Intrasubstituent Isotope Effect Studies of Oxidative N-Demethylations Catalyzed by Secondary Amine Monooxygenase. Comparison to Cytochrome P-450

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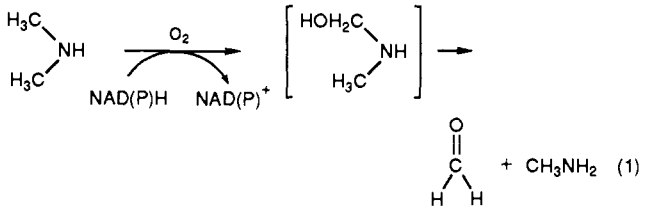
Abstract: Intermolecular, intramolecular, and intrasubstituent kinetic deuterium isotope effect studies have been carried out on secondary amine monooxygenase, the only heme-containing monooxygenase other than cytochrome P-450. Secondary amine monooxygenase exhibits an active site structure similar to myoglobin but has a reactivity, oxidative N-dealkylation, in common with those demonstrated by P-450. An *inter*molecular isotope effect was not observed for N-dealkylation of dimethylamine, indicating that C-H bond cleavage is not the rate limiting step. The *intra*molecular isotope effect is also masked by a high commitment to catalysis and/or the absence of substrate methyl group interchange at the active site. A net intrinsic kinetic deuterium isotope effect of 1.76 has been revealed via an *intrasubstituent* isotope effect study of competition between D and H atoms within each methyl group of dimethylamine in 1,1,1′,1′-tetradeuteriodimethylamine. This value is essentially identical to that previously reported for P-450 and is much smaller than values observed for other histidine-ligated heme proteins such as myoglobin and the peroxidases (Miwa, G. T. et al. *J. Biol. Chem.* **1983**, *258*, 14445–14449). The small isotope effect is most consistent with a deprotonation mechanism in which significant H-atom tunneling is absent, rather than a H-atom abstraction mechanism. It has been shown that the identity of the eventual proton acceptor is important in determining the magnitude of the intrinsic isotope effect for deprotonations (Dinnocenzo, J. P.; Banach, T. E. *J. Am. Chem. Soc.* **1989**, *111*, 8646–8653). Therefore, a relatively nonpolar proton acceptor environment within secondary amine monooxygenase, like that of cytochrome P-450, is implicated by close agreement in the isotope effects of these two proteins. Since cytochrome P-450 has a thiolate proximal ligand and secondary amine monooxygenase has a histidine proximal ligand, the identity of the heme proximal ligand appears to be less important than the nature of the distal active site environment in these N-dealkylation reactions. Thus, secondary amine monooxygenase resembles myoglobin and the peroxidases in its heme ligation and spectroscopic properties but catalyzes N-dealkylations via a mechanism that is similar to that employed by the other heme-containing monooxygenase, cytochrome P-450.

Introduction

Secondary amine monooxygenase from *Pseudomonas aminovorans*[1] and the ubiquitous cytochrome P-450[2] are the only known heme-containing monooxygenases. Both contain a protoporphyrin IX prosthetic group, catalyze O_2-dependent N-dealkylation reactions, and are inhibited by CO.[1,2] Recent spectroscopic studies from our laboratory, however, clearly reveal that the two enzymes have rather different active site structures.[3] The ability of P-450 to activate dioxygen for insertion into unactivated C-H bonds has often been attributed to the presence of a cysteinate proximal ligand to its heme iron.[2c] Secondary amine monooxygenase, while sharing with P-450 the ability to catalyze oxidative N-dealkylation reactions, has a proximal histidine ligand.[3]

The oxidative N-dealkylation of alkylamines catalyzed by secondary amine monooxygenase and P-450 is a two-step process involving addition of an oxygen atom to the carbon α to the heteroatom to yield a carbinolamine followed by nonenzymatic breakdown to give an aldehyde and the dealkylated amine (eq 1).[4] Myoglobin, hemoglobin, catalases, and peroxidases also



(1)

catalyze this reaction but do so in a peroxide-dependent manner, not requiring dioxygen and not inhibited by CO.[5] The several possible mechanistic pathways (Figure 1) that have been proposed for the first step of eq 1 can be differentiated based on whether C-H bond cleavage involves H-atom abstraction or deprotonation. We wish to report the determination of the *net*[6] deuterium kinetic isotope effect for this type of C-H bond cleavage catalyzed by secondary amine monooxygenase. Comparison of these results with those previously described for P-450 catalyzed N-demethylations suggests there are close mechanistic similarities between the two monooxygenases despite their different heme ligation, and that these enzymes have rather similar active site environments. Accordingly, measurement of the intrinsic isotope effect exhibited by secondary amine monooxygenase represents an important step in our effort to elucidate the range of oxygen activation mechanisms and examine the active site structures of heme-containing monooxygenases.

