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REVIEW PAPER

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The role of active-site residues in naphthalene dioxygenase

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Abstract The three-component naphthalene dioxygenase enzyme system catalyzes the first step in the degradation of naphthalene by *Pseudomonas* sp. strain NCIB 9816-4. A member of a large family of bacterial Rieske non-heme iron oxygenases, naphthalene dioxygenase is known to oxidize over 60 different aromatic compounds, and many of the products are enantiomerically pure. The crystal structure of the oxygenase component revealed the enzyme to be an $\alpha_3\beta_3$ hexamer and identified the amino acids located near the active site. Site-directed mutagenesis studies have identified the residues involved in electron transfer and those responsible for controlling the regioselectivity and enantioselectivity of the enzyme. The results of these studies suggest that naphthalene dioxygenase can be engineered to catalyze a new and extended range of useful reactions.

Keywords Rieske non-heme iron oxygenases · Aromatic hydrocarbons · Biodegradation · Dioxygenase · Naphthalene

Introduction

Naphthalene dioxygenase (NDO) is a member of a large family of bacterial Rieske non-heme iron oxygenases that initiate the degradation of a wide range of aromatic compounds in aerobic environments. These multicomponent enzyme systems are capable of oxidizing aromatic hydrocarbons, aromatic acids, chlorinated aromatic compounds, as well as nitroaromatic and amino aromatic compounds. Many of these chemicals are naturally occurring components of crude oil; others are components of plant material, while others, such as nitroarenes and polychlorinated biphenyls, are synthetic chemicals that have been introduced into the environment relatively recently by human activities. NDO catalyzes the first step in the aerobic degradation of naphthalene (Fig. 1) [6] and is the most well-characterized of the naphthalene family of enzymes [12]. The pathway for naphthalene degradation in *Pseudomonas* sp. strain NCIB 9816-4 [20] is plasmid-encoded [35] and the genes encoding NDO have been cloned and sequenced [9, 27, 36]. The three components of the NDO system from NCIB 9816-4 have been purified and characterized [8]. An iron–sulfur center-containing flavoprotein reductase [14] and a Rieske [2Fe–2S] ferredoxin [13] transfer electrons from NAD(P)H to the oxygenase component [7] (Fig. 2). The oxygenase component contains a Rieske iron–sulfur center and mononuclear iron at the active site. Both redox centers are located in the α-subunit of the oxygenase. The reaction requires molecular oxygen, and both atoms of O_2 are added to the aromatic ring in a stereospecific fashion to form enantiomerically pure (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-dihydrodiol) ([20, 21]; Fig. 2).

The study of Rieske non-heme iron oxygenases is important for two reasons. First, these enzyme systems catalyze essential reactions in bacterial pathways for the degradation of many aromatic compounds that are considered to be serious environmental pollutants. Naphthalene, for example, has been defined as a priority pollutant by the US Environmental Protection Agency. Therefore, bacterial strains carrying these pathways may be useful for the development of bioremediation technologies for the clean-up of contaminated environments. In addition, enzymes such as NDO have two useful features: many of them have broad substrate ranges (NDO, for example, is known to oxidize over 60 different aromatic substrates [34]), and many of the products are enantiomerically pure compounds that are difficult to generate using standard chemical syntheses. With chiral *cis*-dihydrodiols as starting materials, chemists have been able to synthesize a wide range of

