Site-directed mutagenesis of an extradiol dioxygenase involved in tetralin biodegradation identifies residues important for activity or substrate specificity

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The sequence of the extradiol dioxygenase ThnC, involved in tetralin biodegradation, was aligned with other extradiol dioxygenases involved in biodegradation of polycyclic compounds, and a three-dimensional model of ThnC, based on the structure of the previously crystallized 2,3-dihydroxybiphenyl dioxygenase from Burkholderia fungorum LB400, was built. In order to assess the functional importance of some non-active-site residues whose relevance could not be established by structural information, a number of positions surrounding the substrate-binding site were mutated in ThnC. Ten mutant proteins were purified and their activity towards 1,2-dihydroxytetralin, 1,2-dihydroxynaphthalene and 2,3-dihydroxybiphenyl was characterized. N213H, Q198H, G206M, A282R and A282G mutants increased \( k_{cat}/K_m \) at least twofold using 1,2-dihydroxytetralin as the substrate, thus showing that activity of ThnC is not maximized for this substrate. N213H and Q198H mutants increased \( k_{cat}/K_m \) using any of the substrates tested, thus showing the relevance for activity of these two histidines, which are highly conserved in dihydroxybiphenyl dioxygenases, but not present in dihydroxynaphthalene dioxygenases. Different substitutions in position 282 had different effects on general activity or substrate specificity, thus showing the functional importance of the most C-terminal \( \beta \)-sheet of the protein. A251M and G206M mutants showed increased activity specifically for a particular substrate. N213H, G206M, A282R, A282G and Y177I substitutions resulted in enzymes more tolerant to acidic pH, the most striking effect being observed in mutant Y177I, which showed maximal activity at pH 5.5. In addition, Q198D and V175D mutants, which had altered \( K_m \), also showed altered sensitivity to substrate inhibition, thus indicating that inhibition is exerted through the same binding site. This mutational analysis, therefore, identified conserved residues important for activity or substrate specificity, and also shed some light on the mechanism of substrate inhibition exhibited by extradiol dioxygenases.

INTRODUCTION

Extradiol dioxygenases catalyse the cleavage of the aromatic ring of catechol derivatives in a position adjacent to the hydroxyl substituents, in a reaction involving incorporation of two atoms of oxygen. These enzymes play a key role in the metabolism of aromatic compounds. In bacteria, they are responsible for the cleavage of aromatic rings during aerobic catabolism of compounds such as toluene and xylenes, naphthalene or biphenyl derivatives, some of which are environmental pollutants of serious concern. In humans, extradiol dioxygenases are associated with genetic disorders such as alkaptonuria or Huntington’s disease (La Du et al., 1958; Schwarcz et al., 1988).

Because of their importance in degradation of aromatic rings, extradiol dioxygenases have received a lot of attention. A large number of genes encoding extradiol dioxygenases have been sequenced from many different bacteria. Sequence and crystallographic data suggest that these enzymes arose from at least two different evolutionary origins. Most of them are type I enzymes, which contain the PROSITE extradiol dioxygenase fingerprint, whilst a few, represented by LigAB, cannot be aligned with type I enzymes.
and are grouped as type II (Eltis & Bolin, 1996; Sugimoto et al., 1999). In spite of the phylogenetic divergence, all types of extradiol dioxygenases appear to have similar catalytic mechanisms (Bugg & Lin, 2001; Que & No, 1996; Solomon et al., 2000), which involve non-haem Fe(II) in the active site. The role of some strictly conserved residues as metal ligands or active-site residues may be inferred from structural and biochemical data (Han et al., 1995; Senda et al., 1996, 1997; Shu et al., 1995; Uragami et al., 2001). However, even for the best-characterized enzymes, the 2,3-dihydroxybiphenyl dioxygenases (DHBDs), the reaction mechanism has not been fully established, nor has the exact role of some active-site residues. Of course, the role of other conserved residues, which may be relevant for enzyme function or substrate specificity, has not been characterized either.

Within the two-domain extradiol dioxygenases bearing the catalytic site in the C-terminal domain, sequence comparison shows that evolutionary relationships correlate with specificity of substrate. These enzymes can be divided in two separate clades: those showing preference for monoaromatic compounds and those showing preference for polycyclic substrates (Eltis & Bolin, 1996; Harayama & Rekik, 1989). More recent sequence comparison revealed that, among enzymes cleaving polycyclic compounds, 1,2-dihydroxynaphthalene dioxygenases (DHNDs) cluster together within the subfamily I.3.E, and that two groups within this subfamily can be established (Andújar et al., 2000). Members of this subfamily of DHNDs are less well characterized and biochemical data are not available or are very scarce for many of them. They share a number of conserved residues but it is not known whether this conservation is functionally important or merely reflects a common evolutionary origin.

