Biphenyl dioxygenase from an Arctic Isolate Is Not Cold Adapted

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Biphenyl dioxygenase from the psychrotolerant bacterium Pseudomonas sp. strain Cam-1 (BPDOCam-1) was purified and found to have an apparent $k_{\text{cat}}$ for biphenyl of 1.1 ± 0.1 s$^{-1}$ (mean ± standard deviation) at 4°C. In contrast, BPDO$_{LB400}$ from the mesophile Burkholderia xenovorans LB400 had no detectable activity at this temperature. At 57°C, the half-life of the BPDO$_{Cam-1}$ oxygenase was less than half that of the BPDO$_{LB400}$ oxygenase. Nevertheless, BPDO$_{Cam-1}$ appears to be a typical Pseudomonas pseudoalcaligenes KF707-type dioxygenase.

The cold-tolerant bacterium Pseudomonas sp. strain Cam-1, previously isolated from Arctic soil, transforms polychlorinated biphenyls (PCBs) with up to four chloro substituents at 7°C at higher rates than Burkholderia xenovorans LB400, a well-characterized PCB-degrading mesophilic bacterium (2, 14). At 50°C, PCB removal by Cam-1 was diminished, but that by LB400 was not (14). The degradation of PCBs by these two strains is further distinguished by their respective congener preferences. LB400 preferentially degrades para-substituted PCBs with up to six chloro substituents (6). In contrast, the congener preference of Cam-1 is similar to that of Pseudomonas pseudoalcaligenes KF707, preferentially degrading para-substituted PCBs with up to six chloro substituents (6). A third well-characterized mesophilic PCB-degrading bacterium, Pandoraea promenusa B-356 (previously Comamonas testosteroni B-356), preferentially transforms meta-substituted congeners with up to four chloro substituents (1).

The first enzyme of the biphenyl/PCB-degrading pathway, biphenyl dioxygenase (BPDO), is one of the major determinants of the aerobic PCB-degrading capabilities of bacteria (17). Accordingly, the PCB-degrading capabilities of strains LB400 and B-356 largely reflect those of BPDO$_{LB400}$ and BPDO$_{B356}$, respectively (7). BPDO is a three-component dioxygenase comprising a reductase, a ferredoxin, and an oxygenase component (iron-sulfur protein [ISP]) of αβ$_{3}$ constitution. The α subunit contains a Rieske FeS cluster and, within the active site, a mononuclear iron center. BPDO utilizes NADH to activate O$_{2}$ and catalyze the 2,3-dihydroxylation of biphenyl. A crystal structure of ISP$_{B356}$, the oxygenase component of BPDO$_{B356}$ in complex with 2,6-dichlorobiphenyl has provided insight into the determinants of congener preference in BPDOs (7). Moreover, BPDOs have been engineered to improve their PCB-degrading capabilities (3, 15).

The oxygenase component of BPDO$_{Cam-1}$ shares 99%, 95%, and 70% amino acid sequence identities with those of BPDO$_{KF707}$, BPDO$_{LB400}$, and BPDO$_{B356}$, respectively. Indeed, the α-subunits of ISP$_{Cam-1}$ and ISP$_{KF707}$ differ by a single residue at position 78 (Ala in ISP$_{Cam-1}$ and Val in ISP$_{KF707}$) (13). To better understand the differences between the various BPDOs and their influence on the temperature dependence of PCB degradation by the parent strains (14), BPDO$_{Cam-1}$ was heterologously produced and purified. The biphenyl- and PCB-transforming activities of BPDO$_{Cam-1}$ were evaluated and compared with the data for BPDO$_{LB400}$, BPDO$_{B356}$, and BPDO$_{KF707}$. The impact of temperature on the activities and stabilities of BPDO$_{Cam-1}$ and BPDO$_{LB400}$ were also compared to evaluate whether the former is cold adapted.