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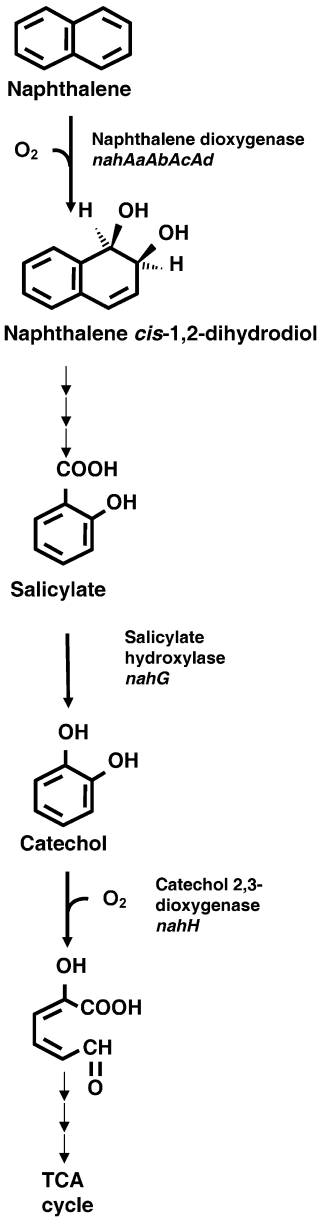

Fig. 1 Pathway for the degradation of naphthalene in *Pseudomonas* sp. NCIB 9816-4. Key intermediates, enzymes, and the genes encoding these enzymatic steps are indicated

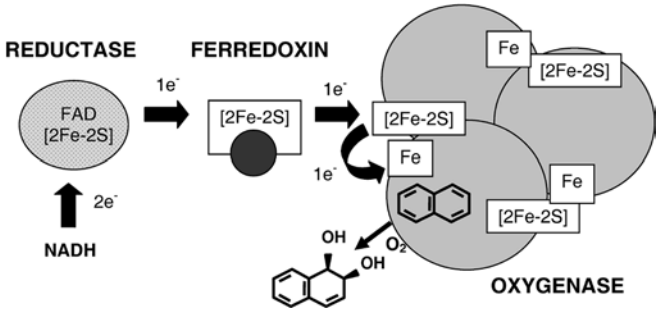

Fig. 2 Reaction catalyzed by the multicomponent naphthalene dioxygenase system

natural products that include sugars, alkaloids, and pharmaceuticals (reviewed in [3, 4, 18]). These types of chemoenzymatic syntheses are likely to become very useful, especially with the current push toward the development of single-enantiomer drugs [37, 38], and the recent interest in "green chemistry" [1]. A particularly interesting feature of NDO is its ability to catalyze different types of reactions besides *cis*-dihydroxylation. In addition to dioxygenation, NDO has been shown to catalyze monooxygenation, desaturation, sulfoxidation, and dealkylation reactions (reviewed in [34]).

Role of the oxygenase α-subunit

The first crystal structure of a Rieske non-heme iron oxygenase, that of NDO, was solved in 1998 [25]. The oxygenase is an $\alpha_3\beta_3$ hexamer. Each α-subunit consists of two domains, a Rieske domain and a catalytic domain. The Rieske domain contains a Rieske [2Fe–2S] center coordinated by Cys-81, Cys-101, His-83, and His-104 (Fig. 3A). Each α-subunit also contains one atom of mononuclear ferrous iron at the active site, which is coordinated by His-208, His-213, Asp-362 and a water molecule (Fig. 3B). Substitution of an alanine at position 362 in NDO completely eliminated enzyme activity [30]. Site-directed mutagenesis of the corresponding histidines in toluene dioxygenase also resulted in inactive enzymes [23]. To date, these seven ligand-binding residues are conserved in all Rieske non-heme iron oxygenases whose sequences have been determined. The 2-His-1-carboxylate motif (Fig. 3B) for the coordination of iron has recently emerged in a large number of unrelated non-heme iron-containing enzymes that catalyze a variety of reactions [16, 33].

Subunit switching experiments with NDO and the closely related enzymes 2-nitrotoluene dioxygenase and 2,4-dinitrotoluene dioxygenase have shown that, in these enzymes, the α-subunit controls substrate specificity, determining the range of substrates oxidized, the position of oxygen attack, and the stereochemistry of the products [28, 29]. In addition, the C-terminal portions of 2-nitrotoluene and 2,4-dinitrotoluene dioxygenases were shown to carry the only determinants of substrate specificity [28]. Consistent with these results, the structure of NDO revealed the presence of 17 C-terminal amino acids lining the active-site pocket [5]. The majority of these amino acids are hydrophobic, providing an appropriate environment for the binding of aromatic substrates (Fig. 4).

