Naphthalene 1,2-dioxygenase (NDOS) is a three-component enzyme that catalyzes cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene formation from naphthalene, O₂, and NADH. We have determined the conditions for a single turnover of NDOS for the first time and studied the regulation of catalysis. As isolated, the αβ oxygenase component (NDO) has up to three catalytic pairs of metal centers (one mononuclear Fe₃⁺ and one derrick Rieske iron-sulfur cluster). This form of NDO is unreactive Rieske [2Fe-2S] clusters. The results show that NDO produced NDOS ferredoxin component (NDF) increases the turnover number. UV-visible and electron paramagnetic resonance spectroscopies show that during catalysis, one mononuclear iron and one Rieske cluster are oxidized per product formed, satisfying the two-electron reaction stoichiometry. The addition of oxidized or reduced NDOS ferredoxin component (NDF) increases both the product yield and rate of oxidation of formerly unreactive Rieske clusters. The results show that NDO alone catalyzes dioxygenase chemistry, whereas NDF appears to serve only an electron transport role, in this case redistributing electrons to competent active sites.

Rieske nonheme iron dioxygenases catalyze a remarkable reaction in which dioxygen is cleaved and both atoms are inserted across a double bond of an unactivated aromatic nucleus to yield a cis-dihydrodiol (1–4). These enzymes initiate the biodegradation of some of the most recalcitrant aromatic compounds that enter the environment from both natural and industrial sources, making their study of great importance for progress in bioremediation practices (5, 6). In addition, the inherent enantio- and regiospecificity of these enzymes make them useful for synthetic applications (7, 8). Several of these multicomponent dioxygenases are known that differ in the number of components and the number and type of subunits in the oxygenase component that contains the active site. However, all of the enzyme systems share the common features of a reductase component that can accept and pass on two electrons from reduced pyridine nucleotide, an electron transport system that may be encompassed into the reductase, and an oxygenase that has both Rieske [2Fe-2S] clusters and mononuclear iron centers.

One of the most thoroughly studied of the Rieske nonheme iron dioxygenases is naphthalene 1,2-dioxygenase (NDOS) isolated from Pseudomonas sp. NCIB 9816-4, which catalyzes the reaction shown in Scheme 1 to yield (±)-cis-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (9).

The complete NDOS consists of three protein components: the 36-kDa reductase protein (NDR) that contains a FAD molecule and a plant-type [2Fe-2S] cluster, the 14-kDa ferredoxin electron transfer protein (NDF) that contains a [2Fe-2S] Rieske cluster, and the 210-kDa αβ oxygenase component (NDO) that contains the Rieske and mononuclear iron centers essential to catalysis.

Recently, the crystal structure of NDO was solved, representing the first structure from this enzyme class (10). It showed that even though both the Rieske and mononuclear iron centers are bound in the α subunit, they reside in widely separated sites (44 Å). However, the quaternary structure of the protein places each Rieske [2Fe-2S] cluster within 12 Å of the mononuclear iron site in the adjacent α subunit. Experimental evidence suggests that it is these pairs of sites that interact during catalysis via a conserved Asp residue that couples the centers (10, 11). The crystal structure also showed that the alternative NDO substrate indole binds near the mononuclear iron, suggesting that this is the oxygen-activating site (12). In support of this proposal, site-directed mutagenesis studies on NDO demonstrated that substitution of the hydrophobic residues lining the substrate binding pocket alter both the stereo- and regioselectivity of NDO (13). Also, the ligands of the mononuclear iron are one solvent, two histidines and one bidentate aspartic acid forming a 2-His 1-carboxylate “facial triad,” as observed in many other O₂-activating iron-containing enzymes (14, 15). This coordination potentially allows one or two exogenous ligands to bind. Accordingly, it has been observed for the phthalate dioxygenase (PDOS) that small molecules mimicking oxygen binding, such as N₃⁻, can bind to the mononuclear iron and form binary enzyme-N₃⁻ and ternary enzyme-substrate-N₃⁻ complexes (16, 17). Taken together, the data provide a good structural model for...
how Rieske nonheme iron dioxygenases bind substrates in the oxygenase component. However, the chemistries of O$_2$ activation and substrate hydroxylation are less well understood. Indeed, to date essentially all mechanistic hypotheses have been based on diagnostic chemistry rather than kinetic analysis or direct observation of intermediates with the consequence that no consensus mechanism has emerged. An essential step in the search for intermediates in the mechanism of cis-diol formation is to determine which protein components are essential for the reaction and what redox state of each of the enzyme cofactors yields a catalytically competent entity. Scheme 1 shows that the balanced chemical equation requires two electrons for substrate hydroxylation. However, little is known about where these electrons must reside at the onset of catalysis or the pathway of electron transfer during O$_2$ activation. Previously, a mechanism was proposed whereby an essential complex forms between the phthalate dioxygenase reductase and oxygenase components during O$_2$ binding/activation (18–20). The authors suggest that the electron transfer component is required for efficient catalysis as a direct source of electrons for oxygen activation and possibly as an effector to tune the structure of the oxygenase for catalysis. Similar roles for essential effector proteins have been proposed for several other oxygenases. For example, putidaredoxin in the P450$_{cam}$ system and component B in the MMO system are thought to assume essential roles as effectors during the catalytic cycle of these monoxygenases (21–24).