Reconstitution of BPDO$_{Cam-1}$, ISP$_{Cam-1}$ and ISP$_{LB400}$, the oxygenase component of BPDO$_{Cam-1}$ and BPDO$_{LB400}$, respectively, were produced in Escherichia coli strain C41(DE3) (15) by using derivatives of pT7-6a and pT7-7a, respectively (8, 13). The ISPs were purified and handled anaerobically in a glovebox, concentrated to 20 to 30 mg/ml, and flash frozen as beads in liquid nitrogen essentially as described previously (7, 11). The yields of ISP$_{LB400}$ and ISP$_{Cam-1}$ were approximately 15 and 25 mg/liter of cell culture, respectively, and the R values ($A_{280}/A_{323}$) and iron and sulfur content of these preparations indicated that the ISPs contained essentially their full complement of Rieske-type [2Fe-2S] clusters and mononuclear iron centers (11). BPDO$_{Cam-1}$ and BPDO$_{LB400}$ were reconstituted by using His-tagged ferredoxin from LB400 (Ht-BphF$_{LB400}$) and His-tagged reductase from B-356 (Ht-BphG$_{B356}$) (4, 10). The specific activities of BPDO$_{Cam-1}$ and BPDO$_{LB400}$ were 3.1 ± 0.4 (mean ± standard deviation) and 0.27 ± 0.01 U/mg, re-

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TABLE 1. Effect of temperature on the reactivities of purified BPDOs with biphenyl

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>BPDO&lt;sub&gt;Cam-1&lt;/sub&gt;</th>
<th>BPDO&lt;sub&gt;LB400&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;app&lt;/i&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>&lt;i&gt;K&lt;/i&gt;&lt;sub&gt;app&lt;/sub&gt; (μM)</td>
</tr>
<tr>
<td>4</td>
<td>1.1 (0.1)</td>
<td>18 (3)</td>
</tr>
<tr>
<td>10</td>
<td>2.2 (0.1)</td>
<td>11 (1)</td>
</tr>
<tr>
<td>25</td>
<td>2.2 (0.2)</td>
<td>29 (3)</td>
</tr>
</tbody>
</table>

Values in parentheses represent standard errors (<i>n</i> = 3 or 4). ND, not detected. 

Data taken from Gomez-Gil et al., in which assay mixtures contained 0.6 μM ISP, 3.6 μM Ht-BphF<sub>LB400</sub>, 1.8 μM Ht-BphG<sub>B356</sub>, 350 μM NADH, and 0.1 to 150 μM biphenyl (7).

A comparative analysis of BPDO<sub>Cam-1</sub> reactivity. The steady-state parameters of BPDO for biphenyl were evaluated by following the consumption of O<sub>2</sub> as previously described (7, 11). All experiments were performed by using air-saturated 50 mM morpholineethanesulfonic acid (MES), pH 6.0, and the assay mixtures contained 0.6 μM ISP, 3.6 μM Ht-BphF<sub>LB400</sub>, 1.8 μM Ht-BphG<sub>B356</sub>, 320 μM NADH, and 0.5 to 160 μM biphenyl. Under all conditions studied, the steady-state consumption of O<sub>2</sub> by BPDO as a function of biphenyl concentration obeyed Michaelis-Menten kinetics with the fitted parameters shown in Table 1. Moreover, the hydroxylation of biphenyl was well coupled to O<sub>2</sub> activation. That is, in the presence of 160 μM biphenyl and excess NADH, the amount of O<sub>2</sub> consumed by BPDO<sub>Cam-1</sub> corresponded to the amount of biphenyl utilized (Table 2) and no H<sub>2</sub>O<sub>2</sub> was detected upon the addition of catalase to these reaction mixtures. At 25°C, the steady-state utilization of biphenyl by BPDO<sub>Cam-1</sub> was remarkably similar to that of BPDO<sub>B356</sub> (apparent<sub>cat</sub><i>k</i><sub>app</sub> = 4.1 ± 0.2 s<sup>-1</sup> and<sub>app</sub><i>K</i><sub>app</sub> = 20 ± 4 μM [7]) and different from that of BPDO<sub>LB400</sub> previously measured under similar conditions. More particularly, the<sub>app</sub><i>k</i><sub>app</sub> values of BPDO<sub>B356</sub> and BPDO<sub>LB400</sub> for biphenyl are 10 times higher than that of BPDO<sub>LB400</sub> while the apparent specificities (<i>k</i><sub>app</sub>/<i>K</i><sub>app</sub>) of the former are approximately 1/10 that of BPDO<sub>LB400</sub>. This is true of BPDO<sub>Cam-1</sub> and BPDO<sub>LB400</sub> at 10°C as well. However, BPDO<sub>LB400</sub> did not detectably transform biphenyl at 4°C.

