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KINETIC SOLVENT DEUTERIUM ISOTOPE EFFECT ON THE OXYGENATION OF N,N-DIMETHYLANILINE WITH THE PIG LIVER MICROSOMAL FAD-CONTAINING MONOOXYGENASE

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Abstract: Both the maximum velocity and the Michaelis constant of the oxygenation of N,N-dimethylaniline with the pig liver microsomal FAD-containing monooxygenase (EC 1.14.13.8) to N,N-dimethylaniline N-oxide appear 1.7 folds greater in aqueous buffer solution of pH 7.4 than those in deuterium oxide buffer solution of pD 7.4.

Liver microsomal FAD-containing monooxygenase (EC 1.14.13.8), which was first isolated and characterized by Ziegler and coworkers,[1] is known to add oxygen to xenobiotic amines (eq.1).[1] The enzyme contains flavin adenine

$$R_3N: + O_2 + NADPH + H^+ \xrightarrow{\text{FAD-containing monooxygenase}} R_3N{\rightarrow}O + H_2O + NADP^+ \quad (1)$$

dinucleotide at the active site. The catalytic mechanism shown in Scheme I has been proposed by Poulsen and Ziegler[2] and Beaty and Ballou.[3] The actual oxidant of this enzymic oxygenation is believed to be 4a-hydroperoxyflavin, E(FlOOH), which oxidizes nucleophilic amines and sulfides to amine N-oxides and sulfoxides, respectively.[2,3] The oxygen addition step involves an electrophilic attack of the terminal peroxidic oxygen of E(FlOOH) on nucleophilic substrates.[4,5] Beaty and Ballou suggested that the rate determining step of oxygenation of N,N-dimethylaniline by this enzyme is not the electrophilic oxidation with E(FlOOH) but is the dehydration of 4a-hydroxyflavin intermediate, E(FlOH), to regenerate the oxidized form of the flavin at the enzyme active site, $E(Fl_{ox})$.[3] We also observed that the oxygen addition step is not the rate determining step of this enzymic oxygenation.[4,5] However, no information about the mechanism of the dehydration of E(FlOH) has been known at all.

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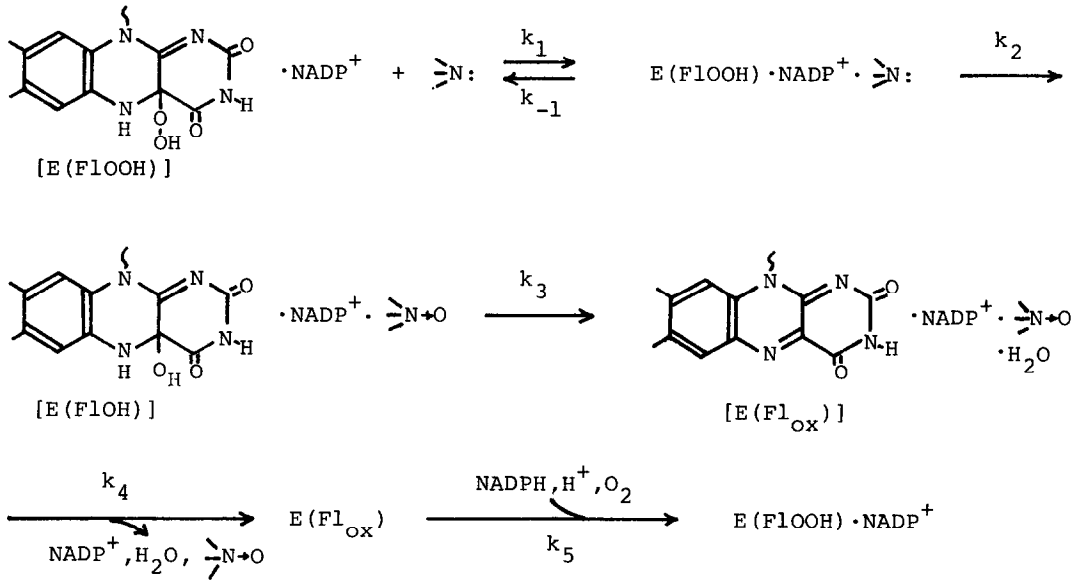

