Intersubunit interaction and catalytic activity of catechol 2,3-dioxygenases

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Catechol 2,3-dioxygenases (C23Os; EC 1.3.11.2) form a large protein family that is divided into several subgroups. Amino acid sequences of C23Os belonging to subgroup I.2.A and those belonging to I.2.B are found to be approx. 50% identical. When the central parts of the C23O sequences belonging to I.2.B were fused with the N-terminal and C-terminal sequences of I.2.A C23O, the hybrid enzymes were not active. To understand why these hybrid C23Os were inactive, hybrids between XylE_p (C23O found in a *Pseudomonas* strain; subgroup I.2.A) and XylE_s (C23O found in a *Sphingomonas* strain; subgroup I.2.B) were constructed. HB3-C23O consisted mostly of the XylE_s sequence, except that its C-terminal end was derived from XylE_p. While HB3-C23O was not active, HB4-C23O, carrying shorter C-terminal XylE_p sequences than HB3-C23O, was active. This observation indicated that certain amino acid residues at the

C-terminus were crucial for C23O activity in the hybrid forms of enzymes between XylE_p and XylE_s. According to the crystal structure of XylE_p, the C-terminal region is involved in the formation of a quaternary structure. Amino acid differences between HB3-C23O and HB4-C23O included the specific β -strand for oligomerization. Thus the quaternary structures of active C23Os, XylE_s, XylE_p and HB4-C23O, as well as that of inactive HB3-C23O, were examined. Active enzymes XylE_s, XylE_p and HB4-C23O were homotetrameric, while HB3-C23O existed only as a monomer. We concluded that hybrids of subgroups I.2.A and I.2.B were often inactive because of a defect in their oligomerization.

Key words: catechol 2,3-dioxygenase, DNA diversity, DNA library, hybrid enzyme, PCR.

INTRODUCTION

Bacteria have adaptively evolved by developing catabolic pathways to utilize a variety of compounds that are available in the natural environment. Many soil bacteria possess the metabolic potential to degrade a variety of aromatic compounds. Although the metabolic pathways of these aromatic compounds are very diverse, they are channelled into a limited number of key intermediates, such as catechol and substituted catechols.

Catechol 2,3-dioxygenase (C23O) is a member of the superfamily of extradiol dioxygenases and catalyses the ring cleavage of catechol and substituted catechols. Therefore, C23O is the key enzyme of many bacterial pathways for the degradation of aromatic compounds [1–4]. This ring-cleavage reaction has been studied intensively, and a general mechanism for oxidative cleavage of catechol by extradiol dioxygenases has been proposed [5]. The two atoms of the oxygen molecule are incorporated into the catechol substrate on two adjacent carbon atoms of the aromatic ring, one of which already carries a hydroxyl substituent of the diol and the other of which is unsubstituted. As with other extradiol dioxygenases, C23O requires ferrous ions for its activity [6]. At the active site, a single ferrous ion binds the substrate and the oxygen and participates in the catalytic cycle. The iron atom is bound to the polypeptide through covalent linkages with the side chains of three residues. Amino acid residues involved in ferrous ion binding and active sites were determined based on the three-dimensional structure of one of C23Os in *Pseudomonas putida* [7]. According to the solved structure, C23O forms a homotetramer.

In the active enzyme, the iron atom is in the ferrous state and is assumed to stay reduced throughout the catalytic cycle.

However, in the presence of certain substituted catechols as substrates, such as chlorocatechols and 4-methylcatechol, oxidation of the iron atom occurs, accompanied by inactivation of the enzyme [8]. Therefore, the reactions catalysed by different C23Os are often the rate-limiting step for the decomposition of aromatic compounds, notable examples being the degradation of 3-chlorocatechol and 4-methylcatechol [9–15].

We had developed an effective method to create hybrid genes for C23O in which the 5'- and 3'-ends of each hybrid gene consists of known C23O gene sequences (e.g. *nahH* from the NAH7 plasmid of *P. putida* [16,17]), whereas the central part of the hybrid gene is derived from C23O gene sequences in DNA extracted from environmental samples [18]. When such DNA was isolated from phenol-digesting bacterial consortia and used to make hybrid C23O genes with *nahH*, almost all the hybrid genes were functional, i.e. they produced active C23Os [18].

