Suspended and Immobilized Chymotrypsin in Organic Media: Structure–Function Relationships Revealed by Electron Spin Resonance Spectroscopy

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Abstract: Comparing the behavior of freely suspended and immobilized enzyme in organic media with low water contents can yield insights into interactions among the biocatalyst, solvent, and support that influence protein structure and function. Immobilized chymotrypsin has higher activity than the suspended enzyme (by 1–2 orders of magnitude) in anhydrous organic solvents ranging from nonpolar *n*-octane to polar acetonitrile. In anhydrous tetrahydrofuran (THF), glass-adsorbed chymotrypsin is ca. 10 times more active than the suspended enzyme, and electron spin resonance (ESR) spectra of an active-site spin-label reveal greater local flexibility. Upon adding up to 0.5% v/v water, increased catalytic efficiency of the immobilized enzyme is accompanied by a sharp rise in active-site polarity but no apparent change in active-site conformation or dynamics. Under the same conditions the activity of the suspended enzyme also increases; however, the active-site polarity remains nearly constant while the spin-label reflects increasing molecular flexibility. For both preparations, further changes in protein structure occur as enzyme activity increases with 0.5–7% v/v added water. Computer simulations of the room-temperature ESR spectra suggest that different initial conformational states contribute to the different behavior of the two enzyme systems over the entire range of added water. These findings show that the structural properties of suspended and immobilized enzyme can differ markedly and that these differences are important to enzyme activity in organic media.

Introduction

That enzymes can function in nonaqueous media is well established;[1] however, the catalytic efficiencies of enzymes in organic solvents are, in general, orders of magnitude lower than those in aqueous systems.[2] Although effective strategies for greatly improving enzyme activity in organic solvents have been demonstrated for select systems,[3] low turnovers and high saturating substrate concentrations remain obstacles to the widespread application of enzymes in organic media. The growth of nonaqueous biocatalysis as a practical technology will no doubt be fostered by an improved understanding of enzyme catalysis in organic media and by the development of biocatalyst design criteria for nonaqueous systems.

Immobilization can facilitate fundamental studies of enzymes in organic solvents as well as offer important practical advantages. Because enzymes are insoluble in most organic solvents, they are prone to aggregate into particles of nonuniform size that may be subject to diffusional limitations.[4] This problem can be mitigated by immobilizing the enzyme to porous particles with pores large enough to allow relatively rapid transport of substrate to the enzyme. Spreading the enzyme onto a solid surface can also increase the interfacial surface area between the protein and solvent, thus leading to higher reaction rates.[5] Moreover, the chemical nature of the immobilization support can change the partitioning of water and/or substrates.[6] In water-miscible media, for instance, attaching the enzyme to hydrophilic beads might increase the retention of the low levels of water essential for activity.[7] In effect, the choice of an appropriate immobilization material can help compensate for water stripping by highly polar solvents.[8]

For example, Reslow *et al.* showed that immobilizing chymotrypsin to supports that readily retain water resulted in high enzymatic activity in acetonitrile.[7b] On the other hand, less hydrophilic supports gave greater activity in the water-immiscible solvent diisopropyl ether. In general, the physicochemical properties of the enzyme, support material, and solvent strongly influence the interactions among them.[9] Little research has been done, however, on the specific nature of these interactions and on how they manifest in the conformation and other structural properties of immobilized enzymes in organic solvents. Such information will aid the design of effective enzyme systems with improved catalytic properties. The present study seeks to explore how a glass-adsorbed enzyme differs

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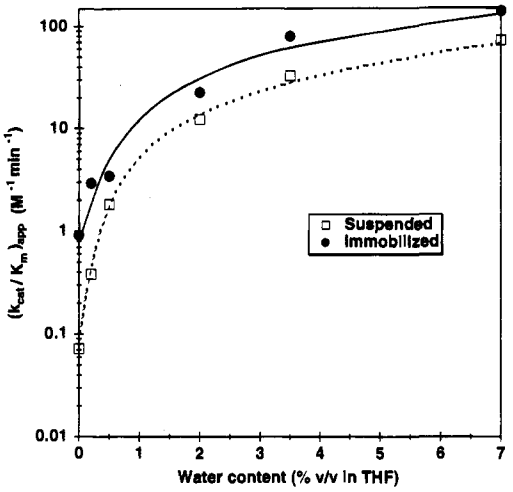

