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Benzene-Induced Uncoupling of Naphthalene Dioxygenase Activity and Enzyme Inactivation by Production of Hydrogen Peroxide

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Naphthalene dioxygenase (NDO) is a multicomponent enzyme system that oxidizes naphthalene to (+)-*cis*-(1*R*,2*S*)-1,2-dihydroxy-1,2-dihydronaphthalene with consumption of O_2 and two electrons from NAD(P)H. In the presence of benzene, NADH oxidation and O_2 utilization were partially uncoupled from substrate oxidation. Approximately 40 to 50% of the consumed O_2 was detected as hydrogen peroxide. The rate of benzene-dependent O_2 consumption decreased with time, but it was partially increased by the addition of catalase in the course of the O_2 consumption by NDO. Detailed experiments showed that the total amount of O_2 consumed and the rate of benzene-induced O_2 consumption increased in the presence of hydrogen peroxide-scavenging agents, and further addition of the terminal oxygenase component (ISP_{NAP}) of NDO. Kinetic studies showed that ISP_{NAP} was irreversibly inactivated in the reaction that contained benzene, but the inactivation was relieved to a high degree in the presence of catalase and partially relieved in the presence of 0.1 mM ferrous ion. Benzene- and naphthalene-reacted ISP_{NAP} gave almost identical visible absorption spectra. In addition, hydrogen peroxide added at a range of 0.1 to 0.6 mM to the reaction mixtures inactivated the reduced ISP_{NAP} containing mononuclear iron. These results show that hydrogen peroxide released during the uncoupling reaction acts both as an inhibitor of benzene-dependent O_2 consumption and as an inactivator of ISP_{NAP}. It is proposed that the irreversible inactivation of ISP_{NAP} occurs by a Fenton-type reaction which forms a strong oxidizing agent, hydroxyl radicals (·OH), from the reaction of hydrogen peroxide with ferrous mononuclear iron at the active site. Furthermore, when [^{14}C]benzene was used as the substrate, *cis*-benzene 1,2-dihydrodiol formed by NDO was detected. This result shows that NDO also couples a trace amount of benzene to both O_2 consumption and NADH oxidation.

Dioxygenases which contain mononuclear iron and Rieske-type [2Fe-2S] clusters play a critical role in the bacterial aerobic degradation of aromatic hydrocarbons and many xenobiotic compounds. They are unique in terms of their ability to catalyze the enantiospecific addition of oxygen (O_2) to substrates with π-electron systems to form *cis*-dihydrodiols (11). For example, naphthalene dioxygenase (NDO; EC 1.14.12.12) from *Pseudomonas* sp. strain NCIB 9816-4 catalyzes the addition of O_2 to naphthalene to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) in a reaction that requires NAD(P)H (20, 21). In addition, NDO has been shown to catalyze other diverse oxidative reactions, including monohydroxylation, desaturation, sulfoxidation, N and O dealkylation, and vinyl group dihydroxylation, depending on the substrate (reviewed in reference 43). It has also been shown that NDO catalyzes an alcohol oxidation reaction that requires NADH and O_2 (28) and a partial uncoupling reaction with ethyl phenyl sulfide, methyl *p*-nitrophenyl sulfide, and acetophenone (27, 28). NDO is a multicomponent enzyme system (7) consisting of a 36.3-kDa iron-sulfur flavoprotein ($reductase_{NAP}$ [Rd_{NAP}]) which contains flavin adenine dinucleotide and a chloroplast-type [2Fe-2S] redox center (16), a 13.6-kDa iron-sulfur protein ($ferredoxin_{NAP}$ [Fd_{NAP}]) which contains a single Rieske-type [2Fe-2S] redox center (15), and a terminal oxygenase component (ISP_{NAP}) which has two different subunits (α = 55 kDa; β = 20 kDa) (6). Each α subunit contains a Rieske-type [2Fe-2S] redox center and a mononuclear iron binding site (22, 25, 50). Most preparations of ISP_{NAP} require exogenous iron for maximum activity (50), suggesting that mononuclear iron plays an important role in the reaction catalyzed by NDO. The β subunit was shown to be essential for activity but to play no major role in determining the substrate specificity of NDO (39, 40). ISP_{NAP} has been recently crystallized (30), and the crystal structure has been solved (25). It has been shown that ISP_{NAP} has an $\alpha_3\beta_3$ subunit structure and that mononuclear iron in the large subunit is coordinated by two histidine residues and one aspartate. The nucleotide sequences for all the structural genes of NDO have been determined (38, 46) and show that ligands are conserved throughout the family of NDO structural genes. The organization and electron transport of NDO are shown in Fig. 1. Several multicomponent enzyme systems that utilize oxygenase components similar to ISP_{NAP} have been described previously (2, 33).

Initial studies on the mechanism of action of cytochromes P-450 (P-450) have shown that certain substrates uncouple electron transfer from substrate oxygenation, and the resulting oxidase activity can lead to the formation of superoxide anion (47), hydrogen peroxide (12, 36), and water (1, 12). It has been reported in a few P-450-catalyzed reactions that hydrogen peroxide formed during the partial uncoupling reaction irreversibly inactivated the oxygenase components (23, 24). In addition, 4-methoxybenzoate monooxygenase is an oxygenase that contains [2Fe-2S] redox centers and mononuclear iron and produces hydrogen peroxide when incubated with different substrate analogs that can serve as partial or total uncoupling

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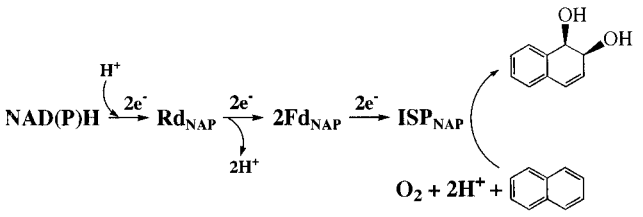

FIG. 1. Proposed electron transport chain for NDO leading to the oxidation of naphthalene to *cis*-naphthalene dihydrodiol by the terminal oxygenase component, ISP_{NAP}. e^- represents the number of electrons transferred at a time.

agents (54). However, the effects of hydrogen peroxide formed during the uncoupling reaction have not been determined.