The extradiol dioxygenase ThnC, required for biodegradation of the organic solvent tetralin by Sphingomonas sp. strain TFA, has been recently purified and characterized (Andújar et al., 2000). It catalyses the extradiol cleavage in a position proximal to the alicyclic ring of tetralin (Fig. 1). Alignment of its deduced amino acid sequence with those of other two-domain enzymes showed that ThnC lies within group 2 of DHNDs (Andújar et al., 2000) (Fig. 2), a group composed of enzymes able to transform a broad range of substrates, some of which were in fact initially identified as DHBDs. Biochemical properties of the enzyme have been fully characterized. Like many other extradiol dioxygenases (Adams et al., 1992; Asturias & Timmis, 1993; Happe et al., 1993; Kim & Zylstra, 1995), ThnC shows a very evident inhibition by the substrates tested, although it is particularly sensitive to inhibition by the catechol intermediate of the tetralin biodegradation pathway, 1,2-dihydroxytetralin (1,2-DHT) (Andújar et al., 2000). Activity of the enzyme against different substrates showed that, as its sequence suggested, ThnC is a true DHND, although it can also efficiently transform 1,2-DHT and 2,3-dihydroxybiphenyl (2,3-DHB) (Andújar et al., 2000).

In an attempt to establish the role of some conserved residues in the function of the enzyme or in substrate specificity, a number of mutations consisting of single amino acid substitutions were constructed and the activity against different substrates of the resulting altered proteins analysed.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Escherichia coli DH5α [F− lacZAM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK− mK−) supE44 thi-1 gyrA relA1] (Hanahan, 1983) was used for cloning and isolation of DNA for sequencing. E. coli strains were routinely grown in Luria–Bertani (LB) medium.

An 830 bp Apal–HindIII fragment from pIZ592 (Andújar et al., 2000) was cloned between the Apal and HindIII sites of the multiple cloning sequence of pBluescript II SK− (+) (Stratagene) to yield pIZ593. Site-directed mutagenesis to construct the mutations V175D, Q198D, N213H, A251M, A282R and A282P was performed following the method of Kunkel et al. (1987), using the mutagenic oligonucleotides shown in Table 1. To construct the mutations Y177I, Q198H, G206M, A282R and A282P, an 830 bp Apal–HindIII fragment was cloned between the Apal and HindIII sites of the multiple cloning sequence of pBluescript II SK− (+) (Stratagene) to yield pIZ593. Site-directed mutagenesis to construct the mutations V175D, Q198D, N213H, A251M, A282R and A282P was performed following the method of Kunkel et al. (1987), using the mutagenic oligonucleotides shown in Table 1. The mutations were confirmed by sequencing, and the mutated Apal–HindIII fragments of 830 bp were used for substitution of the wild-type sequence in the plasmid pIZ591, a plasmid bearing a His-tag fused to thnC (Andújar et al., 2000).

**Protein purification.** For overexpression of wild-type and thnC mutants, E. coli NCM631/pIZ227 (Govantes et al., 1996) was transformed with the plasmids pIZ591, pIZ595, pIZ596, pIZ598, pIZ599, pIZ1100, pIZ1101, pIZ1102, pIZ1103, pIZ1104 or pIZ1105, bearing the different alleles of thnC fused to a His-tag. The resulting transformants were grown in LB liquid medium at 26°C to OD600 0.6–0.8. Then, they were induced with 1 mM IPTG overnight (8–10 h). Cells were harvested by centrifugation and suspended in 0.5 vol. buffer (20 mM sodium/Imidazole (0.1 M) was used to elute the protein.

**Enzyme assay.** Since specific activity of different preparations of ThnC may vary because of oxidation or loss of Fe²⁺ during purification (Andújar et al., 2000), all purified enzymes were reactivated by incubation on ice with 2 mM ammonium ferrous sulfate for 20 min, immediately before any enzyme assay.

