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Naphthalene Dioxygenase: Purification and Properties of a Terminal Oxygenase Component

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Naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816 is a multicomponent enzyme system that oxidized naphthalene to *cis*-(1*R*, 2*S*)-diohydroxy-1,2-dihydronaphthalene. The terminal oxygenase component B was purified to homogeneity by a three-step procedure that utilized ion-exchange and hydrophobic interaction chromatography. The purified enzyme oxidized naphthalene only in the presence of NADH, oxygen, and partially purified preparations of components A and C. An estimated M_r of 158,000 was obtained by gel filtration. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed the presence of two subunits with molecular weights of ca. 55,000 and 20,000, indicative of an $\alpha_2\beta_2$ quaternary structure. Absorption spectra of the oxidized enzyme showed maxima at 566 (shoulder), 462, and 344 nm, which were replaced by absorption maxima at 520 and 380 nm when the enzyme was reduced anaerobically by stoichiometric quantities of NADH in the presence of the other two components of the naphthalene dioxygenase system. Component B bound napthalene. Enzyme-bound napthalene was oxidized to product upon the addition of components A and C, NADH, and O_2. These results, together with the detection of the presence of 6.0 g-atoms of iron and 4.0 g-atoms of acid-labile sulfur per mol of the purified enzyme, suggest that component B of the naphthalene dioxygenase system is an iron-sulfur protein which functions in the terminal step of napthalene oxidation.

The initial reaction in the degradation of naphthalene by soil pseudomonads involves the enzymatic incorporation of one molecule of oxygen into the aromatic nucleus to form *cis*-(1*R*, 2*S*)-dihydroxy-1,2-dihydronaphthalene (6, 15, 16). Preliminary studies have shown that in *Pseudomonas* sp. strain NCIB 9816 this reaction is catalyzed by a multienzyme system termed naphthalene dioxygenase. This system consists of three protein components, A, B, and C, which are essential for enzymatic activity (12). In this respect, naphthalene dioxygenase is similar to the multicomponent enzyme systems that form *cis*-dihydrodiols from benzene (2, 10), toluene (9, 13, 20, 21, 26), and 5-amino-4-chloro-2-phenyl-2*H*-pyridazin-3-one (19). Each of these enzyme systems consists of a flavoprotein reductase and a plant-type ferredoxin which are required for the transfer of electrons from NADH to the terminal dioxygenase or to other electron acceptors such as cytochrome *c*. The dioxygenases are iron-sulfur proteins that require exogenous iron for optimum activity. However, naphthalene dioxygenase is not stimulated by exogenous iron. In addition, one of its components (A) is an iron-containing flavoprotein that will transfer electrons directly from NADH to cytochrome *c* (unpublished data). These properties are similar to those reported for the two component benzoate dioxygenase (23–25) and 4-methoxybenzoate O-demethylase (4–5) enzyme systems. The only multicomponent system that requires an iron flavoprotein and two additional proteins for activity is the methane monooxygenase from *Methylococcus capsulatus* Bath (8, 9).

We now report the purification and some properties of component B in the naphthalene dioxygenase enzyme system. The oxidized enzyme binds naphthalene and can accept two electrons from NADH in the presence of components A and C. The results presented suggest that component B functions as the terminal dioxygenase component in naphthalene dioxygenase. Preliminary characterization studies show that component B is an iron-sulfur protein that we have designated ISP_{NAP}.

MATERIALS AND METHODS

Pseudomonas sp. strain NCIB 9816 was provided by W. C. Evans, The University College of North

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Wales, Bangor, Wales. The maintenance and growth of the organism, as well as the preparation of crude cell extracts, were as described previously (12). To ensure enzyme stability, crude extracts were prepared in 50 mM Tris-hydrochloride buffer (pH 7.8) containing 10% (vol/vol) ethanol, 10% (vol/vol) glycerol, and 0.5 mM dithiothreitol (TEG buffer).