In the present study, benzene is shown to be a partial uncoupling substrate for NDO, resulting in the formation of hydrogen peroxide and *cis*-benzene 1,2-dihydrodiol. Evidence also shows that hydrogen peroxide formed during the reaction acts both as an inhibitor of benzene-dependent O_2 consumption and as an inactivator of ISP_{NAP}.

(A preliminary report of this work has been published previously [29].)

MATERIALS AND METHODS

Materials. Catalase (19,900 U/mg) from bovine liver, Mn-containing superoxide dismutase (SOD; 3,300 U/mg) from *Escherichia coli*, 30% solution of hydrogen peroxide, [^{14}C]benzene (58.2 mCi/mmol), and [^{14}C]naphthalene (49.8 mCi/mmol) were obtained from Sigma (St. Louis, Mo.). FerroZine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid, monosodium salt hydrate, 97%] was obtained from Aldrich (Milwaukee, Wis.). Lactoperoxidase (72 U/mg) was obtained from Worthington Biochemical Co., Freehold, N.J. All commercially available chemicals were used without further purification. Deionized water and membrane-filtered (0.22-μm pore size) buffers were used for fast-performance liquid chromatography (FPLC; Pharmacia LKB).

Bacterial strains and growth conditions. *E. coli* JM109(DE3)(pDTG141) (49), which contains the cloned *nahAaAbAcAd* genes encoding the NDO components (Rd_{NAP}, Fd_{NAP}, and ISP_{NAP}) from *Pseudomonas* sp. strain NCIB 9816-4 (5), was used for the purification of Rd_{NAP}. *E. coli* JM109(DE3)(pDTG135) (51), which contains the cloned *nahAb* genes encoding the Fd_{NAP}, was used for the purification of Fd_{NAP}. *E. coli* JM109(DE3)(pDTG121) (51), which contains the cloned *nahAcAd* genes which encode the large and small subunits of ISP_{NAP}, was used for the purification of ISP_{NAP}. Growth conditions for the recombinant *E. coli* strains and expression of the genes have been described previously (31).

Purification of NDO components. Crude cell extracts were prepared as described previously (14). The buffer used for breakage was BTGD buffer (50 mM bis-Tris [pH 6.8], 5% glycerol, 1 mM dithiothreitol [DTT]) containing 0.1 mM phenylmethylsulfonyl fluoride and 1 μg of DNase/ml. All columns were operated with a Pharmacia FPLC system at 5°C. ISP_{NAP} was purified to homogeneity as described previously (30). Rd_{NAP} and Fd_{NAP} were purified to homogeneity (26) by modifications of the methods described previously (15, 16). BTGD buffer was used for the purification of all three NDO components.

Biotransformation with whole cells and GC-MS analysis. Biotransformation with isopropyl β-D-thiogalactopyranoside (IPTG)-induced cells of *E. coli* JM109 (DE3)(pDTG141) has been described previously (31). Fifty microliters of benzene was used for a 100-ml reaction volume. Ethyl acetate extracts were prepared and analyzed by gas chromatography-mass spectrometry (GC-MS) as described previously (44).

Oxygen uptake studies. The rates and stoichiometries of O_2 consumption by NDO with benzene and naphthalene were determined polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) as described previously (27). Each reaction mixture contained in 1.0 ml of 50 mM 2-(*N*-morpholino)ethanesulfonate (MES) buffer (pH 6.8), NADH (0.35 μmol), Rd_{NAP} (10.5 μg of protein), Fd_{NAP} (48 μg of protein), and ISP_{NAP} (40 μg of protein). Information about the addition of other components to the reaction mixtures is indicated in the figure legends. The initial slopes formed by the addition of each substrate were used to determine the initial rates of O_2 consumption by NDO.

Time course of ISP_{NAP} inactivation. Time course of ISP_{NAP} inactivation during reactions in the presence or absence of benzene was determined as follows. Reaction mixtures (0.5 ml) contained Rd_{NAP} (5.25 μg of protein), Fd_{NAP} (25.6 μg of protein), and ISP_{NAP} (52.5 μg of protein) in 50 mM MES (pH 6.8). When appropriate, catalase (1,000 U), SOD (82.5 U), benzene (0.1 mM), and Fe$(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 mM) were added into the reaction mixtures. Reactions were initiated by the addition of 10 μl of 50 mM NADH to give a final concentration of 1 mM. Reactions were conducted in capped 1-dram (3.7-ml) tubes at 22°C and incubated horizontally with shaking (150 rpm). At appropriate time points, aliquots of 10 μl (each) were withdrawn from the reaction mixtures and rapidly suspended in the following assay mixtures to determine the remaining ISP_{NAP} activity. The assay mixtures contained Rd_{NAP} (5.25 μg of protein), Fd_{NAP} (16 μg of protein), catalase (1,000 U), and Fe$(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 mM) in 50 mM MES (pH 6.8). The reactions were initiated by the addition of 20 μl of 5 mM [^{14}C]naphthalene to give a final concentration of 0.25 mM (1.11×10^6 dpm/ml), followed by the addition of 2 μl of 50 mM NADH. The final reaction volume was 0.4 ml. Aliquots of 10 μl (each) were withdrawn from the assay mixtures after 1, 2, or 3 min and quickly mixed with 15 μl of a quenching solution containing 25 mM nonradioactive naphthalene dissolved in methanol in a 600-μl Eppendorf tube. Samples were applied to plastic-backed sheets of silica gel F_{254} (1 by 1.5 cm, 0.2-mm thickness; E. Merck Co.) and air dried in a fume hood for 30 min to remove volatile naphthalene. Radioactivity of nonvolatile *cis*-naphthalene dihydrodiol remaining in each plate was determined by scintillation counting with a Ready Safe cocktail solution (Beckman) as described previously (7). Initial NDO activity was determined from the linear portion of the reaction, and all experiments were conducted in duplicate.

