

Kinetic Isotope Effects as Mechanistic Probes of Flavoprotein Oxidases

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Kinetic isotope effects on rapid reaction and steady state kinetic parameters have been used to study the mechanisms of CH bond cleavage by several flavoprotein amine oxidases. To date, mechanisms involving removal of the substrate hydrogen as a proton, a hydrogen atom, or a hydride have all been proposed; in some cases substrate-flavin adducts are also proposed as intermediates. These mechanisms make different predictions regarding the formation of a flavin intermediate prior to cleavage of the substrate CH bond and to the relative timing of CH bond cleavage and rehybridization of the amine nitrogen. Our general strategy has been to identify substrates for which the intrinsic primary deuterium isotope is fully expressed in the rate constant for flavin reduction and in the k_{cat}/K_m value for the amino acid. This establishes that isotope effects on these parameters report on the transition state for CH bond cleavage. Stopped-flow spectroscopy with deuterated substrates is then used to look for flavin intermediates, and ^{15}N kinetic isotope effects (kies) are used to determine the timing of the amine rehybridization. D-Amino acid oxidase (DAAO) and N-methyltryptophan oxidase (MTOX) are both members of the same structural family. D-Serine was found to be an appropriate substrate for DAAO, while sarcosine was selected for MTOX. Tryptophan monooxygenase (TMO) is an L-amino oxidase and a member of the monoamine oxidase structural family; L-alanine was selected as the substrate for TMO. In no case was an intermediate detected during flavin reduction with deuterated substrate. With DAAO, the ^{15}N kinetic isotope effect increases with pH to a value of 0.994 at pH 10.6. With TMO, correction of the ^{15}N kinetic isotope effect at pH 8.3 for the ^{15}N effect on the amine pK_a yields an ^{15}N kie of 0.991. With MTOX, the pH dependence of the ^{15}N kie on the k_{cat}/K_m value yields a value of 0.994-0.995 for the ^{15}N kie on CH bond cleavage. The isotope effects corresponding to several proposed mechanisms were calculated at the B3LYP/6-31+G** level. Mechanisms involving nucleophilic addition of the amine to the flavin yield ^{15}N kies of 1.013-1.015, mechanisms involving a radical yield ^{15}N kies no more inverse than 0.996, while mechanisms involving hydride transfer yield ^{15}N kies of 0.993-0.994. In addition, the energetics for hydride transfer are far more favorable than for radical formation. Overall, the results are consistent with hydride transfer as the mechanisms of substrate oxidation for both structural families of flavoprotein amine oxidase.