Catalytic mechanism of 5-chlorohydroxyhydroquinone dehydrochlorinase from the YCII superfamily of largely unknown function

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Background: TftG is a YCII-superfamily dehydrochlorinase that catalyzes conversion of 5-chlorohydroxyhydroquinone to hydroxybenzoquinone.

Results: The TftG crystal structure in complex with product analog 2,5-dihydroxybenzoquinone illustrated the catalytic residues and mechanism.

Conclusions: A His-Asp dyad and other conserved signature residues are implicated for catalysis and substrate binding.

Significance: This is the first elucidation of a YCII-superfamily protein mechanism, helping to understand their obscure nature.

ABSTRACT

TftG, 5-chloro-2-hydroxyhydroquinone (5-CHQ) dehydrochlorinase, is involved in the biodegradation of 2,4,5-trichlorophenoxyacetate by Burkholderia phenoliruptrix AC1100. It belongs to the YCII superfamily, a group of proteins with largely unknown function. In this work, we utilized structural and functional studies, including the apo-form and 2,5-dihydroxybenzoquinone binary complex crystal structures, computational analysis, and site-directed mutagenesis, to determine the dehydrochlorination mechanism. The His-Asp dyad, which initiates catalysis, is strongly conserved in YCII-like proteins. In addition, other catalytically-important residues such as Pro76, which orients the His-Asp catalytic dyad; Arg17 and Ser56, which form an oxyanion hole; and Asp9, which stabilizes the oxyanion hole, are among the most highly conserved residues across the YCII superfamily members. The comprehensive characterization of TftG helps not only for identifying effective mechanisms for chloroaromatic dechlorination, but also for understanding the functions of YCII superfamily members which we propose to be lyases.

2,4,5-Trichlorophenol (2,4,5-TCP) is a toxic recalcitrant pollutant introduced into the environment through its extensive use as a herbicide, insecticide, and wood preservative (1,2). Like other chlorophenols, the major toxicity of 2,4,5-TCP results from its ability to uncouple mitochondrial oxidative phosphorylation, leading to subsequent convulsions, hyperthermia and possible death (3). 2,4,5-TCP is a product of the biodegradation of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (4,5).

Early investigation of Burkholderia phenoliruptrix illustrated that it could utilize 2,4,5-T as a sole carbon source. A 2,4,5-T-negative mutant, PT88, was also characterized and found to accumulate 5-chloro-2-hydroxyhydroquinone (5-CHQ) when grown in the presence of glucose and 2,4,5-T (4,6). Complementation of PT88 for growth on 2,4,5-T as a sole source of carbon identified a cluster of genes (tftEFGH) essential for the metabolism of the 5-CHQ intermediate (4-6). The revealed biodegradation pathway of 2,4,5-T B. phenoliruptrix begins with the conversion of 2,4,5-
T to 2,4,5-TCP by the oxygenase enzyme encoded by the tftA and tftB genes. 2,4,5-TCP is subsequently converted to 5-CHQ (4,6,7) by TftC and TftD. We previously reported the crystal structures and reaction mechanisms of TftC and TftD, which are reductase and monooxygenase enzymes respectively (8). 5-CHQ is then dehydrochlorinated into hydroxybenzoquinone (HBQ) by the enzyme TftG. The resulting HBQ is enzymatically reduced to hydroxyhydroquinone by a quinone reductase and then to β-ketoacidpate by TftH and TftE respectively (4). β-ketoacidpate, a common metabolite in the biodegradation of aromatic compounds, is further channeled into the tricarboxylic acid (TCA) cycle for complete mineralization (Fig. 1).

TftG, a relatively small (11,166 Da) protein, catalyzes aromatic dehydrochlorination, but its mechanism has been unknown. Sequence analysis suggests that TftG belongs to the YCII superfamily. All members of YCII share a conserved His-Asp dyad, of which the putative role has been suggested. This rapidly expanding family also contains strongly conserved Arg and Ser residues. The structural genomics effort has determined several crystal structures from this family, which confirmed similar placement of these residues, but functions of those proteins remain still unclear. Thus a structural and mechanistic understanding of TftG, a critical enzyme in the biodegradation of 2,4,5-T is useful not only for future informed bioremediation strategies, but also in better understanding the largely uncharacterized YCII superfamily. Here we report the crystal structures for both apo-form and the binary complex of TftG with the product analog 2,5-dihydroxybenzoquinone (2,5-DHBQ). This information together with accompanying enzymatic assays for site-directed mutants illustrates the biological function and potential reaction mechanism, which sheds light on the functions of YCII superfamily enzymes.

EXPERIMENTAL PROCEDURES

Chemicals - Chemicals were obtained from Sigma Aldrich or Fisher Scientific. Crystallization screens were obtained from Hampton Research.