Role of the oxygenase β-subunit

The role of the β-subunit is not yet clear; its main function appears to be structural [25]. The β-subunits contain no prosthetic groups. In the related enzyme toluene dioxygenase, the β-subunit was shown to be essential for activity. Purified α-subunit had no activity on its own,

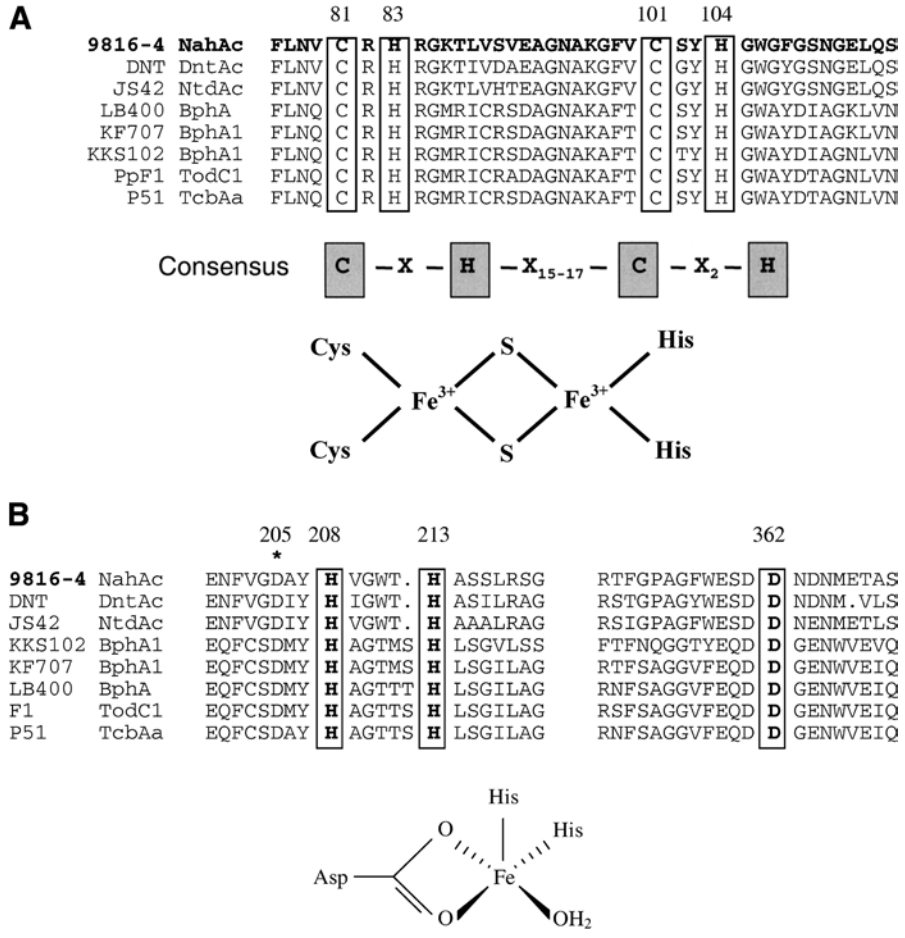

Fig. 3A, B Iron binding motifs in the α-subunits of Rieske non-heme iron oxygenases. **A** Rieske center binding-site alignment and consensus. The Rieske iron–sulfur center is coordinated by conserved cysteines and histidines. **B** 2-His-1-carboxylate binding of mononuclear at the active site. The mononuclear iron is coordinated by His-208, His-213, Asp-362, and a water molecule in a 2-His-1-carboxylate facial triad. Asp-205 (*star*) is involved in electron transfer (see text). The alignments of α-subunit sequences from the following enzyme systems are shown: *9816-4 NahAc* Naphthalene dioxygenase (NDO) from *Pseudomonas* sp. NCIB 9816-4 [27], *DNT DntAc* 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp. DNT [39], *JS42 NtdAc* 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42 [27], *LB400 BphA* biphenyl dioxygenase from *Burkholderia* sp. LB400 [10], *KF707 BphA1* biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 [40], *KKS102 BphA1* biphenyl dioxygenase from *Pseudomonas* sp. KKS102 [11], *PpF1 TodC1* toluene dioxygenase from *Pseudomonas putida* F1 [44], *P51 TcbAa* trichlorobenzene dioxygenase from *Pseudomonas* sp. P51 [42]. NDO α-subunit numbering is shown

