BIOORGANIC CHEMISTRY 7, 15–33 (1978)

On the Mechanism of Action of Methyl Chymotrypsin[1]

L. D. BYERS[2] AND D. E. KOSHLAND, JR.

Department of Biochemistry, University of California, Berkeley, California 94720

Received May 19, 1977

The properties of α-chymotrypsin methylated at histidine-57 were examined to explain the mechanism of this enzyme which is about 10^5 times less active than chymotrypsin. Studies on the protein showed (i) an alteration in the acyl and leaving group specificity, (ii) decreased binding of some protein protease inhibitors by methyl chymotrypsin, (iii) lack of dimerization of methyl chymotrypsin at low pH, (iv) decreased stability of methyl chymotrypsin in urea, (v) a larger solvent deuterium isotope effect with methyl chymotrypsin, and (vi) decreased binding of a tetrahedral intermediate analog to methyl chymotrypsin. These properties suggest that while only subtle alterations occur in the active site upon methylation of His-57, the transition state and the tetrahedral intermediate are destabilized but not to the same extent. General base catalysis remains an integral feature of the hydrolytic mechanism of the modified chymotrypsin, and the base appears to be the methylated nitrogen of the imidazole moiety of His-57.

INTRODUCTION

The catalytic power of enzymes is a subject of great fascination, particularly in the current era when crystallographic structures are available and a great deal is known about the physical organic chemistry of enzyme catalysis. One of the most intriguing clues to the mechanism of enzyme action was contributed by the synthesis of methylated chymotrypsin [where the enzyme is specifically methylated at the N-3 ($N^{\epsilon 2}$ or N^{τ})-position of His-57] by Nakagawa and Bender (*1*) and the findings of Henderson (*2*) that this methylated chymotrypsin has some activity, albeit very small. In view of the central role of histidine-57 in all theories of chymotrypsin action, the rationalization of the properties of this modified enzyme are particularly important. They not only demand explanation in terms of any general theory of enzyme action, but have the capability of assigning quantitative values to the various components in the mechanism of chymotrypsin. Despite extensive and excellent investigations by a number of workers (*3*) it seemed that some further studies of this protein were in order, and therefore a number of experiments were performed as described below. These experiments, together with the studies previously published, have allowed us to propose an explanation for the two crucial observations: viz., (a) methyl chymotrypsin is far less effective than chymotrypsin as a catalyst and (b) methyl chymotrypsin retains significant catalytic power.

[1] A preliminary report of this work was presented at the 168th American Chemical Society, National Meeting (1974). This work was supported by USPHS Grant AM09765 and National Science Foundation Grant BMS 71-0133A03.

[2] Present address: Department of Chemistry, Tulane University, New Orleans, Louisiana 70118. USPHS Fellow (5 FO2 GM 49094-02).

0045-2068/78/0071-0015$02.00/0

MATERIALS AND METHODS

Materials

Enzymes. α-Chymotrypsin was obtained from Worthington Biochemical Corp. (Lot CDI 2DB, sp. act. = 69 U/mg). Solutions of enzymes were freshly prepared in appropriate buffers before use. Methyl chymotrypsin was prepared essentially by the Henderson (*2*) modification of the procedure of Nakagawa and Bender (*1*) except that the methyl chymotrypsin was separated from PMS-chymotrypsin[3] and denatured protein by affinity chromatography by the method of Ako *et al.* (*4*) and lyophilized. Anhydro chymotrypsin was prepared by the method of Strumeyer *et al.* (*5*) and purified by the method of Ako *et al.* PMS-chymotrypsin was prepared by the method of Fahrney and Gold (*6*). Chymotrypsinogen (Worthington) was treated with PMSF to remove trace contaminants of active enzyme.

Protein inhibitors. Lima bean trypsin inhibitor was obtained from Worthington Biochemical Corp. (Lot LBF 33P648). Bovine pancreatic trypsin inhibitor (Kunitz) was obtained from Sigma Chemical Co. Potato inhibitor I was a generous gift of Dr. C. A. Ryan.

Substrates. Ac-TyrOEt and Z-TyrOEt were obtained from Mann Research Laboratories. ρ-Nitrophenylacetate was obtained from Aldrich Chemical Co. Z-L-PheONP was obtained from Sigma Chemical Co., and Z-L-TrpONP was obtained from Cyclo Chemical Co. ρ-Nitrophenyl ρ-trifluoromethyl benzoate was a gift of Dr. J. F. Kirsch. Bis(ρ-nitrophenyl)carbonate was obtained from Sigma Chemical Co.

Inhibitors. Proflavin was obtained from Mann Chemical Co. and purified by the method of Marini and Caplow (*7*). *N*-Methyl-*N*-acetyl-L-tyrosine methyl ester was prepared by the method of Peterson *et al.* (*8*) and had a mp[4] = 132–133°C (lit. mp = 132.5–133°C). Phenylethaneboronic acid was prepared as described by Koehler and Lienhard (*9*) and found to have mp = 87–88°C [lit. (*10*) mp = 88°C], *Anal*: C, 63.55%; H, 7.42 (theoretical: C, 64.06; H, 7.39). *N*-Acetyl-L-tyrosine hydroxamic acid was prepared by the method of Kurtz and Niemann (*11*) and found to have a mp = 190–191°C *d* (lit. mp = 190.5–191°C *d*). Methyl ρ-nitrobenzene sulfonate was prepared by the general method of Morgan and Cretcher (*12*) as described by Nakagawa and Bender (*1*) and found to have a mp = 91–92°C (lit. mp = 91.5–92°C).

Other reagents. Deuterium oxide (99.85%) was obtained from Bio-Rad Laboratories, and deuterium chloride (38% in 99% D_2O) was obtained from Stohler Isotope Chemicals. Acetonitrile (Eastman Kodak Co.) was purified by the method of Steinhardt (*13*). Urea was purified by ion-exchange chromatography. Other reagents were of highest grade commercially available.

Methods

Enzyme assays. Esterolytic assays of chymotrypsin and methyl chymotrypsin were performed at 25.0°C in a Gilford 2000 recording spectrophotometer by following the

[3] Abbreviations used: CT, α-chymotrypsin (EC 3.4.4.5): MeCT, His-57-N^{τ}-methyl chymotrypsin; PMSF, phenylmethane sulfonyl fluoride; Ac-, acetyl-; Z-, benzyloxycarbonyl-; -OEt, -OCH_2CH_3; -OMe, -OCH_3 -ONP, -ρ-nitrophenyl; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

[4] All melting points were determined on a Thomas Hoover capillary apparatus and are uncorrected.

change in optical density accompanying the hydrolysis of Ac-TyrOEt, Z-L-PheONP, or Z-L-TrpONP. Acylation of MeCT and deacylation were followed by the proflavin displacement of technique of Bernhard *et al.* (*14*) as described by Henderson (*2*). Solvent deuterium isotope effects were followed in buffers prepared by drying the buffering reagents, dissolving in D_2O, and evaporating to dryness. Methyl chymotrypsin was also dissolved in D_2O and lyophilized before use. Na_4EDTA (1 m*M*) was added to both H_2O and D_2O solutions to avoid interference by any divalent cations which may be present in one solvent and not the other. The pD values were estimated from the formula pD = pH (meter reading) + 0.41 (*15*). The pH values were measured on a Radiometer pH meter-26 with a combination electrode (Radiometer GK 2322C).

