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 XylEₚ (C23O found in a Pseudomonas strain; subgroup I.2.A) and XylEₚ (C23O found in a Sphingomonas strain; subgroup I.2.B) were constructed. HB3-C23O consisted mostly of the Xyle sequence, except that its C-terminal end was derived from XylEP. While HB-C23O was not active, HB4-C23O, carrying shorter C-terminal XylEP 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 XylEP and XylEP. According to the crystal structure of XylEP, 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, XylEP, XylEP, and HB4-C23O, as well as that of inactive HB3-C23O, were examined. Active enzymes XylEP, XylEP, 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 (xylEp [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.
The central parts of the genes encoding C23O 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 C23O gene were amplified either from xylEP or xylES. The 5'-end of xylEP or xylES gene was amplified by using the primer sets 5F and 5R or 5SF and 5SR, respectively, while the 3'-end of the xylEP or xylES 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 3'-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 xylEP, the central C23O gene segments amplified by CF and CR, and the 3'-end segment of xylES was subjected by PCR without a primer, full-length genes in the form (5'-xylEP)-(central C23O)-(3'-xylES) were amplified. Similarly, the second PCR using a mixture of the 5'-end of 5R and 5'-end fragment of either xylEP or xylES 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. The 5'- and 3'-end fragments of xylEP 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 pJJZ1522 (10 ng) containing xylEP [21] was used as a template to amplify the 5'- and 3'-end fragments of xylES; 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 (NH4)2SO4, 10 mM MgCl2, 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 C23O genes, the 5'-end fragment of either xylEP or xylES, and the 3'-end fragment of either xylEP or xylES) 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-hydroxyxymuconic 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 analysis with a 373A DNA sequencer (PE Biosystems).

Preparation of the cell-free extract and purification of C23Os

Two parental C23O genes, xylE<sub>P</sub> and xylE<sub>E</sub>, and hybrid genes between xylE<sub>P</sub> and xylE<sub>E</sub>, 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<sub>600</sub> 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 centrifugation for 15 min at 5500 g, washed with 10 mM ethylenediamine/H<sub>2</sub>SO<sub>4</sub> (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/cm<sup>2</sup>, and centrifuged at 16500 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 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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) NaN<sub>3</sub>. 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 Prism™ instrument (Dainippon Pharmaceutical). Although the absorbance maximum of the yellowish product was at 375 nm [25], 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<sub>P</sub>, XylE<sub>E</sub> 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 assays 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-hydroxyxymuconic semialdehyde; <i>e</i> = 33 mM<sup>−1</sup> cm<sup>−1</sup>) was determined spectrophotometrically at 375 nm [25]. The <i>K<sub>m</sub></i> and <i>k<sub>cat</sub></i> 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<sub>P</sub> antibody was raised against recombinant XylE<sub>P</sub>. The xylE<sub>P</sub> gene was cloned into the pTYB1 plasmid (New England BioLabs). pTYB1 harbouring the xylE<sub>P</sub> 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<sub>600</sub> value of 0.5. The expression of XylE<sub>P</sub> 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 % (w/v) 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 16500 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<sub>P</sub> 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<sub>P</sub> several times. Serum bleeds were collected and the antibody was purified with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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), para-amysin (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<sub>P</sub> 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
Figure 2  Alignment of the amino acid sequences of various C23Os

XylEs and XypEs are C23Os of *S. yanoikuyae* 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 XylEP. Dots indicate amino acid residues identical to those of XylES. Residues different from those of both XylES and XylEP 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 *xylEp* 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 *xylEp* was fused in-frame to the central C23O gene sequences and 3′-end sequence of *xylEp*. All the essential
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Figure 3 Structural details of C23Os

(A) Structures of the hybrid C23O enzymes. Sequences derived from XylEP and XylES are shown as open and shaded boxes, respectively. (B) Alignment of the amino acid sequences of XylEP and XylES. 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 XylEP 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 XylEP; they had greater similarity to XylES, with an identity of more than 90% (Figure 2). The 5'- and 3'-end fragments of xylE 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 were formed functional hybrid genes.
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 XyIEP and XyIES were detected in fractions 31–36, mainly in fractions 32–34, by the anti-XyIES 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 XyIEP and XyIES (148 kDa). The XyIEP and XyIES proteins were not detected in any other fractions. C23O activity was also found only in fractions 32–34 of XyIEP and XyIES (Figure 4B). These results clearly indicated that almost all the synthesized C23O polypeptides of parental C23Os, XyIEP and XyIES, 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 xyIEP belonging to subgroup I.2.B of the extradiol dioxygenase gene superfamily. They showed less than 50 % identity to the sequence of xyIES 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 xyIEP sequences, the hybrid C23O genes did not synthesize the active enzymes. We constructed various hybrids between xyIEP and xyIES to investigate the relationship of hybrid structure and enzyme activity. Two very similar constructs that contained XyIEP on most N-terminal parts, HB3-C23O and HB4-C23O, showed different enzyme activity. HB4-C23O, which contained longer XyIES sequences, was active, while HB3-C230, which was composed of more XyIEP 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], XyIES consists of four \( \beta \alpha \beta \beta \beta \) modules: one module is composed of \( \beta \)-sheet, \( \alpha \)-helix, \( \beta \)-sheet, \( \beta \)-sheet and \( \beta \)-sheet (i.e. module 1 is \( \beta \)1, \( \beta \)1, \( \beta \)2, \( \beta \)3 and \( \beta \)4). The fourth module contains the extended secondary structures \( \beta \alpha \alpha \). One of the \( \beta \)-sheet structures in module 4, \( \beta \)5 (Figure 3B), is shown to be important for the formation of the quaternary structure. The \( \beta \)-sheet of one monomer interacts with three residues (Asp-133, Val-134 and Asn-135) in XyIES 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 \( \beta \)5 \( \beta \)-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 homotetramers formed from two planar tetramers. Two parallel \( \beta \)-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 \( \beta \)-sheets are located in the
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Figure 4  Analysis of the quaternary structures of C23Os

(A) Proteins in the cell-free extracts were separated by SDS/PAGE and transferred to membranes. C23O proteins detected by anti-XylEp antibody are indicated by the arrow. Cell-free extracts were prepared from E. coli BL21 carrying C23O genes shown above the each lane: P. putida xylEp (XylEP), HB2-C23O (HB2), HB3-C23O (HB3) and S. yanoikuiye xylEs (XylEs). (B) Proteins in the cell-free extracts (CFE) from cells expressing XylEP, XylEs, HB3-C23O (HB3) and HB4-C23O (HB4) were separated by Superdex 200 HR gel filtration column and fractions were collected every minute. Upper panels: C23O protein in each fraction was monitored by Western blotting with anti-XylEp antibody. T, D and M indicate the fractions corresponding to the molecular masses of the homotetramer, homodimer and monomer of C23O, respectively. Lower panels: C23O 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 C23O 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 XylEp. We then concluded that HB1-C23O, HB2-C23O and HB3-C23O were inactive because two crucial regions for the subunit interaction, the projecting loop and the 4S5 β-sheet, were derived from different origins, either XylEp or XylEs, 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<sub>P</sub> and XylE<sub>R</sub>) 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<sub>P</sub> and the N-terminal part was XylE<sub>R</sub>. Even though this hybrid enzyme contained amino acid sequences of XylE<sub>P</sub> 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 $\beta$-sheet modules, suggesting that C23O has evolved through the duplication and fusion of an ancestral $\beta$-sheet 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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