In this study, however, we found that the hybrid C23O genes thus constructed did not always produce active enzymes. When DNA isolated from phenanthrene- or dibenzothiophene-digesting bacterial consortia was used to make hybrid C23O genes with *xylE* from *P. putida* mt-2 (*xylE*_p [19]), none of the genes expressed C23O activity. Through this study we elucidated why C23Os synthesized from the hybrid genes were not functional and concluded that intersubunit interaction between monomers was important in enzyme activity of hybrid C23Os.

EXPERIMENTAL

Oligonucleotide primers

Two pairs of degenerate primers were used for PCR amplification of the central parts of the C23O genes (Figure 1). The first set was

Abbreviations used: C23O, catechol 2,3-dioxygenase; IPTG, isopropyl β -D-thiogalactoside.

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The nucleotide sequence data reported will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AB074513 and AB074514.

Figure 1 The principle of cassette PCR

The central parts of the genes encoding C230 were PCR-amplified from DNA that had been isolated from mixed cultures of phenanthrene- or dibenzothiophene-degrading micro-organisms using primers CF and CR or SCF and SCR. The 5'- and 3'-end sequences of the C230 gene were amplified either from *xyIE_p* or *xyIE_s*. The 5'-end of the *xyIE_p* or *xyIE_s* gene was amplified by using the primer sets 5F and 5R or 5SF and 5SR, respectively, while the 3'-end of the *xyIE_p* or *xyIE_s* gene was amplified by using the primer sets 3F and 3R or 3SF and 3SR, respectively. The 5'-end of 5R is complementary to the 5'-end of CF (indicated by double lines), while the 5'-end of CR is complementary to the 5'-end of 3F (indicated by dotted lines). Therefore, when a mixture of the three PCR-amplified segments, the 5'-end segment of *xyIE_p*, the central C230 gene segments amplified by CF and CR, and the 3'-end segment of *xyIE_p*, was subjected by PCR without a primer, full-length genes in the form (5'-*xyIE_p*)-(central C230)-(3'-*xyIE_p*) were amplified. Similarly, the second PCR using a mixture of the 5'-*xyIE_s* segment, the central C230 gene segments amplified by SCF and SCR, and the 3'-*xyIE_s* segment yielded full-length genes in the form (5'-*xyIE_s*)-(central C230)-(3'-*xyIE_s*).

CF and CR, which was designed according the amino acid sequences of known C230 genes belonging to subgroup I.2.A [20] or to the nucleotide sequence of *xyIE_p*. The respective CF and CR sequences were 5'-**GCC GAT TCT GAG CCA GGT ATG GAY TTY ATG GGN TTY AA**-3' and 5'-**ACT TCG TTG CGG TTA CCG GAN TCR AAR AAR TAH ATN GT**-3'. The 5'-end sequences in bold letters are identical to those of *xyIE_p*. The other primer pair was SCF and SCR, which were based on the amino acid sequences of known C230 genes belonging to subgroup I.2.B or on the nucleotide sequence of *xyIE_s*, the C230 gene from *Sphingomonas yanoikuaye* B1 [21]. Their respective sequences were 5'-**GCC GAT TCT GGC GGG CTC GAY CGN ATG GGN TTY AA**-3' and 5'-**TCG TTC CGG TTG CCC GAG GGR TCR AAR AAR TAD AT**-3'. The 5'-end sequences in bold letters are identical to those of *xyIE_s*. The 5'-end sequences of CF, CR, SCF and SCR indicated by bold letters were also complementary to the sequences of primer 5R, 3F, 5SR and 3SF, respectively, as shown below.

The primer set 5F and 5R was used to PCR-amplify the 5'-end region of *xyIE_p*, while the primer set of 3F and 3R was used for amplifying the 3'-end region of *xyIE_p* (Figure 1). The sequences of these primers were as follows: 5'-**GGC GGC CGC** TGA AGA GGT GAC GTC ATG AA-3' (5F); 5'-CAT ACC TGG CTC ATC GGC TT-3' (5R); 5'-**GGG AAT TCT AAG TCG TAC CGG ACC ATC**-3' (3R); and 5'-TCC GGT AAC CGC AAC GAA GT-3' (3F). Two more primer sets, 5SF and 5SR,

and 3SF and 3SR, were used for PCR amplification of the 5'- and 3'-end regions of *xyIE_s*, respectively. The sequences of these primers were 5'-**GGC GGC CGC** AAG GAC AGG GAG AAC TGA AA-3' (5SF); 5'-TCG AGC CCG GCA GAA TCG GC-3' (5SR); 5'-**GGG AAT TCC** GGC ACT CCG TTC ATG CTC A-3' (3SR); and 5'-CCC TCG GGC AAC CGG AAC GA-3' (3SF). The underlined sequences in primers 5F and 5SF are *NotI* restriction sites, while those in primers 3R and 3SR are *EcoRI* restriction sites.

