Members of the antibiotic biosynthesis monooxygenase family catalyze O$_2$-dependent oxidations and oxygenations in the absence of any metallo- or organic cofactor. How these enzymes surmount the kinetic barrier to reactions between singlet substrates and triplet O$_2$ is unclear, but the reactions have been proposed to occur via a flavin-like mechanism, where the substrate acts in lieu of a flavin cofactor. To test this model, we monitored the uncatalyzed and enzymatic reactions of dithranol, a substrate for the nogalamycin monooxygenase (NMO) from *Streptomyces nogalater*. As with flavin, dithranol oxidation was faster at a higher pH, although the reaction did not appear to be base-catalyzed. Rather, conserved asparagines contributed to suppression of the substrate pKact. The same residues were critical for enzymatic catalysis that, consistent with the flavoenzyme model, occurred via an O$_2$-dependent slow step. Evidence for a superoxide/substrate radical pair intermediate came from detection of enzyme-bound superoxide during turnover. Small molecule and enzymatic superoxide traps suppressed formation of the oxygenation product under uncatalyzed conditions, whereas only the small molecule trap had an effect in the presence of NMO. This suggested that NMO both accelerated the formation and directed the recombination of a superoxide/dithranyl radical pair. These catalytic strategies are in some ways flavin-like and stand in contrast to the mechanisms of urate oxidase and (1H)-3-hydroxy-4-oxoquinidine 2,4-dioxygenase, both cofactor-independent enzymes that surmount the barriers to direct substrate/O$_2$ reactivity via markedly different means.

Reactions between organic molecules and O$_2$ are among the most important in biology, powering aerobic life. These reactions also present a distinct mechanistic challenge, as O$_2$ has two unpaired electrons (‘O$_2$, ↑ ↑), although most organic substrates (S) have all their spins paired (‘S, ↑ ↓). Uncatalyzed reactions between the two violate the so-called spin rule and are consequently slow (1). The sluggishness of these reactions protects biological organisms from potentially destructive oxidations and necessitates the use of catalysts for activating and directing O$_2$ reactivity.

Enzyme-associated cofactors, flavins, pterins, or redox-active metals that are capable of sequentially donating electrons to O$_2$, provide a pathway of microscopic chemical steps, each of which adheres to the spin rule. Cofactors have therefore long been assumed to serve an obligate role in the catalytic activation of O$_2$ in biological systems. Over the last 2 decades, however, O$_2$-activating enzymes that defy chemical expectation by not requiring cofactors have been discovered (2). These enzymes are biologically diverse, coming from several structural classes and evolutionary families and deriving from all kingdoms of life. They act upon a variety of substrates that nonetheless tend to be highly conjugated good reducing agents. Because they use O$_2$ from air, they require no exogenous metals or redox-sensitive organic components, and they have all of the benefits of biocatalysis, cofactor-free oxidases and oxygenases provide a model for “green” oxidation chemistry.

In part because of the nature of their substrates, the O$_2$-activating mechanisms of many cofactor-independent oxygenases have been proposed to be flavoprotein-like (3–8). Flavoprotein-dependent oxidases and oxygenases are able to accelerate the rate of the flavin/O$_2$ reaction by 10$^4$–10$^6$-fold over the reaction of free flavin in aqueous solution (9, 10) according to principles that are well described (summarized in Scheme 1). The flavin acts as an intermediary, activating O$_2$ and using it either to hydroxylate an exogenous substrate or to dispose of substrate-derived electrons (as H$_2$O$_2$). In the cofactor-free enzymes, the substrate itself has been proposed to reductively activate O$_2$ via the same series of steps used by the flavin cofactor. The activated substrate-O$_2$ complex subsequently converts to the oxidized product. Such a motivating model, although plausible, has not been widely tested.

The antibiotic biosynthesis monooxygenase family (ABMs,2 Pfar family PF03992) contains several small, structurally simple, and intrinsically solvent-solvent and temperature-stable enzymes known to catalyze O$_2$-dependent and cofactor-independent oxidations or monooxygenations (5–8, 11–15). Many ABMs are part of the biosynthetic pathways for polyketide antibiotics, including tetracenomycin (14, 16), daunomycin (17), actinorhodin (8, 12), alamunic (18), and aclacinomycin (11), also known as nogalamycin (19). Additionally, a subset of ABMs

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2 The abbreviations used are: ABM, antibiotic biosynthesis monooxygenase; NMO, nogalamycin monooxygenase; CMH, 1-hydroxy-3-methoxybenzyl-2,2,5,5-tetramethylpiperrolidone; HOD, (1H)-3-hydroxy-4-oxoquinoline 2,4-dioxygenase; ME, 2-methoxyethanol; TEMPO, 2,2,6,6-tetramethyl-1-piperidinylx; SOD, superoxide dismutase; Tricine, N-2-hydroxy-1,1-bis (hydroxymethyl)ethylglycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; BES, 2-(bis-2-hydroxyethyl)aminoethanesulfonic acid.
known as IsdGs catalyze the ring-opening oxygenation of the metallosubstrate heme (20) in many heme-feeding pathogenic bacteria.

Here, we have tested three essential components of the flavin model for the reaction catalyzed by the cofactor-independent nogalamycin monooxygenase (NMO), an ABM from Streptomyces nogalater (gene locus, SnoaB). We have compared the properties of the reaction in both the presence and absence of the enzyme to understand how the latter contributes to catalysis. Finally, we have interpreted the results in light of mechanistic work with IsdG (from Staphylococcus aureus) (21) and the emerging model for catalysis by the increasingly well characterized cofactor-independent oxygenase, (1H)-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (HOD) (4, 22–27).

**Results**

**Characterization of the Enzyme, Substrate, and Products—**

His_{6}-NMO was purified in yields of ~25 mg/liter culture. Pure enzyme had a measured subunit molecular mass of 16.85 kDa (MS); calculated 16.98 kDa. The discrepancy was attributed to loss of part of the histidine tag before or during MS analysis. The enzyme retained full activity for at least 25 min in the ME-containing buffers, after which activity began to decline.

UV-visible spectra for dithranol were monitored as a function of pH (Fig. 1A). Plots of absorbance at 354 and 389 nm versus pH yielded sigmoidal curves that were fit to Equation 3, yielding an average pK_{a} = 8.5 ± 0.3 (Fig. 1A, inset), ascribed to the conversion of neutral dithranol to the monoanionic enolate (dithranolate) (Scheme 2) (28). UV-visible features were identified under acidic and alkaline conditions in the buffer/ME solvent (Fig. 1B). Well resolved HPLC peaks for dithranol and its oxidation products were obtained with retention times of 5.9 min (dithranol), 5.2 min (dithranone), and 8.5 min (bianthrone) (data not shown).

**Uncatalyzed Oxidation of Dithranol in Air—** The spontaneous conversion of dithranol to products was monitored in air (280 μM O_{2}) 2 units below dithranol’s pK_{a} (pH 6.3) via time-resolved UV-visible spectroscopy. The dithranol peak red-shifted over a period of hours to 392 nm as the yellow starting solution turned brown (Fig. 2A). The lack of isosbestic behavior was consistent with the formation of more than one product. The reaction was subsequently monitored over time by discontinuous HPLC ([dithranol]_{initial} = 500 μM, Fig. 2B). The curve indicating the progress of reaction for dithranol disappearance fit well to a single exponential equation, yielding k = 0.0028 ± 0.0001 min^{-1} (t_{1/2} = 240 min, see Table 1). This exactly matched the first-order rate of bianthrone formation. The major product (90%) was bianthrone, with 10% dithranone (HPLC quantification errors were ±10%).