Spectroscopic analysis. Comparison of the UV/Vis spectra of ISP_{NAP} after catalytic turnover with benzene and naphthalene was performed as follows. Reaction mixtures (0.4 ml) contained 0.5 mM NADH, substrate (8 μl of a 25 mM stock in methanol to yield a final concentration of 0.5 mM), Rd_{NAP} (4.2 μg of protein), Fd_{NAP} (22.4 μg of protein), and ISP_{NAP} (520 μg of protein) in 50 mM MES buffer (pH 6.8). Reactions were conducted in capped 1-dram (3.7-ml) tubes and incubated horizontally with shaking (60 rpm) at 22°C. At the incubation time of 2 h, the protein was concentrated and desalted twice with 50 mM MES buffer (pH 6.8) containing 1 mM DTT by centrifugation over an Amicon YM100 Microcon at 5°C. The retentants were resuspended in BTGD buffer and subjected to the measurement of UV/Vis absorbance. The filtrates had no absorbance at 340 nm, indicating that NADH was completely oxidized in both reactions. The ISP_{NAP} activity remaining in the retentants was also determined by oxygen uptake experiments as described in the paragraph entitled oxygen uptake studies. The reported results shown are the averages of duplicated measurements.

Removal of mononuclear iron from ISP_{NAP} by FerroZine. Removal of iron from ISP_{NAP} by FerroZine was based on a modification of a published method (41). Briefly, purified ISP_{NAP} (1,275 μg of protein) was added to a solution containing 5 mM FerroZine and 5 mM ascorbic acid in BTGD buffer (final preparation, 0.5 ml). The reaction mixture was incubated on ice for 63 h, and then the protein was concentrated and desalted three times with BTGD buffer by centrifugation over an Amicon YM100 Microcon. This preparation yielded 1,044 μg of ISP_{NAP}.

Hydrogen peroxide-mediated ISP_{NAP} inactivation. The effect of hydrogen peroxide on the activity of either oxidized or reduced ISP_{NAP} was determined with the following reaction mixtures. The reaction mixtures (0.25 ml in 1.5-ml Eppendorf tubes) used for the oxidized ISP_{NAP} contained ISP_{NAP} (26.2 μg of protein) and hydrogen peroxide at final concentrations of 0.1 to 0.6 mM in 50 mM MES (pH 6.8). Reaction mixtures that were performed with reduced ISP_{NAP} contained Rd_{NAP} (2.62 μg of protein), Fd_{NAP} (12.8 μg of protein), and ISP_{NAP} (26.2 μg of protein) with hydrogen peroxide in the same range as that used for the oxidized ISP_{NAP} in 50 mM MES (pH 6.8). Stock solutions of hydrogen peroxide were made fresh prior to use. Actual concentrations of hydrogen peroxide were determined based on an extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm (3). Reactions were initiated by the addition of 5 μl of 50 mM NADH to give a final concentration of 1 mM. The reaction mixtures were incubated at 22°C. At the incubation time of 10 min, aliquots of 20 μl were withdrawn from the reaction mixtures, and the remaining ISP_{NAP} activity was determined as described above in the paragraph entitled time course of ISP_{NAP} inactivation. The same activity assays were also conducted in the absence of 0.1 mM Fe$(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to determine the requirement of ferrous ion for the activity. The results shown are the averages of duplicated measurements.

Identification of *cis*-benzene 1,2-dihydrodiol. In order to detect the benzene oxidation product formed by NDO, [^{14}C]benzene was used as the substrate. Reaction mixtures (1 ml each) contained Rd_{NAP} (10.5 μg of protein), Fd_{NAP} (48 μg of protein), ISP_{NAP} (40 μg of protein), and [^{14}C]benzene (102.2 μM; 1.32×10^7 dpm/ml) in 50 mM MES buffer (pH 6.8). When appropriate, NADH (0.5 mM), Fe$(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.1 mM), and catalase (1,000 U) were added to the reaction mixtures. The same reaction was also conducted with corresponding amounts of NADH, [^{14}C]benzene, Fe$(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and purified toluene dioxygenase components from *Pseudomonas putida* F1 (27). Reactions were conducted in 7.4-ml screw-cap vials, with an agitation of 150 rpm at 22°C. After 1 h, aliquots of 20 μl were withdrawn and suspended in 10 μl of 25 mM cold benzene in methanol. The solutions were loaded onto silica gel plates (1.5 cm^2; 0.2-mm thickness) and dried in the hood for 30 min. The amount of the radioactive polar product remaining on each plate was determined by scintillation counting. In addition, the reaction mixtures after incubation were extracted with 2 ml of NaOH-neutralized ethyl acetate, and each 1 ml of the extracts was concentrated over a stream of nitrogen to 25 μl. Aliquots (5 μl each) were subjected to thin-layer chromatography (TLC) on silica gel F_{254} plastic sheets with a developing solvent of chloroform-acetone (8:2), followed by autoradiography. X-ray films were exposed at −70°C for 5.5 days before development. The

