The Tautomeric Half-reaction of BphD, a C-C Bond Hydrolase

KINETIC AND STRUCTURAL EVIDENCE SUPPORTING A KEY ROLE FOR HISTIDINE 265 OF THE CATALYTIC TRIAD*

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BphD of Burkholderia xenovorans LB400 catalyzes an unusual C-C bond hydrolysis of 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA) to afford benzoic acid and 2-hydroxy-2,4-pentadienoic acid (HPD). An enol-keto tautomerization has been proposed to precede hydrolysis via a gem-diol intermediate. The role of the canonical catalytic triad (Ser-112, His-265, Asp-237) in mediating these two half-reactions remains unclear. We previously reported that the BphD-catalyzed hydrolysis of HOPDA (λmax is 434 nm for the free enolate) proceeds via an unidentified intermediate with a red-shifted absorption spectrum (λmax is 492 nm) (Horsman, G. P., Ke, J., Dai, S., Seah, S. Y. K., Bolin, J. T., and Eltis, L. D. (2006) Biochemistry 45, 11071–11086). Here we demonstrate that the S112A variant generates and traps a similar intermediate (λmax is 506 nm) with a similar rate, 1/τ ~ 500 s−1. The crystal structure of the S112A:HOPDA complex at 1.8-Å resolution identified this intermediate as the keto tautomer, (E)-2,6-dioxo-6-phenyl-hexa-3-enolate. This keto tautomer did not accumulate in either the H265A or the S112A/H265A double variants, indicating that His-265 catalyzes tautomerization. Consistent with this role, the wild type and S112A enzymes catalyzed tautomerization of the product HPD, whereas H265A variants did not. This study thus identifies a keto intermediate, and demonstrates that the catalytic triad histidine catalyzes the tautomerization half-reaction, expanding the role of this residue from its purely hydrolytic function in other serine hydrolases. Finally, the S112A:HOPDA crystal structure is more consistent with hydrolysis occurring via an acyl enzyme intermediate rather than a gem-diol intermediate as solvent molecules have poor access to C6, and the closest ordered water is 7 Å away.

Bacteria use meta-cleavage pathways to degrade a large variety of aromatic compounds, and some alicyclic compounds, such as steroids (1). Although each pathway has its own substrate specificity, they all employ the same underlying logic: vicinal dihydroxylation of an aromatic ring enables dioxygenase-catalyzed extradiol (or meta) ring opening. The resulting meta-cleavage product (MCP) is degraded by an MCP hydrolase, which adds water across a carbon-carbon bond to generate a dienolate and a carboxylic acid (Fig. 1). Enzymes of the meta-cleavage pathway have been of interest because of their roles in biodegradation. For instance, the biphenyl (Bph) catabolic pathway transforms a number of polychlorinated biphenyls (PCBs). This process is limited by the inhibitory effects of PCBs or their chlorinated metabolites (2, 3). More recently, it was discovered that Mycobacterium tuberculosis catalyzes cholesterol via a meta-cleavage pathway (4) that is essential for pathogen survival in the macrophage (5). A better understanding of the meta-cleavage pathway enzymes should accelerate the development of their potential for biocatalysis and biodegradation, as well as facilitate the design of novel therapeutics for the treatment of tuberculosis.

MCP hydrolases, exemplified by BphD of the Bph pathway and HsAD of the cholesterol catabolic pathway, contain the canonical structural fold and Ser-His-Asp catalytic triad characteristic of the α/β-hydrolase enzyme superfamily (6–9). This triad is associated with the classical hydrolytic mechanism typified by the serine proteases, in which the Asp-His dyad first activates Ser to nucleophilic attack at the substrate carbonyl, and subsequently activates water to release the resulting acyl-enzyme covalent intermediate. Although the catalytic Ser and Asp residues vary within the nucleophile-His-acid framework, all known α/β-hydrolase active sites contain the catalytic His and the oxyanion hole. The latter, which stabilizes the negative charge of the tetrahedral intermediate oxyanion, is created by the partial positive charges of backbone amide protons.

The proposed mechanism of MCP hydrolases differs from that of other α/β-hydrolases in two important respects: (i) it invokes an enol-keto tautomerization prior to hydrolysis (Fig. 1) (10, 11) and (ii) it asserts hydrolysis via a gem-diol intermediate rather than an acyl-enzyme (12–16). The two best studied

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The atomic coordinates and structure factors (codes 2PU5, 2PU6, aPU7, 2PUH, and 2PUU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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§ The abbreviations used are: MCP, meta-cleavage product; HOPDA, 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid; HPD, 2-hydroxy-2,4-pentadienoic acid; Bph, biphenyl; PCB, polychlorinated biphenyl.
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MCP hydrolases are BphD from *Burkholderia xenovorans* LB400, which hydrolyzes 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) to benzoic acid and 2-hydroxy-2,4-pentadienoic acid (HPD), and MhpC involved in phenylpropanoid acid degradation. Kinetic and structural studies of both enzymes have indicated that the catalytic His may mediate tautomeration, as well as help catalyze hydrolysis (13, 17, 18). For instance, stopped-flow studies showed that substitution of the catalytic histidine decreased the rate of a process proposed to represent tautomeration in MhpC (18) and a polyhistidine-tagged BphD (Ht-BphD) (13). In the crystal structure of an MhpC:substrate analog binary complex, the catalytic His was 3.2 Å from the C2 carbonyl and 3.9 Å from C5 of the analog (17), consistent with histidine’s role in abstracting a proton from the C2 hydroxyl and protonating C5. More convincingly, the crystal structure of the S112C variant of BphD incubated with HOPDA revealed a complex with the product HPD and an interaction between the catalytic His-265 and the C-2 hydroxy/ carboxyl substituent of the dieneoate, consistent with a general base catalytic contribution of each residue based on the accumulation of different intermediates, such as E:S\text{Red}. In parallel, substrate complexes of the BphD variants were characterized at high resolution by x-ray crystallography. Monitoring the ability of enzyme variants to catalyze tautomeration of HPD provided further insight into the catalytic roles of the substituted residues in the tautomeric half-reaction. Implications for catalysis in MCP hydrolases are discussed.