The same reactions were subsequently monitored for the alkaline form of dithranol (pH 9.8, Fig. 3A). The dithranol peak red-shifted over a period of hours to 392 nm as the yellow starting solution turned brown (Fig. 2A). The lack of isosbestic behavior was consistent with the formation of more than one product. The reaction was subsequently monitored over time by discontinuous HPLC ([dithranol]_{initial} = 500 μM, Fig. 2B). The curve indicating the progress of reaction for dithranol disappearance fit well to a single exponential equation, yielding k = 0.0028 ± 0.0001 min^{-1} (t_{1/2} = 240 min, see Table 1). This exactly matched the first-order rate of bianthrone formation. The major product (90%) was bianthrone, with 10% dithranone (HPLC quantification errors were ±10%).

The same reactions were subsequently monitored for the alkaline form of dithranol (pH 9.8, Fig. 3A). The dithranol peak red-shifted over a period of minutes to a product spectrum closely resembling that of dithranone. Dithranolate disappearance monitored by HPLC (500 μM) was linear with time and significantly faster than under acidic conditions (20
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(±1) μM min⁻¹, see Fig. 3B and Table 1), converting to a mixture of the alkaline forms of dithranone (60%) and bisanthrone (40%). The shift to an apparent zero-order reaction (in dithranol) was observed when [dithranol]₀ > [O₂] and appeared to result from the rapid depletion of O₂ relative to the rate of re-equilibration of the solution with air. When the reaction was carried out at [dithranol]₀ = 30 μM, the dithranol versus time curve was single exponential (k = 0.02 (± 0.05) min⁻¹, data not shown), indicating that the reaction was first order in both O₂ and dithranol as in the acidic case.

To avoid complications due either to very slow equilibration of O₂ (pH 9.8) or reaction times (pH 6.3), the reaction order and rate constant for the dithranol reaction were measured by the method of initial rates using HPLC to monitor dithranol disappearance. This also allowed for the simplest direct comparison between the uncatalyzed and catalyzed reactions (Fig. 4A). Consistent with the expected second-order rate law, rate = k [O₂] [dithranol], the dependence of initial rate of dithranol disappearance on concentration was linear. From the slope of the line (k [O₂]), the second-order rate constants k = 1.2 (± 0.005) x 10⁻⁴ and 3.6 (± 0.005) x 10⁻⁴ μM⁻¹ min⁻¹ were computed at pH 6.3 and 9.8, respectively (Table 1).

Slower apparent rates of O₂ consumption relative to dithranol disappearance were observed for the same reactions. This is qualitatively consistent with a proposed mechanism where the O₂ product disproportionate to form O₂⁻ and H₂O₂ (Scheme 2), thereby suppressing the apparent rate of O₂ consumption. The second-order rate constants measured via the HPLC method were therefore reported (Fig. 4A and Table 1).

Enzymatic Oxidation of Dithranol in Air—Dithranol disappearance was monitored via O₂ consumption under acidic and alkaline conditions identical to those used for determining the uncatalyzed second-order rate constants (see above) but in the presence of catalytic NMO (4 μM) (Fig. 4A and Table 2). Enzymatic rates were corrected for the background uncatalyzed reaction.

Under acidic conditions, the plot of initial rate versus [dithranol] fit well to the Michaelis-Menten model (Equation 4), yielding kₐₐₜ/Kₘₐₜ = 0.050 ± 0.01 μM⁻¹ min⁻¹ (pH 6.3). This is ~400-fold higher than the counterpart second-order rate constant measured under identical conditions but with no enzyme present.

Under basic conditions (pH 9.8), the initial rates fit to the Michaelis model with substrate inhibition. Fitting the data in Fig. 4A to Equation 5 yielded kₐₐₜ/Kₘₐₜ = 0.58 ± 0.04 μM⁻¹ min⁻¹, or about a 1600-fold increase relative to the uncatalyzed second-order rate constant. These values for kₐₚ/Kₘₚ are apparent because the O₂ concentration (air, 280 μM) was not saturat-
range of pH values (5–11) using various concentrations of dithranol to the enzyme suppresses further dithranol consumption. This suggests that, at high pH, bisanthrone (green circles) converts to bisanthrone (brown circles, k cat = 20 µM min −1), monitored discontinuously by HPLC, was linear over time (t 1/2 = 13 min), converting into the alkaline forms of dithranone (red circles, k = 13 µM min −1) and bisanthrone (brown circles, k = 6.7 µM min −1).

FIGURE 2. Uncatalyzed dithranol oxidation under acidic conditions (pH 6.3) is very slow. A, dithranol (green) converts to bisanthrone (brown) as its major product at pH 6.3 in air (100 µM starting dithranol, 20 °C, 0.1 M citrate/ME, gray spectra, measured at 30-min increments). The lack of an isosbestic point suggests the generation of more than one product. B, dithranol (500 µM) conversion monitored discontinuously over time by HPLC (green circles) clearly shows the appearance of the major product, bisanthrone (brown circles, 90% yield), as well as a small amount of dithranone (red circles, 10% yield) (0.1 M citrate/ME (pH 6.3), air). The disappearance of dithranol and appearance of bisanthrone occurred with first-order kinetics, t 1/2 = 240 min.

FIGURE 3. Uncatalyzed oxidation of the dithranol anion is much more rapid under basic conditions (pH 9.8) and gives a different product distribution. A, dithranol anion (green dashes) converts to approximately equal amounts of bisanthrone and dithranone (red dashes) over a period of 40 min at pH 9.8 in air (100 µM starting dithranol, 20 °C, 0.1 M CAPS/ME, gray traces measured at 5-min increments); B, dithranolate (500 µM) disappearance (green circles, k = 20 µM min −1), monitored discontinuously by HPLC, was linear over time (t 1/2 = 13 min), converting into the alkaline forms of dithranone (red circles, k = 13 µM min −1) and bisanthrone (brown circles, k = 6.7 µM min −1).

TABLE 1

| pH  | k  | t 1/2 | Bisanthrone | Dithranone |
|-----|----|-------|-------------|------------|
| 6.3 | 1.2 × 10⁻⁴ | 240° | 90 ± 4 | 10 ± 4 |
| 9.8 | 3.6 × 10⁻⁴ | 13° | 40 ± 2 | 60 ± 2 |

* Reaction was first-order in dithranol; k = 0.0028 min⁻¹ and t 1/2 = 0.693/k.

** Reaction was pseudo-zero-order in dithranol under conditions where [dithranol] initial > [O₂] (air-saturated solvent). The value for t 1/2 is reported for [dithranol] initial = 500 µM, where k = 20 µM min⁻¹ and t 1/2 = [dithranol] initial /2k.

required the use of the substrate inhibition model (Equation 5). Only the anionic form of dithranol appeared to bind ES to generate the inhibitory SES complex.

Plots of both log k cat and log k cat/K m(dithranol) increased markedly with pH (Fig. 4, B and C) and had slopes of ~0.6, suggesting that a single deprotonation event controlled the conversion between a less reactive acidic and more reactive alkaline form. Fitting the data to Equation 7 yielded pK a = 6.8 ± 0.05 (k cat and 6.7 ± 0.2 (k cat/K m[dithranol]). These values are more than 1.5 units smaller than the pK a value measured for the substrate (8.5) in the same solvent system.

