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Purification and Characterization of a Novel Naphthalene Dioxygenase from *Rhodococcus* sp. Strain NCIMB12038

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We report here the characterization of the catalytic component (ISP_{NAR}) of a new naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038. The genes encoding the two subunits of ISP_{NAR} are not homologous to their previously characterized counterparts in *Pseudomonas*. The deduced amino acid sequences have only 33 and 29% identity with the corresponding subunits in *Pseudomonas putida* NCIB 9816-4, for which the tertiary structure has been reported.

Members of the genus *Rhodococcus* are found in many environmental niches and have a remarkable ability to metabolize a wide variety of xenobiotic compounds (6, 25). Naphthalene is released into the environment as coal tar and coal tar products such as creosote (17), and bacteria which degrade naphthalene are widely distributed in nature (3). The first step in the catabolism of naphthalene by bacteria has been elucidated for *Pseudomonas* spp. and involves the oxidation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (naphthalene *cis*-dihydrodiol), which is catalyzed by naphthalene 1,2-dioxygenase (NDO) (EC 1.14.12.12) (11). To date only one NDO has been well characterized, namely, that of *Pseudomonas putida* NCIB 9816-4. In this sense, therefore, NDO is a paradigm for further studies of this important group of enzymes.

The *Pseudomonas* NDO is a member of a family of over 40 multicomponent, non-heme iron oxygenases (2). Initially, an iron-sulfur flavoprotein transfers electrons from NAD(P)H to a putative Rieske [2Fe-2S] iron sulfur center in a ferredoxin protein. The electrons are then transferred to the catalytic component of the enzyme to facilitate the addition of dioxygen to the substrate, naphthalene. The catalytic component of the enzyme, ISP_{NAP}, consists of two nonidentical subunits (α and β) needed for the reaction. Recently, the three-dimensional structure has been elucidated by X-ray crystallography and shown to be a 3α and 3β structure (12). The role of the β subunit has not been determined; however, the α subunit is directly involved in catalysis. Electrons are passed to a Rieske [2Fe-2S] center of one α subunit and then to a mononuclear iron at the active site in an adjacent α subunit. The apparent presence of a Rieske [2Fe-2S] center and a mononuclear iron moiety at the active site is typical of such dioxygenases (2). The catabolism of naphthalene by *Rhodococcus* strain B4 to the metabolites salicylic acid and gentisic acid has been reported (7). Many putative NDO sequences are available in gene data banks; however, most are not based on biochemical confirmation of their induced expression and function. Also, they are all very closely homologous at the DNA level to either the *Pseudomonas* NDO (23) or the 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT (24).

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We have previously reported that *Rhodococcus* strain NCIMB12038 degrades 1-naphthol to salicylic acid and gentisic acid (15). This strain has been shown also to catabolize naphthalene and to express NDO enzyme activity in cell extracts, with naphthalene *cis*-dihydrodiol confirmed as the product (1). We report here the purification and characteristics of a new naphthalene-induced NDO ISP_{NAR} from *Rhodococcus* sp. strain NCIMB12038 and the complete nucleotide sequence of the genes encoding the α and β subunits.

Characterization of the *Rhodococcus* NDO. The conditions used to grow *Rhodococcus* sp. strain NCIMB12038 were as described by Allen et al. (1). The NDO assay used was based on that of Ensley et al. (5), where [^{14}C]naphthalene is converted to a relatively nonvolatile product, *cis*-1,2-dihydroxy-1,2-dihydro[^{14}C]naphthalene (naphthalene *cis*-dihydrodiol) (1). An optimum NADH concentration of 2 mM was reported for NDO activity in *P. putida* NCIB 9816-4 (5). However, our results suggest that with the *Rhodococcus* sp. enzyme, the optimum concentration is much higher; we assayed NDO in the presence of 5.5 mM NADH.

The effect of other coenzymes on NDO activity was determined, and NADPH was found to be usable as an alternative electron donor to NADH. When flavin adenine dinucleotide (FAD) or flavin mononucleotide was added, enzyme activity also increased. A similar effect was observed with *P. putida* NCIB 9816-4 (5), and the data suggest that a flavoprotein is also required for NDO activity in *Rhodococcus* sp. Subsequently, 5 μM FAD was added routinely to NDO assays.