Cloning and enzyme purification - The tftG gene was cloned into the pET30-LIC vector, and expressed as a C-terminal HIS6X fusion protein. 100 mL of Luria broth (LB) supplemented with 30 μg/mL kanamycin was inoculated with a freezer stock of pET30A TftG in BL21 (DE3) cells and incubated overnight at 37 °C with constant shaking at 250 rpm. The 100 mL culture was then used to inoculate 1.5 L of LB medium. TftG expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 0.3 mM final concentration at mid-log phase (A600 = ~0.6). Following induction, the cells were further incubated for 12 hr at 22 °C with constant shaking at 250 rpm. The cells were then harvested by centrifugation (3,000 × g), after which the pellet was frozen to promote cell lysis. The pellet was thawed at room temperature and suspended in a minimal volume of lysis buffer (50 mM Tris, 300 mM NaCl, 20 mM imidazole, and 1 mM dithiothreitol, pH 8.0). The cell suspension was sonicated 5 times for 10 seconds each using a model 450 Sonifier® (Branson Ultrasonics), and the resulting lysate cleared by centrifugation (20,000 × g for 30 min). Lysate was applied to a nickel-nitrilotriacetate column and washed with several column volumes of lysis buffer. Elution buffer consisted of lysis buffer supplemented with 250 mM imidazole. Eluted fractions containing TftG were combined, concentrated, and buffer-exchanged into 20 mM Tris (pH 8.5) with 1 mM DTT by ultrafiltration in an Amicon 8050 cell with a 5-kDa cutoff polyethersulfone membrane (Millipore), loaded onto a Mono Q™ GL10/100 anion-exchange column (GE Healthcare), and eluted at 200 mM NaCl with a linear NaCl gradient of 0 to 2 M NaCl using a preparative HPLC (Akta Explorer, GE Healthcare). Fractions containing TftG were pooled, concentrated, and exchanged into 20 mM Tris (pH 7.5) with 1 mM DTT. Final homogeneity of purified TftG was estimated over 99 %.

Selenium auxotroph B834 cells were also transformed with the same tftG pET30-LIC plasmid. Expression and purification of selenium-substituted TftG was carried out by the same protocol as native TftG.

Data collection and structure determination - TftG at 50 mg/mL in 20 mM Tris, 1 mM DTT, pH 7.5 was crystallized at 4 °C using the hanging drop vapor-diffusion method. 1.5 μl of TftG was mixed with 2.5 μl of the precipitant solution containing 0.2 M potassium citrate, 0.1 M Sodium citrate, pH 5.0. Crystals were cryoprotected by equilibrating against the same solution supplemented with 30% (25% for selenium analog) glycerol and flash cooled with liquid nitrogen.
with 1.5 μl of a reservoir solution containing 30 % (w/v) polyethylene glycol 1500 and equilibrated against the same reservoir solution. This condition was used for both native TftG and selenium-substituted TftG crystals. Diffraction-quality crystals appeared after three weeks. Selenomethionyl-TftG diffraction data was collected on a Rigaku FR-E+ Superbright rotating anode dual wavelength x-ray source. Cryoprotectant consisted of 20 % (v/v) glycerol in reservoir buffer. Initial data collection for Selenium-SAD phasing was conducted using chromium radiation (2.29 Å) at 100 K and processed using d*TREK (9). Experimental SAD phasing was conducted using PHENIX AutoSol followed by one cycle of model-building in PHENIX Autobuild (10), resulting in an electron density map in which 94 out of 100 residues were correctly modeled. Higher resolution (1.6 Å) apo-form data was collected at the Advanced Light Source (ALS) beamline 8.2.1. A binary complex crystal of TftG with 2,5-dihydroxybenzoquinone was formed by soaking an apo-form TftG crystal with 2,5-dihydroxybenzoquinone and followed by cryoprotection in 30 % (w/v) polyethylene glycol 1500 supplemented with 20 % glycerol. Binary complex diffraction data was collected at the ALS (BL8.2.1) and processed using HKL2000 (11). The atomic model provided by the selenomethionyl-TftG chromium dataset, was used for refinement with the ALS data described above. The TftG-Apo structure had 100 % of residues in Ramachandran favored regions. Selenomethionyl-TftG had 99.19 % of residues in preferred regions and 0.84 % in allowed regions with no outliers. The TftG-2,5-DHBQ binary complex structure had 97.94 % of residues in favored regions, 1.03 % in allowed and 1.03 % outliers. The coordinates for TftG-apo, selenomethionyl-TftG and TftG-2,5-DHBQ binary complex have been deposited in the Protein Data Bank under PDB ID 4LBH, 4LBI and 4LBP, respectively.

Multi-angle light scattering (MALS) and Isothermal titration calorimetry (ITC) - The weight-average molecular mass of TftG was measured by combined size exclusion chromatography and multi-angle laser light scattering as described previously (12). Briefly, 200 μg of TftG was loaded onto a BioSep-SEC-S 2000 column (Phenomenex) and eluted isocratically with a flow rate of 0.5 ml min⁻¹. The eluate was passed through a tandem UV detector (Gilson), Optilab DSP interferometric refractometer (Wyatt Technology), and a Dawn EOS laser light scattering detector (Wyatt Technology). Scattering data was analyzed using the Zimm fitting method with software (ASTRA) provided by the instrument manufacturer.