One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol substrate min⁻¹. Cell extracts or purified protein were added to a solution (final volume, 1 ml) containing 50 mM sodium/
Table 1. Mutants constructed and conserved amino acids in the different subfamilies of family I.3 at positions subjected to mutagenesis

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Plasmid</th>
<th>Oligonucleotide</th>
<th>Conserved amino acids in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I.3.E.1</td>
</tr>
<tr>
<td>V175D</td>
<td>pIZ595</td>
<td>5′ GGAGTGTGACGAATACC 3′</td>
<td>Val</td>
</tr>
<tr>
<td>Y177I</td>
<td>pIZ596</td>
<td>5′ GTGTGAAACTCCACCTGC 3′</td>
<td>Tyr</td>
</tr>
<tr>
<td>Q198D</td>
<td>pIZ598</td>
<td>5′ ACGATGGGACACGGCGGT 3′</td>
<td>Asp</td>
</tr>
<tr>
<td>Q198H</td>
<td>pIZ599</td>
<td>5′ GATCGGCCACACCCGGGT 3′</td>
<td>Met</td>
</tr>
<tr>
<td>G206M</td>
<td>pIZ1100</td>
<td>5′ TCGGCTCATGTGCATGCC 3′</td>
<td>Asn</td>
</tr>
<tr>
<td>N213H</td>
<td>pIZ1101</td>
<td>5′ AGGCCATCATACCTGAT 3′</td>
<td>Ala</td>
</tr>
<tr>
<td>A251M</td>
<td>pIZ1102</td>
<td>5′ ACAGCAGATCTGACCTT 3′</td>
<td>Gly</td>
</tr>
<tr>
<td>A282R</td>
<td>pIZ1103</td>
<td>5′ ATTACAAGGCGACCTGTT 3′</td>
<td></td>
</tr>
<tr>
<td>A282G</td>
<td>pIZ1104</td>
<td>5′ TTAACAGGCGACCCGT 3′</td>
<td></td>
</tr>
<tr>
<td>A282P</td>
<td>pIZ1105</td>
<td>5′ ATTACAAGGCGACCTGTT 3′</td>
<td></td>
</tr>
</tbody>
</table>

NC: Not conserved.
*Ala is specific to ThnC.

potassium phosphate buffer (pH 6.8) and 25 μM 1,2-DHT at 25 °C. 1,2-DHND activity was assayed by measuring substrate consumed in 50 mM acetate buffer (pH 5.0), as previously described (Kuhn et al., 1991), using the following absorption coefficient: \( \varepsilon = 331 \text{ nm} \). 

Extradiol dioxygenase activity with other substrates was assayed by measuring the formation of the corresponding ring-fission products. The following absorption coefficients were used (in 50 mM sodium/potassium phosphate buffer pH 6.8): 1,2-DHT (Andújar et al., 2000), \( \varepsilon = 336 \text{ nm} \); 2,3-DHB (Eltis et al., 1993), \( \varepsilon = 434 \text{ nm} \). Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as the standard. All colorimetric assays were quantified using a Beckman DU 640 spectrophotometer.

The ranges of substrate concentrations used in enzyme assays for determination of the kinetic parameters were: [1,2-DHT] = 5–250 μM; [2,3-DHB] = 7.5–4 mM; [1,2-dihydroxynaphthalene (1,2-DHN)] = 10–300 μM.

To perform enzyme assays at different pHs, the buffers were used at a concentration of 50 mM: citric acid pH 4.0; pH 4.5 and pH 5.0; MES pH 5.5 and pH 6.0; PIPES pH 6.5 and pH 7.0; TRIZMA pH 7.6; pH 8.0; pH 8.5 and pH 9.0. Absorption coefficients of 1,2-DHT at different pHs were previously calculated (Andújar et al., 2000).

**Sequence analysis comparison and structural model.** The amino acid sequence of ThnC was initially compared to those in the databases using the BLASTp program (Altschul et al., 1997). Amino acid sequences which showed high similarity to that of strain TFA were aligned using the CLUSTAL W program (Thompson et al., 1994) using default parameters.

The computer program Swiss PDB Viewer (Guex & Peitsh, 1997) was used to build the structural model of ThnC using the crystal coordinates of BphC from Burkholderia fungorum LB400 (Han et al., 1995) as the reference.

