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ORIGINAL PAPER

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Functionality of biphenyl 2,3-dioxygenase components in naphthalene 1,2-dioxygenase

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Abstract Naphthalene 1,2-dioxygenase (Nap dox) and biphenyl 2,3-dioxygenase (Bph dox) are related enzymes that have differentiated during evolution as their specificity has changed. Although their component arrangement is similar, the structure of each component has been modified quite extensively. The purpose of this work was to determine the catalytic capacity of purified Nap dox toward chlorobiphenyls and to investigate the functionality of Bph dox components in the Nap dox system. Both enzyme systems were purified by affinity chromatography as histidine-tagged fused proteins. Data show for the first time that Nap dox can catalyze the oxygenation of all three monochlorobiphenyl isomers, but it is unable to hydroxylate 2,5-, 2,2′-, 3,3′-, 4,4′-di- and 2,2′,5,5′-tetrachlorobiphenyl. The rates of cytochrome *c* reduction by the ferredoxin components of the two enzymes were identical when the Bph dox reductase component was used in the assay, showing an efficient electron transfer between the Bph dox reductase component and the Nap dox ferredoxin. However, when the Bph dox ferredoxin was used to reconstitute a hybrid Nap dox, the enzyme was only 22% as active as the parental enzyme. These data are discussed in terms of the potential use of Nap dox for the development of enhanced chlorobiphenyl-degrading dioxygenases.

Introduction

Aryl-group-hydroxylating dioxygenases catalyze the first enzymatic step for most bacterial catabolic pathways involved in the degradation of aromatic compounds (Mason and Cammack 1992). They catalyze a dihydroxylation of vicinal carbons of the aromatic ring. These enzymes have been classified into three classes on the basis of the types of components associated and the chemical nature of their substrate (Batie et al. 1991; Mason and Cammack 1992). A comparison of the predicted amino acid sequences of these oxygenases identified three genetic clusters, which coincide with class IB, II and III dioxygenases (Sylvestre et al. 1996b; Werlen et al. 1996).

Despite their evolutionary specialization aryl-group-hydroxylating dioxygenases can catalyze the oxygenation of a broad range of analogs. Thus biphenyl dioxygenase (Bph dox) can catalyze the oxygenation of chlorobiphenyls (Haddock et al. 1995), toluene (Furukawa et al. 1993), hydroxybiphenyls (Sondossi et al. 1991) and dichlorophenyltrichloroethane (Massé et al. 1989). Benzene/chlorobenzene dioxygenase can hydroxylate toluene, biphenyl and naphthalene (Werlen et al. 1996). Naphthalene dioxygenase (Nap dox) can catalyze the oxygenation of several polycyclic aromatic hydrocarbons (Menn et al. 1993; Kiyohara et al. 1994) and it can oxygenate biphenyl (Barriault et al. 1998). The three-component Nap dox system has been purified from *Pseudomonas putida* NCIB 9816 (Haigler and Gibson 1990a, b; Ensley and Gibson 1983). It comprises an iron-sulfur flavoprotein reductase (Red_{Nap}) and a ferredoxin (Fer_{Nap}), which are involved in the electron transfer from NADH to the oxygenase component, an iron-sulfur protein (ISP_{Nap}). The latter comprises two dissimilar subunits α and β (Kauppi et al. 1998). In strain NCIB 9816 $nahA_a$ codes for Red_{Nap}, $nahA_b$ codes for Fer_{Nap} and $nahA_c$ and $nahA_d$ code for the ISP_{Nap} α and β subunits respectively. This arrangement was found to be the same for the *P. putida* G7 Nap dox system ($Nap\ dox_{G7}$) (Simon et al. 1993). Except for the fact that Red_{Nap} is an iron-sulfur flavoprotein, the arrangement of enzyme components of Nap dox (a class III dioxygenase) is similar to that of Bph dox (a class II dioxygenase) purified from *Comamonas testosteroni* B-356 ($Bph\ dox_{B\text{-}356}$) (Hurtubise et al. 1995, 1996). BPH

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$dox_{B\text{-}356}$ components comprise a reductase (Red_{Bph}), a ferredoxin (Fer_{Bph}) and a terminal oxygenase (ISP_{Bph}).

