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José R. Tormos, Marina B. Suarez, Paul F. Fitzpatrick

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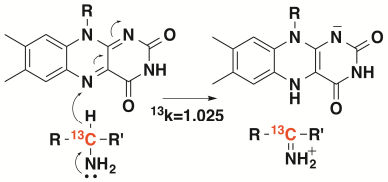


^{13}C Kinetic Isotope Effects on the Reaction of a Flavin Amine Oxidase Determined from Whole Molecule Isotope Effects

José R. Tormos,[†] Marina B. Suarez,[¶] and Paul F. Fitzpatrick[‡,*]

[†]Department of Chemistry and Biochemistry, St. Mary's University, San Antonio, TX 78228

[¶]Department of Geological Sciences, University of Texas-San Antonio, San Antonio TX 78249

[‡]Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229

*Corresponding author. E-mail: fitzpatrickp@uthscsa.edu. Phone: (210)-567-8264.

Abbreviations used: PAO, N-acetylpolyamine oxidase PAO; MAO, monoamine oxidase; DBDB, N, N'-dibenzyl, 1,4-diaminobutane IRMS, isotopic ratio mass spectrometry

Abstract

A large number of flavoproteins catalyze the oxidation of amines. Because of the importance of these enzymes in metabolism, their mechanisms have previously been studied using deuterium, nitrogen, and solvent isotope effects. While these results have been valuable for computational studies to distinguish among proposed mechanisms, a measure of the change at the reacting carbon has been lacking. We describe here the measurement of a ^{13}C kinetic isotope effect for a representative amine oxidase, polyamine oxidase. The isotope effect was determined by analysis of the isotopic composition of the unlabeled substrate, N, N'-dibenzyl-1,4-diaminopropane, to obtain a pH-independent value of 1.025. The availability of a ^{13}C isotope effect for flavoprotein-catalyzed amine oxidation provides the first measure of the change in bond order at the carbon involved in this carbon-hydrogen bond cleavage and will be of value to understanding the transition state structure for this class of enzymes.

KEYWORDS

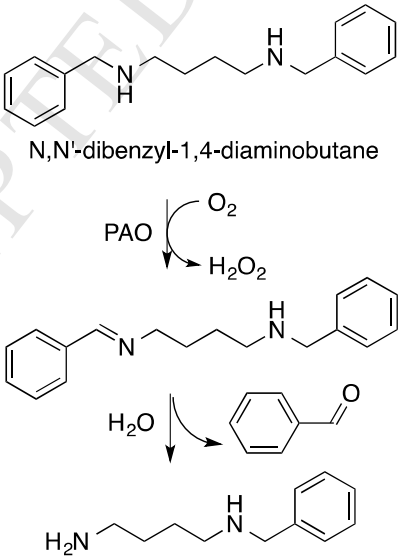
polyamine oxidase, enzyme mechanisms, flavoprotein, dehydrogenase

Kinetic isotope effects provide an extremely powerful probe of enzyme mechanisms, in that isotopic substitution does not perturb substrate structure while allowing one to probe the reaction at the level of individual atoms in the substrate [1]. Analysis of the effects of substituting each of the atoms in the substrate involved in the reaction on the intrinsic rate constant for the chemical reaction can allow one to calculate the change in bond order at each reactive atom when the transition state forms [2]. Comparison of these values with those determined from computation for different mechanisms is frequently sufficient to establish the catalytic mechanism. Indeed, as computational methods have improved, experimentally determined kinetic isotope effects have come to provide necessary constraints on the structures of transition state structures derived from computation [3, 4].

The mechanisms of cleavage of carbon hydrogen bonds by enzymes have been heavily studied using kinetic isotope effects due to the metabolic importance of such reactions and the readily measured primary deuterium isotope effects. Several classes of flavoproteins catalyze carbon-hydrogen bond cleavage during the oxidation of alcohols to the corresponding aldehyde or ketone, while others do so during the oxidation of aliphatic amines and amino acids to the corresponding imines. Both deuterium and solvent isotope effects have been measured for the former, and the results are consistent with hydride transfer from the alkoxide [5]. Oxidation of amines has been analyzed using deuterium and nitrogen isotope effects, and the results are consistent with hydride transfer from the neutral amine [6]. A number of computational studies support these mechanisms [7-12].