Figure 1. Apparent catalytic efficiencies of freely suspended and immobilized chymotrypsin as a function of water content in THF. For the dispersed enzyme the numerical values plotted, M^{-1} min^{-1}, are 0.072 at 0%, 0.38 at 0.2%, 1.82 at 0.5%, 12.2 at 2.0%, 33.0 at 3.5%, and 72.5 at 7.0% v/v water in THF. Values of $(k_{cat}/K_m)_{app}$ for glass-adsorbed enzyme are 0.91 M^{-1} min^{-1} in dry solvent and 2.92 at 0.2%, 3.44 at 0.5%, 22.5 at 2.0%, 79.9 at 3.5%, and 140.4 at 7.0% v/v water added to THF.

Table 1. Values of $(k_{cat}/K_m)_{app}$ (M^{-1} min^{-1}) × 100 for Freely Suspended and Immobilized Chymotrypsin in Various Solvents[a]

solvent	freely suspended chymotrypsin	immobilized chymotrypsin	$(k_{cat}/K_m)_{app,immobilized}/(k_{cat}/K_m)_{app,suspended}$
n-octane	221	19120	87
carbon tetrachloride	15	2150	141
toluene	7.7	557	72
diethyl ether	33	1370	42
THF	7.2	91	13
acetonitrile	0	56	

[a] Note: $(k_{cat}/K_m)_{app}$ values are based on active-site titration results obtained in aqueous solution.

from a freely suspended preparation in activity, flexibility, and active-site polarity in nearly anhydrous media.

Results and Discussion

For the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) and propanol in dry tetrahydrofuran (THF), the apparent catalytic efficiency, $(k_{cat}/K_m)_{app}$, of freely suspended chymotrypsin is 0.072 M^{-1} min^{-1}, whereas the value for the glass-adsorbed enzyme is 0.91 M^{-1} min^{-1} (Figure 1). The difference in apparent rate constants for the two systems exceeds an order of magnitude and is maximal in the anhydrous solvent. Immobilized chymotrypsin has higher activity than the lyophilized, suspended enzyme in a variety of other media, ranging from nonpolar *n*-octane to polar acetonitrile (Table 1). In each case $(k_{cat}/K_m)_{app}$ for the glass-bound biocatalyst is 1−2 orders of magnitude larger than that of the free enzyme.

A cursory examination produces a few potential reasons for the higher activities of the immobilized enzyme. One explanation might be that glass-bound chymotrypsin has a higher hydration level than freely suspended enzyme. Karl Fischer titration indicates, however, that the amount of water present in the lyophilized enzyme powder is 10.0% w/w (the average of multiple measurements) in the absence of solvent; the water content of the immobilized chymotrypsin sample is 2.2%, not much different from the value of 2.0% for the glass beads alone prepared under similar conditions. While the glass-adsorbed enzyme system contains more water overall, the water appears

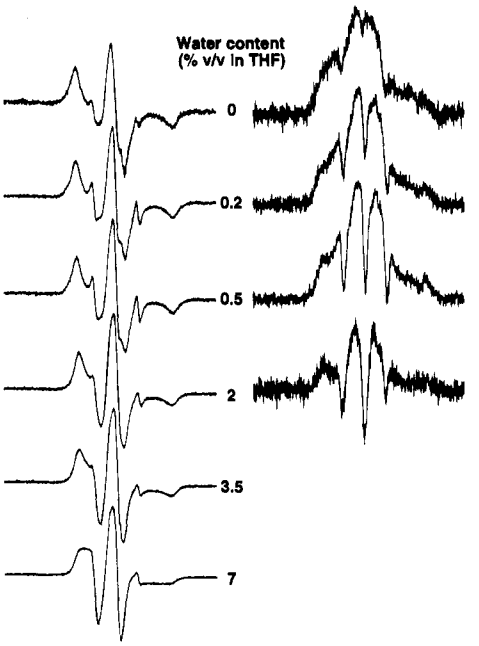

Figure 2. Conventional (left) and saturation transfer (right) ESR spectra of freely suspended chymotrypsin labeled with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy at Met-192.

to be associated with and distributed throughout the support material. In the immobilized system the hydrophilic support probably competes with the enzyme for the available water.

