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Isolation and Preliminary Characterization of the Subunits of the Terminal Component of Naphthalene Dioxygenase from *Pseudomonas putida* NCIB 9816-4

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The terminal oxygenase component (ISP_{NAP}) of naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4 was purified to homogeneity. The protein contained approximately 4 g-atoms each of iron and acid-labile sulfide per mol of ISP_{NAP}, and enzyme activity was stimulated significantly by addition of exogenous iron. The large (α) and small (β) subunits of ISP_{NAP} were isolated by two different procedures. The NH_2-terminal amino acid sequences of the α and β subunits were identical to the deduced amino acid sequences reported for the *ndoB* and *ndoC* genes from *P. putida* NCIB 9816 and almost identical to the NH_2-terminal amino acid sequences determined for the large and small subunits of ISP_{NAP} from *P. putida* G7. Gel filtration in the presence of 6 M urea gave an α subunit with an absorption maximum at 325 nm and broad absorption between 420 and 450 nm. The α subunit contained approximately 2 g-atoms each of iron and acid-labile sulfide per mol of the subunit. The β subunit did not contain iron or acid-labile sulfide. These results, taken in conjunction with the deduced amino acid sequences of the large subunits from several iron-sulfur oxygenases, indicate that each α subunit of ISP_{NAP} contains a Rieske [2Fe-2S] center.

Naphthalene dioxygenase initiates the degradation of naphthalene by incorporating both atoms of molecular oxygen into the aromatic nucleus to form *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) as the first detectable product (20, 21). Subsequent studies (8) have shown that naphthalene dioxygenase is a multicomponent enzyme system consisting of an iron-sulfur flavoprotein ($reductase_{NAP}$; 15), a [2Fe-2S] ferredoxin ($ferredoxin_{NAP}$; 16), and an iron-sulfur protein (ISP_{NAP}; 9) which serves as the terminal oxygenase component (Fig. 1). ISP_{NAP} has an $\alpha_2\beta_2$ subunit composition and contains approximately 6 g-atoms of iron and 4 g-atoms of acid-labile sulfide per mol of enzyme. These findings suggest that ISP_{NAP} contains two [2Fe-2S] centers and mononuclear iron which are required for catalytic activity (9).

We now report the separation of the α (large) and β (small) subunits of ISP_{NAP} and provide evidence to show that each α subunit contains a Rieske-type [2Fe-2S] center (27).

MATERIALS AND METHODS

Organism and growth conditions. *Pseudomonas* sp. strain NCIB 9816 was originally isolated from soil by virtue of its ability to grow with naphthalene as the sole source of carbon and energy (7). This strain is designated *Pseudomonas putida* 9816-4 (35). The organism was maintained at 30°C on mineral salts basal medium (30) agar with naphthalene supplied in the vapor form from crystals in the lid of the plate. Large quantities of cells were obtained by growing the organism on mineral salts basal medium containing 0.1% naphthalene in a 250-liter fermentor.

Purification of naphthalene dioxygenase components. The $reductase_{NAP}$ and $ferredoxin_{NAP}$ components of naphthalene dioxygenase were separated from each other by Blue Sepharose CL-6B chromatography as described previously (15). $Ferredoxin_{NAP}$ and ISP_{NAP} were not resolved by this system and were separated from each other by DEAE-cellulose chromatography (16). Fractions that contained ISP_{NAP} activity were pooled and applied to a column (2.0 by 12.5 cm) of Octyl-Sepharose CL-4B. The column was equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 10% (vol/vol) ethanol, 10% (vol/vol) glycerol, and 0.5 mM dithiothreitol (TEG buffer) and 40% ammonium sulfate. The column was washed with 100 ml of TEG buffer containing 40% ammonium sulfate. During this time, ISP_{NAP} formed a dark-brown band at the top of the column. ISP_{NAP} was eluted from the column with 200 ml of TEG buffer containing 30% ammonium sulfate. The flow rate was 1.0 ml/min. Fractions containing ISP_{NAP} were pooled and applied to the top of a column (1.6 by 92 cm) of Sephacryl S-200. The column was equilibrated with TEG buffer, and the flow rate was 10 ml/h. ISP_{NAP} eluted from this column was applied to a small column (0.9 by 10 cm) of DEAE-cellulose. ISP_{NAP} was eluted from the column with a linear gradient of 0.0 to 0.3 M potassium chloride in TEG buffer. The brown protein solution was concentrated by ultrafiltration, dialyzed against three changes of TEG buffer at 4°C for 3 h, divided into aliquots, and stored at −70°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the ISP_{NAP} obtained by this procedure was homogeneous.