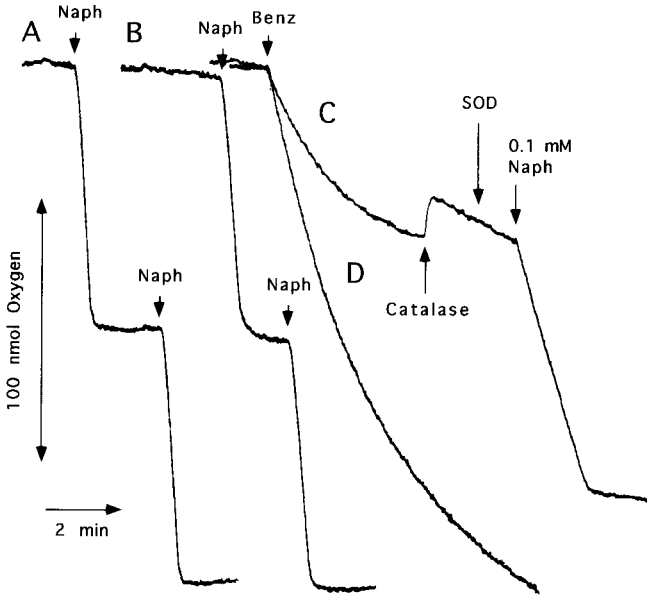

FIG. 2. Oxygen consumption by NDO in the presence of naphthalene (Naph) and benzene (Benz). Additions of substrates (4 μl of 25 mM in methanol), catalase (1,000 U), and SOD (82.5 U) were made at the times indicated by arrows. Experimental details are described in Materials and Methods. Additions of 0.1 mM naphthalene (A); 0.1 mM naphthalene in the presence of 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (B); 0.1 mM benzene, followed by additions of catalase, SOD, and 0.1 mM naphthalene (C); and 0.1 mM benzene in the presence of 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (D).

R_f value of the product was compared to those of phenol and *cis*-benzene 1,2-dihydrodiol formed by toluene dioxygenase (10).

RESULTS

Uncoupling of O_2 consumption and formation of hydrogen peroxide in the presence of benzene. NDO catalyzed the rapid and stoichiometric consumption of O_2 when incubated with naphthalene (Fig. 2A). In addition, approximately identical O_2 consumption rates were observed in the presence of 0.1 mM ferrous ion in the reaction mixture (Fig. 2B). Both had averages of 5.3 and 4.8 μmol of O_2 consumed/min/mg of ISP_{NAP} for the first and second additions of 0.1 mM naphthalene (Fig. 2A and B). GC-MS analysis of the reaction mixtures at the end of the experiment showed that *cis*-naphthalene dihydrodiol was the only product formed. In contrast, NDO catalyzed the steadily decreasing O_2 consumption in the presence of benzene, with an initial rate of 0.96 μmol of O_2 consumed/min/mg of ISP_{NAP} (Fig. 2C). This reaction was tightly coupled to NADH oxidation and dependent on all three NDO components (data not shown). GC-MS analysis of the reaction mixtures did not reveal the presence of oxidation products. Since NDO did not incorporate a detectable amount of O_2 into benzene, the reaction mixture was examined for reactive oxygen species that might account for the observed O_2 consumption. Addition of catalase at the time shown in Fig. 2C increased the dissolved O_2 level in the reaction mixture by 15.7 nmol, showing that part of the O_2 consumed by NDO in the presence of benzene was converted to hydrogen peroxide with an uncoupling reaction (reaction 1) as shown below. According to the stoichiometry of reactions 1 and 2, the amount of O_2 formed by the addition of catalase is equivalent to 50% of the O_2 consumed by NDO during the reaction.

$$O_2 + 2e^- + 2H^+ \xrightarrow{NDO} H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + 1/2O_2 \quad (2)$$

The amount of hydrogen peroxide equivalent to 40 to 50% of the O_2 consumed by NDO accumulated in the reaction mixture during the first 5 to 7 min of the reaction. The accumulation of hydrogen peroxide decreased over time (data not shown). Furthermore, the addition of increasing amounts of benzene to the reaction mixture showed that similar amounts of O_2 were consumed by NDO as in the presence of 0.1 mM benzene, but the initial rates of O_2 consumption increased slightly (data not shown). In spin trapping experiments with a hydroxyl radical ($^{\cdot}OH$) trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (4), the electron spin resonance (ESR) spectrum of DMPO and $^{\cdot}OH$ adduct (DMPO-OH) was detected with sodium phosphate buffer under the conditions when hydrogen peroxide did not accumulate in the medium due to lesser amounts of NDO components (26). The ESR spectrum did not form in the presence of catalase, indicating that hydrogen peroxide released into the reaction medium decomposes to $^{\cdot}OH$ under the reaction conditions. Analogous experiments were also conducted in the presence of 1,3-diphenylisobenzofuran, a singlet oxygen acceptor (17). No decrease in absorbance at 415 nm due to the reaction of singlet oxygen with the acceptor was observed.