Cassette PCR

The method used for cassette PCR has been described previously [18] and is summarized in Figure 1. We immersed 1 g of wet sand from a Kuwait beach in 200 ml of a basal salt medium [BSM; 0.1% (w/v) NH_4NO_3 , 0.1% (w/v) K_2HPO_4 , 0.05% (w/v) KH_2PO_4 , 0.002% (w/v) $\text{FeC}_6\text{H}_5\text{O}_7 \cdot n\text{H}_2\text{O}$, 3% (w/v) NaCl, 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.03% (w/v) KCl] supplemented with 0.2% (w/v) phenanthrene or dibenzothiophene, and incubated at 40 °C while shaking. The two-week culture suspension (10 ml) was used to inoculate 200 ml of fresh medium of the same composition, which was further cultivated under the same conditions. The cells were harvested and genomic DNA from the mixed cultures was prepared.

Cassette PCR was conducted in two steps (Figure 1). In the first step, the central parts of the C230 genes were amplified from 1 µg of extracted DNA. The PCR conditions were 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min. The 5'- and 3'-end fragments of *xyIE_p* were prepared by PCR with DNA isolated from a *P. putida* strain harbouring TOL plasmid pWW0 [22]; the PCR conditions were 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min. Plasmid pGJZ1522 (10 ng) containing *xyIE_s* [21] was used as a template to amplify the 5'- and 3'-end fragments of *xyIE_s*; the PCR conditions were 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Each PCR mixture contained 5 µl of a 10× reaction buffer [50 mM Tris/HCl (pH 8.8), 15 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM MgCl_2 , 0.45% (v/v) Triton X-100 and 200 µg/ml BSA], 4 µl of dNTPs (2.5 µM each), 100 pmol each of the appropriate primers, 0.2 µl of *Taq* polymerase (1 unit; Hoffman La Roche), one of the templates already described and sterile distilled water to adjust the total volume to 50 µl. The PCR products were separated by electrophoresis on 0.8% (w/v) agarose gel, excised from the gel and purified by a QIA gel extraction kit (Qiagen).

In the second step, 100 ng each of three PCR products (the central fragments of the C230 genes, the 5'-end fragment of either *xyIE_p* or *xyIE_s*, and the 3'-end fragment of either *xyIE_p* or *xyIE_s*) were mixed, and the second PCR amplification was carried out. The PCR conditions were 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min with 50 pmol of primers 5F and 3R, or 5FS and 3RS.

Cloning, screening and sequencing

After digesting the second PCR products with the *NotI* and *EcoRI* restriction enzymes, the fragments were ligated to pBlue-script SK (–) (Stratagene) digested by the same enzymes. The ligated DNA was used to transform *Escherichia coli* TOP10F', and the recombinants were selected on LB agar plates containing 100 µg/ml ampicillin, 20 µg/ml 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-gal) and 0.1 mM isopropyl β-D-thiogalactoside (IPTG). To screen the clones that expressed the active

C23O genes, 100 mM catechol was sprayed on the colonies. Catechol, the substrate of C23O, is colourless, while the product, 2-hydroxymuconic semialdehyde, is bright yellow [23]. The transformed colonies of *E. coli* expressing active C23O enzymes showed a yellow colour when catechol was applied.

Plasmids were purified from the transformants. Nucleotide sequences were determined on both strands by using an ABI Prism™ Dye Terminator cycle sequencing kit (PE Biosystems) according to the manufacturer's instructions, followed by analysing with a 373A DNA sequencer (PE Biosystems).

Preparation of the cell-free extract and purification of C23Os

Two parental C23O genes, *xylE_p* and *xylE_s*, and hybrid genes between *xylE_p* and *xylE_s* were cloned into the pTrc99A vector [24], and the resulting recombinant plasmids were used to transform *E. coli* BL21. The transformants were grown in 250 ml of LB containing 100 µg/ml of ampicillin until the cell density gave a D_{600} value of 0.5–0.8. The expression of C23O was then induced by adding 1 mM IPTG and cultivating overnight at 30 °C. The cells were harvested by centrifuging for 15 min at 5500 *g*, washed with 10 mM ethylenediamine/H₂SO₄ (pH 7.5) containing 10% (v/v) isopropanol and resuspended in a 1/20 culture volume of the same buffer at 4 °C. The cells were then disrupted by a French press (Ohtake) at 300 kg/cm³, and centrifuged at 16 500 *g* at 4 °C for 60 min. The supernatants were used as cell-free extracts and applied to further purification of C23Os by the method described previously [10,18].

