The Molecular Basis for Inhibition of BphD, a C–C Bond Hydrolase Involved in Polychlorinated Biphenyls Degradation

LARGE 3-SUBSTITUENTS PREVENT TAUTOMERIZATION*

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The microbial degradation of polychlorinated biphenyls (PCBs) by the biphenyl catabolic (Bph) pathway is limited in part by the pathway’s fourth enzyme, BphD. BphD catalyzes an unusual carbon–carbon bond hydrolysis of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), in which the substrate is subject to histidine-mediated enol-keto tautomerization prior to hydrolysis. Chlorinated HOPDAs such as 3-Cl HOPDA inhibit BphD. Here we report that BphD preferentially hydrolyzed a series of 3-substituted HOPDAs in the order H > F > Cl > Me, suggesting that catalysis is affected by steric, not electronic, determinants. Transient state kinetic studies performed using wild-type BphD and the hydrolysis-defective S112A variant indicated that large 3-substituents inhibited His-265-catalyzed tautomerization by 5 orders of magnitude. Structural analyses of S112A:3-Cl HOPDA and S112A:3,10-diF HOPDA complexes revealed a non-productive binding mode in which the plane defined by the carbon atoms of the dienoate moiety of HOPDA is nearly orthogonal to that of the proposed keto tautomer observed in the S112A-HOPDA complex. Moreover, in the 3-Cl HOPDA complex, the 2-hydroxy group is moved by 3.6 Å from its position near the catalytic His-265 to hydrogen bond with Arg-190 and access of His-265 is blocked by the 3-Cl substituent. Nonproductive binding may be stabilized by interactions involving the 3-substituent with non-polar side chains. Solvent molecules have poor access to C6 in the S112A:3-Cl HOPDA structure, more consistent with hydrolysis occurring via an acyl-enzyme than a gem-diol intermediate. These results provide insight into engineering BphD for PCB degradation.

Polychlorinated biphenyls (PCBs)4 were manufactured extensively in the 20th century for industrial and commercial applications, including use in electrical transformers, hydraulics, and plasticizers.5 Although banned in the United States since 1977, environmentally persistent PCBs have been linked to cancer (2), childhood neurodevelopmental deficits arising from prenatal exposure (3), and a host of other effects attributed to endocrine disruption (4). Indeed, concerns about high concentrations of PCBs and other contaminants in some freshwater fish have prompted a recent comprehensive risk-benefit analysis of fish consumption (5).

The observation that bacteria partially degrade PCBs has motivated research into microbial bioremediation as an alternative to traditional remediation approaches, which typically require the costly and invasive removal of contaminated soil. Bacteria use the biphenyl (Bph) pathway to aerobically degrade many PCBs in a strain-dependent manner. The upper Bph pathway consists of four enzymes that transform biphenyl into benzoic acid and 2-hydroxyxypenta-2,4-dienoic acid (6). Nevertheless, certain congeners are not efficiently degraded by this pathway, leading to the accumulation of these congeners or their chlorinated metabolites and even inhibition of Bph enzymes. For example, 2’,6’-diCl,3,3-dihydroxybiphenyl (2’,6’-diCl-DHB) inhibits the third pathway enzyme, DHB dioxygenase (DHBD) in the potent PCB-degrading strain Burkholderia xenovorans LB400 (7). Similarly, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acids (HOPDAs) that are chlorinated at the 3 and 4 positions inhibit the fourth enzyme, BphD, of B. xenovorans LB400 (8) and Rhodococcus globulatus P6 (8–10) as described below. Interestingly, a glutathione S-transferase in B. xenovorans LB400, BphK, catalyzes the dehalogenation of 3-Cl HOPDA (11) and DxnB2, a homolog of BphD from the dibenzofuran catabolic pathway of Sphingomonas wittichii RW1, catalyzes the hydrolysis of 3-Cl HOPDA (8–10). Nevertheless, chlorinated HOPDAs accumulated in whole cell studies of PCB degradation (12–14), suggesting that BphD represents a bottleneck for PCB degradation.

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4 The abbreviations used are: PCB, polychlorinated biphenyl; Bph, biphenyl; DHB, dihydroxybiphenyl; DHBD, DHB dioxygenase; HOPDAs, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; WT, wild type.

5 U. S. Environmental Protection Agency (Oct. 24, 2007) Polychlorinated biphenyls (PCBs) (Oct. 24, 2007) http://www.epa.gov/epaoswer/hazwaste/pcbs/index.htm.
The inhibition of Bph enzymes has recently taken on additional significance in light of discoveries in Mycobacterium tuberculosis, the etiological agent of tuberculosis. Specifically, homologues of the Bph enzymes are involved in cholesterol catabolism (15) and are critical for the survival of the pathogen in the human macrophage (16). The lack of human homologues of the cholesterol-degrading enzymes suggests that they are promising drug targets. Understanding the mechanism of BphD inhibition will facilitate the engineering of the enzyme to improve its activity toward chlorinated substrates and should inform the development of inhibitors of HsaD, the cholesterol-degrading homologue in M. tuberculosis.