Furthermore, the activation factor does not correlate with the polarity of the solvent, as would be expected if water distribution and stripping were the dominant factors.[8b] That catalytic enhancement occurs in a variety of media (Table 1) also eliminates the specific chemical properties of the solvent as a likely cause. Possible diffusional effects within the lyophilized, dispersed enzyme samples should also be considered. However, Zaks and Klibanov have demonstrated that, for the exact same reaction, the transport of substrate is not rate limiting for freely suspended chymotrypsin in *n*-octane, the solvent in which the highest activities are observed.[10] Remaining factors that might account for the observed phenomenon are the intrinsic properties of the enzyme and interactions of the protein with the support.

The active-site dynamics of the enzyme preparations were investigated by electron spin resonance (ESR) spectroscopy using a spin-label bound near the catalytic Ser-195, in the P_1 binding pocket. Met-192, the residue to which the probe is attached, shifts during the activation of the zymogen from a completely buried position out to the surface of the enzyme.[11] The thioester side chain of Met-192 forms the lid to the specificity cavity and interacts with the aromatic side chain of the substrate in unmodified chymotrypsin.[12] For a spin-label in this position, the flexibility of the environment might give an indication of the active site's accessibility to APEE and/or the active site's ability to orient the substrate optimally for catalysis. Conventional ESR spectra reveal that the active-site environments of immobilized and suspended chymotrypsin are not the same even under identical initial solvent conditions. The suspended enzyme produces a very broad line shape indicative of highly immobile spin-label (Figure 2). By comparison, the spectrum of spin-labeled immobilized enzyme reflects much

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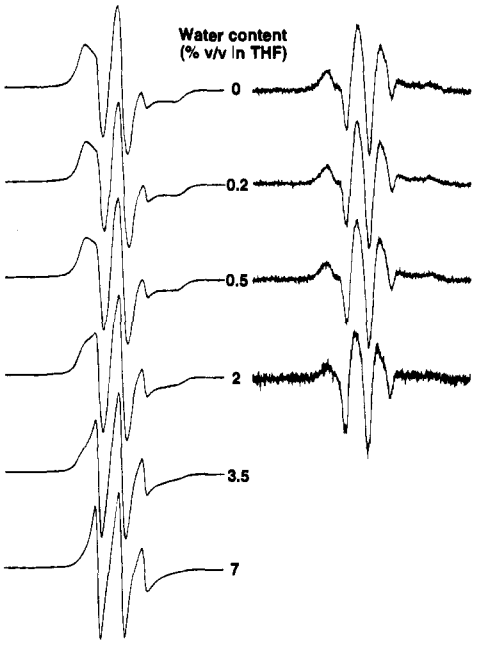

Figure 3. Conventional (left) and saturation transfer (right) ESR spectra of spin-labeled chymotrypsin, immobilized by physical adsorption on glass at an enzyme loading of 5.0% (w/w).

more rapid motion (Figure 3). The dynamics of immobilized chymotrypsin in dry THF are similar to those of freely suspended enzyme in THF containing 3.5–7% v/v water. As mentioned previously, glass-adsorbed chymotrypsin does not contain more water per weight than the suspended enzyme, but the hydrophilic surface of the support could potentially induce a reorganization of water molecules, e.g., the migration of water from more interior regions of the enzyme to the interface between the glass and protein.

