The Catalytic Mechanism of Microsomal Epoxide Hydrolase Involves Reversible Formation and Rate-Limiting Hydrolysis of the Alkyl−Enzyme Intermediate

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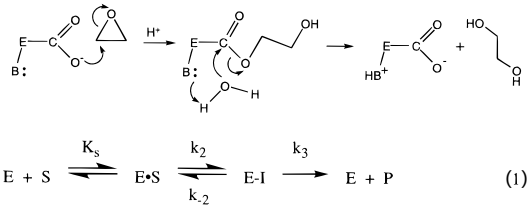
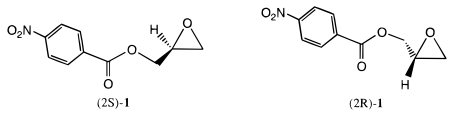
Microsomal epoxide hydrolase (MEH) catalyzes the addition of water to epoxides and arene oxides to give vicinal diol products.[1] The reaction occurs in two steps involving initial attack of an active-site carboxylate at one of the oxirane carbons of the substrate to give an alkyl−enzyme (ester) intermediate that is subsequently hydrolyzed by attack of water at the carbonyl carbon of the ester (Scheme 1).[2] Unlike many hydrolytic or group transfer reactions that occur via covalent intermediates, the bond made in formation of the intermediate is not the same as the bond cleaved in its decomposition. A simplified kinetic mechanism for the enzyme is shown in eq 1 where rapid equilibrium binding (K_s) of the substrate to form a Michaelis complex is followed by reversible formation (k_2/k_{-2}) and essentially irreversible hydrolysis (k_3) of the intermediate (E-I). The rate-limiting step in the enzyme-catalyzed reaction has not been established for any epoxide or arene oxide substrate.

Scheme 1



Here we provide presteady state and steady state kinetic evidence that the rate-limiting step in the hydration of the 2*R*- and 2*S*-enantiomers of glycidyl-4-nitrobenzoate (**1**) is hydrolysis of the alkyl−enzyme intermediate. A comparison of the steady state and presteady state kinetic constants suggests that formation of the ester is reversible with $k_2 \gg k_{-2}$ and $k_{-2} \approx k_3$. Although the enantioselectivity of the enzyme is found to be manifest in both the alkylation and hydrolysis steps, it is most pronounced in the hydrolysis of the intermediate. The regiospecificity for the reaction is the same for each enantiomer with exclusive attack of the carboxylate at the primary oxirane carbon.



The active-site nucleophile of MEH has been tentatively identified by sequence alignments with other C−X bond hydrolases such as haloalkane dehalogenase as Asp226 that precedes Trp227 in the sequence.[2,3] The proximity of the carboxylate to Trp227 and to Trp150 located in the oxyanion hole suggested that formation of E-I with an appropriate epoxide might be accompanied by quenching of the intrinsic tryptophan fluorescence of the protein. Rapid mixing[4] of recombinant MEH[5] with either enantiomer of **1** results in a rapid initial decrease in the intrinsic fluorescence of the enzyme (Figure 1A) which, after a lag, recovers to the initial value (Figure 1B). The rate of the initial decrease in fluorescence as well as the length of the lag before recovery is dependent on both the initial concentration and the chirality of the substrate. The presteady state decay in fluorescence follows a single exponential. The dependence of the rate constant (k_{obs}) for approach to the steady state on the substrate concentrations differs for the two enantiomers. The 2*R*-enantiomer exhibits saturation behavior (Figure 2) as expected for a kinetically significant preassociation of the substrate and the enzyme prior to formation of the alkyl−enzyme (eq 1).[6] In contrast, a Michaelis complex is not detected with (2*S*)-**1** in the concentration range of 0.05−1.0 mM. As a consequence, only lower limits can be set for the values of k_2 and K_s for this enantiomer (Table 1). The enzyme is rapidly alkylated by either substrate with apparent rate constants that are 10^2- to 10^3-fold greater than that for hydrolysis of the ester (k_3). Extrapolation of the data for both substrates to [S] = 0 indicates a small but significant rate (k_{-2}) for reformation of

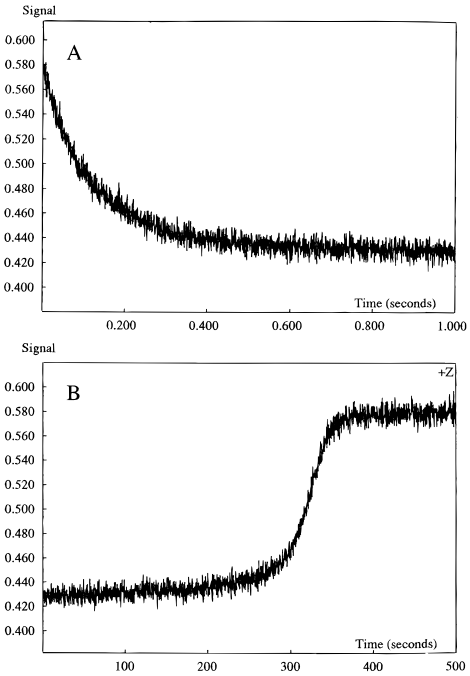


Figure 1. (A) Presteady state decrease in intrinsic fluorescence of MEH (5.1 μM) when mixed with 100 μM (2*S*)-**1**. (B) Poststeady state recovery of fluorescence upon consumption of the substrate.