Gel-filtration column chromatography

Proteins in the cell-free extracts were precipitated by (NH₄)₂SO₄ at 70% saturation at 4 °C and then dissolved in the same volume of 10 mM Tris/HCl (pH 7.5). The protein suspension was passed through a Millex filter (0.22 µm pore size; Millipore) and charged on to a Superdex 200 HR 10/30 column (Amersham Biosciences), which was pre-equilibrated with 50 mM potassium phosphate (pH 7.4) containing 100 mM NaCl and 0.02% (w/v) NaN₃. The charged proteins were then separated at a flow rate of 0.4 ml/min. Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa) and cytochrome *c* (12.4 kDa) were used as molecular mass standards (Amersham Biosciences). Microtitre plate assay for the C23O activity of each fraction was carried out in a 100 mM potassium phosphate buffer (pH 7.5) at 25 °C with 330 µM catechol as the substrate. The yellow ring-cleavage product was detected spectrophotometrically at 405 nm by a Titertek™ Twinreader PLUS instrument (Dainippon Pharmaceutical). Although the absorption maximum of the yellowish product was at 375 nm [25], absorbance at 405 nm was proportional to the amount of yellowish products. The relative C23O activity was calculated from the absorbance at 405 nm.

Determination of the kinetic parameters of C23Os

The cell-free extracts were prepared from 500 ml of *E. coli* BL21 cells expressing XylE_p, XylE_s or HB4-C23O (see the Results section for a description of this hybrid). Either of C23Os was purified to homogeneity by the method described previously [10,18]. Standard assay for C23O activity of the purified enzymes was carried out in a 100 mM potassium phosphate buffer (pH 7.5) at 25 °C with 330 µM catechol as a substrate, and the amount of the ring-cleavage product of catechol (2-hydroxymuconic semialdehyde; $\epsilon = 33 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) was determined spectrophotometrically at 375 nm [25]. The K_m and k_{cat} values of the C23Os for catechol were determined under the standard conditions,

except that the concentration of catechol was varied in the range of 0.5–128 µM. Curve fitting was carried out using GraphPad Prism version 3.03 for Windows (GraphPad Software), employing non-linear regression based on a one-site binding equation.

Antibody production

Anti-XylE_p antibody was raised against recombinant XylE_p. The *xylE_p* gene was cloned into the pTYB1 plasmid (New England BioLabs). pTYB1 harbouring the *xylE_p* gene was introduced into *E. coli* BL21(DE3). The transformants were grown in 250 ml of LB containing 100 µg/ml ampicillin until the cell density gave a D_{600} value of 0.5. The expression of XylE_p was then induced by adding 0.5 mM IPTG for 3 h at 37 °C. Cells were harvested by centrifugation for 15 min at 5500 *g*, washed with column buffer [20 mM Na-Hepes (pH 8.0), 500 mM NaCl, 0.1 mM EDTA and 0.1% Triton X-100] and resuspended in a 1/20 culture volume of the same buffer at 4 °C. Cells were disrupted by a French press and centrifuged at 16 500 *g* at 4 °C. The supernatant was loaded on to a chitin column (New England BioLabs) pre-equilibrated with column buffer. After washing with column buffer, XylE_p was eluted with cleavage buffer [20 mM Na-Hepes (pH 8.0), 50 mM NaCl, 0.1 mM EDTA and 30 mM dithiothreitol] from the chitin column using the manufacturer's instructions. A rabbit was immunized with recombinant XylE_p several times. Serum bleeds were collected and the antibody was purified with (NH₄)₂SO₄ precipitation followed by dialysis against PBS.