**RESULTS**

**Sequence and structural alignment of ThnC**

Alignment with CLUSTAL W of ThnC and sequences of extradiol dioxygenases of the I.3 family, as defined by Eltis & Bolin (1996), confirmed strict conservation of the six active-site residues and three other residues previously identified. In addition, our analysis identified 41 highly conserved residues in the I.3 family. Also, 74 positions were highly conserved in the I.3.E subfamily of DHND and contained residues different from those found in other extradiol dioxygenases. Of these, 50 positions were not particularly conserved among the members of other subfamilies, and 24 were conserved but contained a different amino acid. Finally, 31 conserved positions in the I.3.E subfamily allowed discrimination between the two groups of this subfamily since residues in these positions were different in the members of the two groups, although conserved within each group. Fig. 2 shows the alignment of the catalytic C-terminal domain of these enzymes.

The structural model of ThnC using the crystal coordinates of BphC of LB400 as a reference (Han et al., 1995) showed a good fit with that of BphC and revealed the conservation of the two structural domains and the two motifs ββββββ in the catalytic C-terminal domain. The three-dimensional model (Fig. 3) showed that each of the two additional β-sheets at the C-terminal end may interact with each ββββββ motif of the C-terminal domain, as shown for the two crystallized BphC enzymes (Han et al., 1995; Senda et al., 1996; Uragami et al., 2001).

Crystalllographic data of both enzymes together with the alignment of ThnC allowed identification of a number of potentially important positions based on their location around the entrance site of the substrate, their interaction with active-site residues or their residue conservation among other enzymes. His-209 (numbering of BphC from LB400) is highly conserved in extradiol dioxygenases (Eltis & Bolin, 1996) and forms a hydrogen bond with the Fe(II) ligand His-210. However, this position (213 in ThnC) is occupied by Asn in all but one member of the subfamily of DHNDs. His-194 of LB400 BphC forms a hydrogen bond...
with the active-site His-195 and is conserved in all members of the I.3 family other than DHNDs. However, this position (198 in ThnC) discriminates between the two groups of DHND, containing Asp those enzymes of the group 1, and Gln all but one of the group 2 enzymes. Asp-171, conserved among all members of the I.3 family other than DHNDs, also forms a hydrogen bond with His-194 of BphC from LB400. The equivalent position (175 in ThnC) contains Val in all DHNDs. The residues Ile-173 and Met-246 help to define the substrate-binding site in BphC from LB400 and are conserved in all other members of the I.E family. The equivalent positions (177 and 251 in ThnC) are occupied by Tyr and Ala, respectively, in all DHNDs. Other positions such as 202 and 280 may also form the substrate-binding site in BphC from LB400 but are not particularly conserved in the I.3 family. However, conservation is observed in equivalent positions of DHND (206 and 282 in ThnC), which contribute to discriminate between the two groups of the I.3.E subfamily. In position 282, ThnC contains Ala, a different residue from those found in the other enzymes aligned. The locations of the residues in these positions are highlighted in the three-dimensional model of the C-terminal domain of ThnC, which shows a frontal view of the substrate entrance to the active site (Fig. 3). Residues in all these positions were substituted by site-directed mutagenesis.

Mutagenesis of thnC and purification of the modified gene products

In order to test the relevance of the residue identity in the selected positions for ThnC activity, the C-terminal domain of ThnC was modified. To this end, 10 substitutions were made on a DNA fragment consisting of the C-terminal domain of ThnC, which contains Val in all DHNDs. The residues Ile-173 and Met-246 help to define the substrate-binding site in BphC from LB400 and are conserved in all other members of the I.E family. The equivalent positions (177 and 251 in ThnC) are occupied by Tyr and Ala, respectively, in all DHNDs. Other positions such as 202 and 280 may also form the substrate-binding site in BphC from LB400 but are not particularly conserved in the I.3 family. However, conservation is observed in equivalent positions of DHND (206 and 282 in ThnC), which contribute to discriminate between the two groups of the I.3.E subfamily. In position 282, ThnC contains Ala, a different residue from those found in the other enzymes aligned. The locations of the residues in these positions are highlighted in the three-dimensional model of the C-terminal domain of ThnC, which shows a frontal view of the substrate entrance to the active site (Fig. 3). Residues in all these positions were substituted by site-directed mutagenesis.

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Mutational analysis of an extradiol dioxygenase

region of thnC. The substitutions, together with information about the residues in equivalent positions of different extradiol dioxygenases, are summarized in Table 1. Mutations were confirmed by sequencing. The mutated fragments were used for substitution of the wild-type sequence in the plasmid pZ591, which contains thnC His-tagged at its N-terminus, and expressed from the T7 promoter (Andújar et al., 2000).