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(4) Enzyme (5−8 μM final concentration) buffered at pH 7 with 50 mM morpholinopropane sulfonate and substrate (0.05−1.0 mM final concentration) in the same buffer containing 10% CH_3CN were mixed at 25 °C in an Applied Photophysics SX-17MV stopped-flow spectrometer. Total fluorescence with excitation at 290 nm was observed through a 320 nm filter.

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(6) The fact that saturation is observed with (2*R*)-**1** indicates that the change in fluorescence is not associated with formation of the Michaelis complex but rather a subsequent slower step. It is assumed that the failure to observe saturation with (2*S*)-**1** is due to a much higher dissociation constant for this substrate.

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Table 1. Presteady State and Steady State Kinetic Constants for the Enzyme-Catalyzed Hydration of the Enantiomers of Glycidyl-4-nitrobenzoate

	pre-post steady state[a]					steady state[b]	
substrate	k_2 (s^{-1})	k_{-2} (s^{-1})	K_s (mM)	$10^5(k_2/K_s)$ (M^{-1} s^{-1})	k_3 (s^{-1})	$10^4(k_{cat}/K_m)$ (M^{-1} s^{-1})	k_{cat} (s^{-1})
(2*R*)-**1**	330 ± 50	4.0 ± 1.8 3.7 ± 1.5[c]	2.0 ± 0.5	1.7 ± 0.3	0.8 ± 0.1	3.0 ± 0.8	0.57 ± 0.07
(2*S*)-**1**	≥250	0.45 ± 0.13 0.44 ± 0.16[c]	≥3	1.1 ± 0.1	0.07 ± 0.01	1.5 ± 0.2	0.057 ± 0.006

[a] Values of k_2, k_{-2}, K_s, and k_2/K_s were obtained from the dependence of k_{obs} on the concentration of substrate (Figure 2). Values of k_3 were obtained from the fitting the presteady state and poststeady state lag phase (Figure 1B) by numerical integration of the kinetic mechanism (eq 1) using the KINSIM program.[6] [b] Obtained from the substrate dependence of initial velocities. [c] Calculated from $k_{cat}/K_m = k_2/K_s(k_3/(k_3 + k_{-2}))$.

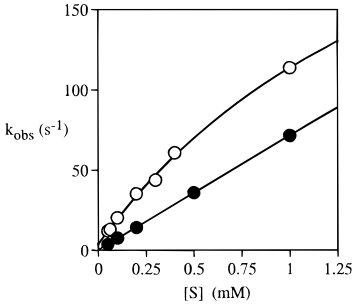

Figure 2. Dependence of k_{obs} for the presteady decrease in fluorescence on the concentration of (2*R*)-**1** (○) and (2*S*)-**1** (●). The solid curve for (2*R*)-**1** is a least squares fit of the data to a hyperbola, $k_{obs} = k_{-2} + k_2([S]/(K_s + [S]))$ with $k_2 = 330 \pm 50$ s^{-1}, $k_{-2} = 4 \pm 2$ s^{-1}, and $K_s = 2.0 \pm 0.5$ mM. The solid curve for (2*S*)-**1** is a least squares fit to the equation $k_{obs} = k_{-2} + k_2([S]/K_s)$ with $k_2/K_s = (1.1 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and $k_{-2} = 0.4 \pm 0.1$ s^{-1}. It was not possible to collect data at higher substrate concentrations due to the limited solubility of **1** (ca 1 mM).

enzyme-bound epoxide from the ester intermediate. Although the errors in the estimates of k_{-2} are substantial, it appears that k_{-2} is as large as, if not larger than, k_3.