BphD catalyzes the hydrolytic C–C bond cleavage of HOPDA (Fig. 1) and has features typical of the αβ-hydrolyase superfamily (17, 18), including the fold and conserved active site catalytic triad composed of serine, histidine, and aspartate residues. To expel the electron-rich dienoate moiety of the substrate, the enzyme first employs a His-265-mediated enol-keto tautomerization to generate a hydrolyzable keto-intermediate (E-S\(^{\text{lev}}\)) (19–24). Indeed, His-265-dependent formation of an intermediate with a red-shifted absorbance spectrum (E-S\(^{\text{red}}\)) is formed at a similar rate in both WT and S112A BphDs (19). Crystallographic data for the E-S\(^{\text{red}}\) intermediate trapped in the S112A-HOPDA complex are most consistent with the bound HOPDA being ketonized (19), although a non-planar, distorted conformation of the enol/enolate could not be completely ruled out. Hydrolysis then proceeds via either a gem-diol intermediate (24–28) or an acyl-enzyme intermediate (19, 20). As noted above, HOPDAs that are chlorinated on the dienoate moiety are poorly transformed by BphD (8–10). Specifically, 5-chlorination reduced the maximal rate of BphD by 3-fold, and chlorination at the 3 or 4 positions reduced the maximal rate by \(10^3\) and \(10^4\)-fold, respectively. Although 4-Cl HOPDA is the least efficiently transformed monochlorinated HOPDA, 3-Cl HOPDAs represent a more significant roadblock to PCB degradation in two respects. First, 3-Cl HOPDA \((t_{1/2} \sim 500\) h) is more stable than 4-Cl HOPDA, which undergoes a non-enzymatic transformation to 4-OH HOPDA \((t_{1/2} = 2.8\) h) followed by degradation to products that include acetophenone \((t_{1/2} \sim 180\) h) (8). Second, 3-Cl HOPDA is a very poor substrate of BphD\(_{L,\text{B4000}}\), effectively inhibiting the hydrolysis of HOPDA \((K_i = 0.57\) \(\mu M\)) more potently than either 4-Cl HOPDA \((K_i = 3.6\) \(\mu M\)) or 4-OH HOPDA \((K_i = 0.95\) \(\mu M\)). It has been unclear whether the 3-Cl substituent impairs tautomerization or hydrolysis and whether the defect is due to an electronic or steric effect.

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**FIGURE 1.** The reaction catalyzed by BphD.

Herein we investigated the basis for inhibition of BphD by 3-Cl HOPDA. First, the ability of BphD to hydrolyze a series of 3-substituted HOPDAs was studied using steady-state kinetics to compare steric versus electronic effects. Second, single turnover stopped-flow kinetic analysis was used to probe the effects of 3-substitution on E-S\(^{\text{red}}\) formation in both WT and hydrolytically impaired Ser112Ala variants. Finally, crystal structures of the S112A-3-Cl HOPDA and S112A-3,10-diF-HOPDA complexes were analyzed to reveal the structural correlates of the kinetic behavior.

**MATERIALS AND METHODS**

**Chemicals**—HOPDA, 3-Cl HOPDA, 3-Me HOPDA, and 3,10-diF HOPDA were enzymatically generated from DHB, 4-Cl DHB, 4-Me DHB, and 4,4’-diF DHB, respectively, using DHBD as previously described (8, 20). The preparation of DHB and chlorinated DHBs has been described elsewhere (29). All other chemicals were of analytical grade.

**Preparation of 4,4’-diF-DHB**—A plate of W medium (30) was streaked with frozen stock of Pandoraea pnomenusa (formerly Comamonas testosteroni) B-356 and incubated with biphenyl crystals in the lid at 30 °C until colonies were visible (~5 days). Several colonies were added to 3 ml of W medium containing 1 mg biphenyl and incubated at 30 °C and 250 rpm until cloudy (~4 days). Alternatively, this step could be shortened to ~2 days if a 50-\(\mu\)l aliquot of frozen stock was used to inoculate the 3 ml of culture. One liter of W medium containing 0.5 g of biphenyl in a 2-liter flask was inoculated with 1 ml of starter culture, and the mixture was incubated as above. When the optical density at 600 nm reached 1, the culture was carefully decanted to remove biphenyl crystals and then centrifuged for 10 min at 7000 \(\times\) g. The pellet was washed twice with potassium phosphate buffer \((I = 0.1\) m, pH 7.5) to remove residual biphenyl and resuspended in 500 ml of buffer supplemented with 100 mg of 4,4’-dif-biphenyl and 7 mg of 3-chlorocatechol. The latter was included to inactivate DHBD (31) and thereby prevent enzymatic transformation of the produced 4,4’-dif-DHB. The culture was incubated as above, and 50-\(\mu\)l aliquots were analyzed by high-performance liquid chromatography at 30-min intervals using a Prodigy ODS Prep column, 2.1 \(\times\) 250 mm (Phenomenex, Torrance, CA), operating at a flow rate of 1.5 ml/min. The mobile phase initially consisted of a 30:70 ratio of solvent A (0.5% aqueous H\(_3\)PO\(_4\)) to solvent B (methanol) for the first 5 min of the run, then a gradient was used to achieve 100% B at 10 min. The retention time of 3-chlorocatechol was 2.6 min, 4,4’-diF-DHB eluted at 5.4 min, and 4,4’-dif-biphenyl eluted at 13 min. When the maximum concentration of 4,4’-dif-DHB in the culture was reached as judged by high-performance liquid chromatography (after ~3 h), the culture was filtered to remove undissolved starting material, and then extracted three times with ~200 ml of ethyl acetate. The pooled fractions were dried over anhydrous MgSO\(_4\) and rotary evaporated to dryness. The crude extract was dissolved in an appro-
priate volume of mobile phase (20:80 A:B ratio) and purified by preparative high-performance liquid chromatography using a Prodigy ODS Prep column, 21.2 × 250 mm (Phenomenex, Torrance, CA) operating at a flow rate of 8.5 ml/min. The 4,4′-difluoro-DHB (retention time ~ 8 min) was collected, extracted into ethyl acetate, dried, and evaporated as above. The purity was estimated to be >95% by high-performance liquid chromatography. The 4,4′-difluoro-DHB was dissolved in 10% ethanol, 80% H2O, and 10% D2O to collect NMR spectra at 25 °C using a 600-MHz spectrometer at the Department of Chemistry, University of Rochester. The 1H NMR reference compound was 100 μM 2,2-dimethyl-2-silapentane 5-sulfonate and was used as an indirect reference for 19F NMR (found by 19F(1H) NMR: −3.6 (s, 1F), −23.9 (s, 1F)) and 1H NMR (6.75–6.82 (m, 2H), 7.21 (t, 2H, J = 6 Hz), 7.52 (t, 2H, J = 6 Hz)).

