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Purification and Properties of NADH-Ferredoxin$_{NAP}$ Reductase, a Component of Naphthalene Dioxygenase from *Pseudomonas* sp. Strain NCIB 9816

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Cells of *Pseudomonas* sp. strain NCIB 9816, after growth with naphthalene or salicylate, contain a multicomponent enzyme system that oxidizes naphthalene to *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. We purified one of these components to homogeneity and found it to be an iron-sulfur flavoprotein that loses the flavin cofactor during purification. Dialysis against flavin adenine dinucleotide (FAD) showed that the enzyme bound 1 mol of FAD per mol of enzyme protein. The enzyme consisted of a single polypeptide with an apparent molecular weight of 36,300. The purified protein contained 1.8 g-atoms of iron and 2.0 g-atoms of acid-labile sulfur and showed absorption maxima at 278, 340, 420, and 460 nm, with a broad shoulder at 540 nm. The purified enzyme catalyzed the reduction of cytochrome *c*, dichlorophenolindophenol, Nitro Blue Tetrazolium, and ferricyanide. These activities were enhanced in the presence of added FAD. The ability of the enzyme to catalyze the reduction of the ferredoxin involved in naphthalene reduction and other electron acceptors indicates that it functions as an NAD(P)H-oxidoreductase in the naphthalene dioxygenase system. The results suggest that naphthalene dioxygenase requires two proteins with three redox groups to transfer electrons from NADH to the terminal oxygenase.

Naphthalene dioxygenase, a multicomponent enzyme system which oxidizes naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene, is induced in *Pseudomonas* sp. strain NCIB 9816 during growth on naphthalene or salicylate (2, 12, 20). After growth on naphthalene, the organism also oxidizes acenaphthalene (31), indole (13), and indan (39). Indan is apparently oxidized by naphthalene dioxygenase in a monooxygenation reaction similar to that described for toluene dioxygenase (39). This suggests that naphthalene dioxygenase can function as either a monooxygenase or a dioxygenase, depending on the substrate.

Naphthalene dioxygenase consists of a terminal iron-sulfur-containing oxygenase (ISP$_{NAP}$) and two other proteins which function as a short electron transfer chain (11, 12). Thus, naphthalene dioxygenase is similar to the three-component enzyme systems involved in the oxidation of benzene (1, 16), toluene (32–34), and pyrazon (29). Each of these enzyme systems requires a flavoprotein reductase and a ferredoxin to transfer electrons to a terminal dioxygenase which converts its aromatic substrate to a *cis*-dihydrodiol. Previous studies indicated that one of the three protein components of naphthalene dioxygenase functioned as an NAD(P)H oxidoreductase (11, 12) that reduced cytochrome *c* without the requirement for an additional protein. This suggested that the reductase component of naphthalene dioxygenase was similar to the iron-sulfur flavoproteins that function in the two-component benzoate dioxygenase (41–43), phthalate dioxygenase (3), and 4-methoxybenzoate *O*-demethylase (5, 6) enzyme systems. The requirement for an iron-sulfur flavoprotein in naphthalene dioxygenase suggests that it has characteristics in common with a wide range of two- and three-component oxygenase systems.

In a separate report we presented the purification and properties of a small iron-sulfur protein (ferredoxin$_{NAP}$) that functions as the intermediate electron carrier in naphthalene dioxygenation (20). Here we describe the purification and properties of ferredoxin$_{NAP}$ reductase and present evidence that this enzyme is an iron-sulfur flavoprotein that transfers electrons from NAD(P)H to ferredoxin$_{NAP}$.

(A preliminary report of this work has been presented [B. E. Haigler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, K101, p. 163].)

MATERIALS AND METHODS

Growth of cells. *Pseudomonas* sp. strain NCIB 9816 was provided by W. C. Evans, The University College of North Wales, Bangor, Wales. The organisms were maintained and grown as described previously (12). Large quantities of cells were obtained by transferring two 10-liter log-phase cultures of naphthalene-grown cells into 180 liters of a minimal salts medium. Cultures were incubated at 30°C in a 250-liter fermentor (New Brunswick IF 250) containing 180 liters of a sterile minimal salts medium (pH 7.2) containing (per liter) 2.0 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 10.0 mg of $FeSO_4 \cdot 7H_2O$, with 0.05% yeast extract and 0.1% arginine. Cells were grown at 30°C with constant stirring at 150 rpm with air supplied at a rate of 180 liters/min. When the A_{600} reached 0.3, sterile sodium salicylate was added to give a final concentration of 0.025% (wt/vol). Salicylate was provided as an inducer and growth substrate in order to circumvent the problem of possible damage to the fermentation equipment by the use of a highly volatile compound such as naphthalene. Salicylate has been shown to be an inducer of the naphthalene dioxygenase system (2). As growth proceeded, sodium salicylate was added in similar concentrations until the time of cell harvest.

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By that time, 0.2% (wt/vol) sodium salicylate had been added. After 12 h of growth, the cells were harvested at an A_{600} of 1.2 with a Sharples (type AS 16) centrifuge. The cell paste was weighed and stored at −20°C until used.