In the past, we have been able to resolve questions related to the component and reactant requirements for efficient catalysis in systems such as P450$_{cam}$ and MMO by studying single turnover reactions (21, 24, 25). Here we employ this strategy to determine the requirements for protein component interactions and electron transfer processes for catalysis by NDOS. By combining the techniques of product analysis, transient kinetics, and spectroscopy, it is shown that reduced NDO is capable of carrying out all aspects of the chemical reaction catalyzed by NDOS without the other two components. Moreover, the full rate of the reconstituted system is attained by NDO alone. This work provides a new protocol for the study of this enzyme class as well as insight into the intermediates of the catalytic sequence.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were purchased from either Sigma, Aldrich, or Matheson and used without purification. (+)-cis-(1R,2S)-Di-hydroxy-1,2-dihydroxynaphthalene (naphthalene cis-dihydrodiol) was prepared as described previously (26). Water was distilled and further purified using a Millipore reverse osmosis system.

**Cell Growth and Protein Purification**—All experiments were performed using NDO purified from either *Pseudomonas* sp. NCIB 9816–4 or from *Escherichia coli* JM109(DE3)pGDTG141) that contains the cloned genes for NDOS (27). NCIB 9816–4 was grown on naphthalene (0.075 g/liter) in mineral salts base medium (28) as described previously (29) with the addition of 5 mM salicylate to yield 600 g of wet cell paste from 240 liters of culture. Growth of *E. coli* and induction were performed as described previously (30). NDR and NDF were purified as described previously (31). ND was purified from both NCIB 9816–4 and *E. coli* using the procedure described previously (32) with the omission of the final S-300 size exclusion column.

**Enzyme Assays**—NDO activity was routinely assayed polarographically at 23 °C by monitoring O$_2$ consumption with a Clark-type electrode as described previously (33). The standard reaction mixture contained air-saturated 50 mM MES buffer, pH 6.8, 50 mM NaCl, 300 μM NADH, and ~250 μM naphthalene. NDOS components were added to concentrations of 0.1 and 1.0 μM for NDR and NDF, respectively, whereas NDO was maintained below 50 nM. Enzyme concentrations were routinely determined by UV absorption with extinction coefficients that were determined from purified protein and its Bradford activity (NDR $e_{380}$ = 86.0 $\text{mM}^{-1} \text{cm}^{-1}$; NDF $e_{380}$ = 16.1 $\text{mM}^{-1} \text{cm}^{-1}$; NDO $e_{380}$ = 129 mM (αβ)$^{-1}$ cm$^{-1}$). Iron was added to the assay as Fe(NH)$_2$(SO$_4$)$_2$$\cdot$6H$_2$O to a final concentration of 100 μM where indicated. One unit of NDO activity is defined as the amount of NDO required to consume 1 μmol of O$_2$ per min.

**Iron Quantitation**—The concentration of iron in NDO samples was determined either by the method of Fish (34) or by atomic absorption spectroscopy using a Varian SpectAA 100 system. The mononuclear iron occupancy was determined by subtracting the Rieske iron content determined by EPR spectroscopy (each S = 1/2 spin = 2.0 Fe) from the total iron. For example, the enzyme used for the experiments described in Table I contained 0.95 spin/molecule and 2.7 Fe/molecule to yield 0.8 mononuclear iron per αβ. However, for NDO preparations containing less than 1 Rieske cluster(αβ), it is likely that some mononuclear iron occupies sites that do not contain Rieske cluster.

**Single Turnover Reactions**—Single turnover reactions were completed in either a quartz cuvette equipped with a mixing chamber and Teflon-sealed cap, a Teflon-sealed reaction vial, a stopped-flow instrument, or a septum-sealed EPR tube as appropriate. Enzyme components were made anaerobic in 100 mM MES buffer, pH 6.8, in a septum-sealed vial and transferred to the appropriate reaction vessel with a gas-tight syringe. The reduction/oxidation states of the components were monitored using a Hewlett Packard 4855 diode-array spectrophotometer.

For product analysis, each reaction contained ~25 nmol of NDO active sites (αβ) and/or ~25 nmol of NDF or NDR where indicated. The reaction was initiated by the addition of an equal volume of buffer saturated with naphthalene (~250 μM) and O$_2$ (~1.4 mM). Each reaction was performed in a volume that maintained the naphthalene/active site ratio at no less than 1 (typically 4). Reaction mixtures were incubated at 23 °C for the times indicated. The reaction was quenched by transferring the mixture to an Eppendorf tube, which was then heated by immersion in a 90 °C water bath for 2 min. This method completely denatured the protein, as judged by a complete loss of the characteristic red-brown color of the enzyme(s) and total loss of activity. Control reactions using authentic naphthalene cis-dihydrodiol verified that no breakdown of product occurs during heat quenching. Samples were vortexed briefly and frozen in liquid N$_2$ before HPLC analysis.

