Evidence That Dioxygen and Substrate Activation Are Tightly Coupled in Dopamine β-Monooxygenase

IMPLICATIONS FOR THE REACTIVE OXYGEN SPECIES*

Oxygen activation occurs at a wide variety of enzyme active sites. Mechanisms previously proposed for the copper monooxygenase, dopamine β-monooxygenase (DbM), involve the accumulation of an activated oxygen intermediate with the properties of a copper-peroxo or copper-oxo species before substrate activation. These are reminiscent of the mechanism of cytochrome P-450, where a heme iron stabilizes the activated O₂ species. Herein, we report two experimental probes of the activated oxygen species in DbM. First, we have synthesized the substrate analog, β,β-difluorophenethylamine, and examined its capacity to induce reoxidation of the pre-reduced copper sites of DbM upon mixing with O₂ under rapid freeze-quench conditions. This experiment fails to give rise to an EPR-detectable copper species, in contrast to a substrate with a C–H active bond. This indicates that the reoxidation of the enzyme-bound copper sites in the presence of O₂ is tightly linked to C-H activation or that a diamagnetic species Cu(II)-O₂ has been formed. In the context of the open and fully solvent-accessible active site for the homologous peptidylglycine-α-hydroxylating monooxygenase and by analogy to cytochrome P-450, the accumulation of a reduced and activated oxygen species in DbM before C-H cleavage would be expected to give some uncoupling of oxygen and substrate consumption. We have, therefore, examined the degree to which O₂ and substrate consumption are coupled in DbM using both end point and initial rate experimental protocols. With substrates that differ by more than three orders of magnitude in rate, we fail to detect any uncoupling of O₂ uptake from product formation. We conclude that there is no accumulation of an activated form of O₂ before C-H abstraction in the DbM and peptidylglycine-α-hydroxylating monooxygenase class of copper monooxygenases, presenting a mechanism in which a diamagnetic Cu(II)-peroxo complex, formed initially at very low levels, abstracts a hydrogen atom from substrate to generate Cu(II)-hydroperoxo and substrate-free radical as intermediates. Subsequent participation of the second copper site per subunit completes the reaction cycle, generating hydroxylated product and water.

Dopamine β-monooxygenase (DbM) along with peptidylglycine-α-hydroxylating monooxygenase (PHM) comprise a unique class of enzymes that contain only copper as a cofactor and catalyze the cleavage of O₂ to form hydroxylated product and water. DbM is of central importance in the catecholamine biosynthetic pathway, catalyzing the conversion of dopamine to norepinephrine (Scheme 1, top), where both substrate and product serve as neurotransmitters within the central nervous system (1). Primarily localized within the secretory granules of adrenal chromaffin cells and neurons, DbM is a large, tetrameric glycoprotein (75 kDa per monomer) consisting of two disulfide-linked dimers.

Although no crystal structure has been reported, extensive structural data exist for DbM. Extended X-ray absorption fine structure was used to characterize the ligand environment of the two copper atoms per subunit in both oxidized and reduced forms of DbM; in the absence of any evidence for back scattering between the metal sites, the distance between the two coppers per subunit was concluded to exceed 4 Å (2, 3). EPR spectroscopy also failed to detect any spin coupling between metal sites in oxidized, resting enzyme (4) and in a catalytically generated product complex (5). These findings provided early evidence against a reactive binuclear center and, instead, implicated separate functions for the two copper centers. The Cu₄ (Cu₄ in PHM), liganded by three histidines (DbM: His-255, His-256, His-326; PHM: His-107, His-108, His-172) and water, has historically been assigned to the electron transfer site, and Cu₂ (Cu₂ in PHM), liganded by two histidines (DbM: His-405, His-407; PHM: His-242, His-244) and water together with a long bond to methionine (DbM: Met-480; PHM: Met-314), has historically been assigned to the substrate binding and hydroxylation site.