MATERIALS AND METHODS

**Chemicals**—HOPDA was enzymatically generated from 2,3-dihydroxybiphenyl (DHB) using DHB dioxygenase (DHBD) as previously described (19). The preparation of DHB has been described (23). HPD was generated together with benzoic acid by BphDLB400-catalyzed hydrolysis of HOPDA. Upon reaction completion (monitored by absorbance at 434 nm), the solution was acidified to pH ~ 3 with 2 N HCl, extracted 3 times with 0.3 volumes of ethyl acetate, dried over anhydrous MgSO\textsubscript{4} and rotary evaporated to dryness. All other chemicals were of analytical grade.

**Mutagenesis, Protein Expression, and Purification**—BphD from *B. xenovorans* LB400 was produced and purified as previously described (19, 24). Ser-112 of BphD was substituted with alanine (S112A) using the Transformer site-directed mutagenesis method (Clontech Laboratories, Palo Alto, CA). Briefly, the S112A mutagenic primer (primer S112A: 5’-GGCCGCCCCATATGGCGTTGGGCGCAATGG-3’) and a second mutagenic selection primer to remove an EcoRI site (primer ODM: 5’-AGCTCGAAATTGGTAATCATGG-3’) were mixed with pSS184, the pEMBL18 vector carrying bphD (24). After second strand synthesis and ligation using T4 DNA polymerase and T4 DNA ligase (GE Healthcare, Uppsala, Sweden), respectively, the DNA was digested with EcoRI to linearize wild-type plasmid, which pSS184SA, carrying the mutated gene, was isolated. The mutated gene was cloned into pVTL31 using XbaI and HindIII restriction sites, and the resulting plasmid, pSS134SA, was used for protein production. Substitution of His-265 with alanine was performed using a 5’-phosphorylated primer (H265A: CTTCAAGTGCGCCTTGGGCGCAATGG-3’) and the QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). Genes encoding the single (H265A) and double (S112A/H265A) variants were generated using pSS184 and pSS184SA, respectively, yielding pSS184HA and pSS184SAHA. These constructs were used directly for protein production. The nucleotide sequences of variants were confirmed using an
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ABI 373 Stretch (Applied Biosystems, Foster City, CA) and BigDye v3.1 terminators.

Enzyme Activity Measurements—All enzyme kinetic experiments were performed using potassium phosphate buffer, \( I = 0.1 \) M, pH 7.5, at 25 °C. Steady-state enzyme activities were obtained by monitoring the decrease in absorbance at 434 nm of the HOPDA enolate versus time using a Varian Cary 5000 spectrophotometer (Varian Canada, Mississauga, ON, Canada). The latter was equipped with a thermostatted cuvette holder maintained at 25.0 ± 0.5 °C, controlled by Cary WinUV software version 3.00. To measure enzyme activity toward HPD, a small volume (<0.5% v/v) of HPD/benzoic acid in ethanol was added to a buffered solution, and absorbance at 270 nm was monitored before and after addition of enzyme.

The half-life of the S112A:HOPDA complex was determined by mixing 25 μM S112A and 5 μM HOPDA, and recording the absorption spectrum at 0.5–1 h intervals. The absorbance at 506 nm was plotted against time, and described by a first order decay using Excel (Microsoft, Redmond, WA).

Stopped-flow Spectrophotometry—Experiments were conducted using an SX.18MV stopped-flow reaction analyzer (Applied Photophysics Ltd., Leatherhead, UK) equipped with a photodiode array detector. The drive syringe chamber and optical cell were maintained at 25 °C by circulating water bath. Multiple wavelength data from the time courses of single turnover experiments were acquired using the Xscan software (Applied Photophysics Ltd.) and exported to Excel where replicate measurements from at least three shots were averaged. To obtain more data at earlier time points, a single wavelength was monitored using the SX18MV software. The data from at least three shots were averaged and equations for single or double exponentials were fit using the same software to obtain reciprocal relaxation times and amplitudes. Good fits were characterized by random variation in the fit residuals.

Crystallization of BphD and Preparation of Substrate Complexes—A 1.6-Å resolution structure of BphD was previously determined using crystals grown from a solution containing 1.6 M ammonium sulfate (19, 24). These crystals had space group \( P6_5 \), with \( a = 135.0 \) Å, \( c = 66.7 \) Å, and a sulfate ion bound in the active site. The failure of many attempts to prepare crystalline complexes by incubation of crystals with substrates suggested sulfate binding or the crystal form restricts formation of E:S complexes leading us to seek alternative crystallization procedures.

New protocols were established for both wild type and variant enzymes using both commercial (Hampton Research) and non-commercial sodium malonate grid screens and the vapor diffusion method. Sitting drops (1–μl each of reservoir and protein solutions) were equilibrated at 20 °C against 500- or 1000-μl reservoirs. The protein sample contained 9–28 mg/ml protein in 20 mM HEPES pH 7.5. Crystals or crystalline precipitates were obtained between 1.5 and 2.4 M sodium malonate within a pH range of 6.0 and 7.0.

Two crystal forms were obtained. Crystals of wild-type enzyme had space group \( P6_5 \), \( a = 135.2 \) Å, \( c = 66.3 \) Å, whereas crystals of the S112A and the S112A/H265A variants had space group \( I4_1 \) with \( a = 117.3 \) Å, \( c = 87.3 \) Å. The hexagonal crystals are essentially isomorphous with the previously reported form obtained from ammonium sulfate and contain two protein monomers per asymmetric unit; the tetragonal form has one monomer in the asymmetric unit. Crystals of wild-type BphD grew over 12 weeks as hexagonal prisms in 2.0 M sodium malonate, pH 6.0 or 6.5. Crystals of S112A and S112A/H265A grew in 4–6 days as tetragonal rods in 1.9 M sodium malonate. The best crystals of the S112A variant were obtained at pH 7.0, whereas the best S112A/H265A crystals grew at pH 6.5.