The pH dependence in Fig. 4 could be attributable to an active site base. Two possible candidates were identified by inspection of the NMO structure (Fig. 5). The enzyme is relatively small, containing a single open cavity between a series of α-helices and β-sheets where ethylene glycol from the crystallization solvent binds. His-49 points inward toward the cavity, whereas His-85 is on a stretch of helix that could conceivably rotate, positioning the side chain in the vicinity of the cavity. No other potential acid-base residues are present in the pocket. The pH-dependent kinetics of the H49A and H85A mutants were characterized as above (Fig. 4, B and C). The lack of any change in k cat and k cat/K m(dithranol) over the entire pH range suggests that neither serves as an active site base residue and indeed neither is directly involved in the reaction. These conclusions are consistent with the finding that neither residue is well conserved among ABMs, although His-85 is conserved within the NMO-containing subfamily (see below).

Although not basic residues, two well conserved asparagines (Asn-18 and Asn-63) are within hydrogen bonding distance of a solvent molecule in the NMO active site (Fig. 5). Mutation of either (non-polar alanine) was previously shown to strongly
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We hypothesized that Asn-18 or Asn-63 could suppress the pK_a value of dithranol by stabilizing its anionic form or acting as a conduit for released H^+. At pH 6.3, when the substrate is in its neutral form, both mutations strongly suppressed k_cat (120- and 50-fold for the N18A and N63A mutants, respectively). By contrast, the values for K_m were actually lower than for WT NMO. This suggests that reducing polarity at Asn-18/Asn-63 facilitates ES formation when the substrate is unchanged, but it impairs subsequent chemical steps.

The situation at pH 9.8, with dithranol in its anionic form, was the opposite. A relatively smaller (20-fold) effect was observed for k_cat for either mutant. However, K_m values for N18A and N63A were elevated 5- and 3-fold, respectively. For the anionic substrate, reduction in polarity in the active site strongly impairs ES formation with a proportionally smaller impact on chemistry (k_cat). These results suggest that polar residues Asn-18 and Asn-63 are most important for ES formation (K_m) when S is already in the anionic state and for catalysis (k_cat) when it is neutral/protonated, as it would primarily be at physiological pH. The k_cat effect could be due to either residue serving as a proton conduit.

Dependence of Substrate Affinity on pH—To further probe the effects of pH, the affinity of WT NMO and the N18A/N63A mutants for dithranol was measured. Under acidic conditions (pH 6.3), K_m = 0.73 ± 0.04 μM. This decreased roughly 10-fold to 0.069 ± 0.03 μM at pH 9.8, indicating that NMO binds the substrate anion with greater affinity. For the N63A mutant protein, K_m = 0.69 ± 0.02 μM (pH 6.3) and 0.20 ± 0.01 μM (pH 9.8). For the N18A mutant, K_m = 0.33 ± 0.009 μM (pH 6.3) and 1.4 ± 0.2 μM (pH 9.8). These results showed that N18A and N63A have approximately the same affinity for neutral dithranol as the WT NMO (pH 6.3). However, both Asn-18/Asn-63 are important for the pronounced NMO/substrate affinity observed specifically at high pH.

Dependence of the Steady State Enzymatic Reaction on O_2 Concentration—Initial rates of the reaction between the NMO-substrate complex and O_2 were measured in the presence of dithranol (250 μM) with various concentrations of O_2 using the O_2 consumption assay (Fig. 6A and Table 2). Under acidic conditions (pH 6.3), fitting Equation 4 to the data yielded k_cat = 13 (±0.9) min^{-1}, K_m(O_2) = 430 μM, and k_cat/K_m(O_2) = 0.030 ± 0.003. Above the observed enzymatic pK_a (pH 9.8), the rate of the reaction increased substantially. Although a saturating [O_2] could not be reached, the apparent K_m from fitting the data to Equation 6 was 410 μM. This fit also predicted a Hill coefficient of 1.8, indicating positive cooperativity. This suggests that the O_2 reaction at one of the two subunits of NMO potentiates a reaction at the second by an unknown mechanism.

The measured k_cat/K_m(O_2) = 0.26 (±0.01) min^{-1} was 730-fold greater than the second-order rate constant for the reaction of dithranol and O_2 determined under identical conditions but with no enzyme present (Fig. 4A and Table 1). The k_cat/K_m(O_2) was furthermore 2-fold less than the k_cat/K_m(dithranol) measured in air. The k_cat/K_m values can be regarded as a second-order rate constant encompassing all microscopic chemical steps involving a given substrate up to and including the one that is rate-limiting. Because k_cat/K_m(O_2) < k_cat/K_m(dithranol),

impair the activity of the enzyme, although both mutant enzymes retain their structures (5). Although the data were near the limits of detection, the pK_a values observed for plots of both log k_cat and log k_cat/K_m(dithranol) for N18A and N63A NMO clearly shifted away from the WT values and toward the pK_a values for free dithranol: pK_a = 8.6 ± 0.1 (k_cat) and 7.2 ± 0.05 (k_cat/K_m(dithranol)) (N18A); pK_a = 7.6 ± 0.1 (k_cat) and 7.0 ± 0.2 (k_cat/K_m(dithranol)) (N63A). This suggests that both residues contributed to the pH dependence in the steady state reaction for WT NMO.

FIGURE 4. Steady state NMO-catalyzed reaction is faster and exhibits substrate inhibition at higher pH. A, Initial rate of substrate (O_2) disappearance was measured as a function of dithranol concentration at pH 6.3 (circles) and 9.8 (triangles) in the presence and absence (inset) of catalytic amounts of NMO (4 μM, 20 °C, air). Under moderately acidic conditions, the enzymatic reaction is slow and follows Michaelis-Menten kinetics (k_cat = 10 ± 0.3 min^{-1}, K_m = 0.050 ± 0.001 μM). Under basic conditions, the reaction is substantially faster and exhibits substrate inhibition: k_cat = 60 ± 6 min^{-1}, K_cat/K_m(dithranol) = 0.58 ± 0.04 μM min^{-1}, and K_m = 100 ± 20 μM. B, log k_cat/K_m(dithranol) vs pH. C, log k_cat/K_m(dithranol) vs pH.
it was concluded that a step involving O$_2$ must limit the overall rate of the NMO-catalyzed reaction.

**Transient Kinetics of the Reaction between the NMO-Dithranol Complex and O$_2$**—Stopped-flow experiments were carried out by mixing the anaerobic enzyme-substrate complex in one syringe with O$_2$-equilibrated solutions in the other (pH 9.8). NMO was highly concentrated and present in excess of dithranol to ensure that substrate would be enzyme-bound. The reaction occurred in two readily identifiable phases, each of which fit well to a single exponential curve (Fig. 6, B and C). Under air, the initial phase was complete within $\pm 5$ s, whereas the second slower phase took on the order of 300 s (Fig. 6B). Carrying out it was concluded that a step involving O$_2$ must limit the overall rate of the NMO-catalyzed reaction.