Experimental Section

Reagents and Syntheses. Common reagents were obtained commercially and used without further purification except as noted. Dimethylamine·HCl (Aldrich) was recrystallized twice from absolute ethanol. 1,1,1-Trideuteriodimethylamine·HCl was the kind gift of J. R. Durig, University of South Carolina. 1,1,1′,1′-Tetradeuteriodimethylamine was purchased from MSD Isotopes as the gas and converted to the HCl salt. 1,1,1,1′,1′,1′-Hexadeuteriodimethylamine·HCl (Aldrich) was recrystallized once from absolute ethanol. 1-Deuterioformaldehyde and 1,1-dideuterioformaldehyde were obtained from MSD Isotopes as

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(6) The *net* intrinsic isotope effect reported herein is the unresolved combination of primary, α-secondary, and magnetic isotope effects. The magnitude of the latter two isotope effects is expected to be a modest perturbation of the primary isotope effect.

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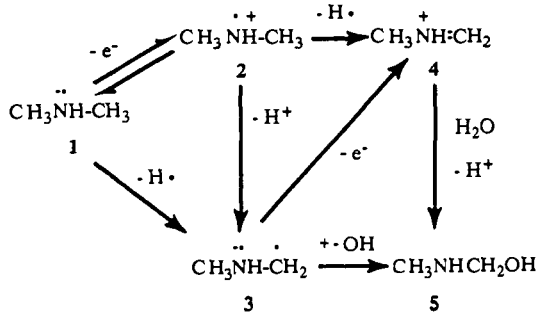

Figure 1. Possible mechanisms for the oxidative N-demethylation of dimethylamine catalyzed by secondary amine monooxygenase.

the polyoxymethylene polymers. Deuterium enrichment at the specified positions for all deuteriated compounds was greater than 98%. 5,5-Dimethyl-1,3-cyclohexanedione (dimedone, Aldrich) was recrystallized twice from ethanol/diethyl ether and once from water. NADPH was obtained from Sigma Chemical Co. 5-(*N*,*N*-Di-*n*-butyl)aminonaphthalene sulfonyl chloride (bansyl chloride) was obtained from TCI, Japan. Authentic trideuteriomethylamine–bansyl and methylamine–bansyl were prepared through reaction of 50 mg of bansyl chloride with 1 equiv of the appropriate amine in 1:1 water/acetone in excess Na_2CO_3. Remaining bansyl chloride was derivatized with proline. The amine–bansyl adducts were extracted, chromatographed, and isolated as previously described.[7] Dimedone adducts of formaldehyde-d_1 and formaldehyde-d_2 were prepared by dissolving the appropriate polyoxymethylene polymer in aqueous base and adding a 5-fold excess of dimedone based on aldehyde concentration. The resulting precipitate was extensively washed with water and dried. Formaldehyde-d_0 (aqueous solution; Fisher) was used to prepare the dimedone-d_0 adduct in an analogous manner.

Biochemical Preparations. Secondary amine monooxygenase was purified and assayed as previously described.[3a] Enzyme preparations used were of specific activity >9.0 μmol min^{-1} mg^{-1}. Protein concentrations were determined by the Lowry method.[8] Assays and reactions were carried out in 50 mM potassium phosphate buffer, pH 7.00 (assay buffer) at 4 °C.

Inter- and Intramolecular Isotope Effect Studies. The intermolecular ^DV and $^D(V_{max}/K_m)$[9] were determined by Michaelis-Menton analysis from the absorbance decrease at 340 nm; 0.95 μM enzyme in assay buffer, 160 mM NADPH. A Cary 210 spectrophotometer coupled with an IBM/XT was used for data acquisition. $^D(V_{max}/K_m)$ was also determined by substrate competition. Dimethylamine and dimethylamine-d_6 were prepared in equimolar concentration (33 μM each) and reacted with secondary amine monooxygenase under the conditions cited above. The reaction was quenched with solid Na_2CO_3. The methylamine products were derivatized in a manner analogous to the preparation of the authentic amine adducts (vide supra). Samples and standards were analyzed with a Finnegan 4521 C gas chromatograph/mass spectrometer on an RTX 5 30 m column: mass selection 348.105 ± 0.500 and 351.104 ± 0.500. Authentic samples of methylamine-d_3– and methylamine-d_0–bansyl were run as external standards in various proportions. The ratio of integrated peak areas were determined for the samples and correlated with the standard curve. Intramolecular isotope effect studies were performed under the same conditions as the intermolecular substrate competition experiments, except that 1,1,1-trideuteriodimethylamine·HCl was used as substrate. Methylamine product deuterium content was determined in the same manner.

