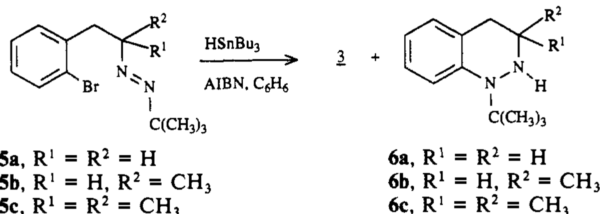
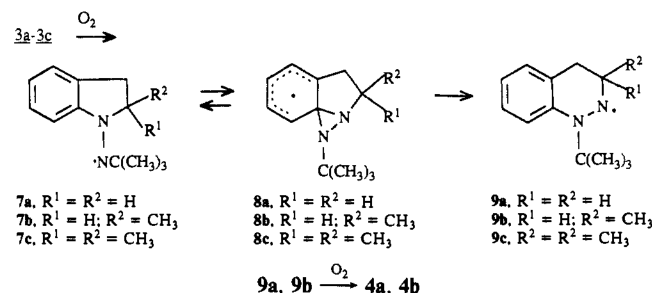
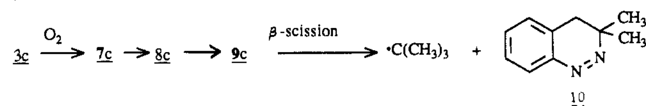
Scheme I



Scheme II



Scheme III



hydrazyl (**9**) readily accounts for the observations.

The mechanism of Scheme II is supported strongly by the behavior of **3c**, which afforded 3,4-dihydrocinnoline (**10**), Scheme III. Hydrazyl **9c** would be expected to lose the *tert*-butyl radical, rather than the CH_3 radical, by β-scission. There is precedent for facile loss of *tert*-butyl from a hydrazyl[10] except in cases of fixed and unfavorable geometry.[11]

It was possible to observe hydrazyl **7c** by irradiation of a solution of **3c** at −40 °C in a mixed solvent consisting of di-*tert*-butyl peroxide and CH_2Cl_2 (1:1 by volume), in the cavity of an EPR spectrometer. Although the initial spectrum was complex, probably because of an impurity present, continued irradiation led quickly to a steady five-line spectrum (intensity ratios within 20% of 1:2:3:2:1 at $g = 2.0038$). The g value is close to those of typical hydrazyls,[12] and the observation of five-lines as described implies that $a_{N(1)} \simeq a_{N(2)} \simeq 11.8$ G. In most hydrazyls,[12] $a_{N(1)} > a_{N(2)}$ but the reverse is known,[13] and it is therefore not surprising that $a_{N(1)} \simeq a_{N(2)}$ should be encountered, as is the case for **7c**. The signal faded rapidly when the light was turned off.

Increasing the temperature did not lead to a change in the spectrum, indicating either that neophyl rearrangement is slow or that the rearranged radical **9c** loses the *tert*-butyl group very rapidly. A strong indication that the latter is true came from attempts to prepare **9c** directly from **6c**, by the method described above for the generation of **7c** from **3c**. No signal at all was observable. Since it is highly unlikely that *tert*-butoxyl radicals abstract efficiently from **3c** but not from **6c**, the conclusion is that loss of *tert*-butyl from **9c** is very much faster than the neophyl rearrangement of **7c** to **9c**.

The sequence of reactions of **3**, induced by oxygen, represents the first examples of neophyl rearrangements of hydrazyls to isomeric hydrazyls. Rearrangement of radicals **7** is surprising because hydrazyls are normally stabilized by three-electron π-bonding. Recent results, that add to an extensive body of literature on the subject of bonding in hydrazyls, include the observation that the NH_2 hydrogens of 1-phenylhydrazyl are nonequivalent[14] and calculations of torsional and inversion barriers for hydrazyl itself.[15] In the case of radicals **7**, hydrazyl resonance is probably less important because it requires the *tert*-butyl group to be held, with entropic cost, in or near the molecular plane, where it must interact sterically with either the CR^1R^2 moiety or with the "peri" hydrogen at C-7. Although radicals **9** also have a "peri" interaction of the type mentioned above, hydrazyl resonance comes at lower entropic cost because both N atoms are in a ring. Rearrangement of **7** to **9** can therefore be expected to be exothermic and effectively irreversible.

Neophyl rearrangement could conceivably be involved during the synthesis of **3** and **6** (Scheme I). Beckwith and co-workers[16] have found that 5-exo cyclizations of alkenylaryl and (alkenyloxy)aryl radicals are followed by neophyl rearrangements that can compete with H-abstraction from $HSnBu_3$. In the present cases, the ratios (**3**:**6**) were independent of the initial concentration of $HSnBu_3$ in the range 0.20–1.15 M. That result suggests that the neophyl rearrangements of **7** are relatively slow, as expected,[17] for it is unlikely that the neopentyl-like **7** abstracts from $HSnBu_3$ with rate constants much larger than those of the Beckwith[16] radicals.