For rapid chemical quench analysis of product formation, the enzyme solution was anaerobically transferred to one syringe of a chemical quench instrument (Update Instruments, Inc.), and the other syringe was loaded with O$_2$- and naphthalene-saturated buffer. After rapid mixing, the solutions were passed through calibrated aging tubing and then sprayed into an equal volume of 6 N NaOH. The estimated dead time of the experiment was 10 ms. Loss of product under the chemical quench conditions was reduced by immediately neutralizing the solution by the addition of equal volumes of 3 N HCl and 0.5 M EDTA, pH 6.8. A small amount of product decay is seen during chemical quench experiments; therefore, the standard curve was generated using naphthalene cis-dihydrodiol treated in the same manner as the chemical quench of NDO turnover.

Reactions monitored by EPR spectroscopy were performed in sealed reaction vials. After initiation of the reaction, the mixture was rapidly transferred to an EPR tube and frozen in liquid nitrogen. Single turnover reactions performed for the determination of reoxidation rates were completed using an Applied Photophysics (model SX.18MV) stopped-flow instrument. The syringes were loaded with solutions as described above (with or without naphthalene), but the reaction was monitored with a diode array detector or a single wavelength detector at 280 nm. The concentration of iron in NDO samples was determined by EPR spectroscopy using a Varian SpectAA 100 system. The mononuclear iron occupancy was determined by subtracting the Rieske iron content determined by EPR spectroscopy using a Varian SpectAA 100 system. The mononuclear iron occupancy was determined by subtracting the Rieske iron content determined by EPR spectroscopy using a Varian SpectAA 100 system. The mononuclear iron occupancy was determined by subtracting the Rieske iron content determined by EPR spectroscopy (each S = 1/2 spin = 2.0 Fe) from the total iron. For example, the enzyme used for the experiments described in Table I contained 0.95 spin/molecule and 2.7 Fe/molecule to yield 0.8 mononuclear iron per αβ. However, for NDO preparations containing less than 1 Rieske cluster(αβ), it is likely that some mononuclear iron occupies sites that do not contain Rieske cluster.
Ultrasphere ODS C18 reversed-phase column (4.6 mm × 25 cm) equilibrated in 20% methanol in H₂O. Two min after injection, a linear gradient of methanol (20–100%) was applied over 20 min. Absorption was monitored at 262 nm (naphthalene cis-dihydriodiol product, ε₉₀ = 8.11 m⁻¹ cm⁻¹). One product peak was identified as naphthalene cis-dihydriodiol and quantified by comparison to authentic standard.

EPR Spectroscopy—X-band EPR spectra were collected on a Bruker E500 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat. The nitrosoyl complexes of NDO were prepared by briefly bubbling the enzyme solution with nitric oxide (NO) passed through 6 n NaOH. EPR spectra of spin S = 5/2, and spin 5/2 complexes were analyzed according to the following spin Hamiltonian equations, respectively.

\[
\hat{H}_1 = g_\beta \mu_\beta S \cdot H + D(S_z^2 - 5/4) + E/D(S_x^2 - S_y^2) \quad \text{(Eq. 1)}
\]

\[
\hat{H}_2 = g_\beta \mu_\beta S \cdot H + D(S_0^2 - 35/12) + E/D(S_x^2 - S_y^2) \quad \text{(Eq. 2)}
\]

where D and E/D are zero field-splitting parameters, and the other parameters have their usual definitions. The term E/D is a measure of the departure of the electronic environment of the iron from axial symmetry. E/D values were determined for this study using the software program RHOMBO, which was provided by Wilfred Hagen (Department of Biotechnology, Delhi University of Technology, The Netherlands). Spin quantification was completed as described by Aasa and Vängård (35) using 1 mM CuCl₂, 1 mM Fe³⁺-EDTA, or 1 mM Fe²⁺-EDTA-NO as standards (35). Spin quantitation of the g = 4.3 species formed during single turnover was performed by using the Fe³⁺-EDTA standard and collecting both the experimental and standard spectra at 20 K. At this temperature, for S = 5/2 species with E/D = 1/5 and <1 < D < 1, the middle Kramer’s doublet (M₇ = ±3/2) is approximately maximally occupied. Under these conditions, the g = 4.3 species represents ~1/3 of the total spins. D for Fe²⁺-EDTA was taken as 0.83 cm⁻¹ (36). Using the temperature dependence of the g = 4.3 species of the NDO single turnover, we have determined D to be 0.76 ± 0.02 cm⁻¹.