PHM, the second enzyme compromising this class of monooxygenase, catalyzes the first step in the C-terminal amidation of glycine-extended peptides and yields a peptidyl α-hydroxylglycine intermediate (Scheme 1, bottom). This is followed by dealkylation (catalyzed by peptidylglycine-α-amidating lyase) to produce glyoxylate and C-terminal-amidated neuropeptide/hormone. PHM is active as a 35-kDa monomer, designated PHMcc, or as a covalently linked, bifunctional protein with peptidylglycine-α-amidating lyase, designated peptidylglycine-α-amidating monooxygenase (6, 7). Despite their large difference in size, DbM and PHM share a 28% sequence identity extending through a common catalytic

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The abbreviations used are: DbM, dopamine β-monooxygenase; PHM, peptidylglycine-β-hydroxylating monooxygenase; PHMcc, catalytic core of PHM; EPR, electron paramagnetic resonance; DPFA, β,β-difluorophenethylamine; Cu₄⁺, electron transfer copper; Cu₂⁺, oxygen binding copper; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
domain of ~270 residues, which includes the conserved copper ligands (8). In addition both enzymes require two copperers per subunit for full activity (9–11) and are believed to utilize ascorbate as the in vivo two-electron donor (12).

The crystal structure of PHM, solved for both oxidized (13) and reduced (14) enzyme, confirmed many of the earlier spectroscopic data. Important features determined from the crystal structure are (i) a two-domain structure in which each domain binds a single copper atom, (ii) a distance of ~10.6 Å (oxidized PHMcc with bound peptide substrate) between the two copper sites, (iii) the absence of closure of the copper binding domains in either enzyme form studied, and (iv) the identification of a water-filled cavity that is at the solvent interface and “links” the two copper binding domains. Extensive debate has taken place in the recent literature regarding the pathway for electron transfer between copper sites and the nature of O₂ activation in DβM and PHM (14, 15).

Comparison of the kinetic parameters for DβM and PHM with substrates of comparable reactivity indicates the same intrinsic hydrogen/deuterium isotope effect (~11) for the C-H activation step (16). Additionally, similar O-18 isotope effects for these two enzymes that decrease with substrate deuteration imply a chemical mechanism for substrate oxidation that is likely to be identical (17). Studies of the kinetic mechanism indicate that both DβM (in the presence of the dianion activator fumarate) and PHM proceed in a preferred ordered mechanism with substrate binding to enzyme before O₂ (16, 18). Thus, all available data imply that DβM and PHM can be regarded interchangeably with respect to mechanism and active site structure.

In early studies of DβM with either substrates or substrate analogs it was concluded that functionalization of substrate involved hydrogen atom abstraction to yield a free radical intermediate (19, 20). Identification of the oxygen species catalyzing hydrogen abstraction from substrate has proven far more elusive. The observation of pH-dependent isotope effects for DβM provided evidence for the involvement of a single proton in the chemical conversion process, leading to the proposal of a copper hydroperoxide as the reactive oxygen intermediate (III in Scheme 2) (18). However, a detailed analysis of the effects of substrate structure and deuteration on O-18 isotope effects (21) found to be inconsistent with the earlier proposed Cu(II)–OOH² and suggested a reductive cleavage of this intermediate to generate copper-oxo as the hydroxylating agent (IV in Scheme 2). This interpretation assumed classical behavior of hydrogen during transfer from substrate to oxygen, which has now been shown in the case of PHM to be dominated by hydrogen tunneling (22). Additionally, recent site-specific mutagenesis studies with PHM unambiguously eliminates a role for the most plausible active site candidate (PHM: Y318; Y484) in reductive activation of Cu(II)–OOH (17). The lack of extensive pH studies for the PHM reaction together with a failure to identify a proton donor from the crystal structure has led to the proposal of mechanism II in Scheme 2 (14). A common feature of all previously proposed mechanisms for PHM and DβM (cf. II, III, and IV in Scheme 2) is that oxidation of both copper centers occurs before substrate activation and leads to the accumulation of a partially reduced form of dioxygen.

DβM: Y484 in reductive activation of Cu(II)–OOH (17). The lack of extensive pH studies for the PHM reaction together with a failure to identify a proton donor from the crystal structure has led to the proposal of mechanism II in Scheme 2 (14). A common feature of all previously proposed mechanisms for PHM and DβM (cf. II, III, and IV in Scheme 2) is that oxidation of both copper centers occurs before substrate activation and leads to the accumulation of a partially reduced form of dioxygen. Blackburn et al. (23) observe significant changes in the copper coordination structure during oxidation, which would appear to preclude a rapid inter-copper electron transfer. They proposed an alternative possibility based on the finding that CO binds to the electron transfer copper in the presence of peptide substrate; this involves a role for superoxide as the electron carrier between Cu₆ and Cu₇ (15).