With the addition of very small amounts of water, i.e., 0.2–0.5% v/v, the differing behavior of the two systems becomes more apparent. The value of $(k_{cat}/K_m)_{app}$ for suspended chymotrypsin increases 25-fold over this range. This sharp rise in activity is accompanied by an increase in motion in the slower regimes, with spin-label rotational correlation times on the order of hundreds of nanoseconds (Figure 2). Since these τ_R values are near the lower detection limit for conventional ESR, these spectra show little difference except for small amounts of unbound spin-label, corresponding to at most 5% of the signal. However, the ST-ESR (ST = saturation transfer) spectra of suspended enzyme show a continuous increase in spin-label mobility (Figure 2, right column).

Rigid limit computer simulations of the suspended enzyme samples frozen at 140 K reveal no significant changes in the hyperfine splitting constant A_0, the average of the diagonal elements of the Hamiltonian **A** matrix (Figure 4). A_0 is governed by the degree of partial charges in the resonance structures of the nitroxide and hence reflects the "polarity" of the spin-label's environment, i.e., its ability to stabilize charged species. Although the measurements of A_0 are performed with frozen suspensions, the differences observed between samples should nevertheless reflect trends that exist at room temperature. That the value of A_0 remains constant for chymotrypsin suspended in THF with added water indicates either that water cannot access the active site of the enzyme or that any water molecules present there are outside the environment of the spin-label probe. The slow but increasing spin-label motion along with minimal differences in the chemical nature of the environment suggest that 0.2–0.5% v/v added water acts primarily as a lubricant

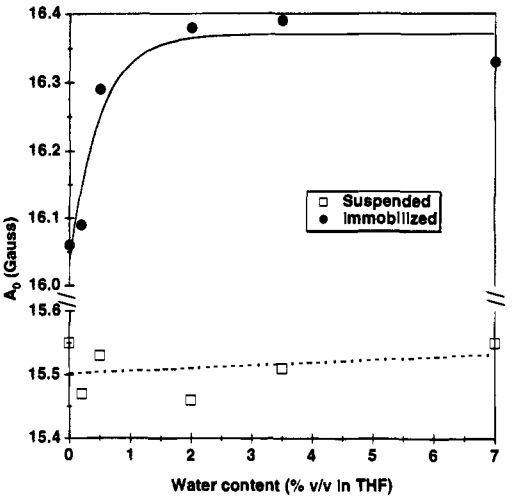

Figure 4. Active-site polarity of freely suspended and immobilized chymotrypsin, as measured by the hyperfine splitting constant A_0 of the spin-label attached to Met-192. Values of A_0 were determined from rigid limit computer simulations of ESR line shapes recorded at 140 K. Spectra of firmly frozen samples were required to minimize motional averaging of the electron–nuclear dipolar interaction.

for freely suspended chymotrypsin and that small increases in active-site flexibility result in major changes in catalytic activity.

This observation is consistent with earlier studies of hydrated biocatalyst powders that helped explicate how low amounts of water affect enzyme function. The general picture that emerged is that lyophilized enzymes are catalytically active provided that all ionic groups and some polar residues are hydrated.[13] Approximately 0.20 g of water/g of protein is necessary for the completion of certain small conformational shifts, which coincides with the onset of enzymatic function.[14] Additional water results in coverage of more surface area, which leads to greater electrostatic shielding and further increases in the active-site flexibility of the enzyme.[15] The enhanced dynamic behavior ultimately results in improved catalytic ability.

While the catalytic efficiency of glass-adsorbed chymotrypsin also increases between 0.2% and 0.5% v/v added water, the changes are not nearly as large. At 0.5% v/v added water, the immobilized enzyme preparation is less than twice as active as the suspended sample. In addition, neither conventional nor saturation transfer ESR detects any differences in spin-label mobility between the dry and partially hydrated (0.5% v/v added water) samples (Figure 3). One factor that might account for the rise in activity is higher active-site polarity for the chymotrypsin on glass. The initial value of the hyperfine splitting constant is significantly higher than that of the dispersed enzyme. Over the range of 0–0.5% v/v added water, the hyperfine splitting constant A_0 increases sharply from 16.07 to 16.28 G, roughly two-thirds its total change (Figure 4). One explanation for the different behavior in active-site flexibility and polarity between the immobilized and freely suspended chymotrypsin is the conformational state of the protein. Physical adsorption of less than a monolayer on glass may reduce unfavorable protein–protein interactions, responsible for structural distortion and/or aggregation, and render the binding pocket more open to small hydrophilic molecules such as water.