**Steady-state Kinetic Measurements**—Initial rates of BphD-catalyzed hydrolysis at varying substrate concentrations were obtained by monitoring the substrate absorbance maximum versus time in potassium phosphate buffer (I = 0.1 M, pH 7.5) using a Varian Cary 5000 spectrophotometer equipped with a thermostatted cuvette holder (Varian Canada, Mississauga, Ontario, Canada) maintained at 25.0 ± 0.5 °C, controlled by Cary WinUV software version 2.00. Reactions were carried out in a 1-ml volume and were initiated by the addition of 5 μl of an appropriately diluted enzyme solution. The 3-Me HOPDA was generated in situ by adding 80 μg of polyhistidine-tagged DHBD to the cuvette containing 4-Me DHB. After obtaining the rate of background decay (0.5–1 min), BphD was added and the activity of the enzyme was determined by correcting for the background. The Michaelis-Menten equation was fit to the data using LEONORA (32). The molar absorptivity of 3,10-difluoro-HOPDA (ε348 = 37.9 ± 2.1 mm−1 cm−1) was calculated from its absorption spectrum after quantification by measuring the amount of dioxygen consumed in the ring-opening reaction of 4,4′-difluoro-DHB catalyzed by DHBD. Dioxygen consumption was measured using a Clark-type polarographic oxygen electrode (Yellow Springs Instruments model 5301, Yellow Springs, OH) as previously described for other HOPDAs (33). The molar absorptivity of 3-Me HOPDA (ε340 = 18.7 ± 0.4 mm−1 cm−1) was determined by recording the absorption spectrum of a known quantity of 4-Me DHB immediately after cleavage by DHBD.

**Stopped-flow Spectrophotometry**—Single turnover reactions (BphD = 8 μM; substrate = 4 μM) in the phosphate buffer described above were monitored 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 a recirculating water system. Multiple wavelength data from the time courses of single shots were acquired using the Xscan software (Applied Photophysics Ltd.), then saved as CSV files in the RISC Pro-K software and exported to Excel where replicate measurements from at least four shots were averaged. Selected single wavelength datasets were then imported into the SX18MV software (Applied Photophysics Ltd.) where multiple exponential equations were fit to the data to obtain reciprocal relaxation times and amplitudes. Good fits were characterized by random variation in the residuals.

**Crystallization and Preparation of Complexes**—Crystals of the substrate-free S112A variant of BphD were grown at 20 °C in 1.9 M sodium malonate, pH 7.0, by sitting drop vapor diffusion, as previously reported (19). Complexes were prepared by incubating crystals for 30 min in 60 μl of reservoir solution supplemented with ~10 mM 3-Cl-HOPDA or ~65 mM 3,10-difluoro-HOPDA. Crystals were prepared for flash freezing by serial transfer into solutions containing higher concentrations of sodium malonate (3.4 M and 3.7 M, pH 7.0) augmented with ~5 mM of 3-Cl HOPDA or trace amounts of 3,10-difluoro-HOPDA. The incubation time was 3–6 s per step. Crystals were frozen by immersion in liquid N2.

**Diffraction Experiments and Structure Analysis**—All diffraction data were acquired by the use of the SERCAT facilities at the Advanced Photon Source, Argonne National Laboratory. The x-ray wavelength was 1.0 Å, and crystals were maintained at ~100 K during data collection. Diffraction images were recorded by a MarMosaic 300 charge-coupled device 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 intensities were merged and scaled using SCALEPACK; both programs were from the HKL2000 program suite (34).

