The efficient synthesis of hPTH (70–84) on the new support will allow its application on rapid and parallel synthesis of multiple peptide analogues, and we are now completing such a synthesis of melittin analogues using labeled sheets of 285 wt % long-chain polystyrene-grafted polyethylene film.

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Registry No. hPTH, 9002-64-8; hPTH (80-84), 122425-31-4; hPTH (75-84), 122443-10-1; hPTH (70-84), 98151-37-2; Boc-Gln-$OCH_2C_6H_4$-*p*-CH_2COOH, 83714-72-1; BOC-Ser(Bzl)-OH, 23680-31-1; BOC-Lys(2Cl-Z)-OH, 54613-99-9; BOC-Ala-OH, 15761-38-3; BOC-Thr(Bzl)-OH, 15260-10-3; BOC-Leu-OH, 13139-15-6; BOC-Val-OH, 13734-41-3; BOC-Asp(OBzl)-OH, 7536-58-5; polystyrene-grafted polyethylene, 106826-12-4.

Free Energy Relationships of Substrate and Solvent Hydrophobicities with Enzymatic Catalysis in Organic Media

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While there is little question that enzymes can function in nonaqueous media,[1] the effects of organic solvents on the catalytic activity and substrate specificity of enzymic catalysis are not well understood. Relatively few kinetic studies have been carried out to date.[2] Such quantitative analyses are crucial for the development of kinetic models that can be used to predict optimal solvent and substrate choices for enzymatic reactions in organic media. In the present study, we have examined horseradish peroxidase catalyzed oxidation of phenols in organic solvents as a model to elucidate solvent-induced kinetic alterations of enzymatic catalysis, particularly with respect to substrate and solvent hydrophobicities. Peroxidase is an ideal enzyme for this study as it catalyzes identical reactions in aqueous and organic solvents,[3] and phenols are highly soluble in organic media. In all cases, the enzymatic reactions take place in monophasic organic solutions[5] with diffusional limitations eliminated by adsorption of the peroxidase onto nonporous glass beads.[6]

Our experimental strategy was to determine the steady-state kinetic constants, V_{max}, K_m, and V_{max}/K_m in water and a variety of organic solvents with varying degrees of hydrophobicity.[7] Phenols with para substitutions that differed in hydrophobicities were employed.[9] Such peroxidase substrates do not induce steric hindrance around the phenolic moiety and are similar in electronic factors.[10a,b] Parts A and B of Figure 1 depict the dependence of catalytic efficiency (V_{max}/K_m) on the substrate hydrophobicity, π, in aqueous buffer and several nonaqueous solvents. The catalytic efficiency was profoundly lower (up to 4 orders of mag-