**TABLE 2**

Kinetic constants for the steady state enzymatic oxidation of dithranol

| pH   | $k_{cat}$ | $K_m$ (dithranol) | $k_{cat}/K_m$(dithranol) | $k_{cat}/K_m$(dithranol)$^a$ | $k_{cat}/K_m$(O$_2$)$^b$ | $K_m$ | Bisanthrone$^c$ | Dithranone$^c$ |
|------|-----------|------------------|------------------------|-----------------------------|------------------------|------|----------------|----------------|
| 6.3  | 10 ± 0.3  | 220 ± 60         | 0.050 ± 0.01           | 430                         | 0.030 ± 0.003          | N/A  | 50 ± 3         | 50 ± 3         |
|      | 13 ± 0.9$^a$ |                   |                        |                             |                        |      |                |                |
| 9.8  | 60 ± 6    | 100 ± 20         | 0.58 ± 0.04            | 410                         | 0.26 ± 0.01            | 1100 ± 300$^a$ | 10 ± 2        | 90 ± 2         |

$^a$ Reaction conditions are as follows: varied concentrations of O$_2$ were used in constant dithranol (250–500 $\mu$M), 4 $\mu$M NMO, 20 °C, citrate/ME (pH 6.3) or CAPS/ME (pH 9.8) buffers.

$^b$ Reaction conditions are as follows: 500 $\mu$M dithranol, air (280 $\mu$M), 1 mM NMO, 20 °C, 30 min incubation, citrate/ME (pH 6.3) or 500 $\mu$M dithranol, air (280 $\mu$M O$_2$), 50 $\mu$M NMO, 10 min incubation. CAPS/ME (pH 9.8).

**FIGURE 5.** Domain and active site structures of NMO (violet), ActVA-Orf6 (cyan), and Isdl (green) (Protein Data Bank codes 3KGO, 1NST, and 3LGN). A, overlay of substrate-binding domain of all three proteins, rendered as schematics. B–D, active site residues conserved within each subfamily are shown, with the protein in the same orientation as in A. Interactions that could be hydrogen bonds (distances $\leq 3.5$ Å) are indicated with dashed lines. Conserved residues are shown and their labels color-coded. Cyan indicates conservation among all three subfamilies; red indicates NMO and ActVA-Orf6; purple indicates NMO and Isdl; black indicates conserved only within the subfamily shown. Note that His-85 (NMO numbering used) is conserved within the NMO subfamily but not the ActVA-Orf6 subfamily. His-49 is highly but not strictly conserved, even within the NMO cluster, but it was examined nonetheless because of its key position. Asn-63 is conserved in both NMO and ActVA-Orf6 subfamilies. Additionally, the functionally important asparagine in the IsdG/Is (Asn-6) is conserved in the NMO subfamily (as Asn-18) but not among ActVA-Orf6 and its close relatives (8). Notably, no positively charged or acid-base residues are conserved between the NMO and ActVA-Orf6 clusters, arguing against their involvement in a common reaction mechanism. The iron-ligating histidine residue that is strictly conserved in IsdG/Is is not conserved in either the NMO or ActVA-Orf6 clusters.

**FIGURE 6.** NMO-catalyzed steady state and single turnover reactions of dithranol and O$_2$ show that an O$_2$-dependent step limits the overall reaction rate. A, initial rate of O$_2$ disappearance was monitored as a function of O$_2$ concentration at pH 6.3 (triangles) and 9.8 (circles). Fitting the data at pH 6.3 (500 $\mu$M dithranol, citrate/ME, 20 °C) to the Michaelis-Menten model (Equation 4) yielded the following: $k_{cat} = 13 \pm 0.9$ min$^{-1}$, $K_m(Dithr) = 430$ mM, and $K_m(O_2) = 0.03 \pm 0.003$ min$^{-1}$. At pH 9.8 (250 $\mu$M dithranol, CAPS/ME, 20 °C), a model including positive cooperativity yielded $k_{cat} = 110 \pm 6$ min$^{-1}$, $K_m(Dithr)/K_m(O_2) = 0.26 \pm 0.01$ min$^{-1}$, $K_m = 410$ mM, and $n = 1.8$ (Hill coefficient). B, reaction between the anaerobic 30 $\mu$M NMO-dithranolate complex and 608 $\mu$M O$_2$ (CAPS/ME (pH 9.8), 20 °C) was monitored over time following rapid mixing. The initial conversion of the starting complex (green spectrum) to a species with a spectrum resembling the alkaline form of dithranone (red) was complete within ~3.6 s. This species subsequently converted very slowly to the final observed photoproduct (purple) within ~300 s. The gray spectra were measured at 0.3-s increments. C, single wavelength traces (389 nm and 66, 137, 262, 392, and 608 $\mu$M O$_2$) plotted versus time illustrate the two kinetic phases shown in A. Data were fit to the sum of two exponentials to obtain values of $k_{on}(1)$ and $k_{on}(2)$, and the arrow shows the direction of increasing O$_2$ concentrations. Inset, values for $k_{on}(2)$, were plotted versus O$_2$ concentration. The initial kinetic phase exhibited a strong linear dependence on [O$_2$], yielding the second-order rate constant $k_s = 0.14 \pm 0.005$ min$^{-1}$ . The second phase, ascribed to the photodegradation of the product, was far slower and independent of O$_2$ concentration.
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the reactions with various concentrations of O2 showed that the first phase was clearly O2-dependent with a second-order rate constant $k = 0.14 \pm 0.005 \mu M^{-1} min^{-1}$ (Fig. 6C, inset). This number is similar to the measured value for $k_{cat}/K_m(O_2)$ (0.26 $\pm$ 0.01 $\mu M^{-1} min^{-1}$), consistent with the expectation that both constants are kinetically limited by the same step of the reaction. The second phase was independent of the concentration of O2. Comparison of the data to a no-enzyme control indicated that the slow phase is due to conversion of dithranone to a photoproduct on exposure to the intense white light of the xenon lamp and that the rate of this phase is directly dependent on the intensity of incident light on the reaction mixture. This is consistent with the known photoreactivity of anthracenone compounds (29).

Radical Pair Formation and Recombination—Prior work has suggested that bisanthrone forms via the initial generation of a dithranyl radical/O2− pair that does not undergo recombination (29, 30–32). Consistent with that expectation, when dithranol, O2, and CMH were incubated at pH 6.3 (major product: bisanthrone), the continuous slow conversion of CMH to CM∗ was observed by EPR for $\geq$120 min (data not shown). Because the half-life of CM∗ under these conditions is on the order of 6 h (31), experiments were confined to the initial phase of the dithranol reaction. Notably, in control experiments carried out in the presence of 2500 units/ml SOD (see below), no CM∗ was observed by EPR. This confirmed that CM∗ was generated by reaction with O2− rather than dithranyl radical.

A related mechanism for dithranone formation can be proposed in which a dithranyl/O2− pair forms (↑↑↓↓), undergoes a spin transition (↑↑↑↓↓), and subsequently recombines (↑↑↓↓) (Schemes 1 and 2). Breakdown of the resulting (hydro)peroxy-adduct by heterolytic cleavage of the O−O bond yields dithranone and H2O. If this mechanism is correct, superoxide scavengers could in principle disrupt the dithranyl/O2− pair, leading to the release of free dithranol. Two neutral dithranyl radicals could then couple to form bisanthrone, biasing the product ratio (bisanthrone/dithranone) toward bisanthrone.