For purification of the protein required for NDO activity, which was subsequently designated ISP_{NAR}, naphthalene-grown cells were resuspended in 50 mM TEG buffer (50 mM Tris-HCl [pH 7.8] containing 10% [vol/vol] ethanol, 10% [vol/vol] glycerol, and 0.5 mM dithiothreitol) (4), disrupted with a French pressure cell, centrifuged (40,000 × *g* for 1 h), and applied to a Blue Sepharose (heparin-Sepharose CL-6B and Pharmacia FPLC system) affinity column eluted with TEG buffer containing 1 mM NAD (8). The nonbinding NDO active fraction was concentrated (100-kDa ultrafiltration membrane; Vivascience), applied to an anion-exchange column (Pharmacia Q-Sepharose LKB), and eluted with a continuous potassium chloride salt gradient (0 to 1 M). Two fractions were needed to restore enzyme activity: fraction A (yellow) eluted between 0.41 and 0.43 M KCl, and fraction B (brown) eluted between 0.44 and 0.46 M KCl. Both fractions were concentrated by using a 5-kDa ultrafiltration membrane (Viva-

TABLE 1. Purification of the ISP_{NAR} protein from *Rhodococcus* sp. strain NCIMB12038

Purification stage[a]	Protein (mg)	Activity (U)[b]	Sp act ($U \cdot mg^{-1}$)	Recovery (%)
(i) Cell extract	198	144.7	0.731	100
(ii) Blue Sepharose column	137	113.8	0.831	79
(iii) Q-Sepharose column	23	119.6	5.225[c]	82
(iv) Superose 12 column	13	77.4	5.971	53

[a] See text for details.
[b] One unit represents production of 1.0 nmol of *cis*-dihydrodiol $\cdot$ min^{-1}.
[c] Fractions A and B were both necessary for enzyme activity. When either fraction was assayed alone, coenzyme-dependent specific activity was reduced to approximately 1 and 14%, respectively, of the combined activity. In enzyme assays used for stages iii and iv, 0.3 mg of fraction A and 0.1 mg of fraction B or ISP_{NAR} were combined in 200 μl of assay mixture.

science). Fraction B was further purified by gel filtration chromatography (Pharmacia Superose 12 HR) and contained a protein that had NDO activity and which could be reconstituted in the presence of fraction A. The data show that 8.2-fold purification of the protein was achieved with 53% recovery of enzyme activity (Table 1). Surprisingly, NDO activity was present in the first fraction eluted from the Blue Sepharose column without added NAD^+. This observation was unexpected, as it was shown previously that one of the components of the NDO complex from *P. putida* NCIB 9816-4 bound to this type of affinity column (8).

Although the fold purification may seem low, the results also indicate that the recovered ISP_{NAR} protein constituted 6.6% of the total protein in the cell extract. This should be compared with *P. putida* NCIB 9816-4, where the analogous ISP_{NAP} protein constituted only 0.9% of the total cell-free protein (4). However, too much emphasis should not be placed on the importance of specific activity measurements in this case. The specific activity of multicomponent enzymes such as NDO is dependent on protein concentration and the relative amounts of the different enzyme components in the assay mixture (i.e., that of the purified ISP_{NAR} protein and the components present in fraction A, as indicated in Table 1). This was confirmed when specific activity was measured while varying the amount of fraction A in the assay but with a constant amount of purified ISP_{NAR} protein (data not shown). These factors explain why there is an apparent increase in the recovery of enzyme activity between steps ii and iii of the purification procedure (Table 1): the components of the dioxygenase enzyme complex were separated at this point.

Polyacrylamide gel electrophoresis (PAGE) of the active ISP_{NAR} protein under nondenaturing conditions (10% [wt/vol] acrylamide gel) revealed the presence of a single band that stained for protein (not shown). PAGE (sodium dodecyl sulfate [SDS] and native gels) was performed by published procedures (14). The molecular mass of the ISP_{NAR} protein was estimated to be 155 kDa from the retention time of the protein on the Superose 12 column used in the purification protocol. Analysis of the purified protein by SDS-PAGE revealed that it was comprised of two subunits, of 55 and 23 kDa, as indicated in Fig. 1. The larger protein is here designated the α subunit, and the smaller is designated the β subunit. The data suggest that in solution, the ISP_{NAR} complex has an $\alpha_2\beta_2$ configuration. Although in similar experiments the *P. putida* NCIB 9861-4 ISP_{NAP} α and β proteins appeared to form a dimeric $\alpha_2\beta_2$ structure (4), the three-dimensional structure indicated a more plausible $\alpha_3\beta_3$ configuration (12). Hence, the $\alpha_3\beta_3$ subunit configuration should also be considered for the ISP_{NAR} enzyme.

