(m, 1 H), 3.37 (dd, 1 H), 3.28 (dd, 1 H), 2.63–0.76 (m, 32 H, C_2-H's, C_3-H's, C_4-H, C_5-H's, C_6-H, C_7-H's, C_8-H, C_{12}-H, C_{13}-H's, C_{14}-H, C_{15}-H's, C_{18}-H, C_{20}-H, C_{22}-H's, C_3'-H's, C_4'-H's, C_3''-H's, C_4''-H's, OH), 1.29 (d, 3 H), 1.25 (d, 3 H), 1.21 (d, 3 H), 1.19 (d, 3 H), 1.15 (d, 3 H), 1.12 (d, 3 H), 1.09 (s, 3 H), 1.08 (d, 3 H), 0.88 (s, 3 H), 0.61 (d, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$, c 10 mg/mL) δ 195.31, 193.69, 182.71, 131.94, 130.66, 100.53, 87.46, 84.45, 82.82, 82.79, 80.89, 76.69, 69.57, 46.86, 42.85, 42.07, 41.81, 41.47, 40.50, 39.95, 39.64, 39.48, 36.59, 34.29, 33.59, 32.99, 32.44, 28.99, 28.52, 27.90, 26.44, 26.34, 23.53, 21.75, 21.17, 21.01, 19.85, 19.45, 19.40, 18.46, 12.20; $[\alpha]_D$ = +31.5° (c 0.232, MeOH). The ultraviolet light absorption spectrum (in 3% aqueous 0.1 M calcium chloride/MeOH) has a maximum at 294 nm. This product co-eluted with an authentic sample of the calcium salt of ionomycin on reverse-phase HPLC (Vydac reverse-phase C_{18} column, 3% 0.1 M aqueous calcium chloride/MeOH, flow rate 2.0 mL/min, t_r = 4.9 min). Anal. Calcd for $C_{41}H_{70}O_9Ca$: C, 65.91; H, 9.44. Found: C, 66.04; H, 9.32.

Acknowledgment. Support has been provided by the National Institutes of Health, the National Science Foundation, and Merck. The NIH BRS Shared Instrumentation Grant Program 1 S10 RR01748-01A1 is acknowledged for providing NMR facilities.

Enzymes as Synthetic Catalysts: Mechanistic and Active-Site Considerations of Natural and Modified Chymotrypsin†

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Abstract: This paper describes the mechanistic investigation of α-chymotrypsin and [Met_{192}-sulfoxide]-α-chymotrypsin-catalyzed peptide synthesis in a kinetically controlled process (i.e., aminolysis) and the relative stabilities of both enzymes in different conditions. Partitioning parameters for various nucleophiles (including D- and L-amino acids) competing with water for the acyl enzyme intermediate were determined. These parameters provide insights into the active-site geometries of both the native and the oxidized enzymes. α-Chymotrypsin with D-isomer selectivity in the hydrolysis of α-methyl-α-nitro esters was used for the synthesis of a D–L pseudopeptide. Molecular modeling together with kinetic results was used to explain the unusual phenomena in hydrolysis and synthesis catalyzed by the native and modified enzymes. α-Chymotrypsin methylated at the ϵ_2-N of the active-site histidine was shown to be an effective catalyst for peptide synthesis in the kinetically controlled process. No peptide bond hydrolysis was observed. Energy diagrams for hydrolyses of activated substrates catalyzed by the native, the methylated, and the organic cosolvent modified enzymes are constructed to understand the effects of methylation and organic cosolvents on catalysis and binding.

In previous papers,[1-8] we have examined various enzymatic systems and attempted to assess these systems for the efficiency in synthesizing certain peptides. These enzymatic systems allow for peptide bond formation in a catalytic regimen under mild conditions, without detectable racemization, and with minimal functional protection.[9] Several problems, however, still remain that hinder the wide acceptance of enzymatic syntheses. High enzymatic specificity often limits the residues between which bonds can be synthesized. Undesired hydrolysis of peptide bonds catalyzed by the enzyme is always troublesome. The optimal conditions for synthesis can be deleterious to the stability of the enzymes, limiting their reuse as catalysts. In addition, enantioselectivity varies or may even be reversed, depending on the substrate employed[10] or the condition used.[9]

One possible solution to some of these problems lies in enzyme derivation, either through chemical or biological means. One example of the use of a derivatized enzyme has already been reported,[3] where methylation of the enzyme active-site histidine converted α-chymotrypsin to a peptide ligase. Both our work and the earlier kinetic work on this protein[11-13] demonstrated that after the methylation the acyl donor binding site (the S_1 subsite) and the nucleophile binding site (the S_1' subsite, see below) are unchanged. The methylated chymotrypsin has been used as a catalyst for synthesis of peptides in a kinetic approach (i.e., aminolysis of amino acid or peptide esters).[3]

To investigate the kinetics of enzyme-catalyzed peptide synthesis, a technique for quantifying the efficiency of amino acid nucleophiles in a kinetic approach is used to examine the reaction of acyl intermediate with available nucleophiles (eq 1). The efficiency of a nucleophile is determined by p as shown in eq 2, where [H] and [P] are the final concentrations of the hydrolysis product (acid) and peptide product, respectively, and [N] is the nucleophile concentration. Determination of p for a given nucleophile at various concentrations allows for the calculation of a partition ratio for the nucleophile, as well as an affinity constant of the nucleophile for the enzyme. Knowing these parameters for different enzyme derivatives also allows for mapping of small

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† This research was supported by NSF Grant CHE 8806182 and DOE (DE-AC0376SF00098) to C.H.W. and Welch Foundation Grant A-328 to E.F.M. A portion of this paper has been taken from the Ph.D. dissertation of J.B.W. from the Department of Chemistry, Texas A&M University.

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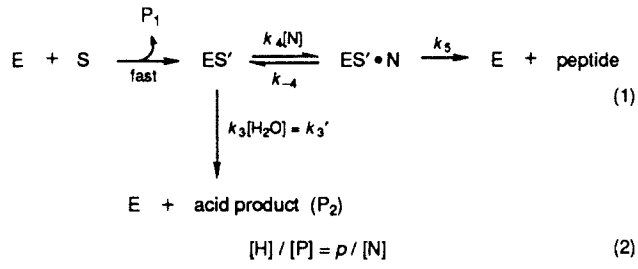

$$[H]/[P] = p/[N] \quad (2)$$

changes in active-site geometries, which may have direct bearing on the synthetic reaction being examined. The kinetics in eq 1 are different from that reported previously,[14] where hydrolyses of both the acyl enzyme and that bound to the nucleophile are considered.