To test this hypothesis, experiments were carried out using CMH and SOD as O2−-scavenging reagents at pH 9.8, where dithranone was the observed major product for both the catalyzed and uncatalyzed reactions (Tables 1 and 2). We expected that either SOD or CMH could compete with dithranyl/O2− recombination in free solution. However, because of its size, SOD should have no effect on reactions occurring inside the NMO-active site. The products of the uncatalyzed reaction (500 $\mu M$ dithranol, air) at pH 9.8 in the presence of increasing equivalents of CMH (with respect to dithranol) were analyzed. The dithranone/bisanthrone ratio decreased roughly linearly from 60:40 to 25:75 as CMH eq increased from 0 to 10 ($\sim$3.2% per CMH, Fig. 7A). SOD had an analogous influence; a concentration of 1250 units/ml gave results comparable with 10 eq CMH. The SOD effect saturated near 5000 units/ml, with $\geq$80% bisanthrone. Notably, the presence of O2−-trapping agents did not appreciably extend the incubation time needed to deplete the entire initial concentration of dithranol. This observation is consistent with a slow dithranol/O2− reaction to form dithranyl/O2− with faster subsequent steps.

FIGURE 7. Superoxide is generated during the catalyzed and uncatalyzed reactions and is an intermediate to the monoxygenase product, dithranone. A, products of the uncatalyzed reaction at pH 9.8 (CAPS/ME, 500 $\mu M$ dithranol, 22 °C) were analyzed by HPLC after the reactions had gone to completion in the presence of increasing concentrations of CMH (red squares) or SOD (red triangles). The dithranone fraction is plotted, and the remainder was bisanthrone. The products of the catalyzed reaction (0.01 M NMO) were analyzed after the reaction had gone to completion in the presence of increasing concentrations of CMH (blue squares). The enzymatic reaction (90% dithranone product) was largely unaffected by even the highest concentration of SOD; hence the data are not shown. Lines or extrapolated smooth curves illustrate the trends in the data. B, reaction of 200 $\mu M$ dithranol (used as limiting reagent due to lack of sample equilibration with air), air, 280 $\mu M$ O2, 5 $\mu M$ NMO, and 10 eq CMH (2 mM) to yield CM∗ was monitored over time by EPR (pH 9.8, CAPS/ME, 22 °C). Data were measured every minute until the spectrum stopped growing in intensity. Spectra measured at selected time points with their y axis normalized to TEMPO standard concentrations are shown. C, time-resolved spectra were measured as in 8 in the presence (blue symbols) and absence (red symbols) of 5 $\mu M$ NMO. Circles represent reactions carried in the presence of CMH as the means for superoxide detection, and triangles are for reactions carried out with both CMH and added SOD (2500 units/ml). The catalyzed reaction generates CM∗ more quickly than the uncatalyzed. Only the uncatalyzed reaction is significantly affected by competition with SOD, suggesting that O2− is protected from SOD inside the NMO active site.

Under the same conditions but with added NMO (50 $\mu M$), 5000 units/ml SOD (a saturating amount in the uncatalyzed reaction, Fig. 7A) had only a modest effect on the NMO-catalyzed reaction, lowering the dithranone/bisanthrone ratio from 90:10 to 80:20. The relative insensitivity of the ratio to SOD suggested that either superoxide was not involved in the NMO-catalyzed reaction or that dithranone is primarily generated from a dithranyl/O2− pair that is stERICALLY protected inside
NMO. By contrast, CMH suppressed the dithranone/bisanthrone ratio as in the uncatalyzed case, although to a lesser extent (10 eq CMH led to 70:30 dithranone/bisanthrone). Truncation of the effect may due to the limited affinity of CMH for NMO, competitive effects between the substrate and CMH, or both. The production of $O_2^-$ during the dithranol oxidation reaction was subsequently monitored over time via EPR at pH 9.8 (Fig. 7B). Superoxide trapping (detected via CM$^\bullet$) was linear with time ($1.2 \mu M$ min$^{-1}$) over the initial portion of the reaction. In the presence of 2500 units/ml SOD, the same reaction yielded CM$^\bullet$ at just above the baseline level although the dithranone/bisanthrone ratio was 25:75. This suggested that $O_2^-$ was preferentially scavenged by SOD. In the presence of 5 $\mu M$ NMO, more rapid CM$^\bullet$ production was observed during the initial phase of the reaction ($3.0 \mu M$ min$^{-1}$). Moreover, in contrast to the uncatalyzed reaction, the rate of CM$^\bullet$ production was modestly affected by 2500 units/ml SOD. These results are consistent with the conclusion that $O_2^-$ generated inside NMO is accessible to CMH but not SOD.

Analysis of Sequence and Structure—The ABM family (45,000 known sequences) is large and contains biochemically characterized oxidases (QuMo (7)) and oxygenases (ActVA-Orf6 (8), NMO, IsdGs (20)), as well as a large number of undescribed proteins that have no documented reactivity. Network analysis was carried out to divide the family into functionally cohesive subfamilies (Fig. 8). At a stringency sufficient to separate the well characterized IsdGs as a discrete cluster ($e^{-15}$), at least 20 distinct subfamilies are observed. Notably, although both catalyze monooxygenase reactions on polyketide antibiotic substrates, ActVA-Orf6 and NMO partition to separate subfamilies. Residues conserved within and between clusters are shown in Fig. 5.

Discussion

ABMs catalyze oxygenation reactions using no metal or organic cofactors, depending instead on the protein environment alone for catalysis. The highly activated nature of many ABM substrates has led to the suggestion that they act in lieu of cofactors, and a specific but largely untested analogy to flavoprotein chemistry has been drawn (2). The environment inside flavin-dependent oxidases and oxygenases indeed has an extraordinary influence over the cofactor, enhancing the rate of its oxidation by $10^4$–$10^6$-fold over the reaction in free solution (10). Here, using the ABM from the nogalamycin biosynthetic pathway (NMO), we examined whether flavoprotein-like catalytic strategies (Scheme 1) were at work, comparing the uncatalyzed and catalyzed reactions.

At least three well described features of the flavin/O$_2$ reaction were considered in the context of NMO. First, both the flavin cofactor and the substrate that it oxygenates react faster in their anionic deprotonated forms (33, 34). Although removal of the substrate proton is often base-catalyzed in flavoproteins (35), the relatively acidic flavin ($pK_a$ of flavin-N$_1$H $\approx$ pH 7) is stabilized in its anionic form by a strategically positioned positive charge or a hydrogen bonding partner (36). In keeping with this model, the pH-rate profiles for the NMO reaction ($k_{cat}$ and $k_{cat}/K_m$) showed that the alkaline enzyme-substrate complex reacts more rapidly than the acidic form (Fig. 4) by nearly 2 orders of magnitude with a $pK_a$ of 6.8 (dithranol $pK_a$ = 8.5). The structure and sequence of NMOs were examined for evidence of base catalysis, identifying well (His-85) and poorly (His-49) conserved potential bases in the open NMO cavity (Fig. 5). However, when either was mutated to alanine, there was no observable effect on either the magnitude or the pH dependence of the steady state constants (Fig. 4). Given the absence of any other plausible residue, this suggested that NMO function does not depend on general base catalysis.

This stands in sharp contrast to HOD, the recently characterized cofactor-free dioxygenase, which undergoes a 5500-fold diminution in $k_{cat}$ when its active site base is eliminated (23). Given the relative acidity of the NMO substrate, we considered the possibility that the protein may exert a more subtle effect. Specifically, Asn-63 and Asn-18 are strictly conserved within the NMO sequence cluster (Fig. 8) and are obvious candidates for hydrogen bonding to the substrate (Fig. 5). Consistent with prior observations (5), N63A and N18A mutant proteins exhibited activity that was strongly suppressed over the entire pH range examined. However, both still bound the neutral/proto-
nated substrate with affinity similar to that of the WT enzyme (pH 6.3). At pH values greater than the pK\textsubscript{a} of the substrate, the binding affinity diminished by 3- and 20-fold, respectively, for the N63A and N18A mutants. Parallel effects were observed for K\textsubscript{m}. This suggested that an interaction with both residues helps to selectively stabilize the anionic form of the substrate and might be partly responsible for lowering its pK\textsubscript{a}. Suppressed values for K\textsubscript{m} further suggested that these residues play an additional catalytic role.

