Xanthine Oxidase Reactivity in Reversed Micellar Systems: A Contribution to the Prediction of Enzymatic Activity in Organized Media

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Abstract: Kinetic parameters are reported for the oxidation of a range of substituted benzaldehydes, differing in their electronic and hydrophobic properties, as catalyzed by xanthine oxidase in different reversed micellar media. For uncharged substrates in water and in the three surfactant systems investigated (DTAB/hexanol/heptane/water, Triton X-100/hexanol/cyclohexane/water, and AOT/isooctane/water), the parameters k_{cat} and K_{MA} and the ratio k_{cat}/K_{MA} were correlated by linear free energy relationships (LFER's). k_{cat} was found to depend mostly on the substituent constant σ, while $1/K_{MA}$ could be correlated with the partitioning constant π. The proportionality constants to σ and π scaling the hydrophobic and electronic forces do not change significantly from one medium to the other, providing a strong argument for identical mechanisms and similar transition state structures in water and reversed micellar systems. The specificity ratio k_{cat}/K_{MA} is lowered in reversed micelles compared to water. Medium effects on solvation were found to be on the order of 5−10 kJ/mol, the same magnitude as substrate substituent effects. For charged substrates, the kinetic data fit a model allowing for partitioning of substrates between the two domains of water core and organic continuum. If the effective volume fraction available to charged substrates and products was restricted to be that offered by the water core, kinetic constants in water and in reversed micellar systems were found to match closely. It is concluded that reactivity in microheterogeneous systems such as reversed micelles is as accessible to analysis as reactivity in aqueous systems.

1. Introduction

In recent years, the use of enzymes as catalysts for organic synthesis reactions has proliferated.[1] A good part of organic synthesis is carried out in organic solvents, for reasons of substrate solubility (e.g. of steroids or long-chain acids, aldehydes, or alcohols), and favorable reaction and partitioning equilibria, as in the case of peptide or ester bond formation.[2] Correspondingly, several methods have been developed to utilize enzymes in organic solvents, such as use of water-miscible solvents,[3] suspension of enzymes in water-immiscible solvents,[4] immobilization on hydrophilic supports,[5] attachment of stabilizing groups to the enzyme surface,[6] and encapsulation in reversed micelles.[7] This last method is of particular interest in this paper as many enzymes have been found to remain active when solubilized in reversed micelles, which are water droplets of nanometer size surrounded by a surfactant monolayer in an organic solvent, as illustrated schematically in Figure 1.

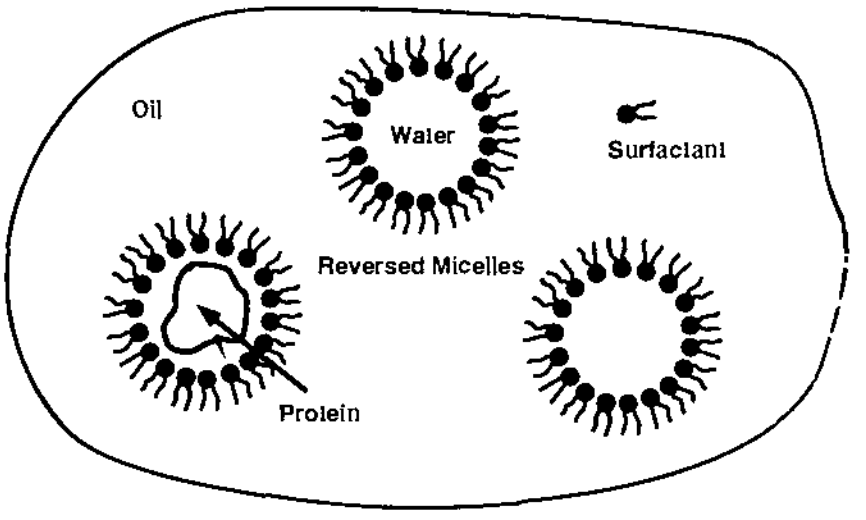

Figure 1. Schematic of an enzyme-containing reversed micellar system.

There are a number of advantages to exploiting enzymes in reversed micellar media. The solubility limitations of hydrophobic substrates are overcome because the capacity limit of the mostly organic medium for such substrates is much higher than that of water and the mass transfer between water droplets and organic continuum is fast, owing to the large surface area per unit volume and short distances for transfer between continuum and water pool. Moreover, while enzymes often aggregate in suspensions with water-immiscible organic solvents, they are molecularly dispersed in reversed micelles, the

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(1) (a) Whitesides, G. M.; Wong, C.-H. *Angew. Chem., Ind. Ed. Engl.* **1985**, *24*, 617−38; *Angew. Chem.* **1985**, *97*, 617−38. (b) Whitesides, G. M.; Wong, C.-H. *Aldrichimica Acta* **1983**, *16*, 27−34. (c) Wong, C.-H. *CHEMTRACTS Org. Chem.* **1990**, *3*, 91−111. (d) Jones, J. B. *Tetrahedron* **1986**, *42*, 3351−3403.

(2) (a) Jakubke, H.-D.; Kuhl, P.; Könnecke, A. *Angew. Chem. Int. Ed. Eng.* **1985**, *24*, 85−93. (b) Semenov, A. N.; Berezin, I. V.; Martinek, K. *Biotechnol. Bioeng.* **1981**, *23*, 355−360. (c) Martinek, K.; Semenov, A. N. *Biochim. Biophys. Acta* **1981**, *658*, 90−101. (d) Yokozeki, K.; Yamanaka, S.; Takinami, K.; Hirose, Y.; Tanaka, A.; Sonomoto, K.; Fukui, S. *Appl. Microbiol. Biotechnol.* **1982**, *14*, 1−5. (e) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192−3196.

(3) Dordick, J. A. *Enzyme Microb. Technol.* **1989**, *11*, 194−211.

(4) (a) Klibanov, A. M. *CHEMTECH* **1986**, *16*, 354−9. (b) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141−4. (c) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114−20.

(5) (a) *Immobilized Enzymes*; Mosbach, K., Ed. In *Meth. Enzymol.* **1976**, *44*. (b) Sharma, B. P.; Bailey, L. F.; Messing, R. A. *Angew. Chem.* **1982**, *94*, 836−52; *Angew. Chem., Ind. Ed. Engl.* **1982**, *21*, 837−54.

(6) Takahashi, K.; Nishimura, H.; Yoshimoto, T.; Saito, Y.; Inada, Y. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 261−5.

(7) (a) Martinek, K.; Levashov, A. V.; Klyachko, N. V.; Pantin, V. I.; Berezin, I. V. *Biochim. Biophys. Acta* **1981**, *657*, 277−94. (b) Luisi, P. L. *Angew. Chem.* **1985**, *97*, 449−61; *Angew. Chem., Ind. Ed. Engl.* **1985**, *24*, 439−50. (c) Shield, J. W.; Ferguson, H. D.; Bommarius, A. S.; Hatton, T. A. *Ind. Eng. Chem. Fundam.* **1986**, *25*, 603−12.

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maximum occupancy of enzyme per micellar core typically being equal to one (for an exception, see ref 8).[8]

This paper deals with the quantification and comparison of enzymatic reactivity in several reversed micellar systems and in water. For reasons outlined below, the oxidation of aromatic aldehydes by xanthine oxidase was selected as a model enzymatic reaction. The reaction system was probed in water and three surfactant systems with interfaces of different polarity: the cationic surfactant DTAB (dodecyltrimethylammonium bromide), the anionic surfactant AOT (Aerosol OT, 2-ethylhexylsulfosuccinate), and the nonionic surfactant Triton X-100 [(*p*-isooctylphenyl)polyoxyethylene glycol ether]. AOT is very well studied and forms reversed micelles with water and an organic solvent in a three-component system.[9] Both the AOT and the DTAB system, recently characterized,[10] were used to study the influence of surfactant charge on the enzymatic reaction and to compare with the system based on the nonionic Triton X.[11] Both AOT and Triton X-100 are biocompatible surfactants, the latter often used as a membrane replacement in biological studies.