SDS/PAGE and Western blot analysis

Proteins in the fractions separated by gel-filtration column chromatography were subjected to SDS/PAGE. Pre-stained broad-range protein markers [β -galactosidase (175 kDa), paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β -lactoglobulin A (25 kDa), lysozyme (16.5 kDa) and aprotin (6.5 kDa); New England BioLabs] were used as standards. After electrophoresis, the proteins were transferred to a ProBlot™ membrane (PE Biosystems), which had been soaked in 5% (w/v) non-fat milk in T-TBS buffer [20 mM Tris/HCl (pH 7.6), 150 mM NaCl and 0.05% (v/v) Tween 20], and incubated for 1 h at room temperature with vigorous shaking. The membrane was incubated overnight in the same buffer containing the anti-XylE_p antibody. The membrane was then washed three times with T-TBS buffer for 10 min at a room temperature and subsequently incubated with the horseradish peroxidase-labelled goat anti-rabbit IgG antibody (New England BioLabs) in 5% (w/v) non-fat milk in T-TBS buffer for 1 h at room temperature. After the membrane had been washed twice with T-TBS buffer for 10 min, the proteins that had reacted with the antibody were detected by enhanced chemiluminescence (ECL) with the ECL Plus Western blotting detection system (Amersham Biosciences). The membrane was exposed to an imaging screen for 10 min and then analysed using the Multi-analyst Molecular Imaging system (Bio-Rad).

RESULTS

Cassette PCR to construct hybrid C23Os

To construct the hybrid C23O genes, the central part of the C23O genes were PCR-amplified from genomic DNA that had been isolated from mixed bacterial populations digesting phenanthrene or dibenzothiophene. The catabolic pathways for the degradation of these aromatic hydrocarbons frequently contain

XylE _S	1:MALTGVLRPGYVQLRVLDLDEAIQHYRDRIGL-NLVSVEGGRAFFQAFDEFDRHSIILRE	59
DT11	1:.N-K..M...H.....MSK.LE..VELL..IEMDRDDQ..VYLK.WT.V.KF.LV...	59
DT216	1:.N-K..M...H.....MSK.LE..VELL..IEMDRDDQ..VYLK.WT.V.KF.LV...	59
Phn173	1:.N-K..M...H.....MSK.LE..VELL..IEMDRDDQ..VYLK.WT.V.KF.LV...	59
XylE _P	1:.N-K..M...H.....MSK.LE..VELL..IEMDRDDQ..VYLK.WT.V.KF.LV...	59
XylE _S	60:ADSAGLDRMAFKVARDADLDHFAERLLDIGVHVDVIAAGEDPGVGRKIRFNTPTSHVFDL	119
DT11	60:..EP.M.F.G...VK.....P..S.....Q...E.	119
DT216	60:..EP.M.F.G...K.S.....L.....VP.....E...E.	119
Phn173	60:..EP.M.F.G...VK.....PS.....Q...E.	119
XylE _P	60:..EP.M.F.G...VDEDA.RQLERD.MAY.CA.EQLP...LNSC..RV..QA.SG.H.E.	119
XylE _S	120:YAEMEL-SESGPAVRNPDVWIAEPRGMRAFRFDHCALNGVDISASAKIFVEALDFSVAEE	178
DT11	120:.....-AAT...K.....VV.....D.....	178
DT216	120:.....-AT...TK.....VV.....D.....T..	178
Phn173	120:.....-AT...N.....VI.....D...T..	178
XylE _P	120:..DK.YTGKW.LNDV..EA.PRDLK..A.V...ALMY.DELP.TYDL.TKV.G.YL..Q	179
XylE _S	179:LVDETSGVRMGIFLSCSNKAHDVAFLGYPEDGRIHHTSFFLDSWHDVGAADIISRYDIS	238
DT11	179:.....T.L.....K.....N.E.....	238
DT216	179:....G..T.L.....H.....K.....N.E.....	238
Phn173	179:....G..T.L.....K.....N.E.....	238
XylE _P	180:VL..-N.T.VAQ...L.T.....IHH..K..L..V..H.ET.E.LLR...L..MT.T.	238
XylE _S	239:LDIGPTRHGITRGQTIYFFDPGSGNRNETFSGGYTY-YPDNPRRMQAENAGKAIFYEKA	297
DT11	239:.....V.C.-D.N...HKPVT.TTDQL.....HDRI	297
DT216	239:.....V.C.-D.N...HKPVT.TTDQL.....HDRI	297
Phn173	239:.....V.C.-D.N...HKPVT.TTDQL.....HDRI	297
XylE _P	239:I.....L.H.K.....V.C.-D.N...HKPVT.TTDQL.....HDRI	297
XylE _S	298:LNDRFMTVNT	307
DT11	298:..E....L.	307
DT216	298:..E....L.	307
Phn173	298:..E....L.	307
XylE _P	298:..E....L.	307