**RESULTS**

**NDO Single Turnover**—As purified, resting NDO contains a nearly fully occupied oxidized Rieske cluster and a partially occupied reduced (Fe²⁺) mononuclear center (see below) in each αβ protomer. This form of the enzyme also has a small amount of the naphthalene cis-dihydriodiol product (<6% of active sites) associated with it (Table I). The concentration of product observed is within the error of the measurement and does not change with incubation of the resting enzyme with O₂ and naphthalene for up to 1 h. Furthermore, the addition of Fe³⁺ to this reaction mixture to increase the population of the mononuclear iron site results in no increase in the amount of product observed within experimental error. Thus, despite having nearly fully occupied metal sites and high concentrations of the two substrates naphthalene and O₂, resting NDO does not appear to catalyze substrate dioxygenation.

Treatment of NDO with a stoichiometric (1 electron per αβ subunit) amount of dithionite results in the one-electron reduction of the Rieske cluster (see below). Because this form of NDO differs from resting NDO primarly by the reduction of the Rieske cluster, we will refer to it as reduced NDO. Table I shows that the addition of naphthalene and O₂ to reduced NDO results in the formation of ~0.7 eq of the stereo- and regio-chemically correct product per αβ active site at the conclusion of the reaction. We have found that the method of analysis is important for these reactions. If the product yield was assessed by ultrafiltration, the reaction mixture rather than complete denaturation of the enzyme, more than 80% of the product remained bound to the enzyme.

The addition of Fe³⁺ to the steady state reaction of NDO and other Rieske nonheme iron oxygenases increases activity (18, 37, 38). Likewise, the addition of 100 μM ferrous ion to the single turnover reaction increases the product yield, as shown in Table I, suggesting that some of the added iron is incorporated into unoccupied sites. These data indicate for the first time that NDO in which both the Rieske and mononuclear iron centers are reduced is capable of converting naphthalene to its cis-dihydriodiol in high yield in the absence of NDOS components NDF and NDR.

The addition of 1 eq of reduced NDF (one NDF/NDN αβ) to the single turnover reaction increases the amount of product formed by 56% (Table I), consistent with NDF transferring its electron to the active site of the oxygenase. Since two electrons are required per product formed and each reduced NDF carries one electron, a maximum increase of 50% would be expected. However, ~30% of reduced NDO Rieske clusters have not reacted to yield product after an initial single turnover (see Table I, reduced NDO turnover). Thus, it appears that a portion of the electrons in unreduced NDO Rieske clusters are used to form additional product. Support for this conclusion is seen in the reaction of reduced NDO with oxidized NDF, naphthalene, and O₂. As shown in Table I, the addition of oxidized NDF increases the amount of product formed, yet the yield does not exceed that expected based on the total number of electrons initially present in the Rieske and mononuclear iron centers of NDO, suggesting that the oxidized NDF facilitates the utilization of these electrons. This can be exploited more explicitly using spectroscopic approaches as shown below. In contrast, it appears that neither oxidized nor reduced NDR increases the yield of NDO turnover, indicating that NDR is incapable of transferring electrons to NDO or transferring electrons between oxygenase active sites in the absence of NDF. This observation is consistent with the proposed role of NDF in coupling electron transfer from NADH-reduced NDR to NDO (39, 40). Neither reduced NDF nor reduced NDR alone catalyze

| Sample | Electrons added | Nmol of product | Percent |
|--------|----------------|-----------------|---------|
| NDO as isolated | 0 | 1.3 | 5 |
| Resting NDO | 0 | 0.8 | 3 |
| Reduced NDO | 1 | 17.0 | 68 |
| + Fe³⁺ | 1 | 19.8 | 79 |
| + oxidized NDF | 1 | 19.3 | 77 |
| + reduced NDF | 2 | 26.5 | 106 |
| + oxidized NDF | 1 | 17.1 | 68 |
| + oxidized NDF | 4 | 16.6 | 68 |
| Reduced NDF | 0 | 0.0 | 0 |
| Reduced NDR | 3 | 0.0 | 0 |

*For reactions that included NDO, the electron associated with the mononuclear Fe³⁺ site is not counted as an added electron, but the results show that it is used in the chemical reaction that yields product. We also do not include the added Fe³⁺ as added electrons because this iron apparently just fills the vacant mononuclear sites, and any excess is unreactive as observed for the reaction of resting NDO with Fe³⁺ added. The number of electrons added is entirely based on full reduction of the theoretical number of reducible cofactors in the reaction (NDO = 1, NDF = 1, NDR = 2). The mononuclear iron occupancy was determined as described under “Experimental Procedures.”

**Table I Single turnover of Pseudomonas sp. NCIB 9816-4 Naphthalene 1,2-dioxygenase**

Turnover experiments were conducted as described under “Experimental Procedures” with a ratio of naphthalene:NDO αβ = 4:1 and NDO αβ to NDF or NDR ratios of 1:1 where indicated. All samples were allowed to incubate for 1 h at 23 °C. The reactions were terminated by heat-denaturing the enzyme, which allows the release of any products that may be tightly bound in the active site. Each reaction condition was completed 3–8 times with a S.E. less than 10%.

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cis-dihydroxylation of naphthalene.