Crystals of HOPDA complexes were obtained by incubating crystals grown in the absence of HODPA in 30–60 μl of reservoir solution augmented with ~15 mM HOPDA for 30–60 min at 20 °C.

Diffraction Data Measurements and Processing—Crystals were prepared for flash-freezing by sequential transfer into solutions containing higher concentrations of sodium malonate. A mounting loop was used to transfer crystals from the growth drop into 60-μl volumes of reservoir solution, then into similar solutions containing 3.4 M and, finally, 3.7 M sodium malonate. The pH was held at the growth value; the incubation time was 3–6 s per step. After the last transfer crystals were flash-frozen by immersion into liquid nitrogen. For enzyme-substrate complexes, each solution was supplemented with HODPA (~5–10 mM).

Preliminary diffraction patterns were acquired with a typical laboratory instrument based on a rotating anode (Cu) generator equipped with focusing mirror or multilayer optics and an imaging plate detector (Rigaku/MSC). The diffraction data used for refinements of atomic models were acquired at SER-CAT beamline 22-ID-D at the Advanced Photon Source, Argonne National Laboratory. For the latter experiments, crystals were maintained at ~100 K, and diffraction images were recorded by a MarMosaic 300 CCD detector (Mar USA, Inc., Evanston, IL). For each crystal, ~100 frames were collected with a 1° rotation per frame; exposure times were 1–10 s per degree. All images were processed using DENZO, and then intensities were merged and scaled using SCALEPACK; both programs were from the HKL2000 program suite (25).

Structure Determination and Refinement—Programs from the CCP4 suite (26) were used for phasing and refinement. The crystal structure of wild-type BphD (PDB code 2OG1, Ref. 19, 24) served as a search model for phasing by molecular replacement using the program MOLREP (27). Rigid body refinement was followed by iterative cycles of restrained atomic parameter refinement using REFMAC (28) and manual density fitting using the molecular graphics program O (29). PRODRG (30) was used to develop structures of substrates and malonate for density fitting and establishment of refinement restraints. The deviation of restrained torsion angles from their expected values was used to evaluate the compatibility of the x-ray data with different tautomers of HOPDA. In the final refinement cycles of HOPDA complexes, the torsion angles in the non-aromatic portion of HOPDA were not restrained; bond lengths and bond angles were restrained to values expected for the keto (S112A: HOPDA) or enol (S112A/H265A: HOPDA) forms. The stereochemical properties of the models and the hydrogen bonding were analyzed by programs PROCHECK (31) and REDUCE (32).
TABLE 1

Kinetic data for BphD variants

| BphD variant | 1/τ₁ | 1/τ₂ | 1/τ₃ |
|--------------|------|------|------|
| S112A/H265A  | 22 s⁻¹ (69%) | 22 s⁻¹ (22%) | 0.34 s⁻¹ (9%) |
| H265A        | 78 s⁻¹ (82%) | 1.3 s⁻¹ (18%) | 0.0058 s⁻¹ (100%) |
| S112A        | ~500 s⁻¹ (~85%) | 76 s⁻¹ (~11%) | 0.92 s⁻¹ (~4%) |

a Measured using a Cary 5000 spectrophotometer as an exponential decay in absorbance at 434 nm.

RESULTS

Kinetic Analysis of Variant Enzymes—Catalytic triad residues His-265 and Ser-112 were substituted to construct three variants: S112A, H265A, and S112A/H265A. The extremely low activity of the S112A and S112A/H265A variants prevented steady state kinetic measurements. Transformation of HOPDA by H265A as measured by the decay of absorbance at 434 nm could only be detected using large quantities of enzyme (~1 mM), and the progress curve was biphasic. In an experiment in which 4 μM HOPDA was mixed with 1.3 μM H265A, the first phase could be described by a single exponential decay with a rate constant of 5.8 (±0.4) × 10⁻³ s⁻¹. In Table 1 this is presented as the third rate of decay, or reciprocal relaxation time (1/τ₃), because it is preceded by two events observed by stopped-flow spectrophotometry (see below). The value of 1/τ₃ is similar to that of HOPDA tautomerization in solution, as observed by deuteron exchange NMR (19). The slope of the linear second phase approximately doubled upon increasing the H265A concentration to 2.6 μM. By contrast, doubling enzyme concentration did not affect the value of 1/τ₁. The apparent burst is consistent with first order decay of an E:S complex followed by steady-state turnover. Curiously, the amplitude of the first phase corresponded to only 9% of the total enzyme added, suggesting only this fraction of the active sites were functional. Similar behavior was observed in stopped-flow experiments (see below). Correcting for the active fraction of enzyme provides a rate of 0.0009 (±0.0002) s⁻¹ for the steady state phase, which is about half of the k_cat measured for the H265A variant of Ht-BphD (13). This is in reasonable agreement considering the current experiments were performed using a substrate concentration below the K_m of 37 μM measured for Ht-BphD.

To better characterize the catalytic impairment of the variants, stopped-flow spectrophotometry was employed under single turnover conditions (E = 8 μM, S = 4 μM) at 25 °C. The S112A variant rapidly generated an intermediate with similar kinetics as the E:S_{Red} intermediate transiently observed in wild type BphD (1/τ₁ ~ 500 s⁻¹, Ref. 19). The spectrum of E:S_{Red} was more red-shifted and more intense in S112A (λ_{max} = 506 nm; Table 1, Fig. 2A) than in the wild type (λ_{max} = 492 nm, Fig. 2C). Moreover, E:S_{Red} decayed extremely slowly in S112A, and was effectively trapped as an orange-colored complex. The half-life of this complex (S112A = 25 μM, HOPDA = 5 μM), determined by monitoring its absorbance at 506 nm, was 4.4 h at 25 °C.