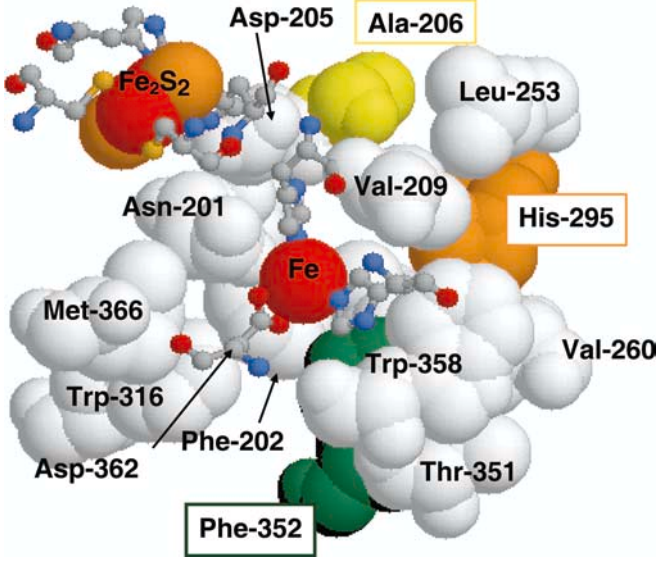

Fig. 4 Active site of NDO showing the junction between the two α-subunits. Residues coordinating the Rieske center and active-site iron are shown in ball and stick configuration (only Asp-362 is labelled). Other residues lining the active-site pocket are shown in space-fill format

but activity was restored after reconstitution of α- and β-subunits [22]. Subunit swapping studies with NDO, 2-nitrotoluene dioxygenase, and 2,4-dinitrotoluene dioxygenase indicated that the β-subunit plays no role in determining the substrate specificity of these enzymes [28, 29]. Consistent with these results, the crystal structure of NDO revealed that there are no β-subunit residues near the active site of NDO [25]. In contrast, there is evidence for β-subunit participation in substrate specificity determination in the more distantly related enzymes toluate dioxygenase, toluene dioxygenase, and biphenyl dioxygenases [15, 17, 19]. However, results indicating that β-subunits do not control specificity in benzene, biphenyl, and tetrachlorobenzene dioxygenases have also been

published [2, 41]. At this time, it is unclear whether the β-subunits play different roles in the various subfamilies of Rieske non-heme iron oxygenases.

Electron transfer within the oxygenase

The relative positions of the redox centers in the $\alpha_3\beta_3$ hexamer suggested that each of the three active sites resides at the junction between adjacent α-subunits. Within a single α-subunit, the distance between the Rieske center and the mononuclear iron is approximately 43Å, but the Rieske center and the mononuclear iron in adjacent α-subunits are only 5Å apart. Site-directed mutagenesis studies in which the conserved Asp-205 residue located at the α–α junction (Fig. 3B, Fig. 4) was replaced with other amino acids (Ala, Glu, Gln, Asn) resulted in the elimination of enzyme activity. These studies suggest that Asp-205 is either directly involved in electron transfer from the Rieske center to iron at the active site, or is essential to position the two α-subunits in a precise conformation to allow for efficient electron transfer [31]. The Rieske center in the purified mutant protein (NDO-D205Q) was reduced in the presence of NADH and catalytic amounts of ferredoxin and reductase, indicating that electron flow from NADH to the Rieske center of the mutant oxygenase was unimpaired. However, benzene, an effective uncoupler of wild-type NDO [26], did not stimulate oxygen uptake by the mutant form of the enzyme, which is consistent with the idea that electrons are not transferred from the Rieske center to iron at the active site in the mutant proteins [31].