The steady state kinetic constants[8] are in reasonable agreement with those obtained in the stopped-flow experiments. For the mechanism described in eq 1, the condition that $k_2 \gg k_3$ and $k_{-2} \neq 0$ requires the steady state $k_{cat}/K_m = k_2/K_s(k_3/(k_3 + k_{-2}))$. The discrepancy between the values of k_{cat}/K_m and k_2/K_s can be used to estimate k_{-2} and provides further evidence that $k_{-2} \neq 0$. The calculated values (Table 1) probably provide a bit more accurate estimate of k_{-2}. The most interesting observation is that the kinetic barriers for hydrolysis of the ester and reformation of enzyme-bound epoxide are roughly equivalent ($k_{-2} \approx k_3$). The steady state values of K_m for (2*R*)-**1** (19 μM) and (2*S*)-**1** (3.7 μM) are much smaller than the apparent dissociation constants of the substrates (K_s > 1 mM) due to extensive accumulation of the intermediate in the steady state.

The enantioselectivity of the enzyme is manifest in the kinetics of both half-reactions but is most pronounced in the hydrolysis of the ester intermediate. The fact that alkylation by (2*R*)-**1** exhibits saturation behavior (and (2*S*)-**1** does not) is indicative of a more specific preassociation of the (2*R*)-enantiomer with the enzyme prior to alkylation. Interestingly, the slower hydration of (2*S*)-**1** is due to the formation of a more stable alkyl−enzyme intermediate. The apparent equilibrium constant for formation of E-I from E·S (k_2/k_{-2}) is at least 5-fold more favorable for the slower substrate (2*S*)-**1**. Product isolated from multiple-turnover reactions of each substrate in $H_2{}^{18}O$ show that ^{18}O is incorporated exclusively in the terminal carbons.[9] Thus, the alkylation reactions with both (2*R*)-**1** and (2*S*)-**1** are essentially regiospecific with >95% attack at the primary (least hindered) oxirane carbon, as has been previously observed with other substituted epoxides.[10] The much slower hydrolysis of the ester formed by reaction with (2*S*)-**1** is not due to a change in regiochemistry (i.e., increased steric hindrance to hydrolysis of the alternative secondary ester) but rather to an alternate binding mode of the 4-nitrobenzoyl group that hampers attack of water or formation of the tetrahedral intermediate at the carbonyl carbon of the ester. The exact nature of this hindrance is not known.

The kinetic mechanism of MEH has important implications for interpretation of experimental results concerning the chemical mechanism of catalysis. For example, the pH dependence of k_{cat} with polycyclic aromatic hydrocarbons has been interpreted to reflect the ionization behavior of the active site histidine (His 431) which acts as a general base in the hydrolysis of the ester.[11] This will only be true when $k_2 \gg k_3$. In addition, the potent alternative substrate inhibition of MEH by 1,1,1-trichloropropylene 2,3-oxide[1a] is probably due to rapid alkylation of the enzyme followed by very slow hydrolysis of the ester. The relatively low steady state K_m observed for many arene oxide substrates are likely to be the result of extensive accumulation of the alkyl−enzyme intermediate ($k_2 \gg k_3$) rather than a high affinity for the substrate in the Michaelis complex.

In summary, we have provided quantitative evidence that the rate-limiting step in the enzyme-catalyzed hydration of glycidyl-4-nitrobenzoates is hydrolysis of the alkyl−enzyme intermediate. Although this also appears to be true for phenanthrene 9,10-oxide and 1,10-phenathroline 5,6-oxide,[12] it need not be true for all oxirane substrates. Finally, the kinetic equivalence of the barriers for partitioning the intermediate between hydrolysis or reformation of the epoxide is remarkable considering that the transition states for the two reactions are quite different. The reversibility of ester formation indicates that the enzyme can stabilize the oxyanion in the alkylation reaction in much the same way that the "oxyanion hole" stabilizes the tetrahedral intermediate in the hydrolysis step, though the exact catalytic device may be different.

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(8) Reactions were carried out with 0.03−0.15 μM enzyme under the same buffer and temperature conditions as the stopped-flow experiments. Initial velocities were calculated from the extent of conversion of substrate to product as determined after separation of substrate and product by HPLC (4.6 × 250 mm MICROSORB C-18 column eluted with 35% CH_3CN/H_2O at 1 mL/min) monitored at 260 nm.

(9) The regioselectivity was determined from the distribution of ^{18}O in product obtained from multiple-turnover reactions in 50 mol % $H_2{}^{18}O$. The extent of isotope incorporation at the primary and secondary carbons was estimated by ^{13}C NMR spectroscopy from the relative intensities of ^{13}C resonance located at 63.7 and 70.1 ppm and isotope-perturbed ($^{13}C-^{18}O$) resonances located 1.4 Hz upfield.

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(12) With these substrates, ester formation can be detected directly by characteristic changes in UV absorption associated with opening of the oxirane ring. The observed rate constants for alkylation are at least 10-fold larger than the corresponding turnover numbers. Tzeng, H.-F.; Lin, S.; Lacourciere, G. M.; Armstrong, R. N. Unpublished results.