This approach examines the synthetic reaction directly and has several advantages over determining efficiency parameters from the hydrolysis kinetics of the peptide products. First, it is possible to synthesize peptides that are not hydrolytic substrates.[7] Traditional hydrolytic kinetic parameters may not be useful in explaining changes in the enzymes and the effect these changes may have on the synthetic reactions. Second, the conditions we often use (high pH, high concentration of organic cosolvent) may alter the enzyme mechanistically, which may invalidate information derived purely from hydrolytic studies. For example, recent work[1] has demonstrated that the addition of organic cosolvents can selectively lower (or completely eliminate) the amidase activity of serine or cysteine proteases, while considerable esterase activity remains and is useful for peptide synthesis via aminolysis. Previous investigation has indicated that the main reasons for this change are the increase of binding of peptide products to the enzyme in the presence of organic solvents and the increase of partition ratio of aminolysis to hydrolysis of the acyl enzyme intermediate.[5]

One problem encountered in our work with α-chymotrypsin-catalyzed peptide synthesis is the instability of the enzyme to the high pH (>8) necessary for the reaction (so that the nucleophile will be unprotonated). High pH is evidently responsible for a conformational change leaving the enzyme inactive.[15-18] However, an α-chymotrypsin derivative, [Met-192 sulfoxide]-α-chymotrypsin, which remains active and stable up to pH 9, has been shown to be useful for peptide synthesis.[8]

In this paper, the comparative stabilities of α-chymotrypsin and [Met-192 sulfoxide]-α-chymotrypsin are assessed at high pH and in the presence of a high concentration of organic cosolvent. The partitioning method mentioned above is used to map changes in the nucleophilic binding site due to the oxidation of Met-192. Molecular modeling studies are employed to elucidate some of the probable causes for the observed partitioning data. Chymotrypsin with D-isomer selectivity discovered in the hydrolysis of α-methyl-α-nitro esters[10] is used to prepare a pseudodipeptide containing a D-α-methyl-α-nitro subunit; the nitro group is subsequently reduced to the amine via catalytic reduction (Scheme I). Molecular modeling of the active-site interactions of α-chymotrypsin and each of the enantiomers of the α-methyl-α-nitro ester is studied to determine a mechanistic rationale for the observed reversal of enantioselectivity. Finally, α-chymotrypsin methylated at the ϵ_2-N of active-site histidine is prepared, and the kinetic parameters and energetics for hydrolyses of activated substrates catalyzed by both the native and the methylated enzymes are determined to explain the effect of active-site-directed methylation on binding and catalysis. These results are compared to that obtained with the native enzymes in the presence of 50% dimethylformamide.

Experimental Section

General Procedures. α-Chymotrypsin was purchased from Sigma and used as received. Active-site titrations were performed using *N*-β-*trans*-cinnamoylimidazole.[19] All enzyme and derivative preparations were found to be at least 80% active. Computer-aided molecular modeling was conducted using the program FRODO[20] in conjunction with an Evans and Sutherland PS 330 graphic terminal. α-Chymotrypsin methylated at the ϵ_2-N of active-site histidine was prepared according to the procedure described previously.[3]

Enzyme Assays. α-Chymotrypsin activity was assayed spectrophotometrically by measuring the absorbance increase at 405 nm due to the release of the *p*-nitrophenoate ion from Z-L-Tyr-*p*-nitrophenyl ester (Z-L-Tyr-ONp). The assay buffer contained 0.2 M tris-maleate (pH 6.8), 0.1 M $CaCl_2$, and 10% dioxane. The substrate solution was 2.4 mM Z-L-Tyr-ONp in dioxane and a typical assay contained 2.8 mL of buffer, 0.1 mL of substrate solution, and 0.02 mL of enzyme solution.

HPLC Analysis. All yields were determined by HPLC on a Gilson Model 43 chromatograph equipped with a Data Master gradient programmer/data manipulation package. The chromatograph was equipped with a Vydac C-18 column (25 × 0.46 cm) and a variable-wavelength UV detector. The flow rate in all experiments was 1.0 mL/min. Peaks were detected at 280 nm for tyrosine-containing peptides and at 254 nm for all others. Peak identification was made by coelution with compounds authenticated by NMR and/or amino acid analysis. Peak intensities were used to calculate relative concentrations. With a given buffer preparation, retention times varied by no more than 3%. Aliquots of enzymatic reactions (20 μL) were diluted with methanol (at least 20-fold), heated to 60 °C for 2 min to coagulate the enzyme, and centrifuged (10000*g*) for 1 min. The supernatant (15 μL) was then injected. Racemization analysis was performed using an isocratic system. The mobile phase was 0.1 M ammonium acetate (pH 4.6)/methanol (56:44, v/v).

Synthesis of [Met-192 sulfoxide]-α-chymotrypsin. [Met-192 sulfoxide]-α-chymotrypsin was prepared by reaction of 1 g of α-chymotrypsin in 50 mL of water with 2 mL of 50 mM trichloromethanesulfonyl chloride in acetone.[21] The pH of the reaction was adjusted to 3.4, and the reaction mixture was refrigerated overnight. The preparation was repeatedly dialyzed against 1 mM HCl (Spectrapor 2, MW cutoff 12000) and then lyophilized.

Alternatively, the reaction mixture was applied to a BioRad P-2 column and then eluted with 1 mM HCl. Fractions containing active enzyme were identified by rapid color development in a standard Z-L-Tyr-ONp-containing solution. Elution volumes 91–210 mL were collected and then lyophilized.

Characterization of [Met-192 sulfoxide]-α-chymotrypsin. (A) FPLC. Derivatized enzyme preparations were analyzed by FPLC equipped with a Pharmacia phenyl-Sepharose column (1 × 10 cm). Samples were loaded in 1.7 M $(NH_4)_2SO_4$. Gradients of 1.4 M $(NH_4)_2SO_4$ in 20 mM tris-HCl, pH 7.7, to 0 M $(NH_4)_2SO_4$ were run over 35 min.

(B) Cyanogen Bromide Cleavage Analysis.[22] Five milligrams each of α-chymotrypsin and [Met-192 sulfoxide]-α-chymotrypsin were dissolved in 1 mL of 70% formic acid. To each was added 0.01 mL of 20 mg/mL tryptophan and 0.01 mL of 120 mg/mL cyanogen bromide, both in 70% formic acid. Each reaction was stirred to mix and then stored at room temperature for 24 h in the dark. Each reaction was then diluted with 20 mL of water and lyophilized.

Aliquots were analyzed on discontinuous SDS gels (15% acrylamide separating gel, pH 8.8, 2.5% acrylamide stacking gel, pH 6.8). Samples were boiled in loading buffer containing 1% SDS, β-mercaptoethanol, and glycerol. Bands were visualized by Coomassie Blue staining.[23]

Partitioning Analysis. Peptide syntheses were conducted in 0.1 M tris-HCl, (pH 8.9)/50% DMSO. Each reaction contained 5 mM Z-L-Tyr-OMe, 25 mL of a 2 mg/mL enzyme solution (in 1 mM HCl), and 20–150 mM of the nucleophile being analyzed. Ionic strength was maintained at 0.5 by the addition of 5 M NaCl. After 2 min, 40 μL of the reaction was diluted into 0.5 mL of methanol, and the sample was heated to 80 °C to quench the reaction. The sample was centrifuged to remove denatured enzyme and analyzed by reverse-phase HPLC (linear gradients of MeOH in 0.1 M NH_4OAc, pH 5.6, optimized for the particular peptide). Peaks were identified by coelution with standards, the identity of which were confirmed by amino acid analysis. Product ratios