Figure 2 Alignment of the amino acid sequences of various C23Os

XylE_S and XylE_P are C23Os of *S. yanoikuaye* B1 and *P. putida* mt-2, respectively. DT11 and DT216 were amplified from DNA of a mixed culture of dibenzothiophene-degrading micro-organisms, while Phn173 was from a culture of phenanthrene-degrading micro-organisms. Both the N- and C-terminal sequences of DT11, DT216 and Phn173 are from XylE_P. Dots indicate amino acid residues identical to those of XylE_S. Residues different from those of both XylE_S and XylE_P are shown in bold. The ferrous-ion-binding sites conserved in C23Os are marked with arrows. Amino acid residues corresponding to the PCR primers used for amplification of the central segments are boxed.

a step catalysed by C23O [1–4]. DNA fragments of the expected molecular size, corresponding to the central parts of the C23O genes, were successfully amplified from DNA of the phenanthrene-digesting or dibenzothiophene-digesting bacteria. The amplified DNA fragments were then inserted between the 5'- and 3'-end fragments of *xylE_P*, to assemble into full-length C23O genes. The PCR products corresponding to the full-length C23O genes were cloned into pBluescript SK(–) and introduced

into *E. coli*. Although most of the white colonies grown on the selective plates harboured plasmids with the correct-size inserts, none of them showed C23O activity after being sprayed with catechol.

The nucleotide sequences of several independent clones were determined. All the clones exhibited in-frame fusion; namely, the 5'-end sequence of *xylE_P* was fused in-frame to the central C23O gene sequences and 3'-end sequence of *xylE_P*. All the essential

Figure 3 Structural details of C230s

(A) Structures of the hybrid C23O enzymes. Sequences derived from XylE_P and XylE_S are shown as open and shaded boxes, respectively. (B) Alignment of the amino acid sequences of XylE_P and XylE_S. Boxes show junction points for each hybrid; the hybrids with switched amino acids at these points are shown under the boxes. The secondary-structural elements for XylE_P were cited from Kita et al. [7]. β -Strands and α -helices are shown as arrows and black bars, respectively. Asterisks indicate the amino acid residues contributing to the formation of a dimer in the projecting loop.

amino acid residues required for C23O activity [1,20] were conserved in these sequences (Figure 2); nevertheless, these hybrid genes did not express C23O activity.

The deduced amino acid sequences of the central segments showed less than 50% similarity to those of XylE_P; they had greater similarity to XylE_S, with an identity of more than 90% (Figure 2). The 5'- and 3'-end fragments of xylE_S were then used

to make hybrids with C23O gene sequences that had been amplified from the phenanthrene- or dibenzothiophene-digesting bacterial consortia. When such hybrid genes were used to transform *E. coli*, almost all the white colonies on the selective plates turned yellow after being sprayed with catechol, indicating that the C23O gene sequences fused to the 5'- and 3'-end sequences of xylE_S formed functional hybrid genes.

Table 1 Michaelis–Menten kinetic parameters

Kinetic parameters of each C23O were calculated by curve fitting of data from three measurements and indicated as means $\pm$ S.E.M. The enzyme assays were performed in 2.4 ml of 100 mM potassium phosphate (pH 7.5) containing catechol (0.5–128 μ M) and the purified C23O. The amounts of purified C23O were 0.1–1 μ g (XylE_p), 0.3 μ g (XylE_s) and 0.7 μ g (HB4-C23O).

	K_m (μ M)	k_{cat} (s ⁻¹)
XylE _p	2.9 $\pm$ 0.5	493 $\pm$ 15
XylE _s	3.8 $\pm$ 0.4	238 $\pm$ 6
HB4-C23O	19.8 $\pm$ 1.7	148 $\pm$ 4

Constructing the hybrid C23Os between XylE_p and XylE_s

To understand the structure–function relationships of the hybrid C23Os so far described, various hybrid genes between *xylE_p* and *xylE_s* were constructed (Figure 3). The HB1-C23O gene, in which the *xylE_s* sequence was sandwiched by the *xylE_p* sequences, did not express C23O activity (Figure 3A). Similarly, the hybrid C23O gene, in which the *xylE_p* sequence was sandwiched by the *xylE_s* sequences, did not express any detectable C23O activity (results not shown). The HB2-C23O gene consisted of most of the *xylE_p* sequence, except that one-fifth of 3'-end sequence was derived from *xylE_s*. This hybrid gene did not express any C23O activity either (Figure 3A). The same lack of C23O activity was true for the HB3-C23O gene, in which the majority of its sequence consisted of *xylE_s*, except that its 3'-end sequence was derived from *xylE_p* (Figure 3A). The HB4-C23O gene was a fusion product similar to the HB3-C23O gene, except that it possessed a shorter *xylE_p* sequence on the 3'-end. HB4-C23O, containing 14 amino acids from XylE_p at the C-terminus (Figure 3B), showed enzyme activity (Figure 3A).