It should be further noted that the addition of catalase to the reaction mixture (Fig. 2C) increased the rate of O_2 consumption from 0.11 to 0.22 μmol/min/mg of ISP_{NAP}. The increase of NDO activity caused by the removal of hydrogen peroxide indicates that accumulated hydrogen peroxide acts as an inhibitor in benzene-dependent O_2 consumption by NDO. In the time course of the O_2 consumption, the addition of 0.1 mM naphthalene at the incubation time as indicated in Fig. 2C resulted in 1.18 μmol of O_2 consumed/min/mg of ISP_{NAP}, with a tight coupling to the amount of substrate added. The rate of O_2 consumption was equivalent to 24.6% of second additions of 0.1 mM naphthalene in Fig. 2A and B. The addition of SOD to the reaction mixture in the presence of catalase did not affect the level of dissolved O_2 in the reaction mixture (Fig. 2C), indicating that superoxide anion did not accumulate under the uncoupling reaction. Interestingly, the presence of 0.1 mM ferrous ion in the reaction mixture containing benzene increased the rate of O_2 consumption (initial rate, 1.54 μmol/min/mg of ISP_{NAP}) as well as the total amount of O_2 consumed (Fig. 2D). This result suggests that ferrous ion may play another role in addition to supplementing iron ion at the active site of ISP_{NAP}.

Effect of the hydrogen peroxide-scavenging agents on benzene-induced O_2 consumption by NDO. The possible involvement of ferrous ion as the substrate of the Fenton reaction (reaction 3) was considered as an explanation for the increase of O_2 consumption in the presence of benzene (Fig. 2D).

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\cdot}OH + Fe^{3+} + {}^{-}OH \quad (3)$$

In reaction 3, the hydrogen peroxide formed could be removed from the reaction mixture by forming a strong oxidizing agent, $^{\cdot}OH$, that could react with the components of the reaction mixture, such as MES in large excess, and even with the NDO components. Increases of the benzene-dependent O_2 consumption in the presence of an enzyme such as catalase or the lactoperoxidase/KI system (34) that utilizes hydrogen peroxide as the substrate corroborated the proposed role of ferrous ion (Fig. 3). Benzene-induced O_2 consumption of NDO increased slightly in the presence of ferric chloride and chelating agents such as diethyenetriaminepentaacetic acid (DETAPAC) and EDTA. DETAPAC and EDTA are known to enhance the

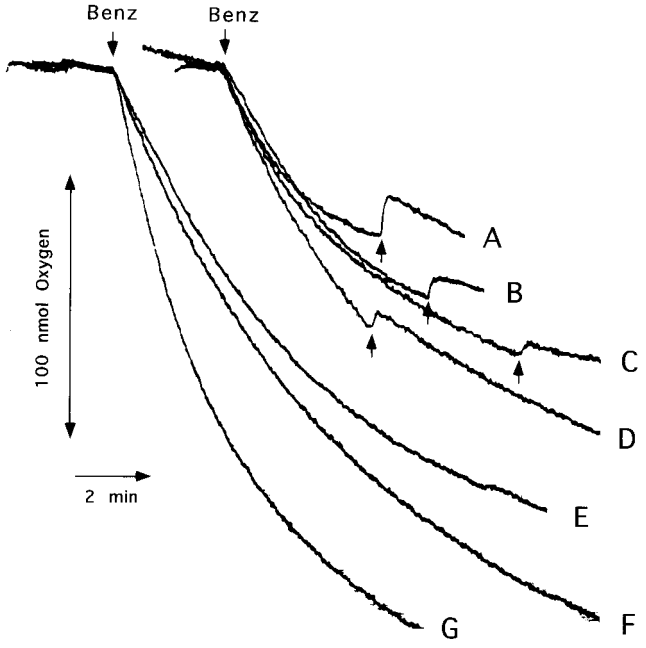

FIG. 3. Benzene-induced oxygen consumption by NDO in the presence of hydrogen peroxide-scavenging agents. Additions of benzene (Benz; final concentration, 0.1 mM) and catalase (1,000 U) were made at the times indicated by upward arrows. The reaction mixtures contained the same amounts of NADH and NDO components in 50 mM MES (pH 6.8) as described in Fig. 2. No addition (A) or addition of 0.1 mM $FeCl_3 \cdot 6H_2O$ (B), 1 mM DETAPAC (C), 1 mM EDTA (D), 10 mM KI and lactoperoxidase (9 U) (E), catalase (1,000 U) (F), and 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (G).

Fenton reaction (56). Levels of hydrogen peroxide accumulation in the presence of hydrogen peroxide-removing agents were also determined by the addition of catalase (Fig. 3). The addition of the ·OH scavengers (17), including DMPO, thiourea, and mannitol, to the reaction mixture in a final concentration of 5 mM did not increase benzene-dependent O_2 consumption.

Inactivation of ISP_{NAP} during benzene-induced O_2 consumption by NDO. The decreasing rate of benzene-dependent O_2 consumption by NDO with time was not completely reversible by the addition of catalase (Fig. 2C), indicating that NDO might be inactivated during the reaction. In order to determine the NDO component inactivated during the reaction, each NDO component was added back to the reaction mixture so that the stimulation of O_2 consumption in the presence of catalase could be observed. Addition of Rd_{NAP} or/and Fd_{NAP} to the reaction mixture did not stimulate the reduced benzene-induced O_2 consumption; however, the addition of ISP_{NAP} resulted in increased O_2 consumption (data not shown). This result shows that the ISP_{NAP} component is inactivated in benzene-induced O_2 consumption. Furthermore, the levels of naphthalene-dependent O_2 consumption by NDO in the presence of different concentrations of hydrogen peroxide were determined to examine the effect of peroxide on NDO activity. The initial rates of the O_2 consumption by NDO were steadily reduced in the presence of increasing amounts of hydrogen peroxide, and the O_2 consumption activities were not stoichiometric in the presence of more than 0.5 mM hydrogen peroxide (data not shown). For instance, in the presence of 0.5 mM hydrogen peroxide the initial rate of naphthalene-dependent O_2 consumption by NDO was 1.1 μmol/min/mg of ISP_{NAP}. O_2 consumption steadily decreased and ceased after 6 min. The addition of naphthalene, Rd_{NAP}, and Fd_{NAP} to the reaction mixture did not stimulate O_2 consumption, but the addition of ISP_{NAP} restarted O_2 consumption. This result shows that hydrogen peroxide inactivates ISP_{NAP}.

