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# Dramatic Solvent and Hydration Effects on the Transition State of Soybean Peroxidase

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The fact that enzymes function in nonaqueous media is well established.[1] Nevertheless, relatively little information is available on the influence of the organic solvent as well as its associated water content on the properties of the enzymatic transition state.[2] This is unfortunate as such knowledge is essential to gain a fundamental understanding of enzymatic catalysis in low-water environments.[3] In the present study, we have used soybean hull peroxidase (SBP)-catalyzed oxidation of phenols as a model reaction to evaluate the influence of solvent and hydration on enzymatic catalysis in nonaqueous media. Classical Hammett analysis has been employed using a series of phenolic substrates with differing electronic and hydrophobic properties[4] to assist in investigating the role of water on enzyme function at the level of the transition state. Our results suggest that the interplay of solvent and water on the transition state of SBP is complex and gives rise to dramatic differences in reactivity and substrate specificity.

The water content of the reaction medium (as reflected in the thermodynamic water activity, $a_w$[5]) strongly influences SBP-catalyzed oxidation of *p*-cresol in $CH_3CN$ and ethyl acetate (Figure 1). The reactivity of SBP in $CH_3CN$ is more than 100-fold higher than in ethyl acetate at the lowest $a_w$ employed; yet this difference becomes less significant as $a_w$ is increased tending to the value in aqueous buffer. Overall, $V_{max}/K_m$ of SBP for the oxidation of *p*-cresol in these systems ranges over 5 orders of magnitude, indicating a considerable change in catalytic efficiency of SBP as a result of solvent choice and level of hydration. The $V_{max}/K_m$ for *p*-cresol oxidation in $CH_3CN$ having a water activity of 0.72 (7%, v/v $H_2O$) is within an order of magnitude of the catalytic efficiency in aqueous buffer, suggesting that the enzyme is highly active in selected organic solvent systems.

The relatively high activity of SBP in $CH_3CN$, particularly at higher values of $a_w$, prompted us to explore further the influence of water on the enzymatic transition state with specific focus on substrate specificity. Using a series of phenolic substrates (with substituents differing in their electronic properties, as represented by $\sigma$ values), we determined the Hammett coefficient, $\rho$, (eq 1) as a function of the $a_w$ in $CH_3CN$ (Figure 2). Plots of $\log(V_{max}/K_m)$ versus $\sigma$ were linear (Figure 1 of Supporting Information), while no correlation was obtained using $\sigma^+$ or $\sigma^-$ values (data not shown). These results are consistent with peroxidase reactions in aqueous media.[6] As the $a_w$ is increased, $\rho$ tends toward the aqueous value of $-1.2$.[7]

$$\log\left(\frac{V_{max}}{K_m}\right) = \rho\sigma + c \qquad (1)$$

Upon reduction of $a_w$ below 0.35, a dramatic change in SBP catalysis is observed, marked by a shift to positive $\rho$ values indicative of the formation of a fundamentally different transition state than in more hydrated $CH_3CN$ and a change in the observed

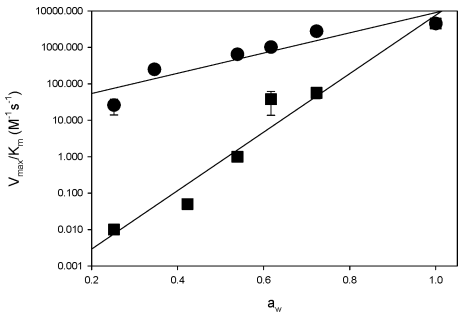


***Figure 1.*** Catalytic efficiency of SBP-catalyzed oxidation of *p*-cresol in acetonitrile (●) and ethyl acetate (■) as a function of thermodynamic water activity (see Supporting Information for experimental details). The units of $V_{max}/K_m$ were transformed into $mM^{-1}s^{-1}$ (SBPs molecular weight of 37 000). Each data point represents the average of three measurements, with the error in most cases smaller than the size of the symbols. The lines represent the best fits through the data, including $H_2O$.

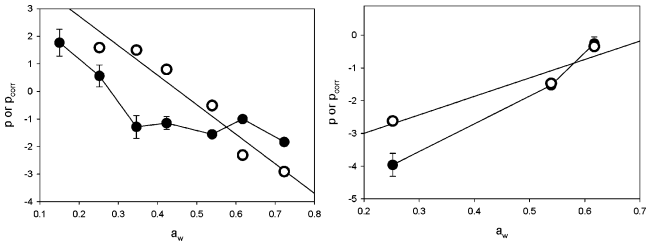


***Figure 2.*** Effect of water activity on the Hammett coefficient $\rho$ (●) and $\rho_{corr}$ (○) in $CH_3CN$ (A) and MeOH (B).

substrate specificity of the enzyme. For example, at high $a_w$ (0.72) *p*-ethoxyphenol ($\sigma = -0.24$) is 34-fold more reactive than *p*-chlorophenol ($\sigma = 0.23$), whereas at low $a_w$ (0.15), this selectivity is reversed, with *p*-chlorophenol being 4-fold more reactive than *p*-ethoxyphenol. This phenomenon represents an inversion of enzyme selectivity of over 2 orders of magnitude, indicating that the substrate specificity of SBP is strongly affected by solvent hydration.