Intrasubstituent Isotope Effect Studies. Secondary amine monooxygenase was reacted with 1,1,1′,1′-tetradeuteriodimethylamine·HCl in the same manner as described above, but the reaction was quenched with NaOH. The formaldehyde products were derivatized by addition of a 10-fold excess of dimedone in aqueous NaOH (pH > 12). The aliquots were acidified with HCl and extracted with benzene. The samples were subjected to GC/MS as before, except that a 25 m DB5 column was used. Peak integrations at m/e ± 0.500 at integral m/e 291–296 were carried out. A curve was prepared from analysis of various ratios of the formaldehyde-d_1– and formaldehyde-d_2–dimedone adducts as external standards.

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Table I. Isotope Effect Studies for Oxidative N-Dealkylations

system	$^D(k_H/k_D)$	ref
N-donor ligated proteins:[a]		
hemoglobin, myoglobin, various peroxidases	8.72–10.1	13
S-donor ligated proteins:[a]		
various P-450 isozymes, chloroperoxidase	1.61–3.05	13
secondary amine monooxygenase:[b]		
$D_3C\text{-}NH\text{-}CD_3$, $H_3C\text{-}NH\text{-}CH_3$	1.07 ± 0.08	this work
$D_3C\text{-}NH\text{-}CH_3$	1.04 ± 0.08	this work
$HD_2C\text{-}NH\text{-}CHD_2$	1.76 ± 0.14	this work

[a] N-Dealkylation of *N*-methyl-*N*-trideuteriomethylaniline. [b] Expressed as mean ±SD with four, five, and eight determinations for the intermolecular, intramolecular, and intrasubstituent isotope effects, respectively. Methylamine product analysis (ref 7).

Table II. Lineweaver–Burke Analysis of N-Demethylation by Secondary Amine Monooxygenase

substrate	K_M,[a] μM	V_{max},[a] μM min^{-1} mg^{-1}	$^DV_{max}$	$^D(V_{max}/K_m)$
dimethylamine	66.5 ± 2.2	1.79 ± 0.16	1.01 ± 0.13	1.04 ± 0.11
perdeuteriodimethylamine	68.3 ± 2.3	1.81 ± 0.17		

[a] Regression analyses: >0.99. K_m determined from the slope; V_{max} from the *y*-intercept.

Results and Discussion

*Inter*molecular $^D(V_{max}/K_m)$ product analysis experiments for N-demethylation catalyzed by secondary amine monooxygenase revealed a value of k_H/k_D = 1.0 (Table I). The *inter*molecular $^DV_{max}$ isotope effect determined via the Michaelis-Menton formalism is 1.0, as it is for $^D(V_{max}/K_m)$ (Table II). These results are not surprising because multistep enzymatic reactions are particularly prone to masking of the intrinsic isotope effect by precatalytic or postcatalytic rate-limiting steps.[10] Nevertheless, lack of an intermolecular isotope effect indicates that C–H bond cleavage is not the rate limiting step in the overall reaction.

The *intra*molecular isotope effect demonstrated no preference for hydrogen versus deuterium (Table I). This observation, however, is consistent with a high commitment to catalysis;[9b,10] the substrate simply binds and remains in one orientation throughout the catalytic process without interchanging methyl groups. This prevents the enzyme intermediate responsible for C–H bond cleavage from "choosing" between C–H and C–D bonds, thereby masking the isotope effect.

The intrinsic kinetic deuterium isotope effect was determined through competition between deuterium and hydrogen atoms within *each* methyl group of dimethylamine, an *intrasubstituent* isotope effect.[11] With 1,1,1′,1′-tetradeuteriodimethylamine as substrate, this experiment yielded an overall net isotope effect for the reaction of 1.76.[12] Masking of the intrinsic isotope effect is overcome by the rapid rotation of the methyl group about the C–N bond. This allows the key intermediate to select between C–H (easier to break) and C–D (harder to break) bonds. Masking of the intrinsic kinetic deuterium isotope effect by precatalytic rate limiting events or high forward commitments to catalysis has been completely eliminated through this novel intrasubstituent isotope effect study.