To date, there have been no reports of ^{13}C kinetic isotope effects on the reactions of flavoproteins oxidizing amines and alcohols, in contrast to the situation with pyridine nucleotide-dependent enzymes catalyzing similar reactions [1]. A measure of the change in bond order at

the carbon atom undergoing carbon-hydrogen bond cleavage is needed for a complete description of the transition state for enzyme-catalyzed oxidation of amines. We report here the measurement of the ^{13}C kinetic isotope effect for mouse N-acetylpolyamine oxidase (PAO). Mammalian PAOs catalyze the oxidation of a carbon-hydrogen bond in N-acetylspermine and N-acetylspermidine [13]; nonenzymatic hydrolysis of the imine product yields aminopropanaldehyde and spermidine or putrescine, respectively [14]. PAOs are members of the heavily studied monoamine oxidase (MAO) family of flavoprotein amine oxidases[15], with three-dimensional structures similar to that of MAO[16]. Mechanistic studies of PAO previously identified N, N'-dibenzyl-1,4-diaminobutane (DBDB, Scheme 1) as a substrate for which carbon-hydrogen bond cleavage is the rate-limiting step [17], in contrast to the situation with the physiological substrates[18]. As a result isotope effects on the k_{cat}/K_m value for DBDB are equal to the intrinsic isotope effect for cleavage of the substrate carbon-hydrogen bond. Consequently, DBDB was selected as the substrate for the present analyses.



Scheme 1

Experimental Procedures

Materials. DBDB was purchased from Prime Organics, Inc. (Mumbai, India). Hydroxylamine, sarcosine (*N*-methylglycine), potassium pyrophosphate, trifluoroacetic acid and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Glycerol was purchased from Fisher Scientific (Pittsburg, PA). Catalase was from USB Corporation (Cleveland, OH).

Protein Expression and purification. PAO was expressed in *E. coli* and purified as previously reported [19]. The enzyme concentration was determined using a ε_{458} value of 10,400 M^{-1} cm^{-1}.

Isotope effects. Starting conditions were typically 8 mM DBDB, 25 mM hydroxylamine, 100 µg/mL of catalase, 10% glycerol and 6 µM PAO in 20 mL. From pH 7.0-10.0, samples were buffered using 0.1 M potassium pyrophosphate, while at pH 11.0 0.1 M sarcosine was used. Reactions were kept within 0.1 pH units of the desired value by constantly monitoring the pH and adjusting as necessary. Additional enzyme was added periodically to keep the reaction progressing. Reactions were carried out at 25° C with wet oxygen blowing over the surface. The reaction progress was monitored by HPLC. A 3 mL sample was withdrawn at every hour and mixed with 0.3 ml of concentrated hydrochloric acid. Denatured protein was removed by filtering the sample through a 0.20 µm nylon sterile filter. The sample was then injected onto a Waters µBondapak C-18 column (19 x 300 mm) using a 2.5 mL loop and eluted isocratically with 97.5% acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 7 mL/min. DBDB and benzaldehyde were detected by monitoring the absorbance at 254 nm. The fractional consumption of DBDB was determined from the peak absorbances of DBDB and benzaldehyde. The DBDB-containing fractions were collected and dried on a rotary evaporator, resuspended in 2 mL of water, lyophilized, and submitted for isotopic ratio mass spectrometry (IRMS) analysis.

IRMS Analysis. Lyophilized samples were weighed (1 to 1.5 mg) into tin capsules and combusted on a Costech 4010 Elemental Analyzer. CO_2 and N_2 were separated via gas chromatography and transferred to a Thermo Finnigan DELTAplus XP isotope ratio mass spectrometer via a Thermo Conflo III interface. Samples were first corrected for any mass dependency and then corrected to the international reference scale, Vienna Pee Dee Belemnite ($^{13}C/^{12}C$ = 0.0112372)[20], via linear regression of accepted and measured delta values of international references analyzed with the samples. Corrected delta values were then used to calculate the $^{13}C/^{12}C$ ratio.