Protein concentrations were estimated from absorbances at 280 nm with $\varepsilon_{280} = 50\,000\ M^{-1}\ cm^{-1}$ (*2*), and operational normalities (active-site concentration) of the enzymes (at high protein concentrations) were determined by analysis of *p*-nitrophenolate burst obtained with Z-L-TrpONP. Under the usual assay conditions, the pK_a of *p*-nitrophenol was 7.14. Absorbances were measured in a Zeiss PMQII spectrophotometer, and spectra were recorded on a Cary 14 double-beam recording spectrophotometer.

Henri–Michaelis–Menten kinetic parameters (V_{max}, K_m, K_i) were determined by computer fit to the Michaelis–Menten equation based on the program of Hanson *et al.* (*16*). First-order kinetic data were analyzed by a computer program for a least-squares reduction.

Inhibitor binding. Inhibitor binding was measured by the proflavin displacement technique (*7*, *14*). The dissociation constant of the dye, K_D, and the change in extinction coefficient at 465 nm, $\Delta\varepsilon$, were determined for methyl chymotrypsin under conditions of the individual experiment and found to be identical to the values for chymotrypsin. At pH 7.9, $K_D = 2.3\ (\pm 0.2) \times 10^5\ M$ and $\Delta\varepsilon = 1.9\ (\pm 0.05) \times 10^4\ M^{-1}\ cm^{-1}$. Inhibitor dissociation constants, K_i, were determined from the equation (*17*):

$$K_i = \frac{K_D(ED)(I)}{(E_T)(D) - (ED)[K_D + (D)]},$$

where (I), the concentration of inhibitor, is much greater than E_T, the total enzyme concentration determined by active site titration with Z-L-TrpONP. (D) is the concentration of free proflavin and (ED) is the concentration of the enzyme–dye complex determined from the change in absorbance at 465 nm. For most of the inhibitors investigated (D) was also greater than (E_T). Inhibitor–dye interactions were negligible under these conditions. In the case of acetylated amino acids the large K_i values necessitated high inhibitor concentrations, and interaction between the inhibitor and proflavin resulted in significant increases in absorbance at 465 nm; e.g., Ac-L-Phe forms a 1 : 1 complex with proflavin with a dissociation constant of 0.45 ($\pm$0.06) *M* and a $\Delta\varepsilon_{465} = 2.9 \times 10^3\ M^{-1}\ cm^{-1}$ (*18*). In these cases proflavin concentrations employed were less than (E_T) to minimize interference due to dye—inhibitor interaction. K_i values obtained from the proflavin displacement technique agreed, within experimental error, with kinetically determined values from the competitive inhibition of methyl chymotrypsin-catalyzed hydrolysis of Z-L-TryONP. With the protein inhibitors (lima bean inhibitors, pancreatic trypsin inhibitor, and potato inhibitor), the enzyme was preincubated for at least 5 min to insure attainment of equilibrium. The second-order

rate constant for attainment of inhibition of CT by lima bean inhibitor, determined kinetically by assaying enzymic activity at various times of preincubation (25°C, 0.7 *M* P_i, pH 7.8), is greater than $6 \times 10^5\ M^{-1}\ sec^{-1}$. The observation that inhibition of methyl chymotrypsin activity by the protein inhibitors is competitive indicates equilibrium conditions prevail.

Ultracentrifugal Analysis. The sedimentation experiments were carried out at 18 or 20°C in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. The samples were spun at ~68 000 rpm in a titanium rotor. A partial specific volume of 0.736 ml/g for chymotrypsin (*19*) was assumed for methyl chymotrypsin as well.

Denaturation. The rate of denaturation of the enzymes was determined by following the first-order increase in fluorescence at 356 nm ($\lambda_{ex} = 290$ nm) with a Hitachi MPG-2A recording fluorescence spectrophotometer equipped with a constant-temperature circulating bath. Measurements were made at 24°C in a 1-cm-path length cuvette. Protein concentrations were ~0.1 mg/ml in solutions of various urea concentrations containing 0.25 *M* phosphate adjusted to pH 7.3.

Hydroxyaminolysis. The rates of production of *N*-acetyl-L-tyrosine hydroxamic acid by the enzymes were followed by the method of Caplow and Jencks (*20*). The reaction was carried out in 0.04 *M* Tris–HCl buffer, pH 7.8, containing 15% CH_3CN and 0.4 *M* $CaCl_2$ with methyl chymotrypsin = 4×10^{-5} *M* and chymotrypsin = 5×10^{-8} *M*. Ac-L-TyrOEt was 4.5 m*M* and NH_2OH 0.2–0.4 *M*. All reaction rates were corrected for the slow nonenzymic conversions of Ac-L-TyrOEt to the hydroxamate. The ferric chloride complex of the hydroxamic acid was found to have an $\varepsilon_{540} = 800\ M^{-1}\ cm^{-1}$ under our assay conditions. The production of Ac-L-tyrosine hydroxamic acid in the methyl chymotrypsin experiments is the result of methyl chymotrypsin and not due to reactivation of any PMS-chymotrypsin which may be present. Negligible regeneration of chymotrypsin activity from PMS-chymotrypsin in 0.4 *M* NH_2OH was observed during the time course for the reaction (2–3 hr), consistent with the results of Radomsky *et al.* (*21*).

RESULTS

Relative Reactivity

Studies on the relative specificities and chemical reactivities of chymotrypsin (CT) and methyl chymotrypsin (MeCT) are shown in Table 1. The dramatic decrease in catalytic efficiency of methyl chymotrypsin does not eliminate its catalytic activity. These studies confirm the figure of approximately 10^5 for the decreased efficiency of the enzyme (*2*) and confirm that it is indeed a catalyst, albeit an inefficient one. More importantly, the data in Table 1 show that the specificity of the enzyme is significantly altered in an interesting way. The relative efficiency of the enzyme for specific unactivated substrates is altered far more drastically by methylation than is its activity toward activated substrates. The alteration in leaving-group specificity and H_2O vs H_2NOH reactivity reminds one of the alteration in specificity when the substrate is methylated, as shown by Caplow and Harper (*22*). The methylated enzyme reacts with specific substrates much the same way the native chymotrypsin reacts with methylated substrates.

Methyl chymotrypsin does not detectably hydrolyze activated nonspecific substrates such as *p*-nitrophenylacetate or amides. However, the ratio of activities of methyl chymotrypsin and chymotrypsin could be for these substrates similar to that of the normal esters and the undetectable hydrolysis could be simply the result of analytical limitations.