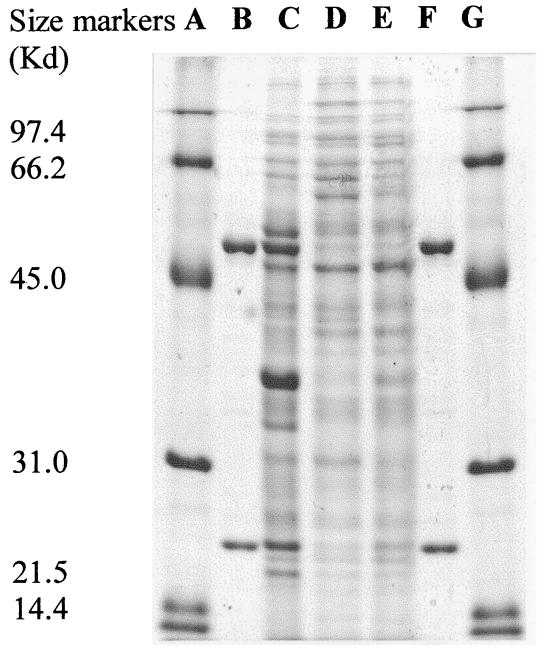

FIG. 1. Expression of NDO ISP_{NAR} α and β subunits in protein extracts from *Rhodococcus* sp. strain NCIMB12038 grown on naphthalene (C), salicylate (D), and pyruvate (E). Purified ISP_{NAR} α and β subunits are also shown (B and F). Size markers are low-molecular-weight standards (A and G) (Bio-Rad).

The N-terminal sequences of the ISP_{NAR} α and β subunits (sequence data were obtained by Brian Dunbar at the Protein Sequencing Facility, University of Aberdeen, Aberdeen, United Kingdom) were found to be Met Leu Ser Asn Glu Leu Arg Gln Thr Leu Gln Lys Gly Leu His Asp Val Asn Ser Asp Trp Thr Val Pro Ala and Met Asn Thr Gln X Val Ser Asp Thr Thr Val Arg Glu Ile Thr Glu Trp Leu Tyr Met Glu Ala Glu Leu, respectively. These sequences facilitated analysis of the genes encoding the ISP_{NAR} subunits (see below).

In a previous report (1), it was noted that NDO activity is expressed by strain NCIMB12038 during growth on naphthalene as the sole carbon source but not when grown on salicylate or pyruvate. We analyzed cell extracts of NCIMB12038 when it was grown on these substrates as the sole carbon source and by SDS-PAGE compared the proteins expressed under the various conditions with the purified ISP_{NAR} α and β subunits (Fig. 1). The results show clearly that proteins of the same molecular weight as the purified components are expressed in naphthalene-grown cells only. This evidence strongly supports the proposal that the purified enzyme α and β subunits are directly associated with naphthalene metabolism and expression of NDO activity.

In order to further confirm the identity of the ISP_{NAR} proteins as the catalytic components of NDO, we incubated the purified protein (combined α and β subunits) with radioactive [^{14}C]naphthalene in a substrate binding experiment. The bound [^{14}C]naphthalene was then separated from unbound substrate by gel filtration chromatography. A similar method was used to show that the ISP_{NAP} component from *P. putida* 9816-4 binds naphthalene (4). Here, a solution of [^{14}C]naphthalene (5.6 μl of 10 mM dimethylformamide [8.1 μCi/mmol]) was added to 100 μl of purified ISP_{NAR} enzyme solution (0.69 mg) in Tris buffer (50 mM, pH 7.5). After incubation for 20 min at room temperature, the mixture was applied to a Sephadex G-25 gel filtration column, and fractions (1 ml) were

eluted with the same buffer. The collected fractions were then assayed for radioactivity (Beckman Rackbeta 1217 scintillation counter, Amersham BCS scintillation fluid) and protein concentration (BCA assay; Pierce Inc., Rockford, Ill.). By analysis of the protein and radioactivity present in fractions collected from the gel filtration column, we were able to show that only the ISP_{NAR} protein bound to the [^{14}C]naphthalene. In a control experiment, the ISP_{NAR} protein was replaced with an equal amount of bovine serum albumin and naphthalene binding was not detected.