Spectroscopic studies. Possible change of the Rieske [2Fe-2S] redox clusters of ISP_{NAP} that reacted with benzene was determined by examining the change in the UV/Vis absorption spectrum ranging from 250 to 700 nm. As a control, the same reaction was also conducted in the presence of naphthalene. The observed differences between the two spectra were minimal (data not shown), and the R factors (A_{280}/A_{450}) of the naphthalene- and benzene-reacted ISP_{NAP} were 17.6 and 17.0, respectively. However, the specific activities for naphthalene- and benzene-reacted ISP_{NAP} were 2.7 and 0.88 μmol of O_2 consumed/min/mg, respectively, by an assay in the presence of 0.1 mM ferrous ion. The specific activities for naphthalene- and benzene-reacted ISP_{NAP} were 1.72 and 0.52 μmol of O_2 consumed/min/mg, respectively, by an assay in the absence of ferrous ion.

Protective effect of hydrogen peroxide-scavenging agents on ISP_{NAP} inactivation. In order to determine the activity of each NDO component remaining during the uncoupling reaction, all three NDO components were incubated in the presence of NADH and benzene. After a 30-min incubation period, aliquots were withdrawn and assayed for the activities remaining for each individual component when there was an excess of the other two components. Under these conditions, Rd_{NAP}, Fd_{NAP}, and ISP_{NAP} had 100, 94, and 20% of their original activities, respectively, indicating that benzene-dependent NDO inactivation was due primarily to the inactivation of the ISP_{NAP} component as shown above. Figure 4 shows the time course of ISP_{NAP} inactivation in the reaction mixtures containing benzene alone or benzene plus other hydrogen peroxide-scavenging agents. The presence of benzene resulted in a significant time-dependent inactivation of ISP_{NAP}. For example, ISP_{NAP} lost approximately 60% of the original activity when incubated with benzene for 10 min. ISP_{NAP} activity was not lost under incubation conditions without either Rd_{NAP} or Fd_{NAP} (data not shown). Catalase greatly protected ISP_{NAP} from inactivation. The same protection was also observed in the presence of both catalase and SOD (data not shown). This result shows that hydrogen peroxide released into the reaction medium during

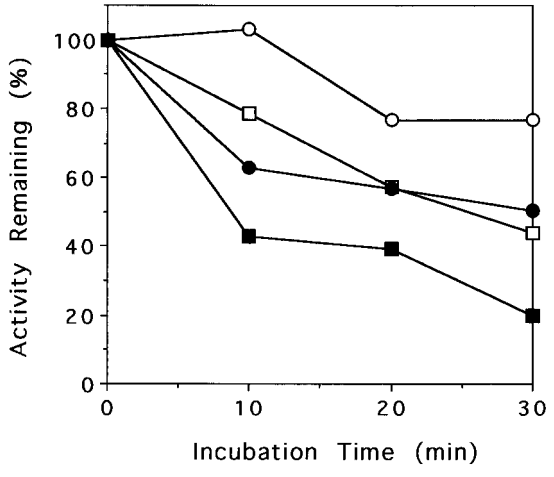

FIG. 4. Time course of ISP_{NAP} inactivation in NDO-catalyzed reaction mixtures containing benzene. Details of the experimental conditions are described in Materials and Methods. Reaction mixtures contained NADH and all three NDO components in 50 mM MES (pH 6.8) with added benzene (■), benzene and catalase (○), or benzene and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (●) or with no addition (□).

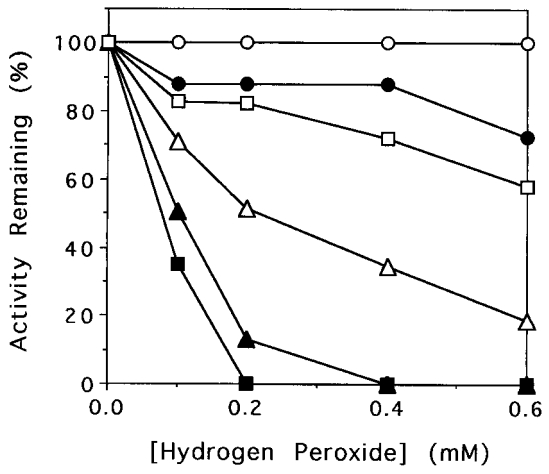

FIG. 5. ISP_{NAP} inactivation in the presence of hydrogen peroxide. Details of the experimental conditions are described in Materials and Methods. Reaction mixtures contained oxidized ISP_{NAP} and the amount of hydrogen peroxide indicated (circles); NADH, all three NDO components, and the amount of hydrogen peroxide indicated (triangles); or NADH, Rd_{NAP}, Fd_{NAP}, and FerroZine-treated ISP_{NAP} and the amount of hydrogen peroxide indicated (squares). The activity of treated ISP_{NAP} was determined under normal assay conditions in the presence of Rd_{NAP} and Fd_{NAP} as described in Materials and Methods. Open and filled symbols represent percentages of remaining ISP_{NAP} activity assayed in the presence and absence of 0.1 mM added $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, respectively.

the reaction is largely responsible for ISP_{NAP} inactivation. Partial relief of ISP_{NAP} inactivation was also observed in the presence of 0.1 mM ferrous ion. Interestingly, in the absence of benzene, ISP_{NAP} was also significantly inactivated in a prolonged incubation, but inactivation was fully prevented by the presence of catalase (data not shown). This result indicates that hydrogen peroxide released into the medium is also responsible for the inactivation of ISP_{NAP} during very slow O_2 consumption by NDO in the absence of substrate.

