ceptionally good agreement with a recent ab initio calculation, 2.04 eV (47.0 kcal/mol).[14]

The results obtained here raise questions concerning the relative reactivity of the ^6D and ^4F states of Fe^+ with other molecules. In previous studies, Fe^+ has been formed by surface ionization,[1] electron impact,[2] and laser desorption ionization,[3] all of which probably produce significant populations of both of these states. Further investigations in our laboratories will seek to determine whether other systems exhibit the substantial difference in reactivity observed here. Preliminary studies indicate that exothermic reactions with larger molecules may not be as sensitive to the state of the ion.

Acknowledgment. This work is supported by the National Science Foundation, Grant CHE-8306511.

Substrate Specificity of Enzymes in Organic Solvents vs. Water Is Reversed

Aleksey Zaks and Alexander M. Klibanov*

Department of Applied Biological Sciences
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received November 21, 1985

The ultimate challenge in the area of biocatalysis is the alteration of substrate specificity of enzymes at will. Redesigning enzymes by site-directed mutagenesis is emerging as a powerful strategy toward that goal.[1] Substrate specificity of enzymes stems from their ability to utilize the free energy of bonding with substrates to facilitate the reaction.[2,3] Since the net binding energy is the difference between the binding energies of the substrate with the enzyme and with water,[4] an alternative approach to changing substrate specificity (in addition to modification of the enzyme's active center) would be replacing water with another reaction medium.

As a result of our recent studies, it is becoming clear that enzymes can vigorously function as catalysts in organic solvents, provided that some basic rules are followed.[5] In the present work we have discovered that substrate specificities of several enzymes in organic solvents are radically different from, and sometimes opposite to, those in water.

α-Chymotrypsin[7] has been selected as the initial target because the nature of its substrate specificity is well established.[8] We have found that the enzyme can catalyze the transesterification reaction between *N*-acetyl-L-phenylalanine ethyl ester[9] (**1**) and primary alcohols in a variety of anhydrous organic solvents, with paraffins affording the highest reactivities.[10] For example, when a 1 mg/mL suspension of chymotrypsin in octane containing 5 mM **1** and 1 M propanol was shaken for 10 h,[10] 50% of **1** was converted into the corresponding propyl ester. The enzyme inactivated by the active-site-directed inhibitor phenylmethanesulfonyl fluoride[12] exhibited no activity in octane, thus confirming the nonartifactual origin of the transesterification reaction.

Table I. Substrate Specificity of Chymotrypsin and Subtilisin in the Reactions of Hydrolysis in Water and Transesterification in Octane

	k_{cat}/K_M, M^{-1} s^{-1}			
	chymotrypsin		subtilisin	
substrate	hydrolysis[a]	trans-esterification[b]	hydrolysis[a]	trans-esterification[b]
N-Ac-L-Phe-OEt	4.0×10^4	0.72	1.3×10^4	1.7
N-Ac-L-His-OMe	2.0×10^2	15.0	5.5×10^2	3.1
N-Ac-L-Ser-OMe	0.87	2.5	1.6×10^2	4.5

[a] Enzymatic hydrolysis of the three esters in water was followed potentiometrically with a Radiometer pH-stat. Conditions: 0.1 M KCl, pH 7.8, 20 °C, 10^{-7} to 10^{-4} M enzymes, and 0.3–5.0 mM esters. [b] Initial rates of the enzymatic reactions were measured as described in ref 10 at the concentration of the enzymes and *n*-propyl alcohol of 1 mg/mL and 1 M, respectively; the ester concentrations were varied in the range from 2 to 30 mM. The kinetics of the enzymatic transesterification strictly followed the compulsory order mechanism with no ternary complexes whose conventional treatment has been used to calculate kinetic parameters (Dixon, M.; Webb, E. C. *Enzymes*, 3rd ed.; Academic Press: New York, 1979; pp 92–93). Both chymotrypsin and subtilisin were lyophilized prior to use in the same manner[10] except that for the latter 0.25% phosphate buffer (pH 7.8) was used instead of *N*-Ac-L-Phe. Octane (99+% purity) contained[11] less than 0.02% water.