Many of the paradigms for the copper monooxygenase mechanism have come from the very detailed chemical analyses of cytochrome P-450, which has been shown to release hydrogen peroxide as product either in the absence of a substrate or in the presence of a poor substrate (24). Additionally, activated oxygen at the active site of cytochrome P-450 has been shown to be capable of further reduction to water (25). These side reactions of cytochrome P-450 lead to an uncoupling of substrate hydroxylation from uptake of O₂ and occur despite the sequestration of the active site from bulk solvent (26). For enzymes like DβM and PHM, whose active sites are fully exposed to solvent, it is expected that extensive uncoupling of substrate hydroxylation and O₂ uptake may occur. In the case of DβM, the size of the primary isotope effects on kcat and kcat/Km for certain phenethylamine substrates indicates that C-H cleavage is rate-determining, with the implication of a steady state accumulation of an enzyme complex that contains the activated

² Note that the charge on oxygen species complexed to copper is omitted when a solid line is drawn between metal and oxygen, e.g. representation of Cu(II)–OOH (Scheme 2) as Cu(II)–O–OH in text.
oxygen intermediate. Thus, if the copper monooxygenase mechanism occurs with a build-up of any type of activated oxygen intermediate, we would expect to observe some degree of uncoupling between oxygen reduction and substrate consumption that would increase significantly as the chemical reactivity of the substrate diminishes. The stoichiometry of the DβM (27) and PHM (28) reactions with their respective substrates, dopamine and ß-Tyr-Val-Gly, was previously shown to be 1 eq of dioxygen consumed for each substrate, with their respective substrates, dopamine and D-Tyr-Val-Gly, contained at 0.2 M with NaCl. The concentration of the substrates (tyramine, dopamine, and phenethylamine) ranged from 0.05 to 10 mM, whereas the ionic strength was maintained at a constant value of 0.15M with the 1 cm optical path and PHM while indicating the inappropriateness of inferences derived from the family of heme-iron-dependent monooxygenases.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—** Soluble DβM was isolated as previously described (5) from bovine adrenal glands. The protein concentration was estimated from the absorbance at 280 nm (ε280 = 1.24 ml mg⁻¹ cm⁻¹). A monomer mass of 75 kDa was used in calculations of enzyme concentration. Trace metal analysis of enzyme-bound copper was performed on a PerkinElmer 3000DV Inductively Coupled Plasma-Atomic Emission Spectrophotometer using commercially available metal standard solutions. Catalase (65,000 units/mg) was from Roche Applied Science. All other materials were of reagent grade. The compounds 4-hydroxyphenylethylamine (tyramine), 3,4-dihydroxyphenylethylamine, and 4-hydroxyphenylglycolic acid (octopamine), and phenethylamine were purchased from Sigma and used as the hydrochloride salts. [1-14C]tyramine hydrochloride, with a specific activity of 55 mCi/mmol, was purchased from American Radiolabeled Chemicals, Inc. 4-(Trifluoromethyl)phenethylamine hydrochloride was synthesized from the commercially available nitrile (purchased from Aldrich) as previously described (31). A saturated solution of sodium metabisulfite (896 mg, 4.7 mmol) was added to 4-(trifluoromethyl)benzaldehyde (1g, 5.7 mmol), and the solution was stirred on ice. A layer of ether (5 ml) was added on top, and an ice-cold saturated solution of sodium cyanide (782 mg, 15.9 mmol) was added. The aqueous layer was diluted with a small amount of water and combined. After separation, other solvent was substituted with bisulfite solution and then with water. The resulting cyanohydrin was isolated by silica-gel flash chromatography (20% ethyl acetate, 80% hexane) and reduced with LiAlH₄ as described in Poos et al. (32) without further purification. The amine was converted to the hydrochloride salt with HCl in ether. Elemental analysis of the hydrochloride salt was found to contain 44.58% C, 4.29% H, 5.80% N (calculated: 44.74% C, 4.59% H, 5.80% N).

