Comparison of Enzymatic and Non-Enzymatic Nitroethane Anion Formation: Thermodynamics and Contribution of Tunneling

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Comparison of an enzymatic reaction with the equivalent non-enzymatic reaction can provide critical insight into the nature of enzyme catalysis. Given that a fundamental mechanism of enzymatic catalysis is transition-state stabilization,[1] comparison of the thermodynamics of transition state formation for the enzymatic and non-enzymatic reaction provides insight into protein−substrate interactions in the transition state.[2] In the case of reactions in which a hydrogen is transferred, there is growing evidence for the importance of quantum mechanical tunneling in enzyme-catalyzed reactions.[3−8] A fundamental and unresolved question is whether enzymes utilize increased tunneling to increase reaction rates.[9,10] Evaluation of the contribution of tunneling to enzymatic catalysis will require comparison of the contribution of tunneling to both the enzymatic and non-enzymatic reactions.

The flavoenzyme nitroalkane oxidase (NAO) catalyzes the oxidation of nitroalkanes to their corresponding aldehyde or ketone products with release of nitrite.[11] As illustrated in Scheme 1, the reaction is initiated by the abstraction of a proton from the neutral nitroalkane substrate by Asp402.[12−14] The non-enzymatic deprotonation of nitroalkanes is a well-studied chemical reaction that has served as a general model for the ionization of carbon acids.[15−17] NAO thus affords an excellent opportunity to compare the enzymatic and non-enzymatic ionization of a carbon acid and to gain insight into the nature of catalysis by this flavoenzyme.

For studies of the non-enzymatic ionization of nitroalkanes, acetate was selected as a base to provide a mimic of Asp402. The phosphate dianion-catalyzed reaction was also examined to have a base with a pK_a value close to that of Asp402, 7.0.[18] The choice of nitroalkane was restricted by the substrate specificity of the enzyme. While NAO will utilize a large number of primary and secondary nitroalkanes as substrates,[19] nitroethane is the only substrate identified to date for which deprotonation is fully rate-limiting in the reductive half-reaction.[13,18,20]

The effect of temperature on the second-order rate constants for deprotonation of nitroethane by acetate and phosphate dianion is shown in Figure 1, and the thermodynamic and kinetic data are summarized in Table 1. The effects of temperature on the deuterium isotope effect with [1,1-^{2}H$_2$]nitroethane for the non-enzymatic reactions are shown in Figure 2A,B, and the resulting values of ΔE_a and the isotope effect on the Arrhenius prefactor, A_H/A_D, are also given in Table 1. The temperature dependence of the k_{cat}/K_m value for nitroethane as a substrate for NAO and of the deuterium isotope effects on the k_{cat}/K_m value are shown in Figures 1 and 2C, and the data derived from these plots are summarized in Table 1.

The second-order rate constant for deprotonation by NAO at 25 °C is 1.2×10^9-fold greater than the rate constant for the acetate reaction. This rate enhancement places NAO in the mid-to-low range of rate accelerations that have been reported for enzymes[26] and corresponds to a difference in the free energies of activation of 12.3 kcal/mol for enzymatic and non-enzymatic nitroethane anion
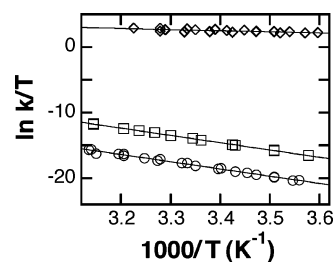

Figure 1. Temperature dependence of the rate constants for nitroethane anion formation by acetate (○) and phosphate dianion[21] (□) and the k_{cat}/K_m values for nitroethane as a substrate for NAO[24,25] (◇). The lines are from fits of the data to $\ln(k/T) = \ln(k_B/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT$.
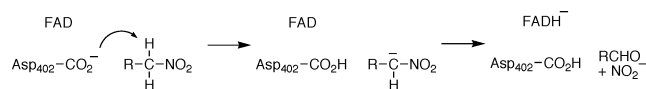
Scheme 1

Table 1. Kinetic and Thermodynamic Parameters for Nitroethane Anion Formation

	acetate	phosphate	NAO
k_H (M^{-1} s^{-1})^a	$4.14 \pm 0.01 \times 10^{-6}$	$1.93 \pm 0.02 \times 10^{-4}$	4800 ± 200^b
$k_H/k_D{}^a$	7.8 ± 0.1	8.8 ± 0.1	9.2 ± 0.4^b
$\Delta G^{\ddagger a,c}$	24.8 ± 0.2	22.4 ± 0.2	12.5 ± 1.0
$\Delta H^{\ddagger c}$	21.9 ± 0.2	22.0 ± 0.2	3.3 ± 0.4
$-T\Delta S^{\ddagger a,c}$	2.9 ± 0.1	0.4 ± 0.1	9.2 ± 0.9
$\Delta E_a{}^c$	1.38 ± 0.26	1.20 ± 0.32	1.35 ± 0.17
A_H/A_D	0.81 ± 0.35	1.22 ± 0.66	0.89 ± 0.25

