Naphthalene 1,2-dioxygenase (NDOS) catalyzes the NAD(P)H and O₂-dependent oxidation of naphthalene to (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene. NDOS consists of three protein components: a flavo-[2Fe-2S] reductase (NDR), a ferredoxin electron transfer protein (NDF), and an (αβ)₂ oxygenase (NDO) containing a mononuclear iron site and a Rieske-type [2Fe-2S] cluster in each α-subunit. The active site is built across a subunit-subunit boundary, and each subunit contributes a mononuclear nonheme iron site and a Rieske-type [2Fe-2S] cluster in each α-subunit. Our previous studies have shown that NDO with both metal centers reduced is capable of an O₂-coupled single turnover to yield the correct cis-diol product in the absence of the NDR and NDF components (Wolfe, M. D., Parales, J. V., Gibson, D. T., and Lipscomb, J. D. (2001) J. Biol. Chem. 276, 19545-19550). It is shown here that addition of H₂O₂ to NDO allows reaction with naphthalene to rapidly yield the correct product in a “peroxide shunt” reaction that does not require a reduced Rieske cluster. The mononuclear Fe³⁺ center is oxidized during turnover, while the Rieske cluster remains in the oxidized state. Peroxide shunt turnover in the presence of [18O]-labeled H₂O₂ shows that both oxygen atoms in the product derive primarily from H₂O₂. The peroxide shunt halts after one turnover despite the presence of excess H₂O₂ and naphthalene, but this is not the result of enzyme inactivation. Rather, it appears that the product cannot be released when the mononuclear iron is in the Fe³⁺ state, blocking a second turnover. This work supports the hypotheses that the cis-dihydroxylation activity of NDOS requires only the NDO component, that a peroxy intermediate is formed during normal catalysis, and that product release requires an additional reducing equivalent beyond those necessary for the first turnover.

Naphthalene 1,2-dioxygenase (NDOS) is a Rieske nonheme iron oxygenase system that catalyzes the dihydroxylation of naphthalene to (+)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene.
Naphthalene 1,2-Dioxygenase Peroxide Shunt

and electrons together in the form of superoxide or hydrogen peroxide directly to the resting enzyme. Indeed, in the cases of P450 and MMO, it has proven possible to substitute hydrogen peroxide for an electron source and O₂, in this way allowing O₂-independent enzymatic reactions to be catalyzed (12-14). These systems appear to be catalytic, both in the sense of accelerating the reaction with hydrogen peroxide and undergoing several turnovers, although P450 is rapidly inactivated during this process.

In this study, we explore reduced oxygen species as the source of both oxygen and electrons for the NDO-catalyzed dihydroxylation of naphthalene. This obviates the need for internal electron transfer from the Rieske cluster allowing the reaction at the mononuclear iron site to be characterized in greater detail. It is shown here for the first time that a “peroxide shunt” is functional in NDO as observed for P450 and MMO, thereby implicating a peroxy intermediate of some type in the O₂ activation process. However, it is also observed that NDO is limited to a single turnover in the peroxide shunt reaction. The basis of this phenomenon is shown to offer new insight into the requirements for continuous turnover by the enzyme. Moreover, the peroxide shunt represents a new reactivity for a nonheme iron dioxygenase.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents—**Naphthalene dioxygenase enzyme components and protocatechuate 3,4-dioxygenase were purified as previously described (5, 15). Xanthine oxidase and bovine liver catalase was purchased from Roche Molecular Biochemicals and Sigma Chemical Co., respectively. Isotopically enriched compounds were purchased from Isotec: H₂O (95 atom %), O₂ (99 atom %), and H₂¹⁸O (90 atom %). Nitric oxide was purchased from Matheson Inc. (+)-cis-(1R,2S)-Dihydroxy-1,2-dihydronaphthalene (naphthalene cis-diol) was prepared as previously described (1). All other chemicals were purchased from Sigma and used without purification except for protocatechue acid, which was further purified by recrystallization. Distilled water was purified using a Millipore reverse osmosis system before use.