Purification of NADH-ferredoxin$_{NAP}$ reductase. All procedures were carried out at 4°C, in TEG buffer (50 mM Tris hydrochloride buffer [pH 7.5] containing 10% [vol/vol] ethanol, 10% [vol/vol] glycerol, and 0.5 mM dithiothreitol) (11, 12). Cell extract was prepared from 50 g of frozen cells as described previously (11), and the volume was adjusted to 150 ml with TEG buffer prior to use.

Crude cell extract (7.05 g of protein) was applied to a Blue Sepharose CL-6B column (2.6 by 28 cm) previously equilibrated with TEG buffer. ISP$_{NAP}$ and ferredoxin$_{NAP}$ did not bind to the column and were collected as a single fraction. The column was washed with 800 ml of TEG buffer, and proteins were eluted with a continuous KCl gradient (0.0 to 220 mM) in 1 liter of buffer. Fractions (8.5 ml) were collected and assayed for cytochrome *c* reductase activity and A_{280}. Fractions exhibiting maximum activity in the presence of flavin adenine dinucleotide (FAD) were pooled and concentrated by ultrafiltration over an Amicon PM-10 filter under nitrogen at 35 lb/in^2. The salt concentration was diluted by the addition of two buffer changes of equal volume at the time of concentration. The concentrated solution was applied to a DEAE-cellulose column (2.3 by 23 cm). The column was washed with 500 ml of TEG buffer, followed by elution with 500 ml of a continuous KCl gradient (0 to 120 mM). Fractions exhibiting maximum cytochrome *c* reductase activity were combined, dialyzed, and concentrated by ultrafiltration prior to storage at −20°C. The purification of ferredoxin$_{NAP}$ and ISP$_{NAP}$ after Blue Sepharose CL-6B chromatography is described elsewhere (20).

Enzyme assays. Naphthalene dioxygenase activity was determined by measuring the rate of formation of [^{14}C] naphthalene dihydrodiol from [^{14}C]naphthalene as described previously (11). NADH-cytochrome *c* reductase activity was measured in the presence of 1.0 nmol of FAD as described previously (12). NADH-ferricyanide reduction, NADH-2,6-dichlorophenolindophenol reduction, and Nitro Blue Tetrazolium reduction were measured as described previously (33). The assay system for measuring these activities was the same as that described for measuring cytochrome *c* reduction except that cytochrome *c* was replaced by 0.2 μmol of ferricyanide, 0.1 μmol of 2,6-dichlorophenolindophenol, or 87 nmol of Nitro Blue Tetrazolium, respectively. One unit of activity was defined as the amount of NADH-ferredoxin$_{NAP}$ reductase required to reduce 1 μmol of the substrate per minute.

SDS-PAGE and molecular weight determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% resolving gel was carried out with the discontinuous system described by Laemmli (24). Standard proteins used for molecular weight determinations of ferredoxin$_{NAP}$ reductase were conalbumin, bovine serum albumin (BSA), ovalbumin, the alpha subunit of RNA polymerase, carbonic anhydrase, chymotrypsinogen A, trypsin inhibitor, and lysozyme. Proteins were stained by the method of Fairbanks et al. (14). For native gels, the procedures were similar except that SDS was omitted from the gels and the protein was not denatured prior to electrophoresis. Activity staining of native gels with Nitro Blue Tetrazolium was performed by cutting lanes from native polyacrylamide gels. The gel lanes were incubated at room temperature in 50 ml of the Nitro Blue Tetrazolium reduction assay mixture with 1 μM FAD. The gels were removed after color formation, rinsed once with buffer, and stored at 4°C for comparison with gels stained for protein.

The molecular weight of ferredoxin$_{NAP}$ reductase was determined by gel filtration on a Sephacryl S-200 superfine column (1.6 by 98 cm). The column was calibrated with cytochrome *c*, chymotrypsinogen A, carbonic anhydrase, ovalbumin, and BSA as described by Laurent and Killander (25).

The molecular weight of ferredoxin$_{NAP}$ reductase was determined by native PAGE by a modification of the method of Bryan (8) as described by Sigma Chemical Co. (Technical Bulletin MKR-137). Slab gel electrophoresis was accomplished with separating gels of 7, 8, 9, and 10% polyacrylamide. The protein standards used for molecular weight determinations were α-lactalbumin, carbonic anhydrase, ovalbumin, and BSA.

Iron and sulfur determinations. The iron content of ferredoxin$_{NAP}$ reductase was determined by using the iron-chelating reagent Ferene S [3-(2-pyridyl)-5,6-bis(2-(5-furyl sulfonic acid))-1,2,4,-triazine, disodium] by a procedure derived from Zabinski et al. (45). Stock Ferene S reagent solutions were prepared (18), and iron concentrations were determined as follows. Different concentrations of purified ferredoxin$_{NAP}$ reductase were diluted to 0.8 ml with TEG buffer, and 0.1 ml of 12 N HCl was added to each solution. The solution was incubated at room temperature with occasional shaking for 10 min, and 0.1 ml of 80% (wt/vol) trichloroacetic acid was added. Precipitated protein was removed by centrifugation, the supernatant was transferred to a glass tube, and 0.2 ml of 45% iron-free sodium acetate was added with mixing. Ferene S reagent (1.8 ml) was added, the tubes were agitated, and the A_{593} versus a buffer blank was determined. A standard curve for calibration of the Ferene S-iron complex was obtained by using stock iron solutions.