In studying the congener preference of BPDO<sub>Cam-1</sub>, it was found that the enzyme depleted biphenyls in the following order of apparent maximal rates at 25°C: biphenyl >> 3,3’-dichlorobiphenyl > 4,4’-dichlorobiphenyl ≈ 2,2’-dichlorobiphenyl (Table 2). Thus, as with the steady-state kinetic parameters of biphenyl utilization, the congener preference of BPDO<sub>Cam-1</sub> is similar to that of BPDO<sub>B356</sub> (biphenyl > 3,3’- dichlorobiphenyl > 2,2’-dichlorobiphenyl ≈ 4,4’-dichlorobiphenyl) and distinct from that of BPDO<sub>LB400</sub> (biphenyl > 2,2’-dichlorobiphenyl > 3,3’-dichlorobiphenyl > 4,4’-dichlorobiphenyl) (7, 11). Consistent with what has been reported for BPDO<sub>B356</sub> and BPDO<sub>LB400</sub>, the utilization of O<sub>2</sub> by BPDO<sub>Cam-1</sub> in the presence of 68 μM 2,2’-dichlorobiphenyl, 80 μM 3,3’-dichlorobiphenyl, or 40 μM 4,4’-dichlorobiphenyl was not fully coupled (Table 2). The extent of uncoupling between O<sub>2</sub> and dichlorobiphenyl utilization was dependent on both the isozyme and the PCB congener. For example, BPDO<sub>Cam-1</sub> was significantly more uncoupled in the presence of 2,2’-dichlorobiphenyl than BPDO<sub>LB400</sub> (Table 2). Moreover, the uncoupling was not necessarily lowest for dichlorobiphenyls that were depleted at the highest rates.

In general, the congener preference data reported for the purified BPDOs are consistent with those reported for the parent strains in whole-cell assays, except that whole cells of strain Cam-1 and B-356 depleted 2,2’-dichlorobiphenyl very poorly, if at all. Thus, in contrast to the purified BPDOs, cells of strain Cam-1 did not detectably transform 2,2’-dichlorobiphenyl (14) and B-356 cells depleted 4,4’-dichlorobiphenyl better than 2,2’-dichlorobiphenyl (1, 11). While these data might reflect the low sensitivity of the whole-cell assay, it is possible that PCB transformation by whole cells is determined by other factors, such as PCB transport across cell membranes and the presence of other PCB-transforming enzymes in the cell. Finally, differences in apparent congener preference between whole cells and purified BPDO might reflect the enzyme’s relative protection against oxidative damage in whole cells. Notably, whole-cell assays are typically conducted over much longer periods of time (hours) than enzyme assays (minutes).

To investigate the poor transformation of 2,2’-dichlorobiphenyl by whole cells and to facilitate comparisons of results for BPDO<sub>Cam-1</sub> with whole-cell data for BPDO<sub>KF707</sub> (18), PCB removal by <i>E. coli</i> C41(DE3) cells freshly transformed with pT7-7a (<i>bphAFEFG<sub>Cam-1</sub></i>) was tested (Table 3) as described previously (7). These data are consistent with the results of pre-

TABLE 2. Reactivities of purified BPDOs with selected biphenyls at 25°C

<table>
<thead>
<tr>
<th>Congener</th>
<th>BPDO&lt;sub&gt;Cam-1&lt;/sub&gt;</th>
<th>BPDO&lt;sub&gt;LB400&lt;/sub&gt;</th>
<th>BPDO&lt;sub&gt;B356&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of congener removal (nmol·min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>&lt;i&gt;H&lt;/i&gt;&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Rate of congener removal (nmol·min&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>95 (9)</td>
<td>ND</td>
<td>12 (4)</td>
</tr>
<tr>
<td>2,2’-Dichlorobiphenyl</td>
<td>3.2 (0.3)</td>
<td>0.36 (0.01)</td>
<td>15 (4)</td>
</tr>
<tr>
<td>3,3’-Dichlorobiphenyl</td>
<td>8 (2)</td>
<td>0.40 (0.05)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>4,4’-Dichlorobiphenyl</td>
<td>2.6 (0.1)</td>
<td>0.26 (0.05)</td>
<td>3.9 (0.3)</td>
</tr>
</tbody>
</table>

<i>O</i> consumption (<i>H</i><sub>2</sub>O<sub>2</sub>/<i>O</i><sub>2</sub> ratios) values are based on the assumption that the amount of <i>O</i><sub>2</sub> detected upon catalase addition corresponds to 50% of <i>H</i><sub>2</sub>O<sub>2</sub> produced. The rates of congener removal and <i>H</i><sub>2</sub>O<sub>2</sub>/<i>O</i><sub>2</sub> ratios are based on data obtained after 1.5 min for biphenyl and after 3 min for dichlorobiphenyls. Values in parentheses represent standard errors (<i>n</i> = 3 or 4). ND, not detected.