D_2O Effect

The rate of hydrolysis of carbobenzoxy-L-tryptophan methyl chymotrypsin occurs 4.2 times more slowly in D_2O than H_2O. This result is consistent with involvement of

TABLE 1

REACTIVITY OF CHYMOTRYPSIN AND METHYL CHYMOTRYPSIN[a]

	Substrates	CT	MeCT
$pK_a(k_2/k_s)$	Specific substrates	~7, ~9[b]	~7, ~9[b,c]
$pK_a(k_3)$	Ac-L-Tyr-E	~7[b]	~7[b]
$k_{cat}(min^{-1})$	Ac-L-TyrOEt	1200[b]	0.23[b]
k_{cat}/k_{OH}(pH 7)	Ac-L-PheONP	10^9[b]	2000[b]
	Ac-L-TyrOEt	10^9[b]	3000[b,c]
$(k_2/K_s)_A/(k_2/K_s)_B$	A = Z-L-PheONP B = Ac-L-TyrOEt	170[b]	1700[b]
$(k_3)_A/(k_3)_B$	A = Z-L-Phe-E B = Ac-L-Tyr-E	0.26[b]	2.6[b]
$(k_3)_{A'}$ (min^{-1})	Z-L-Trp-E	1500 (±100)[c]	0.045 (±0.001)[c]
$(k_3)_{B'}$ (min^{-1})	Z-L-Phe-E	3100[b]	0.65 (±0.08)[b,c]
$(k_3)_{A'}/(k_3)_{B'}$		0.5	0.07
k_{H_2NOH} $(M^{-1}\ min^{-1})$	Ac-L-TyrOEt	5700 (±100)[c]	2.8 (±0.1)[c]
$k_{H_2NOH}/k^*_{H_2O}$	Ac-L-TyrOEt	0.475[c]	11.8[c]
$k_3^{H_2O}/k_3^{D_2O}$	Z-L-Trp-E	2.9 (±0.2)[c]	4.2 (±0.4)[c]

[a] k_2 is the acylation rate constant of the enzyme saturated with substrate and k_3 is the deacylation rate constant. $k^*_{H_2O}$ is based on the activity of water = 1. E = enzyme. The reactions were carried out at 25°C and pH = 7.8–7.9.

[b] Values from Henderson (*2*).

[c] Values determined in this study.

general base catalysis in the deacylation reaction. In water, the deacylation depends on a basic group with $pK_a = 7.0$ at 25°C. In deuterium oxide it depends on a group with $pK_a = 7.5$ (Fig. 1). A shift of about 0.5 pK_a units going from water to D_2O is consistent with the D_2O effect measured for other groups with a pK_a of 7 (*23*). The kinetic isotope effect, however, is somewhat larger than that generally encountered in hydrolytic enzymatic reactions (*2*, *24*, *25*). Caplow and Jencks (*20*), however, have reported a solvent deuterium isotope effect of 3.6 for deacylation of a nonspecific enzyme, benzoyl chymotrypsin, at pH 8.2. The limiting isotope effect for deacylation of Z-L-Try-α-chymotrypsin is 2.9, based on k_{cat} measurements of the Z-L-TryONP under the same conditions for which the k_{H_2O}/k_{D_2O} effect for deacylation of the methylated enzyme was observed. The above results are an indication of the existence of general base catalysis because of the size of the effect and because alternative mechanisms seem to be very unlikely.

No deuterium isotope effect is observed for reactions of chymotrypsin with many activated nonspecific substrate where the rate-limiting step is believed to be nucleophilic attack by histidine (*26*), nor is there any effect observed in the alkylation of chymotrypsin by TPCK (*27*). Deacylation of *p*-nitrophenylcarbonyl chymotrypsin occurs about 50% via a general base pathway and 50% via nucleophilic pathway, and the deuterium isotope effect is 1·5 (*28*). Variation of the fraction of D_2O solvent led Pollock *et al.* (*29*) to conclude that the solvent deuterium isotope effect in deacylation of acetyl chymotrypsin is due to transfer of a single proton in the transition state.

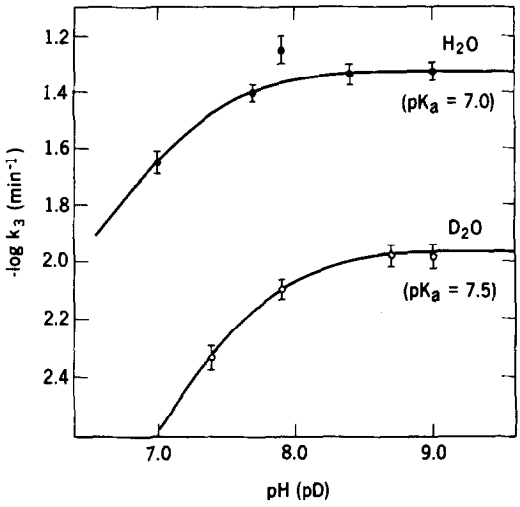


FIG. 1. Solvent deuterium isotope effect on the deacylation of *N*-benzyloxycarbonyl-L-tryptophanyl methyl chymotrypsin ($S_0 = 1.4 \times 10^{-5}$ *M*, $E_0 = 2.7 \times 10^{-5}$ *M*, 86 m*M* P_i, 0.86 m*M* EDTA, 0.128 m*M* proflavin, 1.4% (v/v) CH_3CN, 25°C) followed by the proflavin displacement technique as described under Methods. The solid lines are the theoretical curves for groups with $pK_a = 7.0$ (in H_2O) and $pK_a = 7.5$ (in D_2O).

Binding of Inhibitors

A variety of inhibitors were investigated to detect possible alterations in the binding site of methyl chymotrypsin, and these are summarized in Table 2. Most specific substrates and analogs bind equally well to methyl chymotrypsin and to chymotrypsin. The *p*-chloroanilide of *N*-acetyl-L-tyrosine is anomalous in that it binds more tightly to chymotrypsin than to methyl chymotrypsin (*30, 31*).

Potato inhibitor binds as well to methyl chymotrypsin as to chymotrypsin and with the same stoichiometry of four enzyme molecules to one inhibitor molecule (*32*). Ako *et al.* (*33*) obtained similar binding constants for the inhibitor to chymotrypsin and to anhydro chymotrypsin. It is unlikely that the affinities of the protein inhibitors are related to acylation of the enzyme in view of the evidence in regard to anhydro chymotrypsin with these same inhibitors. Moreover, the K_i determined with methyl chymotrypsin was independent of the length of preincubation time (up to 35 hr).

Since the position of the methyl group in the enzyme is of some interest, the binding of the methylated substrate (*N*-methyl-*N*-Ac-L-TyrOME) to the methylated enzyme was tested and found to be relatively unchanged from that of the same substrate to

chymotrypsin. This suggests that there is no steric interference between the methyl group on the enzyme and the methyl group on the modified substrate, consistent with model-building assuming that the *N*-methylated ester is bound in the productive mode.

