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Nitrogen Kinetic Isotope Effects for the Monoamine Oxidase B-Catalyzed Oxidation of Benzylamine and (1,1-$^2H_2$)Benzylamine: Nitrogen Rehybridization and CH Bond Cleavage Are Not Concerted

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Supporting Information

ABSTRACT: Nitrogen kinetic isotope effects for the oxidation of benzylamine and (1,1-$^2H_2$)benzylamine by recombinant human monoamine oxidase B show that cleavage of the CH bond is not concerted with rehybridization of the nitrogen atom.

Monoamine oxidases (MAOs) are flavin-dependent enzymes that catalyze the oxidation of many amines to their corresponding protonated imines. The protonated imines are then nonenzymatically hydrolyzed to afford the final carbonyl compounds. The enzyme itself is regenerated to its active form by molecular oxygen, which in turn is reduced to hydrogen peroxide. In mammals, MAO exists in two different isozymes, A and B, which differ partly in their substrate and inhibitor specificities.[1] Many different approaches have been employed to gain insights into how MAO effects catalysis, but the mechanism has yet to be unambiguously established.

It is assumed that MAO A and B act by the same mechanism and that the differences in substrate and inhibitor specificities stem from structural features of the active sites of the respective isozymes. In light of the three-dimensional structures determined for human liver MAO A and B, this seems to be a reasonable assumption. Several mechanisms have been proposed for amine oxidation by flavoenzymes over the years. These mechanisms include as key steps either single electron transfer reactions, direct hydride transfer from the amine to the flavin, or nucleophilic addition of the amine to the flavin.[1]

For MAO, the main attention has been given to the aminium cation radical mechanism according to Silverman[2] and the polar nucleophilic mechanism as put forth by Edmondson.[3] The radical mechanism (Scheme 1) is initiated by a reversible single electron transfer from the unprotonated amine to the flavin to produce a flavin radical and an aminium cation radical intermediate. The radical intermediate undergoes proton abstraction, and then further oxidation takes place directly by electron transfer or via radical recombination and adduct formation. Alternatively, the initial electron transfer and proton abstraction steps might be concerted.[2,4]

According to the polar nucleophilic mechanism, the initial step is a nucleophilic attack by the unprotonated amine on the flavin C4a (Scheme 2). The resulting adduct then decomposes to form reduced flavin and protonated imine. The proton abstraction may be concerted with either the adduct formation (route on the right side) or the product formation (route on the left side). Recently performed semiempirical calculations on the isoalloxazine ring system and some para-substituted benzylamines undergoing the polar nucleophilic mechanism suggest that adduct formation is the rate-limiting step of the reaction.[5]

Fitzpatrick and co-workers have reported kinetic isotope effect (KIE) investigations for several flavoprotein oxidases, including D-amino acid oxidase,[6] L-tryptophan monooxygenase,[7] and *N*-methyltryptophan oxidase.[8] Interpretation of the nitrogen KIEs for flavoprotein amine oxidation reactions were aided by quantum-chemical calculations using alloxazine and dimethylamine as a model system.[8] Their overall conclusion was in support of a hydride transfer or a single electron transfer, thus dismissing the nucleophilic addition mechanism. Moreover, it was suggested that MAOs operate by the same mechanistic pathway, given their structural similarities to flavoprotein amino acid oxidases.[9]

Many of the mechanistic studies performed have included the use of KIEs. The substantial magnitudes of the KIEs observed for MAO B turnover ($^Dk_{cat}$ = 4.7; $^Dk_{cat}/K_m$ = 4.5) and flavin reduction ($^Dk_{red}$) are in keeping with the cleavage of the α-C−H bond being the rate-limiting step, fully or to a large degree.[10] Further mechanistic insight regarding bond changes in the substrate during catalysis may be gained by employing heavy-atom KIEs. A normal ^{15}N KIE signals decreased bonding to the nitrogen atom, whereas an inverse (less than unity) KIE shows that increased bonding to the nitrogen atom is partially rate-limiting.

The nitrogen KIE of recombinant human liver MAO B acting on benzylamine was determined under steady-state conditions with respect to substrate concentration. Competitive kinetics were applied, and a chromatographic procedure in combination with isotope-ratio mass spectrometry at natural abundance provided the fraction of reaction and isotopic composition of the isolated substrate.

The finding of a significant nitrogen KIE (Table 1) demonstrates that a step involving nitrogen rehybridization is at least partially rate-limiting. As the kinetic experiments were performed at pH 7.5, the observed KIE was corrected for the degree of protonation of the substrate, which in itself is dependent on the isotopic composition.[8] The corrected $^{15}(V/K)$ value was found to be 0.9846 ± 0.0005 (Table 1). The inverse nitrogen KIE supports mechanistic scenarios involving an increase in the