**Synthesis of 4-(Trifluoromethyl)phenethylamine Hydrochloride—** 4-((Trifluoromethyl)benzaldehyde was reacted with sodium metabisulfite and sodium cyanide as described previously (31). A saturated solution of sodium metabisulfite (896 mg, 4.7 mmol) was added to 4-(trifluoromethyl)benzaldehyde (1g, 5.7 mmol), and the solution was stirred on ice. A layer of ether (5 ml) was added on top, and an ice-cold saturated solution of sodium cyanide (782 mg, 15.9 mmol) was added. The aqueous layer was diluted with a small amount of water and combined. The solvent was then substituted with bisulfite solution and then with water. The resulting cyanohydrin was isolated by silica-gel flash chromatography (20% ethyl acetate, 80% hexane) and reduced with LiAlH₄ as described in Poos et al. (32) without further purification. The amine was converted to the hydrochloride salt with HCl in ether. Elemental analysis of the hydrochloride salt was found to contain 44.58% C, 4.29% H, 5.80% N (calculated: 44.74% C, 4.59% H, 5.80% N).

**Synthesis of β,β-Difluorophenethylamine Hydrochloride—** Styrene was first converted to the azirine (2-phenyl-1-azirine) as described previously (33). To a solution of styrene (52.19 g, 57.5 ml, 0.50 mol) in 400 ml of CCl₄ in an ice bath was added a solution of bromine (88 g, 0.25 mol) through an addition funnel over 30 min. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated under vacuum to yield 1,2-dibromo-2-phenylbenzene, a white solid that was immediately dissolved in 750 ml of dry Me₂SO and placed in a 1-liter 3-necked round-bottomed flask. While on an ice bath Na₂S₂O₃ was added (49 g, 0.75 mol) with stirring and under nitrogen. After 12 h the reaction mixture was cooled on an ice bath, and 20 g of NaOH (0.5 mol) in 20 ml of H₂O was added. After 1 h of stirring only one spot was visible by TLC (n-hexane). The mixture was diluted to a volume of 2 liters with 2% NaHCO₃ in H₂O and extracted with petroleum chloride 4 × 200 ml, and the combined extracts were washed with H₂O, filtered, and concentrated in vacuo to yield a brown powder. The crude product was dissolved in concentrated H₂SO₄ (20% anhydrous Al₂O₃) column with petroleum ether as the eluent. This gave 72.8 g of a light yellow oil, which was dissolved in 1.25 liter of toluene. 1.2 liters of the α-azido styrene in toluene was placed in a round-bottom flask. The mixture was heated under reflux, and progress of the reaction was monitored by TLC (n-hexane,ethyl acetate). After 1 h and 20 min the reaction was quenched with the addition of 60 ml of water at 78 °C. Finally the azide was converted to the hydrochloride salt by heating in an HCl solution under reflux for 0.5 h. The solvent was evaporated and solid-washed with acetone. The white solid was recrystallized 3 times from a mixture of ethanol and ether to give 1.26 g (7%...
yield of the hydrochloride salt. The final product was found to have a melting point of 181–183 °C, and the elemental analysis gave 49.50% C, 5.15% H, 7.35% N (calculated: 49.63% C, 5.21% H, 7.23% N).

Freeze-quench Experiments with DjBM—A detailed description of the set-up as well as the experimental procedures can be found in Brenner et al. (5). A dead time of 4 ms was estimated as described previously. The samples used for EPR analysis were quenched at various time points ranging from 9 to 470 ms. The manner in which reaction solutions were mixed for a sample with tyramine was the following; initially, equal volumes of the enzyme solution consisting of 60 μM DjBM monomer, 100 mM potassium Pi (pH 6.19), 30.6 mM ascorbic acid, 10 mM fumarate, and 50 mM potassium Pi (pH 6.0) were added. Then, either 75 mM DFPA, 25 mM KCl, or 2 mM CuCl2 was added. The concentration of substrate stock solution, 10 mM tyramine, 90 mM KCl, 10 mM fumarate, and 50 mM potassium Pi (pH 6.0) failed to yield the final conditions. These contained 15 μM DjBM (2 Cu2+/subunit), 100 mM total chloride adjusted with KCl, 15 μM ascorbate, 10 mM fumarate, 50 mM potassium Pi (pH 6.0), 30 μM Cu2+, and either 5 mM tyramine or 40 mM β,β-difluorophenethylamine (DFPA) at 25 °C. Air oxidation of ascorbate-reduced enzyme was studied under identical conditions as described above but lacking tyramine or DFPA substrate.