We attempted to determine if the purified ISP_{NAR} protein was also necessary for conversion of naphthalene to naphthalene *cis*-dihydrodiol. The enzyme assay was performed with the purified protein, as shown in Table 1. The reaction was terminated after 5 min by diluting the assay mixture (200 μl) with methanol (800 μl), and 30 ml of this mixture was analyzed by thin-layer chromatography (0.25 mM silica gel plates with a mobile phase comprising 8% methanol in dichloromethane). It was noted after autoradiography that only one radioactive metabolite was present, and it had the same R_f (0.5) as an authentic standard of naphthalene *cis*-dihydrodiol. The autoradiography procedures are described elsewhere (1). In control experiments, the assay was repeated with both the purified ISP_{NAR} protein and the fraction A protein alone. In these cases, no radioactive naphthalene *cis*-dihydrodiol was detected. This confirmed that at least two proteins (one being ISP_{NAR} and present in fraction B and the other in fraction A) were necessary for naphthalene *cis*-dihydrodiol formation.

Biochemical evidence that ISP_{NAR} is a Rieske-type iron-sulfur protein. A typical feature of the catalytic component of dioxygenase enzymes is that they are iron-sulfur proteins with Rieske-type [2Fe-2S] centers (9). We therefore sought to determine the iron and sulfur content of the purified ISP_{NAR} protein and found that 2.4 g-atoms of iron (± 0.2 [$n = 3$]) and 2.1 g-atoms of sulfur (± 0.9 [$n = 3$]) were present per αβ unit of protein. Published methods were used to determine the iron (8) and sulfur (22) contents of the purified proteins, and the sulfur assay was calibrated with ferredoxin from *Clostridium pasteurinum* (Sigma).

The UV spectrum of the oxidized ISP_{NAR} protein was also obtained in 50 mM Tris buffer (pH 7.5) by using a Beckman DU 640B spectrophotometer (4). Absorbance maxima of 334 nm ($\varepsilon = 23.9\ mM^{-1} \cdot cm^{-1}$), 462 nm ($\varepsilon = 14.1\ mM^{-1} \cdot cm^{-1}$), and 566 nm ($\varepsilon = 7.5\ mM^{-1} \cdot cm^{-1}$ [shoulder]) were characteristic of a Rieske-type [2Fe-2S] iron sulfur center being present in the protein (2). These absorbance maxima are also identical to those observed with the ISP_{NAP} protein from *P. putida* NCIB 9816-4 (4). This conclusion was further supported by the fact that when the protein was reduced with sodium dithionite, the absorbance maxima at 462 and 566 nm disappeared and a new maximum was observed at 519 nm. To obtain the reduced UV spectrum of the ISP_{NAR} protein, the method of Latimer et al. (16) was used. The enzyme (170 μg of protein in 50 μl) was added to a 1 M solution of oxygen-free sodium dithionite (2.5 ml in 50 mM Tris buffer, pH 7.5) in a sealed quartz cuvette. The mixture was then purged with nitrogen before spectroscopy. A similar observation was noted for the purified ISP_{NAP} protein when it was reduced enzymatically (4). When air was purged through the reduced ISP_{NAR} mixture, the oxidized spectrum was partially restored. Overall, these observations indicate that ISP_{NAR} is a redox protein, with shifts in absorbance maxima of this type being also observed for other Rieske-type [2Fe-2S] oxygenases and flavoproteins (10, 16, 20).

Nucleotide sequence analysis of the *narAa* and *narAb* genes. N-terminal sequences of the ISP_{NAR} α and β subunits were used to design primers for analysis of the NDO (*narA*) locus. It was assumed that the order of the genes encoding these subunits in *Rhodococcus* is the same as was shown for *Pseudomonas* species. The likely codon usage preference of *Rhodococcus* (assuming a high G+C content) was taken into account for the design of three degenerate primers: F200 (forward primer, amino acid residues 16 to 20 of the ISP_{NAR} α subunit), 5′-GACGTSAACWSSGACTGGAC-3′; F201 (forward primer, amino acid residues 4 to 9 of the ISP_{NAR} α subunit), 5′-AACGAGCTSCGSCAGAC-3′; R202 (reverse primer, amino acid residues 24 to 18 of the ISP_{NAR} β subunit), 5′-TCSGCCTCCATGTASAGCCA-3′. Total DNA from the *Rhodococcus* strain was isolated as described by Kulakova et al. (13), and amplification of *Rhodococcus* sequences was done with *Taq*$^+$ DNA polymerase (Stratagene). Reactions were carried out in volumes of 25 μl with deoxynucleoside triphosphates at 200 μM concentrations and primers at 0.6 μM each. The following temperature profile was used: denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 40 s, 55°C for 30 s, and 72°C for 1 min. Both pairs of primers (F200-R202 and F201-R202) yielded fragments of about 1.5 kbp, analysis of which revealed that they contained sequences corresponding to the N-terminal amino acid sequences of the larger, α subunit and the smaller, β subunit of NDO. Nucleotide sequences upstream and downstream of this region were obtained by the inverse PCR approach. For this, total DNA was digested with either the *Mlu*I, *Xho*I, or *Sac*II restriction enzyme. Then, the preparations were ligated with T4 DNA ligase and used in PCRs with different sets of divergent primers (conditions as above except that the primer concentration was 0.15 μM).