Computer simulations of the conventional room-temperature ESR spectra (Figure 5) support the hypothesis that the structural characteristics of the enzyme vary between the freely suspended

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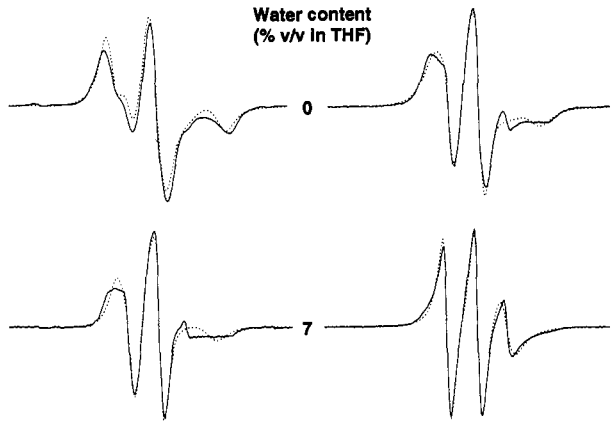

Figure 5. Simulated (dotted lines) and experimental (solid lines) room-temperature ESR spectra of spin-labeled suspended (left) and immobilized (right) chymotrypsin. For experimental details, see the text.

and immobilized preparations. Although the calculated values of the rotational correlation time τ_R in anhydrous THF are similar, both 21−23 ns, the value for suspended enzyme is most likely higher. As mentioned previously, the spectrum of lyophilized chymotrypsin in dry solvent is near the slow-motional limit for the technique; thus, a better method of estimating τ_R involves comparing the central region of the ST-ESR spectrum to the calibration curve for spin-labeled hemoglobin prepared by Thomas *et al.*[16] This analysis yields a τ_R value of about 100 ns (Table 2).

The relative values of the two components of the overall rotational correlation time are also revealing. For immobilized chymotrypsin, $\tau_{\|}$, describing motion parallel to the nitrogen p orbital, is ca. 20 times greater than $\tau_\perp$, the rotational correlation time along the remaining two axes of the molecular coordinate system. In contrast, for the lyophilized enzyme, $\tau_{\|}$ corresponds to rotational diffusion about the N−O bond of the nitroxide, and $\tau_{\|}/\tau_\perp = 6.9$. The differing symmetry axes and values of $\tau_{\|}/\tau_\perp$ suggest that the range of possible spin-label motions varies considerably between the two samples. Hence, the distinct line shapes for the immobilized and suspended enzyme in dry solvent and in organic media with 0.2−0.5% v/v added water appear to arise from conformational differences as well as from variations in protein flexibility.

In an effort to obtain better agreement between the theoretical and experimental spectra, the model of microscopic order and macroscopic disorder (MOMD) was also employed. This model assumes that the spin-labeled species exhibits some degree of order on a local level; however, on a larger scale, the ordered populations of spin-label are randomly distributed in space.[17] The local ordering of spin-label is defined with respect to a preferred direction of alignment in a local region of space, and is governed by an ordering potential. Increased order corresponds to a smaller range of orientations sampled by the motion of the spin-label. For each of the spectra simulated, the ordering potentials were varied and in each case yielded values near zero. As a result MOMD led to no improvement of the simulated line shape fits. Adding a second component also failed to provide uniformly better agreement between the experimental and theoretical spectra. Hence, the simplest model of homogeneous spectra without MOMD was used to fit the line shapes and to obtain estimates of the rotational correlation times.

The two enzyme preparations behave similarly with higher levels of water in the solvent. From 0.5% to 2% v/v water in THF, A_0 for immobilized chymotrypsin increases slightly and then levels off for greater amounts of added water. The polarity for suspended enzyme remains constant, as it did for 0−0.5% v/v added water. Although the catalytic efficiency of the free enzyme is still lower, its increase with added water no longer outpaces that of the glass-adsorbed chymotrypsin. The activities of both samples change by comparable factors, increasing 40-fold between 0.5% and 7% v/v water in THF. Higher mobility of the nitroxide becomes evident for both the suspended and immobilized enzyme. Slow motional computer simulations of these spectra suggest that conformational changes are also occurring.