Enzyme assays. Naphthalene dioxygenase activity was determined by measuring the accumulation of nonvolatile metabolites from [^{14}C]naphthalene (12). During purification of the terminal component of the naphthalene dioxygenase system (component B), this procedure was used except that partially purified component A and partially purified component C were added to the reaction mixture. Reactions (0.4 ml each) contained 50 mM Tris-hydrochloride buffer (pH 7.5), 100 μg of component C, 60 μg of component A, 1.0 nmol of flavin adenine dinucleotide, 1.0 μmol of NADH and appropriate amounts of component B. The reaction was started by the addition of 100 nmol of [1(4,5,8)-^{14}C]naphthalene (1.1×10^6 dpm) in 10 μl of dimethylformamide. One unit of enzyme activity is defined as that amount of protein required to convert 1.0 nmol of naphthalene to nonvolatile metabolites in 1 min. NADH-cytochrome *c* reductase activity was measured as described previously (12). Protein content in cell extracts and enzyme fractions was determined by a microbiuret procedure (14). Bovine serum albumin (BSA) was used as a protein standard.

Enzyme-substrate binding measurements. The binding of naphthalene to purified component B (ISP_{NAP}) was measured by diluting 0.1 ml of TEG buffer containing 2.9 nmol of the enzyme with 0.9 ml of 50 mM Tris-hydrochloride buffer, pH 7.5. The enzyme solution was incubated with 50 nmol of [^{14}C]naphthalene (55×10^5 dpm) in 5.0 μl of dimethylformamide for 20 min at room temperature. Unbound naphthalene was separated from the protein by passing the solution over a column of Sephadex G-25 (1 by 9 cm) which had been previously equilibrated in 50 mM Tris-hydrochloride buffer, pH 7.5. Fractions of 1.1 ml were collected, and ISP_{NAP} was detected by measuring the absorbance of each fraction at 460 nm. The radioactivity in each fraction was determined by transferring 0.1 ml into 5.0 ml of Aquasol scintillation cocktail. Measurements were made in a liquid scintillation counter. Control experiments utilized the same procedure, with ISP_{NAP} replaced by BSA. The presence of BSA in column fractions was detected by measuring the absorbance at 280 nm.

The oxidation of enzyme-bound naphthalene was measured by incubating 1.0 ml of a column fraction prepared in the above procedure with the other two components of the naphthalene dioxygenase system and NADH. A 1.0-ml column fraction, which contained 2.3 nmol of [^{14}C]naphthalene bound to 2.5 nmol of purified ISP_{NAP}, was incubated with 48 μg of partially purified component A, 60 μg of partially purified component C, and 0.5 μmol of NADH for 10 min at room temperature. The reaction mixture was then rechromatographed, and fractions containing radioactivity were pooled and extracted twice with equal volumes of ethyl acetate. The organic phases were combined, dried over anhydrous sodium sulfate, and evaporated to dryness under a stream of air at room temperature. The residue was dissolved in 100 μl of ethyl acetate, and 50 μl was applied to the origin of a precoated silica gel chromatography plate. Authentic samples of *cis*-naphthalene dihydrodiol, 1-naphthol, and naphthalene were applied to the same spot on the chromatogram, which was developed twice in a solvent system of chloroform-acetone (8:2, vol/vol). The location of the standards was detected by viewing under UV light. Areas of the chromatogram corresponding to each standard were transferred into 7.0 ml of scintillation fluid, and the amount of radioactivity in each of the areas was determined by standard scintillation counting procedures.

Spectrophotometric measurements. All spectrophotometric measurements were made with an Aminco DW-2a double-beam spectrophotometer. Samples were contained in quartz cuvettes with a 1.0-cm light path. Analyses of anaerobic samples were made in anaerobic cuvettes (Hellma Cells, Inc., Jamaica, N.Y.) fitted with rubber septa. The samples were rendered anaerobic by alternate evacuation of the cuvettes and sparging with argon which had been scrubbed over a column of hot copper pellets.