When TPCK was tested with the methylated enzyme, it was found to displace proflavin rapidly, consistent with a dissociation constant of 8×10^{-5} *M*. Hydrocinnamoyl nitrile is found to be a good competitive inhibitor of chymotrypsin, binding

TABLE 2

INHIBITOR BINDING TO CHYMOTRYPSIN AND TO METHYL CHYMOTRYPSIN

Compound	K_i^{MeCT} (*M*)[a]	$\frac{K_i^{MeCT}}{K_i^{CT}}$	$\Delta\Delta G'$ (kcal/mol)[b]	Reference
Proflavin	2.3×10^{-5} (±4%)	1	0	*2*, this work
Ac-L-TyrOEt	1.6×10^{-3}	1	0	*2*
Ac-L-TyrNHØ-OMe-*p*	1.2×10^{-2}	1	0	*47*
Ac-GlyNHØ-Cl-*p*	9.6×10^{-4}	1	0	*64* (5% DMF)
Formyl *p*-chloroanilide	5.1×10^{-4}	1	0	*64* (5% DMF)
Ac-L-Phe (pH 7.2)	9.3×10^{-2} (±17%)	1	0	This work
Ac-L-TyrNHØ-Cl-*p*	1.1×10^{-2}	13	1.5	47
	4.8×10^{-3}	6.1	1.1	*64* (5% DMF)
TPCK (reversible phase)	7.9×10^{-5} (±7%)	0.86	−0.8	This work, *65*
N-Methyl-*N*-Ac-L-TyrOMe (5% CH_3CN)	1.1×10^{-3} (±15%)	1.6	0.3	This work
$ØCH_2CH_2CN$ (5% CH_3CN)	2.5×10^{-3} (±18%)	5	1	This work
$ØCH_2CH_2B(OH)_2$	1.8×10^{-2} (±20%)	400	3.5	This work
Lima bean inhibitor (5% CH_3CN)	9.5×10^{-7} (±30%)	50	2.3	This work
Bovine pancreatic trypsin inhibitor (Kuntz)	$>3.4 \times 10^{-5}$	>50	>2.3	This work
Potato inhibitor	9.3×10^{-7} (±40%)	1	0	This work
Turkey ovomucoid inhibitor	1.1×10^{-7}	60	2.4	*66*

[a] Dissociation constant of inhibitor from methyl chymotrypsin. Values obtained at pH = 7.8 and 25°C (organic solvent less than 1.5% (v/v) unless otherwise indicated). The numbers in parentheses are the standard errors of the mean (SEM) of at least three determinations.

[b] Binding energy of inhibitor to methyl chymotrypsin (ΔG_{MeCT}) minus binding energy of inhibitor to chymotrypsin (ΔG_{CT}) at pH 7.8.

more tightly to the native enzyme than do the analogs 3-phenylproprionate and 3-propionamide (*34*). Since its affinity might be a result of covalent bond formation, it was tested with chymotrypsin on the basis that an intermediate imidate ester would be expected to be quite labile. No spectral evidence for decomposition of the nitrile was obtained, and the K_i value was time independent with both the native enzyme and methyl chymotrypsin.

The compound, 2-phenylethaneboronic acid, is a particularly potent competitive inhibitor of chymotrypsin (*9*, *35*). If this compound is a perfect transition state analog it should bind about 10^5 times less tightly to methyl chymotrypsin than to chymotrypsin since chymotrypsin is 10^5 times better as a catalyst and the K_s values for the substrates

in the ground state are identical for both enzymes (*36*, *37*). In fact, the phenylethaneboronic acid binds 400 times more tightly to chymotrypsin, suggesting that it more closely resembles a reactive intermediate than a substrate, but it is not a perfect transition state analog for the deacylation of chymotrypsin.

Physical Properties

Dimerization of chymotrypsin was measured under various conditions. Neet *et al.* (*38*) have observed no dimerization at pH 4, but since we have found that the preparation of Nakagawa and Bender (*1*) contains some denatured enzyme, the work was repeated with chromatographically pure methyl chymotrypsin. We confirmed the results that methyl chymotrypsin dimerizes little, if any. Neet *et al.* (*38*) demonstrated that at pH 6, however, where association of α-chymotrypsin is more complex, the sedimentation behaviour of methyl chymotrypsin is very similar to chymotrypsin.

The ultraviolet absorption spectrum of methyl chymotrypsin is very similar to that of chymotrypsin, both at pH 4.1 and 7.8 with a λ_{max} of 282 nm and $\varepsilon_{282} = 5 \times 10^4\ M^{-1}$ cm^{-1} at the higher pH. [See also Nakagawa and Bender (*1*)]. The fluorescent properties of the two enzymes were also found to be identical. Measurements of the rate of denaturation showed that the methylated enzyme is kinetically less stable by about 1 kcal/mol at urea concentrations greater than 5 *M*. This is about the order of magnitude expected for a single hydrogen bond (*39*). The one hydrogen bond between histidine and serine that is missing in methyl chymotrypsin is also missing in anhydro chymotrypsin and the latter was also found to be more unstable in urea than chymotrypsin. The results with methyl chymotrypsin were similar to those obtained by following the denaturation by proflavine binding ability and changes in the CD spectrum (*40*).

DISCUSSION

Structural Alterations and Their Effect at the Active Site of Chymotrypsin

X-ray diffraction studies indicate that there are only small changes in the crystal structure of methyl chymotrypsin compared to chymotrypsin (*2*, *25*). The methylated histidine N-3 moiety moves out about 0.3 Å from its position in chymotrypsin but the other nitrogen ($N^{\delta 1}$) remains hydrogen-bonded to aspartic 102. The γ-oxygen of serine 195 can occupy two alternative positions, resulting in a slight displacement relative to its position in chymotrypsin. These small changes may, however, reflect extremely important thermodynamic changes on the binding constants, and therefore it was important to examine the binding of substrates and inhibitors to the active site.

The examination of these substrates and inhibitors indicates that the binding site is essentially intact and has changed very little in its properties. The data reported above in Table 2 show very consistent dissociation constants for almost all the substrates examined including such special ones as *N*-methylated tyrosine esters. The identity of the proflavine binding is particularly interesting since anhydro chymotrypsin, DFP-treated chymotrypsin, tosyl chymotrypsin, and chymotrypsinogen all bind proflavin an order of magnitude less tightly than does chymotrypsin (*41*), and yet methyl chymotrypsin binds the dye with essentially the same binding constant. These results indicate that the binding site itself is changed very little and the main effect is on the catalytic steps.

Henderson (*2*) had previously reported that the methyl chymotrypsin is acylated as is chymotrypsin and that the values of k_2 (the acylation rate constant) and k_3 (the deacylation rate constant) are decreased relative to those of the native enzyme. Since K_s values are essentially the same for both enzymes, the reduction of the second-order acylation rate constant is a result of reduction only in k_2. This argues against a major role of differential strain (i.e., the ES complex being more strained with chymotrypsin than with methyl chymotrypsin) in accounting for the lower catalytic effect of methyl chymotrypsin. If strain were important in catalysis it should be observed in a differential effect between K_s for the methylated versus the native enzyme, and therefore a concordance of these values indicates that differential strain is not important in accounting for the diminished reactivity of methyl chymotrypsin.

Possible Mechanisms for the Methylated Enzyme

Even though the methylated enzyme has substantially reduced k_2 and k_3 values, methyl chymotrypsin remains a good catalyst. If a rate constant for alkaline hydrolysis of acetyltyrosine ethyl ester is assumed to be similar to that of hydrolysis of acetylphenylalanine ethyl ester [$k_{OH} = 116\ M^{-1}\ min^{-1}$ (*43*, *44*)], then methyl chymotrypsin is 2×10^4 times more effective than hydroxide ion at pH 7 in catalyzing the hydrolysis of this ester. This means that two problems confront us in regard to the properties of methyl chymotrypsin: (a) why it is reduced in catalytic power by a factor of 10^5 from the native enzyme and (b) why it is 10^4 times better than no catalyst at all. Three different possibilities arise.