For immobilized chymotrypsin in THF with 7% v/v water, τ_R is approximately 1.6 ns, an order of magnitude lower than that for the same preparation in anhydrous media. Furthermore, the values of $\tau_{\|}$ and $\tau_\perp$ differ by less than a factor of 2, a small deviation compared to the 20-fold difference seen in dry solvent. The motion of the spin-label has thus become much faster and more isotropic.

The rotational correlation time for freely suspended chymotrypsin in THF with 7% v/v water is roughly 7 ns, and $\tau_{\|}/\tau_\perp = 4.1$. However, the simulated spectrum does not fit the experimental data closely, on the basis of both visual inspection and values of the "goodness-of-fit" parameter χ^2. The assumption of axially symmetric nitroxide motion thus works well for suspended chymotrypsin in the absence of water but not in its presence, implying that the conformational states of the enzyme in the two solvent systems are indeed distinct. Therefore, for both freely suspended and glass-bound enzyme in THF with 2−7% v/v water added, modifications within the protein result in distinct structures with greater active-site flexibility and increased catalytic activity.

Over the range of added water studied, the behavior of enzyme powder and immobilized chymotrypsin in THF differs markedly. The trends observed are summarized in Table 3. In anhydrous solvent, the transesterification activity of the glass-adsorbed biocatalyst is over 10-fold higher than that of the suspended enzyme. Introducing very small amounts of water, up to 0.5% v/v, results in greater catalytic efficiencies for both systems, but that of the freely suspended enzyme increases more dramatically. Physicochemical and dynamic changes accompany the increased activity. For immobilized chymotrypsin, the active site becomes more polar, as measured by the hyperfine splitting constant A_0. Freely suspended enzyme shows instead an increase in flexibility for slow motions on the time scale of tens to hundreds of nanoseconds. The two samples also appear to have different initial structural configurations; that of the immobilized enzyme may involve extensive hydrogen bonding between the support (and/or glass-bound water) and the enzyme. A similar mechanism was originally investigated by Carpenter *et al.* to explain the effect of carbohydrates on proteins upon drying.[18] Using FTIR they showed that such solutes bind to the protein, effectively replacing the water molecules removed and enabling the retention of the protein's native structure. For immobilized chymotrypsin the glass support may provide similar stabilization by occupying water binding sites on the protein surface, freeing water molecules to enter the active site. Immobilization material may also minimize deleterious protein−protein interactions that distort the structure of the enzyme in the suspended state. For higher amounts of added water, from 0.5% to 7% v/v in THF, both enzyme preparations display further, though comparable, improvements in activity due to less restricted enzyme conformations. These results demonstrate

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Table 2. Calculated Rotational Correlation Times for the Nitroxide Spin-Label Attached to Met-192 of Immobilized and Suspended Chymotrypsin in THF

enzyme sample	τ_R (ns)	$\tau_{\|}$ (ns)	$\tau_\perp$ (ns)	$\tau_{\|}/\tau_\perp$	symmetry axis
immobilized, dry	23	108	4.8	22	2p π orbital
immobilized, 7.0% v/v water	1.6	1.3	2.0	0.65	2p π orbital
suspended, dry	21−100	55	8.0	6.9	N−O bond
suspended, 7.0% v/v water	7	14	3.4	4.1	2p π orbital

Table 3. Summary of Suspended and Immobilized Chymotrypsin Behavior in THF with Added Water

amt of water added	suspended enzyme	immobilized enzyme
0% v/v	less active than immobilized enzyme by a factor of ca. 12	more active than suspended enzyme
	less mobile spin-label environment	more mobile spin-label environment
0−0.5% v/v	activity increases sharply	activity increases gradually
	increase in flexibility indicated by ST-ESR	no changes in dynamics evident
	no change in active-site polarity	sharp increase in A_0 spanning 2/3 of total change
0.5−2% v/v	activity increases by the same factor as that of immobilized enzyme	activity increases by the same factor as that of freely suspended enzyme
	no polarity changes	gradual increase and leveling off of A_0
0.5−7% v/v	conformational changes	conformational changes
7% v/v	less active than immobilized enzyme by a factor of ca. 2	

that physicochemical interactions between the enzyme and support play an important role in enzyme structure and activity. Moreover, such interactions are solvent sensitive.