Enzyme assays. The enzyme assays used to measure $reductase_{NAP}$, $ferredoxin_{NAP}$, and ISP_{NAP} activities were described previously (9, 15, 16). One unit of ISP_{NAP} activity is defined as the amount of protein required to form 1.0 μmol of *cis*-naphthalene dihydrodiol per min. Specific activity is given as milliunits per mg of protein. Protein concentrations were determined by the method of Bradford (3) with bovine serum albumin as the standard.

Subunit separation by liquid chromatography. Purified

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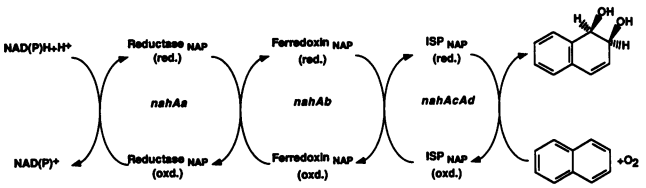

FIG. 1. Proposed sequence for the transfer of electrons from NAD(P)H to the terminal oxygenase component (ISP_{NAP}) of naphthalene dioxygenase. The individual components $reductase_{NAP}$ (15), $ferredoxin_{NAP}$ (16), and ISP_{NAP} (9) have been purified to homogeneity. The redox state of each protein is indicated as reduced (red.) or oxidized (oxd.). The gene designations *nahAa*, *nahAb*, and *nahAcAd* are given for $reductase_{NAP}$, $ferredoxin_{NAP}$, and the α and β subunits of ISP_{NAP}, respectively.

ISP_{NAP} (1 mg) in 0.25 ml of TEG buffer was frozen at −70°C and lyophilized. Trifluoroacetic acid (1%) equal to the sample volume prior to lyophilization was added, and the subunits of ISP_{NAP} were separated by high-performance liquid chromatography (HPLC) on a Vydac C_4 column with a continuous 27 to 48% acetonitrile gradient in 0.1% trifluoroacetic acid. Two proteins were detected by their A_{220}s, and fractions corresponding to the peaks were collected.

ISP_{NAP} subunits were also separated by fast-performance liquid chromatography on a Superose 6 gel filtration column. Purified ISP_{NAP} in TEG buffer was incubated with an equal volume of Tris-HCl buffer (50 mM, pH 7.5) containing 6 M urea for 30 min. The protein sample was then injected into a prepacked Superose 6 gel filtration column and eluted with the same buffer. Proteins were detected by their A_{280}s, and 1-ml fractions were collected.

Analytical methods. The iron and acid-labile sulfide contents of protein samples were determined as previously described (15). SDS-PAGE was done as described by Laemmli (23). Standard proteins used for molecular weight determinations were bovine serum albumin (M_r, 66,000) ovalbumin (M_r, 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000), carbonic anhydrase (M_r, 29,000), trypsinogen (M_r, 24,000), soybean trypsin inhibitor (M_r, 21,100), and α-lactalbumin (M_r, 14,000). Absorption spectra were recorded with an Aminco DW-2A double-beam recording spectrophotometer. HPLC was performed with a Waters Associates 6000A solvent delivery system with a U-6K septumless injector and a 440 absorbance detector.