The main driving force of the enzyme–substrate binding in the case of chymotrypsin is hydrophobic interactions between the side chain of the amino acid substrate and the binding pocket of the enzyme.[4,8] This is illustrated by the data in the first column of Table I: upon transition from hydrophobic Phe to hydrophilic Ser in the series of esters of *N*-acetyl-L-amino acids (specific model substrates for chymotrypsin[4,8]), k_{cat}/K_M for the enzymatic hydrolysis drops nearly 5×10^4 fold. Since hydrophobic interactions owe their existence to water as the reaction medium,[13] one might expect that the substrate preference of chymotrypsin in organic solvents should be entirely different because hydrophobic bonding will play no part. The results in the second column of Table I confirm that prediction: in the transesterification reaction in octane, the ester of *N*-Ac-L-Ser is 3 times more reactive than that of *N*-Ac-L-Phe. Furthermore, *N*-Ac-L-His-OMe, whose reactivity in water was 0.5% of that of **1**, in octane becomes a 20-fold more reactive substrate.[14]

(1) Ulmer, K. *Science* (*Washington, D.C.*) **1983**, *219*, 666–671. Fersht, A. R.; et al. *Nature* (*London*) **1985**, *314*, 235–238. Craik, C. S.; et al. *Science* (*Washington, D.C.*) **1985**, *228*, 291–297.

(2) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; Chapter 5. Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219–410.

(3) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman: New York, 1985; Chapters 12 and 13.

(4) Reference 3, Chapter 11.

(5) These rules include:[6] (i) a proper choice of the solvent (with hydrophobic ones being the best as they do not strip the essential layer of water from the enzyme molecule); (ii) the use of an enzyme recovered from an aqueous solution of the pH optimal for enzymatic activity; (iii) elimination of diffusional limitations by vigorous agitation and fine dispersion of the enzyme powder in an organic solvent.

(6) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192–3196. Kazandjian, R. Z.; Klibanov, A. M. *J. Am. Chem. Soc.* **1985**, *107*, 5448–5450. Klibanov, A. M. *CHEMTECH* **1986**, *16*, No. 6.

(7) Bovine pancreatic α-chymotrypsin (EC 3.4.21.1) was purchased from Sigma Chemical Co. as a lyophilized powder with a specific activity of 58 BTEE units per mg of protein.

(8) Bender, M. L.; Kezdy, F. *Annu. Rev. Biochem.* **1965**, *34*, 49–76. Hess, G. P. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1970; Vol. 3, pp 213–248. Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145–152.

(9) One of the best classical substrates for α-chymotrypsin.[8]

(10) The optimal mode of preparation of chymotrypsin for these experiments (used throughout this study) was lyophilization of 5 mg/mL enzyme from an aqueous solution, pH 7.8, containing the ligand *N*-Ac-L-Phe (0.25%) (Zaks, A.; Klibanov, A. M., unpublished results). The lyophilized sample contained[11] about 2.5% (w/w) water, i.e., no more than 50 molecules of water per enzyme molecule. An enzyme suspension in a solution of substrates in an organic solvent was placed in a stoppered flask and shaken on an orbit shaker at 250 rpm and 20 °C. Periodically, aliquots were withdrawn and assayed by gas chromatography using a 530-μm fused silica capillary column (Hewlett-Packard). The rates of chymotrypsin-catalyzed transesterification were not controlled by diffusion of the substrate through the enzyme bead: ultrasonication of a suspension of the enzyme in octane (resulting in a decrease of an average enzyme particle from 270 to 5 μm) had no appreciable effect on the reaction rate.

(11) The water concentration both in the organic media and in the enzymes was measured by the optimized Fischer method (Laitinen, H. A.; Harris, W. E. *Chemical Analysis*, 2nd ed.; McGraw-Hill: New York, 1975; pp 361–363).

(12) Fahrney, D. E.; Gold, A. M. *J. Am. Chem. Soc.* **1963**, *85*, 997–1000.

(13) Kauzmann, W. *Adv. Protein Chem.* **1959**, *14*, 1–63. Tanford, C. *The Hydrophobic Effect*; Wiley: New York, 1980.

(14) It should be stressed that although in water the values of k_{cat}/K_M in most instances are much greater than in octane, this does not necessarily mean that chymotrypsin and subtilisin are less efficient catalysts in octane than in water. This is because two entirely different processes are being compared, hydrolysis in water and transesterification in octane, for which the rate constants of nonenzymatic reactions are also totally different.

0002-7863/86/1508-2767$01.50/0

Qualitatively similar data were obtained for subtilisin[15] (the last two columns in Table I)[16] and porcine liver carboxyl esterase.[17] In the latter case, k_{cat}/K_M in the enzymatic hydrolysis of hydrophobic ethyl hydrocinnamate was nearly 8 times as high as for hydrophilic ethyl L-lactate. However, in octane, ethyl lactate's k_{cat}/K_M for the carboxyl esterase catalyzed transesterification with propanol was 47 times as high as for ethyl hydrocinnamate. Hence for all three enzymes a complete reversal of substrate specificity occurs upon replacement of water with octane as the reaction medium. The described phenomenon is expected to be general since water plays a critical role in all types of enzyme–substrate interactions and therefore its substitution should have a major impact on substrate specificity of enzymes.