Experimental Methods

Materials. Bovine pancreatic α-chymotrypsin type II, controlled pore glass PG1000-400 (200−400 mesh, 1000 Å pore size), and APEE were obtained from Sigma Chemical Co., St. Louis, MO. 4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxy, dicyclohexylcarbodiimide (DCC), and iodoacetic acid, all reagents in the synthesis of the spin-label, came from Aldrich, Milwaukee, WI. The solvents in the study were THF, *n*-octane, carbon tetrachloride, diethyl ether, toluene, and acetonitrile. All were manufactured by Fisher (Fair Lawn, NJ) and were dried over 3 Å molecular sieves for at least 24 h before use. After such treatment the water contents of the solvents were below 0.02% v/v.

Determination of the Water Content of the Catalyst. The amount of water bound to the lyophilized chymotrypsin powder and the glass-bound enzyme preparation was determined by Karl Fischer titration of the dry solid.[19]

Kinetic Studies. For the lyophilized, suspended enzyme samples, α-chymotrypsin was dissolved in 10 mM sodium phosphate buffer, pH 7.0, at a concentration of 18.3 mg/mL. The pH, which shifted into the acidic range when the protein dissolved, was readjusted to 7.0 with dilute sodium hydroxide. The solution was then freeze dried for 24 h. In this preparation the content of active enzyme in the aqueous solution was 72% of the total protein, as determined by the method of Schonbaum *et al.*[20]

To immobilize the enzyme on controlled pore glass, 100 mg of chymotrypsin was dissolved in 80 mL of 1 mM sodium phosphate (a lower buffer concentration was used in order to obtain approximately the same salt-to-enzyme weight ratio as in the lyophilized preparation, between 7% and 10% w/w), and the pH of the solution was adjusted to 7.0. After 2.0 g of porous glass was added, the slurry was agitated gently on a shaker for 30 min at room temperature. The immobilized enzyme preparation, with a protein loading of 5% w/w, was dried for 5 h under vacuum in a rotary evaporator at 42 °C. Using 1 M KCl to remove the chymotrypsin from the porous glass and titrating the resultant solution yielded an active site concentration of 40%.

The typical reaction mixture contained 10 mg of lyophilized or 250 mg of immobilized enzyme in 5 mL of THF containing 10 mM APEE, 0.85 M 1-propanol, and the desired amount of water. The mixture was placed in a gas-tight vial and incubated at 30 °C with constant shaking. At specified intervals aliquots of the liquid phase were removed for analysis. The total reaction time was about 10 h.

For conditions containing even low amounts of water, hydrolysis of APEE to *N*-acetyl-L-phenylalanine (AP) is a potential competing reaction to transesterification, which produces *N*-acetyl-L-phenylalanine propyl ester (APPE). Both possible products were monitored, the former by HPLC and the latter by GC, and concentrations were calculated using individual calibration curves. Therefore, $(k_{cat}/K_m)_{app}$ was measured as the second-order rate constant for combined hydrolysis and transesterification. To quantify the appearance of any AP, a C_{18} μBondapak HPLC column (Waters Associates, Milford, MA) was used with a flow rate of 1 mL/min and spectrophotometric detection at 257 nm. A linear gradient was run with 1:1 v/v acetonitrile/water and 10 mM ammonium phosphate, pH 2.3. The GC conditions for following the concentration of APPE involved a capillary HP-1 column (25 m long, 0.2 mm i.d.), injector and detector temperatures of 250 °C, and a temperature gradient program from 180 to 250 °C at 25 °C/min.