Materials and chemicals. The following materials and chemicals were obtained from the sources indicated: DEAE-cellulose (Whatman DE-52), Whatman, Inc., Clifton, N.J.; Octyl-Sepharose CL-4B, Blue-Sepharose CL-6B, Sephacryl S-200, and Superose 6 (prepacked column), Pharmacia Inc., Piscataway, N.J.; NADH, flavin adenine dinucleotide, dithiothreitol, and protein molecular weight standards, Sigma Chemical Co., St. Louis, Mo.; [U-^{14}C]naphthalene (specific activity, 4.7 mCi/nmol), Pathfinder Laboratories Inc., St. Louis, Mo.

RESULTS AND DISCUSSION

Purification and properties of ISP_{NAP}. A modified procedure for the purification of ISP_{NAP} was developed to purify all three components of naphthalene dioxygenase from a single cell extract. This involved initial separation of $reductase_{NAP}$ from $ferredoxin_{NAP}$ and ISP_{NAP} by chromatography on a Blue Sepharose CL-6B column (15). The eluate from this column contained $ferredoxin_{NAP}$ and ISP_{NAP}, which were separated from each other by DEAE-cellulose chromatography (16). Fractions containing partially purified ISP_{NAP} were purified by the procedure described in Materials and Methods. The purified protein gave two bands which stained for protein when analyzed by SDS-PAGE (Fig. 2, lane 2). The molecular weights of the two proteins, 55,000 and 20,000, are the same as those reported previously for the α and β subunits of ISP_{NAP} (9). However, the specific activity of the ISP_{NAP} purified in the present study (270 mU/mg) was five times lower than the value (1,370 mU/mg) reported previously (9). The latter preparation of ISP_{NAP} contained approximately 6 g-atoms of iron and 4 g-atoms of acid-labile sulfide per mol of enzyme, and the ability of this preparation to oxidize naphthalene was not enhanced by the presence of exogenous iron. In contrast, the ISP_{NAP} purified in the present study contained approximately 4 g-atoms each of iron and acid-labile sulfide per mol of ISP_{NAP} (Table 1), and naphthalene dioxygenase activity was significantly stimulated by addition of ferrous iron (Table 2). The specific activity of this ISP_{NAP} preparation (1,085 mU/mg) is closer to the activity reported previously for ISP_{NAP} in the absence of added ferrous iron.

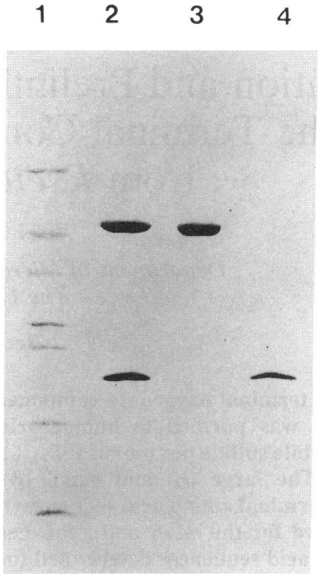

FIG. 2. SDS-PAGE of purified ISP_{NAP} (lane 2) and its α (lane 3) and β (lane 4) subunits. Lane 1 contained molecular weight standards. These were, from the top, bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, and α-lactalbumin.

The absorption spectrum of oxidized ISP_{NAP} (Fig. 3) shows maxima at 334 462, and 566 nm (shoulder). Upon reduction with dithionite, new absorption maxima were

TABLE 1. Iron and acid-labile sulfide contents[a] of ISP_{NAP} and its α and β subunits[a]

Protein	Iron (g-atoms/mol)	Acid-labile sulfide (g-atoms/mol)
ISP_{NAP}	4.0 ± 0.2[b]	3.8 ± 0.4
α subunit	1.9 ± 0.3	1.8 ± 0.3
β subunit	0	0

[a] Mean ± standard deviation (n = 3).
[b] Experimental conditions are described in Materials and Methods.