Determination of kinetic parameters of C23Os

The K_m and k_{cat} values of wild-type and hybrid C23Os were determined by examining their initial velocities at substrate concentrations between 0.5 and 128 μ M. Both the affinity for the substrate, catechol, and k_{cat} value of HB4-C23O were lower than those of two wild-type C23Os, XylE_p and XylE_s (Table 1). The K_m value of HB4-C23O was about 5-fold higher than that of one of the parents, XylE_s. The k_{cat} value of HB4-C23O was about half that of XylE_s, indicating that not all the hybrid molecules in HB4-C23O were active forms.

Characterization of the hybrid C23Os

In order to examine whether C23O proteins had been synthesized from the hybrid constructs, cell-free extracts were prepared from various *E. coli* recombinant cells carrying wild-type and hybrid C23O genes, separated by SDS/PAGE and blotted on to membranes. The anti-XylE_p antibody was used for C23O detection. Protein with a molecular mass of 37 kDa was clearly detected in cell-free extracts of XylE_p (Figure 4A). Although the amino acid similarity of XylE_s to XylE_p is about 50%, the antibody could recognize XylE_s (Figure 4A). All three hybrid C23Os, HB2-C3O, HB3-C23O and HB4-C23O, contained both XylE_p and XylE_s, in different ratios, and also reacted with the antibody (Figure 4). Even though the antibody reactivity to the hybrid C23Os was different, very small amounts of HB2-C23O and HB3-C23O were detected (Figure 4A). This might reflect the fact that both hybrids were unstable and easily degraded because they could not proceed with proper oligomerization (see below).

Proteins in cell-free extracts were separated by gel-filtration chromatography. Each fraction was subjected to SDS/PAGE, and the C23O proteins were detected by Western blotting. At the same time, the C23O activity in each fraction was determined. Both XylE_p and XylE_s were detected in fractions 31–36, mainly in fractions 32–34, by the anti-XylE_p antibody (Figure 4B). The molecular mass of fraction 32 was estimated to be about 150 kDa by comparing with the eluted volumes of the molecular-mass markers, which matched the calculated molecular masses of the tetramers of both XylE_p and XylE_s (148 kDa). The XylE_p and XylE_s proteins were not detected in any other fractions. C23O activity was also found only in fractions 32–34 of XylE_p and XylE_s (Figure 4B). These results clearly indicated that almost all the synthesized C23O polypeptides of parental C23Os, XylE_p and XylE_s, formed homotetramers to express enzyme activity. The hybrid protein HB4-C23O was also detected only in the tetramer fractions, fractions 32–34. C23O activity was detected in these fractions too (Figure 4B). In contrast, the hybrid protein HB3-C23O was detected only in fractions 37 and 38, which corresponded with the molecular mass of the monomer (37 kDa). No C23O enzyme activity was found in any fractions of the HB3-C23O preparation (Figure 4B).

DISCUSSION

The central parts of the C23O genes amplified from DNA of the phenanthrene- or dibenzothiophene-digesting bacterial consortia were similar to that of *xylE_s* belonging to subgroup I.2.B of the extradiol dioxygenase gene superfamily. They showed less than 50% identity to the sequence of *xylE_p* belonging to subgroup I.2.A (Figure 2). The most striking observation was that when the PCR-amplified central parts were combined with less similar *xylE_p* sequences, the hybrid C23O genes did not synthesize the active enzymes. We constructed various hybrids between *xylE_p* and *xylE_s* to investigate the relationship of hybrid structure and enzyme activity. Two very similar constructs that contained XylE_s on most N-terminal parts, HB3-C23O and HB4-C23O, showed different enzyme activity. HB4-C23O, which contained longer XylE_s sequences, was active, while HB3-C23O, which was composed of more XylE_p sequences, was inactive. We speculated that this region (i.e. amino acid residues 266–287) was important for demonstrating C23O activity in the hybrid enzymes of different subgroups.