Kinetic constants were determined in *each* of the four media for a series of substituted benzaldehyde substrates with varying electronic and hydrophobic properties. The kinetic constants for uncharged substrates are interpreted in terms of observable or predictable parameters derived from linear free energy relationships (LFERs), which include (i) electronic forces, correlating data with the Hammett equation;[12] (ii) hydrophobicity (Hansch approach[13-17]) based on the substrate partitioning constant π;[14] (iii) dispersion forces,[18] correlating data with molar refractivity R_m, often obtained from the Lorentz–Lorenz equation, $R_m = (n^2 - 1)/(n^2 + 2)(MW/d)$; and (iv) a steric influence, expressed by the molecular volume MW/*d*, which is often used instead of R_m. While LFER's in enzymology are well-known,[19] this approach has not yet been used for reversed micellar systems. Depending on the relative importance of reaction and binding phenomena with respect to change of substrate, either k_{cat} (the catalytic constant), K_{MA} (the Michaelis constant), or the ratio k_{cat}/K_{MA} gives the best fit to LFER's. Most of the studies on k_{cat} reported for other enzymes have been consistent with a Hammett-type correlation,[20,21,22a] although exceptions have been noted.[23] The K_{MA} and K_i (inhibition constant) data for several enzymes have been interpreted by Hansch's $\sigma-\pi-R_m$ analysis,[22] and it is often found that K_{MA} correlates with π. Except for the K_i values for xanthines,[22a] no data exist for xanthine oxidase. Medium effects of both water-immiscible[24] and water-miscible[25] solvents on enzyme activity have been analyzed by such an approach. Table 1 lists all measured or calculated substituent parameters that are relevant for the LFER correlations in this study.

Table 1. LFER Parameters for Para-Substituted Benzaldehydes

substituent	MW	σ	π	R_m	MW/*d*
p-NO_2	151.12	0.78	−0.28	38.61	s[a]
p-CN	131.13	0.63	−0.57	37.58	s
p-CF_3	174.12	0.54	0.88	36.27	136.56
p-$OCOCH_3$	164.16	0.52	−0.64	43.95	140.55
p-CHO	134.14	0.45	−0.65	38.13	s
p-COOH	150.14	0.27	−0.32	38.18	s
p-Cl	140.57	0.23	0.71	37.28	117.53
p-COO^-	149.14	0.23	−4.36	37.30	s
p-$CH(OEt)_2$	208.11	0.23	−0.19	59.05	198.77
H	106.13	0	0	32.28	101.90
p-CH_3	120.16	−0.17	0.56	37.12	117.92
p-OCH_3	136.16	−0.27	−0.02	40.09	121.68
p-OH	122.13	−0.36	−0.67	34.10	s
p-$N(CH_3)_2$	149.19	−0.83	0.18	46.80	s

[a] s = solid. The correlation coefficient r^2 is high (0.865) between R_m and MW/*d* but low (≪0.5) for any other pair of molecular constants.

2. Model Enzyme System: Xanthine Oxidase–Catalase

Xanthine oxidase[26] catalyzes hydroxylation reactions of the type

$$RH + H_2O + acc_{ox} \rightarrow ROH + acc_{red}H_2 \quad (1)$$

at an electrophilic, sp^2-hybridized carbon atom in the substrate RH. For instance, aldehydes are oxidized to carboxylic acids according to the reaction[27,28]

$$RCHO + H_2O + O_2 \rightarrow RCOOH + H_2O_2 \quad (2)$$

Since the byproduct H_2O_2 deactivates the enzyme,[29] reaction 2 is coupled with the decomposition of hydrogen peroxide by catalase

$$H_2O_2 \rightarrow H_2O + {}^1/_2O_2 \quad (3)$$

so that the net reaction reads

(8) Brochette, P.; Petit, C.; Pileni, M. P. *J. Phys. Chem.* **1988**, *92*, 3505–15.

(9) (a) Zulauf, M.; Eicke, H. *J. Phys. Chem.* **1979**, *83*, 480–486. (b) *Surfactants in Solution*, Mittal, K. L., Lindman, B., Eds.; Plenum Press: New York, 1984; see especially contributions pp 1533–1549, 1745–1758, and 1759–1779.

(10) Petit, C.; Bommarius, A. S.; Pileni, M. P.; Hatton, T. A. *J. Phys. Chem.* **1992**, *96*, 4653–58.

(11) (a) Kumar, C.; Balasubramanian, D. *J. Colloid Interface Sci.* **1979**, *69*, 271–9. (b) Kumar, C.; Balasubramanian, D. *J. Colloid Interface Sci.* **1980**, *74*, 64–70. (c) Kumar, C.; Balasubramanian, D. *J. Phys. Chem.* **1980**, *84*, 1895–1899.

(12) Hammett, L. P. *Chem. Rev.* **1935**, *17*, 125.

(13) (a) Meyer, H. *Arch. Exp. Pathol. Pharmakol.* **1899**, *42*, 110. (b) Overton, E. *Studien über die Narkose*; Fischer: Jena, 1901.

(14) (a) Hansch, C.; Fujita, T. *J. Am. Chem. Soc.* **1964**, *86*, 1616–26. (b) Hansch, C.; Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; John Wiley & Sons: New York, 1977.

(15) Fujita, T.; Iwasa, J.; Hansch, C. *J. Am. Chem. Soc.* **1964**, *86*, 5175–80.

(16) Rekker, R. F. *The Hydrophobic Fragmental Constant*; Elsevier: Amsterdam, 1977.

(17) (a) Hilhorst, R.; Spruijt, R.; Laane, C.; Veeger, C. *Eur. J. Biochem.* **1984**, *144*, 459–66. (b) Hilhorst, R. *Enzymatic Reactions in Reversed Micelles*. Ph.D. Thesis, University of Wageningen The Netherlands, 1984.

(18) Pauling, L.; Pressman, D. *J. Am. Chem. Soc.* **1945**, *67*, 1003.

(19) (a) Jencks, W. P. *Structure-Reactivity Correlations and General Acid-Base Catalysis in Enzymic Transacylation Reactions*; Cold Spring Harbor Symp. Quant. Biol. **1971**, *36*, 1–11. (b) Kirsch, J. F. *Linear Free Energy Relationships in Enzymology*. In *Advances in Linear Free Energy Relationships*; Chapman,N. B., ShorterJ., Eds.; Plenum Press: London, 1972. (c) Fersht, A. *Enzyme Structure and Mechanism*; 2nd ed.; Freeman & Company: London, 1985; p 147.

(20) Bender, M. L.; Nakamura, K. *J. Am. Chem. Soc.* **1962**, *84*, 2577–82.

(21) Lowe, G.; Yuthavong, Y. *Biochem. J.* **1971**, *124*, 107–15.

(22) (a) Silipo, C.; Hansch, C. *J. Med. Chem.* **1976**, *19*, 62–71. (b) Yoshimoto, M.; Hansch, C. *J. Org. Chem.* **1976**, *41*, 2269–73. (c) Hansch, C.; Grieco, C. Silipo, C. Vittoria, A. *J. Med. Chem.* **1977**, *20*, 1420–35. (d) Hansch, C.; Smith, R. N.; Rockoff, A.; Calef, D. F.; Jow, P. Y. C.; Fukunaga, J. Y. *Arch. Biochem. Biophys.* **1977**, *183*, 383–92.

(23) (a) Klinman, J. P. *J. Biol. Chem.* **1972**, *247*, 7977–87. (b) Klinman, J. P. *Biochemistry* **1976**, *15*, 2018–26.

(24) Kanerva, L. T.; Klibanov, A. M. *J. Am. Chem. Soc.* **1990**, *111*, 6864–5.

(25) Ryu, K.; Dordick, J. A. *J. Am. Chem. Soc.* **1990**, *111*, 8026–7.

(26) (a) Bray, R. C. Xanthine Oxidase. In *The Enzymes*, 3rd ed.; Boyer, P. D., et al., Eds.; Academic Press: New York, 1975; Vol. 12, pp 368–9. (b) Coughlan, M. P. *Molybdenum and Molybdenum-Containing Enzymes*; Coughlan, M. P., Ed.; Pergamon Press: Oxford, 1980; Chapter 4.

(27) (a) Booth, V. H. *Biochem. J.* **1938**, *32*, 494–502. (b) Booth, V. H. *Biochem. J.* **1938**, *32*, 503–7.

(28) Pelsy, G.; Klibanov, A. M. *Biochim. Biophys. Acta* **1983**, *742*, 352–7.