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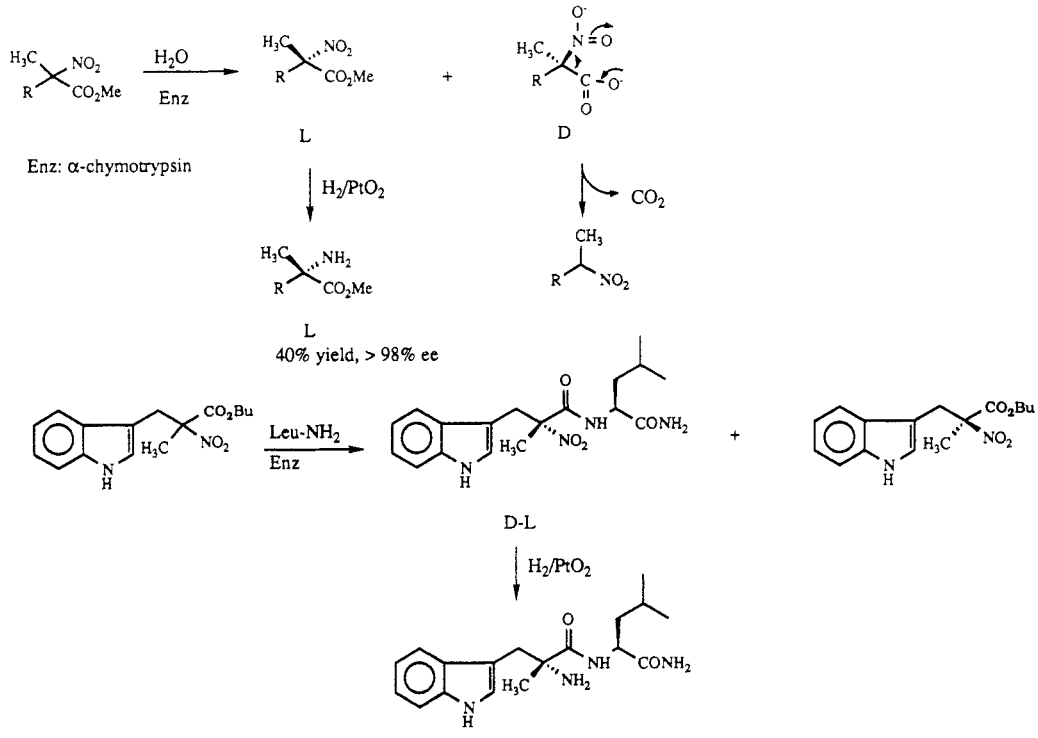
Scheme I



were determined from peak area ratios, scaled to account for different absorbance at 280 nm.

Synthesis of *N*-[D-3-(3-Indolyl)-2-methyl-2-(nitropropionyl)]-L-leucine Amide. To a suspension of butyl 3-(3-indolyl)-2-methyl-2-nitropropionate (47 mg, 150 mmol) in 3 mL of 50 mM phosphate (pH 8.9)/DMF (1:1, v/v) was added L-leucine amide (free base; 39 mg, 300 mmol) and α-chymotrypsin (35 mg, 1750 U). After 14 h, HPLC analysis showed 28% of the desired dipeptide; 21% of the hydrolyzed, decarboxylated byproduct 2-(3-indolyl)nitroethane, and 51% of the unreacted ester. The solvents were removed in vacuo and the residue was triturated with ethyl acetate and filtered. The ethyl acetate solution was dried over sodium sulfate, filtered, and evaporated. The residue was triturated with toluene solution and streaked onto a preparative TLC plate. The plate was developed with benzene/ethyl acetate (1:1, v/v). The product band (R_f 0.24) was removed and the product eluted from the silica gel with chloroform/methanol (1:1, v/v). Evaporation of the solvents yielded 14 mg (51%, based on consumed ester): ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.84 (d, 3 H, J = 4.0 Hz, Leu δ-H), 0.87 (d, 3 H, J = 4.4 Hz, Leu δ-H), 1.40–1.65 (m, 3 H, Leu β-H and γ-H), 1.76 (s, 3 H, Prop α-CH_3), 3.71 (s, 2 H, Prop β-H), 4.39–4.54 (m, 1 H, Leu α-H), 6.07 and 6.28 (2 s, 2 H, Leu NH_2), 6.98 (d, 1 H, J = 1.9 Hz, indolyl H-2), 7.06–7.22 (m, 3 H, indolyl H-5, H-6, and amide NH), 7.25–7.38 (m, 1 H, indolyl H-7), 7.50–7.59 (m, 1 H, indolyl H-4), 8.48 (s, indolyl H-1); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.69, 21.90, 22.86, 24.84, 33.42, 40.65, 52.27, 95.34, 107.58, 111.76, 118.99, 120.33, 122.63, 124.89, 128.15, 136.29, 167.56, 174.75; $[\alpha]^{23}_D$ −34.3° (c 2.3, $CHCl_3$); LRMS, m/z 360 ($M^{\bullet+}$), 204, 184, 130; HRMS, m/z $M^{\bullet+}$ 360.17743, calcd for $C_{18}H_{24}N_4O_4$ 360.17975.

Synthesis of α-Methyl-D-tryptophanyl-L-leucine Amide. A solution of *N*-[D-3-(3-indolyl)-2-methyl-2-(nitropropionyl)]-L-leucine amide (128 mg, 355 μmol) in 10 mL of dry methanol was placed in a 100-mL hydrogenation bottle equipped with a stirring bar. The bottle was purged with argon and 55 mg of fresh platinum dioxide (Adam's catalyst) was added to the reaction mixture. The bottle was then attached to a hydrogenation apparatus, purged five times with hydrogen gas, and pressurized at 50 psi with hydrogen gas. After stirring for 12 h, the excess hydrogen gas was released. The solution was filtered through a Celite pad and evaporated in vacuo. The residue was dissolved in chloroform and applied to a preparative TLC plate. The plate was developed twice with chloroform/methanol (9:1, v/v). The product band (R_f 0.3) was removed and the product eluted from the silica gel with chloroform/methanol (1:1, v/v). After filtration and evaporation, 70 mg (60%) of α-methyl-D-tryptophanyl-L-leucine amide was obtained: ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.89 (d, 3 H, J = 6.0 Hz, Leu δ-H), 0.92 (d, 3 H, J = 6.2 Hz, Leu δ-H), 1.46 (s, 3 H, Tryp α-CH_3), 1.40–1.80 (m, 3 H, Leu β-H and γ-H), 1.82 (br, s, 2 H, Tryp, α-NH_2), 2.80 (d, 1 H, J = 14.3 Hz, Tryp β-H), 3.57 (d, 1 H, J = 14.3 Hz, Tryp β-H), 4.28–4.37 (m, 1 H, Leu α-H), 5.64 and 5.92 (2 s, 2 H, Leu NH_2), 7.01 (d, 1 H, J = 2.0 Hz, indolyl H-2), 7.05–7.20 (m, 2 H, indolyl H-5 and H-6), 7.32–7.37 (m, 1 H, indolyl H-7), 7.59–7.64 (m, 1 H, indolyl H-4), 7.89 (d, 1 H, J = 10.0 Hz, amide NH), 8.72 (s, 1 H, indolyl H-1); ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.65, 24.91, 27.61, 36.44, 40.12, 51.66, 59.29, 109.98, 111.62, 118.59, 119.44, 121.81, 124.05, 128.25, 136.08, 175.48, 177.72; $[\alpha]^{23}_D$ −2.2° (c 0.88, $CHCl_3$); HRMS, m/z $M^{\bullet+}$ 330.2063, calcd for $C_{18}H_{26}N_4O_2$ 330.2056.

Substrate Kinetics. All the substrate kinetics were determined by measuring the initial rate of substrate turnover. For the enzymatic hydrolysis of Suc-Ala-Ala-Pro-Phe-SBzl, each reaction was carried out in 0.1 M sodium phosphate buffer containing 0.1 M NaCl (pH 8.4) with 4% v/v DMF. Each reaction was observed at 324 nm (Beckman DU-70 spectrophotometer) in order to monitor the release of the thiobenzyl leaving group, which reacts with 4,4′-dithiodipyridine[24] (570 μM in each reaction) to form a complex with ε = 15 000 M^{-1} cm^{-1}. The initial rates were then fit directly to the Michaelis–Menten equation using ENZFITTER[25] to generate the kinetic constants. The hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA was carried out in the same way except that to each reaction was added 4% v/v DMF, and the release of the *p*-nitroaniline was monitored at 410 nm (ε = 8638 M^{-1} cm^{-1}). These initial rates were analyzed with ENZFITTER also. For hydrolyses in 50% DMF, the phosphate buffer (0.2 M) was added to an equal volume of DMF and the pH was adjusted to 8.4; the reaction was monitored at 324 nm for the thioester (ε = 17 144 M^{-1} cm^{-1}) and at 400 nm for the amide substrate (ε = 7937 M^{-1} cm^{-1}).