The initial model for both complexes included only the protein atoms from the crystal structure of the S112A-malonate complex (PDB code 2PU6) (19). Rigid body refinement performed in REFMAC (35) from the CCP4 package (36) was followed by iterative cycles of restrained atomic parameter refinement via REFMAC and manual density fitting using the molecular graphics program O (37). PRODRG (38) was used to construct structures of 3-Cl HOPDA, 3,10-difluoro-HOPDA, and malonate for density fitting and establishment of refinement restraints. The bond lengths and bond angles of the ligands were restrained to values expected for the enol isomer. Torsion angles in the non-aromatic portion of the substrates were unrestrained. The stereochemical properties of the models and the hydrogen bonding were analyzed by using the programs PROCHECK (39) and REDUCE (40).

**RESULTS**

**Steady-state Kinetics**—To assess the basis of inhibition of BphD by 3-Cl HOPDA, the enzyme-catalyzed hydrolysis of 3,10-difluoro-HOPDA and 3-Me HOPDA was studied using steady-state kinetics. The 10-fluoro substituent is not expected to greatly affect catalysis because HOPDAs with small, electron-withdrawing substituents (e.g., -Cl and -CF3) at this position are hydrolyzed by BphD with kcat values within 25% of that for HOPDA (8, 28). Significantly, reduction of the volume of the electronegative 3-substituent by 40% upon chlorine-to-fluorine substitution resulted in a 150-fold increase in kcat (Table 1), indicating that larger 3-substituents interfere with catalysis. This conclusion was supported by the observation that the substrate with the largest 3-substituent, 3-Me HOPDA, had the lowest apparent kcat (Table 1). Although a kcat value was reported for 3-Cl HOPDA (8), kcat could not be determined for 3-Me HOPDA due to a combination of lower activity, lower molar absorptivity, and higher background decay rates, which together prevented reliable initial rate measurements at low substrate concentrations. Finally, we also determined the stability of HOPDAs in the enzyme reaction buffer (Table 1), but we discerned no obvi-
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TABLE 1
Steady-state parameters of BphD hydrolysis of 3-substituted HOPDAs

| 3-X HOPDA     | Half-life | Electronegativity | Volume | C–X bond length | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|---------------|-----------|-------------------|--------|-----------------|-----------|-------|--------------|
| H             | 58        | 2.2               | 7.2    | 1.08            | 6.5       | 0.2   | 32           |
| F             | 11        | 4.1               | 13.3   | 1.34            | 1.4       | 4.8   | 0.29         |
| Cl            | 500       | 2.8               | 22.5   | 1.73            | 0.0089    | 0.54  | 0.016        |
| Me            | 30        | 2.3               | 28.4   | 1.51            | 0.0036    | ND    | ND           |

Errors are less than 15%.

In an attempt to observe $ES^{red}$ accumulation for 3-Cl HOPDA, the S112A variant was used. This variant catalyzes $ES^{red}$ formation at the same rate as WT BphD but possesses severely impaired hydrolytic activity (19). For 3-Cl HOPDA, $ES^{red}$ did not detectably accumulate in this variant (Fig. 2A). Under single turnover conditions ([BphD] = 8 μM, [3-Cl HOPDA] = 4 μM), an initial decrease (1/$\tau_1$ = 15 s$^{-1}$) occurred at the absorbance maximum of the 3-Cl HOPDA enolate (432 nm) (Table 2). This decay corresponded to a ~20% loss in absorbance and occurred together with a slight blue shift to 427 nm. This may correspond to formation of an enzyme-bound planar 3-CHOPDA enolate, $ES^{red}$. This species very slowly decayed (1/$\tau_2$ = 0.0077 s$^{-1}$) at a rate that matches the previously measured $k_{cat}$ value (Table 1). Interestingly, this rate is similar to that of tautomerization of HOPDA in solution as measured by deuterium exchange experiments (20, 22). The absence of $ES^{red}$ accumulation suggests that 3-Cl HOPDA tautomerization is slower than hydrolysis.

The absence of $ES^{red}$ accumulation for 3-Cl HOPDA, the S112A variant was used. This variant catalyzes $ES^{red}$ formation at the same rate as WT BphD but possesses severely impaired hydrolytic activity (19). For 3-Cl HOPDA, $ES^{red}$ did not detectably accumulate in this variant (Fig. 2B). Similar to the behavior of WT, S112A caused an initial decrease (1/$\tau_1$ = 26 s$^{-1}$) corresponding to ~15% loss in 3-Cl HOPDA absorbance (Table 2), albeit without a correlated blue shift. This was followed by a very small (<1% of total 3-Cl HOPDA absorbance) and relatively slow (1/$\tau_2$ = 0.55 s$^{-1}$) increase in absorbance at 432 nm. Subsequent decay was not detected on the time scale examined (<1 min). In summary, an $ES^{red}$ spe-
cies of 3-Cl HOPDA was not detected in either wild type or S112A, suggesting that an ES$^*$ complex is the predominant enzyme-bound species and that catalysis is limited by formation of ESS$^{\text{red}}$.