Oxygen Activation in DjBM

RESULTS

Kinetic Studies of β,β-Difluorophenethylamine—The coupling of O2 and substrate activation can be tested by the use of substrate analogs that are unable to undergo C-H activation. Fluorinated substrates appear to be well suited for this approach, and the analog, β,β-difluorophenethylamine was synthesized as described under “Experimental Procedures.” We find that the analog is a weak competitive inhibitor of substrate (Ki = 36 ± 3 mM) for DjBM (data not shown). Under steady state conditions, up to 75 mM difluoro analog and 10 μM DjBM failed to support any uptake of O2 using a Clark oxygen electrode. Steady state assays indicated that there was no consumption of oxygen above the ascorbate background in the presence of DFPA. As a control it was shown that preincubation of enzyme with substrate plus DFPA gave a similar stability profile to incubation with substrate alone, ruling out any enzyme inactivation by DFPA.

We, therefore, turned to pre-steady state conditions to see if a stoichiometric amount of pre-reduced enzyme could be reoxidized in the presence of the difluoro analog. These experiments contained 40 mM of the analog and 15 μM DjBM, leading to an estimated 60% occupancy of the enzyme sites. This concentration was a balance between achieving significant occupancy of the enzyme active sites and having sufficient analog to measure reaction at many time points. In the course of these experiments, enzyme is first pre-reduced with ascorbate under anaerobic conditions and then mixed with either tyramine or DFPA. As reported earlier (5), rapid mixing of anaerobically reduced enzyme with tyramine and dioxygen led to full reoxidation of both coppers within 500 ms of mixing (Fig. 1). By contrast, mixing of the anaerobically reduced Cu(I) form of enzyme with DFPA and O2 failed to give rise to a detectable Cu(II) EPR signal within 500 ms (Fig. 1). The time course for DFPA appears similar to that in the absence of substrate or analog, consistent with earlier reports that substrate is required for reoxidation of reduced copper either under steady state (35) or pre-steady state conditions (5). After 20 ± 43% of the copper was reoxidized in the DFPA-incubated samples compared with 52% of nonspecific oxidation for enzyme-bound copper in the absence of substrate or substrate analog; these are considered within experimental error. Even after 20 min only 53% of the total copper had been reoxidized to Cu(II) in the air-incubated sample.

The failure of DFPA to support copper reoxidation in a single turnover experiment could be due to two explanations. The first is that copper reoxidation is tightly coupled to C-H activation and that no activated oxygen species is formed at the level of detection of the EPR (limits of detection in these experiments, ~10% of total enzyme). The second possibility is that an oxidized copper, spin-coupled species had formed. One candidate is a Cu(II)–OOH substrate intermediate that is anti-ferromagnetically coupled to the second copper at the CuA site. This seems highly unlikely in view of the absence of any spin coupling in catalytically generated enzyme-product complexes with DjBM where both coppers are in the +2 oxidation state (36). As an alternative, we propose that the addition of O2 leads to oxidation of only a single copper site (CuB) to produce a spin-coupled cupric-superoxide complex, CuB(II)–O2−. Though not considered in previous investigations of PHM and DjBM mechanisms, this offers an alternate view of O2 activation that is described under “Discussion.”

Tight Coupling of Substrate and O2 Activation—Two experimental approaches have been used in this study to quantify the stoichiometry of substrate hydroxylation to O2 uptake. Using fast substrates, end-point assays were performed to compare the stoichiometry of dioxygen consumption (by Clark O2 electrode) to that of product formation (by HPLC). In the case of slower substrates, where achievement of reliable end-points becomes extremely difficult, we compared initial rates of O2 uptake
consumption and product formation. Experiments were reproducible over multiple experiments, as shown in Table I, for the slowest substrate, 4-(trifluoromethyl)phenethylamine.