A second defining feature of flavoprotein/O\textsubscript{2} catalysis is the formation of a semiquinone/O\textsubscript{2}\textsuperscript{-} radical pair in a rate-limiting step (Scheme 1A). The barrier to this important step is lowered by the enzyme, sometimes via a well positioned positive charge that stabilizes superoxide and thereby minimizes the reorganization energy following electron transfer (37–40).

An analogous substrate radical/O\textsubscript{2}\textsuperscript{-} pair can be proposed at the NMO-active site (Scheme 1B); however, such an intermediate is not obligate. As an alternative, the protein could stabilize the substrate or an intermediate in a radical doublet (↓) state, which could react directly with triplet O\textsubscript{2} (↑↑) to form a doublet substrate/O\textsubscript{2} adduct (↓). Such a reaction would not violate the spin rule and hence could occur rapidly. In fact, Trp-66, one of a handful of strictly conserved residues in the heme-degrading IsdG family (Fig. 5), has been shown to deform the normally planar heme inside these enzymes. Such ruffling induces unpaired electron density in the meso-carbons of the tetrapyrole (20, 41, 42), thereby facilitating the direct reactions between O\textsubscript{2} and heme (43, 44). A mechanism involving induction of radical character in the substrate has also been proposed for the oxidation of urate by the cofactor-independent urate oxidase (45, 46). Catalysis could alternatively involve formation of a triplet state di-radical adduct between the singlet substrate and triplet O\textsubscript{2}. Recent experimental and computational evidence suggested that the anion of the substrate for HOD reacts directly with O\textsubscript{2} to form such an intermediate in a step that limits the overall reaction rate (22).

In investigating the O\textsubscript{2} reactivity of NMO, we found that, consistent with the flavoenzyme paradigm, the reaction between dithranol and O\textsubscript{2} occurs as a slow step (Fig. 6). We then tested the hypothesis that a dithranyl/O\textsubscript{2}\textsuperscript{-} radical pair is the key catalytic intermediate that forms in the slow step, finding three kinds of evidence in support. First, a stable substrate radical inside the anaerobic NMO-substrate complex was not observed by EPR, arguing against a urate-oxidase-like activation of the substrate (data not shown). Second, using enzymatic (SOD) and small molecule (CMH) superoxide-trapping agents (Fig. 7A), the intermediacy of superoxide was confirmed for the uncatalyzed dithranone-forming pathway. Both were able to reverse the 60:40 ratio of dithranone/bisanthrone (pH 9.8) toward near-complete formation of bisanthrone, presumably due to the scavenging of O\textsubscript{2}\textsuperscript{-} away from a dithranyl/O\textsubscript{2}\textsuperscript{-} pair. A similar although smaller effect on the product ratio was observed for the NMO-catalyzed reaction carried out in the presence of CMH (Fig. 7A) but not SOD, suggesting that O\textsubscript{2}\textsuperscript{-} generated inside NMO could be quenched by reaction with the small molecule CMH. Third, the amount of CM\textsuperscript{+} formed by the dithranol/O\textsubscript{2} reaction, monitored by EPR, diminished in the presence of SOD for the uncatalyzed reaction, suggesting that CMH and SOD could compete for O\textsubscript{2}\textsuperscript{-}. However, SOD had no effect on CMH/O\textsubscript{2}\textsuperscript{-} trapping in the presence of NMO (Fig. 7C). Collectively, these experiments point toward a dithranyl/O\textsubscript{2}\textsuperscript{-} pair as a key intermediate on either the catalyzed/uncatalyzed pathway leading to the oxygenated product. Distinct from many flavoenzymes, there is no stable positive charge in the active site, although the amide side chains of Asn-63 and/or Asn-18 have charged resonance forms. Either of these residues could play a role in accelerating the formation of the radical pair.

A third and final role for the enzyme in flavin-dependent oxidases/oxygenases is in steering highly reactive intermediates toward a single set of products. This role asserts itself around at least two major decision points in the mechanisms of flavoenzymes (10). First, the initially formed flavin semiquinone/O\textsubscript{2}\textsuperscript{-} radical pair is in the triplet state (two unpaired electron spins) (9). The radicals in the pair are able to drift apart, generating the semiquinone and O\textsubscript{2}\textsuperscript{-} as the ultimate products. This is the observed outcome for flavin-dependent electron transferases as well as cryptochromes, light-receptor flavoprotein oxidases that use the resulting superoxide as a diffusible signaling agent (47–49). It is also a principal reaction pathway for free flavin in solution, where autocatalytic reactions between the flavin semiquinone and O\textsubscript{2} or O\textsubscript{2}\textsuperscript{-} compete with the enzymatically driven recombination (10). Alternatively, the two electron spins can pair and the radicals rejoin, forming the flavin-C4a-OO\textsuperscript{-} adduct. With the addition of a proton, this adduct becomes the central C4a-OOH reactive species of flavin monoxygenases and some oxidases. The pendant peroxy group can either depart from the flavin as H\textsubscript{2}O\textsubscript{2} (oxidases) or the O–O bond can cleave heterolytically as the terminal oxygen is incorporated into a substrate (oxygenses). In the latter case, the enzyme selects the appropriate substrate and tunes the oxidant toward either nucleophilic or electrophilic attack.

Because there is no adjacent imine nitrogen on dithranol to act as an electron sink, the analogous dithranyl-OOH adduct would appear to obligatorily follow the pathway in Schemes 1 and 2. This path predicts movement of the C10 hydride to the C10 oxygen, heterolytic O–O cleavage, and release of water. The critical steps controlling the outcome of the reaction therefore appear to be those that mediate the propensity of the radical pair to recombine or part ways. For the uncatalyzed reaction (Table 1 and Figs. 2 and 3), pH is clearly a key factor in this decision. First, the O\textsubscript{2}\textsuperscript{-}-dependent disappearance of dithranol accelerates dramatically with pH, with reaction time scales moving from hours to minutes. The acceleration is attributable to the faster formation of a dithranyl/O\textsubscript{2}\textsuperscript{-} pair as the pH increases. Second, the product distribution becomes more markedly biased toward dithranol, the oxygenase product, with increasing pH.

The NMO-catalyzed reaction exhibited the same pH-dependent trends in rate and product distribution, but to an even more marked extent. Together, these results suggested that the enzyme has two major functions. First, it catalyzes the formation of the radical pair, in a reaction that is faster when the substrate is deprotonated and where Asn-63 and/or Asn-18 may serve key roles. Second, it directs the radical pair away from the path of mutual dissociation and toward that of the
recombination product, dithranone. The fact that bisanthrone is still a product of the enzymatic reaction at pH 6.3 suggests that these two functions are not strictly coupled; the radical pair can form under catalyzed conditions and then leave the active site without recombining, leading to bisanthrone on a time scale of minutes rather than hours (uncatalyzed reaction).

How NMO selectively enforces the recombination at higher pH values is not clear. However, recombination, whether in the catalyzed or uncatalyzed process, appears to be the preferred route as the pH increases. Under these conditions, the substrate radical is neutral, rather than a protonated radical cation (Scheme 2). The possible connection between the protonation state of the dithranyl and its subsequent reactivity will be examined in future work.