(29) Altomare, R. E.; Kohler, J.; Greenfield, P. F.; Kittrell, J. R. *Biotech. Bioeng.* **1974**, *16*, 1659–73.

$$RCHO + {}^1/_2O_2 \rightarrow RCOOH \quad (4)$$

The oxidation of benzaldehydes with xanthine oxidase was selected as a model of enzymatic reactivity in reversed micellar systems for the following reasons:

(i) The *broad substrate specificity* of xanthine oxidase, ascribed to loose steric requirements around the active sites,[30] permits systematic variation of substrate properties to test for influences of the reversed micellar environment. In the reductive half-reaction, the natural electron acceptor acc_{ox} in eq 1, O_2 (with H_2O_2 as $acc_{red}H_2$), can be replaced by redox dyes such as 2,6-dichloroindophenol (DCIP).[31] In the oxidative part, in addition to aldehydes, xanthine oxidase catalyzes the oxidation of many purines, such as the natural substrate hypoxanthine, or pyridine dinucleotides.[32]

(ii) Xanthine oxidase is known to follow a two-step transfer or *ping-pong mechanism.*[26a] Since release of acid product in the oxidative reaction is the rate-determining step,[33] the reductive half-reaction can be assumed to be fast in comparison. The cofactors are tightly bound to the enzyme so that *no externally added cofactors are required.*

(iii) In vivo, xanthine oxidase is active in *both soluble and membrane-associated form* (lipid globule membrane[34] in bovine milk), so with this incipient hydrophobicity, the enzyme can be expected to be solubilized into and remain active in most reversed micellar systems. In contrast to some enzymes in previous studies in reversed micelles,[35] xanthine oxidase is sufficiently stable over the time scale of the experiment so as not to affect intrinsic activity as the investigated parameter.

The simple steric demands of the active site and the wide choice of substrate structure in the xanthine oxidase system are of value in the investigation of the intrinsic biocatalyst influence on reactivity in aqueous medium, while the influence of substituent properties on kinetic parameters facilitates the characterization and comparison of medium effects in different reversed micellar media.

3. Materials and Methods

Surfactants. AOT from Sigma (St. Louis, MO) and Triton X-100 from Serva (Heidelberg, Germany) were used as received. DTAB from Eastman Kodak (Rochester, NY) was recrystallized with ether/methanol.

Solvents. Cyclohexane and *n*-heptane (reagent grade) and isooctane (spectrophotometric grade) were from Mallinckrodt (St. Louis, MO); *n*-octane was gold label from Aldrich (Milwaukee, WI). Hexanol was purum grade from Fluka (Buchs, Switzerland). All were used as received. Water was deionized and distilled.

Enzymes. Xanthine oxidase from bovine milk was obtained either from Boehringer Mannheim 0.8 unit/mg (Indianapolis, IN) or from Serva 0.5 unit/mg (New York, NY) as a 2.3 M $(NH_4)_2(SO_4)$ suspension. Catalase (80% protein, activity 5000 units/mg) was from Sigma.

Substrates and Products. Benzaldehydes and benzoic acids were obtained at the highest grade commercially available and used as received if they were of purity higher than 99%. Otherwise, the liquids were distilled at reduced argon pressure over a 15 cm Vigreux column and kept sealed from light at 4 °C under argon; the solids were recrystallized from ether/methanol and kept desiccated.

Dialysis of Enyzmes. A 0.5−2 mL sample of xanthine oxidase suspension was dialyzed within a 10 000 MWCO polycarbonate membrane from Spectrapor (Waltham, MA) in a stirred 750 mL solution against 0.1 M phosphate buffer of pH 7.5 at 4 °C for 24−48 h and subsequently sterile-filtered (0.22 μm). The filtered solution was stored at 4 °C for up to 4 weeks with no significant drop in xanthine oxidase activity. Before use, the solution was diluted with sterile-filtered phosphate buffer solution to the desired enzyme concentration level, usually around 3 mg/mL.

Preparation of Catalase Solution. A 1−2 mg/mL catalase solution was prepared as described for the xanthine oxidase solution, mainly to remove the nonproteinaceous glycosidic impurities (20 wt % of the preparation). It was diluted to 0.02 mg/mL before use.

Sample Preparation. Benzaldehydes were dissolved in 0.1 M phosphate buffer and sonicated up to 10 min in a Brandon ultrasonicator. If necessary, up to 20 vol % ethanol was added to improve solubility. The final alcohol content in the aqueous solution was lower than 10%. It was found that xanthine oxidase can tolerate up to 25% alcohol in aqueous solution without decreased activity. In the experiments with reversed micelles, aldehydes were added to the organic surfactant solution.

Experiments in reversed micellar systems were carried out under argon to exclude any influence of oxygen on the results. A test tube covered with parafilm containing an organic solution of surfactant, buffer, and catalase but no xanthine oxidase was blanketed with argon for 5 min. Enzyme solution was added from an argon-sparged solution of xanthine oxidase via a syringe to the test tube, which was stirred on a vortex mixer for 10 s. Subsequently, the micellar solution was poured rapidly into a disposable cuvette in an argon stream and tightly sealed with a snugly fitting rectangular plastic cap.

Kinetic Runs with Benzaldehydes. Reaction progress was monitored on a Perkin-Elmer Lambda 3 UV/vis spectrophotometer either at the aldehyde wavelength or at the wavelength of DCIP, i.e., 535 nm in the AOT/isooctane/water or 620 nm in the DTAB and Triton systems. The cuvettes were thermostated at 25 ± 0.1 °C. All enzymatic activities were standardized against 0.5 mM *p*-hydroxybenzaldehyde in aqueous solution (standard activity 0.8 unit). A typical cuvette volume in aqueous solution consisted of 2.85 mL of aqueous buffer, 150 μL of 10 mM aldehyde solution in aqueous buffer, and 20 μL each of 3 mg/mL xanthine oxidase and 0.02 mg/mL catalase solution. A typical cuvette volume of 3 mL of reversed micellar solution was made from 2.8 mL of organic surfactant solution, 100 μL of organic surfactant solution containing substrates, 100 μL of aqueous buffer, and 20 μL each of catalase and xanthine oxidase solution. The aqueous components were added in 20 μL increments while the reversed micellar solution was stirred on a Vortex mixer for about 10 s. The extinction curves were linear for at least a few minutes while conversion stayed low. The extinction coefficients were measured in the same medium at the same water−surfactant ratios as in the actual runs. The typical enzyme concentrations were 87.5 nM xanthine oxidase in both water and in reversed micellar solution.

4. Results

4.1. Reactivity and Selectivity in Aqueous Solution. Since the easily-monitored DCIP (λ_{max} = 600−620 nm) is a severe inhibitor of xanthine oxidase in water (vide infra), the benzaldehyde substrates were reacted in air-saturated 0.1 M phosphate buffer at pH 7.5 (I = 0.2 M; the reductive half-reaction is not rate-limiting[33]). k_{cat} and K_{MA} for the aldehydes were determined from the kinetic equation for the reaction rate for a two-site ping-pong mechanism,[36]

(30) Skribo, E. B. *Biochemistry* **1986**, *25*, 4189−4194.
(31) Fridovich, I. *J. Biol. Chem.* **1966**, *241*, 3624−9.
(32) (a) De Meesters, J. Synthesis and Oxidation by Xanthine Oxidase from Arthrobacter, M-4 of 6-Aryl-4(3H)-pteridinones and related Compounds. Ph.D. Thesis, University of Wageningen, Wageningen, The Netherlands, 1987. (b) Bergmann, F.; Levene, L. *Biochim. Biophys. Acta* **1976**, *429*, 672−88. (c) Bergmann, F.; Levene, L.; Tamir, I.; Rahat, M. *Biochim. Biophys. Acta* **1977**, *480*, 21−38.
(33) Olson, J. S.; Ballou, D. P.; Palmer, G.; Massey, V. *J. Biol. Chem.* **1974**, *249*, 4363−82.
(34) (a) Briley, M. S.; Eisenthal, R. *Biochem. J.* **1974**, *143*, 149−57. (b) Keenan, T. W.; Heid, H. W.; Stadler, J.; Jarasch, E.-D.; Franke, W. W. *Eur. J. Cell Biol.* **1982**, *26*, 270−6. (c) Levashov, A. V.; Kabanov, A. V.; Khmel'nitskii, Yu. L.; Berezin, I. V.; Martinek, K. translated from *Dokl. Akad. Nauk SSSR* **1984**, *278*, 246−8. (d) Levashov, A. V.; Kabanov, A. V.; Nametkin, S. N.; Martinek, K.; Berezin, I. V. translated from *Dokl. Akad. Nauk SSSR* **1985**, *284*, 755−8. (e) Torchilin, V. P.; Omel'yanenko, V. G.; Klibanov, A. L.; Mikhailov, A. I.; Gol'danskii, V. I.; Smirnov, V. N. *Biochim. Biophys. Acta* **1980**, *602*, 511−21. (f) Goldmacher, V. S. *Biochem. Pharmacol.* **1983**, *32*, 1207−10. (g) Torchilin, V. P.; Klibanov, A. L. *Enzyme Microb. Technol.* **1981**, *3*, 297−304.
(35) Fletcher, P. D. I.; Rees, G. D.; Robinson, B. H.; Freedman, R. B. *Biochim. Biophys. Acta* **1985**, *832*, 204−14.