**Enzyme Assays—**NDO activity assays and protein determinations were performed as previously described (5). All NDO concentrations reported reflect the concentration of theoretical active sites and are referred to as “NDO activity.” Where indicated, KCN was added to 10 mM prior to addition of NDO components. To determine the effects of H₂O₂ (0–200 mM) on the activity of NDO, hydrogen peroxide was added to NDO samples (120 μM NDO αβ) in 100 mM MES buffer, pH 6.8, 250 mM naphthalene (saturated aqueous solution) in the presence or absence of 10 mM KCN. The samples were incubated from 0 to 15 min with mixing prior to adding the enzyme to the assay containing the other NDO components and NADH. KCN was not found to affect the stability or activity of NDO. Single-turnover reactions and all product analyses were performed as described previously (5). The calculated error (S.D.) for all product yield measurements ranged from 6 to 10%.

**Peroxide Shunt—**Peroxide shunt reactions were performed in triplicate at 23 °C in 100 mM MES buffer, pH 6.8, containing 70 μM NDO αβ, 250 μM naphthalene, and 480 mM catalase. Two different methods were used to introduce O₂ into the reaction mixture. In the first method, aliquots of KO₂/Me₂SO were added to the reaction from a stock solution of KO₂ (15 mM). The stock solution was prepared immediately before the experiment by dissolving solid KO₂ in dry Me₂SO under an atmosphere of N₂ in an anaerobic dry box. Five 10-μl additions were made to the enzyme mixture at 1-min intervals with constant mixing (final O₂ concentration = 940 μM). A second method utilized the xanthine oxidase (XO) system to generate O₂ in situ. Reactions contained 40 μM of XO and were initiated by addition of 500 μM. Aliquots of each reaction were transferred at various times to plastic tubes, quenched by immersion into a 90 °C water bath for 2 min and immediately frozen in liquid nitrogen and stored at −80 °C until product analysis was performed. Degradation of authentic naphthalene cis-diol was not observed following identical treatment with O₂. Control reactions lacked the presence of reduced iron in the enzyme were performed in the absence of NDO with the addition of 200 μM Fe(NH₄)₂(SO₄)₂·6H₂O in solutions containing superoxide and naphthalene.

**Peroxide Shunt—**Peroxide shunt reactions were carried out using 100 mM MES buffer, pH 6.8, containing 50–70 μM NDO αβ and 250 mM naphthalene, and were initiated by addition of H₂O₂. In some instances, naphthalene was added to buffered solutions from a naphthalene stock solution prepared in methanol. Reaction mixtures were incubated at 23 °C for 30 s with constant stirring. Longer reaction times (up to 1 h) showed no increase in naphthalene cis-diol yield, and the reaction was complete within 10 s, the shortest time tested. The reactions were then quenched as described above for the superoxide reactions. Alternatively, following the incubation period, protein samples were concentrated in Centricon 30 filtration devices to separate NDO from small molecules without protein denaturation. Control reactions were also performed by replacing NDO with 200 μM Fe(NH₄)₂(SO₄)₂·6H₂O. Authentic naphthalene cis-diol showed no sign of degradation under identical peroxide shunt conditions. All reactions were performed 3–10 times. In all experiments, 10 mM KCN was included to inhibit any catalase activity that might arise from a contaminating heme enzyme. Control experiments showed that the activity of NDO was not affected by KCN at this concentration, and no background catalase activity was detected.

Anisotropic reactions were performed in septum-sealed reaction vials by first flushing the buffered enzyme solution with argon for 10 min. A small volume of naphthalene dissolved in methanol was then added to saturate the solution. Finally, protocatechuate 3,4-dioxygenase and protocatechuate were added to final concentrations of 5 μM and 1 mM, respectively, to remove any trace of remaining oxygen and scavenge any oxygen generated during the reactions. Following a 5-min incubation, H₂O₂ was added to initiate the reaction.