The concentration of the native enzyme was determined by titrating the enzyme with *N-trans*-cinnamoylimidazole.[26] However, since methylchymotrypsin would not turn over this titrant another titrant had to be used. The active ester Z-Tyr-ONp was used and the titration was carried out in a tris buffer containing 50% DMF (0.1 M, pH 7.78, ϵ_{ONp} = 11 942 M^{-1} cm^{-1}).

Boc-Phe-OCH$_2$CN. One gram of Boc-L-Phe-OH was dissolved in 2 mL of triethylamine and 2 mL of chloroacetonitrile. The reaction was stirred for 2 h at room temperature. The mixture was concentrated under vacuum and the residue taken up in 5 mL of ethyl acetate. The ethyl acetate was then washed with 2% Na_2CO_3 (3×), water (2×), 1 N HCl (2×), water (2×), and concentrated brine (1×). The organic phase was dried over $MgSO_4$ and concentrated to dryness. The residue was re-

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Table I. Partitioning Parameters for Nucleophiles for Chymotrypsin and [Met-192 sulfoxide]-α-chymotrypsin-Catalyzed Reactions with Z-L-Tyr-OMe in 50% DMSO, pH 8.9[a]

	α-chymotrypsin		[Met-192 sulfoxide]-α-chymotrypsin	
nucleophile	(k_3'/k_5)[b]	K_N,[c] mM	(k_3'/k_5)[b]	K_N,[c] mM
L-Leu-NH_2	0.06	181	0.09	146
D-Leu-NH_2	0.07	2100	0.23	1900
L-Ala-NH_2	0.09	100	0.18	56
D-Ala-NH_2	1.8	141	3.7	118
D-Met-OMe	0.62	213	2.3	114
Gly-NH_2	0.76	13	0.69	15
Gly-Gly-OMe	0.34	75	0.35	80
GMDA[d]	0.52	520	0.56	285
β-Ala-OMe	4.4	121	5.5	51

[a] All measurements are within 10% accuracy. [b] Where $k_3' = k_3[H_2O]$. [c] Where $K_N = (k_{-4} + k_5)k_4$. [d] Aminoacetaldehyde dimethyl acetal.

crystallized from CH_2Cl_2/hexane to yield Boc-L-Phe-OCH_2CN, 1.0 g (89%): mp 67–68 °C; $[\alpha]_D$ +8.2° (c 2, DMF); ^1H NMR ($CDCl_3$) δ 0.9 (s, 9 H, Boc), 3.0 (m, 2 H, Phe), 4.6 (m, 3 H, αH and cyanomethyl), 5.2 (d, 1 H, amide), 7.2 (m, 5 H, Phe phenyl).

L-Phe-OCH_2CN·HCl. Boc-L-Phe-OCH_2CN (0.5 g) was dissolved in 0.5 mL of a solution of trifluoroacetic acid and anisole (9:1, v/v) under argon and stirred for 30 min. The solvent was concentrated under vacuum, and 3 mL of 4 N HCl/dioxane was added. The solvent was concentrated under vacuum, and anhydrous ether was added to effect crystallization. The solid was collected by filtration and washed with ether. The product was homogeneous by TLC ($CHCl_3$/MeOH/HOAc, 9/1/0.3, (R_f 0.29) as detected by ninhydrin and UV absorption, $[\alpha]_D$ −17.2° (c 1, DMF). This compound was used directly without further purification.

Z-L-Tyr-Gly-Gly-Phe-OCH_2CN. Z-Tyr-Gly-Gly-OH (300 mg, 0.69 mmol) was dissolved in 5 mL of dry THF, and the temperature was lowered to −10 °C in a dry ice/ethylene glycol bath. One equivalent of *N*-methylmorpholine and 1 equiv of isobutyl chloroformate were added with stirring. One equivalent of H-L-Phe-OCH_2CN·HCl (166 mg) and 1 equiv of *N*-methylmorpholine dissolved in 2 mL DMF were added and the solution stirred for 5 min. The reaction was allowed to come to room temperature and then was diluted with 6 volumes of ethyl acetate. The ethyl acetate was washed with cold water (2×), 0.1 N HCl (3×), 0.2 M Na_2CO_3 (2×), water (2×), and concentrated brine (1×). The ethyl acetate was concentrated carefully under vacuum, and the residue was recrystallized from CH_2Cl_2/hexanes to give 260 mg (62%) of Z-Tyr-Gly-Gly-Phe-OCH_2CN. Amino acid analysis: $Tyr_{1.0}Gly_{1.9}Phe_{0.9}$.

Enzymatic Peptide Synthesis. For dipeptide syntheses, 100 mM acyl donor and 200 mM acyl acceptor were dissolved in 1 mL 0.1 M tris-HCl (pH 8.9)/DMSO (1:1, v/v). A 20-mg aliquot of enzyme was quickly added, and the reaction was stirred. At various times, 1.0-mL aliquots were removed, dissolved in 4 volumes of methanol to quench the reaction, centrifuged at 13000*g* for 2 min to remove denatured enzyme, and analyzed by reverse-phase HPLC (C_{18} column) with gradients of methanol in 0.1 M NH_4OAc (pH 5.6). For the synthesis of pentapeptide Z-Tyr-Gly-Gly-Phe-Leu-NH_2 from Z-Tyr-Gly-Gly-Phe-OCH_2CN + Leu-NH_2, these reaction conditions were found to produce only slow acyl donor hydrolysis and little peptide hydrolysis. Z-Phe-Leu-NH_2: amino acid analysis; $Phe_{1.0}Leu_{0.9}$. Anal. Calcd for $C_{26}H_{28}N_3O_4$: C, 70.27; H, 6.53. Found: C, 70.11; H, 6.60. Z-Tyr-Gly-Gly-Phe-Leu-NH_2: amino acid analysis, $Tyr_{0.91}Gly_{1.0}Phe_{1.0}Leu_{0.9}$. Anal. Calcd for $C_{36}H_{44}N_6O_8$: C, 63.34; H, 6.45. Found: C, 63.50; H, 6.60.

Results

Characterization of [Met-192 sulfoxide]-α-chymotrypsin. Hydrophobic interaction chromatography on the Pharmacia phenyl-Sepharose FPLC column proved to be a sensitive assay for the derivatized enzyme. Under the conditions reported, native α-chymotrypsin eluted in 32 min, while the oxidized enzyme eluted in 22 min. The preparation of the derivative showed no detectable native enzyme remaining.

This result was confirmed by cyanogen bromide cleavage analysis. Native α-chymotrypsin consists of three peptide chains, with Me-192 about midway along the C chain (residues 147–245) with a molecular weight of approximately 10000. Cleavage at this methionine will result in two peptides of less than 6000 daltons (Da), and the 10000-Da band will disappear. However, oxidation of this residue to the sulfoxide will render it inert to cyanogen bromide. Thus, with [Met-192-sulfoxide]-α-chymotrypsin the C chain should remain intact. (The only other methionine residue in α-chymotrypsin is at position 180, also on the C chain. However, this residue is not accessible to solvent and should not react in the nondenatured enzyme.) Analysis of the cleavage products by electrophoresis revealed that the C chain was intact in the oxidized preparation, while the band at 10000 MW was completely absent in the reaction with the native enzyme, indicating that the derivatization had occurred as planned.