Registry No. 1, 2361-96-8; *N*-Ac-L-His-OMe, 36097-48-0; *N*-Ac-L-Ser-OMe, 54322-41-7; $Me(CH_2)_6Me$, 111-65-9; ethyl hydrocinnamate, 2021-28-5; ethyl L-lactate, 687-47-8; α-chymotrypsin, 9004-07-3; subtulisin, 9014-01-1; carboxyl esterase, 9016-18-6.

(15) Protease from *Bacillus subtilis*, also known as subtilisin Carlsberg (EC 3.4.21.14) (for a review, see: Ottesen, M.; Svendsen, I. *Methods Enzymol.* **1970**, *19*, 199–215), was obtained from Sigma in a crystallized and lyophilized form with a specific activity of 9.6 casein units per mg of solid.

(16) The magnitude of the effect here is not as dramatic because the hydrophobic binding site in subtilisin is not as pronounced as in chymotrypsin (ref 3, Chapter 1).

(17) This enzyme (EC 3.1.1.1; for a review, see: Krisch, K. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 5, pp 43–69) was purchased from Sigma as a suspension in $(NH_4)_2SO_4$ with a specific activity of 200 ethyl butyrate units per mg of protein. Ammonium sulfate was removed by dialysis, and the esterase was lyophilized from aqueous phosphate buffer (pH 7.8). Basic conditions of the esterase reactions were the same as those employed for chymotrypsin and subtilisin.

(18) This work was financially sponsored by Miles Laboratories.

6-Methylenebicyclo[3.1.0]hex-3-en-2-yl Cation, an Isomer of Benzyl Cation

Ronald M. Jarret, Martin Saunders,* Steven Pikulin, and Jerome A. Berson*

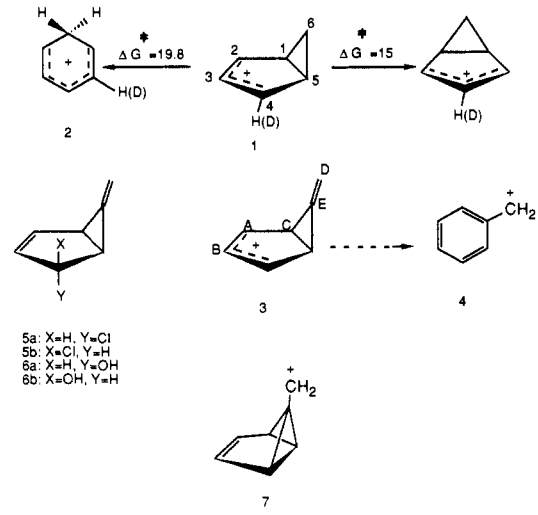
Department of Chemistry, Yale University
New Haven, Connecticuit 06511

Received December 12, 1985

Previous work[1a] reported the preparation and proton NMR observation of the stable bicyclo[3.1.0]hex-3-en-2-yl cation (**1**). Cation **1** was shown by an isotopic labeling experiment to undergo a slow degenerate rearrangement at −90 °C ($\Delta G^{\ddagger}$ = 15 ±1 kcal/mol). At −20 °C, irreversible decomposition to unidentified products competes with ring opening to benzenonium ion (**2**) ($\Delta G^{\ddagger}$ = 19.8 kcal/mol).[1a-f]

Insertion of a methylene group at C_6 of **1** would give cation **3**, whose decyclization to benzyl cation **4** should be exothermic by ~63 kcal/mol (from bond additivities). We hoped that this driving force might facilitate the direct spectroscopic detection of benzyl cation (**4**), which hitherto has not been reported, in the literature, as a long-lived species.[1g]

A 10:1 mixture of chlorides **5a,b**, precursors of cation **3**, was best prepared by treatment of a mixture of epimeric 6-methylenebicyclo[3.1.0]hex-3-en-2-ols, **6a,b**[2] with $SOCl_2$ (4 equiv) and pyridine (1 equiv) in pentane solution at −15 °C. Purification of the chloride mixture was effected by bulb-to-bulb distillation at 0.2 torr followed by careful preparative gas chromatography (5% OV-101, column temperature 45 °C).[3]



Cation solutions were prepared by using the standard method,[4] by codistillation of **5a/5b** and SbF_5 under vacuum into a receiver at −196 °C, followed by addition of SO_2ClF to the distillate and warming to −110 °C. Cation **3**, so formed, shows the ^1H and ^{13}C NMR spectra shown in Figure 1 and interpreted[5] in Table I.