TABLE 2. Iron requirement for oxygenase activity of purified ISP_{NAP}[a]

$FeSO_4 \cdot 7H_2O$ (nmol)	Naphthalene dioxygenase sp act[b] (mU/mg of ISP_{NAP})
0	260
0.68	300
6.80	452
68.00	1,085
136.00	1,055

[a] Reaction mixtures contained in a final volume of 0.4 ml: 50 mM Tris/HCl buffer (pH 7.5); NADH, 1.0 μmol; flavin adenine dinucleotide, 1.0 nmol; [^{14}C] naphthalene, 100 nmol (5.61×10^5 dpm); $reductase_{NAP}$, 10 μg of protein; $ferredoxin_{NAP}$, 10 μg of protein; ISP_{NAP}, 10 μg of protein. The three protein components were incubated with the concentrations of $FeSO_4 \cdot 7H_2O$ shown for 10 min prior to initiation of the reaction with [^{14}C]naphthalene.

[b] Averages of duplicate determinations are shown.

observed at 380, 428, and 520 nm (data not shown). These spectra are identical to those reported previously for purified ISP_{NAP} (9) and are very similar to the spectra of the Rieske protein from *Thermus thermophilus* (11). Rieske proteins (27) contain one or more [2Fe-2S] centers in which one iron atom is ligated to two cysteine residues and the second iron atom is coordinated with the remote N atoms of histidine (4, 11, 14, 33). In addition to the characteristic optical spectra noted above, Rieske proteins give unique electron paramagnetic resonance spectra. The reduced forms are rhombic and give *g* values of 2.02, 1.90, and 1.8 that are observed at high temperatures (4, 11). In a study the results of which will be presented elsewhere, we found that ISP_{NAP} purified from a recombinant *Escherichia coli* strain gave an electron paramagnetic resonance spectrum with *g* values of 2.01, 1.91, and 1.80.

The isolation of ISP_{NAP} that requires exogenous iron for activity is a new finding that contrasts with our previous report on the purification of this protein (9). The reason for the loss of iron during the modified purification procedure and the experimental conditions required for optimization of

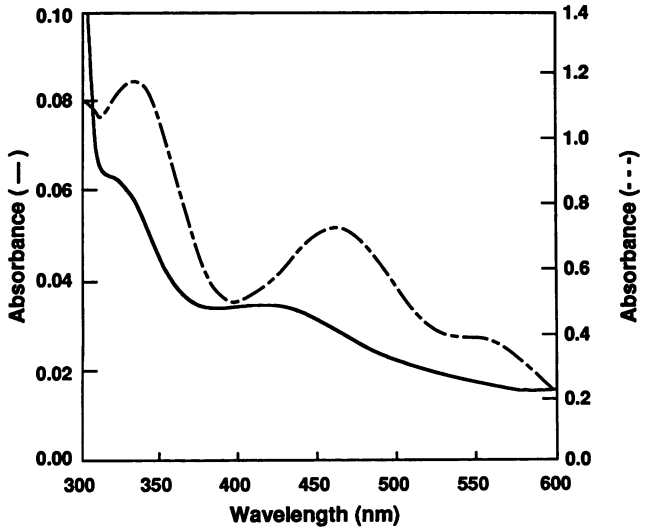

FIG. 3. Absorption spectra of homogeneous ISP_{NAP} (dashed line) and its α subunit (solid line). The concentrations of ISP_{NAP} and the α subunit were 66.5 and 7.3 μM, respectively.

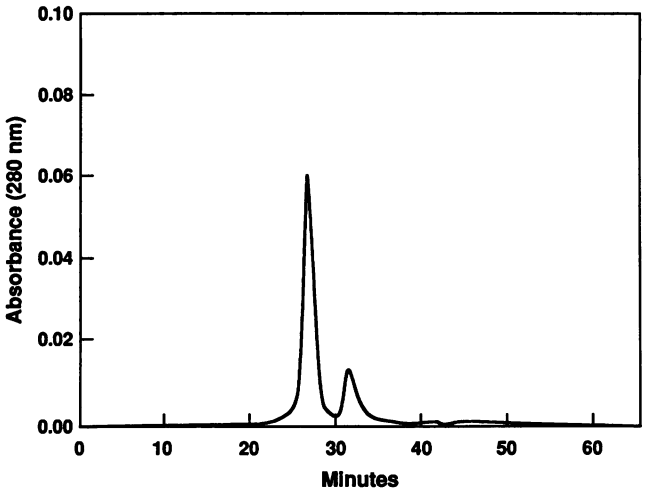

FIG. 4. Separation of the α and β subunits of ISP_{NAP} by gel filtration in the presence of 6 M urea. Chromatographic procedures are described in Materials and Methods.