Table 2. Kinetic Parameters of Para-Substituted Benzaldehyde Oxidation by Xanthine Oxidase in Aqueous Solution

substituent	k_{cat} (s^{-1})	K_{MA} (mM)	k_{cat}/K_{MA} $(M\ s)^{-1}$	$\log_{10}(k_{cat}/K_{MA})$	K_{iP} (mM)	K_{MA}/K_{iP}
p-$N(CH_3)_2$	1.26	0.55	2290	3.36	–	–
p-OCH_3	4.42	0.20	20320	4.31	0.75	0.29
p-CH_3	4.14	1.17	3510	3.55	4.2	0.28
H	1.22	0.96	1270	3.10	0.83	1.16
p-Cl	13.33	1.00	13260	4.12	3.00	0.33
p-$OCOCH_3$	13.89	0.07	191900	5.28	0.83	0.08
p-CF_3	28.88	0.85	33940	4.53	≫4.20	≪0.20
p-NO_2	32.63	0.18	177300	5.25	–	–
p-OH	11.60	0.08	145000	5.16	1.79	0.04
m-OH	1.23	0.11	10480	4.02	–	–
o-OH	1.35	0.28	4800	3.68	–	–
p-COO^- [a]						
p-CHO[a]						
p-$CH(OEt)_2$[a]						
p-CN[a]						

[a] Severe substrate inhibition in aqueous solution. $K_{iP} \ll 0.1$ mM.

$$\frac{v}{v_{max}} = \frac{[A]}{K_{MA}\left(1 + \frac{K_{iO_2}[P]}{[O_2]K_{iP}} + \frac{[P]}{K_{iP}}\right) + [A]\left(1 + \frac{K_{MO_2}}{[O_2]}\right)} \quad (5)$$

(where A and P denote aldehyde and acid product, respectively, where $v_{max} = k_{cat}[E]$) simplified using three assumptions concerning the reductive half-reaction with oxygen: (i) $[O_2]$ was at aqueous saturation level (8 mM), (ii) K_{MO_2} was the same (1.5 μmol) as determined by Fridovich[37] using xanthine as reducing agent, and (iii) inhibition by oxygen was insignificant. Then, the terms containing $K_{MO_2}/[O_2]$ and $K_{iO_2}/[O_2]$ can be neglected, and we obtain

$$\frac{v}{k_{cat}[E]} = \frac{[A]}{K_{MA}\left(1 + \frac{[P]}{K_{iP}}\right) + [A]} \quad (6)$$

Since k_{cat} and K_{MA} are determined at low conversion, $[P]/K_{iP}$ can be neglected; K_{iP} is determined from the Michaelis constant observed when [P] is varied by adding product acid, according to the equation

$$K_{MA,obs} = K_{MA}(1 + [P]/K_{iP}) \quad (7)$$

Results are listed in Table 2.

Correlation of k_{cat}. To find the predominant influence on the kinetic parameters, the results were plotted against σ, π, R_m, and MW/d. The data for several *uncharged* substrates are well-correlated by the substituent constant σ (Figure 2a, $r^2 = 0.983$, $n = 7$) via the equation

$$\log k_{cat} = 0.86 + 0.93\sigma \quad (8)$$

The slope, the reaction constant ϱ, is determined as 0.93 ± 0.06; the intercept, $k_{cat,0}$, is the value for benzaldehyde ($\sigma = 0$, k_{cat} in s^{-1}). k_{cat} depends only on σ, not at all on π ($r^2 = 0.001$), R_m ($r^2 = 0.002$), or MW/d ($r^2 = 0.175$). Since the substituents were picked so that the values for the molecular parameters are linearly independent (see remark in Table 1), correlations of kinetic constants with molecular parameters are based on the influence of the corresponding forces on the reaction and not on the choice of the substituent set. The data points of substrates with substituents CN, CHO, COOH (or COO^-), and $CH(OEt)_2$ were excluded from the correlation in eq 8 because these substrates severely inhibit xanthine oxidase ($K_{iP} \ll 100$ μM). Likewise, charged substrates for which the Hammett equation is not valid were not considered.

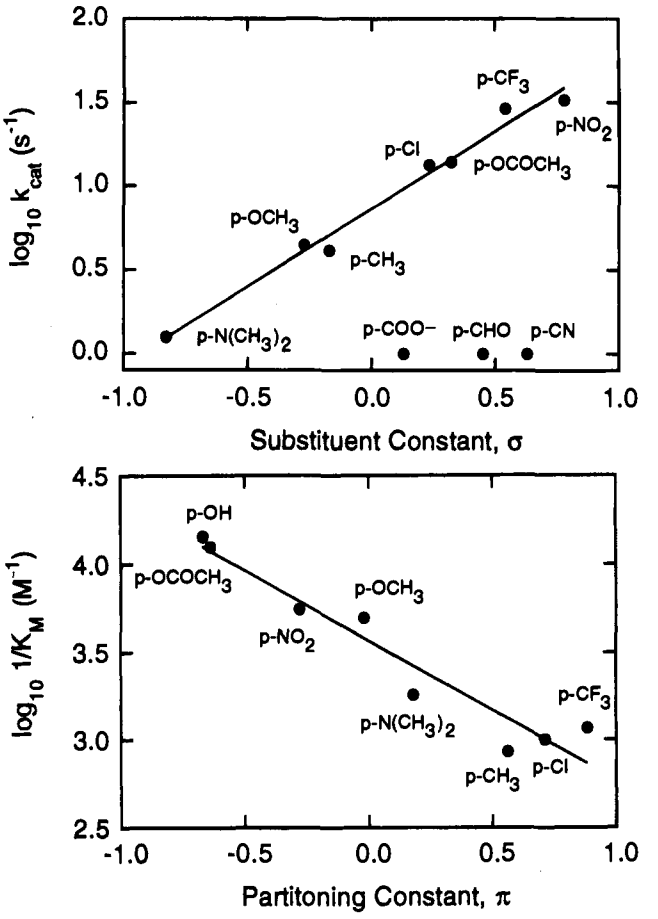

Figure 2. (a) Catalytic constant k_{cat} versus substituent constant σ of benzaldehydes catalyzed by xanthine oxidase in water and (b) correlation of Michaelis constant K_{MA} and partitioning constant π of benzaldehyde oxidation in aqueous solution (0.1 M phosphate buffer, pH 7.5).

Correlation of K_M. The log $1/K_{MA}$ data of uncharged substrates correlate well with the partitioning constant π, as expected[22] (Figure 2b; $r^2 = 0.929$, $n = 7$: for other molecular parameters, $r^2 < 0.2$):

$$\log 1/K_{MA} = 3.56 - 0.79\pi \quad (9)$$

The intercept, log $1/K_{MA,0}$, again is the point for benzaldehyde, ($\pi = 0$, $K_{MA,0}$ in M).

Correlation of k_{cat}/K_M. Using eqs 8 and 9, a regression of the experimental data for k_{cat}/K_{MA} demonstrates that log k_{cat}/K_{MA} depends on both σ and π ($r^2 = 0.930$, $n = 7$):

$$\log(k_{cat}/K_{MA}) = 4.07 + 0.93\sigma - 0.79\pi \quad (10)$$

The inhibition constants K_{iP} and inhibition ratios K_{MA}/K_{iP} tend to decrease with increasing substrate hydrophilicity but no good correlation could be obtained between K_{iP} and substituent parameters σ or π. The inhibition ratios are much less than unity, so inhibition is not a major influence on the reaction even at high degrees of conversion.[38]

4.2. Activity in Reversed Micelles with DCIP as an Electron Acceptor. Reactivity and Partitioning of DCIP in Reversed Micellar Systems. In water, DCIP is a potent inhibitor (K_{iDCIP} of $(1.0-2.8) \times 10^{-6}$ M[39]) as well as electron acceptor of xanthine oxidase. However, in reversed micellar

(36) Segel, I. H. *Enzyme Kinetics*; Wiley-Interscience: New York, 1975; Section K, Chapter 9, p 626.
(37) Fridovich, I. *J. Biol. Chem.* **1970**, *245*, 4053–4057.
(38) Lee, L. G.; Whitesides, G. M. *J. Org. Chem.* **1986**, *51*, 25–36.