Mechanism I: Unassisted nucleophilic attack by serine anion. The observation that the acylation of methyl chymotrypsin by specific substrates is much slower than acylation of chymotrypsin by the same substrates suggests that the slow rate may be due to the small amount of serine anion ($SerO^-$) present at the active site. The second-order rate constant for acylation of methyl chymotrypsin by Z-L-PheONP, k_2/K_s, is $1.5 \times 10^5\ M^{-1}\ min^{-1}$ at pH 7.9 (*2*). The second-order rate constant for hydroxide ion hydrolysis of Z-L-PheONP is $3.24 \times 10^4\ M^{-1}\ min^{-1}$ (*45*, *46*). Bruice *et al.* (*46*) have shown that the anion of acetylserine amide is 41 times more reactive toward *p*-nitrophenylacetate than hydroxide ion. Thus, a reasonable estimate for the second-order rate constant for $SerO^-$ attack on Z-L-PheONP is $k_{SerO^-} = 1.35 \times 10^6\ M^{-1}\ min^{-1}$. If it is assumed that the pK_a of Ser-195 at the active site of MeCT is the same as the pK_a of N-AcSerNH$_2$ (13.6) (*46*), then at pH 7.9 the fraction of the enzyme with serine unprotonated is 4.75×10^{-6}. Thus, if the acylation rate is due to unassisted attack by serine anion one would expect $k_2/K_s = 1.35 \times 10^6\ M^{-1}\ min^{-1} \times 4.75 \times 10^{-6} = 6.4\ M^{-1}\ min^{-1}$. This is lower than the observed value by a factor of 2.4×10^4. Similar results are obtained if one compares the acylation of methyl chymotrypsin by AcTyrOET with the rate of hydroxide ion reaction with the ethyl ester (*43*).

Unassisted nucleophilic attack by serine anion, however, would suggest that the rate of acylation increases as the pH is increased up to the pK_a of serine. Since the pH profile for acylation levels off at a pH corresponding to a group with $pK_a = 7$ (and drops at a pH corresponding to a group with $pK_a = \sim 9.5$) (*47*), this suggests that if the unassisted nucleophilic attack by serine anion is operative the reaction must be inhibited by deprotonation of a group with $pK_a = 7$. A reasonable candidate is methyl-His-57 [the

pK_a of *N*-methyl imidazole is ~7 (*48*)], but it seems unlikely that deprotonation of the *N*-methyl imidazolium moiety will result in a conformational change which will prohibit the attack of serine anion on the substrate, particularly since deprotonation of His-57 (or the charge relay system) in chymotrypsin results in the catalytically *active* species.

Furthermore, this mechanism appears unlikely in view of the solvent isotope effect results. By analogy with the mechanism of action of chymotrypsin, one would expect the acylation reaction (nucleophilic attack by serine) and the deacylation reaction (nucleophilic attack by water) to proceed via the same mechanism (see Scheme I). If acylation of methyl chymotrypsin involves an unassisted nucleophilic attack by the serine anion, then one would predict that deacylation of Z-Trp-methyl chymotrypsin occurs via unassisted attack by hydroxide ion. This mechanism, however, can be ruled out since it predicts an inverse solvent isotope effect in deacylation, and this is not observed.

Mechanism II: Catalysis by the unmethylated nitrogen of histine-57 after rotation of the imidazole moiety. Methylation of the imidazole moiety of histidine-57 will inhibit tautomerization of the ring and effectively freeze the two nitrogens, one resembling a methylated pyrrole nitrogen and the other a pyridine nitrogen. In this situation the pyridine nitrogen will have a pK_a of ~7 and would be an effective general base. However, this nitrogen is now effectively attached to the aspartate residue. If the histidine is rotated so that the pyridine nitrogen is exposed to the serine residue, there would be an appreciable misorientation of the basic nitrogen on histidine with serine-195, since geometric constraints prohibit hydrogen bond formation between serine-195 and histidine-57 even when methyl histidine has rotated. The existence of the charge relay system in several enzymes tends to indicate that the aspartate 102 plays a role in stabilizing the protonated histidine moiety. Although arguments can be made in regard to the importance of the charge on the aspartate group (*49*, *50*), it could reasonably also be argued that the main function of the aspartate is to maintain the orientation of the histidine and to stabilize the positive charge when the histidine becomes protonated. The fact that papain has almost the same structure, but in that case an asparagine residue takes the place of an aspartate (*51*a; also see *51*b), would tend to indicate that any solvating type structure can perform the function. The aspartate group then is providing the same function as water in an aqueous solution. The difference which requires aspartate in the enzyme is that the imidazole is embedded in the hydrophobic region and needs an adjacent polar group to stabilize the generated charge. Since the amount of imidazole which would be rotated must be extremely small and cannot be correctly oriented, this mechanism requires the assumption of an unlikely situation where the rapidly rotating imidazole has essentially the same catalytic power as the highly oriented and juxtaposed imidazole in the native enzyme.

Mechanism III: General base catalysis by the methylated nitrogen of histidine-57. Assuming the major reason for the effectiveness of chymotrypsin is the presence of a general base in proper orientation and juxtaposition to the substrate and that a general base catalyst is needed to explain the small but appreciable catalytic power of methyl chymotrypsin, the most logical candidate for the base is the methylated nitrogen of histidine-57.

This methylated nitrogen is expected to be a very poor base. An approximate estimate of its pK_a can be made. *N*-Methyl pyrrole has a pK_a of −2.9 (*48*). The presence

of a pyridine-type nitrogen in the ring, one carbon away from the nitrogen of interest, is expected to lower the pK_a about 4 units as pyrimidine has a pK_a 4 units lower than that of pyridine (*52*). The the pK_a of the methylated nitrogen in *N*-methyl imidazole is expected to be ~—6.9. Ths is a very poor base but if the Brønsted coefficient is 0.5 [a typical value for hydrolysis of most esters (*24*, *53*)] this would predict that the histidine moiety would be $\sim 10^7$ times less reactive in methyl chymotrypsin than in chymotrypsin. If, however, the Brønsted coefficient is 0.36, one would get precisely the value that is observed for the decreased rate of methyl chymotrypsin.

The Tetrahedral Intermediate

By analogy with nonenzymic hydrolytic reactions both actylation and deacylation of chymotrypsin are expected to involve tetrahedral intermediates. Phenylethaneboronic acid forms an adduct with chymotrypsin which resembles the tetrahedral intermediate (*35*). It is unlikely that the presence of an acetylamide group on the 1-carbon of phenylethaneboronic acid will increase its affinity for chymotrypsin since (a) the K_i values for 2-phenylpropionate, *N*-Ac-Phe, and *N*-Ac-PheOMe are similar, and (b) the K_i values for the aldehydes hydrocinnamaldehyde [3.8×10^{-4} *M* (*35*)] and *N*-Ac-Leu-Leu-phenylalaninal [5.2×10^{-5} *M* (*54*)] are similar. The aldehydes form an adduct with chymotrypsin which is structurally similar to the tetrahedral intermediate (*35*). Since *N*-Ac-PheOMe and other derivatives are better substrates for chymotrypsin than is methyl 2-phenylpropionate by factors of ≥24 (*55*), the similar K_i values of the two aldehydes suggests that the enzyme–tetrahedral intermediate has a significantly lower energy than the enzyme–transition state complex since a larger k_{cat} value implies greater affinity for the substrate in the transition state (*36*, *37*).