The purpose of this work was to determine the catalytic capacity of purified Nap dox_{G7} with chlorobiphenyls and to investigate the functionality of Bph $dox_{B\text{-}356}$ components in the Nap dox_{G7} system. The enzyme components were purified as histidine-tagged (ht-) fused proteins. Results are discussed in terms of the potential use of Nap dox for the development of enhanced chlorobiphenyl-degrading dioxygenases.

Materials and methods

Bacterial strains and general protocols

The bacterial strains used in this study were *Escherichia coli* M15[pREP4] (Qiagen Inc., Chatworth, Calif.), *E. coli* M15 carrying the genes encoding the Bph dox components of *C. testosteroni* B-356 on pQE31 (Hurtubise et al. 1995, 1996), and *P. putida* G7 (harboring NAH7) (Dunn and Gunsalus 1973). Most molecular biology manipulations were done according to protocols described by Sambrook et al. (1989). Polymerase chain reactions (PCR) to amplify $nahA_b$ and $nahA_c/nahA_d$ were performed using *Pwo* DNA polymerase according to procedures outlined previously (Hurtubise et al. 1995, 1996).

Production and purification of ht-Bph $dox_{B\text{-}356}$ and ht-Nap dox_{G7} components

ht-Bph $dox_{B\text{-}356}$ components were produced and purified as described previously (Hurtubise et al. 1995, 1996). ht-Nap dox_{G7} components were produced using the Qiagen expression system, from *E. coli* M15[pREP4] as described previously for ht-Bph $dox_{B\text{-}356}$ (Hurtubise et al. 1995, 1996). The oligonucleotides used for PCR amplification of $nahA_b$ (G7-Fer_{Nap}) from NAH7 DNA, were chosen on the basis of their published nucleotide sequence (Simon et al. 1993) and were as follows: oligonucleotide I (*Bam*HI) 5′-GCGGGATCCGACAGAAAAATGGATT-3′ and oligonucleotide II (*Kpn*I) 5′-CGACGGTACCTTAAAACTCTCCGCT-3′. G7 ht-ISP_{Nap}, carrying a histidine tag on the α subunit, was expressed from a pQE31 construct carrying $nahA_c$ and $nahA_d$ placed in tandem. The following oligonucleotides were used for PCR amplification $nahA_cA_d$: $nahA_c$ oligonucleotide III (*Sac*I) 5′-GCATCGAGCTCGATGAATTACAAAAAC-3′ and $nahA_d$ oligonucleotide IV (*Hin*dIII) 5′-GAGCCAAGCTTTCACAGAAAGACCATC-3′. All constructions were such that the histidine tail was adding 13 amino acids (MRGSHHHHHHTDP) to the protein at its N-terminal portion.

Protein characterization

The protocols used for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel and for the determination of protein concentrations were as described previously (Hurtubise et al. 1995, 1996) except that the published value of ε_{460} = 4920 $M^{-1}\,cm^{-1}$ (Haigler and Gibson 1990a) and ε_{462} = 9000 $M^{-1}\,cm^{-1}$ (Ensley and Gibson 1983) were used to determine G7 ht-Fer_{Nap} and G7 ht-ISP_{Nap} concentrations respectively.

Chemicals

Biphenyl was from Aldrich Chemicals (Milwakee, Wis.). Chlorobiphenyl congeners, 2-, 3- and 4-chlorobiphenyl, 2,5- 2,2′-, 3,3′- and 4,4′-dichlorobiphenyl and 2,2′,5,5′-tetrachlorobiphenyl were obtained from ULTRA Scientific (North Kingstown, R.I.).

Bph dox and Nap dox enzyme assays

Reconstituted ht-Bph $dox_{B\text{-}356}$ was assayed as described previously (Hurtubise et al. 1996). The ht-Nap dox_{G7} assay was performed in 200 µl volume in 50 mM MES buffer, pH 6.5. The reaction mixture contained 100 nmol NADH, 1 nmol $FeSO_4$, 1 nmol G7 ht-ISP_{Nap}, 1 nmol G7 ht-Fer_{Nap} and 1 nmol strain B-356 ht-Red_{Bph}. The reaction was initiated by addition of 100 nmol substrate dissolved in 2 µl acetone.