Some experiments required chelator treatment and iron reconstitution after the peroxide shunt. In this case, following a typical shunt reaction, NDO was treated with 5 mM EDTA and 5 mM o-phenanthroline for 2 h at 4 °C. The mixture was then dialyzed at 4 °C against 100 mM MES buffer, 5 mM EDTA, 10 mM NaCl, and 0.1 mM o-phenanthroline to remove iron and any product associated with NDO. EDTA was then removed by gel filtration over a Sephadex G-25 column. Reconstitution was completed at 23 °C by addition of 2 eq of Fe(NH₄)₂(SO₄)₂·6H₂O in the presence of 250 μM naphthalene and 10 mM KCN. In some cases, a second peroxide shunt reaction was initiated after a 5-min incubation by addition of H₂O₂ to 30 mM.

**¹⁸O Incorporation Experiments—**All ¹⁸O incorporation experiments were completed at 23 °C using 100–200 μM NDO αβ in 100 mM MES buffer, pH 6.8, containing 10 mM KCN and 250 μM naphthalene. Septum-sealed, stirred reaction vials were used for all reactions. Peroxide shunt experiments carried out in either unlabeled or labeled water were completed by dilution of a buffered, concentrated, NDO solution with H₂O or H₂¹⁸O (95 atom %), respectively. KCN and naphthalene were then added, and the reaction was initiated by addition of H₂O₂ to 40 mM. Following dilution, the final calculated H₂¹⁸O enrichment was 45 atom %. ¹⁸O reactions were completed by first flushing the enzyme solution with argon for 10 min. The argon headspace was then replaced with ¹⁸O₂ (90 atom %) by flushing the reaction vial for 1 min followed by a 5-min equilibration period under the 1 atm of ¹⁸O₂. KCN and naphthalene were then added, followed by H₂O₂ to 40 mM. H₂¹⁸O reactions were identical to those completed using H₂O except that the 40 mM H₂O₂ was replaced with 28 mM H₂¹⁸O (90 atom %). A control sample was made by incubating 280 μM authentic naphthalene cis-diol with 40 mM H₂O₂ in the presence of the other components. All reactions were quenched as described above and stored at −80 °C until further processed at which time each sample was thawed and centrifuged to remove precipitated protein. Naphthalene cis-diol was isolated from each sample by HPLC as described previously (5). After fractionation and collection, naphthalene cis-diol samples were dried to constant weight before mass analysis. Each sample was dissolved in 5 μl of methanol. Isotope ratios were determined using a least squares algorithm available at stdin02.niddk.nih.gov/iso.html.

**Spectroscopic and Kinetic Measurements—**UV-visible, EPR, and stopped-flow measurements were performed as described previously (5). Kinetic parameters are shown in the figure legends. Each sample was measured by ESCAP mass spectrometry (ESI-MS) was performed using a JEOL SX102 mass spectrometer operated at an accelerating voltage of 70 eV. Samples were desorbed from the heated probe at a temperature of −80 °C. The instrument was scanned from m/z 40 to 400 every 2 s. The isotopic
and H$_2$O$_2$ Reactions
to NDO in the presence of peroxide shunt reaction was complete in less than 10 s. As (Table I). Addition of an excess of both H$_2$O$_2$ and naphthalene produce a variety of reactive oxygen species, failed to generate correct naphthalene to NDO resulted in the formation of significant amounts of the peroxide shunt reaction was achieved by addition of only a mass spectrometry. Approximately 50% of the total yield from elution of the sample compound.

conditions, and no degradation was observed.

values were determined from the average of spectra acquired during the elution of the sample compound.

RESULTS

O$_2^-$ and H$_2$O$_2$ Reactions—Our previous studies of reduced NDO demonstrated that the two electrons necessary for hydroxylation of naphthalene to naphthalene cis-diol via the normal reductive oxygen activation pathway are derived from 1 e oxidation of both mononuclear iron and Rieske cluster metal centers (5). Because NDO is isolated with its mononuclear iron in the reduced state, half of the electrons required for a single turnover are present. Consequently, reaction between this form of NDO and O$_2^-$ in the presence of naphthalene would, in principle, create a system in which the requisite two electrons and two atoms of oxygen are present, and thus, turnover by a “peroxide shunt” might occur. We tested this idea using two different methods of introducing O$_2^-$ to NDO in the presence of naphthalene. Table I shows that neither superoxide system produced concentrations of product above what is typically observed bound to protein as isolated. As a control, authentic naphthalene cis-diol was exposed to O$_2^-$ under identical reaction conditions, and no degradation was observed.