The oxidative rearrangements of **3a** and **3b** reported here may represent an attractive route to 1-alkyl-1,4-dihydrocinnolines, such as **4a** and **4b**, from 1-(alkylamino)indolines.

Registry No. 3a, 116302-23-9; **3b**, 109638-02-0; **3c**, 109637-95-8; **4a**, 116302-24-0; **4b**, 116302-25-1; **7c**, 116302-26-2; **10**, 116302-27-3.

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Control of Enzyme Enantioselectivity by the Reaction Medium

Takayasu Sakurai, Alexey L. Margolin, Alan J. Russell, and Alexander M. Klibanov*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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Stereoselectivity is one of the hallmarks of enzymatic catalysis.[1,2] In principle, enzyme stereoselectivity could be altered by protein engineering[3] which would be of profound significance for both enzyme-catalyzed preparative synthesis[4] and mechanistic biochemistry. Recently, we have observed that upon a transition from water to organic solvents as the reaction medium the enantioselectivity of the protease subtilisin Carlsberg in the reaction of peptide synthesis dramatically relaxes.[5] If general, this phe-

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Table I. Enantioselectivity of Various Proteolytic Enzymes in Water and in Butyl Ether[a]

enzyme[b]	amino acid substrate[c]	$(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$ in water	in butyl ether
subtilisin Carlsberg	N-Ac-Ala-OEtCl	1800	4.4
subtilisin Carlsberg	N-Ac-Phe-OEtCl	15000	5.4
elastase	N-Ac-Ala-OEtCl	>1000[d]	4.5
α-lytic protease	N-Ac-Ala-OEtCl	>10000[d]	8.3
subtilisin BPN′	N-Ac-Phe-OEtCl	16000	7.3
α-chymotrypsin	N-Ac-Ala-OEtCl	710	3.2[e]
trypsin	N-Ac-Phe-OEtCl	>4000[d]	3.2

[a] In water, enzymatic hydrolysis of the esters was kinetically examined at 30 °C and pH 7.0 as previously described.[7] In butyl ether, enzymatic transesterification of the esters with *n*-propanol (1 M) was kinetically investigated at 30 °C with the previously[7,8] developed protocol. In all cases no appreciable reaction was detected without enzyme or in the presence of proteases preinactivated with phenylmethanesulfonyl fluoride (Fahrney, D. E.; Gold, A. M. *J. Am. Chem. Soc.* **1963**, *85*, 997–1000). [b] Subtilisin Carlsberg (protease from *Bacillus subtilis*, type VIII, EC 3.4.21.14), porcine pancreatic elastase (type I, EC 3.4.21.36), subtilisin BPN′ (protease from *Bacillus amyloliquefaciens*, type XXVII, EC 3.4.21.14), bovine pancreatic α-chymotrypsin (type II, EC 3.4.21.1), and bovine pancreatic trypsin (type III, EC 3.4.21.4) were purchased from Sigma Chemical Co. α-Lytic protease (protease from *Lysobacter enzymogenes*, EC 3.4.21.12) was prepared according to the literature procedure (Bachovchin, W. W.; Roberts, J. D. *J. Am. Chem. Soc.* **1978**, *100*, 8041–8047). All enzymes were lyophilized from aqueous solutions at the pH optimal for catalytic activity prior to their use:[8] pH 7.8, 8.8, 7.8, 7.8, 8.0, and 8.0, respectively. [c] Both enantiomers of the esters were synthesized as previously described (Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 584–589). [d] $(k_{cat}/K_m)_D$ was too low to measure accurately. Thus, the value given is the lower limit of enantioselectivity. [e] In the case of α-chymotrypsin, butyl ether was supplemented with 0.1% distilled water.[8]

nomenon would provide an alternative strategy to protein engineering. Therefore, in the present work we have investigated the enantioselectivity of several different enzymes in a variety of solvents.

The enzymatic reaction studied in water was the hydrolysis of 2-chloroethyl esters of *N*-acetyl-L- and -D-amino acids. In organic solvents,[6] we examined the enzymatic transesterification reaction[7,8] between the same esters and propanol. In both instances, enzymatic reactions obeyed Michaelis–Menten kinetics, and kinetic analysis[7,8] afforded the ratio of specificity constants $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$, which reflects enantioselectivity of an enzyme. For the hydrolysis of *N*-Ac-Ala-OEtCl in water catalyzed by subtilisin Carlsberg, $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$ was determined to be 1800 (the first line of Table I). For all nonaqueous solvents tested, this enantioselectivity factor[9] was found to be 1–2 orders of magnitude lower. Also, there is a linear correlation between the difference in free energy of activation ($\Delta\Delta G^{\neq}$)[10] for subtilisin-catalyzed transesterification of L- and D-enantiomers of *N*-Ac-Ala-OEtCl in 13 different organic solvents and the logarithm of the solvent's octanol–water partition coefficient[11] (Figure 1).