The catalytic oxygenation of chlorobiphenyls was evaluated by monitoring substrate depletion or metabolite production by HPLC analysis as described previously (Hurtubise et al. 1996, 1998) for ht-Bph $dox_{B\text{-}356}$. The reaction rates with biphenyl as substrate were also evaluated spectrophotometrically at 434 nm in a coupled reaction in the presence of excess amounts of purified histidine-tagged 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase (Sylvestre et al. 1996a) and histidine-tagged 2,3-dihydroxybiphenyl 1,2-dioxygenase (Hein et al. 1998), using conditions described previously (Hurtubise et al. 1996). The ε_{434} value used to quantify the amount of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid produced from biphenyl was 22 000 $M^{-1}cm^{-1}$. The cytochrome *c* reductase assay used to compare the electron-transfer activity between B-356 ht-Red_{Bph} and G7 ht-Fer_{Nap} with that between B-356 ht-Red_{Bph} and B-356 ht-Fer_{Bph} was performed as described previously (Hurtubise et al. 1995).

Results

Production and purification of ht-Nap dox_{G7} components

Amplification of $nahA_b$ from NAH7 yielded a DNA fragment of approximately 850 bp. Ligation of the *Bam*HI/*Kpn*I-digested fragment into pQE31 produced plasmid pQE31::$nahA_b$. Purified preparations containing 9.5 mg protein were obtained from 7.5 g cells (wet weight) of *E. coli* harboring pQE31::$nahA_b$. The M_r of ht-Fer_{Nap} encoded from pQE31::$nahA_b$ was estimated from SDS-PAGE gel to be 15 500, which is closed to the predicted M_r of Fer_{Nap} with 13 amino acids added (Simon et al. 1993). The enzyme was estimated to be more than 85% pure by Coomassie-brillant-blue-stained SDS-PAGE (Fig. 1). The absorption spectrum of the preparation was typical of a Rieske-type iron-sulfur protein with maxima at 323 nm and 454 nm (Fig. 2A). These values are close to the one reported for purified preparations of Fer_{Nap} obtained from *Pseudomonas* sp. strain NCIB 9816 (Haigler and Gibson 1990a).

Amplification of $nahA_{cd}$ from NAH7 yielded a DNA fragment of approximately 2 kb. The ligation of the *Sac*I/*Hind*III-digested fragment into pQE31 produced plasmid pQE31::$nahA_{cd}$. Approximately 3.5 g (wet weight) of *E. coli* M15 [pQE31::$nahA_{cd}$] cell paste was required to produce 8.5 mg purified protein. These preparations showed two major bands on SDS-PAGE (Fig. 1). Their M_r values were estimated to be 53 000 and 25 000, which correspond to the deduced M_r values of ht-ISP_{Nap} large (including the histidine-tagged) and small subunits respectively (Simon et al. 1993). The purity of these preparations was estimated to be more than 90% from Coomassie-brillant-blue-stained SDS-PAGE. The absorption spectrum of the preparation was typical of a Rieske-type iron-sulfur protein with maxima

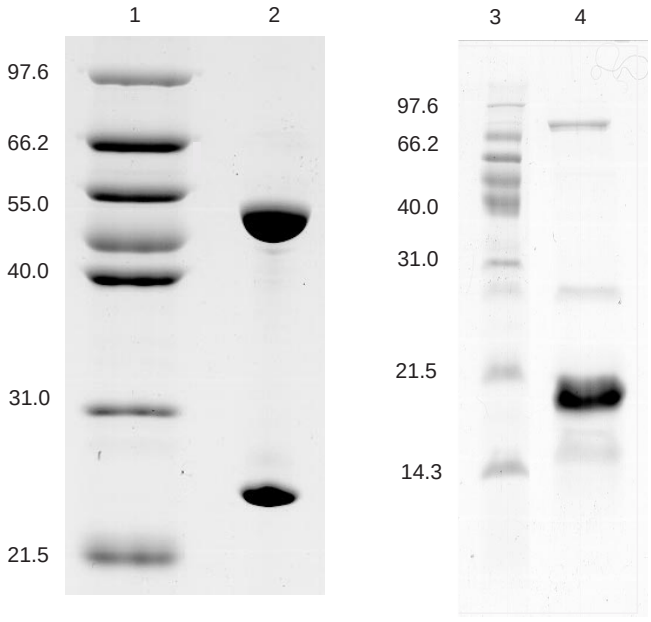

Fig. 1 Sodium dodecyl sulfate/polyacrylamide gel of *Pseudomonas putida* G7 naphthalene 1,2-dioxygenase (Nap dox_{G7}) components. *Lanes: 1* M_r markers, *2* Nickel-nitrilotriacetic-acid-purified preparation of *P. putida* G7 histidine-tagged (ht) iron-sulfur protein (ISP_{Nap}) (0.4 nmol), *3* M_r markers, *4* Nickel-nitrilotriacetic-acid-purified preparation of G7 ht-ferredoxin (Fer_{Nap}) (0.4 nmol)