The data in Table I show a background rate of O₂ consumption before the addition of enzyme that was assumed to be constant over the time course of the experiment. This is largely due to the presence of Cu(II) ions and ascorbate, which are necessary to optimize conditions for DβM turnover. The rate of air oxidation of ascorbate in aqueous solution is very dependent on pH and catalytic metals and produces dehydroascorbate with concomitant production of hydrogen peroxide, superoxide, and hydroxyl radicals (37). These reduced oxygen species, more specifically H₂O₂, are known to inactivate DβM (38) and PHM (39). For this reason catalase was included in the majority of reactions to disproportionate hydrogen peroxide to water and dioxygen and prevent inactivation of DβM over the time course of the experiments. Although the presence of catalase would underestimate DβM uncoupling by a factor of two (for H₂O₂ production) or by a factor of four (for superoxide leakage), the sensitivity and reproducibility of our experiments is very high and indicates a molar ratio of O₂ consumption to product formation of 0.99 ± 0.04. To demonstrate that catalase did not alter the measured stoichiometries with p-(trifluoromethyl)-phenethylamine, a limited number of experiments in the absence of catalase were performed. To avoid high backgrounds, no exogenous copper was added to the assays. Samples of DβM were prepared with 1.9 eq of pre-bound copper, and the ratio of O₂ uptake to product formation was determined. Although the background correction for O₂ consumption was greater, in -

| Presence of catalase | μM | μM | μM | nmol | nmol | nmol | nmol |
|----------------------|----|----|----|------|------|------|------|
| 2                    | 0.88| 0.45| 23.8| 5.47 | 18.3 | 5.97 | 17.8 | 1.03 |
| 2                    | 0.88| 0.45| 20.1| 3.00 | 17.1 | 3.25 | 17.2 | 0.99 |
| 2                    | 2.07| 1.06| 65.2| 3.84 | 61.4 | 5.16 | 65.7 | 0.93 |
| 2                    | 2.07| 1.06| 70.9| 4.80 | 66.1 | 5.16 | 65.1 | 1.01 |
| 2                    | 2.07| 1.06| 81.5| 6.42 | 75.1 | 5.16 | 76.2 | 0.99 |

| Absence of catalase  | 0   | 0.93| 1.79| 59.7| 14.5 | 45.2 | 49.8 | 40.3 | 1.12 |
| Absence of catalase  | 0   | 0.93| 1.79| 58.2| 19.4 | 38.8 | 49.1 | 39.6 | 0.98 |
| Absence of catalase  | 0   | 0.93| 1.79| 58.6| 20.7 | 37.9 | 50.9 | 41.4 | 0.92 |

| Exo(Cu) | [DβM] | [E-Cu] | O₂ uptake | Product formation | Molar ratio of O₂ consumption to product formation |
|---------|-------|-------|-----------|-------------------|--------------------------------------------------|
| Total   | Background | Corrected | Total | Blank | Corrected | nmol | nmol | nmol |

### Table II

**Relationship between substrate structure and coupling of O₂ uptake to product formation**

| Substrate | X | Y | (kcat/Km)substrate (μM⁻¹s⁻¹) | Molar ratio O₂/ Product |
|-----------|---|---|----------------------------|-------------------------|
| dopamine  | OH| OH| 15.6 ± 0.9 | 1.03 ± 0.05 |
| phenethylamine | H | H | 10.4 ± 0.6 | 1.00 ± 0.05 |
| tyramine  | OH| H | 28 ± 3 | 1.04 ± 0.05 |
| p-CF₃-PFA | CF₃| H | 0.008 ± 0.001 | 1.00 ± 0.006 |

*Refers to the structure:*  

\[
\text{X} \quad \text{Y} \quad \text{O} \quad \text{H} \quad \text{CH}_2 \text{CH}_2 \text{NH}_2 \text{Cl}
\]

**Relationship of O₂ Activation to Substrate Hydroxylation in the DβM and PHM Family of Copper Monooxygenases—Two major observations have been made in this study. The first is**
that a large change in substrate reactivity does not perturb the relationship of $O_2$ uptake to substrate hydroxylation (Tables I and II), and the second is the failure to observe any Cu(II) formation from Cu(I) in the presence of $O_2$ with an inert substrate analog (Fig. 1). The aggregate of these experiments point toward an extremely tight coupling between $O_2$ activation and C-H cleavage from substrate.