The relative stabilities of the enzyme–tetrahedral intermediate (Td) complex and the enzyme–substrate complex in the transition state (Tx) can be estimated from the following exchange reactions:

$$\mathrm{Td} + \mathrm{E{-}OH} \overset{K_{Td}}{\rightleftharpoons} \mathrm{EOTd} + {}^{\ominus}\mathrm{OH} \quad (1)$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad {}^{\oplus}\mathrm{H}$$

$$\mathrm{Tx} + \mathrm{E{-}OH} \overset{K_{Tx}}{\rightleftharpoons} \mathrm{E{-}OTx} + {}^{\ominus}\mathrm{OH} \quad (2)$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad {}^{\oplus}\mathrm{H}$$

Thus, from the rate for the enzyme-catalyzed ester hydrolysis and the nonenzymic saponification rate and knowledge of K_i and K_a (the ionization constant) for the phenylethaneboronic acid, Rawn and Lienhard (*35*a) were able to determine that reaction (2) is more favorable than reaction (1) by a factor of 450. This information, along with the observations that (a) phenylethaneboronic acid (which forms an analog of the enzyme–tetrahedral intermediate adduct) binds 400 times more tightly to chymotrypsin than to methyl chymotrypsin and (b) acyl-chymotrypsin is hydrolyzed $\sim 5 \times 10^4$ times faster than acyl-methyl chymotrypsin, provides a basis for the estima-

tion of the relative energies of the enzyme–tetrahedral intermediate complex (E · Td) and the E · S complex in the transition state (E · Tx) for the two enzymes:

$$\begin{aligned}\Delta\Delta G_{Td} &= G^{MeCT}_{E\cdot Td} - G^{MeCT}_{E+Td} - (G^{CT}_{E\cdot Td} - G^{CT}_{E+Td}) \\ &= -RT \ln (1/400) = 3.5 \text{ kcal/mol} \end{aligned} \tag{3}$$

$$\begin{aligned}\Delta\Delta G\ddagger &= G^{MeCT}_{E\cdot Tx} - G^{MeCT}_{E-Ac} - (G^{CT}_{E\cdot Tx} - G^{CT}_{E-Ac}) \\ &= -RT \ln (1/5 \times 10^4) = 6.4 \text{ kcal/mol,} \end{aligned} \tag{4}$$

where E-Ac is the acyl-enzyme. Subtracting Eq. (3) from Eq. (4), rearranging, and noting that $G_{E+Td} = G_E + G_{Td}$, yields

$$\begin{aligned}\Delta\Delta G\ddagger - \Delta\Delta G_{Td} = (G^{MeCT}_{E\cdot Tx} - G^{MeCT}_{E\cdot Td}) - (G^{CT}_{E\cdot Tx} - G^{CT}_{E\cdot Td}) \\ - (G^{MeCT}_{E-Ac} - G^{MeCT}_{E}) + (G^{CT}_{E-Ac} - G^{CT}_{E}). \end{aligned} \tag{5a}$$

The last two parenthetical terms in Eq. (5a) represent the relative stabilities of the two acylated enzymes:

$$\Delta\Delta G_{Ac} = G^{MeCT}_{E-Ac} - G^{MeCT}_{E} - (G^{CT}_{E-Ac} - G^{CT}_{E}) \tag{6}$$

This term can be estimated to be ~0.8 kcal/mol from the ratio of the second-order acylation rate constant to the second-order deacylation (hydrolysis) rate constant for the two enzymes. Identical values are obtained for both Ac-L-TyrOEt and Z-L-PheONP. Thus,

$$\begin{aligned}\Delta\Delta G\ddagger - \Delta\Delta G_{Td} &= (G^{MeCT}_{E\cdot Tx} - G^{MeCT}_{E\cdot Td}) - (G^{CT}_{E\cdot Tx} - G^{CT}_{E\cdot Td}) - \Delta\Delta G_{Ac} \\ &= \Delta G^{MeCT}_{*} - \Delta G^{CT}_{*} - \Delta\Delta G_{Ac}. \end{aligned} \tag{5b}$$

Therefore, $\Delta\Delta G_{*} \equiv \Delta G^{MeCT}_{*} - \Delta G^{CT}_{*} = 6.4 - 3.5 + 0.8 = 3.7$ kcal/mol.

ΔG_{*} is the free-energy difference for the exchange reaction represented by Eqs. (1) and (2). Thus,

$$\begin{aligned}\Delta G^{CT}_{*} &= G^{CT}_{E\cdot Tx} - G^{CT}_{E\cdot Td} - (G_{Tx} - G_{Td}) \\ &= -RT \ln (450) = -3.6 \text{ kcal/mol.} \end{aligned} \tag{7a}$$

Hence,

$$\begin{aligned}\Delta G^{MeCT}_{*} &= G^{MeCT}_{E\cdot Tx} - G^{MeCT}_{E\cdot Td} - (G_{Tx} - G_{Td}) \\ &= \Delta\Delta G_{*} + \Delta G^{CT}_{*} = 3.7 - 3.6 = 0.1 \text{ kcal/mol.} \end{aligned} \tag{7b}$$

The $G_{Tx} - G_{Td}$ for the nonenzymic saponification of methyl acetate is about 8.5 kcal/mol (*56*). Thus, the relative stabilities of the enzyme–substrate complexes in the transition state and the enzyme–tetrahedral adduct can be estimated:

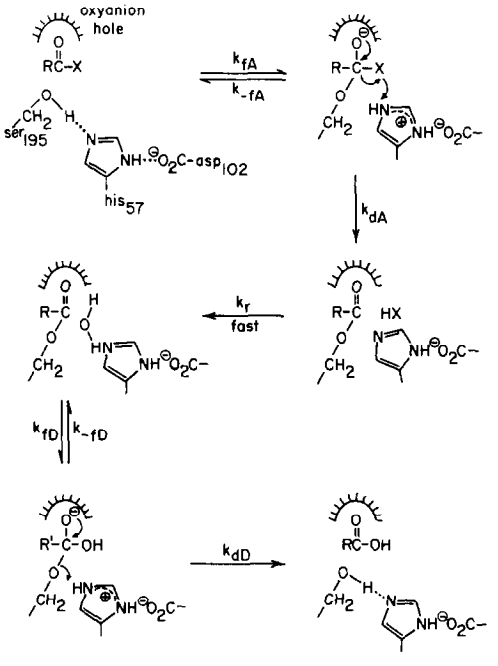
$$\begin{aligned}\Delta G^{CT}_{**} &= G^{CT}_{E\cdot Tx} - G^{CT}_{E\cdot Td} = \Delta G^{CT}_{*} + (G_{Tx} - G_{Td}) \\ &= -3.6 + 8.5 = 4.9 \text{ kcal/mol} \end{aligned}$$

and, similarly

$$\Delta G^{MeCT}_{**} = 0.1 + 8.5 = 8.6 \text{ kcal/mol.}$$

Thus, the relative energy content of the transition state and the tetrahedral intermediate are different for the two enzymes. Methyl chymotrypsin destabilizes the transition state relative to the tetrahedral intermediate more than does chymotrypsin (by about 3.7 kcal/mol). This means that the transition state for deacylation of methyl chymotrypsin is structurally less similar to the tetrahedral intermediate than is the transition state for deacylation of chymotrypsin.