The amount of acid-labile sulfur was determined as described by Chen and Mortenson (9). Samples were incubated with the alkaline-zinc reagent for 2 h prior to addition of *N*,*N*-dimethyl-*p*-phenylenediamine and ferric chloride.

Flavin identification. The flavin cofactor of ferredoxin$_{NAP}$ reductase was dissociated from the enzyme, and the fluorescence spectrum of this solution was determined with an Amicno SPF 500 spectrofluorometer as described previously (33).

The FAD content in purified and reconstituted samples of the reductase was determined after precipitation of the protein by boiling for 10 min or addition of trichloroacetic acid. Protein was removed by centrifugation, and the flavin content of the resulting supernatant was determined spectrophotometrically as described previously (33).

Determination of protein concentration. Protein concentrations in cell extracts and protein solutions were determined by the method of Bradford (7) with BSA as the standard. For quantitative physical determinations, purified ferredoxin$_{NAP}$ reductase was lyophilized to dryness and used as the standard.

Isoelectric focusing. The isoelectric point of purified ferredoxin$_{NAP}$ reductase was determined by electrophoresis in tube gels and vertical slab gels (28). Electrophoresis was performed for 18 to 22 h at 4°C, with ampholytes of pH ranges 3 to 10 and 5 to 7. The location of the protein and the pH in duplicate gel lanes were determined as described previously (33).

Amino acid composition and NH_2-terminal amino acid analysis. The amino acid composition of ferredoxin$_{NAP}$ reductase was determined with a Beckman model 121MB

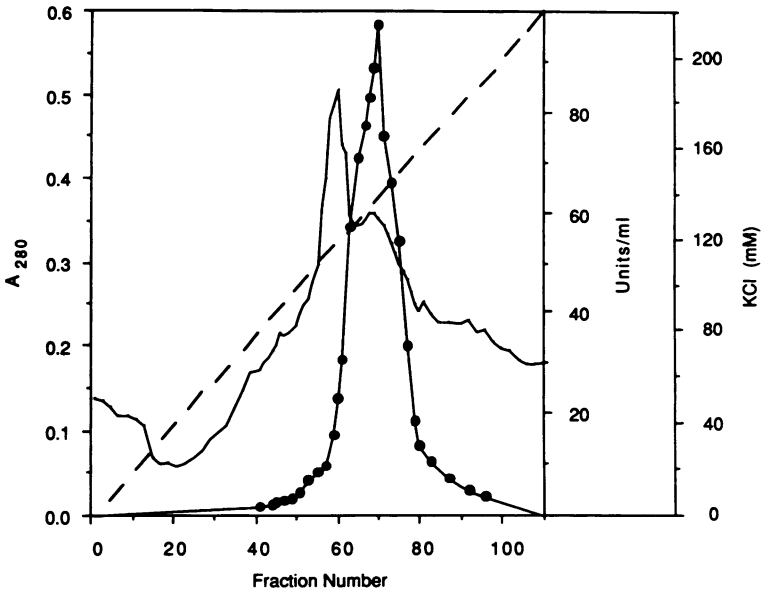

FIG. 1. Blue Sepharose CL-6B chromatography of ferredoxin$_{NAP}$ reductase. Proteins were separated by a 0.0 to 0.22 M KCl gradient (– – –), and fractions were tested for ferredoxin$_{NAP}$ reductase activity (●) and A_{280} (——).

amino acid analyzer. Samples were precipitated with 10% trichloroacetic acid. The precipitate was washed with deionized water and then hydrolyzed in 6 N HCl for 24, 48, and 72 h at 110°C in a vacuum. Tryptophan was destroyed by the hydrolysis procedure and was not determined. The cysteine residues of ferredoxin$_{NAP}$ reductase were determined by the spectrophotometric assay described by Ellman (10). Dithiothreitol was removed by dialysis prior to the assay. The NH_2-terminus of each protein was determined by Burt Ensley, Timothy Osslund, and Mary Simon at Amgen (Thousand Oaks, Calif.).

Chemicals and reagents. DEAE-cellulose (Whatman DE-52) was obtained from Whatman Ltd., Maidstone, Kent, England; Blue Sepharose CL-6B was obtained from Pharmacia Fine Chemicals, Piscataway, N.J.; Trizma base (Tris) and proteins used in molecular weight determinations were from Sigma Chemical Co., Rockford, Ill.; and [U-^{14}C]naphthalene (specific activity, 6.12 mCi/mmol) was obtained from Pathfinder Laboratories Inc., St. Louis, Mo. All other chemicals and reagents were those used in previous investigations (12, 33).

Analytical methods. Spectrophotometric measurements were made with either an Aminco DW-2A double-beam spectrophotometer or a Beckman model 25 spectrophotometer as described previously (11, 12). Scintillation counting was conducted with a Beckman LS6800 scintillation counter. Gel electrophoresis was carried out in a Bio-Rad Laboratories Protean Dual 16-cm slab gel apparatus.