The improved turnover of 3,10-diF HOPDA compared with 3-Cl HOPDA (Table 1) implies that 3,10-diF HOPDA may be more readily ketonized by the enzyme. The reaction of BphD with 3,10-diF HOPDA yielded a detectable ESS$^{\text{red}}$ (λ$\text{max}$ ~ 468 nm, Fig. 2C). The low intensity of this spectrum suggests that only a small amount of ESS$^{\text{red}}$ accumulates, and thus tautomeration may be only partially rate-limiting. Decay of the signal at the absorbance maximum of 3,10-diF HOPDA (438 nm) can be described by three phases (Table 2): (i) a loss in absorbance (1/τ$_1$ = 51 s$^{-1}$) analogous to the initial decay in the 3-Cl HOPDA reaction, whereby a slightly blue-shifted feature (λ$\text{max}$ = 427 nm) was generated together with a ~30% loss in absorbance; (ii) a small (20% of total absorbance decrease) second decay (1/τ$_2$ = 7.6 s$^{-1}$) that generated ESS$^{\text{red}}$ (λ$\text{max}$ ~ 468 nm), and (iii) a final phase, 1/τ$_3$ = 1.3 s$^{-1}$.

Although ESS$^{\text{red}}$ (λ$\text{max}$ ~ 517 nm) accumulated to a greater extent in S112A than in wild type, stoichiometric formation of this species was not observed using 3,10-diF HOPDA (Fig. 2D). ESS$^{\text{red}}$ is formed quickly (1/τ$_1$ = 90 s$^{-1}$), but consumes only ~35% of the substrate based on absorbance. This is followed by a minor relaxation of <1% total absorbance, which occurs as an increase at 438 nm (1/τ$_2$ = 1.1 s$^{-1}$) and a corresponding decrease at 517 nm. The incomplete ESS$^{\text{red}}$ formation suggests that the 3-F substituent destabilizes ESS$^{\text{red}}$ relative to the ES$^*$ complex and/or free substrate. Curiously, 3,10-diF HOPDA ESS$^{\text{red}}$ formation is faster in S112A than wild type, suggesting that the Ser-112 hydroxyl function may impede tautomeration by further stabilizing the ES$^*$ complex relative to ESS$^{\text{red}}$.

In summary, increasing the size of the 3-substituent appears to slow ESS$^{\text{red}}$ formation. The fluoro-substitution apparently slows tautomeration by similarly stabilizing both ESS$^{\text{red}}$ and an alternate ES$^*$ binding mode. Although ESS$^{\text{red}}$ accumulation is minimal, tautomeration is not significantly slowed relative to hydrolysis, and therefore the overall rate of catalysis is not dramatically affected. In contrast, tautomeration is more severely impaired by chloro-substitution, which may preferentially stabilize ES$^*$ relative to ESS$^{\text{red}}$.

Crystal Structure of S112A:3-Cl HOPDA—The crystal structure of the binary complex of S112A with 3-Cl HOPDA was examined at 1.7 Å resolution. Tables 3 and 4 summarize the diffraction data and refinement statistics, respectively. With respect to the fold of the monomer and quaternary structure, the protein structure exhibits no important differences relative to the malonate (PDB entry 2PU6) and HOPDA complexes (PDB entry 2PUH) of S112A; these conclusions are also valid for the structure of the 3,10-diF HOPDA complex described below.

The binding of 3-Cl HOPDA to S112A and related electron density maps are illustrated in Fig. 3A. As shown in Fig. 3A, F$_o$ – F$_c$ maps, calculated before the substrate was added to the model, contained readily interpretable electron density for 3-Cl HOPDA, which was modeled as the enol isomer, (2Z,AE)-3-Cl HOPDA, because the kinetic experiments did not detect accumulation of a species corresponding to S$^{\text{red}}$ in solution. Nevertheless, the nearly coplanar conformation of 3-Cl HOPDA cannot exclude the presence of the keto isomer: the torsion angles do not uniquely predict which bonds are single and which are double.

Fig. 3B shows that 3-Cl HOPDA and HOPDA bind in distinctly different conformations and slightly different overall positions. Thus, although the dienoate moiety, the 6-oxo group, and the 6-phenyl substituent occupy the same binding sites in the two complexes, the root mean square deviation is 0.86 Å for

### TABLE 2

| Substance          | BphD variant | Phase 1 (amplitude) | Phase 2 (amplitude) | Phase 3 (amplitude) |
|--------------------|--------------|---------------------|---------------------|---------------------|
| 3-Cl HOPDA         | WT           | 15 s$^{-1}$ (0.031) | 0.0077 s$^{-1}$ (0.13) | 0.55 s$^{-1}$ (−0.001) |
|                    | S112A        | 26 s$^{-1}$ (0.026) |                     |                     |
| 3,10-diF HOPDA     | WT           | 51 s$^{-1}$ (0.051) | 7.6 s$^{-1}$ (0.028) | 1.3 s$^{-1}$ (0.070) |
|                    | S112A        | 88 s$^{-1}$ (0.055) |                     |                     |