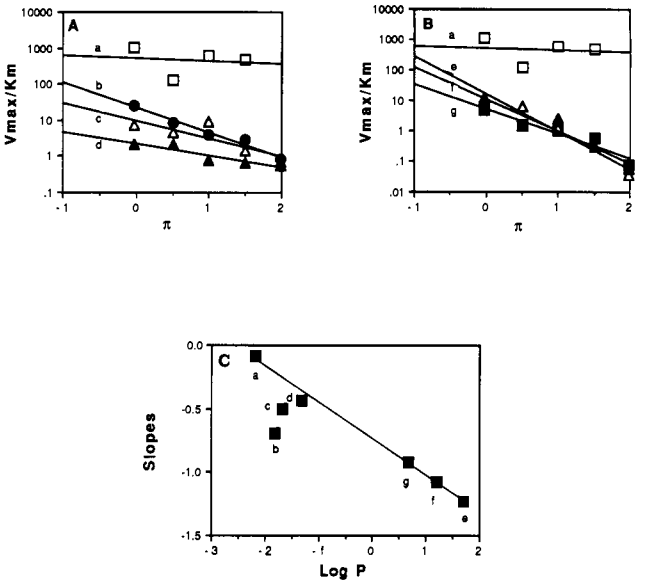

Figure 1. Catalytic efficiency of peroxidase in aqueous and nonaqueous media. (A) Water-miscible solvents: (a) aqueous buffer; (b) dioxane with 30% aqueous buffer; (c) dioxane with 20% aqueous buffer; (d) dioxane with 5% aqueous buffer. (B) Water-immiscible solvents: (a) aqueous buffer; (e) butyl acetate, containing 1% aqueous buffer; (f) propyl acetate, containing 1.5% aqueous buffer; (g) ethyl acetate, containing 2% aqueous buffer. (C) Slopes of (V_{max}/K_m vs π) vs log P. Values of log P for dioxane–water mixtures were calculated by using the following correlation: $\log P = (1 - x) \log P_{solvent} + x \log P_{water}$, where x is the mole fraction of water in the mixture (Reslow, M.; Aldercreutz, P.; Mattiasson, B. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 1–8). Conditions: (i) For phenol oxidation in water, phenol concentrations were varied from 0.05 to 5 mM, the H_2O_2 concentration was fixed at 0.25 mM, and the concentration of peroxidase was 0.01 μg/mL, pH 7.0 (10 mM phosphate buffer), 25 °C. The poor solubility of *p-tert*-butylphenol in aqueous solutions made accurate measurements of kinetic constants impossible. (ii) For phenol oxidation in organic media, phenol concentrations were varied from 10 mM to 1 M, the H_2O_2 concentration was fixed at 0.25 mM, and peroxidase concentrations were in the range from 0.05 to 0.5 μg/mL, 25 °C, shaken at 200 rpm. All kinetic analyses were carried out spectrofluorometrically by following the initial rate of fluorescent dimer production. Kinetic constants were determined by using Eadie–Hofstee graphical representation of duplicate reactions with linear regression analysis. For alkylphenols, excitation and emission were 305 and 350 nm, respectively, while for *p*-methoxyphenol, excitation and emission were 320 and 375 nm, respectively. Correlation of fluorescence intensity with phenol oxidation was carried out by HPLC as described earlier.[2a] The units of V_{max}/K_m are mM^{-1} s^{-1} (peroxidase molecular weight of 42 000[4]).

(1) For general reviews, see: (a) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194–211. (b) Klibanov, A. M. *Trends Biochem. Sci. (Pers. Ed.)* **1989**, *14*, 141–144. (c) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259–270. (d) Butler, L. G. *Enzyme Microb. Technol.* **1979**, *1*, 253–259. (e) *Biocatalysis in Organic Media*; Laane, C., Tramper, J., Lilly, M. D., Eds.; Elsevier: Amsterdam, 1987.

(2) (a) Ryu, K.; Stafford, D. S.; Dordick, J. S. In *Biocatalysis* in *Agricultural Biotechnology*; Whitaker, J. R., Sonnet, P., Eds.; ACS Symposium Series 389; American Chemical Society: Washington, DC, 1989. (b) Zaks, A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1986**, *108*, 2767–2768. (c) Sakurai, T.; Margolin, A. L.; Russell, A. J.; Klibanov, A. M. *Ibid.* **1988**, *110*, 7236–7237.

(3) In the presence of hydrogen peroxide, horseradish peroxidase catalyzes the single-electron oxidation of phenols and aromatic amines.[4] The reaction mechanism is independent of solvent, and it is possible to compare, directly, the kinetics of phenol oxidation in aqueous and organic media.[2a]

(4) Saunders, B. C.; Holmes-Siedle, A. G.; Stark, B. P. *Peroxidase*; Butterworth: London, 1964.

(5) Defined as enzymatic reactions in the absence of a bulk aqueous phase.[1] This includes water-immisible solvents with water contents below saturation and water-miscible solvents with water contents insufficient to cause enzyme solubilization.

(6) Peroxidase was deposited onto 75–150-μm nonporous glass beads (Sigma Chemical Co.) at a loading of 0.1 mg/g of beads.[2a]

(7) Solvents used ranged in hydrophobicities from water (log P = −2.17) to butyl acetate (log P = 1.70); log P is defined as the logarithm of the partition coefficient between 1-octanol and water (Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81–87). Added water was 0–30% (v/v) in dioxane, 0% in dimethylformamide and acetone, 2% in ethyl acetate, 1.5% in propyl acetate, and 1% in butyl acetate. In the water-immiscible ester solvents, the water contents were slightly below saturation levels, an amount proven to be optimal for similar solvent systems[8] and confirmed with peroxidase in this work.

(8) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017–8021.

(9) Phenolic substituents in the para positions and their respective π values (in parentheses)[10c] included methoxy (−0.02), methyl (0.56), ethyl (1.02), propyl (1.53), and *tert*-butyl (1.98).

(10) (a) Gordon, A. J.; Ford, R. A. *The Chemist's Companion*; Wiley: New York, 1972; pp 152–153. (b) Dunford, H. B.; Adeniran, A. J. *Arch. Biochem. Biophys.* **1986**, *251*, 536–542. (c) Hansch, C.; Coats, E. *J. Pharm. Sci.* **1970**, *59*, 731.

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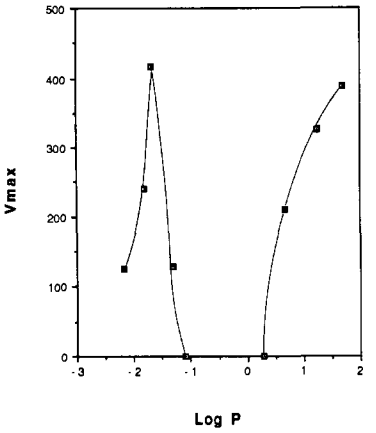

Figure 2. Effect of solvent hydrophobicity on the catalytic activity of peroxidase with *p*-cresol as substrate. The units of V_{max} are μmol (mg enzyme)$^{-1}$ s^{-1}.