RESULTS

Purification of ferredoxin$_{NAP}$ reductase. More than one protein capable of reducing cytochrome *c* was present in the crude cell extracts. However, only one of these was capable of supporting naphthalene dioxygenase activity. Therefore, purification data for the reductase are presented only for the Blue Sepharose CL-6B chromatography step and subsequent procedures. The Blue Sepharose CL-6B chromatography step separates the reductase component from the other two proteins essential for naphthalene dioxygenase activity and the majority of other contaminating proteins (Fig. 1). Final purification of the protein was accomplished by anion-exchange chromatography with DEAE-cellulose (Fig. 2). These procedures led to 74% recovery of the activity present after the Blue Sepharose CL-6B chromatography step (Table 1).

Properties of ferredoxin$_{NAP}$ reductase. PAGE in the presence and absence of SDS revealed a single band that stained for protein (Fig. 3). Staining for activity with Nitro Blue Tetrazolium in the presence of NADH and FAD resulted in the detection of a single band of activity that comigrated with ferredoxin$_{NAP}$ reductase in the native gels.

The molecular weight of purified ferredoxin$_{NAP}$ reductase was determined by gel filtration, SDS-PAGE, and native PAGE. These procedures gave molecular weights of 37,000, 36,000, and 34,900, respectively. Amino acid determinations of ferredoxin$_{NAP}$ reductase gave a molecular weight of 37,104 (Table 2). Consequently, an average molecular weight of 36,300 was used in subsequent calculations. The isoelectric point of ferredoxin$_{NAP}$ reductase on ampholyte gels (pH 5 to 7) was pH 6.3.

Solutions of purified ferredoxin$_{NAP}$ reductase were red and gave absorption maxima at 278, 340, 420, and 460 nm, with a broad shoulder at 540 nm (Fig. 4). The molar extinction coefficients at 340, 420, and 460 nm were calculated to be 23.7, 15.5, and 14.9 mM^{-1} cm^{-1}, respectively.

The purified enzyme was unstable at room temperature. It lost 50% of its cytochrome *c* reductase activity within the first 8 h. At 0 to 5°C, the purified enzyme retained 70% of its cytochrome *c* reductase activity after 5 days. The protein could be stored at −20°C for up to 1 month with a minimal loss of activity. However, storage of the protein for periods in excess of 1 month often resulted in precipitation of the protein upon warming to temperatures above 5°C.

Amino acid composition and NH_2-terminal sequence. The amino acid composition of ferredoxin$_{NAP}$ reductase is shown in Table 2. Determination of the number of sulfhydryl groups by the use of 5,5′-dithiobis(2-nitrobenzoate) (10) indicated that there were six cysteine residues in the protein.

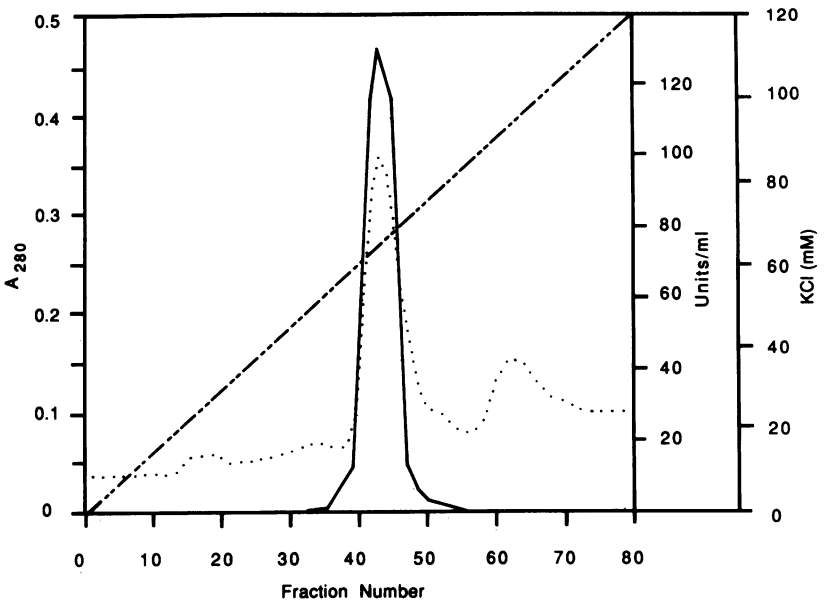

FIG. 2. DEAE-cellulose chromatography of ferredoxin$_{NAP}$ reductase. Proteins were separated by a 0.0 to 0.12 M KCl gradient (- - - - -), and fractions were tested for ferredoxin$_{NAP}$ reductase activity (——) and A_{280} (· · · ·).

The amino acid sequence of the NH_2-terminal end of ferredoxin$_{NAP}$ reductase was Met-Glu-Leu-Leu-Ile-Gln-Pro-Asn-Asn-Arg-Ile-Ile-Pro-Phe-Ser-Ala-Gly-Ala-Asn-Leu-Leu-(Cys)-Val-Leu-Arg-. The parentheses indicate a questionable identification. The NH_2-terminal sequence of the *nahA* gene product from plasmid NAH7 (30), for comparison, is Met-Glu-Leu-Leu-Ile-Gln-Pro-Asn-Asn-Arg-Leu-Ile-Ser-Phe-Ser-Pro-Gly-Ala-Asn-Leu-Leu-Glu-. The sequences exhibit homology with differences at only four positions out of the 22 amino acids sequenced for the *nahA* gene product. This portion of the *nahA* gene product has been putatively identified as the NH_2-terminal end of the naphthalene dioxygenase enzyme system (30).