Isothermal titration calorimetric reactions were carried out in a VP-ITC instrument (MicroCal). The protein was prepared for ITC by extensive buffer exchanging into titration buffer (20 mM Tris, pH 8.5). The concentration of protein in the calorimetric reaction cell was diluted to 100 μM. All titrations were performed at 25 °C with a stirring speed of 300 rpm and 29 injections (10 μL each). Ligands were diluted into the same titration buffer and injected into the cell containing TftG solution, and the heats of binding were recorded. Ligands were also titrated against buffer to account for heats of dilution. Ligand concentrations were adjusted to obtain significant heats of binding. All samples were degassed prior to titration.

Atomic absorption spectroscopy - A standard curve of ZnCl₂ (0 - 100 μM, 20 mM Tris, pH 7.5) was generated using a Shimadzu AA-6200 atomic absorption spectrophotometer at the atomic absorption wavelength of zinc (213.9 nm). TftG samples were measured at 20, 40, 60, and 80 μM protein concentration to detect any bound zinc.

Enzyme assays of mutant TftG - TftG mutants (R17A, H24A, S56A, D75N, and H96A) were generated using a QuikChange Lightning site directed mutagenesis kit (Stratagene, CA) and transformed into XL-10 Gold ultra-competent cells. DNA sequencing was used to confirm mutation, and the resulting plasmids were transformed into BL21(DE3) expression cells. Purification of mutant TftG was the same as that of wild type. 5-CHQ was enzymatically produced by TftD as previously described (13). Briefly, purified TftD (1 mg/ml) and Escherichia coli flavin reductase (Fre) (5 μg/ml) was incubated with 100 μM 2,4,5-trichlorophenol (2,4,5-TCP) in the presence of 10 μM flavin adenine dinucleotide (FAD), 2 mM NADH and 2 mM ascorbate in 100 μl of 40 mM potassium phosphate buffer at 25 °C for 10 min. Fre utilized NADH to reduce FAD to FADH₂, and TftD oxidized 2,4,5-TCP to 5-CHQ with O₂ and...
FADH$_2$ as the co-substrates. The complete conversion of 2,4,5-TCP to 5-CHQ was confirmed by HPLC analysis. Then, the reaction mixture was aliquoted to 20 µl each, and 1 µl of TftG was added and mixed. The reaction was incubated at 25 °C for 1 min, and 20 µl of 10 % acetic acid in acetonitrile was added to stop the reaction. After centrifugation to remove protein precipitates, the supernatant was analyzed by HPLC for 5-CHQ consumption. Assays were run in triplicate and specific activities were calculated for TftG and its mutants.

Circular dichroism spectroscopy – CD spectroscopy was utilized to investigate the effect of mutations on secondary structure. CD spectra for each wild-type and mutant TftG protein was measured from 200 to 300 nm wavelength using an AVIV 202SF spectropolarimeter (AVIV Biomedical) at 25°C. The TftG samples were prepared in 5 mM potassium phosphate (pH 7.0) at a protein concentration of 10 µM.

Quantum mechanics/molecular mechanics calculations – Unless otherwise stated, all calculations were performed in the gas phase using Gaussian 09 (G09) (14) and utilized double-zeta correlation consistent basis sets (15) with augmented functions on all atoms but hydrogen (hereafter referred to as aVDZ). ONIOM (16) calculations were assisted by the Toolkit to Assist ONIOM (17) The residues allowed to move during optimizations were Ile5, Arg7, Asp9, Arg17, Ile18, Tyr21 - Leu25, Lys32, Ile35, Gly38 - Pro40, Met53, Gly55 - Leu58, Phe71, Val72, Asp75 – Phe77, Leu82, Phe83, Gly94 – Asp98; all but the carbon of an additional C-terminal N-methyl (NMe) cap were allowed to move. 5-chloro-2-hydroxyhydroquinone was generated in GaussView 3.09 (18) and optimized with tight convergence criteria, followed by a frequency calculation, at the B3LYP (19-21)/aVDZ level of theory. Ligand harmonic stretch and bend AMBER molecular mechanics (MM) force-field (22) parameters were generated from these frequencies using the parafreq utility (23); chlorine non-bonded parameters were taken from the parm99 parameter set as found in AmberTools 13, (22) and dihedrals/improper torsions involving chlorine were assigned by analogy with the hydroxyl OH atom type. The geometry-optimized ligand was inserted into the NMe-capped TftG product-analogue complex crystal structure with Lys95 converted to Ala and hydrogen atoms added using the PHENIX ReadySet! program, (24) with His24 as Nδ-His and His96 doubly protonated; all other histidines were kept singly-protonated. This model was converted into a dimer via symmetry operations, followed by optimization of its key residues (listed above), the ligand, and all hydrogen atoms using PM7 semi-empirical theory (25) with the MOZYME linear-scaling method (26) as implemented in MOPAC2012 (27). Restricted electrostatic potential (RESP) charges (28) were then fitted to the MOZYME-optimized Arg7/His24/Asp75 and Asp9/Arg17/Ser56 trios via Merz-Kollman-Singh (29) electrostatic potential fitting (overlay 6/33=2) at the CAM-B3LYP (30)/aVDZ level of theory, generating the ONIOM input structure. Hydrogen atom geometries of the input structure were first re-optimized using a two-layer ONIOM scheme with PM6 semi-empirical theory (31) for the model system (defined as the ligand and the side chains of Arg7, Arg17, Asp9, Asp75, Asp96, His24, His96, Ser56, and Tyr21) and the AMBER MM force field for the real system. Following this, the ligand and key residues were optimized using a three-layer ONIOM scheme with CAM-B3LYP/aVDZ for the model system, PM6 for the intermediate system, and the AMBER force field for the real system. Residues included in the model and intermediate systems, with their approximations shown as (model/intermediate), were Arg7 and Arg17 (N-methylguanidinium/N-propylguanidinium); Asp9 and Asp75 (acetate/propanoate); Tyr21 (phenol/4-ethylphenol); His24 (1H-imidazole/4-propyl-1H-imidazole); Ser56 (ethanol/ethanol); the Pro76/Phe77 peptide bond (N-methylacetamide, intermediate layer only); and the C-terminal tail (His96 as imidazolium and Asp98 as acetate/His93 as Ala plus Gly94 through the NMe cap in its entirety).