Data Analysis. The whole molecule ^{13}C kinetic isotope effect ($^{13}k_{obs}$) was calculated using equation 1 [21], where f is the fraction of DBDB consumed, and R_f/R_0 is the $^{13}C/^{12}C$ ratio of the remaining DBDB divided by the initial $^{13}C/^{12}C$ ratio in DBDB. The isotope effect assuming that only one of the 18 carbons in DBDB is responsible for the ^{13}C isotope effect was calculated using equation 2.

$$R_f/R_0 = (1-f)^{1/13kobs-1} \quad (1)$$

$$^{13}k = (^{13}k_{obs} - 1)*18 + 1 \quad (2)$$

Results

The ^{13}C kinetic isotope effect for the reaction catalyzed by PAO was determined as the isotope effect on the k_{cat}/K_M value for the amine substrate using DBDB containing ^{13}C at natural abundance. Reactions were set up with a concentration of DBDB at least 100-fold above the K_m value (15 µM at pH 8.6) and an oxygen concentration of 1.2 mM at 25 ^oC. Aliquots were removed from the reaction before adding enzyme and at times corresponding to 20-80% consumption of the amine. After the remaining DBDB in each aliquot was isolated by HPLC, each sample was combusted to N_2 and CO_2 and injected into an isotope ratio mass spectrometer

to determine the ratio of ^{13}C to ^{12}C. Typically 3-5 different time points were analyzed in a single experiment; all experiments carried out under the same conditions were combined for data analysis. Figure 1 shows the change in the isotopic composition of the DBDB as the reaction progresses at pH 10, near the pH optimum. The data are well-fit by equation 1, yielding an isotope effect of 1.0020 ± 0.00006. Because the isotopic ratio of the intact substrate was determined, this value reflects the change in isotopic composition at all 18 carbon atoms in DBDB. Since the reaction involves oxidation of a single carbon-nitrogen bond, it is reasonable to assume that the isotope effect arises from changes in the bond order to the single carbon in this bond. The observed isotope effect can be corrected to reflect this assumption using equation 2 to obtain a ^{13}C isotope effect of 1.036 ± 0.001 for the reactive carbon.

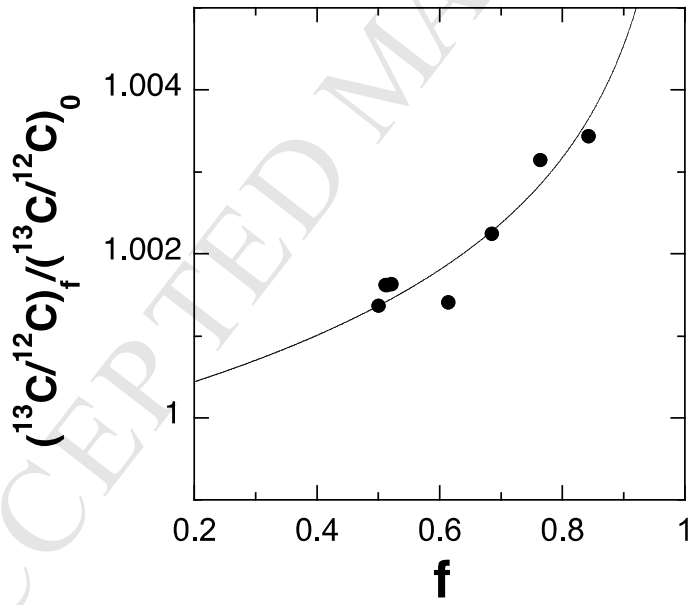


Figure 1. Change in the carbon isotopic composition of DBDB upon oxidation by polyamine oxidase at pH 10. The carbon isotopic composition of the remaining substrate was determined by isotope ratio mass spectrometry at the indicated fractional conversions as described in the Experimental Procedures. The line is from a fit of the data to equation 1.

The ^{13}C isotope effect was also determined as a function of pH over the pH range 7 - 11. As shown in Figure 2, the observed isotope effect is pH independent, with an average value of 1.00140 ± 0.00016; correcting for the number of carbons gives a pH-independent ^{13}C isotope effect of 1.025 ± 0.003. Since the isotope effect is pH-independent, all of the data from pH 7-11 could be fit together to equation 1; this gives an observed isotope effect of 1.00139 ± 0.00011. Correcting for the number of carbons yields a value of 1.025 ± 0.002.