Neither H265A nor S112A/H265A accumulated E:S_{Red} instead yielding a species possessing a more intense, slightly blue-shifted spectrum with respect to the free HOPDA enolate (λ_{max} = 432 nm; Fig. 2B). This species was provisionally identified as the fully deprotonated enolate because, in S112A/H265A, its molar absorptivity matched that of fully deprotonated HOPDA in solution. In contrast, H265A only partially deprotonated HOPDA under these conditions. Indeed, the amplitude of the absorbance increase at 434 nm was only 25% of the same signal in the experiment using S112A/H265A. Increasing the concentration of H265A to 32 μM (4 μM HOPDA) resulted in the same spectrum as observed in S112A/H265A, suggesting that only ~12% of the active sites in H265A supported deprotonation, as observed in the steady-state experiments described above. This phenomenon was observed in two different preparations of H265A. Attempts to rescue E:S_{Red} formation in the double variant with imidazole (1–5 mM) were unsuccessful. In summary, E:S_{Red} formation is His-265-dependent, and its decay requires Ser-112.

Reciprocal relaxation times (1/τ) and amplitudes for each phase observed in the single turnover stopped-flow experiments are summarized in Table 1. The first reciprocal relaxation time (1/τ₁) corresponding to an increase in absorbance at 434 nm and assigned to HOPDA enolate formation, was 220 ± 40 s⁻¹ in S112A/H265A. In H265A, this same relaxation was 78 ± 15 s⁻¹. This deprotonation step in both H265A variants was significantly slower than E:S_{Red} formation in wild type and S112A (1/τ₁ ~ 500 s⁻¹). In all three variants, slower relaxations of smaller amplitude followed the initial, relatively rapid relaxation (Table 1).

Tautomerization of HPD by Variant Enzymes—To further probe the ability of the BphD variants to catalyze the tautomerization half-reaction, we investigated their respective abilities to catalyze the tautomerization of HPD to (E)-2-oxo-3-pentenoate (19). The tautomerization of HPD is observed as decay in absorbance at 270 nm and occurs non-enzymatically in aqueous solution at a slow rate. Wild-type and S112A catalyzed the tautomerization of HPD (~14 μM) with specific activities of 85% and 18%, respectively. The E:S_{Red} intermediate in the S112A variant was found to be ~9% deprotonated, as observed in the stopped-flow experiments (19).

The HOPDA enol has a pK₆ of 7.3 (1). Hence, the ~99% deprotonated form at pH 9.5 (50 mM Na-CHES, 25° C) has a molar absorptivity of 40.1 mM⁻¹ cm⁻¹ at 434 nm.

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Mixing the complexes were corrected based on the isosbestic point occurs at 461 nm.

This tautomerization. Thus, His-265 is necessary for catalyzing hydrolysis of HOPDA.

0.013 units/mg respectively. By contrast, neither H265A nor S112A/H265A detectably catalyzed the slower S112A reaction was easily detectable (0.0067 μmol/m in contrast, neither H265A nor S112A/H265A detectably catalyzes this tautomerization. Thus, His-265 is necessary for catalyzing tautomerization of HPD.

Primary Crystallographic Results—Crystal structures of BphD, its S112A and S112A/H265A variants, and complexes of the variants with HOPDA were determined and refined at resolutions between 1.57 and 2.30 Å. Determination of high resolution structures for HOPDA complexes of the variants was facilitated by a new tetragonal crystal form (see “Materials and Methods”). Table 2 summarizes the properties of the crystals and the diffraction data, which are generally of high quality.

Table 3 characterizes the refined models. The model for wild type BphD lacks the N-terminal methionyl residue in chain A (as expected from mass spectral analysis) and three N-terminal residues in chain B, whereas the models for the variants lacked the three N-terminal residues in all cases. Each model includes several residues in multiple conformations. For all models, >98% of the residues are in favored or allowed regions of Ramachandran plots as defined by PROCHECK. For the outliers, which include the key active site residue S/A112, the backbone conformations are stabilized by hydrogen bonds and are similar in all independent coordinate sets.

Tertiary and Quaternary Structure and Crystal Packing—The monomer of BphD includes two domains: a core domain with the α/β-hydrolase fold (residues 2–145 and 213–286) and a lid domain (residues 146–212) occurring as an insertion in the core domain. The active site is located at the cleft between the two domains (Fig. 3).

BphD and its variants are tetramers in solution, and the crystal structures show the tetramer has 222 (D2) point group symmetry. The 222 symmetry is crystallographic in the I4,22 crystals; thus, all monomers are equivalent. In the P63 crystals, the 222 symmetry is produced by a combination of crystallographic and non-crystallographic operations, and the asymmetric unit includes an A:B dimer. There is a significant difference in the environment of A and B (19), such that the average B factors are 34 and 65 Å2, for A and B, respectively. For these crystals we describe structural details only for molecule A because it is represented by better electron density.

Binding of Malonate: a Partial Mimic of the Substrate—The substrate binding site of BphD is conventionally partitioned in two subsites (33): the P subsite complements the substrate three polar groups, and the NP subsite binds the non-polar phenyl substituent. The active site Ser-112 and the oxyanion hole formed by the backbone NHs of Gly-42 and Met-113, are at the boundary between the P and NP subsites; the guanidinium group of Arg-190 lies at the opposite end of the P subsite.

Malonate (propanedioic acid) binds slightly differently in each of the BphD variants, but in each case mimics the binding of one or more of the HOPDA three polar groups. In wild-type BphD, the C1 carboxylate occupies a site near Arg-190 that is utilized by the HOPDA carboxylate in the substrate complex (see below). One oxygen atom from the malonate C3 carboxylate occupies the site of the HOPDA 2-oxo group near His-265, whereas the second oxygen atom hydrogen bonds with Oγ of Ser-112 and the peptide NH of Gly-42 near the oxyanion hole. The binding mode is similar in the S112A/H265A variant, although the deletion of the imidazole group from residue 265 results in minor adjustments in the position and orientation of the C3 carboxylate.

In the S112A variant, the malonate is ~2.5 Å further away from Arg-190 and closer to the oxyanion hole. One oxygen atom of the C3 carboxylate lies in the oxyanion hole and forms hydrogen bonds with the peptide NHs of Gly-42 and Met-113;
the other oxygen hydrogen bonds with Nε2 of His-265. One oxygen of the C1 carboxylate is near His-265 and the other forms a hydrogen bond with the peptide NH of Gly-43.