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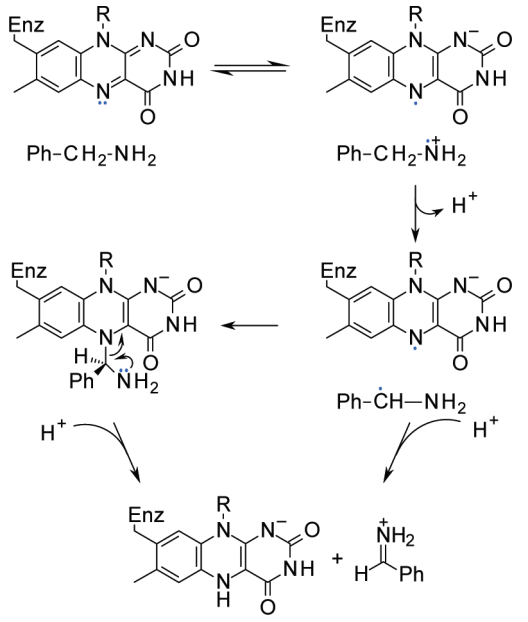
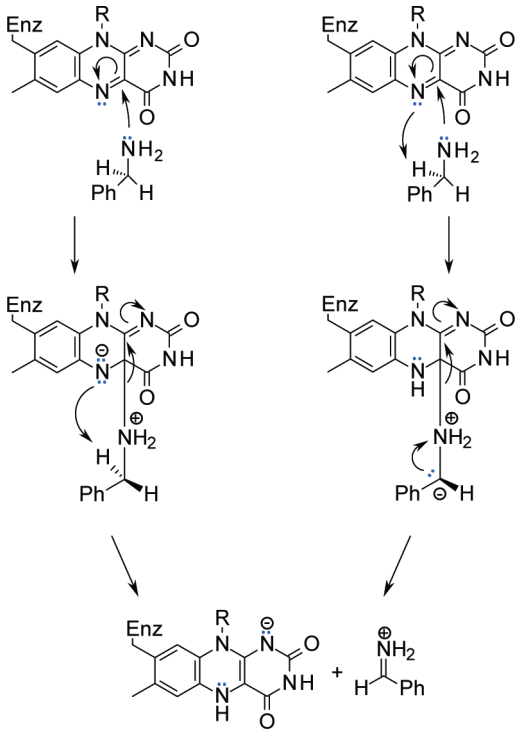
Scheme 1



Scheme 2



overall bond order to the nitrogen that is at least partially rate-limiting. However, it does not give any insight into the timing between the bonding changes at nitrogen and the α-hydrogen. Thus, no discrimination is provided regarding the validity of the mechanistic hypotheses given.

Comparison of the nitrogen KIEs for the α,α-diproteo substrate and the corresponding α,α-dideutero compound offers a means to probe the timing of α-hydrogen abstraction and nitrogen rehybridization.[11] Increasing the barrier for hydrogen abstraction with the deuterated substrate attenuates the nitrogen KIE only if these bonding changes take place in a stepwise fashion. For a concerted α-hydrogen abstraction and nitrogen bond rehybridization, the values of $^{15}(V/K)$ and $^{15}(V/K)_D$ are equal when that step is already fully rate-limiting. For a partially rate-limiting step, $^{15}(V/K)$ increases upon deuteration.

The value of $^{15}(V/K)_D$ was determined by repeating the kinetic experiments but using (1,1-$^2H_2$)benzylamine as the substrate. Deuteration was found to influence the nitrogen KIE; the corrected $^{15}(V/K)_D$ of 0.9901 ± 0.0009 (Table 1) shows an attenuation of the KIE. The reduction of the nitrogen KIE upon deuteration is compatible with the α-hydrogen abstraction either preceding or succeeding the partially rate-limiting nitrogen bond change. A concerted scenario, such as the hydride transfer depicted in Scheme 3, is therefore ruled out. Discrimination between the polar and radical mechanisms (Schemes 1 and 2 respectively) is not possible on the basis of the data presented here. However, the concerted alternatives within the polar and radical

Table 1. Nitrogen KIEs for Recombinant Human Liver MAO B Oxidation of Benzylamine and (1,1-$^2H_2$)Benzylamine at pH 7.5[a]

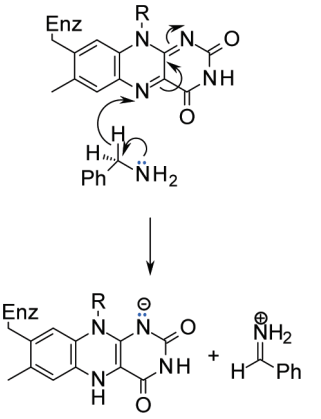
	$^{15}(V/K)$	$^{15}(V/K)_D$
observed	1.0069 ± 0.0005	1.0125 ± 0.0009
corrected for $^{15}K_{eq}$[b]	0.9846 ± 0.0005	0.9901 ± 0.0009

[a] The initial substrate concentration in these experiments was ~6 mM. The pH−(V/K) rate data for MAO B exhibit a single pK of 8.7.[12] The values are reported as mean ± standard deviation of the mean. [b] As MAO acts on the unprotonated substrate, the observed KIE was corrected for the equilibrium isotope effect on the deprotonation, $^{15}K_{eq}$, according to eq 1:

$$KIE_{corr} = \frac{KIE_{obs}}{1 + (^{15}K_{eq} - 1)f_m} \quad (1)$$

where f_m is the mole fraction of the protonated species. The $^{15}K_{eq}$ value used was 1.0229, which is the average of the values for the deprotonations of several amino acids with uncharged side chains.[7] pK_a values of 9.46 and 9.492 were used for benzylamine and (1,1-$^2H_2$)benzylamine, respectively.

Scheme 3



mechanism families are dismissed, which introduces a new constraint for future theoretical calculations.

■ ASSOCIATED CONTENT

Supporting Information. Kinetic data for the individual experiments, kinetic procedures, materials, and data evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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