Effect of inhibitors. The effect of several electron transfer inhibitors on cytochrome *c* reduction by ferredoxin$_{NAP}$ reductase was tested as described previously (33). Of the three sulfhydryl inhibitors tested, *p*-chloromercuribenzoate was the most effective in inhibiting cytochrome *c* reduction by ferredoxin$_{NAP}$ reductase (Table 3). Inhibition with *o*-phenanthroline suggested that iron may play a catalytic role in the purified enzyme. Iron and acid-labile sulfur analysis of ferredoxin$_{NAP}$ reductase revealed the presence of 1.8 g-atoms of iron and 2.0 g-atoms of acid-labile sulfur per molecule of enzyme.

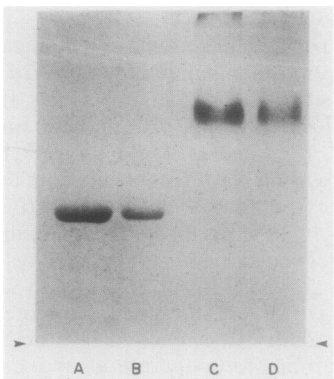

FIG. 3. PAGE of purified ferredoxin$_{NAP}$ reductase. The purity of ferredoxin$_{NAP}$ reductase was examined on polyacrylamide gels in the presence (A and B) and absence (C and D) of SDS. Protein concentrations were 20 μg (A and C) and 10 μg (B and D). The arrows indicate the tracking dye front.

Electron acceptors. In the presence of NADH, the purified enzyme catalyzed the direct reduction of 2,6-dichlorophenolindophenol, ferricyanide, and Nitro Blue Tetrazolium as well as cytochrome *c* (Table 4). FAD markedly enhanced the activity with all four electron acceptors. NADPH was also effective as an electron donor but yielded only 39% of the activity obtained with NADH in the cytochrome *c* reductase assay.

Flavin content of ferredoxin$_{NAP}$ reductase. Naphthalene dioxygenase activity was observed only in the presence of ferredoxin$_{NAP}$ reductase, ISP$_{NAP}$, and ferredoxin$_{NAP}$. The effect of varying the concentration of ferredoxin$_{NAP}$ reductase in the naphthalene dioxygenase assay is shown in Fig. 4.

Previous results with partially purified ferredoxin$_{NAP}$ reductase indicated that ferredoxin$_{NAP}$ reductase-dependent cytochrome *c* reductase activity and naphthalene dioxygenase activity were stimulated in the presence of flavins (12). Addition of FAD or flavin mononucleotide (FMN) to cytochrome *c* reductase assay mixtures containing ferredoxin$_{NAP}$ reductase purified in this investigation stimulated

TABLE 1. Purification of ferredoxin$_{NAP}$ reductase[a]

Purification step	Protein (mg)	Activity (U)	Sp act (U/mg)	Recovery (%)
1. Crude cell extract	7,050			
2. Blue Sepharose CL-6B chromatography	75	9,600	128	100
3. DEAE-cellulose chromatography	18	7,150	397	74

[a] Details of the purification procedures are described in the text. One unit of enzyme activity is defined as the amount of ferredoxin$_{NAP}$ reductase required to reduce 1.0 μmol of cytochrome *c* per minute.

TABLE 2. Amino acid composition of NADH-ferredoxin$_{NAP}$ reductase

Amino acid	No. of residues/mol of enzyme	Amino acid	No. of residues/mol of enzyme
Lysine	10	Histidine	11
Arginine	19	Serine[a]	21
Threonine[a]	19	Proline	21
Glycine	35	Alanine	29
Half-cystine[b]	6	Valine	36
Methionine[c]	6	Isoleucine	24
Leucine	35	Tyrosine	11
Phenylalanine	10	Tryptophan	ND[d]
Aspartate + asparagine	25	Glutamate + glutamine	32

[a] Determined by extrapolation to zero time of hydrolysis.
[b] Determined after 24 h of hydrolysis.
[c] Determined as methionine sulfone.
[d] ND, Not determined.

TABLE 3. Effect of inhibitors on ferredoxin$_{NAP}$ reductase activity

Compound added	Concn (M)	Inhibition (%)[a]
p-Chloromercuribenzoate	5×10^{-8}	18
	1×10^{-7}	60
	5×10^{-7}	94
Iodoacetate	5×10^{-3}	15
	1×10^{-2}	50
N-Ethylmaleimide	1×10^{-4}	7
	2×10^{-3}	30
	1×10^{-2}	67
Sodium azide	1×10^{-2}	21
	2×10^{-2}	27
	4×10^{-2}	46
o-Phenanthroline	1×10^{-3}	3
	5×10^{-3}	26
	1×10^{-2}	63

[a] The activity of the uninhibited enzyme was taken as 0% inhibition.

activity more than 100-fold. Cytochrome *c* reductase activity in the presence of FAD was approximately 1.7 times the activity observed with FMN. Ferredoxin$_{NAP}$ reductase-dependent naphthalene dioxygenase activity was also stimulated in the presence of FAD or FMN, but not riboflavin. In naphthalene dioxygenase assays, the activity with FAD was 1.9 times that observed with FMN. Spectrofluorometric studies of the isolated prosthetic group revealed the presence of a small amount of a flavin cofactor. However, the amounts observed were too low to allow identification of the flavin bound by the protein, which suggested that the enzyme lost most of its flavin cofactor during the purification procedures.