The paradigms for enforcing “cofactorless O2 catalysis” are remarkably diverse. Although urate oxidase stabilizes a substrate radical and HOD a triplet substrate/O2 diradical adduct, NMO follows a third route, where the enzyme clearly plays a major role in both forming and directing the reactivity of a dithranyl/O2 pair. How does NMO lower the barrier (∆G°)? The uncatalyzed oxygenation of the substrate anion (pH 9.8) is orders of magnitude slower than the NMO-catalyzed process, illustrating that substrate deprotonation is not the primary function of the enzyme. At the same time, catalysis must be due to more than desolvation or restriction of substrate translational and rotational motions. Illustrating the point, the N63A and N18A mutants, which have fully intact structures and bind the substrate with reasonable affinity, have strongly impaired activity.

Instead, the principal role in catalysis appears to come ultimately from the ability of NMO to facilitate formation of a dithranyl/O2 pair. According to classic transition state theory (50), if the non-enzymatic and enzyme-catalyzed reactions share a similar transition state (as appears likely here), then dissociation constants for each (K_diss)S, S‡ or, in other words, by lowering K_T. Relating these constants to rates, if k_E describes the rate-limiting chemical transformation of ES to EP (equivalent to k_cat in the Michaelis model) and k_s describes the same step in the uncatalyzed case, then we get Equation 1.

\[
k_s/k_n = K_diss/K_s = K_s/K_T
\]

Inserting values measured under identical conditions for the nonenzymatic and NMO-catalyzed reactions (k_E = 60 min⁻¹, k_diss = 0.02 min⁻¹, K_s = 0.069 s⁻¹, K_T = 0.023 μM (pH 9.8), air, 20 °C, 30 μM S or ES complex) yields K_T = 0.023 μM. The associated extent of transition state stabilization for K_T relative to K_s is, therefore, 19 kJ mol⁻¹. In terms of magnitude, this is on the order of 2–3 hydrogen bonding interactions.

A more detailed investigation of the energy landscape promoting both the formation and recombination of the radical pair in NMO may allow us to understand the nature of the transition state and the enzyme structural features that promote its formation. These investigations may serve to support the development of catalysts that use O2 from the air and obviate the need for fragile, expensive, and nonrenewable cofactors.

Experimental Procedures

Expression and Purification of NMO—The gene encoding the N-terminally His6-tagged NMO (pBad vector) was received as a kind gift from the Schneider laboratory, University of Turku, Finland (5, 6). The NMO was expressed and purified as described previously (6). The purified enzyme mass was verified by electrospray ionization mass spectrometry.

Generation of Site-directed Mutants—Site directed mutagenesis was carried out using a QuickChange lightning kit (Agilent Technologies), the pBAD/His-NMO construct. Cultures of E. coli Top10 cells containing the mutant plasmids were grown, and the proteins were expressed and purified using the same protocol as for WT NMO.

Dithranol Stocks and Reaction Media—Dithranol (1,8-dihydroxy-9,10-dihydroanthracen-9-one, MP Biomedicals) was used as a surrogate for the natural NMO substrate (Scheme 1) (5, 6). Stocks (10 mM) were prepared in N2-equilibrated dimethyl sulfoxide (DMSO) in septum-sealed vials inside an anaerobic chamber (Coy) immediately prior to use. The DMSO stock was diluted into reaction media consisting of 1:2 (v/v) buffer/ME. The organic ME component was essential for solubilizing the substrate and product. All buffers were prepared at 0.1 M, supplemented with 0.3 M NaCl, and adjusted to the desired pH as follows: citrate (pH 4.2–6.3), BES (pH 6.6–7.6), Tricine (pH 7.4–8.4), CHES (pH 8.3–9.3), and CAPS (pH 9.8–10.8).

NMO/Substrate Binding Monitored by Fluorimetric Quenching—NMO/dithranol binding was monitored via quenching of the intrinsic fluorescence of the protein on forming the enzyme-substrate (ES) complex. Dithranol (1.25 mM) was added via air-tight syringe (Hamilton) to a septum-sealed N2-purged cuvette containing 6.5 μM NMO (pH 6.3 or 9.8) and allowed to equilibrate. 1,4-Dithiothreitol (DTT) (1 mM) was added via air-tight syringe (Hamilton) to a septum-sealed N2-purged cuvette containing 6.5 μM NMO (pH 6.3 or 9.8) and allowed to equilibrate. 1,4-Dithiothreitol (DTT) (1 mM) was added to both the dithranol titrant solution and protein solution to remove residual O2 and stabilize thiols Fluorescence measurements were made using a Cary50i fluorometer at room temperature with an excitation wavelength of 295 nm (tryptophan) and emission scanned from 300 to 400 nm (emission λmax = 328 nm). The percentage of quenched fluorescence intensity was plotted against [substrate] and fit to Equation 2 to obtain K_D.

\[
\Delta F_{obs} = \frac{\Delta F_{max}}{2E_T} (L_0 + E_I + K_0 - \sqrt{(L_0 + E_I + K_0)^2 - 4E_T L_0})
\]
Cofactorless Dioxgen Catalysis in ABMs

$V_{op}, E_c, K_D$, and $\Delta F_{max}$ are the ligand concentrations, total protein (subunit) concentration, the equilibrium dissociation constant, and the maximum % fluorescence that was quenched.

**pH Titration of Dithranol**—Aliquots of dithranol (100 μM) (Tris-ME (pH 5.0), 20 °C) were titrated with 1 N NaOH and their pH (Corning 430 pH electrode) and ultraviolet-visible spectra were measured (Cary60i spectrometer). Absorbance values at spectral maxima were plotted versus pH and fit as shown in Equation 3,

$$y = (B \times 10^{-pH} + A \times 10^{-pK_a})/(10^{-pH} + 10^{-pK_a}) \text{ (Eq. 3)}$$

where $A$ and $B$ are the highest and lowest absorbance values, respectively.

**Analysis of Reactants and Products by HPLC**—Dithranol and its quinonoid oxidation product dithranone (Sigma) were dissolved (500 μM) in 1:1 Tris-ME/tetrahydrofuran in an anaerobic chamber. The dimeric product, bisanthranol, was prepared by exposing 500 μM dithranol under acidic conditions to atmospheric O$_2$ (>60 min, 20 °C, Tris-ME (pH 6.3)) (51, 52). An Agilent 1100 series HPLC instrument with diode array UV-visible detection (300–700 nm) was used as follows: flow rate of 1.5 ml/min, 50 °C, 20–μl injection volume, Phenomenex Luna C18 3 μ column, 150 × 4.6 mm. The solvents were 99.9% H$_2$O, 0.1% TFA (solvent A) and 99.9% acetonitrile, 0.1% TFA (solvent B). Separation of the three molecules began with 50% solvent A (0–2 min) followed by a gradient (2–11 min) to 5% solvent A, 95% solvent B and a short isocratic phase (11–14 min). Standard curves were generated from 25–250 μM using integrated HPLC peak intensities. Error bars are ±1 S.D. Dithranol and dithranone were directly quantified relative to standard curves; bisanthranol concentrations were deduced as the difference between the total substrate/product component and the measured amounts of dithranol and dithranone.