**Binding of HOPDA to the S112A Variant**—Our prior crystallographic analysis of the BphDS112C variant after incubation with HOPDA revealed clear electron density for all atoms from the C1 carboxylate through C5 (19). Because marginal electron density was observed for the phenyl group, the final interpretation was a complex of the enzyme with the product, HPD. The electron density maps obtained in the current study reveal the first images of the entire substrate bound to an MCP-hydrolase, albeit catalytically impaired variants missing the Oγ atom of the active site serine.

For the S112A variant, the electron density in unbiased (Fo-Fc) maps calculated before HOPDA was added to the model (Fig. 4) is reasonably compatible with multiple isomers of HOPDA: the monoanionic 2-keto form, (E)-2,6-dioxo-6-phenylhex-3-enoate; the enol form, (2Z,4Z)-2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; and a dianionic, 5,6-enolate variant of the 2-keto form, (3E,5Z)-2-oxo-6-oxido-6-phenylhexa-3,5-dienoate. Although the unbiased maps did not exclude the presence of any of these forms in some fraction of the unit cells, the results of refinements suggest the monoanionic 2-keto form is most compatible with the x-ray data. In these experiments, the torsion angles about the double bonds characteristic of each species were tightly restrained to 0 or 180°, as appropriate. Following refinement, the deviations of the torsion angles from the expected values (Table 4) reflect the compatibility of the x-ray data with each isomer. The results suggest that the 2-oxo,6-oxido isomer is the least compatible inasmuch as the refined torsion angle about the C5–C6 bond is 150°, a significant devi-

**TABLE 2**

Crystal properties and x-ray diffraction data for BphD variants and complexes

| Parameter or statistic | Wild type: malonate | S112A: malonate | S112A/H265A: malonate | S112A: HOPDA | S112A/H265A: HOPDA |
|------------------------|---------------------|----------------|-----------------------|---------------|---------------------|
| Resolution (Å)         | 2.30                | 1.68           | 2.07                  | 1.82          | 1.57                |
| Space group            | P6_4                | I4_22          | I4_22                 | I4_22         | I4_22               |
| a (Å)                  | 135.2               | 117.3          | 116.3                 | 116.8         | 116.8               |
| c (Å)                  | 66.3                | 87.3           | 87.9                  | 87.6          | 87.5                |
| Unique refls           | 30870               | 34855          | 18453                 | 27213         | 42069               |
| Completeness (%) (last shell) | 99.8 (98.6)    | 99.8 (99.3)   | 98.8 (92.4)           | 99.3 (94.3)  | 99.1 (99.1)         |
| R_map (%)              | 12.4 (33.2)         | 12.6 (27.1)    | 8.4 (41.9)            | 5.4 (45.1)   | 8.8 (45.8)          |
| Mean | 12.2 (3.4)         | 18.6 (8.5)     | 23.9 (3.0)            | 36.6 (2.6)   | 21.2 (3.5)          |
| Multiplicity (last shell) | 6.3 (3.5)         | 12.3 (11.8)    | 9.3 (6.7)             | 7.8 (5.7)    | 9.3 (6.9)           |

*a R_sym = Σ|Iobs| - Σ|Icalc| / Σ|Icalc| |

**TABLE 3**

Results of model refinement for BphD variants and complexes

| Parameter or statistic | Wild-type: malonate | S112A: malonate | S112A/H265A: malonate | S112A: HOPDA | S112A/H265A: HOPDA |
|------------------------|---------------------|----------------|-----------------------|---------------|---------------------|
| Resolution range (Å)   | 117.0–2.30          | 83.0–1.68      | 82.2–2.07             | 82.5–1.82     | 82.5–1.57           |
| R (%)                  | 18.0                | 17.4           | 18.8                  | 17.1          | 17.6                |
| R_mol (%)              | 27.0                | 20.0           | 27.0                  | 20.5          | 20.0                |
| Est. coordinate error* | 0.24                | 0.09           | 0.20                  | 0.11          | 0.08                |
| Model content          |                     |                |                       |               |                     |
| Protein atoms          | 4520                | 2264           | 2255                  | 2255          | 2259                |
| Malonate molecules     | 2                   | 2              | 2                     | 0             | 1                   |
| Water molecules        | 107                 | 143            | 71                    | 113           | 156                 |
| Average B factors (Å²) |                     |                |                       |               |                     |
| Protein:chain*         | 35.64               | 18             | 36                    | 30            | 26                  |
| Water atoms            | 34                  | 23             | 37                    | 33            | 32                  |
| All atoms              | 49                  | 18             | 36                    | 30            | 26                  |
| R.m.s.d.from target    |                     |                |                       |               |                     |
| Bond lengths (Å)       | 0.022               | 0.009          | 0.015                 | 0.013         | 0.010               |
| Bond angles (degrees)  | 2.0                 | 1.2            | 1.6                   | 1.3           | 1.2                 |

*a Cruickshank’s diffraction-component precision index as calculated by REFMAC.
*b For the wild-type:malonate complex, the first value is for monomer A and the second is for monomer B.

**FIGURE 3**

Ribbon drawing of the BphD tetramer illustrating the secondary, tertiary, and quaternary structure of the enzyme. For the monomer at the upper right, the hydrolase and lid domains are colored yellow and orange respectively. HOPDA is represented by spherical atoms (CPK style) colored gray for carbon and red for oxygen. The solid oval marks the location of a 2-fold axis perpendicular to the page; arrows indicate two-folds parallel to the plane.
Tautomerization in a C-C Bond Hydrolase

FIGURE 4. Enzyme-substrate interactions in the BphD-S112A:HOPDA complex. A, stereo view showing: the refined model; the (2F_o-F_c) electron density (cyan, contour level = 1σ) of the refined structure; and the (Fo-F_c) electron density before HOPDA was added to the model (blue, contour level = 3σ) of the substrate. C, N, and O atoms are colored gold, blue, and red, respectively. B, schematic representation of most enzyme-substrate interactions. C, N, and O atoms are colored black, blue, and red, respectively. Covalent bonds are drawn as orange sticks for the substrate and gray sticks for the protein residues. Protein residues involved in non-polar interactions are represented by green semi-circles with radial lines directed toward the substrate C atoms with which they interact. Hydrogen bonds are shown as dotted lines overlaid with the distance between the donor and acceptor. Not illustrated are interactions of HOPDA with Gly-41, Gly-43, Ala-46, Met-113, Gly-138, and Gly-139, including two hydrogen bonds involving the peptide NHs of Gly-43 and Met-113 and the HOPDA carboxylate and 6-oxo groups, respectively.