The apparent dissociation constants of ferredoxin$_{NAP}$ reductase for FAD and FMN in the cytochrome *c* reductase assay were determined by the method described by Beadle and Smith (4). A Lineweaver-Burk plot of the results gave an apparent K_d for FAD of 0.29 μM. Identical experiments performed with FMN gave an apparent K_d of 0.35 μM. These experiments indicate that the enzyme can utilize both flavin cofactors but exhibits a slightly higher affinity for FAD.

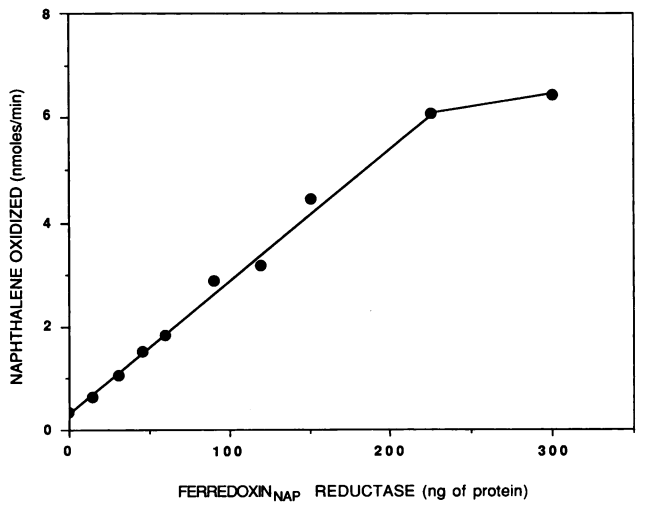

FIG. 4. Requirement of ferredoxin$_{NAP}$ reductase for naphthalene dioxygenase activity. Naphthalene dioxygenase activity was determined as described previously (11), with partially purified ISP$_{NAP}$ (55 μg), partially purified ferredoxin$_{NAP}$ (23 μg), and the indicated amounts of purified ferredoxin$_{NAP}$ reductase.

When purified protein was dialyzed against FAD and then dialyzed against buffer to remove unbound flavin, the absorption spectrum of the protein exhibited a maximum at 460 and shoulders at 325 to 435, 485, and 550 nm (Fig. 5). Dialysis of the reconstituted enzyme for an additional 12 h against TEG buffer resulted in a slight decrease in the FAD content. The difference spectrum between the flavin-reconstituted enzyme and purified protein gave a spectrum typical of a flavoprotein, with absorbance maxima at 395 and 457 nm and a shoulder at 480 nm. The anaerobic addition of a small amount of sodium dithionite to the reconstituted enzyme resulted in a total bleaching of the spectrum. The original spectrum returned upon exposure of the enzyme to air. Similar results were obtained with purified enzyme without flavin reconstitution.

When the flavin cofactor was restored to the purified protein as described above, the molar ratio of FAD to protein increased from less than 0.15 mol of FAD per mol of protein to approximately 1 mol of FAD per mol in the flavin-reconstituted protein. The cytochrome *c* reductase activity of the enzyme also increased from less than 1 U/mg of protein to about 100 U/mg of protein in the reconstituted protein. Addition of FAD to cytochrome *c* reductase assays with the flavin-reconstituted enzyme still resulted in a two- to threefold increase in activity.

Reduction of ferredoxin$_{NAP}$ by ferredoxin$_{NAP}$ reductase. Ferredoxin$_{NAP}$ has been postulated to serve as an electron acceptor for ferredoxin$_{NAP}$ reductase in the naphthalene dioxygenase enzyme system (11, 12). Purified preparations of ferredoxin$_{NAP}$ reductase were low in flavin content and did not catalyze transfer of electrons from NADH to ferre-

TABLE 4. Activity of ferredoxin$_{NAP}$ reductase in the presence of different electron acceptors

Electron acceptor	Sp act (U/mg)	
	No FAD	With 1 nmol of FAD
Cytochrome *c*	0.26	397
Ferricyanide	1.71	1,225
2,6-Dichlorophenolindophenol	0.14	417
Nitro Blue Tetrazolium	0.04	4