In contrast to O$_2^-$ H$_2$O$_2$ was found to readily react with NDO as isolated to initiate catalysis in a “peroxide shunt” reaction (Table I). Addition of an excess of both H$_2$O$_2$ and naphthalene to NDO resulted in the formation of significant amounts of the correct naphthalene cis-diol product as identified by HPLC and mass spectrometry. Approximately 50% of the total yield from the peroxide shunt reaction was achieved by addition of only a stoichiometric amount of H$_2$O$_2$ relative to the $a_b$ subunit concentration (data not shown). At all peroxide concentrations, the peroxide shunt reaction was complete in less than 10 s. As shown in Table I, addition of Fe$_{2+}$ to the reaction mixture increased the yield of naphthalene cis-diol formed, consistent with increasing the occupancy of mononuclear iron centers. However, under no conditions did we observe product yields greater than the concentration of NDO active sites in the assay.

Several molecules were tested as potential effectors of the NDO peroxide shunt (Table II). Removal of O$_2$ from the reaction mixture prior to addition of H$_2$O$_2$ resulted in a slight increase in product yield, whereas addition of oxygen radical scavengers such as superoxide dismutase and mannitol had no significant effect on the peroxide shunt yield. These results indicate that neither O$_2$ nor diffusible oxygen-derived radicals are involved in the product forming reaction. Likewise, a buffered solution of Fe$_{2+}$ and H$_2$O$_2$, the Fenton system known to produce a variety of reactive oxygen species, failed to generate naphthalene cis-diol from naphthalene (Table I). Addition of oxidized NDF to the shunt reaction also had no effect on the yield of product, showing that it does not play a role in the peroxide shunt.

Studies of Limited Turnover in the H$_2$O$_2$ Shunt—The low product yield of the peroxide shunt was unexpected, because both naphthalene and H$_2$O$_2$ (the source of electrons and oxygen) were present in large excess over NDO. In addition, repeated additions of H$_2$O$_2$ or naphthalene failed to increase the peroxide shunt product yield above that of a single turnover yield. The experiment was repeated with several different preparations of NDO only to find that each batch of enzyme had a different product yield associated with the shunt (Table III). In each case, the yield approaches that from a single turnover of the same batch of enzyme using chemical reduction and O$_2$ exposure. Control reactions using authentic naphthalene cis-diol indicate that H$_2$O$_2$ does not destroy the product (data not shown). Thus, the limited product yield from the peroxide shunt is not a result of insufficient quantities of substrates or product degradation.

Lee previously reported that the complete NDOS is slowly inactivated by hydrogen peroxide (16). Thus, loss of NDO activity could also explain the product yield being limited to one turnover. To address this issue, NDO was incubated with different concentrations of H$_2$O$_2$ in the presence of naphthalene to allow the peroxide shunt reaction to occur. Then the enzyme was assayed using the standard NDOS steady-state reaction. Only with long incubation times and high H$_2$O$_2$ concentrations was significant inactivation of NDO observed. For example, a 5-min incubation with 30 mM hydrogen peroxide was required for a 60% decrease in activity. This incubation time is much longer than that required for the peroxide shunt product forming reaction to occur (<10 s). Thus, it appears that enzyme inactivation cannot account for the one-turnover nature of the peroxide shunt.

Previously, we observed that product is tightly associated with NDO at the conclusion of single turnover (5). Likewise, after performing a peroxide shunt reaction and separation of NDO from small molecules by ultrafiltration, 90% of the product formed was retained with the enzyme. A nearly identical amount of naphthalene cis-diol remained bound following single turnover of NDO. In another experiment, following a peroxide shunt reaction, the mononuclear iron (and any product bound to it) was removed from NDO using metal chelators, dialysis, and gel filtration. After this treatment, Fe$_{2+}$ was added back to the apoenzyme (the Rieske cluster remains intact), and naphthalene and H$_2$O$_2$ were added to initiate another peroxide shunt. As shown in Table II, the naphthalene cis-diol yield from the second shunt approached that formed