RESULTS

The apo-form TftG crystallized in a hexagonal space group with one molecule in the asymmetric unit. A crystallographic symmetry operation of monomeric TftG generated a tetramer, which was consistent with the tetrameric nature of TftG in solution (Fig. 2). In order to obtain a binary structure of TftG, a product analogue, 2,5-DHBQ,
Catalytic mechanism of a YCII-like dehydrochlorinase was diffused into an apo-form crystal by a soaking approach.

**Global Structure** - Apo-form TftG resembled a ferredoxin-like core fold. Each subunit consisted of four beta strands (β1-β4) arranged in an antiparallel beta sheet that were flanked by three opposing alpha helices (α1, α3, and α4) (Fig. 3a). The tetrameric assembly of TftG establishes through the interaction of two dimeric α-β barrels (Fig. 3b and 3c). Hydrogen bonding between the β4 strands (Residues 84-88) from two different dimeric α-β barrels forms a continuous 8-stranded anti-parallel β-sheet. There are also several electrostatic interactions such as Lys8-Glu89 and Arg90 with the carbonyl of Gly47, which stabilize the tetramer.

A structural alignment between the apo-form and binary complex with 2,5-DHBQ illustrated no large structural deviations (r.m.s.d = 0.193 Å). One noticeable structural difference was the improved electron density corresponding to residues 95-98 in the binary complex structure due to its hydrogen bonding with the substrate.

**Isothermal titration calorimetry (ITC)** - ITC was employed to find any suitable substrate analog for obtaining binary complex crystals. Tested ligands included hydroquinone, 5-chlorohydroquinone, catechol, and 2,5-DHBQ. The only compound with significant affinity was 2,5-dihydroxybenzoquinone (Fig. 4) with \( K_d = 11.4 \) µM, which led us to pursue a binary complex. The association of 2,5-DHBQ with TftG had a considerable enthalpic contribution, \( \Delta H = -6.5 \text{ kcal mol}^{-1} \), likely due to the observed hydrogen bonds between ligand and residues of TftG, which was later confirmed in the binary crystal structure. In addition, the positive entropic contribution, \( \Delta S = 0.79 \text{ cal mol}^{-1} \text{ degree}^{-1} \) was observed suggesting the liberation of water molecules from the active site upon ligand association.

**Active site** - As indicated in Fig. 3 and Fig. 5a, a distinct solvent accessible pocket was noticed between β2-β3 and α1 in apo-form TftG. Coincidently, the \( F_o-F_i \) maps calculated from the 2,5-DHBQ-soaked crystal diffraction data showed clear electron density for 2,5-DHBQ in this pocket. The polar residues comprising the pocket are Arg17, His24, Ser56, Asp75, His96, and Asp98. The C-terminal residues of the adjacent subunit, His96 and Asp98, close off the active site trapping the ligand inside (Fig. 5b).

The His24 and Asp75 were proximate to each other, establishing a catalytic dyad, such that the Asp75 O\(^\delta_1\) was hydrogen bonded to the His24 N\(^\varepsilon_2\). The His24 N\(^\varepsilon_2\) atom also established a hydrogen bond with the 2-hydroxyl group of 2,5-DHBQ. In addition, the side chains of Arg17 and Ser56 were both within hydrogen bonding distance to each other and to 2,5-DHBQ at its 1-carbonyl oxygen. The His96 N\(^\varepsilon_2\) atom was within hydrogen bonding distance of either the 4-carbonyl oxygen or the 5-hydroxyl group of 2,5-DHBQ. Asp98 was within hydrogen bonding distance of the 4-carbonyl oxygen of 2,5-DHBQ (Fig. 5b). A hydrogen bond network continued away from the His24-Asp75 and Arg17-Ser56 dyads, including a salt-bridge between the side chains of Arg7 and Asp75, and another between the side chains of Asp9 and Arg17.