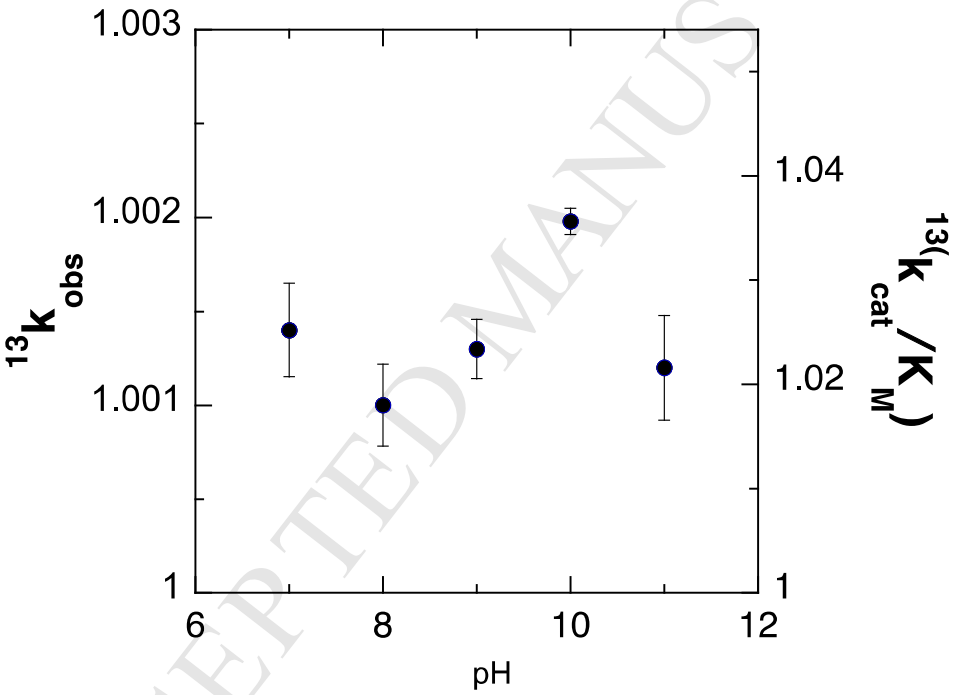


Figure 2. Effect of pH on the ^{13}C kinetic isotope effect for oxidation of DBDB by polyamine oxidase at 25°C. The $^{13}k_{obs}$ values were obtained from analyses of the isotopic compositions of the remaining DBDB as a function of the reaction, as described for Figure 1. The $^{13}(k_{cat}/K_m)$ values were calculated from the $^{13}k_{obs}$ values using equation 2.

Discussion

Typically, isotope effects are measured using molecules containing a single isotopically-

substituted atom at the position of interest. This may involve a second substituted atom at a position not expected to exhibit an isotope effect, a remote label [22]. In either case, the synthesis of the required labeled molecule is often much more demanding than the actual measurement, such that the lack of availability of the appropriately-labeled molecules can effectively preclude the experimental measurement. This limitation is greater for the measurement of isotope effects other than those involving deuterium, due to a combination of commercial availability and synthetic complexity. If the isotope effect is to be determined using IRMS, as is typically done for isotopes of atoms other than deuterium, the need for the degree of isotopic substitution to be close to the natural abundance of the heavy atom can require the synthesis of one molecule containing exclusively the heavy isotope at the position of interest and a separate molecule containing exclusively the light isotope at that position. NMR-based methods provide an alternative to this problem, in that they can be used to determine the isotopic substitution at each atom in the substrate [23, 24], but the amounts of material required typically rule out such methods for measurement of heavy atom isotope effects on enzyme-catalyzed reactions. Alternative methods utilizing NMR spectroscopy have been described that rely on synthetic molecules containing a second “reporter” atom [25, 26], but these typically require complex syntheses.

The need to synthesize isotopically-labeled substrates can be avoided if the isotope effect is expected to arise from only one atom in the substrate. In such a case the change in the isotopic composition of the entire molecule will be determined by the change at the atom of interest. While this makes the measurement of whole molecule isotope effects attractive, the observed isotope effect is decreased from the isotope effect of interest due to the presence of non-reactive atoms. Synthesis of substrates in which the isotopic composition at the atom of interest is

increased above natural abundance has been used to circumvent this limitation [27, 28], but this approach again requires synthesis of isotopically-substituted substrate. The precision of measured isotope effects is much greater if the isotopic composition of the substrate is determined at multiple fractional conversions, allowing the entire progress curve to be fit, instead of calculating the isotope effect from the isotopic composition of the substrate or product at a single fractional conversion. Such an approach of course requires multiple measurements and therefore more material. Still, the growing sensitivity of isotope ratio mass spectrometers [29] suggests that measurement of whole molecule isotope effects using molecules at natural abundance is a viable approach to the measurement of isotope effects on enzyme-catalyzed reactions.