| Shunt system | Reaction time | Naphthalene cis-diol yield (%) |
|-------------|--------------|------------------------------|
| NDO as isolated | 0.03 | |
| + KO$_2$ | 5 min | 0.01 |
| + XOX | 10 min | 0.01 |
| + 22 mM H$_2$O$_2$ | 30 min | 0.01 |
| + 22 mM H$_2$O$_2$ | 10 s | 0.62 |
| + Fe$_{2+}$ + 22 mM H$_2$O$_2$ | 10 min | 0.62 |
| Fe$_{2+}$ + KO$_2$ | 5 min | ND |
| + XOX | 5 min | ND |
| + 22 mM H$_2$O$_2$ | 10 min | ND |

*ND, no product was detected.*

| Reaction condition or treatment | Relative naphthalene cis-diol yield (%) |
|-------------------------------|--------------------------------------|
| NDO + 30 mM H$_2$O$_2$ | 100 |
| − O$_2$ | 106 |
| + SOD | 98 |
| + mannitol | 104 |
| + oxidized NDF | 100 |
| NDO + 30 mM H$_2$O$_2$ | 100 |
| after iron removal | 2 |
| after iron removal, + Fe$_{2+}$ + 30 mM H$_2$O$_2$ | 71 |

*The yield of product from each reaction is referenced to the amount formed in a reaction with NDO and hydrogen peroxide alone.*

*Represents the amount of product remaining associated with NDO following treatment with metal chelators, dialysis, and gel filtration as described under “Experimental Procedures.”*
during the first peroxide shunt reaction, suggesting that a sustained peroxide shunt is prevented by tightly bound naphthalene cis-diol in the active site.\(^2\)

**Spectroscopic and Mechanistic Characterization of the Peroxide Shunt**—To further characterize the NDO peroxide shunt, we studied the reaction using EPR spectroscopy and stopped-flow UV-visible methods. Fig. 1 shows EPR spectra of NDO before (a) and after (b) reaction with H\(_2\)O\(_2\) in the presence of naphthalene. Addition of hydrogen peroxide to substrate-bound NDO leads to the rapid generation of an EPR signal at \(g = 4.3\) arising from \(S = 5/2\) \(\text{Fe}^{3+}\) species with rhombic electronic symmetry (line width = 17 gauss). This signal arose on the same time scale as product formation (<10 s) and is identical to that from the previously characterized mononuclear \(\text{Fe}^{3+}\) formed at the conclusion of the NDO single turnover (5). Quantification of the rhombic species indicates that one mononuclear iron has undergone oxidation per naphthalene cis-diol formed (about 0.31 spin/mol \(\alpha\beta\) for this batch of NDO). No evidence of reduced Rieske cluster is observed in the EPR spectrum. For comparison, Fig. 1c shows the spectrum of NDO as isolated following reaction with H\(_2\)O\(_2\) in the absence of naphthalene. Oxidation of a small portion (\(-0.1\) spin/mol \(\alpha\beta\)) of the starting mononuclear \(\text{Fe}^{3+}\) is evident by the appearance of the derivative resonance at \(g = 4.3\). However, this signal is more asymmetric and broader (line width = 30 gauss) than the resonance from the \(\text{Fe}^{3+}\) formed after the peroxide shunt and single turnover reactions, suggesting that it arises from alteration in the iron environment. This may arise due to the observed slow inactivation of NDO caused by peroxide. A small signal of unknown origin is also observed at \(g = 2.0\) in samples with H\(_2\)O\(_2\) (Fig. 1, b and c); integration shows that this species represents less than 0.02 spin/mol \(\alpha\beta\).

Stopped-flow spectroscopy was also used to monitor the oxidation state of the Rieske cluster during the NDO peroxide shunt reaction. Rapid mixing of a solution of NDO and naphthalene with 10 mM H\(_2\)O\(_2\) produced no change in the optical absorption spectrum of the oxidized Rieske cluster of the isolated enzyme. This contrasts with the single turnover reaction where rapid oxidation of the reduced Rieske cluster is observed during the product-forming reaction (5). Thus, a mechanism involving H\(_2\)O\(_2\) reduction of NDO, followed by the O\(_2\)-dependent single turnover is unlikely.