**Quantum mechanics/molecular mechanics (QM-MM) substrate modelling** - Quantum mechanics/molecular mechanics was employed to investigate placement of the native substrate 5-CHQ and surrounding catalytic residues. The r.m.s.d between the crystal structure and three-layer ONIOM-optimized structures was 0.3 Å. The orientations of the key catalytic residues and other residues forming the active site pocket showed little change, with the major changes being due to movement of the C-terminal tail. The position of 5-CHQ had similar orientation to the crystallographic position of the product analog 2,5-DHBQ with the chlorine atom facing out of the binding pocket towards the C-terminal tail (Fig. 5c).

**Activity assays and CD spectra of TftG mutants** - Activity assays of Arg17Ala, His24Ala, Ser56Ala, and His96Ala site-directed mutants were performed to test their potential catalytic roles (Table 2). The mutant Asp75Asn could not be tested due to solubility issues. The His24Ala mutant exhibited the least amount of activity, which was less than 1 % specific activity relative to wild type. Arg17Ala and His96Ala also displayed quite low activity (~1 %) and Ser56Ala retained 5 % activity.

The overall secondary structure as evaluated by CD spectra appeared unperturbed relative to wild-type for all mutants except His24Ala mutant. (Fig. 6). The crystal structure
illustrated the residue His24 was located in α1 and Asp75 was located at the C-terminal end of α3. The side chains of those residues, contributed from two different helices, were within hydrogen-bond distance and thus appeared to be important for structural integrity of the active site pocket. Considering the location of the functional imidazole ring of His24 and its mutant CD data, it is likely that His24 could be important for both structural and catalytic roles.

DISCUSSION

Structural homologs of TftG - TftG is a recently discovered dehydrochlorinase enzyme that catalyzes a cofactor-independent dehydrochlorination of 5-CHQ to produce 2-hydroxy-1,4-benzoquinone. There has been a critical gap in structural and mechanistic information regarding the dechlorination of chlorocatechols like 5-CHQ, hampering an overall understanding of their catabolic mechanism.

In order to firmly establish a classification of TftG and to find any structural homologs, a DALI (32) search was performed against deposited structures in the Protein Data Bank (PDB). The search found several proteins with similar structures, but relatively low sequence identity to TftG. All those matches had promiscuous or unknown functional roles. The highest match was the HI0828 protein of unknown function from Haemophilus influenza (PDB: 1MWQ) (33), with a Z-score of 14.3 and 27 % sequence identity. The second highest match was 5-chloromuconolactone dehalogenase of Rhodococcus opacus 1Cp (PDB: 3ZNU) (34), with a Z-score of 10, but displayed lower sequence identity (23 %). Another protein of unknown function, TM1266 from Thermotoga maritima (PDB: 2NZC), shared reasonable structural similarity with a Z-score of 8.5 and sequence identity of 11 %. All the following proteins in the DALI search list had substantially decreased Z-scores and sequence identity, which included a bacterial actinorhodin biosynthesis monooxygenase (ActVa-Orf6) (35), bacterial muconalactone isomerase (36) and the C-terminal domain of archaeal LprA (37).

Detailed structural investigation of TftG with a manual superposition of HI0828 protein (PDB: 1MWQ) illustrated a similar active site pocket as shown in TftG with the exception of Arg at position 21 instead of Tyr. However, HI0828 has a zinc ion at the active site and a phosphohistidine has been proposed to be involved in activity. Atomic absorption spectroscopy was conducted to test for the presence of zinc ions in TftG, however, no zinc ions were indicated at all tested concentrations of TftG. The lack of any zinc ion in the crystal structure of TftG and supporting atomic absorption evidence shows that zinc is not involved in TftG catalysis. It is possible that the presence of zinc ion in the HI0828 active site is an artifact from the crystallization condition and HI0828 could be a dehalogenase. However, the HI0828 quaternary structure is a dimer while the TftG quaternary structure is a tetramer (Fig. 7a). Thus, our speculation about HI0828 from Haemophilus influenza needs to be tested.

The next match in our DALI search, 5-chloromuconolactone dehalogenase (PDB: 3ZNU) belongs to the muconolactone delta-isomerase (MIase) [pfam02426] superfamily. Members of this protein family (EC 5.3.3.4) are involved in the metabolism of catechols. 5-chloromuconolactone dehalogenase has a ferredoxin-like fold similar to TftG, but it does not have a conserved His-Asp dyad distancing itself from TftG and YCII superfamily. Instead, it has a conserved Glu27 acting as a catalytic base. The oligomeric structure of 5-chloromuconolactone isomerase was also quite different, existing as a decamer (Fig. 7b).