The Hammett expression (eq 1) does not take into account the effects of substrate hydrophobicity and hence substrate desolvation from the bulk solvent and solvation into the enzyme's transition state, which may also affect observed enzyme reactivity and specificity. To account for differences in substrate desolvation, we used a modified Hammett expression, first proposed by Hansch (eq 2),[8] where $\pi$ is the hydrophobic substituent parameter and $\delta$ is the transition state dependence on substrate hydrophobicity. When several of the data points in Figure 2A are corrected for substrate hydrophobicity, $\rho_{corr}$ becomes strongly linearly related to solvent hydration, which therefore indicates that water has a direct impact on the transition state of SBP. Specifically, assuming that water can penetrate the active site of SBP[9] we may rationalize the aforementioned behavior in terms of the ability of water to promote increased solvation of polar and ionic groups in the enzymatic transition state. At low $a_w$, such solvation is decreased and the

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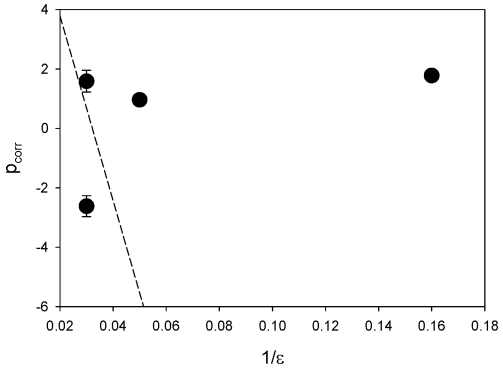


***Figure 3.*** Effect of solvent dielectric on the Hammett coefficient at constant water activity ($a_w = 0.25$). The solvents in increasing order of inverse dielectric are $CH_3CN$, MeOH, 1-propanol, and *tert*-amyl alcohol. For the dashed line, $B_1$ and $B_2$ were calculated from the phenolic oxidation of 1,1-diphenyl-2-picrylhydrazyl radicals and modified for the scale of the data. Similar results (not shown) were seen for other $a_w$ values used in the study.

strength of ionic and dipolar interactions between the enzyme and substrate in the transition state is increased, and this could exaggerate the effects of electron donating/withdrawing substituents. As a result, the magnitude of $\rho_{corr}$ increases. It should be noted that the influence of substrate size does not appear to influence $\rho_{corr}$ even under more rigid conditions of low $a_w$. Specifically, the highly negative $\rho_{corr}$ in MeOH at $a_w = 0.25$ is due to a *higher* reactivity of the more bulky *p*-ethoxyphenol than the less bulky *p*-chlorophenol.

$$\log\left(\frac{V_{max}}{K_m}\right) = \rho\sigma + \delta\pi + c \qquad (2)$$

Because of the strong effect of solvent hydration on the transition state of SBP in $CH_3CN$, we proceeded to evaluate whether similar effects are observed with other solvents. As with $CH_3CN$, the transition state of SBP catalysis in MeOH is strongly dependent on solvent hydration, with an observed linear free energy relationship (Figure 2B). Unlike $CH_3CN$, however, a decrease in $a_w$ results in a *decrease* in $\rho_{corr}$ (again with an increased magnitude of $\rho_{corr}$) such that the specificity of SBP is far different at low $a_w$ than in $CH_3CN$, and the enzyme shows a strong preference for electron-donating phenolic substituents reflective of a decrease in electron density in the transition state. Thus, as was observed with reactivity (Figure 1), there exists a strong solvent effect on the influence of hydration on transition state properties (Figure 2).[10]

To identify the specific property of the solvent that governs SBP catalysis in nonaqueous media, we further examined $\rho_{corr}$ in different solvents at a low level of solvent hydration, $a_w = 0.25$. According to Hammett for the ionization of benzoic acid derivatives, $\rho$ is empirically related to solvent dielectric (eq 3).[11] However, the plot of $\rho_{corr}$ versus $1/\epsilon$ was highly scattered (Figure 3),[12] as was the plot of $\rho$ versus $1/\epsilon$ (data not shown), and far from the linear relationship obtained by Howard and Ingold[13] based on the $B_1$ value of $4.4 \times 10^6$ Å$^2$ K for phenolic oxidation using 1,1 diphenyl-2-picrylhydrazyl radicals. Once again, because $\rho_{corr}$ implicitly incorporates substrate hydrophobicity, the lack of correlation of $\rho_{corr}$ with eq 3 is not a result of differential substrate desolvation in different solvents.

$$\rho = \left(\frac{1}{d^2T}\right)\left(\frac{B_1}{\epsilon} + B_2\right) \qquad (3)$$

Thus, solvent and hydration effects on the transition state of SBP is more complex than for simple chemical reactions, and this must be at least partly due to the complex 3D environment that surrounds the transition state in the former as opposed to the latter. This 3D environment consists of multiple interactions with the transition state that fosters catalysis. Such interactions are expected to be strongly dependent on the location of solvent molecules (including organic solvent and water) that penetrate into the enzyme's active site. Not all of these interactions would be strictly dependent on solvent dielectric. Indeed, our results support the critical importance of hydrogen bonding on the transition state of SBP (e.g., $CH_3CN$ vs MeOH). Along these lines, the presence of H-bond acceptor residues in the active site of the closely related enzyme horseradish peroxidase ($Arg_{183}$ and the proximal His residue coordinated with the heme)[14] is suggestive of an important role of H-bond interactions in the transition state.

In conclusion, our results show that enzyme−solvent−water interactions are complex and can be probed at the transition state level. The combined effects of organic solvent and solvent hydration on SBP catalysis are substantial; differences in observed $\rho$ values from ca. −2 in water and hydrated $CH_3CN$ to +2 in poorly hydrated $CH_3CN$ represent a tremendous variability in the nature of the enzyme's transition state. Even more remarkable is that the factors that cause such a change in the nature of the transition state are not sufficient to disable the enzyme's catalytic function.

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**Supporting Information Available:** Experimental procedure; plots of phenolic oxidation in $CH_3CN$ in a variety of $a_w$'s. This material is available free of charge via the Internet at http://pubs.acs.org.

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