The resulting plasmids, together with pIZ591, were used to transform the overproducing strain NCM631/pIZ227 (Govantes et al., 1996). After induction of the transformed strains with IPTG, overproduction was very evident in all cases. In spite of the different degrees of solubility of the His-tagged proteins, all were successfully purified in a single step by affinity chromatography, and preparations showed a single band after electrophoresis (data not shown).

pH range of the mutant proteins

Because the activity assays using 1,2-dihydroxynaphthalene (1,2-DHN) as the substrate had to be carried out at pH 5–5 (Kuhm et al., 1991), preliminary experiments aimed at characterization of mutant proteins included tests of activity at different pH values, using the substrate for ThnC intermediate in the tetralin biodegradation pathway, 1,2-DHT. Activity of wild-type ThnC showed a strong dependence on pH with an optimum at pH 6–8–7, and just 35–40% of the activity at pH 5–5. Some mutant proteins exhibited activity significantly more tolerant to acidic pH, although their pH optima were the same as that of wild-type ThnC. Activities of these proteins at pH 5–5 relative to their activities at pH 6–8 were the following: 62% for N213H, 69% for G206M or A282R, and 65% for A282G. Strikingly, mutant Y177I exhibited a sharp peak of activity as a function of pH but the optimum pH was displaced from 7.0 to 5.5. Because of this, subsequent characterization of the activity of this mutant was always carried out at pH 5.5, regardless of the substrate used.

Kinetic parameters of the mutant proteins

Activity of each enzyme was assayed at different concentrations of 1,2-DHT. All enzymes exhibited strong activity inhibition at high substrate concentrations. This phenomenon was previously described for ThnC (Andújar et al., 2000) and for other extradiol dioxygenases (Kim & Zylstra, 1995; Kuhm et al., 1991; Kunkel et al., 1987; La Du et al., 1958). The kinetic data fitted well with the polynomial equation $S/V = K_m/V_{max} + (1/V_{max}) [S] + (1/K_c) [S]^2$ previously described for enzymes showing substrate inhibition and it was therefore used to calculate the theoretical kinetic parameters from the empirical data. Kinetic parameters together with the observed activities at the optimum substrate concentration ($V_{obs}$) are shown for all mutant enzymes in Table 2.

Only one of the substitutions, A282P, had a dramatic negative effect on the activity of ThnC, resulting in a protein very inefficient in substrate processing (its $k_{cat}$ is less than 3% of that of wild-type ThnC) (Table 2). Interestingly, two other substitutions in the same position, A282R and A282G, also altered the rate of 1,2-DHT turnover but had an opposite effect, resulting in proteins at least twofold more efficient than wild-type ThnC. These data clearly indicate that this position is important for catalysis of 1,2-DHT cleavage by ThnC.

Three other substitutions, N213H, Q198H and G206M, produced enzymes with significantly higher rates of substrate turnover ($k_{cat}$ at least twofold higher than that of wild-type ThnC), which suggests that residues in these positions also contribute to efficient catalysis of 1,2-DHT cleavage. Of these, G206M was the mutation that most increased substrate affinity, as evidenced by its $K_m$, in addition to the increase in turnover rate. Interestingly, the enzyme bearing this substitution was also more sensitive to substrate inhibition.

The remaining substitutions, Q198D, V175D, Y177I and A251M, had negative effects on the rate of 1,2-DHT turnover. The effects, though significant, were weak and none of the substitutions reduced the enzyme activity more than 50%. On the other hand, Q198D and V175D significantly increased affinity for 1,2-DHT ($K_m$, approximately half of that of wild-type ThnC), thus compensating their lower turnover rate (see their $k_{cat}/K_m$).