6-Phenyl substituent interacts with a number of non-polar side chains including those of Ile-153, Leu-213, Trp-216, and Val-240, but is not “buried” in the sense that the three most distal atoms of the ring, CB3, CB4, and CB5, are solvent accessible to a 1.4 Å radius probe.

Hydrogen bonding between His-265-N=O and the carboxylate of Asp-237 has been observed in all crystal structures of BphD and its S112A variant. In the structure of the S112A:HOPDA complex, His-265-N=O is 3.1 Å from the C2-keto oxygen atom, 3.5 Å from C4, and 4.6 Å from C5. A small rotation about the Cα-Cβ bond would place His-265 in a position and orientation competent for abstraction of a proton from C2-OH.

TABLE 4

| Bond and property       | HOPDA isomer                  |
|-------------------------|-------------------------------|
|                         | 2-Enol | 2-Keto,6-oxo | 2-Keto,6-oxido |
| C2–C3 Bonding Restraint | 180    | 180          | 158           |
| Init. angle a (°)       | 179    | 180          | 165           |
| Ref. angle a (°)        | 175    | 175          | 179           |
| C3–C4 Bonding Restraint | Single | Double       | Double        |
| Init. angle a (°)       | 139    | 180          | 165           |
| Ref. angle a (°)        | 145    | 180          | 178           |
| C4–C5 Bonding Restraint | Double | Single       | Single        |
| Init. angle b (°)       | 0      | 78           | 12            |
| Ref. angle b (°)        | 0      | 69           | 70            |
| C5–C6 Bonding Restraint | Single | Single       | Double        |
| Init. angle b (°)       | 164    | 156          | 178           |
| Ref. angle b (°)        | 178    | 150          | 150           |

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a Double bonds were restrained with an estimated S.D. of 0 degrees; single bonds were not restrained.

b Init. angle is the value in the initial model as fit to the density manually; deviations of up to 15° from the expected restraint were allowed. Ref. value is the value measured after restrained refinement.
Although His-265 is near C5 and closer to the predicted position of the C5 pro-S proton, its position and orientation (relative to HOPDA) suggest a significant change in the conformation/position of the HOPDA and/or of the His-265 side chain would be necessary for proton delivery to C5.

In the vicinity of the scissile bond, the Cβ atom of Ala-112 is in contact with both C5 (3.6 Å) and C6 (3.5 Å). On the opposite side of the binding pocket, side chain atoms of Ile-153 and Leu-156 are ~4.1 Å from C5 and C6, respectively, with the C5 protons directed toward Ile-153. When the two independent wild-type structures (sulfate- or malonate-bound) are superposed onto the S112A:HOPDA complex, they predict the same location (within 0.3 Å) for the Oγ of Ser-112, placing Oγ 1.8 Å (sulfate) or 2.1 Å (malonate) from C6. This placement is apparently stabilized by a hydrogen bond with the peptide NH of Met-113, which is common to both structures and positions Oγ away from His-265 and the plane defined by C5 and its protons. For the superposed BphDwt:malonate and S112A:HOPDA structures, the angle Oγ-C6-O6 is 62°, which is not close to 109°, the Burgi-Dunitz angle for nucleophilic attack at a carbonyl. However, if the χ1 torsion angle is adjusted from +146° to ~−130° so as to place Oγ in the plane of C5 and its protons (equidistant from C4 and C6), the angle Oγ-C6-O6 changes to 103° and Oγ is 2.4 Å from C5. Moreover, in the S112A complex and both superposed wild-type structures, the Cβ atom of residue 112 is in contact with both C6 and Ne2 of His-265 (all distances less than 3.8 Å). Although deletion of the Oγ atom and the flexibility and dynamics of the substrate and enzyme warrant cautious interpretation, the S112A:HOPDA crystal structure suggests there is inadequate room in the active site for a water molecule to approach C6 from either face. In this regard, it is worth noting that the substrate binding site includes no crystallographically ordered waters and no water-sized voids. The closest ordered water is 7 Å away, beyond a nonpolar wall formed by Ile-153, Val-240, and the HOPDA phenyl group.

As has been noted previously, occupation of the active site by different ligands results in the movement or reorientation of several residues. For example, comparison of the wild type structures with the S112A:HOPDA complex reveals coordinated displacements of the Phe-239, Phe-175, and Trp-266 side chains in the vicinity of carboxylate and C2-hydrox/oxo groups. Similarly, interactions of the Arg-190 and Phe-175 side chains with the substrate appear to motivate two major helices of the lid domain to close on the active site. For example, a comparison of the S112A:malonate and S112A:HOPDA structures shows movements of backbone atoms as large as 0.8 Å.

Structure of BphD-S112A/H265A:HOPDA—Based on the solution spectrum of the S112A/H265A:HOPDA complex, HOPDA was modeled in the crystal structure as the enol form, (2E,4E)-2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. The structural evidence for an enol tautomer is not definitive. We note that the C2–C3 (−4°) and C4–C5 (178°) angles deviate from 0°/180° by less than 5°, whereas the angle about the C3–C4 (−167°) deviates by 13°; these observations are consistent with expectations for the enol tautomer, but they are also reasonable for a (3E)-keto tautomer. As in the S112A complex, HOPDA is not in a fully extended conformation, in this case because of the conformation about C2–C3.