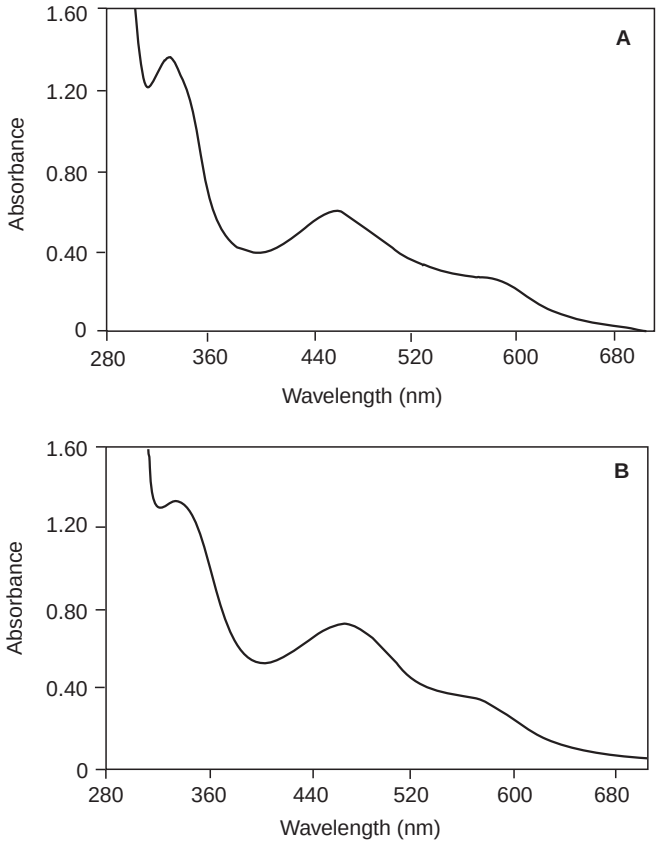

Fig. 2 **A** UV–visible light absorption spectra of oxidized G7 ht-Fer_{Nap} (140 μM); **B** UV–visible light absorption spectra of oxidized G7 ht-ISP_{Nap} (80 μM)

at 330 nm and 460 nm (Fig. 2B), which are close to the values reported for *Pseudomonas* sp. NCIB 9816 ISP_{Nap} (Ensley and Gibson 1983). As shown below, the reconstituted Nap dox, comprised of ht-ISP_{Nap}, ht-Fer_{Nap} and B-356 ht-Red_{Bph}, was active. Its optimal pH of activity was determined to be 6.5.

Functionality of B-356 ht-Red_{Bph} and B-356 ht-Fer_{Bph} in the Nap dox system

The cytochrome-*c*-reducing activities with B-356 ht-Red_{Bph} were comparable for similar concentrations of G7 ht-Fer_{Nap} and B-356 ht-Fer_{Bph}. For example, when B-356 ht-Red_{Bph} was in excess and the ferredoxin concentration was set at 0.6 nmol, the reaction rates for B-356 ht-Fer_{Bph} and G7 ht-Fer_{Nap} were respectively 0.033 μmol cytochrome *c* reduced/min and 0.035 μmol cytochrome *c* reduced/min. On the basis of these data, B-356 Red_{Bph} was used to reconstitute both Bph dox and Nap dox.

The reconstituted ht-Nap dox_{G7} comprised of B-356 ht-Red_{Bph}, G7 ht-Fer_{Nap} and G7 ht-ISP_{Nap} was fairly active when biphenyl was used as substrate (Table 1). However, the substitution of G7 ht-Fer_{Nap} by B-356 ht-Fer_{Bph} to reconstitute Nap dox_{G7} and the substitution of B-356 ht-Fer_{Bph} by G7 ht-Fer_{Nap} to reconstitute Bph $dox_{B\text{-}356}$ greatly hindered the enzyme activity (Table 1). Altogether, the data confirm that Nap dox_{G7} can catalyze the hydroxylation of biphenyl but, more importantly, these results show that although G7 Fer_{Nap} can accept an electron from B-356 Red_{Bph} quite effectively; the transfer of electrons from the ferredoxin to the terminal oxygenase component appears to be much more specific, however.