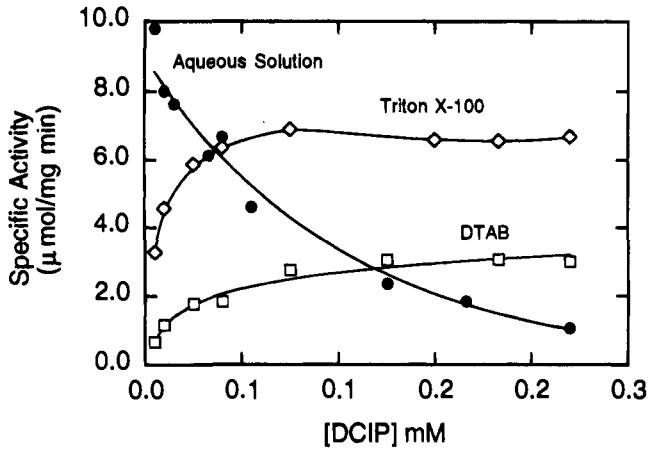

Figure 3. Behavior of DCIP in aqueous and reversed micellar media ([*p*-hydroxyaldehyde]$_{ov}$ = 0.44 mM, pH 7.5, 0.1 M phosphate buffer, 0.2 M Triton X-100/cyclohexane/10% hexanol, or 0.2 M DTAB/*n*-octane/11% hexanol, w_0 = 11.2).

systems the dye displayed normal saturation behavior (Figure 3): at 0.44 mM aldehyde, $K_{MA,ov}$ equals 1.6×10^{-5} (Triton X), 2.2×10^{-5} (DTAB), and 1.12×10^{-4} M (AOT; not shown). No inhibitory effect of DCIP was observed in any surfactant system, suggesting a diminished concentration in the enzyme's active site relative to that in water. Since DCIP is a substrate, however, it cannot partition far away from the enzyme.

The distribution of the DCIP molecules between the continuum, interfacial, and pool domains can be probed by solubilization and solvatochromic studies. DCIP is found to be insoluble in isooctane but to be solubilized in proportion to AOT concentration in reversed micellar solutions of this surfactant. At pH 2−12 and w_0 values from 0 to 50, λ_{max} of DCIP absorption is 535 nm, close to λ_{max} in aqueous solution below the pK_a of DCIP at 5.9.[40] For DTAB and Triton X solutions, λ_{max} of DCIP shifts from 543 nm at $w_0 = 0$ to 600−620 nm at $w_0 > 0$, the λ_{max} value for ionized DCIP in water at pH values higher than the pK_a.

The apparent lack of ionization of DCIP molecules in the AOT system and the higher $K_{MA,ov}$ for DCIP in AOT as compared to DTAB or Triton suggest that DCIP molecules in AOT are located exclusively in the tail region of the interface with no contact with the water pool at any molar water to surfactant ratio (w_0) value,[41] thus reducing the number of molecules available for reaction. In DTAB and Triton, the dye is located in the head group region of the interfaces in predominantly ionized form with contact with the pool domain. The predominant location of DCIP molecules in the tail region of the surfactant with no contact with head group layer or water pool does not seem to present an obstacle to binding to xanthine oxidase molecules and subsequent reaction.

Reactivity of Benzaldehydes in the Reversed Micellar Systems. With the kinetic data for different aldehydes at the solubility limits of DCIP (approximately 3−4$K_{MA,ov}$; for AOT it is 1.4×10^{-4} M, and for DTAB and Triton, 7.8×10^{-4} M; the kinetic data are in Tables 3 for AOT, 4a for DTAB, and 4b for Triton), log(k_{cat}/K_{MA}) could be correlated by the equation

$$\log(k_{cat}/K_{MA}) = \zeta + \varrho\sigma + \kappa\pi \qquad (11)$$

The coefficients are tabulated in Table 5 for each reaction medium. The values reported for water are those given in eqs 8−10. In the DTAB and Triton systems, the catalytic constants for uncharged substrates obey the Hammett law (Figure 5) with $\varrho = 0.91$ in Triton X and $\varrho = 0.79$ in DTAB, as compared to $\varrho = 0.93$ for the oxygen acceptor in water. Likewise, log(1/K_{MA}) follows the Hansch hydrophobicity relation ($\kappa = -0.88$ in DTAB and −0.56 in Triton). The individual contributions to the intercept ζ from the log k_{cat} vs σ (analogous to eq 8) and the log(1/K_{MA}) vs π equations (analogous to eq 9) are 0.25 and 3.34 for DTAB and 0.26 and 3.36 for Triton, reflecting remarkably similar results for both systems.

In contrast to the results obtained for the other three media, for the AOT system neither k_{cat} nor 1/K_{MA} individually

(39) (a) Fridovich, I. *J. Biol. Chem.* **1966**, *241*, 3624−9. (b) Gurtoo, H. L.; Johns, D. G. *J. Biol. Chem.* **1971**, *246*, 286−93.
(40) Armstrong, J. McD. *Biochim. Biophys. Acta* **1964**, *86*, 194−7.
(41) Leodidis, E. B.; Bommarius, A. S.; Hatton, T. A. *J. Phys. Chem.* **1991**, *95*, 5943−56.

Table 3. Kinetic Data for Aldehyde Oxidation in AOT/Isooctane/Water System[a]

substituent	k_{cat} (s^{-1})	$K_{MA,ov}$ (mM)	k_{cat}/K_{MA} $(M\ s)^{-1}$	$\log_{10}(k_{cat}/K_{MA})$	r^2
p-OH	2.10	0.13	15480	4.19	0.96
p-$OCOCH_3$	1.45	0.76	1890	3.28	0.97
p-CN	0.33	0.16	2110	0.33	0.98
p-NO_2	0.89	0.36	2500	3.40	0.94
p-$CH(OEt)_2$	0.52	0.34	1510	3.18	0.96
p-OCH_3	1.09	1.11	980	2.99	0.97
H	0.96	3.22	298	2.47	0.88
p-$N(CH_3)_2$	1.38	1.27	1090	3.04	0.96
p-CH_3	0.60	1.54	391	2.59	0.92
p-Cl	0.24	0.45	536	2.73	0.95
p-CF_3	0.44	1.39	322	2.51	0.92

[a] Experimental conditions: [DCIP]$_{ov}$ = 1.38×10^{-4} M, [E]$_{ov}$ = 8.87×10^{-8} M, w_0 = 10.4, 0.1 M PO_4 buffer, pH 7.5, cuvette volume = 3 mL; assay at 535 nm.

Table 4. Kinetic Constants in the Cationic and Nonionic Micellar Systems

substituent	k_{cat} (s^{-1})	$K_{MA,ov}$ (mM)	$k_{cat}/K_{MA,ov}$ $(M\ s)^{-1}$	$\log_{10}(k_{cat}/K_{MA,ov})$
		DTAB System		
p-NO_2	2.00	0.26	7620	3.88
p-Cl	1.28	1.86	688	2.84
p-CH_3	0.36	1.51	241	2.38
p-OCH_3	0.28	0.46	612	2.79
p-$N(CH_3)_2$	0.13	0.64	203	2.31
		Triton System		
p-NO_2	2.45	0.31	8043	3.91
p-Cl	1.27	1.05	1161	3.07
p-CH_3	0.35	0.92	386	2.59
p-OCH_3	0.26	0.42	618	2.79
p-$N(CH_3)_2$	0.10	0.56	182	2.26

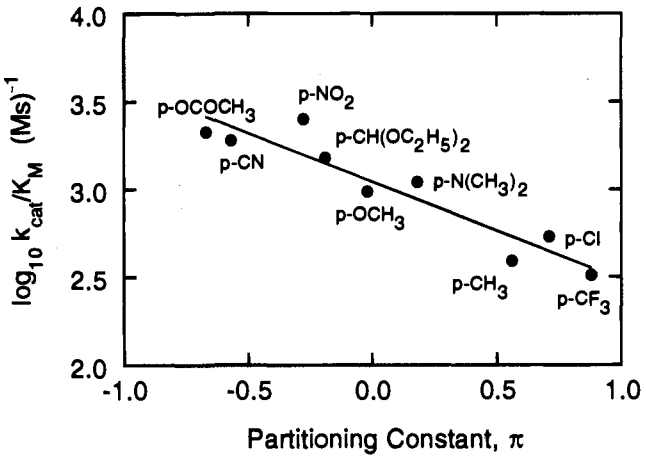

Figure 4. Correlation of k_{cat}/K_{MA} with π in AOT/isooctane/water (0.2 M AOT/isooctane; phosphate buffer, pH 7.5).