Inactivation of ISP_{NAP} in the presence of hydrogen peroxide. Experiments were conducted to determine whether hydrogen peroxide inactivation is specific for oxidized or reduced ISP_{NAP}. As shown in Fig. 5, oxidized ISP_{NAP} was resistant to the range of hydrogen peroxide tested. However, in the presence of excess hydrogen peroxide (10 mM), oxidized ISP_{NAP} had 57% of the original activity under the same incubation conditions. In contrast, reduced ISP_{NAP} was easily inactivated by hydrogen peroxide. For example, reduced ISP_{NAP} had no activity after incubation with 0.4 mM hydrogen peroxide for 10 min by an assay in the absence of added ferrous ion. However, the same incubation had 35% of the original activity by an assay with 0.1 mM ferrous ion. This result indicates that reduced ISP_{NAP} containing the mononuclear iron lost the activity by the treatment of hydrogen peroxide and that reduced ISP_{NAP} without the mononuclear iron which was intact during the incubation gained activity because of the supply of ferrous ion in the assay mixture. The role of mononuclear iron at the active site in the inactivation was further examined with partially mononuclear iron-depleted (FerroZine-treated) ISP_{NAP}, whose specific activities were 1.0 and 4.1 μmol of O_2 consumed/min/mg of ISP_{NAP} by assay without and with 0.1 mM ferrous ion, respectively. As shown in Fig. 5, partially mononuclear iron-depleted ISP_{NAP} was more resistant to hydrogen peroxide than original ISP_{NAP} by an assay of the activity in the presence of 0.1 mM ferrous ion. The effect of FerroZine-treated ISP_{NAP} is illustrated by the fact that ca. 25% of the enzyme that contained mononuclear iron became inactive in a typical manner, while the apoprotein could be reconstituted to achieve near full activity when ferrous ion was supplied in the assay. This result supports the inactivation of reduced ISP_{NAP} containing mononuclear iron by hydrogen peroxide. In addition, the [2Fe-2S] redox centers of FerroZine-treated ISP_{NAP} were also fully reduced in the presence of NADH, Rd_{NAP}, and Fd_{NAP} (data not shown).

Formation of *cis*-benzene 1,2-dihydrodiol by NDO. Since no benzene oxidation product was detected by GC-MS from concentrated ethyl acetate extracts obtained through purified NDO and IPTG-induced *E. coli* JM109(DE3)(pDTG141) biotransformations, the possibility of the formation of a trace amount of product was examined with [^{14}C]benzene. Surprisingly, NDO oxidized benzene to a polar product that stuck on the silica gel plate. To identify the product, the reaction mixtures were further extracted with ethyl acetate, concentrated about 20-fold, and subjected to TLC. The only polar product formed by NDO had an R_f of 0.11, identical to that of *cis*-benzene 1,2-dihydrodiol. The amounts of *cis*-benzene 1,2-dihydrodiol formed by NDO under various reaction conditions are listed in Table 1. The results show that the formation of *cis*-benzene 1,2-dihydrodiol by NDO is highly dependent on the presence of ferrous ion or catalase. For example, in the presence of 0.1 mM ferrous ion, a maximal accumulation of 1.31 μM (equivalent to a turnover ratio of 2.59 [product formed/active site]) was observed for 1 h of incubation.

DISCUSSION

Purified NDO has been shown to oxidize toluene sequentially through benzyl alcohol to benzaldehyde by reactions involving benzylic monooxygenation and alcohol oxidation, respectively (28). In this study the reactions catalyzed by NDO with a smaller substrate, benzene, were investigated to examine the factors involved in the uncoupling of substrate oxidation. As shown in Fig. 2, 40 to 50% of the O_2 consumed by NDO in the presence of benzene was reduced to hydrogen peroxide by an uncoupling reaction. In addition, a trace amount of *cis*-benzene 1,2-dihydrodiol was formed by a coupling reaction (Table 1). These results indicate that in the presence of benzene, NDO catalyzes a partial uncoupling reaction, resulting in the formation of hydrogen peroxide and *cis*-benzene 1,2-dihydrodiol. The sum of the O_2 utilized to form hydrogen peroxide and *cis*-benzene 1,2-dihydrodiol is insufficient to account for the O_2 consumption during the reaction, indicating that some intermediates formed from the consumed O_2 disappeared during the reaction. However, since O_2 consumption was tightly coupled to NADH oxidation, the formation of H_2O resulting from a four-electron reduction reaction (12) was not considered during the uncoupling reaction. Under the experimental con-

TABLE 1. Formation of *cis*-benzene 1,2-dihydrodiol by NDO[a]

Reaction condition	Amt (nmol) of *cis*-benzene 1,2-dihydrodiol formed/ml[b]	Turnover[c]
NADH + benzene	0.23	0.45
NADH + benzene + Fe^{2+}	1.31	2.59
NADH + benzene + catalase	0.90	1.78
NADH + benzene + catalase + Fe^{2+}	1.10	2.17
Benzene + Fe^{2+} + catalase	—[d]	—

[a] Reactions were conducted for 1 h. Details of the experimental conditions and product analysis are described in Materials and Methods.

[b] Values are the averages of two different experiments with two different assays. Standard deviations were less than 5% of the values given.

[c] Ratio of *cis*-benzene 1,2-dihydrodiol to the α and β subunits of ISP_{NAP}.