The significant differences between the conformation and binding of HOPDA in the two crystal structures are most readily described in terms of a transition from the S112A complex to the state observed in the S112A/H265A complex (Fig. 5). Described as a transition, three torsion angles change dramatically: the C1–C2 angle by 135°, the C2–C3 angle by 180°, and the C4–C5 angle by 120°. In addition, the overall orienta-
tion of the HPD portion of HOPDA changes in a way that places the 2-hydroxo group in a completely different binding site. It is as if the HPD plane was rotated by 108°, moving the C2 oxygen substituent by 4.1 Å and also altering the binding interactions of the carboxylate group. In the S112A complex, the two carboxylate oxygens are hydrogen bonded to the N\textsubscript{H}9257\textsubscript{1} and N\textsubscript{H}9257\textsubscript{2} of Arg-190, but in the S112A/H265A complex, one carboxylate oxygen and the 2-hydroxo group hydrogen bond with Arg-190, and the second oxygen hydrogen bonds only with the peptide NH of Gly-43. Moreover, Phe-175 and Trp-266 assume significantly different conformations. Phe-175 must move to prevent a steric conflict with the new position of the C2-hydroxo oxygen and, in turn, Trp-266 must move to free space for Phe-175. The 6-oxo and 6-phenyl groups also change somewhat in position and orientation, but essentially remain in the same binding sites as in the S112A complex.

S112A:HOPDA crystals had a similar spectrum to the solution complex, although it was red shifted by ~14 nm. Considering that a similar shift occurs between wild type and S112A in solution, this appears to be in reasonable agreement, thereby confirming that a $E:S^k$ gives rise to the >490 nm absorbance feature.

Curiously, the spectrum of the S112A/H265A:HOPDA crystal differed significantly from solution. Maximum absorbance occurred at 470 nm, with a large shoulder at 520 nm. This may reflect populations of both enol and keto tautomers in the crystalline complex, in contrast to the enolate form in solution. As noted above, the electron density is compatible with both tautomers. The long incubation (30–60 min) may have provided sufficient time for the keto tautomer to accumulate.

**DISCUSSION**

Through a combination of mutagenesis, kinetic and structural approaches, the current study (i) reports the trapping of an $E:S^{\text{Red}}$ intermediate that we had previously observed (19), (ii) identifies it as the keto tautomer, $E:S^k$, and (iii) demonstrates that the conserved His of the catalytic triad catalyzes its formation (Fig. 6). This study therefore describes the first direct observation of the long-proposed keto-intermediate on the

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**FIGURE 6. The proposed mechanism of BphD.** HOPDA may bind as a strained, or “twisted” enol, which is rapidly tautomerized to generate the 2-keto, 6-enolate intermediate, $E:S^6_e$. The proton ($H^+$) added to the pro-S position of C5 may originate from His-265 to produce the 2,6-diketo intermediate ($E:S^6_{ke}$). Although water may directly attack the C6 carbonyl to generate a gem-diolate, we favor nucleophilic attack by Ser-112, resulting in the first tetrahedral intermediate ($T_{I1}$). Collapse of $T_{I1}$ onto the re face of the C3–C4 double bond generates HPD with the added proton ($H^+$) at the observed H5 position. Loss of HPD from the active site provides room for water to hydrolyze the acyl-enzyme ($E:B$) intermediate.
reaction coordinate of an MCP hydrolase. Previous evidence for His-265-mediated tautomerization was based on its proximity to both proton donor and acceptor positions in the S112C:HPD crystal structure (19), and stopped-flow analysis of variant enzymes showing impaired rates of processes thought to represent ketonization (13, 18). The role of His-265 in tautomerization is further established by the failure of S112A/H265A and H265A variants to accumulate E$^{\text{Sk}}$ as well as their inability to catalyze HPD tautomerization.

The large (60–70 nm) red shift in absorbance observed upon formation of E$^{\text{Sk}}$ is counterintuitive. According to the empirical rules developed by Woodward (34) the keto intermediate, 2,6-dioxo-6-phenyl-3-hexenoate, should be blue-shifted with respect to HOPDA ($\lambda_{\text{max}} \leq 300 \text{ nm}$). A reasonable explanation is that the protein environment perturbs the electronic properties of the bound HOPDA, as has been reported for several other ligands when bound to proteins. For example, the absorbance maximum of an $\alpha,\beta$-unsaturated thiol ester inhibitor was red-shifted 90 nm when bound to the active site of crotonase (20). Similarly, the spectrum of a substituted stilbene derivative was red-shifted 102 nm upon binding to an antibody (21). Recent theoretical calculations suggest that the spectrum of rhodopsin is primarily determined by the interaction of the positively charged chromophore with Glu-113, which may account for a shift as large as 157 nm (22). For BphD, it is possible that Arg-190, which interacts with the carbonylate of HOPDA, may similarly perturb the spectrum of the keto tautomer. Whatever the origin of the perturbation, it is interesting that the spectrum of the HOPDA enolate in the H265A variants is not similarly perturbed, and the crystal structure of the complex of the S112A/H265A:HOPDA complex reveals reduced interactions with Arg-190. Indeed, the similarity of the spectra of the HOPDA enolate bound to each of the H265A variants to that of the enolate in solution further supports the assignment of the red-shifted absorbance feature to the keto form of the substrate.

Assignment of E$^{\text{SRed}}$ in the wild type enzyme relies upon linking it to the E$^{\text{Sk}}$ crystal structure (S112A:HOPDA) via the spectroscopic similarity of E$^{\text{SRed}}$ in both enzymes. Although both spectra have similar peak shapes, E$^{\text{SRed}}$ in S112A is red-shifted 14 nm from that in wild type (Fig. 2C). If the large (~200 nm) red shift of E$^{\text{Sk}}$ from the expected absorbance maximum of the keto tautomer in solution is dictated by active site interactions, it is reasonable to expect a relatively small shift upon substitution of the polar residue Ser-112 with non-polar alanine. Electrostatic interactions between the chromophore and polar amino acids are responsible for similar shifts in visual pigments. For example, the substitution of Ser-94 with Ala in a visual pigment from newt retina caused a 14-nm blue shift (35).