Table 5. $\zeta-\varrho-\kappa$ Matrix for k_{cat}/K_{MA} in Water and the Three Surfactant Systems Investigated[a]

system	no. of data points	ζ	ϱ	κ
water	7	4.07 ± 0.22 (0.93)	0.93 ± 0.06 (0.98)	−0.79 ± 0.09 (0.93)
AOT	10	3.05 ± 0.13 (0.96)	–	−0.56 ± 0.08 (0.55)
DTAB	5	3.17 ± 0.21 (0.97)	0.79 ± 0.11 (0.95)	−0.88 ± 0.11 (0.96)
Triton	5	3.14 ± 0.20 (0.98)	0.91 ± 0.09 (0.97)	−0.56 ± 0.11 (0.92)

[a] Numbers in parentheses are the correlation coefficients for the fitting of the data to the relevant LFERs.

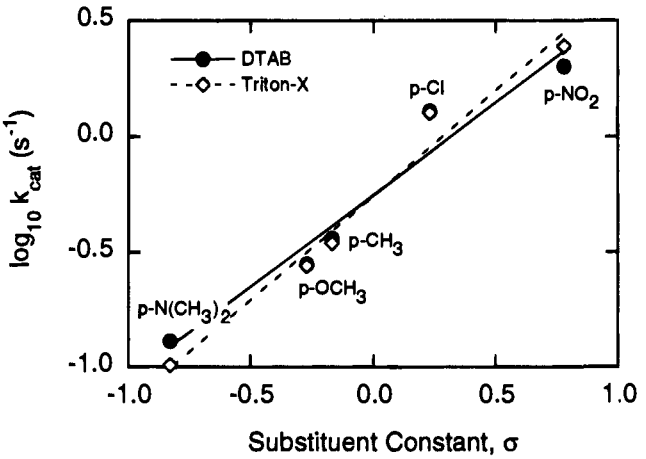

Figure 5. Hammett plot of enzymatic aldehyde oxidation in four-component reversed micelles (pH 7.5, 0.1 M phosphate buffer, 0.2 M Triton X-100/cyclohexane/10% hexanol, or 0.2 M DTAB/*n*-octane/11% hexanol, w_0 = 11.2).

Table 6. Specificity Constants of Hydroxybenzaldehyde in Aqueous and Four-Component Reversed Micellar Systems

	k_{cat}/K_{MA} (mM s)$^{-1}$		
system	ortho	meta	para
water	4.79	10.40	145
Triton	0.48	1.95	332
DTAB	<0.10	0.48	457
AOT	nd	nd	nd

correlated well with these molecular parameters, although the ratio k_{cat}/K_{MA} is well-described by the equation

$$\log(k_{cat}/K_{MA}) = 3.05 - 0.56\pi \qquad (12)$$

with $r^2 = 0.958$ and $n = 10$; see Figure 4. Remarkably, even *p*-cyano- and *p*-(diethoxymethyl)benzaldehyde, severe inhibitors in water, followed saturation kinetics in AOT/isooctane and do not act as inhibitors in this reaction medium.

4.3. Reactivity of Charged Substrates in Reversed Micelles. In aqueous solution, *p*-hydroxybenzaldehyde reacts more rapidly than the meta and ortho regioisomers and more rapidly than calculated from the Hammett equation for the OH group using eq 8 (Table 1). The *p*-OH group (pK_a 7.66) is partially ionized at the reaction pH of 7.5 whereas both the o-OH (pK_a 8.37) and the *m*-OH compounds (pK_a 8.98) are not significantly ionized. The effect of screening substrates based on the degree of charge should be vastly enhanced in reversed micellar systems with their distinct hydrophobic and aqueous domains, which allow for partitioning of substrates and products away from the enzyme, or concentrating them in the vicinity of the enzyme. In DTAB and Triton, *p*-hydroxybenzaldehyde showed marked activity at pH 7.5, in contrast to the ortho and meta compounds (k_{cat}/K_{MA} data in Table 6). Variation of pH between 6.5 and 9.5 (at pH > 9.5, the decline of xanthine oxidase activity is not substrate-specific) illustrates that *o*- and *m*-hydroxybenzaldehyde are also good substrates in the reversed micellar systems (Figure 6) at pH values > 7.7 or 8.3, respectively, corresponding to the

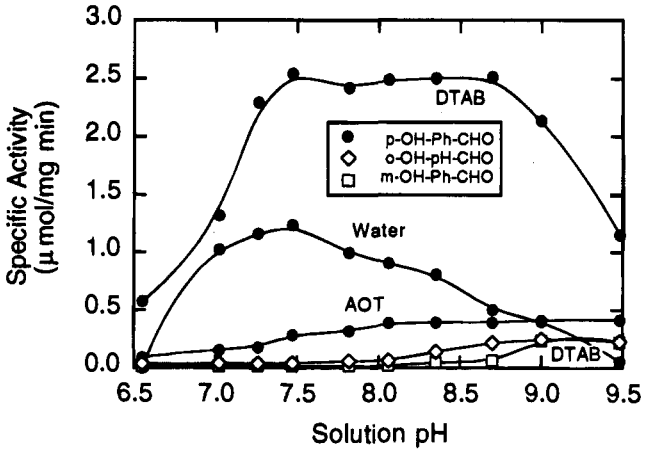

Figure 6. Reactivity−pH profiles for hydroxybenzaldehydes (0.2 M DTAB/*n*-octane/11% hexanol, w_0 = 11.2; 0.2 M AOT/isooctane, w_0 = 10.4).

onset of ionization. Apparently, xanthine oxidase favors negatively charged over uncharged substrates.

Kinetics and Inhibition. In aqueous solution, product inhibition by benzoic acid is insignificant (K_{MA}/K_{iP} < 1; Table 2). The concentration of the product in the vicinity of the enzyme caused by a strong partitioning of the benzoic acid to the water pool away from the organic continuum may render product inhibition important in reversed micellar systems, however. Indeed, *p*-hydroxybenzoic acid (pK_a 4.58) is fully charged in micellar systems at the reaction pH of 7.5 and partitions fully into the water pools at that pH, as expected (data not shown). The oxidation kinetics with oxygen as acceptor have been determined with *p*-hydroxybenzaldehyde as a model-charged substrate in both DTAB and Triton systems.

To interpret the results in reversed micellar systems, eq 5 must be modified to include partitioning effects. We use the two-pseudophase partitioning model first proposed by Martinek et al.[42] to explain enzymatic reactivity in reversed micelles, extended here to incorporate the effect of product inhibition. In this model, solutes are assumed to distribute between the reversed micelles and the continuum, which are considered to be two pseudophases in equilibrium. The kinetic equation is written in terms of micellar quantities (denoted by the subscript mic) as

$$\frac{v_{mic}}{k_{cat,mic}[E]_{mic}} = \frac{[A]_{mic}}{K_{MA,mic}\left(1 + \frac{K_{iO_2,mic}[P]_{mic}}{[O_2]_{mic}K_{iP,mic}} + \frac{[P]_{mic}}{K_{iP,mic}}\right) + [A]_{mic}\left(1 + \frac{K_{MO_2,mic}}{[O_2]_{mic}}\right)} \qquad (13)$$

This can be expressed in terms of the observable overall quantities (denoted by the subscript ov) on recognition of the relationships $[E]_{ov} = \alpha[E]_{mic}$ and $v_{ov} = v_{mic}/\alpha$, where the enzyme

(42) Khmelnitskii, Yu. L.; Levashov, A. V.; Klyachko, N. L.; Martinek, K. *Usp. Khim.* **1984**, *53*, 545−65.

is assumed to reside exclusively in the water pool and α is the water pool volume fraction. To relate the concentrations of substrate (or product) in the two domains to the overall concentration, we use

$$[A]_{ov} = \alpha[A]_{mic} + (1 - \alpha)[A]_{cont} \quad (14)$$

where the concentration in the continuum phase is denoted by the subscript cont. With the partition coefficient P_A defined as the equilibrium concentration ratio $[A]_{mic}/[A]_{cont}$, this relation can be expressed as

$$[A]_{ov} = (\alpha + (1 - \alpha)/P_A)[A]_{mic} \quad (15)$$

Then, the kinetic equation reads in overall quantities:

$$\frac{v_{ov}}{k_{cat,ov}[E]_{ov}} = 1/\left[\frac{K_{MA,mic}(\alpha + 1(1 - \alpha)/P_A)}{[A]_{ov}} \times \left(1 + \frac{K_{iO_2,mic}(\alpha + (1 - \alpha)/P_{O_2})[P]_{ov}}{K_{iP,mic}(\alpha + (1 - \alpha)/P_P)[O_2]_{ov}} + \frac{[P]_{ov}}{K_{iP,mic}(\alpha + (1 - \alpha)/P_P)}\right) + \left(1 + \frac{K_{MO_2,mic}}{[O_2]_{mic}}\right)\right] \quad (16)$$

As was found by Martinek,[42] comparison with eq 13 yields:

$$k_{cat,ov} = k_{cat,mic} \quad (17)$$

$$K_{xX,ov} = \{\alpha + (1 - \alpha)/P_X\}K_{xX,mic} \quad (18)$$

where K_{xX} can be K_{MA}, K_{iP}, or K_{MO_2}. If $P_X \ll 1$, eq 18 is simplified to $K_{xX,ov} = K_{xX,mic}/P_X$, while if $P_X > 1$, $K_{xX,ov} = \alpha K_{xX,mic}$.