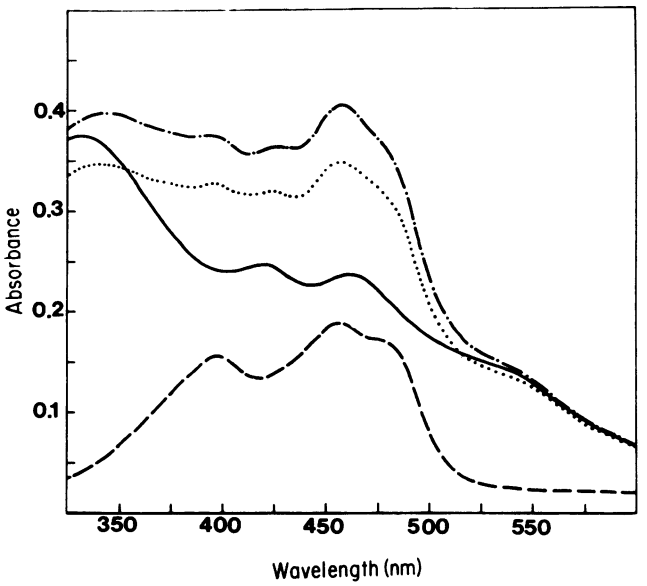

FIG. 5. FAD reconstitution of ferredoxin$_{NAP}$ reductase. The spectrum of purified ferredoxin$_{NAP}$ reductase (15 nmol/ml) was analyzed before (——) and after dialysis against FAD (13.9 μM) (— · —) and after additional dialysis for 12 h against TEG buffer (· · · · ·). The difference spectrum between flavin-reconstituted protein (sample) and purified enzyme (reference) is also shown (– – –).

doxin$_{NAP}$ without the addition of FAD. In the presence of FAD, ferredoxin$_{NAP}$ reductase catalyzed the transfer of electrons from NADH to ferredoxin$_{NAP}$ (Fig. 6). Reduction of ferredoxin$_{NAP}$ resulted in a loss of the 460-nm absorption peak and the appearance of a new peak at 382 nm. These spectral changes were similar to those observed when ferredoxin$_{TOL}$ is reduced by ferredoxin$_{TOL}$ reductase and NADH (33). Exposure of the anaerobic mixture to air resulted in a return of the original spectrum. Control experiments in which either FAD, NADH, or ferredoxin$_{NAP}$ reductase was omitted showed that there were no spectral changes in the ferredoxin$_{NAP}$ solution until the appropriate component was added.

DISCUSSION

A common theme among multicomponent oxygenase systems is the use of short electron transport chains to catalyze electron transfer from an external donor, usually NAD(P)H, to a terminal iron-containing oxygenase. Two redox centers are employed in such electron transfer chains. Systems containing three protein components often use two different proteins as redox centers. Thus, toluene dioxygenase (32–34), pyrazon dioxygenase (29), benzene dioxygenase (1, 16), camphor monooxygenase (23), and the alkane hydroxylase from *Pseudomonas oleovorans* (27) contain two proteins which function in the transfer of electrons to the terminal oxygenase. Two-component systems utilize a single protein to maintain the two required redox centers. Such systems include phthalate dioxygenase (3), benzoate-1,2-dioxygenase (41–43), and 4-methoxybenzoate-*O*-demethylase (5, 6). Evidence from this investigation suggests that naphthalene dioxygenase is a unique multicomponent oxygenase that uses two proteins containing three redox centers to transfer electrons to the terminal oxygenase.

The multicomponent oxygenases discussed above contain a flavoprotein component which is essential for catalytic activity. This protein accepts reducing equivalents from NAD(P)H and catalyzes the transfer of electrons to a second redox center. Results from this investigation indicate that ferredoxin$_{NAP}$ reductase contains a loosely bound molecule of FAD which is readily lost during purification.

The loss of the flavin cofactors from putidaredoxin and NADH-ferredoxin$_{TOL}$ reductase has been reported previously (19, 33). Although the presence of 5% glycerol minimizes the loss of FAD from putidaredoxin, this is not the case with ferredoxin$_{NAP}$ reductase. In this respect, ferredoxin$_{NAP}$ reductase is similar to ferredoxin$_{TOL}$ reductase,

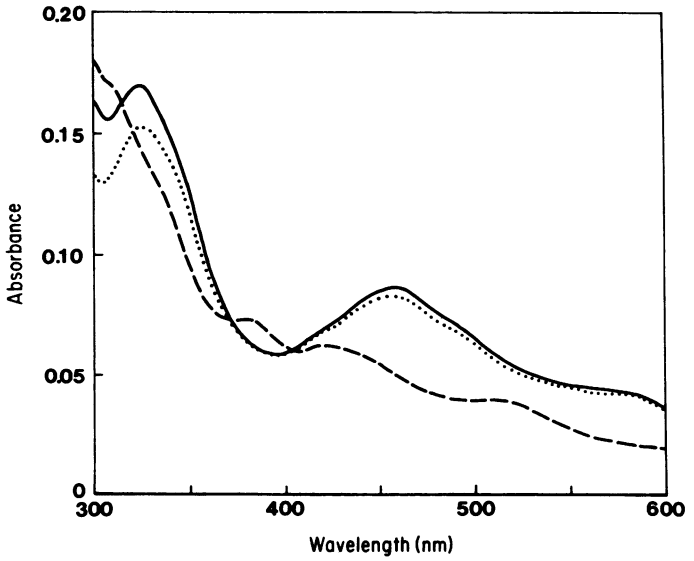

FIG. 6. Reduction of ferredoxin$_{NAP}$ by ferredoxin$_{NAP}$ reductase. A ferredoxin$_{NAP}$ solution (43 nmol in 3 ml of TEG buffer) was made anaerobic by alternately evacuating the air and flushing the cuvette with argon which had been passed over a hot copper coil. Ferredoxin$_{NAP}$ reductase (0.55 nmol) and FAD (0.1 nmol) were added (——), and spectral changes after addition of NADH (25 nmol) (– – –) and after 10 min of exposure to air (· · · · ·) were recorded.