Substrate specificity of the mutant proteins

In order to determine whether the effects of the substitutions on the activity towards 1,2-DHT were general, or specific to the substrate used in the reaction, activity of each enzyme was also tested using different concentrations of 2,3-DHB and 1,2-DHN. Fig. 4 shows the specificity of each protein for Fig. 3. Three-dimensional model of the C-terminal domain of ThnC based on the crystal structure of BphC from B. fungorum strain LB400. Frontal view of the funnel-shaped substrate-binding site. Active-site residues are shown in grey. Residues at the mutated positions are shown in black. The on-line version of this paper (at http://mic.sgmjournals.org) contains a supplementary colour figure of the three-dimensional structure.
Table 2. Kinetic parameters of mutant proteins using 1,2-DHT as the substrate

Each value represents the mean of at least three repetitions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$K_a$ (µM)</th>
<th>$k_\text{cat}$ (s$^{-1}$)</th>
<th>$k_\text{cat}/K_m$ (s$^{-1}$ µM$^{-1}$)</th>
<th>$V_{\text{obs}}$ (U mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>21.94 ± 1.18</td>
<td>45.17 ± 1.14</td>
<td>14.45 ± 0.35</td>
<td>0.66 ± 0.02</td>
<td>24.04 ± 0.58</td>
</tr>
<tr>
<td>N213H</td>
<td>20.91 ± 2.29</td>
<td>63.82 ± 6.13</td>
<td>35.58 ± 1.87</td>
<td>1.73 ± 0.28</td>
<td>59.15 ± 3.11</td>
</tr>
<tr>
<td>Q198D</td>
<td>10.13 ± 2.07</td>
<td>24.98 ± 4.15</td>
<td>8.21 ± 0.63</td>
<td>0.85 ± 0.23</td>
<td>13.65 ± 1.05</td>
</tr>
<tr>
<td>Q198H</td>
<td>16.72 ± 2.31</td>
<td>54.28 ± 4.03</td>
<td>27.17 ± 0.02</td>
<td>1.65 ± 0.22</td>
<td>45.16 ± 0.04</td>
</tr>
<tr>
<td>V175D</td>
<td>9.95 ± 1.29</td>
<td>57.18 ± 6.08</td>
<td>10.01 ± 0.05</td>
<td>1.02 ± 0.14</td>
<td>16.65 ± 0.09</td>
</tr>
<tr>
<td>Y177I*</td>
<td>21.49 ± 4.11</td>
<td>151.42 ± 6.96</td>
<td>8.51 ± 0.81</td>
<td>0.41 ± 0.11</td>
<td>14.74 ± 1.97</td>
</tr>
<tr>
<td>A251M</td>
<td>19.25 ± 1.91</td>
<td>73.5 ± 5.31</td>
<td>7.94 ± 0.34</td>
<td>0.41 ± 0.02</td>
<td>13.20 ± 0.56</td>
</tr>
<tr>
<td>G206M</td>
<td>15.17 ± 3.19</td>
<td>35.75 ± 5.07</td>
<td>28.94 ± 0.91</td>
<td>2.01 ± 0.48</td>
<td>48.10 ± 1.52</td>
</tr>
<tr>
<td>A282R</td>
<td>23.90 ± 2.85</td>
<td>58.97 ± 2.78</td>
<td>32.70 ± 1.56</td>
<td>1.38 ± 0.10</td>
<td>54.35 ± 2.60</td>
</tr>
<tr>
<td>A282G</td>
<td>18.64 ± 0.26</td>
<td>77.22 ± 1.11</td>
<td>47.25 ± 2.34</td>
<td>2.53 ± 0.09</td>
<td>78.53 ± 3.88</td>
</tr>
<tr>
<td>A282P</td>
<td>18.05 ± 1.40</td>
<td>55.89 ± 2.03</td>
<td>0.29 ± 0.05</td>
<td>0.02 ± 0.001</td>
<td>0.49 ± 0.08</td>
</tr>
</tbody>
</table>

*Values obtained at pH 5-5.

Each substrate tested. Specificity for 1,2-DHT or 2,3-DHB is shown at the optimum pH of each protein (pH 5-5 for Y177I and pH 6-8 for all the other protein variants). Activity using 1,2-DHN as the substrate was always tested at pH 5-5.

The negative effect of the substitution A282P was very obvious regardless of the substrate used in the assay (Fig. 4). Therefore, this substitution renders a virtually inactive protein. Mutant A282G, which produced the largest specificity increase for 1,2-DHT, had a negligible positive effect on specificity for the other substrates, thus resulting in an enzyme particularly more specific for the intermediate of the tetralin biodegradation pathway. The last substitution at the same position, A282R, had significant but lower positive effects on the specificity for 1,2-DHB or 1,2-DHN, as compared to that for 1,2-DHT.

The substitution G206M increased threefold the specificity for 1,2-DHT but its specificity for other substrates was very slightly reduced. Therefore, this replacement resulted in an extradiol dioxygenase particularly more specific for the intermediate of the tetralin degradation pathway.