Analytic methods. Polyacrylamide gel electrophoresis was performed on 8 and 10% acrylamide gels in Tris-glycine buffer, pH 8.3, as described by Davis (11). Sodium dodecyl sulfate gels used for molecular weight determinations were prepared according to Weber and Osborn (22). Cytochrome *c*, ovalbumin, catalase, and BSA were used as protein standards. The native molecular weight of the protein was estimated by chromatography over a calibrated Sephadex G-200 column as described by Andrews (1). Inorganic iron measurements were performed with a Perkin-Elmer model 400 atomic absorption spectrophotometer. Acid-labile sulfide was measured by the method of King and Morris (17).

Materials. The following items were from the sources indicated: cytochrome *c*, NADH, flavin adenine dinucleotide, Trizma base, DNase, BSA, and DEAE–Sephadex G-25, Sigma Chemical Co., St. Louis, Mo.; Octyl–Sepharose 4B, Sephadex G-75, and Sephadex G-25, Pharmacia Inc., Piscataway, N.J.; DEAE-cellulose (DE-52), Whatman, Inc., Clifton, N.J.; XM-50 ultrafiltration membranes, Amicon Corp., Lexington, Mass.; [1(4,5,8)-^{14}C]naphthalene (specific activity, 5 mCi/mmol), Amersham Corp., Arlington Heights, Ill.; precoated thin-layer chromatography sheets, Silica Gel 60 F_{254}, MCB Manufacturing Chemists, Inc., Cincinnati. *cis*-Naphthalene dihydrodiol was prepared as described previously (18). All other chemicals were of the highest purity commercially available.

RESULTS

Purification of the terminal oxygenase component. Crude cell extract prepared from 25 g (wet weight) of cells (71.0 ml, 2.20 g of protein) was applied to a column of DEAE–Sephadex G-25 (2.5 by 11.0 cm) which had been previously equilibrated with TEG buffer. Components A and B of the naphthalene dioxygenase system did not bind to the column and were collected as a single fraction. The column was then washed with 200 ml of TEG buffer, followed by 200 ml of TEG buffer containing 0.125 M KCl. Component C was eluted from the column with 0.2 M KCl in

TEG buffer. Fractions of 10 ml were collected and assayed for naphthalene dioxygenase activity in the presence of components A and B. Those fractions containing component C were pooled and stored at −20°C.

Components A and B were separated by ion-exchange chromatography. Protein which did not bind to the DEAE–Sephadex G-25 column (75.0 ml, 2.0 g of protein) was applied to a column (4.0 by 8.0 cm) of DEAE-cellulose (DE-52). The column was washed with 200 ml of TEG buffer. Components A and B were then eluted with a linear gradient (800 ml) of KCl (0.0 to 0.12 M) in TEG buffer. Fractions (11.0 ml each) were collected and assayed for naphthalene dioxygenase and NADH-cytochrome *c* reductase activity. Those fractions that contained both activities were pooled and stored at −20°C as a source of component A. Fractions 70 to 84 were pooled to give a red-brown protein solution (153 ml, 230 mg of protein) that showed naphthalene dioxygenase activity only when assayed in the presence of components A and C. The protein solution obtained from fractions 70 to 84 was concentrated to 26.0 ml over an Amicon XM-50 membrane and then brought to 40% saturation with ammonium sulfate. A slight precipitate formed which was removed by centrifugation at 10,000 × *g* for 15 min. The supernatant solution was applied to a column (1.5 by 20.0 cm) of octyl–Sepharose 4B which had previously been equilibrated with TEG buffer containing 40% ammonium sulfate. During this procedure, a dark-red band of protein was observed to concentrate at the top of the column. The column was washed with 50 ml of TEG buffer containing 40% ammonium sulfate, and this was followed by 200 ml of the same buffer containing 30% ammonium sulfate. Fractions (10.0 ml each) were collected and assayed for naphthalene dioxygenase activity which was detected in fractions 16 to 25. The contents of these tubes were pooled, dialyzed overnight against 2.0 liters of TEG buffer, and concentrated to 9.5 ml over an Amicon XM-50 membrane. A summary of the purification procedure is shown in Table 1. A 36-fold purification of the enzyme was achieved, with a recovery of 30% of the activity present in the crude cell extract.