Table 1 Enantiomeric composition of naphthalene *cis* 1,2-dihydrodiol produced by wild-type and mutant NDO enzymes[a]. *n.d.* Not determined, although trace amounts of naphthalene *cis*-dihydrodiol were detected, – no product detected

NDO mutation(s)	Enantiomeric composition of: naphthalene *cis*-1,2-dihydrodiol
Wild-type NDO	>99% (+)-(1*R*,2*S*)-
A206I	>99% (+)-(1*R*,2*S*)-
L253T	>99% (+)-(1*R*,2*S*)-
V260N	>99% (+)-(1*R*,2*S*)-
H295I	>99% (+)-(1*R*,2*S*)-
F352G	98% (+)-(1*R*,2*S*)-
F352A	96% (+)-(1*R*,2*S*)-
F352T	93% (+)-(1*R*,2*S*)-
F352V	92% (+)-(1*R*,2*S*)-
F352I	94% (+)-(1*R*,2*S*)-
F352L	96% (+)-(1*R*,2*S*)-
A206I H295I	>99% (+)-(1*R*,2*S*)-
V260N F352I	94% (+)-(1*R*,2*S*)-
A206I L253T	>99% (+)-(1*R*,2*S*)-
A206I F352I	70% (+)-(1*R*,2*S*)-
A206I F352I L253T	70% (+)-(1*R*,2*S*)-
A206I H295I V260N	>99% (+)-(1*R*,2*S*)-
A206I H295I F352I	63% (+)-(1*R*,2*S*)-
A206I H295I V260N F352I	n.d.
A206I H295I V260N F352I L253T	–

[a] In addition, single amino acid substitutions at positions 201, 202, 316, 351, 358, and 366 had no effect on the enantioselectivity with naphthalene. Data are from [30, 32, 43].

Active-site amino acids controlling substrate specificity

The crystal structure of NDO identified the amino acids located near the active site (Fig. 4). In addition, structures containing bound substrates (indole, naphthalene) and product (naphthalene *cis*-dihydrodiol) at the active site have been determined [5, 24]. Oxygen bound at the active site is positioned for a side-on attack on the substrate, which is consistent with the conserved *cis*-stereochemistry of dihydroxylation products [24]. Site-directed mutagenesis of 12 residues positioned near the active-site iron has allowed identification of amino acids critical for determining the substrate specificity and enantioselectivity of NDO. As mentioned earlier, substitution of an alanine for the iron-binding aspartate at position 362 resulted in a completely inactive enzyme,