Equation 16 can be simplified by making assumptions similar to those used in the description of the kinetics of the reductive half-reaction with oxygen in water, namely (i) $[O_2]$ within the reversed micelle is at its aqueous saturation level (8 mM) as measured by Almgren;[43] (ii) $K_{MO_2,mic}$ is the same (1.5 μmol) as determined by Fridovich[37] using xanthine as reducing agent; and (iii) inhibition by oxygen is insignificant.

With i and ii, the term $K_{MO_2,mic}/[O_2]_{mic} \ll 1$ and can be neglected. With iii, it follows that the term containing $K_{i,O_2}/[O_2]$ can be neglected too. The charged fraction of the *p*-hydroxybenzaldehyde substrate ($10^{pH-pK_a} = 0.308$) is assumed to partition completely into the pools, as are all benzoic acid molecules, whereas the contribution of uncharged aldehyde to benzaldehyde concentration in the pool is neglected. With $P_P = [P]_{mic}/[P]_{cont} > 1$, $K_{iP,ov} = \alpha K_{iP,mic}$ and eq 16 reduce to

$$\frac{v_{ov}}{k_{cat,ov}[E]_{ov}} = \frac{1}{\frac{K_{MA,ov}}{0.308[A]_{ov}}\left(1 + \frac{[P]_{ov}}{K_{iP,ov}}\right) + 1} \quad (19)$$

Product inhibition of the aldehyde by acid is competitive as expected for the partial rapid-equilibrium−random two-step transfer mechanism of xanthine oxidase.[36] For Triton and DTAB, the lines of a Lineweaver−Burk plot intersect at the origin, so only the specificity ratio $k_{cat}/K_{MA,ov}$ can be determined from the slope, given by $K_{MA,ov}/v_{max}$. Since $k_{cat,mic} = k_{cat,ov}$, it follows that

$$K_{MA,obs} = K_{MA,ov}(1 + [P]_{ov}/K_{iP,ov}) \quad (20)$$

The acid concentration to be used in eq 20 should include partitioning effects. Table 7 lists results for K_{iP} in water and in the reversed micellar systems. With $\alpha = 0.0288$, $K_{iP,ov}$ data (column 1) can be converted to concentrations based on water pool volume, $K_{iP,mic}$. The value for $K_{iP,mic}$ in Triton matches $K_{iP,aqu}$ very well; $K_{iP,mic}$ in DTAB is even higher than the constant in water.

Table 7. Product Inhibition Constants for *p*-Hydroxybenzoic Acid

medium	$K_{iP,ov}$ (mM)	$K_{iP,mic}$ (mM)	r^2
water	1.79	1.79	0.994
DTAB	0.117	4.06	0.993
Triton	0.052	1.82	0.996
AOT	nd	nd	nd

5. Discussion

5.1. Substrate Parameter Study. Influence of Field Effects on Enzyme Reaction. The good fit of the k_{cat} data to the Hammett equation with a positive value for ϱ (Table 5) indicates the dominance of field effects on the substrate, which might be expected[28] given the rate-determining step in the oxidation sequence.[31,33,44] Thus, electron-withdrawing substituents in the para position enhance the δ^+ charge at the carbonyl carbon resulting in a higher rate, with the reverse being true with electron-donating substituents. Since ϱ is a measure of the rate-determining step of the reaction sequence, the statistically insignificant difference in ϱ between aqueous, DTAB, and Triton medium is a very strong argument for the same rate-determining step and for similar transition states for the xanthine oxidase reaction in both aqueous and reversed micellar media. Similar ϱ values and thus rate-determining steps upon changing from oxygen as electron acceptor in water to DCIP in reversed micellar systems demonstrate that the reductive half-reaction does not control the overall reaction rate.[31] As a consequence, effects of structured media do not influence the first-order catalytic step but might influence the second-order binding step. However, in AOT, no dependence of k_{cat} on field effects can be found, and since σ depends on the surrounding solvent,[45] the immediate active site environment in AOT/isooctane seems to be so hydrophobic that inductive forces do not seem to be important. This picture is supported by spectroscopic evidence for an open, spatially loose active site close to the enzyme surface of xanthine oxidase[30] and by the partitioning results which demonstrate that the substrate DCIP resides in the AOT tail layer without contact with the water pool domain.

The common structural feature of all benzaldehyde inhibitors of xanthine oxidase in water (the *p*-CN, *p*-CH(OEt)$_2$, and *p*-COO(H) compounds) is a polarized sp^2-hybridized carbon with a δ^+ charge in the para position to the aldehyde group; substrates with nonpolarized carbon atoms in the para position such as the CH_3 or CF_3 group do not inhibit the enzyme. Structural and electronic similarity with the aldehyde substrate might lead to catalytically nonproductive binding of the δ^- charged polarized groups, as is known to occur with purine substrates,[26a] and thus might cause inhibition.

Influence of Hydrophobicity. K_{MA} of the enzyme reaction was found to correlate very closely with the hydrophobicity of benzaldehyde substrates in all four systems investigated. Both water and the DTAB system have κ coefficients between −0.8 and −0.9, whereas the influence of π on $\log(k_{cat}/K_{MA})$ is smaller in AOT and Triton, for both of which $\kappa = -0.56$. A negative κ value corresponds to a low $K_{MA,app}$, signifying tight binding of hydrophilic substrates for $\pi < 0$, while for $\pi > 0$ loose

(43) Almgren, M.; Grieser, F.; Thomas, J. K. *J. Am. Chem. Soc.* **1980**, *102*, 3188−3193.

(44) Robins, R. K.; Revankar, G. R.; O'Brien, D. E.; Springer, R. H.; Novinson, T.; Albert, A.; Senga, K.; Miller, J. P.; Streeter, D. G. *J. Heterocycl. Chem.* **1985**, *22*, 601−634.

(45) Hammett, L. P. *Physical Organic Chemistry*, 2nd ed.; McGraw-Hill: New York, 1970.

binding of hydrophobic substrates is indicated. In contrast, hydrophobic phenyl-substituted purines strongly inhibit the enzyme, probably owing to a hydrophobic pocket that is located close to the active site of xanthine oxidase.[30,46] Hydrophobic benzaldehydes behave differently, probably because they are too small to be affected by the hydrophobic pocket. From the intercept of $\log(1/K_{MA})$ vs π, $K_{MA,ov}$ for a substrate of average hydrophobicity ($\pi = 0$) was calculated to be somewhat higher in DTAB (0.46 mM), Triton (0.44 mM), or AOT (0.89 mM) than $K_{MA,ov}$ in water (0.27 mM); $K_{MA,ov}$ of xanthine was also significantly higher in a membrane-associated state in milk fat globules than in homogeneous aqueous solution.[34]

The different degree of partitioning between active site and surrounding medium in nonaqueous, structured media compared to water can account for the differences in the apparent Michaelis constants for the different media. If partitioning between the xanthine oxidase active site and water paralleled partitioning between water and octanol, κ would be equal to zero for the K_{MA} correlations. A κ value of -0.88 in water implies an active site considerably less hydrophobic than octanol; the identical κ value (-0.88) in DTAB demonstrates similar hydrophobicity in water and the DTAB head group layer. The reduced κ value of -0.56 in Triton and AOT indicates that medium hydrophobicity is enhanced in both of these head group layers in comparison to water, since decreased hydrophobicity differences mean smaller absolute values of κ.

Interpretation of the Standard Reactivity ζ. The standard reactivity ζ at the intercept of the general reactivity equation for $\log(k_{cat}/K_{MA})$ at $\sigma = \pi = 0$ is about 1 order of magnitude higher in aqueous medium ($\zeta = 4.07$) than for reversed micellar systems ($\zeta = 3.17$ (DTAB), 3.14 (Triton), and 3.05 (AOT)). Comparing the three systems where the contributions to ζ from hydrophobicity (K_{MA}) and electron-donating power (k_{cat}) can be separated (water, DTAB, and Triton), we note that the larger portion of the decrease in ζ can be attributed to a decrease in k_{cat} rather than in $1/K_{MA}$. Changes in ζ, in contrast to electronic or hydrophobic effects, affect all substrates and k_{cat} as well as K_{MA} in equal manner; they are caused by factors such as strain of the enzyme, reduced amount of active enzyme, or nonproductive binding.[47] Although observation of saturation kinetics in AOT of DCIP and some benzaldehydes that are inhibitors in water points to altered (productive) binding in reversed micellar as compared to aqueous media (nonproductive binding), there is insufficient evidence to identify the cause of the decrease in ζ with any particular factor.