[d] Not detected.

ditions, hydrogen peroxide spontaneously decomposed to hydroxyl radicals, interfering with the precise measurement of accumulated hydrogen peroxide. A two-component 2-oxo-1,2-dihydroquinoline 8-monooxygenase which contains Rieske-type [2Fe-2S] clusters and additional iron in its oxygenase component has been shown to consume O_2 in the presence of pseudosubstrates without the oxidation of substrate (45). The decay under the experimental conditions and the present results suggest that hydrogen peroxide decomposes spontaneously under certain reaction conditions.

Since the rate of the benzene-dependent O_2 consumption by NDO decreased with time, the biochemical background for the observation was further studied. The presence of hydrogen peroxide-scavenging agents such as catalase, ferrous ion, lactoperoxidase, EDTA, and DETAPAC increased O_2 consumption (Fig. 3), leading to the conclusion that the hydrogen peroxide released was responsible for the decrease of O_2 consumption in the presence of benzene. Hydrogen peroxide was found to function as an inhibitor (Fig. 2) of O_2 consumption and as an irreversible inactivator of ISP_{NAP}. In the reconstituted system, ISP_{NAP} was shown to be largely protected from benzene-dependent inactivation in the presence of catalase (Fig. 4). A similar result was obtained in the formation of *cis*-benzene 1,2-dihydrodiol (Table 1). ISP_{NAP} inactivation was not related to the destruction of Rieske [2Fe-2S] clusters and was heavily dependent on the reduced form of ISP_{NAP} containing mononuclear iron (Fig. 5). These results suggest that residues at the mononuclear iron-containing active site are the primary reaction target for hydrogen peroxide-dependent ISP_{NAP} inactivation. Some purified (oxidized) ISP_{NAP} is known to contain ferrous ion, based on the ESR spectrum of a Fe^{2+}-NO complex giving $S = 3/2$ spins (55). It has also been shown in the related phthalate dioxygenase that the mononuclear iron of the active enzyme is ferrous (9). The presence of ferrous mononuclear iron at the active site of the oxidized ISP_{NAP} may be the reason part of the ISP_{NAP} is inactivated in the presence of excess hydrogen peroxide. Since the reduced form of ISP_{NAP} is more sensitive to hydrogen peroxide, it may play another role in reactivity to hydrogen peroxide. Many enzymes are reported to be inactivated in the presence of hydrogen peroxide and ferrous ion (8). In most cases, enzyme inactivation was proposed to occur via a Fenton-type reaction (reaction 3), in which the strong oxidizing agent $^{\cdot}OH$ reacts with an amino acid(s) at or near the active site (48). This type of irreversible inactivation resulting from the formation of reactive oxygen species is distinct from oxygenase inactivation resulting from the formation of reactive substrate intermediates, capable of formation of covalent adducts at the functional groups of the enzymes (19, 35, 37). Since only the former type of enzyme inactivation is inhibited by catalase, these two types of enzyme inactivation can be differentiated. ISP_{NAP} inactivation during NDO catalysis in the presence of benzene was also partially prevented when ferrous ion was present in the reaction mixture (Fig. 4). This effect appears to be contradictory to the proposed mechanism of ISP_{NAP} inactivation by a Fenton-type reaction. However, ferrous ion in the reaction mixture seems to participate in the breakdown of hydrogen peroxide that is released and enters into the active site. Hydroxyl radicals formed in the medium can be easily quenched by reacting with excess MES buffer. This explanation can also be applied to the increase in O_2 consumption in the presence of ferrous ion and chelating agents such as EDTA and DETAPAC that help break down hydrogen peroxide by accelerating the Fenton reaction (56) (Fig. 3). In addition, the possibility cannot be excluded that added ferrous ion plays an extra role in protecting ISP_{NAP} from inactivation by hydrogen peroxide.

With respect to the electron transport reaction catalyzed by NDO, a role for hydrogen peroxide formed during the benzene-dependent uncoupling reaction can be proposed. It has been proposed and shown in many studies with iron-containing oxygenases that the reduction of O_2 by NAD(P)H involves the release of hydrogen peroxide at the ferric state in the form of $[FeO_2]^+$ or $Fe^{3+}(O_2^{2-})$ (13, 32, 42). This is reasonable in terms of a known chemistry in which a ferrous peroxo form $[FeO_2]^0$ can undergo a Fenton-type reaction, causing suicide inactivation of the oxygenase in the absence of substrate (17). With a continuous supply of two reducing equivalents from NAD(P)H, O_2 bound at the mononuclear iron site of ISP_{NAP} can be reduced to a peroxide level to be released as hydrogen peroxide by the benzene-dependent uncoupling reaction catalyzed by NDO. During the electron transfer cycle, mononuclear iron undergoes ferric and ferrous states. Released hydrogen peroxide could reversibly bind to ferric ion at the active site to block O_2 binding or could react with ferrous ion at the active site of reduced ISP_{NAP} formed during the catalytic cycle. This could result in inhibition of O_2 consumption by ISP_{NAP} and in ISP_{NAP} inactivation via a Fenton-type reaction, respectively. From this study it can be concluded that a ferric peroxide is an intermediate for NDO-dependent O_2 activation as proposed earlier for a similar oxygenase, 4-methoxybenzoate monooxygenase (52, 54). A ferric peroxide per se, however, cannot participate in all of the reactions catalyzed by NDO (18). In addition, the heterolytic cleavage of an O-O bond from a ferric peroxide intermediate proposed for monooxygenase-type P-450 (13, 42) and methane monooxygenase (32) cannot be a part of the dihydroxylation reaction, which requires both atoms of O_2 incorporated into the substrate. Thus, the mechanism for further activation of the intermediate in NDO catalysis remains to be elucidated; this mechanism should account for dioxygenation, monooxygenation, and radical reactions (43, 53).

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