**Source of the Oxygen Incorporated into the Product**—To determine the source of oxygen utilized in the peroxide shunt reaction, \(^18\)O-isotope studies were initiated. Fig. 2 shows

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\(^2\) Adding one reducing equivalent from a stoichiometric concentration of reduced NDF followed by additional H\(_2\)O\(_2\) to initiate a second peroxide shunt approximately doubled the yield, as expected. However, this treatment might also lead to product formation by the usual reductive oxygen activation pathway due to the fact that NDF can shuttle electrons between active sites that might result in the reduction of both Rieske and mononuclear iron centers (5).
labeled O2 was similar to those shown in the NDOS peroxide shunt.

EI-MS spectra were acquired on the naphthalene cis-diol product generated in NDO peroxide shunt reactions performed in the presence of H2O2/H2O (a), H18O2/H2O (b), and H2O2/H18O (c) as described under “Experimental Procedures.” The mass spectrum for the reaction in which 18O2 was utilized in place of unlabeled O2 is shown in Figure 2. The lower yield in the 18O2 experiment was reproducible but appears to be due to the additional treatments required to remove unlabeled O2, because similar concentrations of unlabeled oxygen were present in all experiments shown.

that the potential of the mononuclear iron site is too high to interact effectively with superoxide. A third possibility follows from fact that the crystal structure shows that the 15-Å substrate entry channel between the surface of the enzyme and the mononuclear iron is lined with hydrophobic amino acids. Thus, small anions such as O2− may have little or no affinity for the active site of NDO.3 Indeed, we have described here that NDOS activity is unaffected by high concentrations of CN−, but uncharged molecules such as NO, O2, and H2O2 can interact with the mononuclear iron of NDO.

The NDO Peroxide Shunt is an Enzyme Catalyzed Reaction—Biochemical studies indicate that the peroxide shunt occurs at the mononuclear iron site of NDO. Although Fe2+ and H2O2 readily react in solution to form highly reactive ‘OH (Fenton chemistry), several observations argue against nonenzymatic activated oxygen species involvement in naphthalene cis-diol formation. These include: (i) control reactions containing only Fe2+ and H2O2 failed to result in oxidized naphthalene, (ii) HPLC and MS indicate that only naphthalene cis-diol is produced in the reaction in contrast to the many singly and doubly hydroxylated products that might have been expected from Fenton chemistry, (iii) neither addition of radical scavenging molecules nor removal of O2 from the reaction altered the product yield, and (iv) 18O labeling experiments indicate that both atoms of oxygen incorporated into naphthalene primarily derive from H2O2 indicating that radical-based chain reactions do not occur as in some Fenton systems. Together, these data strongly suggest that activated oxygen species are generated at the mononuclear iron active site of NDO by reaction with H2O2.

Basis of the One Turnover Nature of the Peroxide Shunt—Despite the fact that both H2O2 and naphthalene are supplied in quantities sufficient for many turnovers, naphthalene cis-diol was found to be generated in yields equivalent to those of a single turnover reaction beginning with reduced NDO and O2. The yields from both a single turnover reaction starting with reduced NDO and H2O2 failed to result in oxidized naphthalene, (ii) HPLC and MS indicate that only naphthalene cis-diol is produced in the reaction in contrast to the many singly and doubly hydroxylated products that might have been expected from Fenton chemistry, (iii) neither addition of radical scavenging molecules nor removal of O2 from the reaction altered the product yield, and (iv) 18O labeling experiments indicate that both atoms of oxygen incorporated into naphthalene primarily derive from H2O2 indicating that radical-based chain reactions do not occur as in some Fenton systems. Together, these data strongly suggest that activated oxygen species are generated at the mononuclear iron active site of NDO by reaction with H2O2.