ISP_{NAP} activity in the presence of exogenous iron were not investigated in the present study. Nevertheless, the homogeneous ISP_{NAP} preparation obtained was considered suitable for preliminary experiments on the separation and characterization of its α and β subunits.

Separation of the α and β subunits of ISP_{NAP}. The initial procedure developed for separation of the α and β subunits of ISP_{NAP} was achieved by HPLC as described in Materials and Methods. The subunits isolated by this procedure were colorless and did not contain iron or acid-labile sulfide. The NH_2-terminal amino sequence of each subunit was determined and shown to be identical to the deduced NH_2-terminal amino acid sequences reported for the *ndoB* and *ndoC* genes from *P. putida* NCIB 9816 (22). These genes, designated *nahAcAd*, have been shown to encode the α and β subunits of ISP_{NAP} in *P. putida* G7 (29).

Separation of the α and β subunits of ISP_{NAP} by fast-performance liquid chromatography on a column of Superose 6 in the presence of 6 M urea gave the results shown in Fig. 4. Fraction 13, which eluted at 26 min, was light brown, and fractions 12 to 14 were pooled and concentrated to 0.5 ml by ultrafiltration. The same procedure was used to concentrate fractions 16 to 18, which were colorless. Both concentrated samples were analyzed by SDS-PAGE (Fig. 2), and the results showed that the brown protein solution (fractions 12 to 14, lane 3) contained the α subunit while the β subunit was present in the concentrated colorless protein solution obtained from fractions 16 to 18 (lane 4).

Solutions of the α subunit showed an absorption maximum at 325 nm and a broad absorption maximum between 420 and 450 nm (Fig. 3). The differences between this spectrum and that given by intact ISP_{NAP} are probably due to denaturation of the α subunit during the isolation procedure. Solutions of the β subunit showed no absorbance between 300 and 600 nm. The iron and acid-labile sulfide contents of the α and β subunits were determined. The results (Table 1) showed that the α subunit contained approximately 2 g-atoms each of iron and acid-labile sulfide per mol of the subunit. Iron and acid-labile sulfide were not detected in the β subunit.

The results obtained suggest that ISP_{NAP} contains two Rieske-type [2Fe-2S] centers. In addition, the absorption spectrum and iron and acid-labile sulfide contents of the α

subunit indicate that each α subunit contains one Rieske-type [2Fe-2S] center. Similar results have been reported for the large α subunit of benzoate dioxygenase from *P. arvilla* C-1 (34). Other bacterial dioxygenases with optical and/or electron paramagnetic resonance spectra indicative of the presence of Rieske-type [2Fe-2S] centers include benzene dioxygenase (5, 12), toluene dioxygenase (13, 31), pyrazon dioxygenase (28), 4-chlorophenylacetate dioxygenase (25), 4-sulfobenzoate dioxygenase (24), and phthalate dioxygenase (1). However, phthalate dioxygenase is the only dioxygenase in which a Rieske-type [2Fe-2S] center has been established by rigorous biophysical techniques (1, 4, 33).