A sequence of 2,619 bp was thus obtained with the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit and an automatic sequencer (Applied Biosystems model 373A). This sequence contained two open reading frames that we have designated *narAa*, corresponding to the α subunit, and *narAb*, corresponding to the β subunit. The *narAa* gene (bp 510 to 1922) encodes a putative protein of 470 amino acids, in which a sequence of 25 N-terminal amino acids is identical to that of the NCIMB12038 NDO ISP_{NAR} α subunit iron-sulfur protein. Similarly, the *narAb* gene (bp 1926 to 2444) encodes a putative protein of 172 amino acids; 23 N-terminal amino acids were identical to that of the NCIMB12038 NDO ISP_{NAR} β subunit iron-sulfur protein (note that residue 5 was not determined with certainty in the N-terminal sequence). Both genes are preceded by putative ribosome binding sites (21).

Database searches (with the FASTA and BLAST programs [19] and the EMBL and GenBank databases) and homology analysis revealed that the putative products of the *narAa* and *narAb* genes have some similarity (31 to 39% amino acid identity) with the α and β subunits of a number of aromatic-ring-hydroxylating dioxygenases (results not presented). The identity of the deduced amino acid sequences of the *narAa* and *narAb* genes with the N-terminal sequences of the α and β subunits of the *Rhodococcus* NDO and the similarity of these amino acid sequences to different aromatic-ring-hydroxylating dioxygenases suggest that the sequenced genes indeed encode the corresponding subunits of NDO.

Alignment of the α subunits of the *Rhodococcus* sp. strain NCIMB12038 NDO and the *P. putida* 9816-4 NDO is shown in Fig. 2. It is worth noting that the β subunits of these enzymes show a slightly lower level of overall similarity than the corresponding α subunits (results not presented). There is little known to date about the catalytic role of the β subunit, which makes it difficult to discuss the significance of the conserved amino acid sequences in these proteins.

Despite significant differences in the amino acid sequences

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NahAc  -------MNYNNKILVSESGLSQKHLIHGDEELFQHELKTIFARNWLFLTHDSLIPAPGD   60
NarAa  MLSNELRQTLQKGLHDVNSDWTVPAAIINDPEVHDVERERIFGHAWVFLAHESEIPERGD   60
                         *       *  * *    *   **   * ** * * **  **

NahAc  YVTAKMGIDEVIVSRQNDGSIRAFLNVCRHRGKTLVSVEAGNAKGFVCSYHGWGFGSNGE  120
NarAa  YVVRYISEDQFIVCRDEGGEIRGHLNACRHRGMQVCRAEMGNTSHFRCPYHGWTYSNTGS  120
       **      *  ** *   * **  ** *****      * **   * * ****     *

NahAc  LQSVPFEKDLYGESLNKKCLGLKEVARVESFHGFIYGCFDQEAPPLMDYLGDAAWYLEPM  180
NarAa  LVGVPAGKDAYGNQLKKSDWNLRPMPNLASYKGLIFGSLDPHADSLEDYLGDLKFYLDIV  180
       *  **  ** **  * *    *       *  * * *  *  *  * *****   **

NahAc  FKHS-GGLELVGPPGKVVIKANWKAPAENFVGDAYHVGWTH----ASSLRSGESIFSSLA  240
NarAa  LDRSDAGLQVVGAPQRWVIDANWKLGADNFVGDAYHTMMTHRSMVELGLAPPDPQFA-LY  240
          *  **  ** *   ** ****  * ********   **       *      *  *

NahAc  GNAVLPPEGAGLQMTSKYGSGMGVLWDGYSGVHSADLVPELMAFGGAKQERLNKEIGDVR  300
NarAa  GEHIHTGHGHGLGII---GPPPGMPLPEFMGL-PENIVEELERRLTPEQVEIFRPTAFIH  300
       *       * **          *       *      * **       *