Identification of E$^{\text{Sk}}$ on the reaction coordinate enables us to refine our view of the catalytic mechanism of MCP hydrolases. In our previous stopped-flow study of wild type BphD, we presented two interpretations of the kinetic data, depending on assignment of the E$^{\text{SRed}}$ intermediate (19). The scheme in which E$^{\text{SRed}}$ was assigned to the enolate, E$^{\text{Se}}$, can now be ruled out in favor of the scheme involving two interconverting enzyme conformations, whereby only one active site per dimer can catalyze C-C cleavage/HPD release. Unfortunately, the present study cannot provide new insight into the two-conformation hypothesis itself, because the crystallographic symmetry forces all active sites to be identical.

Although the two-conformation issue is not addressed, these results advance our understanding of the enzyme-catalyzed tautomeric half-reaction. The E$^{\text{S}}$ structure combined with the critical role of His-265 in tautomerization are consistent with previous proposals (18, 19) whereby His-265 abstracts a proton from the 2-OH of the substrate and then protonates at the C5 position, perhaps via the intermediary of a C6 enolate stabilized in the oxyanion hole (Fig. 6). Protonation by His-265 would direct a proton to the pro-S position of C5. Because BphD (19), Ht-BphD (36) and MhpC (11) incorporate an H$^{\text{5e}}$ proton into HPD, pro-S protonation at C5 must be followed by C5–C6 fragmentation onto the $\pi$ face of the C3–C4 double bond (Fig. 6). The synclinal conformation we observe about the C4–C5 bond of E$^{\text{Sk}}$ (Fig. 4) is fully consistent with this stereochemical course.

Our data do not explicitly rule out two variations on the proposed mechanism of tautomerization. First, it is possible that His-265 is protonated in the substrate-free enzyme and that the enzyme binds the enolate form of the substrate. However, a decrease in rate of the proposed tautomterization step occurred at pH 5 in MhpC, implying that histidine is not protonated at neutral pH (18). Second, it is possible that the S112A variant traps an E$^{\text{Se}}_{\text{en}}$ species and that Ser-112 protonates C5. Although the S112A:HOPDA crystal structure is not rigorously complete with the E$^{\text{S}}_{\text{en}}$ form, the presence of E$^{\text{Sk}}_{\text{en}}$ is more likely for two reasons. First, the refinements suggest rotation about C5–C6 is necessary to fit the x-ray data. Second, the nonplanarity of the conjugated C3–C4 and C5–C6 double bonds in E$^{\text{Sk}}_{\text{en}}$ suggests that minimal stabilization energy would arise from $\pi$ orbital overlap; hence the bond energies of the C-6 keto tautomer (E$^{\text{Se}}_{\text{en}}$) are predicted to be ~18 kcal/mol more stable than the C-6 enol (E$^{\text{Se}}_{\text{en}}$) (37). We are currently performing experiments to distinguish possible C6 tautomers.

The crystal structures also provide insight into several other aspects of catalysis. First, the S112A:HOPDA structure shows that the phenyl ring of the E$^{\text{Sk}}$ intermediate is twisted out of planarity with respect to the C-6 carbonyl, perhaps enhancing the latter’s susceptibility to nucleophilic attack in the subsequent hydrolytic reaction by disrupting overlap between the ring and carbonyl $\pi$-orbital systems. Second, the distance between the carboxylate and oxyanion binding sites is appropriate for the non-planar E$^{\text{Sk}}$ intermediate but not the fully extended, coplanar conformation of HOPDA that presumably predominates in solution (2). It is possible that the observed conformation has additional significance relative to the hypothesis that tautomerization is promoted by destabilizing the substrate in a twisted, non-planar binding mode (10). Notably, model building experiments suggest that the fully extended 2Z,AE dienol configuration could associate with the active site prior to ketonization, but it cannot do so and simultaneously occupy the carboxylate and oxyanion binding sites. Moreover, the enol tautomer in the S112A/H265A:HOPDA crystal structure is not fully extended (Fig. 5).

The 2E,AE conformation of HOPDA in the S112A/H265A complex (Fig. 5) probably does not reflect a catalytically relevant species. The absence of E$^{\text{S}}$ accumulation in both H265A
variants suggests that tautomerization becomes rate-limiting. Instead, a relatively slow (~50% of wild type or S112A tautomerization, Table 1) HOPDA deprotonation occurs en route to a species not spectroscopically observed in the wild type reaction. Although E:S\(^6\) does not accumulate without His-265, the substrate may slowly tautomerize, as in solution, to generate the crystallographically observed 2E:AE isomer, which may be the more stable form within the active site. Interestingly, two additional transient kinetic phases after deprotonation are observed in formation of this complex (Table 1), and may reflect molecular rearrangement to generate the different iso- 

mers of HOPDA.

While His-265 is crucial for the tautomeric half-reaction, Ser-112 clearly catalyzes hydrolysis, because its replacement with alanine impairs this reaction by at least 10\(^5\)-fold (19). Although Ser-112 is catalytically important, its precise role remains uncertain. Several studies have suggested that, in MCP hydrolases, the catalytic serine does not act as a nucleophile as in other serine hydrolases, but as a hydrogen bond donor to stabilize a \(\text{gem}-\text{diolate} \) formed after His-mediated attack of water at the substrate carbonyl (12–16,18). Nevertheless, the present crystal structures are more consistent with a nucleophilic role for Ser-112. For instance, no solvent molecule is necessary. Because Ser-112 appears appropriately poised for nucleophilic catalysis employing the canonical oxyanion hole, it seems unnecessary. Because Ser-112 contributes an additional hydrogen bond to an oxyanion intermediate, as proposed in the \(\text{gem}-\text{diol} \) hypothesis, seems unnecessary. For as discussed previously (19), the \(\text{gem}-\text{diol} \) mechanism also fails to explain the observed release of benzoate from the active site after HPD despite the apparently lower affinity of the enzyme for benzoate and the solvent accessibility of the benzate-binding pocket.

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