**Spectroscopic Properties of NDO**—Fig. 1a shows the X-band EPR spectrum of NDO as isolated from E. coli (NDO\textsuperscript{red} yield = 0.47).\textsuperscript{2} A small resonance is seen at \( g = 4.3 \) that quantitates to less than 0.02 spin/\( \alpha \beta \). No other signal indicative of either high spin or low spin Fe\textsuperscript{3+} is seen in the spectrum, suggesting that most of the mononuclear iron in resting NDO is in the Fe\textsuperscript{2+} state. Furthermore, no observation of an anisotropic signal with \( g_{av} < 2 \) is observed, indicating that the isolated NDO Rieske cluster is in the oxidized, diferric state.

Exposure of resting NDO to NO (Fig. 1a, inset) generates a major S = 3/2 species (\( E/D = 0.025; g = 4.18, 3.88, 2.01 \)) and at least one other minor S = 3/2 species (\( E/D = 0.106; g = 4.63, 3.37, 1.95 \)). Integration of all S = 3/2 species yields \(-0.4 \) spins/\( \alpha \beta \) for the particular sample used (NDO\textsuperscript{red} yield = 0.47). This type of EPR spectrum is known from our past studies to arise from nitrosyl adducts of mononuclear Fe\textsuperscript{3+} (41, 42). This approach has the advantages of making the normally EPR silent Fe\textsuperscript{2+} mononuclear site accessible to a sensitive spectroscopic technique that can differentiate between the types of iron-containing centers in NDO and allows facile quantitation. The substoichiometric S = 3/2 spin quantitation is consistent with the observations that (i) NDO is typically isolated with fewer than 3 Fe/\( \alpha \beta \) (2 Fe per Rieske center, 1 Fe mononuclear site), and (ii) the specific activity increases with the addition of Fe\textsuperscript{3+} to assays. However, the quantitation is slightly lower than we would expect from the known iron content of the enzyme preparations. This may be due to a low affinity of NDO for NO under these conditions or to the binding of two NO molecules to a fraction of the NDO mononuclear iron population. In any event, NDO as purified has at least one site available for small molecule binding, consistent with the proposal that O\textsubscript{2} binds at this site during catalysis.

The ability of the Fe\textsuperscript{2+} site in resting NDO to bind NO suggests that it might also bind O\textsubscript{2}, resulting in oxidation of the site. However, Fig. 1b shows that even after 1 h of incubation of

\[ \text{Fe}^{2+} - \text{NO} \rightarrow \text{Fe}^{3+} \text{NO} \]

NDO with naphthalene and O\textsubscript{2}, there is only a small increase in the g = 4.3 signal due to ferric ion. Accordingly, Fig. 1b, inset, demonstrates that Fe\textsuperscript{2+} is still present because an Fe\textsuperscript{2+}-NO complex is readily formed (0.4 spins/\( \alpha \beta \); \( E/D = 0.031; g = 4.21, 3.84, 2.01 \)). The spectrum in this case is slightly more rhombic and more homogeneous than the spectrum of the NO complex of NDO as isolated, showing that small changes have occurred in the environment of the iron. Similar results are observed upon incubation of NDO in the absence of substrate with O\textsubscript{2} or NO (data not shown). The failure of the mononuclear iron in these forms of NDO to react with O\textsubscript{2} accounts for the observed lack of single turnover catalysis in the absence of an additional reducing equivalent.

**Kinetics of the Single Turnover Reaction**—Fig. 2a shows the X-band EPR spectrum of reduced NDO (NDO\textsuperscript{red} yield = 0.68) before the single turnover reaction. One-electron reduction of the formerly diferric Rieske cluster results in the antiferromagnetic coupling of the S = 5/2 and S = 2 irons to yield the S = 1/2 system observed (\( g_{av} = 1.89 \)). Exposure of the reduced enzyme to naphthalene and O\textsubscript{2} (Fig. 2a, b) oxidizes 63% of the Rieske cluster (\(-1 \) spin/product) within 20 s (the minimum time used...
Using UV-visible spectroscopy, attempts have been made to determine if the oxidation rate of the Rieske cluster during the slow phase is 2nd order with respect to NDO concentration. However, we could not discern any obvious change in reciprocal relaxation time with enzyme concentration.

\[ \text{The complex spectrum near } g = 2 \text{ is believed to result from NO interacting with the Rieske cluster, based on our thorough studies of the interactions of NO with benzene dioxygenase showing that enzyme devoid of mononuclear iron displays identically high field EPR spectra to those shown in Fig. 4B (c and d) (M. D. Wolfe, and J. D. Lipscomb, unpublished observations). The spectra were successfully simulated by summing spectra of reduced NDO, d7 nitrosyl, and d9 dinitrosyl iron complexes (not shown). The resulting } g_{av} \text{ of } 2. 03 \text{ EPR spectra of the d7 and d9 complexes are commonly observed for metalloproteins exposed to NO and have been extensively reviewed in the literature (43–46).} \]
Instrument conditions: microwave power, 200 mW; 23 °C for 30 min.