Polyacrylamide gel electrophoresis of the purified enzyme on 8 and 10% gels gave a single band which stained for protein.

Properties of component B. The molecular weight of purified component B was determined by gel filtration on a standardized column of Sephadex G-200. From the results obtained, the molecular weight of the native protein was calculated to be 158,000. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol revealed the presence of two subunits with molecular weights of 55,000 and 20,000. These observations suggest that the native protein has an $\alpha^2\beta^2$ composition.

TABLE 1. Purification of naphthalene dioxygenase

Purification step[a]	Protein (mg)	Activity (U)	Sp act (U/mg)	Recovery (%)
1. Crude extract	2,215	84,000	37.9	
2. DEAE-Sephadex eluate	1,987	81,000	10.8	96
3. DEAE-cellulose chromatography	230	73,400	319.0	87
4. Octyl-Sepharose	19	26,030	1,370.0	31

[a] Activity determined in the presence of partially purified components A and C and flavin adenine dinucleotide as described in the text.

Preliminary analyses revealed the presence of 4.0 g-atoms of acid-labile sulfur and 5.6 g-atoms of iron per 158,000 g of protein. These results indicate that component B of the naphthalene dioxygenase system is an iron-sulfur protein that we have designated ISP_{NAP}.

Spectral studies. The absorption spectrum of the purified enzyme showed maxima at 566 (shoulder), 462, and 334 nm (Fig. 1). The calculated extinction coefficients at these wavelengths were 3.8, 9.0, and 21.0 mM^{-1} cm^{-1}, respectively. The protein could be enzymatically reduced by NADH in the presence of catalytic amounts of components A and C under anaerobic conditions (Fig. 2), with a loss of absorption at 566 and 462 nm and the appearance of new maxima at 520 and 380 nm (Fig. 2, line 10). When absorbance at 462 nm was plotted as a function of the NADH added (Fig. 3), a linear

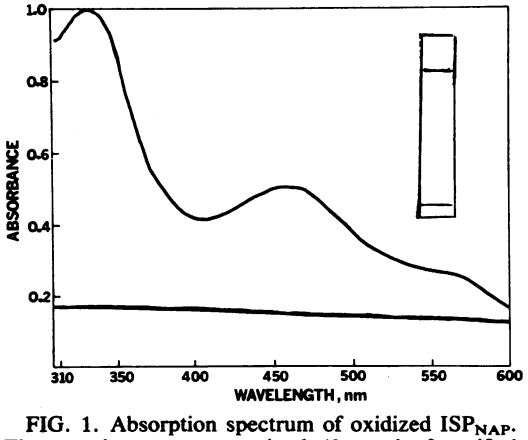

FIG. 1. Absorption spectrum of oxidized ISP_{NAP}. The sample cuvette contained 40 nmol of purified enzyme in 1.0 ml of TEG buffer. The inset shows the result of electrophoresis of purified component B in an 8% polyacrylamide gel at pH 8.3.

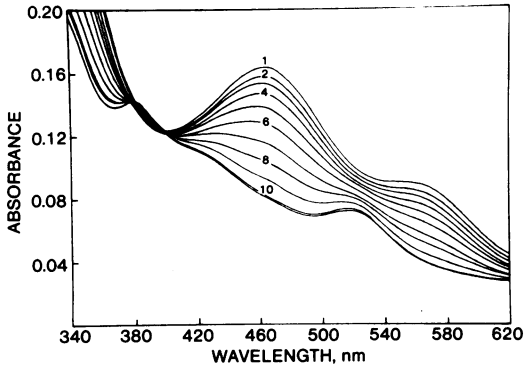

FIG. 2. Anaerobic titration of ISP_{NAP} with NADH in the presence of components A and C. The sample cuvette contained, in a final volume of 1.0 ml of polyethylene glycol buffer, 20.4 nmol of ISP_{NAP}, 48 μg of component A, and 60 μg of component C. Curve 1 shows the absorption spectrum of the oxidized enzyme under anaerobic conditions. Curves 2 to 11 show spectra obtained after separate 2.4-nmol additions of NADH.

decrease in absorbance was observed until the concentrations of enzyme and NADH were equal. A sharp endpoint was noted at this stage, with no absorbance changes occurring after further additions of NADH. These results indicate that ISP_{NAP} can accept two electrons.