These data can be rationalized with the following simple model. When a substrate interacts with the enzyme's active center, water

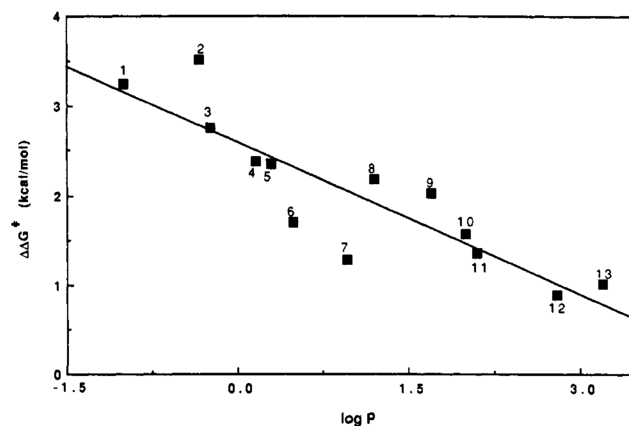

Figure 1. The dependence of the enantioselectivity[10] of subtilisin Carlsberg on the hydrophobicity of the solvent. Solvents: 1, dimethylformamide; 2, acetonitrile; 3, acetone; 4, methyl acetate; 5, butanone; 6, tetrahydrofuran; 7, cyclohexanone; 8, propyl acetate; 9, butyl acetate; 10, benzene; 11, methylene chloride; 12, butyl ether; and 13, cyclohexane. Their hydrophobicities, expressed as the logarithm of partition coefficients between octanol and water, were taken from the references in footnote 11. Kinetic parameters for the enzymatic transesterification reaction between *N*-acetylalanine chloroethyl ester and propanol in different solvents were measured as described in footnotes to Table I. The correlation coefficient R for the linear free energy relationship shown is 0.88.

must be excluded from between them.[12a] In particular, the binding of an *N*-acyl-L-amino acid substrate to the active center of a proteolytic enzyme results in the release of a certain number of water molecules from the hydrophobic binding pocket of the protease; this process is a driving force in the enzyme-substrate interaction.[12b] One would expect that the release of water molecules into the reaction medium will become less thermodynamically favorable with increasing hydrophobicity of the solvent. If the D-enantiomer binds to the active center of a protease in the same manner as the *N*-acyl-L-amino acid substrate, then the scissile bond will not face the enzyme's nucleophile, and hence cleavage cannot occur.[2] In order for the D-isomer to be reactive, it must bind to the enzyme "incorrectly",[2] and this binding will release fewer molecules of water from the hydrophobic binding pocket than that of its L-counterpart.[12b] Therefore, the reactivity of the L-enantiomer should be diminished to a greater extent, and consequently the enzyme's enantioselectivity should decrease, when the hydrophobicity of the solvent increases.

Our theoretical model further predicts that a difference in enzyme enantioselectivity between water and an organic solvent should be greater for a substrate with a bulkier side chain. This effect was indeed observed for subtilisin Carlsberg in anhydrous butyl ether when Ala was replaced with Phe (the first two lines in Table I).

To test the generality of the observed phenomena, we investigated enantioselectivity of five other serine proteases in water and in butyl ether (in which subtilisin Carlsberg had the lowest enantioselectivity, see Figure 1). As seen in Table I, elastase, α-lytic protease, subtilisin BPN′, α-chymotrypsin, and trypsin all exhibit striking enantiomeric selectivity in water but not in the nonaqueous solvent: while in the former $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$ is on the order of 10^3–10^4; in butyl ether it does not exceed even 1 order of magnitude.

In summary, the results of this study demonstrate the predictable and rational control of enantioselectivity of enzymes by changing the reaction medium rather than the enzyme itself. In addition to its theoretical interest, this phenomenon also allows for the enzymatic processing of D-amino acid derivatives which is impossible in water.[5]

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(9) Note that the bimolecular rate constant k_{cat}/K_m describes the reaction of the free enzyme with the free ester substrate (ref 1a, Chapter 4). This terms equals k_2/K_S (where k_2 and K_S are the rate constant of acylation and binding constant, respectively), which corresponds to the first chemical step of the enzymatic reaction and thus is independent of the origin of the nucleophile (water or propanol). Therefore, the ratios of the specificity factors for enzymatic hydrolysis and transesterification can be directly compared.

(10) $\Delta\Delta G^{\neq} = -RT\ln[(k_{cat}/K_m)_L/(k_{cat}/K_m)_D]$.

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