A. Addition.

naphthalene (3d) is after the addition of an equal volume of buffer saturated with 1 s, 1 min, 5 min, and 20 min, with the arrow indicating the extent of oxidation of the mononuclear iron site is nearly matched by the increase in oxidation of the Rieske cluster, about 30%. This suggests one electron transfer from unreacted Rieske sites to mononuclear sites that have undergone reaction and, thus, are oxidized. This process of redistribution of the electrons in the system is apparently facilitated by the NDF component, presumably by accepting electrons from reduced Rieske clusters and shuttling them to other Rieske clusters that are coupled to the higher potential mononuclear iron centers, thereby reducing them. It seems likely that a Rieske cluster might initially be left in the reduced state during a single turnover reaction if its partner mononuclear site is vacant. On the other hand, oxidation of an occupied mononuclear site implies that the associated Rieske cluster is populated and reduced at the beginning of the reaction. Consequently, the only mononuclear sites that could accept a redistributed electron are those that are part of an inherently active pair. Thus, the NDF may occasionally shuttle an electron to a Rieske cluster associated with a newly reduced mononuclear center, allowing a second turnover and increasing the yield as observed.

Fig. 2A, d shows the EPR spectrum of the reaction mixture of reduced NDO with O2 and naphthalene in the presence of reduced NDF. At the conclusion of the reaction, all of the mononuclear iron present was reduced, and all of the NDF was oxidized, but a portion of the oxygenase Rieske cluster remained reduced.5 These results can be understood within the following context. Under the conditions required for observation by EPR (high protein concentration), only enough substrate for one turnover was present. Therefore, at the conclusion of oxygenase turnover, ~0.7 Fe3+ and ~0.7 oxidized Rieske clusters are present (Fig. 2A, b), which leaves ~1.3 electrons/αβ available for reduction of these sites (1.0 from reduced NDF, 0.3 from the unreduced NDO Rieske cluster). Reduction of 0.7 mononuclear sites leaves 0.6 electrons stored in the NDO Rieske cluster. Under the conditions of this experiment in which all the substrate has been utilized, NDO is trapped in a state with fully reduced mononuclear iron and ~60% reduced Rieske cluster, with NDF fully oxidized. This is in complete accord with the proposals that rapid oxidation of the metal centers is strictly correlated with substrate oxidation and that redistribution of electrons is greatly facilitated by NDF. Indeed, the apparent transfer of electrons between sites occurs much faster than the oxidation of unreacted Rieske cluster in NDO single turnover without NDF, suggesting that NDF-facilitated electron transfer from one oxygenase Rieske cluster to another is very rapid.

DISCUSSION

It has been shown here for the first time that the oxygenase component of a Rieske nonheme iron dioxygenase is capable of efficiently catalyzing the cis-dihydroxylation of an aromatic substrate in the absence of the other protein components of the enzyme system. The reaction catalyzed yields the correct product in a concentration that is approximately equal to that of the limiting mononuclear iron center and occurs with a rate constant that is at least as large as the turnover number for the reconstituted system. Importantly, it has been shown that both overall catalysis and rapid reaction with oxygen only occur when both the Rieske and mononuclear iron centers are reduced and organic substrate is present, suggesting a mechanism of regulation based within the oxygenase rather than externally through effector proteins. In accord with this proposal, the ferredoxin, which might potentially serve an effector role based on analogy with other oxygenase systems, appears to

5 The Rieske cluster in NDO can be resolved from the NDF Rieske cluster by the difference in the EPR spectrum of the reduced clusters.
serve only an electron transfer role. Through comparison of product yields, the results presented here imply that single turnover reactions only occur if both of the metal centers of a catalytic pair (apparently involving sites across a subunit boundary) are occupied. Moreover, the effects of the other protein components of NDO on product yield show that electron transfer between different catalytic pairs within a single NDO molecule occurs primarily externally via NDF rather than internally through the protein. These findings hold great significance for catalysis by Rieske nonheme iron oxygenases as discussed in the following sections.