Catalytic activity of ht-Nap dox_{G7} on chlorobiphenyls

Data presented in Fig. 3 show that ht-NAH dox_{G7} catalyzes the oxygenation of all three monochlorobiphenyl congeners to generate the corresponding dihydrodiol metabolites. However, no dihydrodiol metabolites were detected by HPLC and no *meta*-cleavage metabolites

Table 1 Influence of the ferredoxin component on the naphthalene (Nap) 1,2-dioxygenase and biphenyl (Bph) dioxygenase activity. The enzyme activity is expressed in nmol 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid produced/min in the reaction medium described in Materials and methods, which contained 1 nmol each enzyme component. *ISP* iron-sulfur protein; *Fer* ferredoxin

Enzyme composition	Activity (nmol/min)
ISP_{Bph} + Fer_{Bph}	1.4
ISP_{Bph} + Fer_{Nap}	0.034
ISP_{Nap} + Fer_{Bph}	0.2
ISP_{Nap} + Fer_{Nap}	0.9

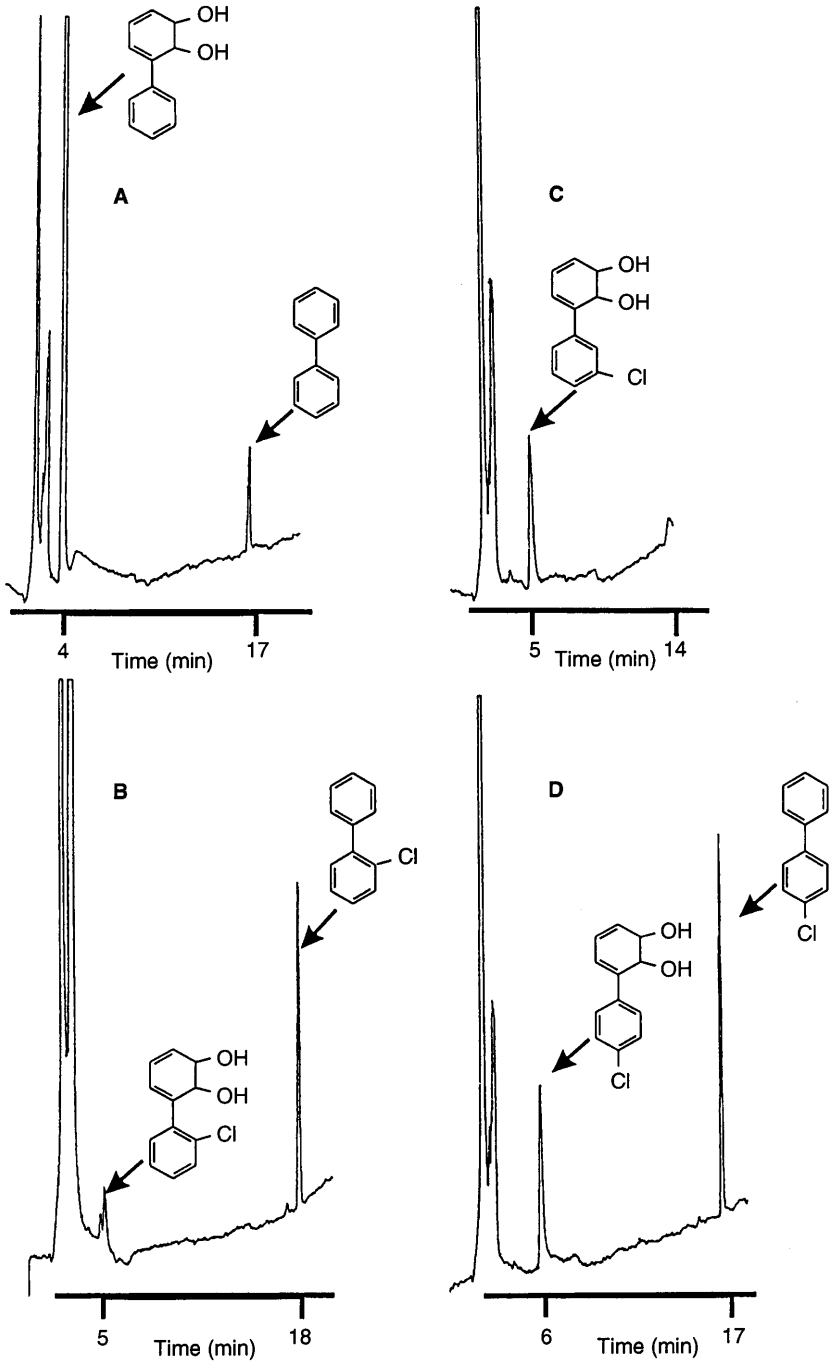

Fig. 3A–D HPLC spectra of the metabolites produced from the Nap dox_{G7} catalytic oxidation of (**A**) biphenyl, (**B**) 2-chlorobiphenyl, (**C**) 3-chlorobiphenyl and (**D**) 4-chlorobiphenyl. The conditions for enzyme assay, extraction and metabolite detection by HPLC are described in Materials and methods. Metabolites were detected at the following wavelengths: biphenyl, 304 nm; 2-chlorobiphenyl, 280 nm; 3-chlorobiphenyl, 307 nm; 4-chlorobiphenyl 306 nm

were detected in the coupled reaction with histidine-tagged 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase plus histidine-tagged 2,3-dihydroxybiphenyl 1,2-dioxygenase when 2,5-, 2,2′-, 3,3′-, 4,4′-dichlorobiphenyls were used as substrate (not shown). Furthermore, although ht-Nap dox_{G7} was shown able to catalyze both the *ortho*, *meta* and the *meta*, *para* dihydroxylation of biphenyl (Barriault and Sylvestre 1999), 2,2′,5,5′-tetrachlorobiphenyl, which requires a *meta*, *para* attack was not transformed by the enzyme. Thus, it appears that the presence of chlorine atoms on the molecule strongly hindered the capacity of Nap dox, more than that of Bph dox to catalyze the hydroxylation of the molecule.

Discussion

On the basis of the genetic analysis (Sylvestre et al. 1996b; Werlen et al. 1996), Nap dox and Bph dox are related enzymes that have differentiated during evolution as their specificity has changed to enhance their activity toward specific substrates. Although their component arrangement remains similar, the structure of