The other two substitutions which increased specificity for 1,2-DHT, Q198H and N213H, also had a positive effect when using any of the other substrates, thus showing that histidines at these positions improve the functionality of ThnC regardless of the substrate to be transformed.

A251M increased specificity for 2,3-DHB while slightly reducing specificity for 1,2-DHT and 1,2-DHN, thus resulting in a protein particularly more specific for 2,3-DHB. On the other hand, V175D showed very slightly increased specificity for 1,2-DHT but significantly lower specificity for the other substrates. The two replacements Q198D and Y177I did not show significant effects which were dependent on the substrate transformed.
DISCUSSION

The sequence of ThnC, the extradiol dioxygenase involved in tetrafluoride biodegradation, was aligned with other extradiol dioxygenases involved in biodegradation of polycyclic compounds, identified in different bacteria. Using crystallographic data of LB400 BphC as a reference, a three-dimensional model was built for ThnC, which indicated that prominent structural features found in two independently crystallized BphC enzymes are conserved in ThnC (Han et al., 1995; Senda et al., 1996; Uragami et al., 2001). With this in hand, a number of positions, which might be important for activity or substrate specificity but whose relevance was not established by structural analyses, were selected for mutational analysis. To this end, a total of 10 amino acid substitutions were constructed in seven different positions, and the resulting enzymes characterized.

ThnC showed significantly high activity towards 1,2-DHT [24 U (mg protein)⁻¹], the substrate in the tetrafluoride biodegradation pathway, and also towards 1,2-DHN and 2,3-DHB, the substrates in the naphthalene or the biphenyl biodegradation pathways (Table 2, Fig. 4). The activity towards 2,3-DHB, 7 U (mg protein)⁻¹, is comparable to or higher than activities shown by other DHNDs. For instance, BphC1 and BphC2 from Sphingomonas sp. strain BN6 showed 3.8 and 6.57 U (mg protein)⁻¹, respectively (Heiss et al., 1995, 1997). This suggests that ThnC could also be efficiently used for degradation of 2,3-DHB. In spite of these data, 5 out of the 10 mutant enzymes showed at least twofold higher specificity for 1,2-DHT (Fig. 4), thus indicating that activity of ThnC is not maximized for its involvement in tetrafluoride biodegradation, and, therefore, that development of an improved catalyst for 1,2-DHT oxidation is feasible.

Asn-213 in ThnC was substituted by His, a residue conserved in equivalent positions of extradiol dioxygenases of all families with the exception of those clustering within the I.3.E subfamily of DHNDs. Substitution resulted in a significantly more active enzyme using any of the substrates tested, thus indicating that His in this position may be important for activity of extradiol dioxygenases, presumably by influencing the environment of the Fe(II)-binding site, since it forms a hydrogen bond with the metal-binding His-210 in LB400 (Han et al., 1995). Similarly, Gln-198 in ThnC was substituted by His, a residue conserved in enzymes cleaving polycyclic substrates not belonging to the subfamily of DHND, which forms a hydrogen bond with the putative O₂-binding His-195 in BphC from LB400 (Vaillancourt et al., 2002). Substitution also increased activity of ThnC towards any of the substrates tested. The effects of the N213H and Q198H substitutions clearly show the relevance of His residues in these positions for activity of ThnC, which is fully consistent with their conservation among extradiol dioxygenases not belonging to the I.3.E subfamily. It is, therefore, to be expected that catalysis by DHNDs in general may be improved by changing the residues in these positions to His. On the other hand, these results are intriguing since there is no obvious explanation for the absence of these histidines among DHNDs (Fig. 2). It is unlikely that absence of these His in DHNDs merely reflects a common evolutionary origin from an ancestor lacking them; their absence might therefore confer some particular characteristics upon DHNDs which are not observable in our assays.

Substitution A251M increased ThnC specificity for 2,3-DHB but reduced its specificity for 1,2-DHT and 1,2-DHN, thus resulting in a protein threefold more specific for 2,3-DHB, in comparison to the other substrates, than wild-type ThnC (Fig. 4). This result indicates that identity of the residue at this position may affect the activity towards a particular substrate, which confirms the interpretation of crystallographic data suggesting that this position (246 in BphC from LB400) could help to define the substrate-binding site (Han et al., 1995). The effect of this substitution may also explain the high degree of conservation of Met in this position among enzymes cleaving 2,3-DHB (Fig. 2).