Reduced ISP_{NAP} could be oxidized by exposure to air in the absence of substrate. The reoxidized spectrum was identical to the original spectrum (Fig. 2, line 1), indicating that a reduction-oxidation cycle of the enzyme in the absence of a substrate had no pronounced effect on the spectral properties of the protein.

The reduction of ISP_{NAP} by NADH required the presence of both of the other components in the dioxygenase system (Fig. 4). No spectral changes, with the exception of increased absorbance near 340 nm, were observed if the enzyme was incubated with component A and NADH under anaerobic conditions (Fig. 4, line 2). A gradual spectral shift to the final reduced form of the enzyme occurred after component C was added to the reaction mixture (Fig. 4, lines 3 to 17). In a similar experiment, ISP_{NAP} was not reduced by component C and NADH, but required the addition of component A before reduction occurred (data not shown).

No changes could be detected in the absolute spectrum of either the oxidized or the enzymatically reduced form of ISP_{NAP} after additions of excess naphthalene to the enzyme. In addition, the base-line spectrum recorded with oxidized or reduced enzyme in both cuvettes was not altered by the addition of excess naphthalene to the sample cuvette.

Substrate binding studies. Incubation of purified ISP_{NAP} with [^{14}C]naphthalene and separation of unbound naphthalene by gel chromatography gave the results shown in Fig. 5. Fractions 3 and 4 contained a total of 2.9 nmol of enzyme and 2.7 nmol of naphthalene. BSA, treated in the same manner, bound minimal amounts of naphthalene.

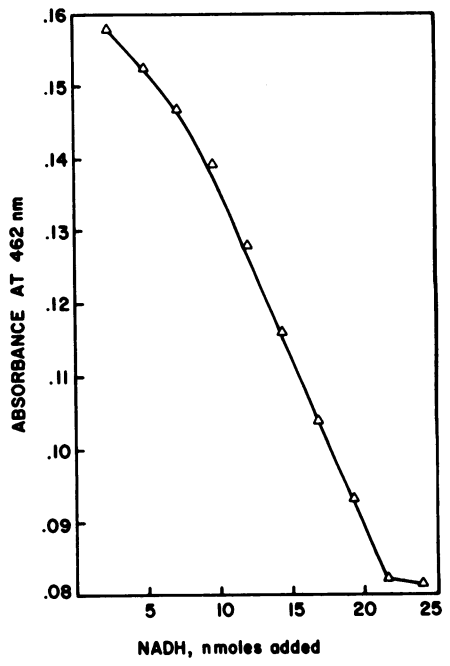

FIG. 3. Anaerobic reduction of ISP_{NAP} by NADH in the presence of components A and C. Results were obtained as described in the legend to Fig. 2.

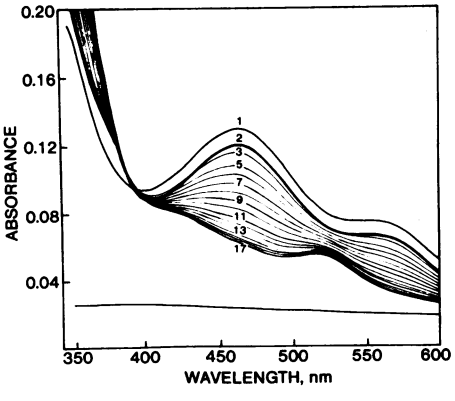

FIG. 4. Requirement of components A and C for the reduction of ISP_{NAP}. Curve 1 shows the oxidized spectrum of ISP_{NAP} (20.1 nmol) in 1.0 ml of Tris-hydrochloride buffer, pH 7.8, under anaerobic conditions. Curve 2 shows the spectrum of ISP_{NAP} after the addition of component A (50 μg of protein) and NADH (0.5 μmol). Curves 3 to 17 show spectral changes recorded at 30-s intervals after the addition of component C (60 μg of protein).