### TABLE 3

| Parameter or statistic | 3-Cl HOPDA | 3,10-diF HOPDA |
|------------------------|------------|---------------|
| Resolution (Å)         | 83.3–1.70  | 83.3–1.57     |
| R (%)                  | 15.5       | 17.2          |
| R$_{free}$ (%)         | 19.3       | 20.1          |
| Est. coordinate error  | 0.09       | 0.078         |
| Model content          |            |               |
| Protein atoms          | 2284       | 2340          |
| Malonate molecules     | 1          | 2             |
| Water molecules        | 166        | 186           |
| Average B factors (Å$^2$) | 21       | 22            |
| Protein                | 21         | 22            |
| Water atoms            | 28         | 31            |
| All atoms              | 21         | 23            |
| Root mean square deviation from target | 0.01 | 0.009 |
| Bond lengths (Å)       | 1.22       | 1.20          |

### TABLE 4

| Parameter or statistic | 3-Cl HOPDA | 3,10-diF HOPDA |
|------------------------|------------|---------------|
| Resolution range (Å)   | 83.3–1.70  | 83.3–1.57     |
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| Est. coordinate error  | 0.09       | 0.078         |
| Model content          |            |               |
| Protein atoms          | 2284       | 2340          |
| Malonate molecules     | 1          | 2             |
| Water molecules        | 166        | 186           |
| Average B factors (Å$^2$) | 21       | 22            |
| Protein                | 21         | 22            |
| Water atoms            | 28         | 31            |
| All atoms              | 21         | 23            |
| Root mean square deviation from target | 0.01 | 0.009 |
| Bond lengths (Å)       | 1.22       | 1.20          |

$^a$ Cruickshank’s diffraction-component precision index (Footnote 1) as calculated by REFMAC.

$^b$ For 3-Cl HOPDA, the total includes 90 atoms from 9 residues modeled in two conformations. For 3,10-diF HOPDA the corresponding values are 202 atoms in 18 residues.
the common atoms of the two ligands. Likewise, several interacting protein side chains, especially those of Asn-111, Met-171, Phe-175, Trp-266, and His-265, are in significantly different positions/conformations in the two complexes. These variations largely reflect a gross difference in the orientation of the dienolate moiety relative to the protein. In essence, the planes defined by atoms C1 through C5 in the two complexes are nearly orthogonal to each other, and the torsion angles about the C2–C3 and C1–C2 bonds differ by 155° and 90°, respectively.

In consequence, the binding interactions of the 1-carboxylate and 2-hydroxo groups of 3-Cl HOPDA are remarkably different relative to those observed in the HOPDA complex. Dominant features of HOPDA binding include hydrogen bonds between both oxygen atoms of the 1-carboxylate group and the guanidinium group of conserved Arg-190, and a hydrogen bond between the 2-oxo/hydroxo oxygen and the side chain of His-265. Although the 1-carboxylate group of 3-Cl HOPDA is in the vicinity of Arg-190, it does not hydrogen bond with that residue. Rather, the 2-hydroxo group is moved by 3.6 Å from its position near His-265 in the S112A/HOPDA complex and hydrogen bonds with Arg-190. The Z conformation about the C2–C3 bond places the 3-Cl atom in an adjacent binding site formed by the non-polar side chains of Leu-156, Phe-175, and Phe-239 (see Fig. 3C), and at longer distances by the side chains of Pro-44 and Met-171, which is found in alternate conformations. Moreover, the chlorine atom and the side chain of Phe-175 lie between His-265 and the 2-hydroxyl group.

Accommodation of the bulky 3-Cl substituent in an appropriate non-polar site appears to be the major cause of the differences in conformation and position of 3-Cl HOPDA relative to HOPDA as well as adjustments of the protein. If 3-Cl HOPDA assumed the conformation and position of HOPDA in the crystal structure of its complex with S112A, the 3-Cl group would be in steric conflict with the backbone nitrogens of Gly-42 and Gly-43 (distances of <2.4 Å) and the 6-oxo-substituent (2.5 Å). Similarly, when the coordinates for 3-Cl HOPDA are mapped onto the HOPDA complex, the chlorine atom is placed 2.1 Å from the Cγ of Phe-175. Relaxation of
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the latter contact may motivate the observed differences in the conformations of His-265, Trp-266, as well as other nearby residues.

At least four characteristic features of the HOPDA complex are reproduced in the 3-Cl HOPDA complex: 1) the 6-oxo group binds in the oxygen hole formed by backbone amides of Gly-42 and Met-113; 2) C5 and C6 remain within 3.8 Å of the Cβ atom of Ala-112; 3) there is inadequate room in the active site for a water molecule to approach C6 from either face; and 4) the 6-phenyl substituent interacts with the same non-polar side chains of the NP-site, including those of Ile-153, Leu-213, Trp-216, and Val-240. With respect to point 3, the substrate binding site includes no crystallographically ordered waters and no water-sized voids. The closest ordered water is 7.5 Å away from C6 and lies in a solvent-accessible pocket beyond the phenyl substituent.

The electron density maps also suggest an additional, minor binding mode in which the phenyl substituent occupies the expected binding site, but in a different orientation, and the rest of the ligand extends away from the catalytic residues toward a possible entrance to the active site near the side chains of Phe-157, Ala-208, and Pro-212. This mode was not modeled.