Although it was not listed in our DALI search due to low level of structural similarity with TftG, LinA from Sphingobium japonicum catalyzes a dechlorination reaction for γ-hexachlorocyclohexane (38,39). LinA belongs to the SnoaL-4 Superfamily (cl17707), which are all polyketide cyclases that share the SnoaL fold. LinA exists as a homotrimer, and each subunit forms a cone-shaped α+β barrel fold. Although LinA and TftG share little similarity (18 % sequence similarity and 4.6 Å r.m.s.d. in their Cα positions), manual Cα chain alignment of LinA and TftG displayed an approximately similar location of the active site. In addition, the exceedingly hydrophobic substrate-binding pocket of LinA contains the same proposed catalytic dyad of His73 and Asp25. The carboxyl group of Asp25 of LinA has been proposed to abstract a proton from imidazole side chain of His73 thereby increasing
the basicity of the imidazole nitrogen (38). The imidazole nitrogen then acts as a catalytic base, abstracting a proton from the carbon of \( \gamma \)-hexachlorocyclohexane ring, ultimately leading to a spontaneous loss of chlorine from the ring (38). The oligomeric structure of LinA was a trimer, further setting it apart from TftG and 5-chloromuconolactone isomerase (Fig. 7c).

LinA, TftG, and 5-chloromuconolactone dehalogenase belong to different enzyme superfamilies and use different substrates. TftG catalyzes proton abstraction from an oxygen atom of an aromatic compound; whereas, 5-chloromuconolactone dehydrohalogenase and LinA catalyze proton abstraction from a carbon atom of non-aromatic substrates.

**Sequence alignment with other YCII family members** - In order to further understand the relation of TftG to other members of the YCII superfamily we conducted a BLAST (40) search of all non-redundant protein sequences. The results revealed many YCII-like proteins, some with high identity (40 - 45 %), but all were of unknown function. Inspection of a sequence alignment of the top 100 proteins illustrated a conserved signature sequence of residues that were both implicated in TftG catalysis and conserved throughout the YCII superfamily. An alignment of the top 20 highest sequence identity proteins illustrates this signature sequence (Fig. 8). As expected, the His24-Asp75 dyad was conserved throughout the family. In addition, Pro76 that is responsible for stabilizing and orienting the catalytic dyad in TftG, was conserved. Another set of residues conserved across the YCII superfamily were Asp9, Arg17, and Ser56. Both Arg17 and Ser56 were responsible for forming an oxyanion hole critical for catalysis in TftG. Asp9 in TftG electrostatically interacts with Arg17, ultimately keeping Arg17 in the proper orientation for oxyanion charge stabilization on the substrate. In summary, Asp9, Arg17, His24, Ser56, Asp75, and Pro76 are conserved throughout the YCII superfamily. Expansion of the BLAST search/sequence alignment to include dozens of lower identity proteins from YCII superfamily still maintains those residues, and thus this conservation of Asp9, Arg17, His24, Ser56, Asp75, and Pro76 is likely quite important for functions throughout the YCII superfamily and could serve as its signature.

**Catalytic mechanism of TftG for 5-CHQ** - The crystal structure of the 2,5-DHBQ binary complex of TftG in combination with the QM:MM results for 5-CHQ, and the site-directed mutagenesis illustrated a plausible catalytic mechanism for TftG. The resting state of TftG likely contains water in the active site as supported by our ITC results and the presence of PEG in its apo-form crystal structure. The QM:MM results confirmed that 5-CHQ would bind in a near identical angular orientation to 2,5-DHBQ as expected from the relatively tight fit of the TftG active site. The computational results also illustrated the 1- and 2-hydroxyl groups of 5-CHQ oriented towards His24 and the Arg17/Ser56, respectively. Upon association of 5-CHQ, all water molecules are liberated from the active site and there is a concomitant closure of the active site by the C-terminal residues of the neighboring subunit. The His24-Asp75 catalytic dyad is responsible for starting the reaction through general base catalysis. The imidazole nitrogen of His24 abstracts a proton from the 2-hydroxyl group of 5-CHQ leading to intramolecular hydrogen bonding between the 2-oxygen and 1-hydroxyl group stabilizing the resulting oxyanion at the 1-position, which is further stabilized by an existing oxyanion hole formed by Arg17 and Ser56 (Fig. 9-I). The other two mutants, Arg17Ala and Ser56Ala, displayed 1.6 % and 5.1 % specific activity, respectively. The higher specific activity of Ser56Ala relative to Arg17Ala is probably due to the fact that Arg17 plays a more dominant role in stabilizing the oxyanion at the 1-position of 5-CHQ.

Ring protonation occurs at the 5-chloro position of 5-CHQ by the general acid role of His96, being followed by deprotonation of the 4-hydroxyl group of 5-CHQ (Fig. 9-II). This was consistent with the specific activity of His96Ala, 1.4 %. The QM:MM result also illustrated the 5-chloro position of 5-CHQ oriented towards His96. The resulting movement of electrons from the 1-oxyanion causes a spontaneous loss of chloride ion (Fig. 9-III). Opening of the C-terminal arm then allows for release of the product, 2-hydroxy-1,4-benzoquinone. The chloride ion leaves as HCl following a proton transfer from Asp98 and the active site of TftG is regenerated (Fig. 9-IV).

**Summary** - TftG is a novel dehydrochlorinase enzyme. The conserved His-Asp dyad proves to be
catalytic mechanism of a YCII-like dehydrochlorinase reaction together with the neighboring polar residues. The active site residues implicated in TftG catalysis form a signature sequence conserved throughout the YCII superfamily. Considering the unique topology and conservation of active site residues in TftG, it is very likely that the proteins in YCII superfamily conduct lyase reactions (e.g., dehydrochlorination) for hydroquinones or related structural analogs.

