Q., Liu, X., Zhang, C., Gu, X., and Luo, M. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 8809–8814
31. Stammers, D. K., Ren, J., Leslie, K., Nichols, C. E., Lamb, H. K., Cocklin, S., Dodds, A., and Hawkins, A. R. (2001) EMBO J. 20, 6619–6626
32. Louie, G. V., Baiga, T. J., Bowman, M. E., Koeduka, T., Taylor, J. H., Spassova, S. M., Pichersky, E., and Noel, J. P. (2007) PLoS ONE 2, e993
33. Min, T., Kasahara, H., Bedgar, D. L., Youn, B., Lawrence, P. K., Gang, D. R., Halls, S. C., Park, H., Hilsenbeck, J. L., Davin, L. B., Lewis, N. G., and Kang, C. (2003) J. Biol. Chem. 278, 50714–50723
34. Mulichak, A. M., Theisen, M. J., Essigmann, B., Benning, C., and Garavito, R. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13097–13102
35. Joernvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jef-fery, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
36. Krispin, E., and Henrick, K. (2007) J. Mol. Biol. 372, 774–797
37. Binkowski, T. A., Naghibzadeg, S., and Liang, J. (2003) Nucleic Acids Res. 31, 3352–3355
38. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
39. Filling, C., Berndt, K. D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jörnvall, H., and Oppermann, U. (2002) J. Biol. Chem. 277, 25677–25684