each of their components has been modified quite extensively to achieve this specialization. In spite of the numerous structural differences between the two enzymes, and especially the quite distinct structural features of their reductase components, our data show that B-356 Red_{Bph} can effectively substitute G7 Red_{Nap} in the electron-transfer activity from NADH to Fer_{Nap}. These results are interesting for two reasons. First they clearly confirm previous observations suggesting that the dioxygenase's reductase component is not very specific to the dioxygenase system to which it belongs (Subramanian et al. 1981; Haigler and Gibson 1990a; Hurtubise et al. 1995). Second, and more importantly, they suggest that if any in vitro mutation program is to be initiated to develop dioxygenases with enhanced activity toward persistent pollutants, the gene coding for the reductase component need not be included in the mutagenesis process. However, the ferredoxin component is more specific. Although the replacement of B-356 Fer_{Bph} by that of *Burkholderia cepacia* LB400 in the B-356 Bph dox system did not affect the enzyme's kinetic parameters (Hurtubise et al. 1998), the substitution of Fer_{Nap} by Fer_{Bph} in the Nap dox system did affect the enzyme's activity. Thus mutations that occur in the ISP component, to modify significantly the enzyme specificity, are likely to bring changes that can affect the electron transfer between the ferredoxin and the oxygenase component. Therefore, achievement of optimal enzyme activity might require concomitant mutations in the ferredoxin component when the oxygenase component is submitted to a mutation program to obtain major changes of its substrate specificity pattern.

Furthermore, for the first time, we are showing that Nap dox can catalyze the oxygenation of monochlorobiphenyl congeners. Crameri et al. (1998) have recently shown that the use of homologous genes to provide functional diversity accelerates the in vitro directed evolution process based on DNA shuffling (Stemmer 1994). Nap dox is able to attack biphenyl and some chlorobiphenyls, and it can catalyze the *meta*, *para* hydroxylation of biphenyl (Barriault and Sylvestre 1999). Therefore, the encoding genes might have a place in the DNA shuffling process, for the development of enhanced dioxygenase systems to degrade persistent chlorobiphenyl congeners. Those observations are being considered in an on-going Bph dox DNA shuffling program.

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