The NMR spectrum of the cation is unchanged below −110 °C. From −110 to −70 °C, the spectrum undergoes reproducible and reversible changes. The peak heights for protons of the five-membered ring (A, B, and C) decrease as the signals broaden into the base line. By −70 °C, these peaks completely disappear; however, recooling returns the peaks to their original heights. At temperatures above −70 °C, the *exo*-methylene signal D at δ 5.34 irreversibly disappears. Recooling once this signal has vanished produces no peaks. If the sample is recooled before the signal from proton D is completely gone, protons A, B, and C reappear in the same relative proportions but in overall diminished intensities (as compared to either tropylium tetrafluoroborate, added to the receiver prior to the preparation of **3**, or to the impurity peak at δ 9.07 presumably resulting from HF or HCl, generated within the sample). The ^{13}C NMR of the cation at −70 °C shows only signals corresponding to D and E. At temperatures above −70 °C cation **3** irreversibly decomposes to unidentified products (even in dilute solution), in contrast to cation **1** which is stable up to −20 °C. Thus far, photolysis of the sample at −150 °C for extended periods of time has led only to decomposition.

Magnetization transfer was observed among the five-membered ring protons A, B, and C. At −110 °C, saturation of proton A causes a decrease in intensity of both peaks B and C, saturation of proton B causes mainly a decrease in intensity of peak A, and saturation of proton C causes mainly a decrease in intensity of peak A. Saturation of proton D does not cause any peaks to decrease in intensity. These results suggest that the methylenecyclopropane ring of cation **3** rapidly migrates around the five-membered ring.

The changes in line width with temperature were used in conjunction with the KUBO[6] program to obtain rates of the ring-walk process over the temperature range of −110 to −70 °C. From −105 to −81 °C, the peak width of A increases from 8.9 to 61.3

(1) (a) Vogel, P.; Saunders, M.; Hasty, N. M., Jr.; Berson, J. A. *J. Am. Chem. Soc.* **1971**, *93*, 1551. See also: (b) Childs, R. F.; Winstein, S. *J. Am. Chem. Soc.* **1968**, *90*, 7146. (c) Koptyug, V. H.; Kuzubova, L. I.; Isaev, I. S.; Manatyuk, V. I. *Chem. Commun.* **1969**, 389. (d) Childs, R. F.; Sakai, M.; Winstein, S. *J. Am. Chem. Soc.* **1968**, *90*, 7144. (e) Childs, R. F.; Parrington, B.; *Chem. Commun.* **1970**, 1540. (f) Swatton, D. W.; Hart, H. *J. Am. Chem. Soc.* **1967**, *89*, 5075. (g) Olah, G. A.; Porter, R. D.; Jeuell, C. L.; White, A. M. *J. Am. Chem. Soc.* **1972**, *94*, 2044.

(2) Pikulin, S.; Berson, J. A. *J. Am. Chem. Soc.* **1985**, *107*, 8274.

(3) Characterized by ^1H and ^{13}C NMR spectroscopy. At temperatures above 55 °C, **5a/b** rearranges smoothly to benzyl chloride in solution (benzene or CH_3CN) or in the gas phase.

(4) Saunders, M.; Cox, D.; Lloyd, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 6656.

(5) (a) The alternative 6-methylenebicyclo[2.1.1]hex-2-enyl cation structure is unlikely on energetic grounds, being more strained than **3** by ~23 kcal/mol[5b] and lacking the resonance energy of an allylic cation (~15 kcal/mol). Moreover it does not account for the ^{13}C or ^1H NMR spectra which, by analogy to the 7-norbornenyl cation, should show a ^{13}C peak for C_2–C_3 near δ 125 and an ^1H resonance for H_2–H_3 near δ 7.5. (b) Greenberg, A.; Liebman, J. F., *Strained Organic Molecules*; Academic Press: New York, 1978; p 94. (c) Olah, G. A.; Liang, G. *J. Am. Chem. Soc.* **1975**, *97*, 6803 and references cited therein.

(6) (a) Saunders, M. *Tetrahedron Lett.* **1963**, 1699. (b) Saunders, M. In *Magnetic Resonance in Biological Systems*; Ehrenberg, A., Malmström, B. G., Vänngård, T., Eds.; Pergamon Press: Elmsford, NY, 1967; p 85.

0002-7863/86/1508-2768$01.50/0