Crystal Structure of S112A 3,10-diF HOPDA—Although the crystal structure of S112A 3,10-diF HOPDA was determined at higher resolution than the 3-Cl HOPDA complex (1.57 Å versus 1.7 Å; see Tables 3 and 4), the development of a definitive model for the ligand proved to be more difficult. As described below, the assessment of many electron density maps and refinement experiments led us to conclude that the C1-carboxylate and C2-hydroxyl substituents bind in multiple, poorly resolved conformations. The final model includes 3,10-diF HOPDA in a single conformation that represents the most reliable observations and is similar to the conformation of 3-Cl HOPDA.

From these observations, 3,10-diF-HOPDA was modeled and refined with the dienoate moiety in the same 2Z,4E conformation as 3-Cl HOPDA. The resulting $F_o - F_c$ maps suggested an alternative location for the carboxylate group consistent with a 2E,4E conformer. To test this observation, maps were calculated with coefficients ($F_{obs}^{S,10-diF-HOPDA} - F_{obs}^{3-Cl-HOPDA}$) and phases from the 3-Cl HOPDA complex using data from two S112A 3,10-diF HOPDA crystals. Both maps had negative features overlapping the carboxylate, 2-OH, and 3-Cl groups of 3-Cl HOPDA, and a positive feature confirming the 10-F substituent of diF-HOPDA (a negative peak is expected at the chlorine atom even if F occupies the same position because of the difference in atomic scattering power). There was no comparable difference density near C5, C6, and most of the atoms of the phenyl ring, indicating these portions of the HOPDA structure are consistently located in the diF-HOPDA and 3-Cl HOPDA complexes. These maps also had minor positive and negative features indicating that malonate was bound in a significant fraction of the active sites in the S112A-diF HOPDA crystals, as it is bound in the previously determined structure of S112A-malonate. The presence of malonate-associated density inhibited confirmation of the location of the 2-OH group as its expected location in the 2E,4E conformer is within 1.3 Å of the location of one of the oxygen atoms of malonate. Maps with coefficients ($F_{obs}^{3,10-diF-HOPDA} - xF_{obs}^{malonate}$) $x = 1.0$, were calculated to obtain the equivalent of an $F_{obs}$ map with the contribution of the malonate removed and to estimate the fractional occupancy of 3,10-diF HOPDA. These maps also failed to establish the location of the 2-OH group or resolve the carboxylate group.

Based on this evidence, 3,10-diF HOPDA was ultimately modeled and refined at 60% occupancy as the 2Z,4E conformer in a conformation similar to that of 3-Cl HOPDA. We also tested all possible 2E,4E and 2Z,4E conformations and found none to be as compatible with the electron density. Taken as a whole, the electron density maps indicate the final model does not provide a full description of the active site structure and lead us to conclude that 3,10-diF HOPDA accesses multiple conformations in the crystal.

DISCUSSION

The molecular basis for the inhibition of BphD by 3-Cl HOPDA was studied by steady-state and transient-state kinetics, and x-ray crystallographic analysis of ES complexes of 3-substituted HOPDAs. These studies revealed that (i) steady-state turnover is limited by steric, not electronic, features of the 3-substituent; (ii) large 3-substituents impair ES$^\text{red}$ formation, and therefore tautomerization; and (iii) 3-substituted HOPDAs bind in a non-productive mode.

Steady-state kinetic studies demonstrated that steric bulk, not electronegativity, of the 3-substituent obstructs BphD catalysis. Incremental increases in the size of the 3-substituent incrementally slowed turnover: the enzyme preferentially hydrolyzed 3-substituted HOPDAs in the order H > F > Cl > Me. By contrast, there was no significant correlation between turnover and electronegativity of the 3-substituent. Stopped-flow spectrophotometry demonstrated that larger 3-substituents impede formation of the ES$^\text{red}$ intermediate. For instance,
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whereas $E:\text{S}^{\text{red}}$ is apparently completely formed from HOPDA, minor accumulation occurred from 3,10-diF HOPDA, and this intermediate was not detected when 3-Cl HOPDA was used as a substrate. Thus, large 3-substituents inhibit the formation of $E:\text{S}^{\text{red}}$ and ultimately disrupt tautomerization.

The kinetic data predict a non-productive binding mode for 3-substituted HOPDAs. For 3-Cl HOPDA, stopped-flow analysis reveals a two-step transformation: initial formation of an $E:\text{S}^\alpha$ intermediate ($1/\tau_1 = 15 \text{ s}^{-1}$), followed by its decay ($1/\tau_2 = 0.0077 \text{ s}^{-1}$) to products, possibly via rate-determining formation of $E:\text{S}^{\text{red}}$. For 3,10-diF HOPDA, a similar initial formation of $E:\text{S}^\alpha$ ($1/\tau_1 = 51 \text{ s}^{-1}$) was followed by two slower relaxations ($1/\tau_2 = 7.6 \text{ s}^{-1}$ and $1/\tau_3 = 1.3 \text{ s}^{-1}$) during which a small amount of $E:\text{S}^{\text{red}}$ appeared to accumulate. The incomplete $E:\text{S}^{\text{red}}$ formation for S112A-3,10-diF HOPDA implies reversibility and suggests that the mechanism may be described by either three-step model in Fig. 4.