Although the position equivalent to 206 of ThnC is conserved within the two groups of the I.3.E subfamily of DHNDs, it is not particularly conserved among other members of the family I.3 (Fig. 2), which suggests that this position tolerates a number of residues. However, substitution G206M, to give the Met residue conserved in DHNDs of group 1, increased specificity particularly for 1,2-DHT (Fig. 4). This shows that identity of the residue in this position also may affect activity towards a particular substrate, at least within the context of ThnC, in agreement with crystallographic data, which indicated that this position surrounds the ‘entranceway’ of the substrate (Senda et al., 1996). On the other hand, since the activity towards 1,2-DHN or 2,3-DHB is not significantly altered by this substitution, the fact that DHNDs of group 1 contain Met in this position whilst those of group 2 contain Gly does not explain the higher specificity of group 2 enzymes for 2,3-DHB.

The position equivalent to 282 of ThnC is conserved within the two groups of the subfamily of DHNDs (Fig. 2). Substitutions in this position, the first in the most C-terminal β-sheet (βR in Fig. 2), revealed its importance for activity of ThnC. On the one hand, the A282P substitution resulted in a virtually inactive protein. Crystallographic data indicated that this C-terminal β-sheet interacts with the last βαβββ motif of the protein. Since Pro provides rigidity to the protein backbone, lack of activity of the mutant protein may be explained by wrong orientation of the C-terminal β-sheet, which could prevent interaction with the last βαβββ motif, thus showing the importance of this interaction for functionality of ThnC. On the other hand, the other two substitutions, A282R and A282G, increased specificity of ThnC for 1,2-DHT, while affecting very little the specificity for the other substrates (Fig. 4). This clearly shows that identity of the residue in this position may specifically affect catalysis of the transformation of particular substrates, and therefore, catalysts with different substrate specificities might be developed from...
DHNDs by changing the residue at this position. It is interesting to note that this and neighbouring positions are not particularly well conserved among extradiol dioxygenases other than DHNDs and that some of them contain Pro in this position. These data suggest that the importance of the identity of the residue in this position shown by the mutational analysis may only be pertinent for DHNDs.

Mutational analysis of ThnC has, therefore, shown that histidines in positions 198 and 213 improve functionality of the protein, consistent with their conservation among extradiol dioxygenases of other subfamilies, that the most C-terminal β-sheet is important for activity and substrate specificity, which had not been shown before, and that identity of the residues in two additional positions, 206 and 251, may also affect catalysis of particular substrates.

Another observation is that some substitutions altered the sensitivity of the enzyme activity to pH, resulting in enzymes more tolerant to low pH. The most dramatic effect was shown by the substitution Y177I, which showed maximal activity at pH 5.5. At pH 5–5.5, the imidazole ring of the metal-binding His, and other potentially important His residues, may be protonated. This has been used to explain loss of activity of BphC from KKS102 below pH 6, since protonated His may easily lose the ability to coordinate to Fe(II) (Senda et al., 1996). The effect of the substitution Y177I may be interpreted as loss of enzyme activity at pH higher than 5–5.5, since its maximal activity is not higher than that of wild-type ThnC at this pH. However, the high relative activity shown by wild-type ThnC (40% of its activity at neutral pH) and the even higher relative activity shown by some mutants (up to 70% of their activity at neutral pH) clearly indicate that extradiol dioxygenases may be active at this acidic pH, possibly because the pKₐ of the essential His residues may be influenced by vicinal residues.

Extradiol dioxygenases show inhibition by the substrate but the mechanism of this inhibition is not known. It has been proposed that the substrate might be involved in negative cooperativity of the subunits, although it is unlikely, since this inhibition has also been reported for a monomeric enzyme (Happe et al., 1993). An alternative explanation could be that the substrate binds to a second site, maybe in the N-terminal domain, and this has a negative allosteric effect on activity. However, substitutions V175D and Q198D, which significantly altered the Kₐ of the enzyme, also altered sensitivity to substrate inhibition (Table 2), thus indicating that inhibition is apparently exerted through the same binding site, as previously suggested by kinetic analysis of the DHBD from LB400 (Vaillancourt et al., 1998). A possible mechanism for the substrate inhibition would be that high concentration of the substrate at the ‘entranceway’ might jam the enzyme, thus preventing proper fitting of the substrate in the active site, or release of the product after catalysis.

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REFERENCES