We selected the oxidation of DBDB by mouse PAO to examine whether reliable ^{13}C isotope effects could be measured using the entire substrate without synthesis of an isotopically-substituted molecule. The enzyme catalyzes the oxidation of a carbon-nitrogen bond, so that any isotope effect should result from the change in the bond order at a single carbon. ^{13}C isotope effects have not been reported previously for flavin amine oxidases, and PAO is a representative of the largest family of these enzymes. The presence of 18 carbons in DBDB makes this a particularly challenging substrate in terms of precision. The data presented here establish the feasibility of this approach.

There do not appear to be any previous reports of a ^{13}C isotope effect for oxidation of an aliphatic amine by hydride transfer. The ^{13}C isotope effect of 1.025 ± 0.002 reported here agrees well with the value of 1.025 ± 0.001 for the NAD-dependent oxidation of benzyl alcohol by liver alcohol dehydrogenase [30], a reaction generally accepted to involve hydride transfer. The magnitude of this value is consistent with a symmetrical transition state with respect to cleavage

of the carbon-hydrogen bond.

The present results complement previous analyses of flavin amine oxidases utilizing kinetic isotope effects. Primary deuterium isotope effects have previously been reported for a number of these enzymes. Oxidation of benzylamine by MAO B provides the closest analogy to the oxidation of DBDB by PAO. With deuterated benzylamine as substrate, the deuterium kinetic isotope effect on the k_{cat} value for that enzyme is 8.1, while that on the rate constant for flavin reduction is 10 [31]; this is likely to be the intrinsic isotope effect. For MAO A the isotope effect on the rate constant for flavin reduction is 8 [32]. The large magnitudes of these effect are consistent with a significant contribution of tunneling to the reactions; this conclusion is supported by the effect of temperature on the oxidation of 4-methoxybenzylamine by MAO B [33]. The deuterium isotope for oxidation of DBDB by PAO is 7 on both the k_{cat}/k_m and the k_{cat} value [14], close to the value for MAO B. The magnitudes of deuterium isotope effects are sensitive to the contribution of tunneling [34-36], and may vary significantly with different substrates for the same enzyme [37].

Solvent isotope effects have been used to probe whether a proton attached to the nitrogen is in flight in the transition state for carbon-hydrogen bond cleavage by flavin amine oxidases [5]. For all enzymes which have been examined, the k_{cat}/K_m value for the amine is unchanged in D_2O, consistent with the neutral amine being the productive substrate.

^{15}N isotope effects have been reported for several flavin amine oxidases; there is less variation in the values than for deuterium isotope effects, likely due to the much smaller contribution of tunneling to heavy atom effects. While mechanisms involving amine radicals have been proposed for flavin amine oxidases [38], ^{15}N isotope effects and the inability to detect a kinetically competent flavin radical are more consistent with a hydride transfer mechanism [6].

For example, Skarpeli-Liati et al. [39] have reported an average ^{15}N isotope effect of 0.998 ± 0.004 for the one electron oxidation of N-methyl- and N, N-dimethylbenzylamines. This is consistent with calculated values of 0.996-0.998 for the formation of the nitrogen radical [8, 40]. In contrast, the average of reported $^{15}(k_{cat}/K_m)$ values for flavin amine oxidases is 0.992 ± 0.005 [40-43], in good agreement with the value of 0.992-0.993 calculated for a hydride transfer mechanism [8, 40].

The results presented here provide a more complete picture of the transition state for amine oxidation by flavoproteins and add to the evidence that oxidation of amines by these enzymes involves hydride transfer. The availability of an experimental ^{13}C isotope for a representative amine oxidation provides an additional benchmark for efforts to use computational methods to further understand the mechanisms of this family of enzymes. The results also demonstrate the feasibility of using whole molecule isotope effects for such analyses.

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