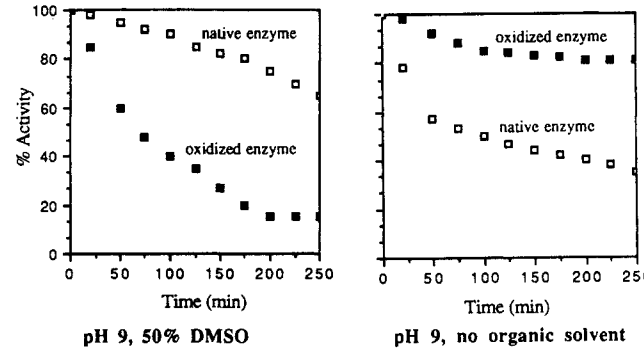

Figure 1. Relative stability of α-chymotrypsin and [Met-192 sulfoxide]-α-chymotrypsin to high pH (pH 9) (right) and high concentration of organic cosolvent (50% DMSO, pH 9) (left). Open squares are the native enzyme and solid squares are the oxidized enzyme.

Partitioning Analysis. This technique is based on the kinetic mechanism presented in eq 1, where binding of the nucleophile is taken into account.[27] The ratio of the acid to peptide products can be expressed as

$$\frac{[H]}{[P]} = \frac{k_3'[N] + k_3'K_N}{k_5[N]} = \frac{p}{[N]} \quad (3)$$

This equation can be rewritten as

$$p = \frac{k_3'}{k_5}[N] + \frac{k_3'K_N}{k_5} \quad (4)$$

A plot of p vs [N] will yield a line of slope k_3'/k_5, ($k_3' = k_3[H_2O]$), which is the ratio of the rates of formation of the hydrolysis product and the peptide product. The line will have a y intercept of $k_3'K_N/k_5$, where K_N is equal to $(k_{-4} + k_5)/k_4$. K_N is very similar to a K_M for a normal enzyme reaction. Notice that when $k_5 \ll k_{-4}$, K_N is roughly equal to k_{-4}/k_4, the dissociation constant for the ES′·N complex. Therefore, the K_N value is a good parameter to use for comparison of enzyme affinity. Comparison of these two parameters for a given nucleophile with different enzyme derivatives allows for direct comparison of the geometries of the S_1' binding site, which in kinetic controlled synthesis is the most important factor influencing the yield of peptide product.[28] The values of these partitioning parameters for various nucleophiles in the α-chymotrypsin-catalyzed reaction with *N*-carbobenzoxy-L-tyrosine methyl ester are listed in Table I. As indicated in Figure 1, [Met-192 sulfoxide]-α-chymotrypsin is more stable to high pH than the native enzyme, as expected, but in the presence of 50% DMSO it is much less stable.

Molecular Modeling of the Synthesis of L–D Dipeptides. In our work, we have demonstrated that the D-amino acid esters (or amides) are effective nucleophiles in kinetic approach synthesis, although less efficient than the L isomers. The L–D dipeptide produced was not a hydrolytic substrate for the enzyme (the rate for hydrolysis of L–D peptides was extremely slow, and the K_m value was higher than 1 M, which was difficult to determine), affording irreversible peptide formation.[18] We have used computer-aided molecular modeling in an attempt to rationalize this result. This allowed us to examine possible conformations of

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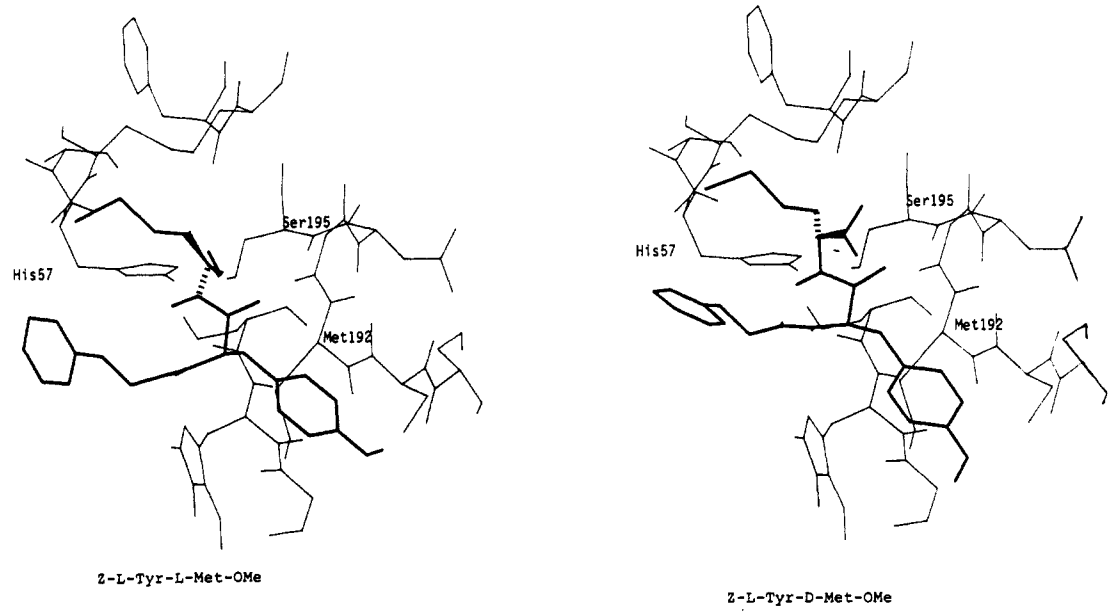

Figure 2. Binding of Z-L-Tyr-D-Met-OMe and Z-L-Tyr-L-Met-OMe to the active site of α-chymotrypsin. The reactive carbonyl is constrained to within 2.5 Å of the serine-195 hydroxyl.

Z-L-Tyr-D-Met-OMe[29] in the active site of α-chymotrypsin. The aromatic side chain was placed in the hydrophobic pocket and in such a way that the tyrosine amide forms the putative hydrogen bond with Ser-214. No conformation could be found for the dipeptide that would allow the labile carbonyl to be brought within 2.5 Å of the Ser-195 hydroxyl, as thought to be necessary for acyl enzyme formation,[30] without forming unacceptably close contacts with some part of the protein, mainly the disulfide bridge between Cys-42 and Cys-58 (Figure 2).

Synthesis of D–L Pseudopeptides. The D-isomer selectivity of α-chymotrypsin in the hydrolysis of α-methyl-α-nitro esters[10] allowed us to use this reversal of enantioselectivity to prepare a pseudodipeptide containing the D-α-methyl-α-nitro subunit. α-Chymotrypsin-catalyzed enantioselective coupling of butyl 3-(3-indolyl)-2-methyl-2-nitropropionate and L-leucine amide in a buffered 50% aqueous DMF solution produced the D–L dipeptide in >90% diastereomeric excess. When the reaction was allowed to continue for 40 h, a small amount of the corresponding L–L diastereomer was isolated. The overlapping resonances for the α-methyl groups were easily separated with base-line resolution by using 100–150 mol % $Eu(hfc)_3$ in deuteriochloroform. Under these conditions the absorbance for the α-methyl group of the L–L diastereomer was observed downfield from the corresponding signal for the α-methyl group of the D–L diastereomer. Catalytic hydrogenation of the nitro group yielded α-methyl-D-tryptophanyl-L-leucine amide.

Molecular Modeling of the Hydrolysis of α-Methyl-α-nitro Esters. Molecular modeling methods were employed to explore structural features of substrate and ligand complexes that could explain the reported reversed enantioselectivity observed for the α-methyl-α-nitro ester substrates based upon the binding constraints of the tryptophan side chain placed in the S_1 pocket and the carbonyl oxygen atom placed in the oxyanion hole. Conversely, the methoxy group would not fit into the oxyanion hole, thus further supporting this orientation. Figure 3 shows the most plausible spatial relationships between the L and D isomers of methyl 3-(3-indolyl)-2-methyl nitropropionate and the active-site residues of α-chymotrypsin. These models indicate that upon binding of the L antipode, the α-nitro group interferes with the enzyme's proton relay system. The serine hydroxyl shows a H-bonding interaction with an oxygen atom of the nitro group. This interaction may prohibit the histidine nitrogen from extracting the serine hydroxy proton, i.e., the general-base catalysis is inhibited. With the D isomer bound, α-chymotrypsin's proton relay system is unimpeded.