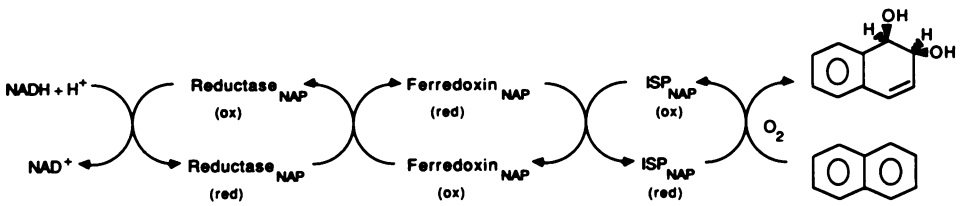

FIG. 7. Proposed electron transport scheme for naphthalene dioxygenase. ox, Oxidized; red, reduced.

which also loses significant amounts of its flavin cofactor during purification in the presence of glycerol (33).

The reduction of cytochrome *c* by flavoproteins requires a second prosthetic group. Typically, this second group can undergo only a one-electron change between its oxidized and reduced forms (22). This second prosthetic group may reside on a separate polypeptide, as in the toluene dioxygenase (17, 33, 34), ω-hydroxylase (36, 37), and the spinach ferredoxin (15, 35) electron transfer systems. In these systems, a flavoprotein functions as the initial electron acceptor that transfers electrons to a second protein of the ferredoxin type. Both proteins are required for cytochrome *c* reductase activity. Alternatively, the second prosthetic group may be arranged on the same polypeptide, as in the reductase components of the benzoate-1,2-dioxygenase (41, 43), the 4-methoxybenzoate-*O*-demethylase (5, 6), and the NADPH-cytochrome *c* (P_{450}) systems (21, 38). In this arrangement, the reductase components are capable of reducing cytochrome *c* directly in the presence of NAD(P)H. Loss of cytochrome *c* reductase activity upon removal of the iron-sulfur clusters from 4-methoxybenzoate-*O*-demethylase (6) and benzoate-1,2-dioxygenase (43) indicates that the iron-sulfur center is involved in electron transfer to cytochrome *c*. Ferredoxin$_{NAP}$ reductase catalyzes cytochrome *c* reduction independently, which suggests that it contains a second prosthetic group.

Purified ferredoxin$_{NAP}$ reductase is a red protein with spectrophotometric properties similar to those of spinach ferredoxin (40) and the iron-sulfur center of component C from methane monooxygenase (26). The absorption spectrum of the purified protein (Fig. 5) is similar to spectra reported for iron-sulfur proteins containing a [2Fe-2S] cluster of the plant-type ferredoxin (26, 35). The spectral characteristics of ferredoxin$_{NAP}$ reductase suggest that the iron and sulfur present in this protein are arranged in a [2Fe-2S] cluster of the ferredoxin type. Thus, ferredoxin$_{NAP}$ reductase appears to be an iron-sulfur flavoprotein similar to the flavoproteins observed in phthalate oxygenase (3), benzoate-1,2-dioxygenase (41, 43), methane monooxygenase (26), and 4-methoxybenzoate monooxygenase systems (6). These proteins contain a [2Fe-2S] cluster in addition to their flavin prosthetic group. Unlike these proteins, however, ferredoxin$_{NAP}$ reductase requires an additional protein in order to transfer electrons to the terminal iron-sulfur oxygenase.

Pseudomonas putida G7 contains a plasmid (NAH7) which encodes the catabolic genes enabling this organism to utilize naphthalene as a carbon source (44). The NH_2-terminal sequence of the naphthalene dioxygenase enzyme system from NAH7 has been identified on the basis of DNA sequencing studies and the location of the transcription start site (30). Twenty-two amino acids of the naphthalene dioxygenase system encoded by the NAH7 genome have been sequenced. With the exception of four amino acids, the sequence is identical to that of the NH_2-terminus of ferredoxin$_{NAP}$ reductase. The results suggest that the naphthalene dioxygenase systems of *P. putida* G7 and *Pseudomonas* sp. strain NCIB 9816 are related.

The results of this investigation support the proposed arrangement of the components in the naphthalene dioxygenase system (11). Ferredoxin$_{NAP}$ reductase will catalyze electron transfer from an electron donor, such as NADH or NADPH, to a variety of one- and two-electron acceptors. Ferredoxin$_{NAP}$ and ISP$_{NAP}$ do not oxidize NADH (11). Ferredoxin$_{NAP}$ can be reduced anaerobically by ferredoxin$_{NAP}$ reductase in the presence of FAD and NADH. The requirement for both ferredoxin$_{NAP}$ reductase and ferredoxin$_{NAP}$ for the reduction of ISP$_{NAP}$ and the binding of naphthalene by ISP$_{NAP}$ (11) indicates that electron flow proceeds as depicted in Fig. 7.

Our results indicate that naphthalene dioxygenase contains a unique and complex electron transfer system requiring three redox centers to transfer electrons from NADH to the terminal oxygenase. Study of this complex protein and the function of each redox center are currently under investigation.

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