Further support for the presence of a Rieske-type [2Fe-2S] center in the α subunit of ISP_{NAP} was obtained by analysis of its deduced amino acid sequence. The nucleotide sequences of the structural genes for the α and β subunits of ISP_{NAP} from *P. putida* NCIB 9816 have been reported (22) and shown to be almost identical to the isofunctional genes from *P. putida* G7 (29). The presence of a Rieske [2Fe-2S] center in the α subunit from each of these organisms can be inferred from the presence of an almost identical conserved sequence: C-R-H-(amino acid)$_{17}$-C-S-Y-H . . . with invariant cysteine and histidine residues. A similar conserved sequence has been identified in the Rieske protein that forms part of the cytochrome bc_1 complex in *Rhodobacter capsulatus*, and site-directed mutagenesis experiments suggest that the consensus cysteine and histidine residues are ligated to the iron atoms in the [2Fe-2S] center (6). Studies on evolutionary relationships among multicomponent oxygenases have shown that the deduced amino acid sequences for the α subunits of benzoate (26), toluate (18), benzene (19), toluene (36), and naphthalene (22, 29) dioxygenases show significant homology with each other in the NH_2-terminal region, and all contain the conserved sequence indicative of a Rieske [2Fe-2S] center (26). Recent additions to this list include the putative α subunits of biphenyl dioxygenases from *P. pseudoalcaligenes* KF707 (32) and *Pseudomonas* sp. strain LB400 (10).

We have previously shown that ISP_{NAP} accepts electrons from $ferredoxin_{NAP}$ in the naphthalene dioxygenase complex (9, 16). $Ferredoxin_{NAP}$ also has physical properties (16) and a deduced amino acid sequence (22, 29) that are consistent with the presence of a Rieske [2Fe-2S] center. Thus, it appears that the function of the [2Fe-2S] centers in the α subunits of ISP_{NAP} is to accept electrons from $ferredoxin_{NAP}$ and transfer them to mononuclear iron at the active site of the enzyme. The location of the iron-binding site was not determined in the present study. Similar results have been reported for benzoate 1,2-dioxygenase. Each α subunit contains a [2Fe-2S] center, and the location of the mononuclear iron required for enzyme activity is not known (34). Neidle et al. (26) have noted that the α subunits of benzene, toluene, naphthalene, benzoate, and toluate dioxygenases each contain three invariant histidines, other than those involved in coordinating iron in the [2Fe-2S] center, and two invariant tyrosine residues. Those investigators have suggested that these amino acids may serve as coordination ligands for mononuclear iron. The location of iron in the α subunits of these dioxygenases would be consistent with the finding that the oxygenase components of 4-methoxybenzoate monooxygenase (2), phthalate dioxygenase (1), and 4-chlorophenylacetate dioxygenase (25) have α_3-, α_4-, and α_3-subunit compositions, respectively. Each of these enzymes contains a Rieske [2Fe-2S] center, and each requires mononuclear iron for oxygenase activity.

The function of the β subunit in ISP_{NAP} is unknown. The deduced amino acid sequences of the β subunits of benzene, toluene, naphthalene, benzoate, and toluate oxygenases show a low but significant degree of homology to each other with nine conserved amino acids present in each subunit. Five of the nine conserved residues are charged amino acids, and it has been suggested that these five amino acids may play a role in the association between the α and β subunits of the oxygenase component (26). In the case of toluate dioxygenase, it has been suggested that the β subunit may play a role in substrate specificity (17). However, we have recently shown that the biphenyl dioxygenases from *Pseudomonas* sp. strain LB400 and *P. pseudoalcaligenes* KF707 show striking differences in the ability to oxidize a wide range of chlorinated biphenyls (12a). The oxygenase components (ISP_{BPH}) of these enzymes have an $\alpha_2\beta_2$ subunit composition, and the deduced amino acid sequences of the β subunits differ by a single amino acid (10, 33). This change of a valine residue in the KF707 β subunit to a leucine residue in the LB400 β subunit seems unlikely to account for the observed differences in substrate specificity in the LB400 and KF707 biphenyl dioxygenases. Further studies are required to elucidate the interactions between the α and β subunits from related aromatic hydrocarbon dioxygenases. In the case of ISP_{NAP}, our current studies are directed towards the construction of recombinant strains that express the individual α and β subunits. These will facilitate future studies on the reconstitution of ISP_{NAP} activity.

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