Data taken from Gomez-Gil et al., in which assay mixtures contained 50 μM of each congener (7).
BPDOCam-1, BPDO KF707, and BPDO B356 are quite similar. These data further indicate that the congener preferences of congeners at significantly higher rates than biphenyl (18). Dichlorobiphenyls and that both enzymes transform these same apparent preference for 3,3'-dichlorobiphenyl and 4,4'-dichlorobiphenyls at a concentration of biphenyl exceeded the BPDOs’ to 60°C (Fig. 1). In the results of the standard assay, the evidence of BPDO activity on temperature was investigated by temperature and entropy of activation. Values in parentheses represent standard errors (n = 3). Values in parentheses represent standard errors (n = 3).

The principal distinguishing characteristic of cold-adapted enzymes is a significant decrease of their enthalpy of activation (ΔH‡) with respect to that of their mesophilic homologues (5 to 42 kJ mol⁻¹), which contributes to a higher kcat at lower temperatures (12). Cold-adapted enzymes also tend to be less thermostable than their isoenzymes, which removed 2,2'-dichlorobiphenyl and 4,4'-dichlorobiphenyls at similar rates. Importantly, these experimental results establish that in whole cells of E. coli, BPDO KF707 and BPDO Cam-1 have the same apparent preference for 3,3'-dichlorobiphenyl and 4,4'-dichlorobiphenyls and that both enzymes transform these congeners at significantly higher rates than biphenyl (18). These data further indicate that the congener preferences of BPDO Cam-1, BPDO KF707, and BPDO B356 are quite similar.

Temperature dependence of BPDO activity. The dependence of BPDO activity on temperature was investigated by performing the standard activity assay at temperatures from 4 to 60°C (Fig. 1). In the results of the standard assay, the concentration of biphenyl exceeded the BPDOs’ Km for this substrate by approximately an order of magnitude. Studies of BPDO B356 indicate that this is also the case for O2 (Km(app) = 28 μM [11]). The relative activities of the enzymes should therefore be essentially unaffected by temperature-dependent changes in the solubility of the two substrates. At any given temperature between 4 and 45°C, the activity of BPDO Cam-1 was higher than that of BPDO LB400. As noted above, at 4°C, the activity of BPDO LB400 did not differ significantly from the background rate, whereas BPDO Cam-1, transformed biphenyl at a significant rate (Table 1). Further, the highest observed activity of BPDO Cam-1 occurred at a lower temperature than that of BPDO LB400 (47°C versus 55°C). The thermodynamic parameters were derived from Arrhenius plots (9, 12) by using the data up to 42.5°C and 35°C for BPDO Cam-1 and BPDO LB400 respectively (Table 4). The free energy of activation (ΔG‡) of BPDO Cam-1 is lower than that of BPDO LB400, consistent with the former’s higher kcat. However, determination of the apparent steady-state parameters of the two BPDOs at 10°C revealed that their respective catalytic efficiencies (kcat/Km) were not significantly different (see Table 1).

Finally, we investigated the respective half-lives of ISP Cam-1 and ISP LB400 at 57°C by following the characteristic absorbance of the iron-sulfur cluster of these proteins. This temperature was chosen because the enzymes lost activity at a significant rate above 50°C. Briefly, a sample of each ISP was diluted to 2.2 μM in MES buffer (pH 6.0) preequilibrated to 57°C. The sample was incubated at 57°C, and its absorbance was continuously monitored at 323 nm, where a decrease in absorbance reflects loss of the Rieske-type [2Fe-2S] cluster and, thus, loss of ISP function. The half-lives of ISP Cam-1 and ISP LB400 were 16 ± 2 min and 38 ± 8 min, respectively. The longer half-life of ISP LB400 at 57°C is consistent with the higher relative kcat of BPDO LB400 that was observed at temperatures above 50°C.