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Catalytic mechanism of a YCII-like dehydrochlorinase

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FIGURE LEGENDS

Figure 1. Conversion of 2,4,5-trichlorophenoxy acetic acid to 2-hydroxybenzoquinone. TftAB converts 2,4,5-trichlorophenoxy acetic acid to 2,4,5-TCP. TftCD converts 2,4,5-TCP to 2,5-dichlorobenzoquinone (not shown) which is reduced to 2,5-dichlorohydroquinone (2,5-DCHQ). TftD further dechlorinates 2,5-DCHQ to 5-chloro-2-hydroxybenzoquinone (not shown), which is reduced to 5-chloro-2-hydroxyhydroquinone, the substrate of TftG. TftG catalyzes the dehydrochlorination of 5-chloro-
2-hydroxyhydroquinone to the product 2-hydroxybenzoquinone, which subsequently enters the TCA cycle after several previously established enzymatic steps. This figure was produced using ChemDraw12.0.2.

Figure 2. Oligomeric nature of TftG in solution: Elution profile for TftG protein (2 mg ml⁻¹) was monitored with multi-angle laser light scattering and was shown as absorbance (left-Y axis) and molecular weight (right-Y axis) versus elution volume (mL). The solid line represents changes in absorption at 280 nm. The thick black cluster in the middle of the peaks indicated the calculated molecular mass, which was extended to the right Y-axis for ease of interpretation. The average molecular weight for the TftG peak was indicated.

Figure 3. Ribbon diagram representing the crystal structure of TftG. (a) TftG monomer. Secondary structural elements have been numbered sequentially as α1-α4 and β1-β4. N and C refer to the N- and C-terminal regions, respectively. α-helices were shown in red and β-strands were shown in green. The product analogue 2,5-dihydroxybenzoquinone in the active site is illustrated in orange. (b and c) TftG tetramer. Two orientations illustrating arrangement of the TftG tetramer and binding sites with 2,5-dihydroxybenzoquinone represented as a molecular surface. This figure was generated by open-source PyMOL 1.1r1.

Figure 4. Measurement of substrate/substrate analog binding through ITC experiments: The trend of heat released by serial injections of substrates into TftG was monitored. 2,5-dihydroxybenzoquinone (diamond) showed a typical heat-releasing pattern. The solid line represents the least square fit of the data. Hydroquinone (square), 5-chlorohydroquinone (circle) and catechol (triangle) did not show any significant affinity to TftG.

Figure 5. TftG active site and ligand binding. (a) Molecular surface of TftG-Apo tetramer. The apo-form structure illustrated a distinct solvent accessible pocket (inside black square) which functions as the active site in TftG. This figure was generated by open-source PyMOL 1.1r1. (b) Active site of TftG – 2,5-dihydroxybenzoquinone binary complex: 2Fo-Fc map covering substrate and hydrogen bonded residues at a contour level of 1.0 σ. (c) Computational results for position of 5-CHQ: An overlay of the crystallographic and three-layer ONIOM-optimized active site catalytic residues and substrate. Carbons are shown in gray for the crystallographic structure with bound 2,5-DHBQ and light blue for the ONIOM-optimized protein and 5-CHQ sites. Catalytic residue-substrate hydrogen bond distances are shown.

Figure 6. CD Spectra for TftG wild-type and mutants. The CD spectra were recorded from 200 to 300 nm for wild-type (diamonds), R17A (triangles), H24A (solid circles), S56A (open circles) and H96A (squares) using an AVIV 202SF spectropolarimeter (AVIV Biomedical) at 25°C, at a concentration of 10 µM.

Figure 7. Monomeric and oligomeric state of known dehydrochlorinases. (a) TftG, (b) 5-chloromuconolactone dehalogenase of Rhodococcus opacus 1Cp (PDB: 3ZNU) and (c) LinA from Sphingobium japonicum (PDB: 3A76). This figure was generated by open-source PyMOL 1.1r1.

Figure 8. Sequence alignment of TftG BLAST search results. The top twenty sequences were aligned with TftG, illustrating several conserved residues (shown in bold) throughout the YCII superfamily. Residues that were conserved and implicated in TftG catalysis were highlighted yellow.

Figure 9. Proposed mechanism for dechlorination of 5-CHQ. The resting state of the enzyme contains water in the active site of TftG. (I) Upon 5-CHQ association, water is liberated from the active site and the C-terminal arm closes. The catalytic dyad His24-Asp75 begins the reaction through general base catalysis as the imidazole nitrogen of His24 abstracts a proton from the 2-hydroxyl group of 5-CHQ leading to intramolecular hydrogen bonding between the 2-oxygen and 1-hydroxyl group and formation of an
oxygen at the 1-position which is stabilized by an oxygen hole formed by Ser56 and Arg17. (II) Ring protonation occurs at the 5-chloro position of 5-CHQ by the general acid His96 followed by deprotonation of the 4-hydroxyl group of 5-CHQ. (III) Electrons from the 1-position oxygen move causing the spontaneous loss of chloride. (IV) The chloride ion leaves following a proton transfer from Asp98, the C-terminal arm opens and the product 2-hydroxy-1,4-benzoquinone is released with subsequent active site regeneration.
Table 1 Data collection and refinement statistics