The S112A-3-Cl HOPDA crystal structure is consistent with the kinetic data, indicating that the 3-Cl HOPDA is bound non-productively. More specifically, the structure reveals a substrate-binding mode (Fig. 3) wherein 3-Cl HOPDA binds in a C1–C6 coplanar conformation with the plane of the dienoate moiety orthogonal to that of the $E:\text{S}^{\text{red}}$ intermediate of the S112A/HOPDA complex (19). Although the crystallographic studies cannot determine the tautomeric state of the ligand in this case, the observed binding mode is consistent with the impaired $E:\text{S}^{\text{red}}$ formation of 3-Cl HOPDA observed by stopped-flow. In addition, the conformation is very similar to that observed for HOPDA in the complex with the S112A/H265A mutant, which does not accumulate $E:\text{S}^{\text{red}}$. The C1-carboxylate and C2-hydroxyl groups of 3-Cl HOPDA are in remarkably different positions relative to those observed for $E:\text{S}^{\text{red}}$ in the same S112A variant. Whereas both oxygen atoms of the HOPDA C1-carboxylate group hydrogen bond with the guanidinium group of Arg-190, this residue instead hydrogen bonds to the 2-oxo/hydroxyl group of 3-Cl HOPDA. As a result, the hydrogen bond between the 2-oxo/hydroxyl oxygen and the side chain of His-265 observed in the S112A complex with HOPDA is not possible for 3-Cl HOPDA. The crystal structure therefore illustrates that the preferred binding mode of 3-Cl HOPDA is incompatible with the proposed His-265-mediated proton transfer of the tautomerization reaction.

In light of the different isomeric states and binding modes of 3-Cl HOPDA and HOPDA to S112A, it is remarkable that the structures of neither complex contain a solvent molecule suitable for attack of the $E:\text{S}^\alpha$ C6 carbonyl in the active site, despite the extra space made available by removal of the serine hydroxyl: the closest water is more than 7 Å away. In this respect, the crystal structure of the S112A-3-Cl HOPDA complex is more consistent with a nucleophile role for Ser-112.

The crystal structures also provide a possible explanation for the greater accumulation of $E:\text{S}^\alpha$ for 3-Cl HOPDA relative to 3,10-diF HOPDA (Fig. 3C). The 3-Cl atom occupies a binding pocket formed by the non-polar side chains of Leu-156, Phe-175, Phe-239, and Met-171. Because these residues do not shift the hydrophobic pocket is fully occupied by the inhibitor's chlorine atom, and substitution with fluorine reduces binding affinity (41, 42). In the crystal, the binding of 3-Cl HOPDA is also monomorphic, whereas the carboxylate, 2-OH, and 3-F moieties of 3,10-diF HOPDA are not well ordered. This may reflect a difference in binding forces and/or a difference in the interaction between the 2-OH and 3X groups in the 2Z conformation.

The proposed effects of 3-substituted HOPDAs on formation of the catalytically competent $E:\text{S}^{\text{red}}$ are summarized in Fig. 5. Unsubstituted HOPDA is rapidly converted to $E:\text{S}^{\text{red}}$ under single turnover conditions ($1/\tau_1 \approx 500 \text{ s}^{-1}$), and the presence of an isosbestic point at ~460 nm suggests direct transformation (19, 20). Large 3-substitu-
ents may stabilize an alternate planar E$S^*$ binding mode in which the 3-substituent occupies a hydrophobic pocket. In contrast, large 3-substituents are predicted to destabilize the non-planar $E S^{red}$ conformation via a steric clash with the backbone of Gly-43 and/or an intramolecular steric conflict with other HOPDA atoms. Thus, access to $E S^{red}$ is more severely impaired for 3-Cl-HOPDA than for 3,10-diF-HOPDA. Interestingly, the possibility of moderate destabilization of both $E S^*$ and $E S^{red}$ by the 3-F substituent is consistent with the S112A:3,10-diF-HOPDA crystal structure indicating multiple binding modes for the carboxylate and 2-OH groups. Nevertheless, the association of the 6-oxo group with the oxyanion hole is maintained, which allows the scissile bond to approach the His-265 and Ser/Ala-112 residues involved in hydrolysis. Although kinetic constants were not determined for 3-Me HOPDA, the volume of the methyl substituent is only ~25% larger than chlorine, so it may also occupy the hydrophobic pocket.

The preference for 3-Cl HOPDA binding in the alternate planar $E S^*$ mode instead of generating the productive, non-planar $E S^{red}$ intermediate has implications for both bioremediation of PCBs and the development of tuberculosis therapeutics. With respect to the former, the ability of the BphD homologue DxnB2 to hydrolyze 3-Cl-HOPDA with a ~13-fold higher specificity may provide an opportunity to overcome this block in the Bph pathway (9). Nevertheless, the structural basis for the different activities of these enzymes is unclear: Gly-42 and Gly-43, which are proposed to clash with the 3-Cl substituent, are conserved between the two enzymes, as is Phe-74, the residue closest in space to Gly-43. Further studies of DxnB2 should reveal the basis for its improved turnover of 3-Cl HOPDA and may further guide protein-engineering efforts.

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