Basis of the One Turnover Nature of the Peroxide Shunt—Despite the fact that both H2O2 and naphthalene are supplied in quantities sufficient for many turnovers, naphthalene cis-diol was found to be generated in yields equivalent to those of a single turnover reaction beginning with reduced NDO and O2. The yields from both a single turnover of the fully reduced enzyme and the peroxide shunt appear to be limited by the concentration of iron populated mononuclear iron sites. In our studies of NDO and benzoate 1,2-dioxygenase (BDZO) single turnover (5, 17), we noted that, upon conclusion of the reaction, cis-diol product remained tightly associated with the enzyme such that protein unfolding (or removal of the mononuclear iron) was required for product release. In the case of BDZO, the bound product complex exhibits a distinctive EPR resonance at

3 We limit our conclusion to small anions for two reasons. First, studies of active site accessibility by cations such as NH4+ have not been performed. Second, larger but less polar anionic molecules such as 2-naphthoic acid do bind to NDO and serve as substrates in the hydroxylation reaction. In this case, hydrophobic interactions between the active site and the aromatic portion of the substrate probably dominate the binding interaction.
Recent observation of a stable, Fe$^{3+}$ state as after single turnover. The NDO peroxide shunt provides further support for this hypothesis, because after the peroxide shunt the mononuclear iron is shown here to be left in the same Fe$^{3+}$ state as after single turnover. Because no source of electrons to reduce the mononuclear iron is available in the peroxide shunt reaction, the reaction is limited to one turnover with naphthalene cis-diol remaining tightly bound to the enzyme. Reduction of the iron either by adding NADH, NDR, and NDF in a subsequent activity assay or by removing the Fe$^{3+}$ and replacing it with Fe$^{2+}$ releases the product and allows one or more additional turnovers. This also shows that the enzyme is not inactivated by the peroxide shunt, eliminating this as the cause of the halt in product formation. The mechanism by which product is retained in the active site when the iron is in the Fe$^{3+}$ state is unknown, but based on the large perturbation in the metal environment caused by the product in the case of BZDO (17), it is possible that naphthalene cis-diol(ate) is bound strongly to the iron in the ferric state of NDO. The recent observation of a stable, Fe$^{3+}$-coordinated styrene diol following reaction between an iron model complex, hydrogen peroxide, and styrene supports our proposal that the naphthalene cis-diol remains bound to the Fe$^{3+}$ following the peroxide shunt (18). In the case of NDO, reduction of the mononuclear iron at the start of the next catalytic cycle would then weaken the Fe-product interaction leading to its release.

Regulation of the Mononuclear Iron Reactivity—Studies of the interactions of NDO and BZDO, with NO and O$_2$, have shown that reactivity at the mononuclear iron center is regulated by both the reoxidation of the Rieske cluster and the presence of substrate (5, 17), With O$_2$, no reaction occurs in the absence of substrate or if the Rieske cluster is oxidized. Such regulation ensures that reducing equivalents are coupled to substrate hydroxylation and that highly reactive species are not generated in the absence of substrate. The nature of the substrate also seems to play a role in regulation, because alternative substrates such as benzene result in significant uncoupling of the reaction to release hydrogen peroxide (16). In the case of BZDO, we have also shown that the rate of electron transfer between metal sites depends upon the specific substrate present in the active site (17). The studies described here demonstrate that the Rieske cluster does not have to be reduced for NDO to facilitate the conversion of substrate to product, thus it apparently does not regulate the reaction by limiting access of small molecules to the mononuclear iron site when it is oxidized. This is consistent with our observation that NO will bind to the mononuclear iron site when the Rieske site is oxidized. It may suggest that the primary role of the Rieske cluster is in reductive oxygen activation, although the fact that even NO will not react with the mononuclear iron when the Rieske cluster is reduced and substrate is absent implies a more significant role in normal catalysis. Substrate appears to regulate aspects of catalysis in the peroxide shunt just as it does in single turnover. The mononuclear iron site is only slowly oxidized in the presence of peroxide if substrate is absent. In contrast, it is completely oxidized to the Fe$^{3+}$ state in less than 10 s in the peroxide shunt reaction. This may mean that substrate helps to form the binding site for small molecules in the active site. Alternatively, the presence of substrate near the mononuclear iron may allow a peroxo complex of the iron to react rapidly (and irreversibly) once it is formed, thereby pulling the reaction forward.