Information about the difference in the primary sequences in this region did not give any clues to solve the structure–function relationship. We found the answer in the secondary structure. According to the crystal structure [7], XylE_p consists of four $\beta\alpha\beta\beta$ modules: one module is composed of β -sheet, α -helix, β -sheet, β -sheet and β -sheet (i.e. module 1 is 1S1, 1H1, 1S2, 1S3 and 1S4). The fourth module contains the extended secondary structures $\beta\alpha\alpha$ (Figure 3B). One of the β -sheet structures in module 4, 4S5 (Figure 3B), is shown to be important for the formation of the quaternary structure. The 4S5 β -sheet of one monomer interacts with three residues (Asp-133, Val-134 and Asn-135) in XylE_p on the projecting loop of another monomer, forming main-chain hydrogen bonds between two subunits [13] (Figure 3B). The critical residues for C23O activity in the hybrid enzymes, residues 266–287, contain 4S5 β -sheet. The crystal structures of two other 2,3-dihydroxybiphenyl 1,2-dioxygenases, members of the extradiol dioxygenase superfamily, have also been solved [26,27]. Their quaternary structures are homooctamers formed from two planar tetramers. Two parallel β -sheets on two subunits corresponding to residues 118–124 and residues 273–279, respectively, interact with each other to form the planar tetramers. These two β -sheets are located in the

Figure 4 Analysis of the quaternary structures of C230s

(A) Proteins in the cell-free extracts were separated by SDS/PAGE and transferred to membranes. C230 proteins detected by anti-XylE_p antibody are indicated by the arrow. Cell-free extracts were prepared from *E. coli* BL21 carrying C230 genes shown above the each lane: *P. putida* xylE_p (XylE_p), HB2-C230 (HB2), HB3-C230 (HB3) and *S. yanoikuyae* xylE_s (XylE_s). (B) Proteins in the cell-free extracts (CFE) from cells expressing XylE_p, XylE_s, HB3-C230 (HB3) and HB4-C230 (HB4) were separated by Superdex 200 HR gel filtration column and fractions were collected every minute. Upper panels: C230 protein in each fraction was monitored by Western blotting with anti-XylE_p antibody. T, D and M indicate the fractions corresponding to the molecular masses of the homotetramer, homodimer and monomer of C230, respectively. Lower panels: C230 activity in each fraction was assayed in 96-well microtitre plates. A portion of each fraction was added to the reaction mixture containing catechol, and incubated for 5 min. The absorbance of yellowish products was measured at 405 nm and the relative C230 activity was calculated. RT, retention time.

vicinity of the sequences corresponding to the projecting loop (residues 130–139) and the 4S5 β -sheet, respectively, of XylE_p. We then concluded that HB1-C23O, HB2-C23O and HB3-C23O were inactive because two crucial regions for the subunit in-

teraction, the projecting loop and the 4S5 β -sheet, were derived from different origins, either XylE_p or XylE_s, in these hybrid enzymes, and could not interact to achieve oligomerization. This inference was demonstrated experimentally. It was found that

the active enzymes, two wild-type C23Os (XylE_p and XylE_s) and HB4-C23O, were tetramers, while HB3-C23O only formed a monomer. We constructed a hybrid enzyme in which the central and C-terminal parts were XylE_p and the N-terminal part was XylE_s. Even though this hybrid enzyme contained amino acid sequences of XylE_p in both the projecting loop and 4S5, it was inactive (results not shown). With Western blotting analysis, only a very weak C23O signal was detected in the cell-free extracts. Another region besides the projecting loop and 4S5 may be related to tetramer formation. We plan to analyse this in detail in the future.

Many enzymes function as oligomers, and their monomeric forms are often not active. Why the oligomeric form is required for some enzymes to exhibit activity is not yet fully understood, although there are few exceptions. Yeast enolase, for example, is active as a dimer. Under high hydrostatic pressure, the dimer form of the enzyme is changed to a monomer and inactivated, because the catalytic magnesium ion appears to be released upon monomerization [28]. The stabilization of proteins by oligomerization, the formation of active sites at the interface of oligomers, and conformational changes upon the oligomerization are possible mechanisms for the oligomerization-dependent activity of enzymes [29]. As described above, the monomer of C23O contains four $\beta\alpha\beta\beta$ modules, suggesting that C23O has evolved through the duplication and fusion of an ancestral $\beta\alpha\beta\beta$ module. During the evolution of C23O, the oligomerization of the modules seems to have created critical regions such as metal-binding sites [30]. In this study, we had demonstrated the *in vitro* evolution of C23O by modifying the properties of the oligomerization.

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