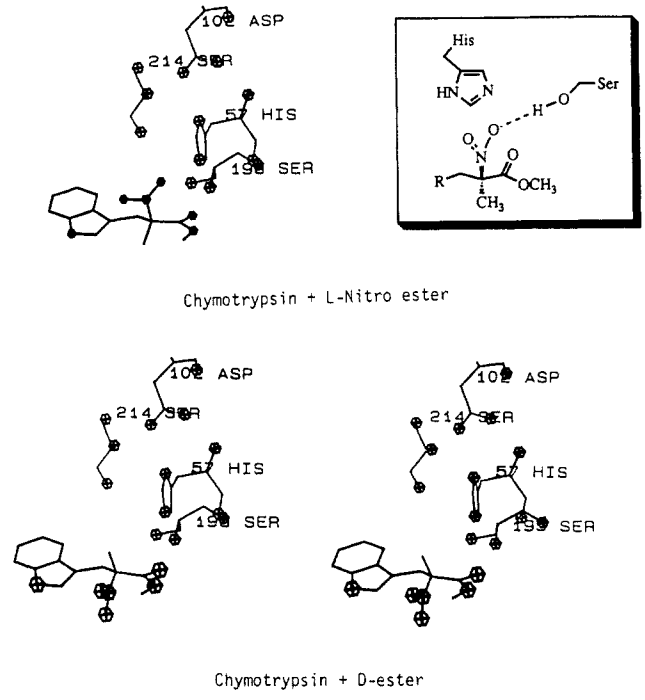

Figure 3. Stereoscopic view of L-nitro acid methyl ester and D-nitro acid methyl ester binding to the active site of α-chymotrypsin. The H-bonding interaction between the nitro group of the L-nitro acid ester and the hydroxyl group of Ser prevents the general-base catalysis required for hydrolysis of the L ester.

Reactions with Methylchymotrypsin. Methylchymotrypsin (MeCT) was prepared and used as catalyst in peptide synthesis. The results obtained in two representative syntheses are shown in eqs 5 and 6.

$$\text{Z-Phe-OCH}_2\text{CN (0.1 M)} + \text{Leu-NH}_2\text{ (0.2 M)} \xrightarrow[40\text{ min}]{\text{MeCT}} \underset{88\%}{\text{Z-Phe-Leu-NH}_2} \quad (5)$$

$$\text{Z-Tyr-Gly-Gly-Phe-OCH}_2\text{CN} + \text{Leu-NH}_2 \xrightarrow[80\text{ min}]{\text{MeCT}} \underset{99\%}{\text{Z-Tyr-Gly-Gly-Phe-Leu-NH}_2} \quad (6)$$

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Table II. Kinetic Parameters for Native, 50% DMF Modified Chymotrypsin, and Methylchymotrypsin Catalyzed Hydrolyses of Suc-Ala-Ala-Pro-Phe-X (Where X = pNA and SBzl)[a]

	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}	k_2, s^{-1}	K_S, M^{-1}
native					
Suc-AAPF-pNA	46 ± 2	86 ± 9	5.35×10^5	76.5	1.43×10^{-4}
Suc-AAPF-SBzl	116 ± 1	30 ± 2	3.92×10^6		
MeCT					
Suc-AAPF-pNA	0.020 ± 0.005	270 ± 15	75	0.0312	4.15×10^{-4}
Suc-AAPF-SBzl	0.059 ± 0.002	415 ± 75	142		
in 50% DMF					
Suc-AAPF-pNA	8.5 ± 2	270 ± 34	3.148×10^4	9.3	2.96×10^{-4}
Suc-AAPF-SBzl	98.2 ± 16	2280 ± 400	4.31×10^4		

[a] k_2 is the rate constant from ES complex to the acyl intermediate and K_S is the dissociation constant of enzyme–substrate complex.[42]

Table III. Free Energy Values for Native, 50% DMF Modified Chymotrypsin, and Methylchymotrypsin Action on Suc-Ala-Ala-Pro-Phe-X (Where X = pNA and SBzl)

	relative free energies,[a] kcal/mol				
enzyme	$\Delta G°$ (ES)	$\Delta G^‡$ (k_2)	$\Delta G^‡$ (k_3)	$\Delta G^‡$ (k_{cat}/K_m)[b]	$\Delta G°$ (ES′)
native	2.94	14.87	14.62	16.63	2.01
MeCT	3.56	19.49	19.11	22.68	3.57
in 50% DMF	3.36	16.12	14.72	19.30	4.58

[a] All free energies calculated are relative to the energy of the free enzyme assigned 0 kcal/mol. Calculations are described in ref 42. [b] For the ester hydrolysis.

Kinetics and Energetics. Kinetic parameters and free energy changes for the native, 50% DMF modified chymotrypsin, and methylchymotrypsin catalyzed hydrolyses of a thioester and an amide substrate were determined and shown in Tables II and III. It was observed that both methylation and addition of DMF have similar effects on the enzyme catalysis and binding. The acyl intermediate in both modification cases becomes less stable, making the aminolysis reaction more favorable (i.e., the energy barrier for the formation of acyl intermediate–amine complex for aminolysis is lower). In the direction of peptide hydrolysis, both modified enzymes, however, experience a higher energy barrier for breaking the peptide bond to form an acyl intermediate. It should be noted that the dissocation constant for the peptide product–enzyme complex depends on the polarity of the peptide product.[6] Charged or hydrophilic peptides bind to the methylated enzyme more tightly than to the native enzyme. The same situation was observed upon addition of DMF to the system. Hydrophobic peptides such as those used in this kinetic study, however, bind to the modified enzymes less tightly than to the native enzyme.

Discussion

α-Chymotrypsin and [Met-192 sulfoxide]-α-chymotrypsin. In traditional enzymology, active-site geometries can be elucidated from the Michaelis–Menten parameters of similar but distinct substrates. The amino acyl subsite specificities of proteases, for example, can be determined from the hydrolytic parameters of various peptide substrates[31] to produce a qualitative picture of this active site prior to the existence of high-resolution X-ray data. In the kinetically controlled peptide synthesis, however, it is possible to produce peptides that are not hydrolytic substrates for the enzyme. Our studies on the hydrolysis of the L–D dipeptide, Z-L-Tyr-D-Met-OMe, with α-chymotrypsin and computer modeling of the interaction indicate that binding of the L–D dipeptide is energetically unfavorable, consistent with the observation made by us and others[32] that various amino acyl nucleophiles exhibit no competitive inhibition on the hydrolysis of Z-L-Tyr-ONp. It is clear, therefore, that when examining the question of synthetic utility of a given protease or derivative, the kinetic parameters derived from hydrolytic reactions must be considered carefully and applied to the synthetic processes properly. The application of the partitioning analysis allows one to determine enzyme parameters that will exactly describe the synthetic reaction.