^{a} 25 °C. ^{b} 27 °C. ^{c} In kilocalories per mole.

formation. The rate constant for nitroethane anion formation by NAO at 25 °C is 2.5×10^7-fold greater than that for the phosphate reaction and corresponds to a similarly large difference in the free energies of activation ($\Delta\Delta G^{\ddagger} = 9.9$ kcal/mol). The energetics of the enzymatic and non-enzymatic reactions establish that a change in the enthalpy of activation is the major source of the enzymatic rate enhancement. These results are in line with the observation of Wolfenden that the rate accelerations observed for enzymatic reactions are largely derived from a decrease in $\Delta H^{\ddagger}$.[2]

The observation of tunneling in an increasing number of enzyme-catalyzed reactions has raised the possibility that enzymes enhance tunneling to increase reaction rates.[27] Evidence for tunneling can be obtained by measuring the deuterium kinetic isotope effect as a function of temperature. The semiclassical values of the Arrhenius parameters for the cleavage of a CH bond relative to a CD bond are $\Delta E_a = 1.4$ kcal/mol and $0.7 < A_H/A_D < 1.7$.[28] Tunneling produces curvature in the Arrhenius plot and results in linear tangents for each isotope over the relatively small temperature range available experimentally and ΔE_a and A_H/A_D values that differ from the semiclassical limits. A recent comparison of enzymatic and non-
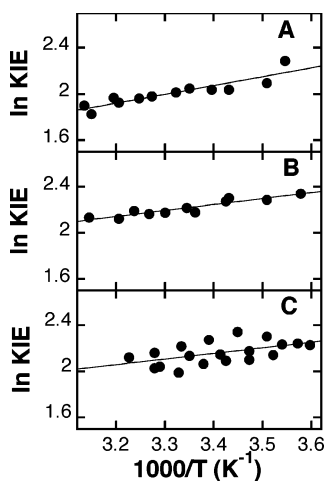
Figure 2. Deuterium kinetic isotope effects for nitroethane anion formation as a function of temperature for acetate (A) and phosphate (B) and the k_{cat}/K_m values of NAO with nitroethane as substrate (C). The lines are from fits of the deuterium kinetic isotope effect at each temperature to $\ln(\text{KIE}) = \ln(A_H/A_D) - \Delta E_a/RT$.

enzymatic hydrogen atom abstraction by adenosylcobalamin found no change in the extent of tunneling in the enzymatic reaction.[9,29]

Evidence for tunneling has previously been reported in studies of the temperature dependence of non-enzymatic nitroalkane deprotonation. These include ionization of phenylnitromethane by several bases, including dianionic phosphate, for which $A_H/A_D <$ 0.7.[30] Unfortunately, phenylnitromethane is a very sticky substrate for NAO, and the ionization step is masked by kinetic complexity.[20] The ΔE_a and A_H/A_D values for the ionization of nitroethane (Table 1) all fall within the semiclassical limits, and the ΔE_a and A_H/A_D values for nitroethane anion formation by acetate and NAO are nearly identical.[31] Thus, the present results provide no evidence for a tunneling contribution to either the enzymatic or non-enzymatic reactions. However, values for these parameters within the semiclassical limits can arise when there is no tunneling or when the amount of tunneling is intermediate.[31] Thus, the data do not rule out comparable contributions of tunneling to both reactions. In either case, the nature of the H-transfer in the enzymatic and non-enzymatic reactions appears to be similar, suggesting that there is no unique contribution of tunneling to the NAO-catalyzed reaction and thus no specific tunneling enhancement by the protein environment. The possibility remains that tunneling does contribute to catalysis in the reactions of NAO substrates with significantly larger k_{cat}/K_m values, such as 1-nitrobutane and phenylnitromethane.

In conclusion, the opportunity to compare the enzymatically and non-enzymatically catalyzed ionization of nitroethane has provided valuable insight into the mechanism of NAO. The major enthalpic contribution to the enzymatic rate enhancement suggests that there are important electrostatic and hydrogen-bonding interactions in the transition state of the enzymatic reaction. Since the ΔE_a and

A_H/A_D values in the enzymatically and non-enzymatically catalyzed reactions are identical and within the semiclassical limits, the extent of quantum mechanical tunneling does not appear to differ in the enzyme-catalyzed and acetate-catalyzed reactions.

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