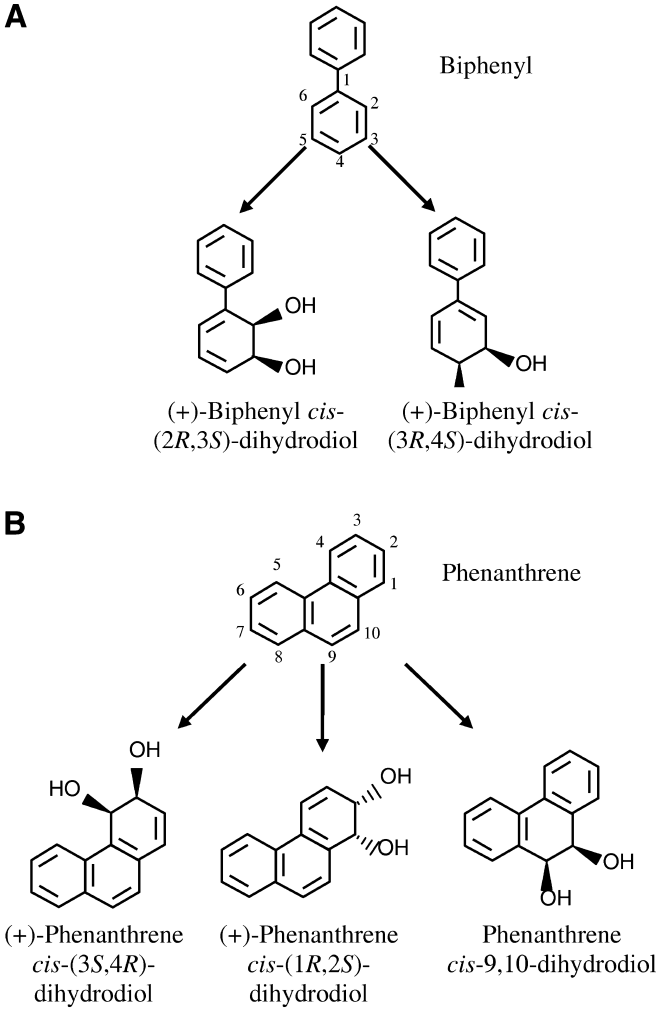

Fig. 5 Products formed by NDO from **A** biphenyl and **B** phenanthrene. Phenanthrene *cis*-9,10-dihydrodiol is not produced by wild-type NDO

Table 2 Products formed from biphenyl by wild-type and mutant NDO enzymes. – No product detected. Data are from [30, 32, 43]

NDO mutation(s)	Regioisomer formed (%)	
	Biphenyl *cis*-2, 3-dihydrodiol	Biphenyl *cis*-3, 4-dihydrodiol
Wild-type NDO	87	13
N201A	trace	–
N201Q	98	2
N201S	81	19
F202V	81	19
F202L	–	–
A206I	95	5
L253T	62	38
V260A	94	6
V260L	80	20
V260N	93	7
H295I	87	13
W316A	96	4
T351R	83	17
T351S	85	15
T351N	90	10
F352G	31	69
F352A	23	77
F352T	8	92
F352V	4	96
F352L	15	85
F352I	17	83
F352Y	–	–
F352W	–	–
W358A	Trace	–
D362A	–	–
M366W	85	15
A206I H295I	92	8
V260N F352I	trace	–
A206I L253T	82	18
A206I F252I	79	21
A206I F352I L253T	58	42
A206I H295I V260N	>99	–
A206I H295I F352I	78	22
A206I H295I V260N F352I	trace	–
A206I H295I V260N F352I L253T	–	–

presumably because the iron at the active site was lost [30]. Other single amino acid substitutions that resulted in inactive enzymes were also obtained. These included the substitutions F202L, F352Y, and F352W [30, 32]. In general, however, the enzyme tolerated a wide range of substitutions near the active site. All active enzymes produced naphthalene *cis*-1,2-dihydrodiol from naphthalene, but in some cases a change in the stereochemistry of the product was seen (Table 1). In particular, all active enzymes with substitutions at position 352 formed significant amounts of the (−)-enantiomer of naphthalene *cis*-dihydrodiol (Table 1). Neither the wild type nor any other single or multiple mutants formed detectable amounts of (−)-naphthalene *cis*-dihydrodiol. However, in the presence of a F352I substitution, enzymes with additional amino acid substitutions formed up to 37% (−)-naphthalene *cis*-dihydrodiol (Table 1). Similarly, the NDO-F352V enzyme demonstrated the same regioselectivity as the wild type with anthracene as a substrate, but unlike the wild type, formed a small amount of the (−)-enantiomer of anthracene-*cis*-1,2-dihydrodiol [32].