The minimal mechanism for the chymotrypsin-catalyzed reaction is summarized in Scheme I:



SCHEME I

The exact rate equation for acylation of the enzyme–substrate complex in the scheme,

$$\mathrm{ES} \underset{k_{-fA}}{\overset{k_{fA}}{\rightleftharpoons}} \mathrm{E \cdot Td} \xrightarrow{k_{dA}} \mathrm{E \cdot Ac},$$

under conditions where the reverse of the k_{dA} step is negligible [i.e., for specific ester substrates (*57*) or initial velocities in general] is given by (*58*):

$$[\mathrm{E \cdot Ac}] = E_T[1 - \lambda_2 (\lambda_2 - \lambda_1)^{-1} \exp(-\lambda_1 t) + \lambda_1(\lambda_2 - \lambda_1)^{-1} \exp(-\lambda_2 t)],$$

where

$$\lambda_1 = \tfrac{1}{2}\{k_{fA} + k_{-fA} + k_{dA} - [(k_{fA} + k_{-fA} + k_{dA})^2 - 4k_{fA}k_{dA}]^{\frac{1}{2}}\}$$

$$\lambda_2 = \tfrac{1}{2}\{k_{fA} + k_{-fA} + k_{dA} + [(k_{fA} + k_{-fA} + k_{dA})^2 - 4k_{fA}k_{dA}]^{\frac{1}{2}}\}$$

Since acylation of the ES complex (as well as deacylation) follows first-order kinetics (i.e., a single exponential process) this implies $\lambda_1 \ll \lambda_2$, and thus the observed rate

constant for acylation and deacylation is λ_1 (after a short induction period $\approx 1/\lambda_2$). There are three limiting conditions for which $\lambda_1 \ll \lambda_2$:

$$k_f + k_{-f} \gg k_d \qquad \lambda_1 \approx \frac{k_f k_d}{k_f + k_{-f}} \tag{i}$$

$$k_{-f} + k_d \gg k_f \qquad \lambda_1 \approx \frac{k_f k_d}{k_{-f} + k_d} \tag{ii}$$

$$k_{-f} \gg k_f, k_d \qquad \lambda_1 \approx \frac{k_f k_d}{k_{-f}} \tag{iii}$$

Condition (iii) is met when the rate-limiting step is breakdown of the tetrahedral intermediate. If the tetrahedral intermediate is at a higher energy state than the ES complex (by >4 kcal/mol), $k_{-fA} \gg k_{fA}$, and thus condition (i) becomes equivalent to condition (iii). For the deacylation of the enzymes and for acylation by esters $k_{dA} \gtrsim k_{-fA}$ and condition (ii) is met (since $k_{dA} \gg k_{fA}$). Thus, the observed acylation rate constants depend in a simple way on whether formation or breakdown of the tetrahedral intermediate is rate limiting. Furthermore, the reaction coordinate diagram for deacylation can be considered similar to the reaction coordinate diagram for acylation (Fig. 2). The dependence of the observed rate constants on the nature of the rate-limiting step is summarized as follows:

Process	Rate-limiting step involving the tetrahedral intermediate	k_{obs}
Acylation	Formation	$k_{fA}k_{dA}/(k_{-fA} + k_{dA})$
Acylation	Breakdown	$k_{fA}k_{dA}/k_{-fA}$
Deacylation	Formation	$k_{fD}k_{dD}/(k_{-fD} + k_{dD})$
Deacylation	Breakdown	$k_{fD}k_{dD}/k_{-fD}$

Since formation of the tetrahedral intermediate with esters is most likely the rate-limiting step in both nonenzymic (*56*) and chymotrypsin-catalyzed hydrolytic reactions (*22*, *59*), the observed rate constant for acylation of the enzyme by esters is expected to be $k_{fA}k_{dA}/(k_{-fA} + k_{dA})$, and the observed rate constant for the deacylation is expected to be $k_{fD}k_{dD}/(k_{-fD} + k_{dD})$ (particularly since the pK_a of the Ser-195 is less than the pK_a of ethanol or methanol).

From a knowledge of the relative energy states of the enzyme–transition state complex and the enzyme–tetrahedral intermediate complex and the rate constants for acylation and deacylation of the enzyme, only an estimate of the ratio k_d/k_{-f} is required for an evaluation of the individual rate constants in Scheme I. An estimate of the value of k_d/k_{-f} is available from the primary ^{18}O isotope effect for acylation of chymotrypsin by *N*-Ac-L-TrpOEt, assuming the mechanism in Scheme I. Since the observed acylation rate constant is given by

$$k_{acyl} = \frac{k_f k_d}{k_{-f} + k_d} \tag{8}$$

and assuming k_f and k_{-f} are independent of isotopic substitution in the ethoxyl oxygen (relative to k_d), the ratio of k_d/k_{-f} can be calculated.[5] Sawyer and Kirsch (*61*) found an isotope effect of 1.018. Since the isotope effect for breakdown of the tetrahedral intermediate is $k_d/k_d^* = 1.066$ (*61*), the observed isotope effect is consistent with a ratio of $k_d/k_{-f} = 2.7$. This is consistent with the value of 2.3 estimated by Guthrie (*56*) for the nonenzymic saponification of methyl acetate.

The conclusion that methyl chymotrypsin destabilizes the transition state ($\Delta\Delta G\ddagger \approx$ 6.4 kcal/mol) to a greater extent than it destabilizes the tetrahedral intermediate

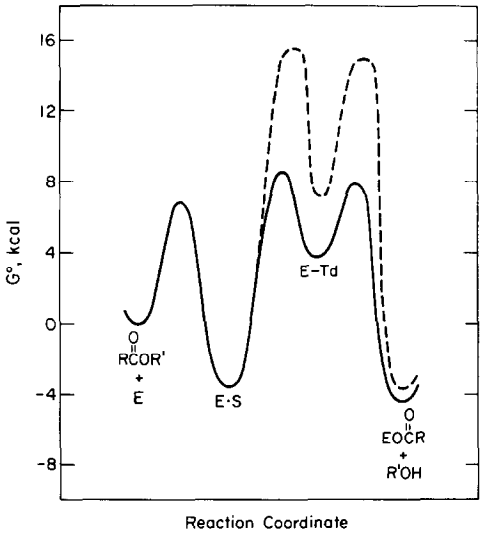


FIG. 2. Reaction coordinate diagram for acylation by esters with chymotrypsin (———) and methyl chymotrypsin (– – – – – –) at pH 7.8, 25°C. The ΔG values (in kilocalories per mole) were calculated from the data in Table 3. The E + S energy value is arbitrarily set at 0. The rate of formation of the ES complex is assumed to be diffusion-controlled. The ΔG^0 for the equilibria $E + S \rightleftharpoons EOCR + R'X$ are from Fastrez and Fersht (*64*).