What mechanisms can be proposed that take into account such tight coupling while also accommodating the extensive mechanistic literature on PHM and D$eta$M? Any proposed mechanism must involve a net hydrogen atom abstraction from substrate to $O_2$ in the initial step of substrate oxidation coupled to some type of activation of the ground state, triplet $O_2$. The detection of sizeable O-18 isotope effects on $k_{cat}/K_m(O_2)$, which are perturbed by substrate deuteration (21), means that any $O_2$ chemistry that occurs before loss of hydrogen from substrate must be a fully reversible process (17). The actual transfer of hydrogen occurs by a tunneling mechanism (22), indicating that the origin of the O-18 isotope effect lies with any pre-equilibrium reduction of $O_2$ together with heavy atom motions within $O_2$ that are necessary for effective tunneling of hydrogen from reactant to the $O_2$ acceptor. Finally, there are no active site residues other than copper and its ligands, which appear to play a direct role in bond cleavage (13, 14, 17).

The above properties rule out a copper-oxo species from further consideration (cf. Ref. 17). Similarly, given the two-electron, one-proton transfer that is necessary for formation of a Cu(II)$-OOH$ intermediate from $O_2$ (Scheme 2, III), it seems very unlikely that this species would be produced at an undetectable level via a reversible, proton-coupled long range electron transfer process. This leaves open the possibility of a copper-peroxo (Scheme 2, II) or copper-superoxo species (Scheme 2, I). The fundamental difference between these two species lies with the number of electrons that are transferred to $O_2$ before C-H activation.

A copper-peroxo mechanism (Scheme 2, II) involves a two-electron reduced form of $O_2$, requiring a long range electron transfer between copper centers before C-H activation. Because $k_{cat}/K_m(O_2)$ is at least partially limited by C-H cleavage (21), this means that electron transfer across a solvent interface must occur, at a minimum, at a rate comparable with C-H cleavage ($680 \ s^{-1}$ for dopamine plus fumarate (19); $1200 \ s^{-1}$ for dopamine minus fumarate (18), and $810 \ s^{-1}$ for hippuric acid with PHM (16)), and as noted above, it must be fully reversible. The suggestion that the long range electron transfer is mediated by $O_2$ itself (as superoxide anion) appears to be ruled out by the observation of complete coupling of $O_2$ uptake to substrate hydroxylation under a variety of conditions (Table II). Thus, the requirement for a rapid and reversible electron transfer between CuA and CuB may also be too great to make copper-
peroxo a viable intermediate. A copper-peroxo mechanism would accommodate the size of the measured O-18 isotope effects; a pre-equilibrium formation of Cu(II)–O²⁻ from O₂ predicts an O-18 isotope effect of −3%, close to the experimentally measured value of 2%. There would be little tendency for the peroxide to dissociate from the metal ion in the absence of an active site proton donor (although bulk water at the solvent-exposed active site might function in this manner). In any case, a copper-peroxo would only be formed at very low levels, given the failure to detect paramagnetic Cu(II) species after incubation of DβM with O₂ and DFFA.

As a second option, a role for copper-superoxo in C-H activation has a great deal of weight. The copper-superoxo species is more electrophilic than peroxo and would be expected to be a reasonable acceptor of the hydrogen atom from substrate. The presence of a diamagnetic, spin-coupled Cu(II)–O₂⁻ (41) would be consistent with the failure of freeze-quench EPR experiments (Fig. 1) to detect any paramagnetic species in the presence of DFFA. Perhaps most appealing is the fact that the second electron transfer from the copper site would not take place until after formation of the substrate-derived free radical and a Cu(II)–OOH. This is expected to be a highly reactive state, capable of driving transfer of an electron from the reduced copper site to the terminal oxygen of the Cu(II)–OOH. This can be modeled to occur through a distance of only 7.5 Å and via a network of three water molecules (Scheme 3). The mechanism of Scheme 2, I and Scheme 3 places the long range electron transfer between copper sites after the initial substrate activation, i.e. in the kcat parameter. Although structure reactivity correlations have implicated a rate-limiting dissociation of copper-bound hydroxylated product in kcat (19), electron transfer may contribute to this parameter as well. This requires that the electron transfer occurs faster than the rate for kcat (12.7 s⁻¹ for dopamine plus fumarate (19), and 39.1 s⁻¹ for hippuric acid with PHM (16)), a far less stringent condition than its contribution to kcat/Km(O₂). Electron tunneling rates through water over a distance of 8 Å have been predicted to occur at a rate of 10³⁹ s⁻¹ (42). However, as pointed out by Blackburn et al. (23) substantial ligand reorganization of the copper sites would decrease this rate, although it should remain significantly faster than the rate for kcat of 12.7 s⁻¹ (dopamine plus fumarate) (19).