Table 3 Enantiomeric composition of products formed from biphenyl by wild-type and mutant NDO enzymes. *n.d.* Not determined. Data are from [32]

NDO mutation	Enantiomeric composition of:	
	Biphenyl *cis*-2, 3-dihydrodiol	Biphenyl *cis*-3, 4-dihydrodiol
Wild-type NDO	>95% (+)-(2*R*,3*S*)-	>98% (+)-(3*R*,4*S*)
F352G	>95% (+)-(2*R*,3*S*)-	60% (+)-(3*R*,4*S*)
F352A	>95% (+)-(2*R*,3*S*)-	65% (+)-(3*R*,4*S*)
F352T	>95% (+)-(2*R*,3*S*)-	60% (−)-(3*S*,4*R*)
F352 V	n.d.	77% (−)-(3*S*,4*R*)
F352I	>95% (+)-(2*R*,3*S*)-	53% (+)-(3*R*,4*S*)
F352L	>95% (+)-(2*R*,3*S*)-	70% (+)-(3*R*,4*S*)

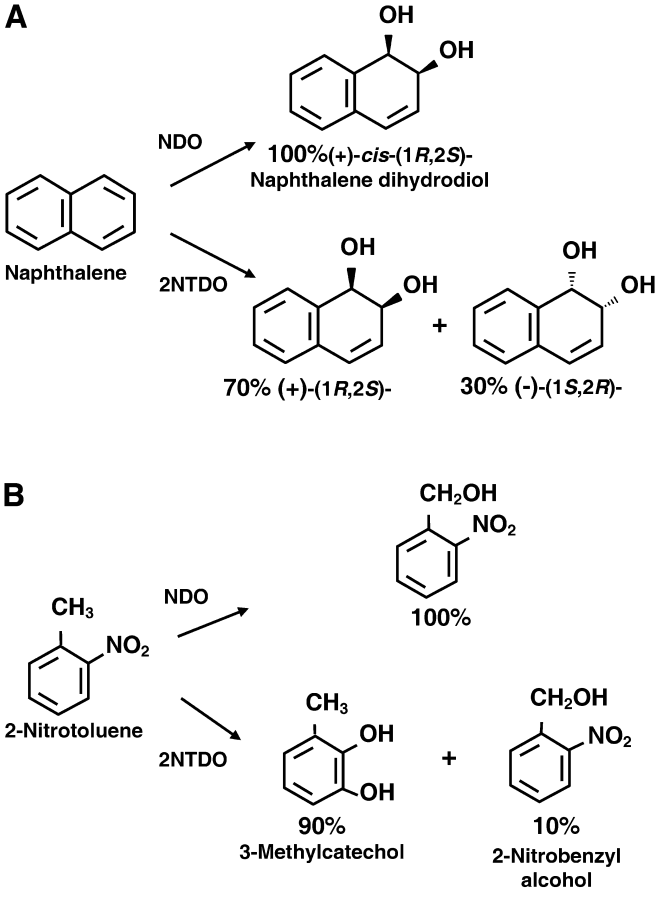

Fig. 6A, B Differences in substrate specificities of NDO and 2-nitrotoluene dioxygenase (2NTDO). **A** Products formed from naphthalene. **B** Products formed from 2-nitrotoluene

With biphenyl and phenanthrene as substrates (Fig. 5), changes in both the regio- and enantioselectivity were seen [30, 32]. Little or no change in the ratio of biphenyl *cis*-2,3- and biphenyl-*cis*-3,4-dihydrodiol formed from biphenyl was observed with most enzymes that contained a single amino acid substitution (Table 2). By far, the most dramatic effects were seen in enzymes with substitutions at Phe-352. Replacement of Phe-352 with smaller amino acids (Gly, Ala, Val, Ile, Leu, Thr)

Table 4 Products formed from phenanthrene by wild-type and mutant NDO enzymes. – No product detected. Data are from [30, 32, 43]