ESR Studies. The spin-label, 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy, which reacts with Met-192 of chymotrypsin, was synthesized by the DCC coupling of 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy with iodoacetic acid.[21] The product was purified by silica gel chromatography with 2:1 v/v ethyl acetate/toluene and crystallized from 1:1 v/v toluene/hexane.[22]

For the freely suspended enzyme preparation, 100 mg of chymotrypsin was dissolved in 4 mL of 10 mM sodium phosphate, pH 7.0. Twenty-eight milligrams of 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy, corresponding to a 20-fold molar excess of spin-label, was dissolved in 1.5 mL of a 1:1 mixture of acetonitrile and 10 mM sodium phosphate. Half (0.75 mL) of the spin-label solution was used immediately, with the other half being added about 6 h later. The reaction was allowed to proceed at room temperature for 24 h. To remove any remaining free spin-label, the reaction solution was loaded on a Sephadex G-50 gel filtration column. The protein peak was collected and concentrated; 4 mL of spin-labeled chymotrypsin was then freeze dried.

The procedure for the immobilized enzyme samples was similar. To minimize hydrolytic release of the nitroxide probe, the spin-labeled enzyme was immobilized directly from the reaction solution, and excess spin-label was removed after the glass-adsorbed chymotrypsin was dried. After 24 h of spin-labeling reaction, 2.0 g of controlled pore glass (Sigma PG-1000-400, 200−400 mesh, 1000 Å pore size) was added along with additional buffer and allowed to stir for 30−45 min. The final enzyme loading was 5% w/w. The mixture was then evaporated *in vacuo* to dryness. The glass beads were washed with acetonitrile until no ESR signal was detectable in the filtrate. The immobilized chymotrypsin, labeled with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy, was then dried in a vacuum desiccator at room temperature for 24 h.

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ESR samples were prepared by suspending either 2.5 mg of lyophilized powder or 50 mg of immobilized enzyme in 0.5 mL of dry THF, sonicating the mixture for 1−2 min, injecting the appropriate water concentration, and then sonicating again. The solid and entrained solvent were transferred to a glass capillary tube using intramedic tubing attached to a syringe, followed by centrifugation. After removal of excess solvent, the process was repeated until a 2 cm pellet remained at the bottom of the tube. Spectra were collected on a Bruker ER200D-SRC spectrometer at 298 K. A microwave power of 12.6 mW, a modulation amplitude of 1.0 G, and a scan range of 150 G were used for the conventional spectra. For ST-ESR spectra a microwave power of 350 mW and a modulation amplitude of 5.0 G were used to record the second harmonic signal, phased by the "self-null" method.[16] To obtain measurements of the active-site polarity, rigid limit computer simulations of ESR spectra recorded at 140 K were performed using a program provided by J. H. Freed and co-workers, Cornell University, Department of Chemistry.

A nonlinear least squares fitting program, developed in the Freed laboratory, was used to simulate the slow-motional room-temperature spectra and to estimate the rotational correlation time of the spin-label, τ_R. The nitroxide is assumed to undergo axially symmetric reorientation described by a rotational diffusion tensor,[23] $R_{X'} = R_{Y'} = R_\perp$, and $R_{Z'} = R_\parallel$, where X', Y', and Z' coincide with the molecular axes of the nitroxide molecule (the mean correlation time is defined by $\tau_R = [6(R_\perp R_\parallel)^{1/2}]^{-1}$). The unique axis of reorientation can thus be defined as being parallel with a principal axis of the nitroxide, and simulations were carried out and compared for all three possibilities. For three samples, the best fit between experimental and calculated spectra, as measured by χ^2, was obtained by assuming that the rotational symmetry axis is parallel with the 2p π orbital of the nitroxide. In one case, however, the best fit corresponds to a symmetry axis parallel to the N−O bond of the nitroxide (Table 2). To ensure that the final result was a stable global minimum, several different sets of initial values of $R_\perp$ and $R_\parallel$ were used, and all gave very similar final results. The model of MOMD was also incorporated in an attempt to obtain better agreement between theory and experiment.[17]

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