Medium and Substrate Effects on Solvation. From the general equation for k_{cat}/K_{MA}, eq 11, the differential Gibbs free energy of activation between two substrates *within a given medium* can be calculated (substrate effects, Table 8) by

$$\Delta\Delta G^{\ddagger}_{\text{substrate}} = -RT \ln \left(\frac{(k_{cat}/K_{MA})_{C-R}}{(k_{cat}/K_{MA})_{C-H}} \right) \qquad (21)$$

where C−H is the parent compound and C−R the derivative. Equation 21 can also be applied to compare differential Gibbs free energies of activation between water and a reversed micellar medium for *the same* substituent (medium effects, Table 9):

$$\Delta\Delta G^{\ddagger}_{\text{medium}} = -RT \ln \left(\frac{(k_{cat}/K_{MA})_{C-R,mic}}{(k_{cat}/K_{MA})_{C-R,water}} \right) \qquad (22)$$

$\Delta\Delta G^{\ddagger}$ values in Table 8 of different substrates based on the parent compound benzaldehyde do not vary much (<8 kJ/mol) between the four investigated media. Table 9 reveals that $\Delta\Delta G^{\ddagger}_{\text{medium}}$ of each substituent between water and the two hydrophilic reversed micellar systems is virtually constant. The change of solvation of the transition state compared to the ground state when going from an aqueous to a reversed micellar medium is small (4−8 kJ/mol) and seems to affect most substrates similarly. In essence, the *medium* effects on solvation are *on the same order of magnitude* as the *substrate substituent* effects, when interpreted in terms of $\Delta\Delta G^{\ddagger}$.

Table 8. Substrate Effects on Solvation: Calculated Differential Gibbs Free Energies of Activation ($\Delta\Delta G^{\ddagger}$) for Benzaldehydes with Respect to the Parent Compound in Each Medium

	$\Delta\Delta G^{\ddagger}$ (kJ/mol)			
substituent	water	AOT	DTAB	Triton
p-NO_2	−6.72	−0.89	−7.31	−7.65
p-CN	−5.91	−1.82	−5.70	−5.09
p-CF_3	−2.63	2.80	1.98	−0.01
p-$OCOCH_3$	−6.92	−2.04	−5.55	−4.74
p-Cl	−0.30	2.26	0.13	−1.66
H	0	0	0	0
p-CH_3	2.99	1.78	1.18	−0.07
p-OCH_3	−1.35	−0.06	−1.28	−1.40
p-OH	−1.11	−2.13	−1.74	−0.26
p-$N(CH_3)_2$	3.21	0.57	2.25	2.15

Table 9. Medium Effects on Solvation: Calculated and Experimental Differential Gibbs Free Energies of Activation ($\Delta\Delta G^{\ddagger}$) for Benzaldehydes in Reversed Micellar Systems Compared to the Same Compound in Aqueous Solution

	$\Delta\Delta G^{\ddagger}$ (kJ/mol)					
	AOT		DTAB		Triton	
substituent	calcd	exp	calcd	exp	calcd	exp
p-NO_2	12.32	10.56	5.21	7.80	5.02	7.66
p-CN	9.91	–	5.34	–	6.13	–
p-CF_3	9.53	11.53	8.01	–	6.21	–
p-$OCOCH_3$	11.41	11.45	7.22	–	8.20	–
p-Cl	9.40	7.95	6.58	7.33	4.95	6.03
H	5.82	3.58	5.13	–	5.31	–
p-CH_3	6.18	5.44	4.89	6.64	3.81	5.47
p-OCH_3	6.41	6.42	4.51	8.67	4.56	8.64
p-OH	4.79	5.54	4.50	–	6.14	–
p-$N(CH_3)_2$	3.18	1.84	4.16	6.00	4.23	6.27

5.2. Charged Substrates. Comparison of Overall and Micellar K_{iP} Inhibition Data To Determine the Validity of the Two-Pseudophase Model. Partitioning effects as calculated by the two-pseudophase model explain the discrepancies between K_{iP} inhibition data based on overall volume in water and in micellar systems (Table 7), especially in the Triton system. A plausible reason for the higher $K_{iP,mic}$ value in the DTAB system is association of acid product with the head group layer, as it would effectively reduce the concentration in the immediate vicinity of the active site. As a conclusion, the two-pseudophase model describes inhibition effects of charged products well, and extension of the model to three pseudophases,[17] incorporating the interface and the water pool as separate domains, does not seem to be necessary here.

Broadened and Shifted pH Optima Resemble Enzyme Immobilization. The broadening of the optimum pH range of *p*-hydroxybenzaldehyde in DTAB reversed micelles (pH 7.5−8.5) and in AOT (pH 8.1−9.5, albeit at lower reactivity) as compared to the aqueous case (0.2−0.3 pH units around pH 7.5, Figure 6) resembles the behavior of enzymes immobilized on solid supports;[5] the broadening might be caused by a decreased dielectric constant in the tail layer of the interface. In AOT, the *p*-hydroxybenzaldehyde curves are also markedly

(46) (a) Baker, R. B.; Wood, W. F. *J. Med. Chem.* **1968**, *11*, 644−61. (b) Bunting, J. W.; Gunasekara, A. *Biochem. Biophys. Acta* **1982**, *704*, 444−449.

(47) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman & Compaby: London, 1985; pp 331ff.

shifted toward more alkaline pH values as compared to the aqueous or DTAB medium. The local H^+ concentration at the AOT interface around the enzyme is higher than in water because at aqueous pH values of 7.5, substrate, surfactant, and enzyme ($pI = 5.4$[26a]) are all negatively charged. The decreased apparent pH value leads to low activity in AOT at a bulk pH of 7.5 only rising to the optimum at a higher pH, where the effective H^+ concentration at the AOT interface matches pH 7.5 in the bulk. For *o*- and *m*-hydroxybenzaldehyde, the optimum is beyond the xanthine oxidase activity limit around pH 9.5. The observed pH shift in AOT of about 1.5 pH units compares with a theoretical shift of 2.3 pH units calculated from a surface potential of 140 mV.[48] For DTAB, any observed pH shift would be much smaller (surface potential data are not available). A pH shift for a micellar enzyme reaction has been described by Jobe et al.[49] who found that the reaction catalyzed by chloroperoxidase is not affected by a SDS/hexanol/water reversed micellar environment except for effects attributable to a changed pH environment in the proximity of the surfactant head group layer.

6. Conclusions

Xanthine oxidase seems to be a good model enzyme for investigation of reversed micellar enzymatic activity: it is active and stable near several surfactant interfaces as well as in water. The main influence on enzymatic reactivity in reversed micelles is exerted by partitioning of charged substrates; in the present case, partitioning explained the differences of enzyme kinetics of charged substrates between water and micellar systems. The enzyme kinetics for uncharged substrates were analyzed with linear free energy relationships. Aldehyde oxidation with xanthine oxidase seems to be a particularly simple case in most systems, for the catalytic constant k_{cat} is correlated by the Hammett equation and the Michaelis constant K_{MA} by the Hansch hydrophobicity relation. The influence of electron-donating power (on catalytic activity) and hydrophobicity (on substrate binding) in reversed micellar systems with comparatively hydrophilic surfactant interfaces is not very different from that on respective forces in aqueous systems. Only in AOT with a very hydrophobic interface were electronic forces found not to be relevant for enzyme catalysis. The specificity ratio k_{cat}/K_{MA} is reduced in all reversed micellar systems compared to water. The evidence for a particular cause is insufficient as of yet, although saturation behavior in reversed micellar systems of several substrates instead of the inhibition pattern found in water is consistent with nonproductive binding in water which is altered in micellar systems. In summary, reactivity in microheterogeneous systems such as reversed micelles seems to be as accessible to analysis as reactivity in more common aqueous systems.

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(48) Leodidis, E. B.; Hatton, T. A. *J. Colloid Interface Sci.* **1991**, *147*, 163–77.

(49) Jobe, D.; Dunford, H. B.; Pickard, M.; Holzwarth, J. F. *Reactions in Compartmentalized Liquids*; Knoche, W., Schomäcker, R., Eds.; Springer: Heidelberg, 1989; pp 39–51.