nitude) in organic solvents as compared to aqueous buffer. Furthermore, in all solvents, catalytic efficiency decreased as substrate hydrophobicity increased. This substrate effect, however, became more pronounced as solvent hydrophobicity increased; the slopes of catalytic efficiency vs π became more negative as a function of log *P* (Figure 1C), and with high linearity (correlation coefficient of 0.98), neglecting the dioxane–water mixtures.[11] These results indicate that a linear free energy relationship exists between catalytic efficiency and *both* substrate and solvent hydrophobicities. Furthermore, substrate hydrophobicity becomes a significant reaction variable as solvent hydrophobicity increases. The above findings may be explained purely by the partitioning behavior of phenols between the bulk reaction medium and the peroxidase active site. This partitioning is likely to diminish as substrate and solvent hydrophobicities increase, thereby necessitating a larger concentration of phenols to saturate the enzyme. This results in an increase in the apparent K_m of the phenols in organic versus aqueous media.[13]

The K_m effect was verified by calculation of the values of apparent K_m. All phenols tested have significantly higher apparent K_m's in organic solvents than in water. In some cases, this increase is over 3 orders of magnitude. For example, in aqueous buffer, the apparent K_m's of *p*-methoxyphenol, *p*-cresol, *p*-ethylphenol, and *p*-propylphenol are 0.63, 0.70, 0.25, and 0.15 mM, respectively. In butyl acetate, however, the apparent K_m's were 28, 42, 110, and 250 mM for the same phenols, respectively. Furthermore, these differences become more pronounced as the phenolic substituent becomes more hydrophobic, as would be expected if substrate partitioning into the peroxidase's active site were disrupted by solvent hydrophobicity.

Taking advantage of the high solubilities of phenols in organic solvents, we investigated the solvent effect on catalytic activity, V_{max}. Inspection of the data for *p*-cresol oxidation in aqueous and organic media (Figure 2) reveals that catalytic activity is *stimulated* in several organic solvents. Hence, peroxidase retains its full catalytic power in a variety of nonaqueous solvents. Peroxidase does require added water for activity, however. No reaction is observed in water-miscible solvents in the absence of added water, and catalysis in water-immiscible solvents requires a small amount of water.[7]

Our findings demonstrate that peroxidase catalysis in organic solvents can occur with full inherent catalytic turnover. The major effect of the solvent is to cause a dramatic increase in apparent K_m values. Furthermore, peroxidase catalysis in organic media follows a linear free energy relationship between catalytic efficiency and substrate and solvent hydrophobicities. This finding, if general (currently under investigation with other enzymes), can be used to develop rational mathematical descriptions of enzymatic catalysis in nonaqueous media in order to optimize enzyme function.

(11) We speculate that the dioxane anomaly is due to a conformational change in the enzyme. While enzymes in water-immiscible solvents appear to retain their native structural integrity[1,2b,c,12] and are rigid, the high water content used in the dioxane solvent systems (5–30%, v/v) may have allowed the peroxidase to become more mobile than in a less hydrated, water-immiscible solvent and thereby adopt an altered conformation.

(12) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A* **1985**, *82*, 3192–3196. Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194–3201. Clark, D. S.; Creagh, L.; Skerker, P.; Guinn, M.; Prausnitz, J.; Blanch, H. In *Biocatalysis and Biomimetics*; Burrington, J. D., Clark, D. S., Eds.; ACS Symposium Series 392; American Chemical Society: Washington, DC, 1989. Kanerva, L. T.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 6864–6865.

(13) Similar increases in apparent K_m have been observed for trypsin catalysis in dioxane–water mixtures (Douzou, P.; Balny, C. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2297–2300).

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A Novel Coordination Mode for Oxygen: Preparation and Properties of $(NBu^n_4)_2[V_4O(edt)_2Cl_8]$ Containing a Square-Planar Oxide Bridge

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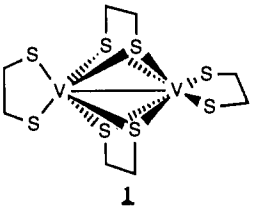
We are currently engaged in a program directed toward the development of V/S chemistry. In earlier reports we described several V/S complexes encompassing nuclearities 1–4 and oxidation levels III–V, including mixed valency.[2-8] Among these is the dinuclear V^{III} complex $(PPh_4)_2[V_2(edt)_4]$ (**1**; edt = eth-



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ane-1,2-dithiolate)[6,8-10] whose diamagnetism and V···V separation (ca. 2.6 Å) suggest a rare example of V^{III} metal–metal bonding. This complex also possesses an unusual metal coordination geometry and a quadruply bridged core. The latter properties are probably the cause of the V···V separation being longer than expected for a d^2–d^2 double bond (ca. 2.4 Å).[11] In subsequent

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(11) Indeed EHT calculations suggest the metal–metal interaction is better described as a single bond. See ref 9b.

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