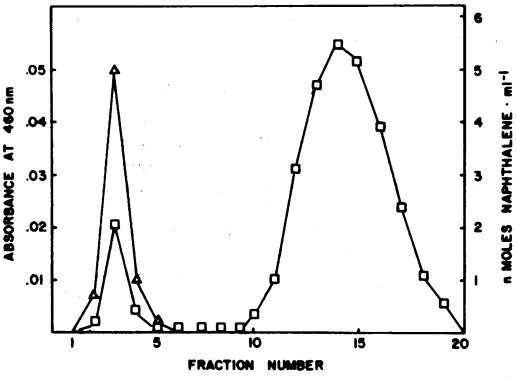

FIG. 5. Binding of [^{14}C]naphthalene to oxidized ISP_{NAP}. Experimental conditions are described in the text. Symbols: △, ISP_{NAP}, absorbance at 460 nm; □, radioactive naphthalene.

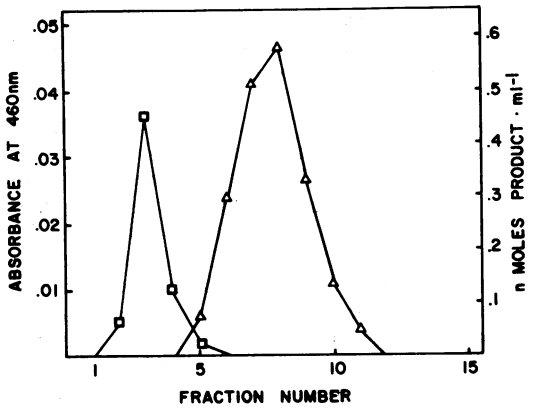

FIG. 6. Oxidation of enzyme-bound naphthalene in the presence of NADH and components A and C. Symbols: □, ISP_{NAP}, absorbance at 460 nm; △, radioactive metabolite. Experimental conditions are described in the text.

After incubation of the enzyme-bound naphthalene with component A, component C, and NADH, followed by rechromatography, the results shown in Fig. 6 were obtained. All of the naphthalene was converted to a product which no longer coeluted with the protein, but eluted sooner than did unreacted naphthalene. Analysis of this compound by thin-layer chromatography revealed that it comigrated with *cis*-naphthalene dihydrodiol.

DISCUSSION

Naphthalene dioxygenase has been shown to be a multicomponent enzyme system which catalyzes the NADH-dependent oxidation of naphthalene to *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (12). Rapid purification of the separate components in this system is essential due to the reported instability of the enzyme (7). The purification scheme presented in this report resulted in the isolation of the terminal component of the naphthalene system in purified form within 4 days. Stability of the enzyme during purification required the presence of 10% ethanol and 10% glycerol (12). The same solvents have been used during the purification of the terminal component of toluene dioxygenase (20).

Purified ISP_{NAP} has several properties in common with the terminal components of the benzene (2, 10), pyrazon (19), and toluene (13, 20) dioxygenase systems. All of these proteins are colored with absorption maxima in the visible region near 550 and 450 nm. The enzymatic reduction of the terminal component in ISP_{NAP} resulted in the loss of absorption at 566 and 462 nm, with the appearance of new maxima at 520 and 380 nm. Similar results were observed with the pyrazon and benzene dioxygenases and the terminal monooxygenase component of the 4-methoxybenzoate O-demethylase system (3–5).

The enzymatic reduction of ISP_{NAP} required the presence of components A and C and NADH. Complete reduction of the protein resulted after the addition of an equimolar amount of NADH, as has been observed with benzene and toluene dioxygenases (10, 13), indicating that two electrons are required for complete reduction. The absorption maxima at 380 and 520 nm, characteristic of the reduced protein, did not begin to appear until after half of the total NADH had been added (Fig. 2, lines 6 to 10). These results suggest that a nonequivalent reduction of iron-sulfur centers occurs in this protein.