Components Required for Turnover—The observation that neither the reductase nor the ferredoxin component of NDO is required for coupled and fast turnover shows that these components do not play direct roles in the activation of O₂ or substrate hydroxylation. Rather, they participate in the fast reduction of the Rieske and mononuclear iron centers at the conclusion of one oxygenase catalytic cycle, regenerating a fully reduced oxygenase for another turnover. Our results differ from those previously reported for PDOS, where it was reported that only minor concentrations of product were formed during reactions of reduced oxygenase with phthalate and O₂ (18). Furthermore, the authors noted that the reoxidation rate of the PDO Rieske cluster during this reaction was only a fraction of V_{max} (>1000-fold slower), suggesting that PDR (reductase component of PDOS) (PDO has no ferredoxin component) was required for efficient turnover. Steady-state analysis of the PDOS reaction also supports the proposal that the reductase interacts with the oxygenase during turnover (19). Nevertheless, our data clearly show that nearly stoichiometric yields of product are formed in the NDO single turnover reaction through the intermediates that could potentially release reactive oxygen species so that the activated intermediate, compound Q, could be efficiently formed and insert an oxygen atom into an unactivated substrate C-H bond, thereby assuring a coupled reaction. P₄₅₀cam also catalyzes this type of reaction, but regulation occurs by two mechanisms that are each unrelated to the MMO regulatory mechanism (21, 22, 51). Both of the mechanisms regulate electron transfer rather than oxygen binding. In the first mechanism, substrate binding to the P₄₅₀Cam hydroxylase component increases the redox potential of the heme such that an electron can be accepted efficiently from putidaredoxin to initiate the catalytic cycle, thus assuring that substrate is present as catalysis begins. In the second mechanism, putidaredoxin acts as an effector by forming a highly specific complex with the reduced hydroxylase component that gates oxygen addition to the enzyme (23, 24). This has the effect of greatly accelerating the reaction through the intermediates that could potentially release reactive oxygen species and thus activating the oxygenase component.

Comparison of Regulatory Mechanisms—Oxygenases generally regulate oxygen activation to prevent the release of reactive oxygen species. Two quite different types of regulation within multicomponent oxygenase systems have been described in past studies, and these are typified by MMO and P₄₅₀cam. In the case of MMO, the regulatory component B acts as an effector by forming a specific complex with the reduced hydroxylase component that gates oxygen addition to the enzyme (23, 24). This has the effect of greatly accelerating the reaction through the intermediates that could potentially release reactive oxygen species so that the activated intermediate, compound Q, could be efficiently formed and insert an oxygen atom into an unactivated substrate C-H bond, thereby assuring a coupled reaction. P₄₅₀cam also catalyzes this type of reaction, but regulation occurs by two mechanisms that are each unrelated to the MMO regulatory mechanism (21, 22, 51). Both of the mechanisms regulate electron transfer rather than oxygen binding. In the first mechanism, substrate binding to the P₄₅₀Cam hydroxylase component increases the redox potential of the heme such that an electron can be accepted efficiently from putidaredoxin to initiate the catalytic cycle, thus assuring that substrate is present as catalysis begins. In the second mechanism, putidaredoxin acts as an effector by forming a highly specific complex with the reduced hydroxylase component that gates oxygen addition to the enzyme (23, 24). This has the effect of greatly accelerating the reaction through the intermediates that could potentially release reactive oxygen species and thus activating the oxygenase component.

Small Molecule Interactions with the NDO Mononuclear Iron Center—We have shown here that the resting NDO can bind the small O₂ analog NO to the mononuclear Fe^{2+} under some conditions. Importantly, O₂ does not appear to react with this site unless substrate is bound and the Rieske cluster is reduced. Similar five-coordinate metal centers are found in extradiol ring cleaving dioxygenases that bind NO but fail to bind O₂ in the absence of substrate (47, 48). These enzymes direct the course of the reaction by binding their catecholate substrate directly to the iron, resulting in a decrease in redox potential and triggering oxygen binding to initiate catalysis (47, 49). Thus, the Fe^{2+} center is balanced by the endogenous ligand set at a potential between those required to bind NO and oxygen, respectively. The crystal structures of these and related enzymes show that the same 2-His, 1-carboxylate facial triad ligand set is employed in each case (14, 15). Consequently, it is not surprising that the similar site of NDO is tuned to react with oxygen in a controlled fashion through substrate interactions. The means by which it does this, however, is not obvious because it is not possible for the aromatic substrate of Rieske nonheme iron dioxygenases to coordinate directly to the metal center to alter the redox potential. It is possible that structural changes caused by reduction of the Rieske cluster in combination with substrate binding alter the environment of the mononuclear iron, allowing access to the required ligand site or causing a change in redox potential. We have detected such changes caused by substrate (and substrate analog) binding and Rieske cluster reduction for the oxygenase components of both naphthalene and benzoate dioxygenases using spectroscopic techniques (50). These data support our proposal that both the substrate and redox state of the Rieske cluster regulate NDO oxygen reactivity.

Regulation in NDOS appears to represent a new class in which oxygen reactivity at one ferrous metal center is triggered by both substrate binding and reduction of a completely separate metal center within the oxygenase component. In contrast to the P₄₅₀ system, this type of regulation does not involve the electron transfer components as effector proteins in a direct way, because the electrons that move the oxygenase component into a reactive state can be supplied by many types of donors. However, the new regulatory scheme does provide a mechanism by which the enzyme can avoid one-electron reduction of oxygen and uncoupled turnover in the absence of naphthalene, since two electrons and substrate must be available before oxygen can react rapidly with NDO. In this way, it is similar to the regulatory schemes for MMO and P₄₅₀Cam, in that it moves the reaction rapidly through the steps where reactive oxygen...
species might be generated and released to participate in non-specific chemistry. Like P450cam, NDO assures that substrate is bound to the enzyme before oxygen is activated. However, like MMO, it apparently accomplishes this by controlling access of oxygen to the reactive metal center rather than using the P450 mechanism of coupling reduction to substrate binding. The remarkable observation that the fully reduced NDO does not allow even the high affinity ligand NO to bind in the absence of substrate implies that O2 access is coupled specifically to substrate binding. Thus, the NDO substrate performs a role similar to that of component B in the MMO regulatory system. Indeed, both may function by inducing a conformational change at the metal center where oxygen is activated, which in the case of NDO is the oxygenase mononuclear iron site.