In the kinetically controlled peptide synthesis, peptide yield is ultimately dependent on the efficiency of the nucleophile in deacylating the acyl enzyme intermediate, relative to water. This efficiency varies greatly and is obviously due to the structure of the nucleophile rather than differences in basicity, as all of the amines considered have comparable pK_a's. Differences in efficiency are evidenced in the two parameters discussed here, affinity of the nucleophile for the enzyme and, once bound, reaction with the acyl enzyme. It is possible for a nucleophile to bind tightly, but in such an orientation that it cannot effectively attack the acyl enzyme, as was found for L-Arg-OMe[33] in chymotrypsin-catalyzed synthesis. Conversely, the nucleophile may bind in an orientation such that it can effectively compete with water, but its affinity for the enzyme may be low. This later case is demonstrated in the comparison of L-Leu-NH_2 and D-Leu-NH_2 in Table I. Both nucleophiles exhibit nearly identical partitioning ratios, but the D-amino acid binds some 10-fold less tightly. This explains the need for higher nucleophile concentrations for high yields of peptide when using D-amino acids as acyl acceptors. The situation is quite different for the two enantiomers of alanine amide, because both isomers have similar binding affinities but the nucleophile-bound acyl enzyme of the L isomer partitions to peptide formation more often than the D isomer, and as a result, the peptide synthesis is more favorable for the L nucleophile. It is not readily apparent why the partitioning for D-alaninamide should be so much farther toward hydrolysis than that for D-leucinamide. As indicated in Table I, aminolysis solely depends on the specific structural feature of the nucleophile rather than its hydrophobicity, as seen in the catalysis of human elastase.[34]

In our experiments, no peptide synthesis was seen for proline methyl ester, even at extreme nucleophile concentrations (>2 M). Peptides with the amino acids proline and sarcosine at the S_1' position are known not to be hydrolytic substrates, due to the consequences of the stereoelectronic effect on the breakdown for the second tetrahedral intermediate (the amide nitrogen pyramidization must invert for the carbon–nitrogen bond to break, and this is impossible for the secondary amine of proline or sarcosine).[35] This is also evident in the synthetic direction at the crucial, irreversible step of bond making and breaking.

These same data can be used to compare changes in active-site conformation brought about by the oxidation of Met-192, which is known to make up part of the S_1' (or leaving group) subsite.[36] Oxidation to the sulfoxide will render the residue more hydrophilic, and it is likely that the side chain will move out toward the solvent;[37] this can be expected to alter the geometry of the S_1' site. This is evident from comparing the parameters for a given nucleophile. The oxidized enzyme, in general, has altered the native chymotrypsin by increasing the affinity of the nucleophile to the acyl enzyme. The oxidized enzyme exhibits twice the affinity for D-Met-OMe as does the native enzyme, and the partitioning ratio is 4 times higher in the hydrolytic direction. On the other hand,

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with the leucine amides, the oxidized enzyme has not changed substrate or nucleophile affinity but rather made the D-isomer reaction more favorable toward hydrolysis. With the alanine amides the partitioning ratio changes equally for each isomer but the affinity is now more in favor of the L isomer. With the glycine nucleophiles there is not much change observed at all, which is not surprising since glycine does not have structural isomers.

Replacing the carboxamide function with a dimethyl acetal (from glycinamide to aminoacetaldehyde dimethyl acetal) lowers the nucleophile affinity for both enzymes, while the partitioning ratio remains fairly constant for both enzymes. By displacing from the carbonyl by one methylene group (β-Ala-OMe) the partitioning ratio is in favor of hydrolysis with little effect on binding. In general, this modification, which gives one advantage (stability of the catalyst at high pH), does not drastically affect the overall synthetic utility; in fact, the efficiency is dependent upon the individual amino acid amides used. We have also determined that [Met-192-sulfoxide]-α-chymotrypsin is generally less stable to the reaction conditions (here, the high concentration of organic cosolvent). Thus, this particular derivative may not be an ideal catalyst for synthesis in many cases.

The use of partitioning analysis allows for the rapid characterization of various peptide synthesis systems from two different directions. With a given protease, the ability of an amino acid nucleophile to compete with water for the acyl enzyme (which in the kinetic approach will determine the yield of peptide) may be directly measured, even in cases where the peptide produced is not a hydrolytic substrate. When closely related proteases, such as a chemical or genetic derivative and the native enzyme, are compared, changes in active-site geometry or chemistry that will have a direct effect on the synthetic reaction can be assessed.

Molecular modeling of the enzyme–substrate interactions provided reasonable explanations for the observed kinetic and enantioselective characteristics of α-chymotrypsin with unusual substrates. Poor binding of L–D peptide products was identified as the probable cause for the irreversible formation of L–D dipeptides in α-chymotrypsin-catalyzed syntheses employing D-amino acid nucleophiles. The proximity of the nitro group of L-α-nitro-α-methyl esters with the α-chymotrypsin proton relay system was inferred to be the cause of the lack of enzyme-catalyzed hydrolysis of these esters. The absence of such interactions between the D-α-nitro-α-methyl esters and α-chymotrypsin explained the ability of α-chymotrypsin to hydrolyze these substrates, albeit at a relatively slow rate.

Catalysis of Chymotrypsin Affected by Methylation and Organic Solvents. This study and previous investigations have proven that MeCT is an effective catalyst for peptide synthesis via aminolysis of peptide cyanomethyl esters.[3] Although the rate of deacylation of MeCT catalysis decreases by a factor $\sim 10^5$ compared to the native enzyme reactions,[11] the ratio of hydrolysis/aminolysis decreases substantially as seen in the synthetic reactions.[3] In this study, the k_3'/k_5 ratio for a specific reaction between Z-Tyr-OMe and Leu-NH_2 was determined to be 0.01 for MeCT and 0.06 for the native enzyme. Similar results were also observed previously for MeCT-[13] and methylsubtilisin[5]-catalyzed aminolysis. In the rate-determining deacylation of the MeCT reaction, a base with $pK_a = 7$ was identified as a general base.[11] Our recent ^{13}C NMR study also indicated that with Z-Tyr-OMe acylation of MeCT did occur at the hydroxyl group of Ser-195.[3] All these results seem to support the general-base-catalysis mechanism, which requires ring flipping of the methylated imidazole (Scheme II). The use of methylated N as a general base ($pK_a \sim -7$), however, was suggested by Byers and Koshland[13] and by the X-ray crystal structure of MeCT.[38] The ring-flipping mechanism in MeCT-catalyzed ester hydrolysis, first suggested by Henderson[11] and later supported by studies on solvent isotope effects,[13] proton inventories,[39] and model systems,[40] is consistent with the results observed

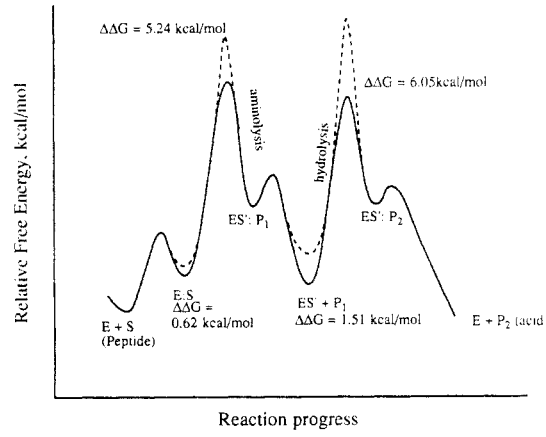
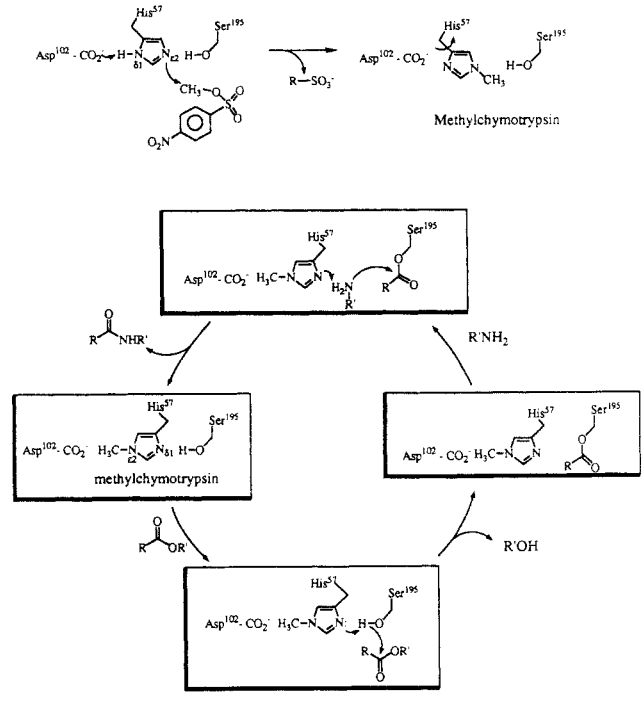