The native and subunit molecular weights of ISP_{NAP} are similar to those reported for toluene dioxygenase (13, 20), whereas the pyrazon and benzene dioxygenases have molecular weights of 180,000 and 215,000, respectively.

Oxidized ISP_{NAP} will bind stoichiometric amounts of naphthalene, whereas the very similar toluene dioxygenase must be reduced before substrate binding can occur (20). The monooxygenase in the 4-methoxybenzoate O-demethylase system will also bind a substrate when the enzyme is oxidized (4), but in marked contrast to this enzyme, ISP_{NAP} does not undergo any spectral changes after incubation with naphthalene. Apparently, substrate binding by ISP_{NAP} does not result in conformational changes which affect the iron-sulfur chromophores in this protein. Complete oxidation of enzyme-bound naphthalene to *cis*-naphthalene dihydrodiol after incubation with components A and C and NADH demonstrates that the bound naphthalene is associated with the catalytic site on ISP_{NAP} and that all of the bound substrate can be oxidized to product. Substrate binding by

ISP_{NAP} is reversible, since enzyme-bound, [^{14}C]naphthalene can be replaced by unlabeled naphthalene in exchange experiments (unpublished data).

ISP_{NAP} was found to contain 5.6 g-atoms of iron and 4.0 g-atoms of acid-labile sulfur per enzyme molecule. This is the highest iron content reported for any of the dioxygenase iron-sulfur proteins. The terminal components of the benzene (10) and toluene (13, 20) dioxygenase systems each contain four atoms of iron and acid-labile sulfur per enzyme molecule, whereas pyrazon dioxygenase contains 1.96 to 2.33 mol of iron and 1.6 mol of inorganic sulfur per mol of enzyme (19). The iron contents reported for benzene, toluene, and pyrazon dioxygenase may not reflect the iron content of the catalytically active protein, since all of these dioxygenases require the addition of exogenous iron to reaction mixtures for maximum activity. In contrast, naphthalene dioxygenase activity is only minimally stimulated by exogenous iron (12).

Putidamonoxin, the monooxygenase in the 4-methoxybenzoate O-demethylase system, has been reported to contain equimolar amounts of iron and acid-labile sulfur (3, 5). Bernhardt and Meisch have recently proposed that this protein contains, in each active site, a 2Fe-2S center and an additional iron bound by a mercaptide group (4). The extra iron is essential for catalytic activity in the proposed model. An analogous catalytic system in ISP_{NAP}, with two iron-sulfur (2Fe-2S) chromophores in each enzyme molecule, would result in a protein which contains six atoms of iron and four atoms of acid-labile sulfur per molecule. Additional studies of the iron-sulfur centers in ISP_{NAP} will be necessary before the interaction of iron and sulfur in the catalytic center(s) of this molecule can be resolved.

The data presented in this study confirm the presumed arrangement of the components in the naphthalene dioxygenase system. Component A will oxidize NADH in the presence of an electron acceptor such as cytochrome *c*, whereas components B and C do not oxidize NADH (12). The requirement of both components A and C for the reduction of ISP_{NAP} and the binding of naphthalene by ISP_{NAP} indicate that the flow of electrons in the naphthalene dioxygenase system follows the pattern: NADH $\rightarrow$ A $\rightarrow$ C $\rightarrow$ ISP_{NAP}. Reduced ISP_{NAP} reacts with bound naphthalene and oxygen to form *cis*-naphthalene dihydrodiol.

The apparent stability of catalytic iron in the terminal component of naphthalene dioxygenase makes this protein particularly useful for studies of the interactions between iron, oxygen, and substrate which are involved in dioxygen fixation. The availability of purified ISP_{NAP} will greatly facilitate efforts to elucidate the mechanism of dioxygenation reactions in microbial enzyme systems.

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