Miwa et al.[13] determined the intramolecular isotope effect for oxidative N-demethylation of *N*,*N*-dimethylaniline by several heme

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(11) A somewhat analogous isotope effect study of in vitro microsomal hydroxylation using toluene-α-d_1 and toluene-α,α-d_2 to resolve primary and secondary isotope effects for hydrocarbon hydroxylation was performed: Hanzlick, R. P.; Hogberg, K.; Moon, J. B.; Judson, C. M. *J. Am. Chem. Soc.* **1985**, *107*, 7164–7167.
(12) In the absence of any isotope effect, one expects $(\text{formaldehyde-}d_1)_{obs}$ = 2 $(\text{formaldehyde-}d_2)_{obs}$ as there are two deuterium atoms and one hydrogen atom at each methyl group. Accordingly, k_H/k_D = 2 [$(\text{formaldehyde-}d_2)_{obs}/(\text{formaldehyde-}d_1)_{obs}$].
(13) Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1983**, *258*, 14445–14449.

proteins in an attempt to distinguish between deprotonation (Figure 1, 2 → 3) and H-atom abstraction (1 → 3 or 2 → 4) mechanisms. The recent model studies of Dinnocenzo and Banach,[14] however, have shown that all of these proteins N-dealkylate tertiary aromatic amines via deprotonation. The magnitude of the isotope effect is controlled by the nature of the proton-accepting distal base at the active site.[14] The peroxidases have a histidine proton donor/acceptor within their more polar active site regions,[2c,15] while cytochrome P-450 has a very nonpolar active site.[16,17] Small isotope effects (~2), therefore, rule out hydrogen radical abstraction events,[18] while large isotope effects (~9) may be due to deprotonation, H-atom abstraction, or H-atom tunneling.[9b,10] Miwa et al. reported (Table I) large isotope effects for heme proteins having histidine N-donor proximal ligands and small values for cysteinate S-donor ligated heme enzymes. Catalase, with a tyrosinate O-donor axial ligand, exhibited an intermediate value.[13] The present results clearly show that the identity of the proximal ligand is not a factor in determining the magnitude of the isotope effect. Instead, the value of the isotope effect for N-dealkylation by secondary amine monooxygenase of 1.76 is consistent with a deprotonation mechanism involving a P-450-like proton donor/acceptor environment.

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Conclusion

In summary, secondary amine monooxygenase appears to catalyze N-demethylation reactions of secondary amines via a deprotonation, rather than an H-atom abstraction, limiting mechanism. Despite their different heme ligation, secondary amine monooxygeanse catalyzes oxidative N-dealkylation reactions by a mechanism like that employed by the only other heme-containing monooxygenase, cytochrome P-450. Thus, the nature of the proximal axial ligand is not a controlling factor in determining the mechanism of N-dealkylation. Furthermore, the distal proton donor/acceptor characteristics of these two proteins appear to be similar.

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Registry No. D_2, 7782-39-0; H_3C-NH-CH_3, 124-40-3; secondary amine monooxygenase, 37367-72-9.

Developing Magnetic and Metallic Behavior in High-Nuclearity Nickel Cluster Carbonyls. A LCGTO-LDF Study of $[Ni_9(CO)_{18}]^{n-}$, $[Ni_{10}Ge(CO)_{20}]^{n-}$, $[Ni_{32}C_6(CO)_{36}]^{n-}$, and $[Ni_{44}(CO)_{48}]^{n-}$ Compounds

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Abstract: We have performed all-electron calculations on the electronic structure of medium- and high-nuclearity bare and carbonylated nickel cluster compounds by means of the linear combination of Gaussian type orbitals (LCGTO) local density functional (LDF) method. The transition from the molecular state to the metallic state as a function of the cluster size was studied by determining the one-electron energy spectra and the magnetic properties of naked and ligated Ni clusters. Medium-size clusters like $[Ni_9(CO)_{18}]^{2-}$ and $[Ni_{10}Ge(CO)_{20}]^{2-}$, where all the Ni atoms are on the surface of the metal cage, show typically molecular features. They do not exhibit any magnetic behavior, and their one-electron spectrum has a discrete nature near the cluster HOMO with a well-defined HOMO–LUMO gap. By contrast, both high-nuclearity clusters, $[Ni_{32}C_6(CO)_{36}]^{n-}$ and $[Ni_{44}(CO)_{48}]^{n-}$, show typical signs of a developing metallic character in agreement with experimental magnetic measurements. In particular, a magnetic behavior connected with the high density of states near the Fermi level is observed. This is entirely connected to the presence of Ni atoms in the interior ("bulk") of the metal cage. The role of the CO ligands and of interstitial atoms, like C or Ge, in quenching the magnetic moment of the bare cluster is elucidated.

1. Introduction

The study of compounds involving transition metal clusters has been one of the most rapidly expanding areas within inorganic and organometallic chemistry in recent years.[1] Most clusters share common framework structures in which metal atoms form a close-packed array; the largest class is composed of clusters in which all the atoms are formally zero-valent and the associated ligands have substantial π-acceptor character, as in metal carbonyl

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