NahAc  ARIYRSHLNCTVFPNNSMLTCSGVF-----------KVWNPIDANTTEVWTYAIVEKDM  360
NarAa  G---------TVFPNLSIGNFLMGKDHLSAPTAFLTLRLWHPLGPDKMEVMSFFLVEKDA  360
                 ***** *                      * *      **     ****

NahAc  PDWFKDESYDSVQRTFGPAGFWESDDNDN-METASQNGKKYQSRDSDLLSNLGFGEDVYG  420
NarAa  PEDLKRRLAKSYLRTFGISGGFEQDDAENWRSITRVMGGQF-AKTGELNYQMGRGVLEPD  420
       *   *     *  **** *  * **  *        *         *    * *

NahAc  DAVYPGVVGKSAIGETSYRGFYRAYQAHVSSSNWAEFEHASSTWHTELTKTTDR--      480
NarAa  PNWTGPGEAYPLDYAEANQRNFLEYWMQLMLAESPLRDGNSNGSGTADASTPAAAK      480
                                 *              *    *    *

NahAc  ---------
NarAa  SKSPAKAEA
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FIG. 2. Amino acid sequence alignment of the ISP_{NAR} α subunits of naphthalene dioxygenases of *P. putida* 9816-4 (NahAc) and *Rhodococcus* sp. strain NCIMB12038 (NarAa). Sequences were aligned by the CLUSTALW program, and manual corrections were introduced to permit alignment of the TVFPN sequences.

of the α subunits, they show conservation of key catalytic residues, as summarized in Table 2. In general, there appears to be more conservation of the amino acid residues within the catalytic regions than in the other regions putatively associated with ferredoxin binding and substrate specificity. It is worth bearing in mind that the sequence of amino acids in the putative ferredoxin protein binding site region of the *Pseudomonas* ISP_{NAP} α subunit is very different from that in the analogous region of the *Rhodococcus* ISP_{NAR} α subunit. In addition to these observations, we note the surprising conservation of a five-amino-acid sequence located in a region of the ISP_{NAR} α subunit which has coordinates close to the substrate cleft, as suggested in the model of Kauppi et al. (12).

In summary, we have isolated a new naphthalene dioxygenase whose catalytic component has a greatly divergent amino acid sequence compared to dioxygenases with similar biochemical properties. Our belief that the ISP_{NAR} protein is the catalytic component of the naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038 is based on a number of biochemical observations. The purified protein binds naphthalene and is necessary for its subsequent conversion to naphthalene *cis*-dihydrodiol. Like the ISP_{NAP} protein from *Pseudomonas*, ISP_{NAR} is also an iron-sulfur protein, with good evidence for the presence of a Rieske [2Fe-2S] center. The ISP_{NAR} protein is comprised of α and β subunits with similar size and configuration ($\alpha_2\beta_2$) to ISP_{NAP} and is clearly expressed only during growth on naphthalene (and not during growth on pyruvate or salicylate) in *Rhodococcus* sp. However,

TABLE 2. Conservation of key amino acids in α subunits of NDOs[a]

Rieske center		Active site		Putative ferredoxin binding region		Electron transfer (Rieske active site)		Region of unknown function	
NahAc	NarAa	NahAc	NarAa	NahAc	NarAa	NahAc	NarAa	NahAc	NarAa
C81	**C88**	**N201**	**N209**	K97	S104	**W106**	**W113**	**T299**	**T297**
C101	**C108**	**H208**	**H216**	G98	H105	**V117**	**V124**	**V300**	**V298**
H83	**H90**	**H213**	**H221**	V100	R107	**R84**	**R91**	**F301**	**F299**
H104	**H111**	**D205**[b]	**D213**	Q115	V122	E200	D208	**P302**	**P300**
		D362	**D372**	S116	G123			**N303**	**N301**
				P118[c]	**P125**[c]				
				W211	M219				

[a] Boldface type indicates conservation of key amino acids.
[b] Asp205 is probably important for electron transfer (12) and is essential for activity (18).
[c] Pro118 (as well as Trp211) is from the catalytic domain.

the low overall similarity of the *Rhodococcus* and *Pseudomonas* NDOs raises an important question concerning the evolutionary origins of these enzymes. They may have diverged from the same ancestral dioxygenase, or they may be the result of convergent evolution of different proteins. If the latter is the case, then the close similarity of the catalytic regions may be due to functional demands rather than actual conservation of amino acid residues.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been assigned GenBank accession no. AF082663.

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