Scheme I

Thus, we determined kinetic solvent deuterium isotope effect following the steady state kinetics of the oxygenation of N,N-dimethylaniline with the purified pig liver microsomal FAD-containing monooxygenase in both aqueous and deuterium oxide buffer solutions. The dehydration of carbinolamines to afford imines is well known to be accelerated by both specific and general acid catalysts.[6] Since E(FlOH) is a kind of carbinolamine, if the dehydration of E(FlOH) is the rate determining step in the enzymic oxygenation of N,N-dimethylaniline, the rate should be accelerated by a general acid catalyst. Therefore, when any general acid is present at the enzyme active site, a moderate size of the kinetic solvent deuterium isotope effect will be observed in the dehydration of E(FlOH).[7]

The pig liver microsomal FAD-containing monooxygenase was purified according to the method reported by Ziegler et al.[1f] with a little modification.[3,4] Rates of the enzymic oxygenation have been determined at 20°C by following the decreasing absorbance at 340 nm due to NADPH.[1a] A deuterium oxide buffer solution was prepared by mixing deuterium oxide solution of KD_2PO_4 and K_2DPO_4 so as to maitain pH meter leading to be 7.0.[8] The maximum velocity (V_{max}) and the Michaelis constant (K_m) have been obtained by Lineweaber - Burk plots of apparent rates measured with various concentration of the substrate. Results are summarized in Table. V_{max}, K_m and V_{max}/K_m of the reaction are expressed by eqs. 2-4, where E_t denotes the concentration of

$$V_{max} = k_2k_3k_4k_5/(k_2k_3k_4 + k_2k_3k_5 + k_2k_4k_5 + k_3k_4k_5)\cdot E_t \qquad (2)$$

Table I Kinetic Solvent Deuterium Isotope Effect on the Oxygenation of N,N-Dimethylaniline with the Pig Liver Microsomal FAD-Containing Monooxygenase at 20°C[a)]

Medium	V_{max}[b)]	K_m[c)]	$V_{max}^{H_2O} / V_{max}^{D_2O}$	$K_m^{H_2O} / K_m^{D_2O}$	$(V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O}$
H_2O	7.09	4.03			
			1.7	1.7	1.0
D_2O	4.27	2.42			

a) The reaction mixture (1 ml) contained 0.1 M phosphate buffer solution of pH or pD of 7.4, 0.5 mg triton X-100, 0.2 nmol of the enzyme, 50μmol of NADPH, and 1-30 μM of N,N-dimethylaniline. b) nmol NADPH-oxidation/nmol enzyme/min. c) μM.

$$K_m = k_3k_4k_5(k_{-1} + k_2)/k_1(k_2k_3k_4 + k_2k_3k_5 + k_2k_4k_5 + k_3k_4k_5) \quad (3)$$

$$V_{max}/K_m = k_1k_2/(k_{-1} + k_2) \quad (4)$$

total enzyme. If the dehydration step of E(FlOH) (k_3) would be the rate determining step (i.e. $k_3 \ll k_2$, k_4 and k_5) as the previous work indicates, the terms, $k_2k_3k_4$, $k_2k_3k_5$ and $k_3k_4k_5$, may be much less than $k_2k_4k_5$, and therefore eqs. 2 and 3 can be simplified as eqs. 5 and 6, respectively.

$$V_{max} = k_3 \cdot E_t \quad (5) \qquad K_m = k_3(k_{-1} + k_2)/k_1k_2 \quad (6)$$

Inspection of the data in Table I reveals that there is no kinetic solvent deuterium isotope effect on V_{max}/K_m, clearly indicating that both substrate binding step and the oxygen addition step to the substrate (k_1, k_{-1} and k_2) are not affected by the change of the medium from H_2O to D_2O (from eq. 4). This means that the conformation of the flavoprotein is not affected by the change of the medium, and hence $K_m^{H_2O}/K_m^{D_2O}$ should be identical to $k_3^{H_2O}/k_3^{D_2O}$ from the eq. 6. Meanwhile, $V_{max}^{H_2O} / V_{max}^{D_2O}$ should also be equal to $k_3^{H_2O}/k_3^{D_2O}$ from the eq. 5. In fact, $V_{max}^{H_2O} / V_{max}^{D_2O}$ and $K_m^{H_2O}/K_m^{D_2O}$ values are identical (1.7) as shown in Table I.

Thus, the moderate solvent kinetic deuterium isotope effect observed in the dehydration step of the 4a-hydroxyflavin intermediate, E(FlOH), is in accordance with that of usual general acid catalyzed reactions[7)] and suggest that the proton transfer from the general acid catalyst to the hydroxy group attached to the 4a-position of the flavin of E(FlOH) is involved in the transition state of the rate determining dehydration of E(FlOH).

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