NDO mutation(s)	Regioisomer formed (%)		
	Phenanthrene *cis*-3, 4-dihydrodiol	Phenanthrene *cis*-1, 2-dihydrodiol	Phenanthrene *cis*-9, 10-dihydrodiol
Wild-type NDO	90	10	–
N201A	78	22	–
N201Q	85	15	–
N201S	76	24	–
F202V	80	20	–
F202L	–	–	–
A206I	57	43	–
L253T	> 99	–	–
V260A	99	1	–
V260L	90	9	< 1
V260N	94	6	–
H295I	83	17	–
W316A	80	20	–
T351R	69	31	–
T351S	88	12	–
T351N	82	18	–
F352G	79	21	–
F352A	53	47	–
F352T	59	41	–
F352V	17	83	–
F352L	64	31	5
F352I	76	24	–
F352Y	–	–	–
F352W	–	–	–
W358A	98	2	–
D362A	–	–	–
M366W	71	29	Trace
A206I H295I	50	50	–
V260N F352I	76	24	–
A206I L253T	14	86	–
A206I F252I	45	23	32
A206I F352I L253T	10	84	6
A206I H295I V260N	–	–	–
A206I H295I F352I	25	34	41
A206I H295I V260N F352I	–	–	–
A206I H295I V260N F352I L253T	–	–	–

resulted in enzymes that produced significantly more biphenyl *cis*-3,4-dihydrodiol (Table 2). In addition, the stereochemistry of the biphenyl *cis*-3,4-dihydrodiol was altered (Table 3). The NDO-F352T and NDO-F352V enzymes formed predominantly (−)-biphenyl *cis*-(3*S*,4*R*)-dihydrodiol, a novel product not made by wild-type NDO. With phenanthrene as a substrate, a major shift in the regioselectivity was also seen with enzymes carrying substitutions at Phe-352 (Table 4). In addition, enzymes with substitutions at position 206 (NDO-A206I and NDO-A206I/L253T) formed significantly more phenanthrene *cis*-1,2-dihydrodiol than wild type. Several of the enzymes formed a new product from phenanthrene, phenanthrene *cis*-9,10-dihydrodiol (Table 4). The F352V substitution also affected the stereochemistry of product formation, and resulted in the production of (−)-phenanthrene *cis*-(1*S*,2*R*)-dihydrodiol with an enantiomeric purity of 91% [32]. In contrast, the wild-type enzyme forms enantiomerically pure (+)-phenanthrene *cis*-(1*R*,2*S*)-dihydrodiol.

An attempt to reconstruct the activity of 2-nitrotoluene dioxygenase in NDO by making the appropriate active-site amino acid substitutions was only partially successful. The α-subunits of the two enzymes are 84% identical, with only five amino acid differences at the active site. Their substrate specificities, however, differ significantly [28] (Fig. 6). Only two amino acid substitutions (F352I, A206I) were required to change the stereochemistry of the naphthalene dihydrodiol formed from naphthalene from that of wild-type NDO (> 99% (+)-(1*R*,2*S*)) to that of wild-type 2-nitrotoluene dioxygenase (70% (+)-(1*R*,2*S*)). However, attempts to generate a variant of NDO that oxidized the aromatic ring of 2-nitrotoluene by site-directed mutagenesis were unsuccessful. In fact, enzymes with four or more amino acid substitutions had negligible activity with all substrates [43]. In general, the mutant enzymes had lower overall rates of product formation with all substrates [30, 32, 43].

Conclusions

It is clear from these studies that a variety of amino acid substitutions are tolerated near the active site of NDO. However, very minor changes in the enzyme resulted in

significant differences in substrate specificity. Various mutant forms of the enzyme had altered regio- and enantioselectivities. Biotransformations with four NDO substrates identified five products not generated by wild-type NDO [(−)-naphthalene *cis*-(1*S*,2*R*)-dihydrodiol, (−)-anthracene-*cis*-(1*S*,2*R*)-dihydrodiol, (−)-biphenyl *cis*-(3*S*,4*R*)-dihydrodiol, (−)-phenanthrene *cis*-(1*S*,2*R*)-dihydrodiol, and phenanthrene *cis*-9,10-dihydrodiol]. Future studies exploiting the crystal structure data and the available active-site information, together with directed evolution should result in engineered NDO enzymes that produce new and useful products.

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