As with the other postulated mechanisms, the copper-superoxo mechanism must accommodate the finding of complete coupling of O₂ uptake to substrate hydroxylation and the size of the O-18 isotope effects. With regard to the former, one possibility is a side-on, by analogy to mononuclear copper-dioxygen species that have been characterized by x-ray crystallography (41) and vibrational spectroscopy (43). The enzyme-bound copper is reported to have a redox potential of 310–380 mV (44, 45), elevated compared with the potential of free copper (160 mV); this will favor an oxygenated species with considerable Cu(I) character. With regard to the size of the experimental kinetic O-18 isotope effects, the observed values of 2% exceed the equilibrium value of only 1%, expected for formation of copper-superoxo. However, this equilibrium limit for the O-18 isotope effect does not take into account any reorganization of the superoxo species that must occur before transfer of hydrogen by tunneling. It is possible that the bond between the copper and superoxo anion undergoes transient weakening to achieve the requisite degeneracy between reactant and product that allows the tunneling process to proceed. The oxygen species at the time of H-tunneling would under these circumstances have binding that lies between Cu(II)–O₂⁻ and free O₂⁻ with the expectation of an O-18 kinetic isotope effect between 1 and 3%. We note that hydrogen atom transfer to superoxo-metal complexes has been demonstrated (46), although with C–H bonds that are weak compared with the benzylic C–H bond of Ca. 95 kcal/mol (19).

As originally detected from extended x-ray absorption fine structure studies of DβM, a change in the ligand environment occurs at the Cuf site upon its reduction such that the coordination number is reduced, and a long bond to methionine becomes significantly shorter (2, 3). This ligand movement has remained an enigma within the context of a mechanism in which copper is first reduced by ascorbate and then reoxidized by O₂ before substrate activation. However, if copper reoxidation and substrate functionalization occur in a tightly coupled step at Cuf, it becomes easier to rationalize the presence of a methionine at this site. Specifically, because methionine is a poor electron donor, it would ensure that the reduced copper remains electrophilic, preventing significant reduction of O₂ in the absence of substrate activation. This implies that the ligand reorganization occurring upon Cuf(I) oxidation (in which methionine is displaced) may contribute to the driving force for substrate oxidation. In this manner, changes in bonding at substrate become linked not only to changes in bonding at O₂ but to alterations in copper ligation as well.

In a recent investigation of the impact of selected mutants on the PHM-catalyzed hydroxylation of peptide substrates, Bell et al. (47) postulate that the substrate undergoes migration from the Cuf to Cuₐ site, reversing the roles of the metal sites such that Cuₐ performs hydroxylation and Cuf electron transfer. The basis for this proposal comes from the finding that elimination of the residue proposed to mediate inter-metal electron transfer (Gln¹⁷⁶) has little effect on rate. Additionally, these authors invoke the earlier demonstration that substrate induces bonding of CO to Cuₐ in PHM and that the CO-stretching frequency is dependent to a small extent on the nature of the bound substrate (15). We point out that although our basic mechanism (Scheme 3) could be accommodated by a reversal in roles of the copper sites, it also eliminates the need to use extreme means (such as substrate and superoxo migration) to achieve an activated complex.

In conclusion, the mechanism in Scheme 2, I and Scheme 3 both allows for the convergence of a large body of kinetic and structural data for DβM and PHM and provides a basis for future theoretical and experimental work on these systems. The data reported herein also argue against invoking cytochrome P-450 as well as other iron-containing monooxygenases as a “universal” paradigm in describing the mechanism of oxygen-activating metallo-enzyme systems. It is evident that the copper monooxygenases use a very different strategy for substrate functionalization than cytochrome P-450. The rules that govern copper and iron reactivity may turn out to be significantly further apart than previously recognized.

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