|                      | TftG-SeMET | TftG-Apo | TftG-Binary complex |
|----------------------|------------|----------|---------------------|
| **Data collection**  |            |          |                     |
| Space group          | C222\textsubscript{1} | P622     | P622                |
| Cell dimensions      |             |          |                     |
| $a, b, c$ (Å)        | 135.24, 158.01, 50.39 | 90.32, 90.32, 51.26 | 90.20, 90.20, 50.92 |
| $\alpha, \beta, \gamma$ (°) | 90.00, 90.00, 90.00 | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 |
| Resolution (Å)       | 23.61-2.21 | 50.00-1.75 | 50.00-1.87          |
| $R_{sym}$            | 0.040 (0.193) | 0.077 (0.266) | 0.089 (0.226)      |
| $I / \sigma I$       | 28.7 (6.3) | 53.96 (12.08) | 66.22 (20.65)      |
| Completeness (%)     | 77.3 (13.5) | 99.8 (96.1) | 99.8 (99.6)        |
| Redundancy           | 6.23 (3.30) | 20.6 (17.3) | 19.7 (15.9)        |
| **Refinement**       |            |          |                     |
| Resolution (Å)       | 23.61-2.21 | 45.17-1.75 | 45.09-1.87          |
| No. reflections      | 21,232     | 12826    | 10,571              |
| $R_{work} / R_{free}$| 0.1704/0.2070 | 0.1859/0.2165 | 0.1887/0.2194      |
| No. atoms            |            |          |                     |
| Protein              | 2992       | 748      | 783                 |
| Ligand/ion           | 0          | 7        | 10                  |
| Water                | 272        | 79       | 78                  |
| **B-factors**        |            |          |                     |
| Protein              | 29.60      | 27.30    | 27.20               |
| Ligand/ion           | N/A        | 44.30    | 28.40               |
| Water                | 32.90      | 35.50    | 33.30               |
| **R.m.s. deviations**|            |          |                     |
| Bond lengths (Å)     | 0.007      | 0.008    | 0.016               |
| Bond angles (°)      | 1.05       | 1.08     | 1.20                |

*Values in parentheses are for highest-resolution shell.
Table 2: Specific activity of TftG active site mutations

| Enzyme | Specific Activity (µmol min⁻¹ mg⁻¹) | % Specific Activity (Relative to WT) |
|--------|-------------------------------------|-------------------------------------|
| WT     | 66.877 ± 0.993                      | 100                                 |
| R17A   | 1.102 ± 0.057                       | 1.6 %                               |
| H24A   | 0.420 ± 0.046                       | <1.0 %                              |
| S56A   | 3.422 ± 0.330                       | 5.1 %                               |
| H96A   | 0.938 ± 0.068                       | 1.4 %                               |

Figure 1

[Diagram of metabolic pathway involving TftAB, TftCD, TftG, and TCA]

Figure 2

[Graph showing elution volume (mL) vs. absorbance (280 nm) with a peak at 48.4 kDa]
Figure 5

Figure 6
Figure 7

Figure 8

|    | D9   | R17   | H24   | SS6  | D75/P76 |
|----|------|-------|-------|------|---------|
| TTd | 1    | 1     | 1     | 1    | 1       |
| M. magnetotacticum | 1    | 1     | 1     | 1    | 1       |
| M. magneticum AMB-1 | 1    | 1     | 1     | 1    | 1       |
| M. gryphiswaldense MSR-1 | 1    | 1     | 1     | 1    | 1       |
| R. rubrum ASCC1170 | 1    | 1     | 1     | 1    | 1       |
| F. gallaeciensis 2.10 | 1    | 1     | 1     | 1    | 1       |
| P. gallaeciensis DSM7395 | 1    | 1     | 1     | 1    | 1       |
| R. bacteriae EM11 | 1    | 1     | 1     | 1    | 1       |
| R. sphaeroides ATCC2705 | 1    | 1     | 1     | 1    | 1       |
| R. capsulatus SS1003 | 1    | 1     | 1     | 1    | 1       |
| J. marina sp. CDS1 | 1    | 1     | 1     | 1    | 1       |
| S. meliloti 1021 | 1    | 1     | 1     | 1    | 1       |
| S. meliloti | 1    | 1     | 1     | 1    | 1       |
| S. fredii USDA257 | 1    | 1     | 1     | 1    | 1       |
| S. fredii USDA257 | 1    | 1     | 1     | 1    | 1       |
| S. fredii USDA257 | 1    | 1     | 1     | 1    | 1       |
| S. fredii USDA257 | 1    | 1     | 1     | 1    | 1       |
| S. fredii USDA257 | 1    | 1     | 1     | 1    | 1       |
Figure 9

Catalytic mechanism of a YCII-like dehydrochlorinase
Catalytic mechanism of 5-chlorohydroxyhydroquinone dehydrochlorinase from the YCII superfamily of largely unknown function
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