Figure 4. Energy diagrams for hydrolyses catalyzed by the native (solid line) and methylated (dashed line) chymotrypsin. See Tables II and III for determination of kinetic parameters and free energy changes. The $\Delta\Delta G$ for aminolysis was determined from the hydrolysis of the activated amide substrate, which may be different from the real value in peptide synthesis.

Scheme II



in the preparative aminolysis. The aminolysis of acyl enzyme intermediate may proceed through abstraction of a proton from the neutral amine (RNH_2) with a concurrent nucleophile attack at the acyl intermediate. The slight change in the orientation of Asp-His-Ser groups after methylation and ring flipping may account for the favorable aminolysis vs hydrolysis for MeCT. Model studies based on the X-ray structure of MeCT did indicate that the water molecule responsible for deacylation would have difficulty approaching the acyl enzyme carbonyl.[35] A direct aminolysis of the acyl enzyme with the neutral amine, however, cannot be ruled out. It has been noted that the acyl enzyme intermediate is much like an active ester as suggested by the Hammett ρ values for the enzyme deacylation and saponification of p-nitrophenyl esters, which are 2.1 and 2.04, respectively.[41] Further studies of solvent isotope effect on the aminolysis may clear this point.

In summary, on the basis of the kinetic data (k_{cat}, K_m, and k_{cat}/K_m) and the corresponding free energy changes of methyl-

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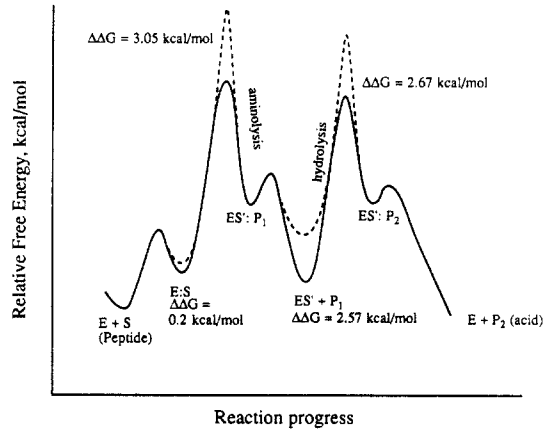

Figure 5. Energy diagrams for hydrolyses catalyzed by chymotrypsin in aqueous solution (solid line) and in 50% DMF solution (dashed line). See Tables II and III for parameters.

chymotrypsin-catalyzed hydrolyses of ester and amide substrates along with aminolysis studies, we begin to understand why the active-site-methylated serine protease is an effective catalyst for peptide synthesis. When the enzyme is methylated, the transition-state energy required for ester hydrolysis increases more than the energy required for aminolysis. The acyl intermediate of methylchymotrypsin is relatively less stable than that of the native enzyme, as calculated from the k_{cat} values of ester hydrolysis and the known transition-state energy change. The energy barrier for the formation of acyl intermediate–nucleophile complex for aminolysis is therefore lower. In addition, the affinity of the peptide product to the methylated enzyme increases for charged or hydrophilic products (data not shown). These two factors cause the peptide formation to be a more irreversible process and account for the favorable aminolysis vs hydrolysis in the methylenzyme-catalyzed aminolysis. Energy diagrams (Figure 4)[42] for hydrolysis of activated substrates (Tables II and III) catalyzed by both the native and methylated chymotrypsin provide some insights into the effect of methylation on catalysis and binding. It should be noted that since synthetic peptides are not substrates for methylchymotrypsin, the $\Delta\Delta G$ for aminolysis was obtained from the hydrolysis of an activated amide. This energy difference for aminolysis, therefore, may not reflect the real value in aminolytic peptide synthesis. Similar situations were observed in the case of organic cosolvent mediated catalysis. As indicated in Figure 5, addition of DMF destabilized the acyl intermediate and enhanced the aminolysis reaction.

Acknowledgment. We thank Dr. Thomas R. Sharp for obtaining the high-resolution mass spectral data and the National Science Foundation (Grant CHE-8705697) for providing funds to purchase the VG analytical high-resolution mass spectrometer.

(42) With serine proteases it has been established that with ester substrates the rate-limiting chemical step is deacylation[43] of the acyl enzyme (ES'), or k_3. By use of k_3 and the k_{cat} determined for the amide substrate, k_2 (from ES complex to the acyl intermediate) can be determined based on $1/k_2 = 1/k_{cat}(\text{amide}) + 1/k_3$. Once k_2 is calculated, K_2, the ES disassociation constant is determined by dividing k_2 by the second-order rate constant, k_{cat}/K_M (for the amide substrate). All the first-order rate constants were converted to free energies of activation by using the Eyring equation or transition-state theory.[44] The second-order rate constants and equilibrium constants were normalized to a 1 μM standard state before relative energies were calculated.[45] The equilibrium constants were converted to calories by using the thermodynamic relationship, $\Delta G^\circ = -RT \ln K$. The relative energy difference for ES', as compared to the free enzyme, can be estimated[46] by subtracting the free energy of activation for the ester k_{cat}/K_M [$\Delta G^\ddagger(k_{cat}/K_M)$] from the free energy of activation for k_{cat} [$\Delta G^\ddagger(k_{cat})$], that is, $\Delta\Delta G^\circ$ (ES') = $\Delta\Delta G^\ddagger(k_{cat}/K_m) - \Delta\Delta G^\ddagger(k_{cat})$.

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Selective Cleavage of Trityl Protecting Groups Catalyzed by an Antibody†

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Received January 11, 1990

Abstract: Monoclonal antibodies have been produced that catalyze the cleavage of trityl protecting groups at neutral pH. These antibodies were induced by immunizing mice with a positively charged tris(4-methoxyphenyl)phosphonium hapten. With a 4,4',4''-trimethoxytrityl ether substrate, a rate enhancement (k_{cat}/k_{uncat}) of 270 was observed. Several other trityl ether derivatives and even a trityl thioether compound were accepted as substrate. The pH–rate profile of the reaction suggests that the observed rate acceleration is not the result of general-acid catalysis in the antibody binding site, but probably derives from electrostatic stabilization of a positively charged transition state.

A relatively large number of different chemical transformations have now been catalyzed in an antibody binding pocket.[1-11] Exquisite and predictable substrate specificity, including stereospecificity, has been observed consistent with the structure of the original immunizing hapten. We now report the production of an antibody capable of catalyzing the cleavage of triphenylmethyl (trityl) protecting groups[12,13] at neutral pH. This work represents the first examples of ether and thioether cleavage catalyzed by an antibody. In theory, a set of chemically similar yet structurally distinct protecting groups can be developed, each one cleavable by a specific antibody at neutral pH. This "recognition-based

† Contribution No. 6226-MB from the Research Institute of Scripps Clinic.

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0002-7863/90/1512-5320$02.50/0