The principal distinguishing characteristic of cold-adapted enzymes is a significant decrease of their enthalpy of activation (ΔH‡) with respect to that of their mesophilic homologues (5 to 42 kJ mol⁻¹), which contributes to a higher kcat at lower temperatures (12). Cold-adapted enzymes also tend to be less thermostable than their isoenzymes, which have a higher ΔH‡.

<table>
<thead>
<tr>
<th>Congener</th>
<th>% Depletion at:</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl</td>
<td></td>
<td>59 (10)</td>
<td>100 (0)</td>
</tr>
<tr>
<td>2,2'-Dichlorobiphenyl</td>
<td></td>
<td>38 (1)</td>
<td>64 (3)</td>
</tr>
<tr>
<td>3,3'-Dichlorobiphenyl</td>
<td></td>
<td>95 (1)</td>
<td>100 (0)</td>
</tr>
<tr>
<td>4,4'-Dichlorobiphenyl</td>
<td></td>
<td>99 (1)</td>
<td>98 (1)</td>
</tr>
</tbody>
</table>

* a Values in parentheses represent standard errors (n = 3).
thermostable than their mesophilic homologues. Thus, comparison of these two characteristics of BPDO_{Cam-1} and BPDO_{LB400} could be interpreted to indicate that BPDO_{Cam-1} is cold adapted. However, several considerations argue that ISP_{Cam-1} is not cold adapted. First, the difference in $\Delta H^\circ$ of the two reactions (10 kJ mol$^{-1}$) is at the low end of which that distinguishes mesophilic from psychrophilic enzymes. Second, the steady-state kinetic parameters of BPDO_{Cam-1} for biphenyl at 25°C are similar to those reported for a mesophilic enzyme, BPDO_{BHE356} determined under similar conditions (7, 11). Third, the specificities of BPDO_{Cam-1} and BPDO_{LB400} for biphenyl ($k_{\text{cat}}/K_{\text{m}}$) are not significantly different at 10°C, contrary to what has been observed in other cold-adapted enzymes (9).

Fourth, the $\alpha$-subunit of ISP_{Cam-1}, which encompasses the BPDO active site, is almost identical to that of another mesophilic enzyme, ISP_{KF707}.

Overall, our data suggest that BPDO_{Cam-1} is a typical KF707-type enzyme. Nishi et al. (16) reported that *Pseudomonas putida* KF715 encodes a KF707-type BPDO on a 90-kbp conjugative element (16). Thus, it is conceivable that such an element was transferred to a psychrotolerant *Pseudomonas* species, such as Cam-1. It is nonetheless possible that ISP_{Cam-1} is less stable than ISP_{KF707} and ISP_{LB400}. In particular, the $\beta$-subunits of ISP_{LB400} and ISP_{KF707} differ by a single residue and they differ from the $\beta$-subunit of ISP_{Cam-1} by the same five residues (Arg16, Pro33, Gln34, Pro40, and Phe79 in ISP_{Cam-1}). Inspection of the crystallographic structure of ISP_{BHE356} reveals that all five residues occur at or very close to subunit interfaces. Perhaps the most intriguing of these is Pro33 in ISP_{Cam-1}, which is an arginine residue in ISP_{LB400}, ISP_{KF707}, and ISP_{BHE356}. In ISP_{BHE356}, this arginine is involved in an extensive network of hydrogen bonds involving residues at the interface of adjacent $\beta$-subunits. The loss of this network in ISP_{Cam-1} could influence this enzyme’s thermostability.

Although the combined data reported here suggest that BPDO_{Cam-1} is not cold-adapted, the differences between BPDO_{Cam-1} and BPDO_{LB400} nevertheless account for the differences between their respective parental strains, Cam-1 and LB400. Thus, whole cells of Cam-1 remove certain PCB congeners at higher rates than LB400 cells at 7°C (14). Furthermore, PCB removal by LB400 increased at high temperature; in contrast, high temperature inhibited PCB removal by Cam-1. Both observations are consistent with the higher $k_{\text{cat}}/K_{\text{m}}$ of BPDO_{Cam-1} for biphenyl at low temperature and the low thermal stability of BPDO_{Cam-1} compared to that of BPDO_{LB400}. Overall, the current data indicate that a distinguishing feature of KF707- and LB400-type enzymes is the former’s superior ability to degrade PCBs at low temperatures.

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### REFERENCES