Electron Stoichiometry—The peroxide shunt reaction results in the same products as normal turnover, and thus it should have the same requirement for reducing equivalents. However, this is not the case for the first turnover, because both of the required equivalents are supplied by peroxide, but the mononuclear iron is also oxidized during the reaction. The fate of the third electron is unknown, but several points suggest that the third electron is not required in the product-forming reaction. First, the same chemistry is catalyzed in both the peroxide shunt and single turnover reaction suggesting that intermediates of equal reactivity are generated in both reactions. Second, if a different reactive species requiring three electrons for formation was generated in the shunt (e.g. an Fe$^{3+}$), the mononuclear iron would be expected to return to the Fe$^{3+}$ state following the two-electron hydroxylation chemistry, which is not observed. Third, EPR studies show that a radical species is not apparent in either the enzyme or the organic product indicating that the odd electron is not associated with the catalyst or hydroxylation reaction. Alternatively, it is possible that the iron is oxidized directly by peroxide in a reaction preceding or following the peroxide shunt reaction to yield water and hydroxyl radical. However, no evidence for reactions of hydroxyl radicals was observed.

Mechanistic Considerations—As in the case of NDO, peroxide shunt reactions of P450 and MMO yield the same types of oxidized products that result from normal turnover (12–14). The peroxide shunts for both of these enzymes occur from the ferric forms of the enzyme, which may also be the case for NDO. However, neither P450 nor MMO yields cis-dihydroxylated products from any aromatic substrate, emphasizing the mechanism-specific nature of the NDO peroxide shunt. The need to both activate molecular oxygen and tightly control both oxygen atoms so that they are incorporated into adjacent positions on the same face of the aromatic ring suggests a highly structured activated oxygen complex. It seems likely that the same intermediate is formed in the peroxide shunt, given the uniqueness and complexity of the catalyzed reaction. Evidently, this intermediate can be formed by either reducing O$_2$ at the active site or by binding peroxide directly, suggesting that a peroxo complex is at least an intermediate in the formation of the reactive species. This is in accord with the conclusions we reached from previous studies of the single turnover reaction (5, 17) as well as studies of isotope effects in the related reaction of the putidamonooxin monoxygenase reaction (19) and recent model studies using cis-diol-forming iron chelate complexes (18, 20, 21). The formation of a peroxo
intermediate does not preclude a further reaction in which a formal Fe$^{5+}$-oxo complex is generated as the reactive species. Indeed, a small amount of $^{18}$O isotope incorporation from solvent as well as exchange of $^{18}$O during reactions with labeled peroxide is noted in the experiments reported in Table IV. This is consistent with an intermediate in which the O–O bond has been cleaved forms before reaction with the substrate. Moreover, isotope exchange experiments (18, 22) as well as density functional theory calculations on the iron chelates (23) strongly suggest that O–O bond cleavage occurs prior to insertion into the carbon–carbon double bond, implying that an Fe$^{5+}$ species is formed. However, if this also occurs in the enzyme-catalyzed reaction, a mechanism to retain the second oxygen from either O$_2$ or, as shown here, peroxide must also be operative.

Scheme 2 summarizes our current understanding of the Rieske oxygenase catalytic cycle. At the beginning of the cycle, both the Rieske and mononuclear iron centers are in the reduced state, and substrate is bound at the mononuclear site (3, 5, 24). Because both components of the regulatory mechanism are in place, the enzyme readily reacts with O$_2$ to form the putative peroxo complex, NDO$^P$. As presented in this report, the reduction of the metal centers and reaction with O$_2$ can be bypassed by reaction with hydrogen peroxide, which also results in oxidation of the Fe$^{2+}$ at some point, probably directly by peroxide. At this stage, either NDO$_1^P$ or NDO$_2^P$ directly interacts with the substrate or, following O–O bond cleavage, becomes the two-electron more oxidized Fe$^{5+}$-oxo complex, NDO$^Q$. In the enzymatic reaction, such an NDO$^Q$ species would probably react quite rapidly with substrate, before significant amounts of solvent could exchange with the intermediate. Once product is formed, it appears to be trapped within the active site to form intermediate NDO$_3^P$ and probably remains bound to the Fe$^{3+}$ until the mononuclear iron is again reduced to Fe$^{2+}$ by NADH, NDR, and NDF to initiate another round of catalysis.

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