**Monitoring Uncatalyzed Reactions Over Time**—Uncatalyzed reactions between dithranol and O$_2$ were analyzed discontinuously over time by HPLC and continuously by UV-visible spectroscopy and O$_2$ consumption assays (pH 6.3 and 9.8). For HPLC, reactions were initiated by exposure of anaerobic dithranol solutions to air at the desired pH (500 μM, 20 °C). Reaction vials were gently stirred. The same reactions were monitored by UV-visible spectroscopy (100 μM dithranol, air, 20 °C) and O$_2$ consumption (2-20 μM dithranol, air, 20 °C), using the methods described below but excluding NMO.

**Monitoring the NMO-catalyzed Reaction Over Time in the Steady State**—Reactions were monitored via both UV-visible spectroscopy and O$_2$ consumption. For determining UV-visible spectra, 1-ml reaction solutions containing 10–200 μM dithranol (in buffer/ME at the desired pH, 20 °C) were equilibrated to air and initiated by the addition of 2–20 μM NMO (all concentrations are given as NMO subunit). For determining Michaelis-Menten parameters, the reaction was monitored in air with [dithranol] varied over 10–200 μM. The initial portion of each curve was fit to a linear equation to obtain the initial velocity ($v_i$, KaleidaGraph). Points were measured in triplicate and averaged (error = ±1 S.D.). Values for $v_i$ were plotted versus [dithranol] and fit to Michaelis-Menten Equation 4, a derivative equation including substrate inhibition (Equation 5), or a derivative accounting for cooperative substrate interactions with dimeric NMO ($h$ = Hill coefficient) (Equation 6),

$$v_i = v_{max}[S]/(K_M + [S]) \text{ (Eq. 4)}$$
$$v_i = v_{max}/(1 + K_M/[S] + [S]/K) \text{ (Eq. 5)}$$
$$v_i = v_{max}[S]^h/(K_M^h + [S]^h) \text{ (Eq. 6)}$$

For O$_2$ consumption, a Clark-type O$_2$ electrode (Yellow Springs International) in a temperature-controlled chamber (2 ml reactions, 20 °C) was used with constant stirring. The rate of background consumption of O$_2$ by dithranol was measured for 1 min in buffer/ME. NMO was subsequently added in catalytic amounts (2–20 μM) to initiate the reaction. Dithranol concentrations were varied from 40 to 1500 μM and [O$_2$] from 0.05 to 1.3 m. The linear background rate of non-enzymatic O$_2$ consumption was subtracted from the rate measured in the presence of NMO. Data were plotted and fit as described above.

**Effects of pH on the Steady State Reaction**—Values of $k_{cat}$ and $k_{cat}/K_M$(dithranol) were measured in air via O$_2$ electrode as a function of pH. Buffers with similar structures and a range of $pK_a$ values (described above) were chosen and evaluated at overlapping pH values to ensure that kinetics were not buffer-dependent. The effects of incubating the enzyme at various pH values before initiating reactions versus adding enzyme to assay mixtures last (“pH jump”) were evaluated. All buffers contained 0.3 mM NaCl to minimize differences in ionic strength. Values of the Michaelis parameters were plotted versus pH and fit to Equation 7 to obtain values for $pK_a$,

$$\log(c/(1 + (10^{-x}/10^{-pK_a}))) \text{ (Eq. 7)}$$

where $c$ is the difference between the minimum and maximum log $k_{cat}$ values, and $x$ is the pH.

**Transient Kinetics of the Reaction between the NMO-Substrate Complex and O$_2$**—Reactions were monitored using a Hi-Tech Scientific stopped-flow spectrophotometer in single mixing mode with diode array detection. The system was sealed from ambient atmosphere and rendered anaerobic by overnight incubation with protocatechuate dioxygenase and its substrate, protocatechuate (method of Ballou) (53). Protocatechuate dioxygenase and protocatechuate were flushed out of the instrument using anaerobic buffer/ME and a baseline spectrum measured (280–700 nm). All reaction solutions were made anaerobic using a double manifold Schlenk line with alternating cycles of argon gas purging and evacuation.

Concentrated solutions of the anaerobic ES (NMO-dithranol) complex were generated by adding 90 μM NMO (CAPS/ME (pH 9.8)) to 60 μM dithranol in an air tight tonometer. These were mixed with CAPS/ME (pH 9.8), equilibrated to various O$_2$ concentrations. Final O$_2$ concentrations after mixing with ES complex (measured via electrode) were 67, 140, 270, 390, and 590 μM. Progress of reaction curves were fit to single or double exponential equations, as described in the text, yielding values for $k_{obs}$ (KinetAssyst).

**Analysis of Reaction Products**—The products of the NMO-catalyzed reaction were quantitatively analyzed by HPLC at various pH values. Reactions were 500 μM dithranol, air (280 μM O$_2$), 22 °C, with either 1 mM NMO (citrate/ME (pH 6.3), 30
min) or 50 μM NMO (CAPS/ME, pH 9.8, 10 min). Products for the un catalyzed reactions were generated under identical conditions, but incubation times sufficient for these reactions to go to ≥90% completion were used, i.e. >3 half-lives: 34 h citrate/ME (pH 6.3) or 40 min CAPS/ME (pH 9.8).

Effects of Superoxide (O2•−) Trapping on Product Distribution—Reactions and product analyses were carried out as described above but in the presence of 0, 0.5, 1.25, 2.5, or 5 mM CMH, a small molecule radical trapping agent with high specificity for O2•− (k = 106–1011 M−1 s−1 (pH 7.4)), yielding CM• and H2O2 (54). Alternatively, SOD (32.5 kDa, 500–5000 units/ml, 0.05–0.2 mg/reaction, Sigma) was used to enzymatically convert 2 O2•− to H2O2 and O2 with greater steric restriction but at a substantially higher rate, k = 6.4 × 109 M−1 s−1 (pH 7.8) (55).

Detection and Quantification of Superoxide via the CM Radical by Continuous Wave EPR Spectroscopy—O2•− trapping was monitored over time via the characteristic three-line EPR spectrum for CM•, a stable nitroso radical that forms following transfer of H+ from CMH to O2•−. EPR spectroscopy was carried out at 22 °C using a Varian spectrometer: 0.2 millilawt, 0.1 milliliter modulation amplitude, 32 ms time constant, 1-min time scans for 20–120 min. CM• was quantified via a standard curve generated using 2,2,6,6-tetramethyl-1-piperidinoloxyl (TEMPO), a stable free radical with a similar three-line EPR signal. Peak height was plotted versus [TEMPO] (25–300 μM TEMPO, CAPS/ME (pH 9.8)), fit to a linear equation, and the slope used to quantify unknowns.

To minimize background autocatalytic reactions between CMH/O2 (54), EPR samples were prepared in buffers from which metals had been removed by treatment with Chelex resin (Sigma). Uncatalyzed reactions contained 200 μM dithranol, 0.1 mM diethylenetriaminepentaacetic acid as a metal chelator, air (280 μM O2), and 1–2 mM CMH. Catalyzed reactions further contained 5 μM NMO. SOD was added to samples at 2500 units/ml, as noted. The reactions were rapidly mixed, injected into a flat cell EPR tube, and placed in the cell holder prior to measurement (manual mixing and sample loading dead time ~2–3 min).

Sequence Analysis—The ABM family was divided into subfamilies using protein network analysis (EFI-EST PFAM/Inter pro Analysis). Members of each of the subfamilies containing NMO, ActVA-Orf6, and IsdG were used to generate sequence alignments (ClustalW) to identify residues conserved within each.

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