Mechanistic Implications—The overall interplay of components necessary for catalysis by NDOS is summarized in Scheme 2. Similar to previous proposals for Rieske nonheme iron oxygenases (2, 52), we believe that reduced NDF interacts with NDO to reduce both the Rieske and mononuclear iron centers. At this point, the studies reported here indicate that the NDF is no longer required. Fully reduced NDO may then bind naphthalene to form the oxygen-reactive complex, although the order of reduction and substrate binding has not been established. The reduced enzyme-substrate complex is able to bind O2, reductively activate it by two electrons, and catalyze cis-dihydroxylation. The cycle continues with product dissociation and the re-reduction of the NDO cofactors by reduced NDF.

Only a few proposals have been made for the chemical mechanism of cis-diol formation at the mononuclear Fe2+ site of Rieske nonheme iron dioxygenases, and these are based on very little supporting experimental evidence. One early proposal whereby an Fe3+-peroxo species attacks the substrate (illustrated in Scheme 3A) derived from studies on putidamonoxygenase. Using uncoupling substrates, which caused the production of hydrogen peroxide, in combination with solvent isotope effects, a ferric-peroxo intermediate was suggested as the species responsible for the demethylation and dihydroxylation reactions catalyzed by this enzyme (53–56). More recently, this general mechanism has found support in the work of Lee (57), which showed that benzene acts as both a substrate and an uncoupler of NDO, generating H2O2 during the reaction. Further evidence supporting the role of an Fe3+-peroxo intermediate in NDO turnover is found in the crystal structure of a putative indole-dioxygen adduct bound to the mononuclear iron (12). These observations suggest that the formation of an Fe3+-peroxo complex is at least an intermediate in the pathway of naphthalene cis-dihydrodiol formation. A second quite different proposal (Scheme 3B) (58) is based on P450 chemistry, which invokes O-O bond cleavage as part of the oxygen activation process and results in the formation of a high valent (formally Fe5+) iron-oxo species to hydroxylate the substrate. We have proposed a similar mechanism for MMO using a nonheme cofactor to generate an electronically equivalent high valent species (24, 59, 60). The P450 or MMO comparison is attractive in light of the variety of reactions NDO catalyzes. In all cases tested, NDO catalyzes the same type of reaction as P450 with one exception, epoxidation of the aromatic nucleus, in which NDO forms arene cis-dihydriodiol from the same substrates. A third hypothesis (Scheme 3C) is to utilize the ferredoxin or reductase component to supply another electron after the oxygen is bound so that a formal Fe5+ species is generated (19). This would presumably facilitate the O-O bond cleavage, avoid the unprecedented step of Fe5+ formation in a mononuclear nonheme iron center, and return the mononuclear iron to the Fe3+-resting state at the end of the reaction. However, a significantly less oxidizing reagent would be generated in the active site.

Chen and Que (61) recently prepared and characterized the first iron-based functional models of Rieske nonheme iron dioxygenases. Two ligand complexes have the ability to transform cyclooctene and cis and trans isomers of hexene to the corresponding cis-dihydriodiol. For one of the complexes, an Fe3+($\eta^2$-OH) intermediate, has been observed, suggesting its participation during catalysis (62, 63). Interestingly, these complexes share a similar labile ligand orientation with that of the mononuclear iron of NDO (64). The authors suggest that this geometry allows for the formation of an Fe3+($\eta^2$-OOH) intermediate that either directly attacks the double bond or matures to the Fe5+ = O, which would then attack the substrate (Scheme 3, A and B, respectively). Evidence supporting the formation of such a high valent nonheme iron has recently been provided by Chen and Que (65).

The results of our work to do not clearly define a mechanism for cis-diol formation; however, they strongly suggest that Scheme 3C is unlikely for two reasons. Both the observation that NDF is not required for a rapid single turnover and the fact that the reaction ends with the enzyme in the Fe3+ rather than the Fe2+ oxidation state are inconsistent with Scheme 3C. Therefore, in agreement with the model chemistry, we propose pathways 3A or 3B to be the catalytic route to cis-diol formation. However, further dynamic and spectroscopic studies are required to determine the exact nature of the reactive species that is responsible for the unique cis-dihydroxylation of unactivated aromatic compounds. We believe that availability of a functional single turnover system will allow direct kinetic approaches to be applied to this problem for the first time.

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Single Turnover Chemistry and Regulation of O$_2$ Activation by the Oxygenase Component of Naphthalene 1,2-Dioxygenase

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