($\Delta\Delta G_{Td} \approx 3.5$ kcal/mol) relative to chymotrypsin indicates the enzyme–tetrahedral intermediate complex is more like the transition state for the chymotrypsin reaction than for the methyl chymotrypsin reaction. Since methylation of the enzyme does not alter the K_s for the substrates there is no effect on the energy of the ES complex. Furthermore, the rate of formation of the ES complex from E + S (at low temperature) is similar for the two enzymes (*62*). Also, methylation results in only a slight effect on the stability of the acyl-enzyme.

Thus the lower catalytic effectiveness of methyl chymotrypsin relative to chymotrypsin is a result of raising the activation energy barrier to the transition state rather than lowering the energy of the ES complex. In the ground state the active site of the two

[5] This assumption is not rigorously valid since the carbon–ethoxyl oxygen bond order in normal esters is larger than 1. However, if the coplanarity of the acyl portion and the alcohol portion of the substrate is reduced on binding of the ester to the enzyme, the ^{18}O isotope effect on k_{fA} and k_{-fA} should be negligible relative to the isotope effect on k_{dA}. Sawyer and Kirsch (*61*), however, emphasize that the observed isotope effect on acylation is not unambiguous.

enzymes are essentially identical, but as the reaction progresses a difference in the active sites develops reaching a maximum in the transition state.

With a knowledge of the acylation and deacylation rate constants for methyl chymotrypsin and for chymotrypsin and an estimate of the energy levels of the E · S complexes in the transition state and as the tetrahedral intermediate, the individual rate constants in Scheme I can now be estimated. For example, the rate constant for acylation (k_2) of chymotrypsin with *N*-acetyl-L-tyrosine ethyl ester is 3×10^5 min^{-1} (*2*). Thus, the transition state for acylation is 12.2 kcal/mol higher in energy than the E · S complex in the ground state. This is readily obtained from transition state theory:

$$\Delta G\ddagger = -RT \ln (k_i h/kT) \tag{9a}$$

where k_i is the rate constant, h is Boltzmann's constant, and k is Planck's constant. For k_i in units of reciprocal minutes and at 25°C Eq. (9a) yields:

$$\Delta G\ddagger = -1.36 \log k_i + 19.8 \text{ kcal/mol} \tag{9b}$$

From Eq. (8) and the relationship $k_d/k_{-f} = 2.7$, $k_{fA} = (3.7/2.7)(3 \times 10^5) = 4.1 \times 10^5$ min^{-1}; substituting this value into Eq. (9b) yields $\Delta G\ddagger = 12.2$ kcal/mol. Assuming a similar value of ΔG^{CT}_{**} ($=G^{CT}_{E\cdot Tx} - G^{CT}_{E\cdot TD}$) for acylation and deacylation, the energy of the enzyme–tetrahedral adduct is expected to be ~4.9 kcal/mol lower than the energy of the E · S complex in the transition state. Thus, $\Delta G\ddagger = -1.36 \log k_{-fA} + 19.8 \approx 4.9$, or $k_{-fA} \approx 9 \times 10^{10}$ min^{-1}. Since $k_d \approx 2.7k_f$, this implies that $k_{dA} \approx 2.4 \times 10^{11}$ min^{-1}. The remaining individual rate constants for Scheme I can similarly be calculated, and these are summarized in Table 3.

TABLE 3

RATE CONSTANTS FOR REACTIONS WITH ESTER SUBSTRATES[a]

Step	Chymotrypsin[b]	Methyl chymotrypsin[c]
k_{fA}	4.1×10^5	2.2
k_{-fA}	9.0×10^{10}	1.7×10^8
k_{dA}	2.4×10^{11}	4.6×10^8
k_{fD}	1.4×10^4	2.7×10^{-1}
k_{-fD}	9.0×10^{10}	1.7×10^8
k_{dD}	2.4×10^{11}	4.6×10^8
$k_{acylation}$ (k_2)	3×10^5	1.6
$k_{deacylation}$ (k_3)	10^4	2×10^{-1}

[a] The rate constants (in reciprocal minutes) are for the individual steps in Scheme I (pH 7.8, 25°C) based on the values of $k_{acylation}$ and $k_{deacylation}$ observed with *N*-acetyl-L-tyrosine ethyl ester (*2*).

[b] The tetrahedral intermediate is assumed to be more stable than the transition state by 4.9 kcal/mol. The value of $k_d/k_{-f} = 2.7$ is based on the ^{18}O isotope effect (*61*).

[c] The tetrahedral intermediate for both steps is assumed to be 7.0 kcal/mol more stable than the transition state. It is assumed that methylation of the enzyme does not alter the k_d/k_{-f} ratio; however, the value of $k_d/k_{-f} = 2.7$ is probably a minimum value (see text).

The deacylation reaction is expected to involve proton transfer from a zwitterionic tetrahedral intermediate to a general base. This species is expected to have a pK_a of approximately 0 (*61*, *63*). In chymotrypsin, the system to which the proton is transferred has a pK_a of 7, yielding a ΔpK_a between residues of approximately 7. If the group to which the proton is transferred results in ΔpK_a of less than 7 (as would be the case in the unrotated methylhistidine-57), then the deuterium isotope of larger than 3 would be expected, and this is observed.

The rate constants summarized in Table 3 are consistent with the overall decrease in rates observed in the methyl chymotrypsin-catalyzed hydrolysis of esters. The rates are also consistent with the decreased catalytic efficiency of the enzyme and the decreased relative rates of nonspecific substrates. The methylated chymotrypsin is not optimally designed for orientation and juxtaposition to the serine moiety. It is capable of picking up a proton from an attacking group and delivering a proton to a departing group in the case of both esters and amides. Thus it is possible to rationalize entirely the properties of this histidine moiety.

It could well be argued that these results provide no special catalytic factor for the charge relay system. In view of the rough value of these calculations, a moderate contribution is not excluded. However, it would be possible to say that the net effect beyond the normal contribution of a histidine in water cannot be very great. The charge relay system provides, as does the oxy-anion hole, a net stabilization which may be slightly greater than the stabilization provided by water in the case of a nonenzymic reaction, but the fact that an asparagine residue can provide essentially the same function in papain suggests that the effect is that provided by the high dielectric constant and high solvating properties of water.

ACKNOWLEDGMENTS

We would like to thank Drs. Jack Kirsch, Michael Caplow, and Barry Zeeberg for helpful discussions regarding this manuscript.

REFERENCES

1. Y. NAKAGAWA AND M. L. BENDER, *Biochemistry* **9**, 259 (1970).
2. R. HENDERSON, *Biochem. J.* **124**, 13 (1971).
3. (a) G. P. HESS, "The Enzymes" (P. Boyer, Ed.), 3rd ed., Vol. 3, p. 213. Academic Press, New York, 1971; (b) D. M. BLOW, *Accounts Chem. Res.* **9**, 145 (1976).
4. H. AKO, C. A. RYAN, AND R. J. FOSTER, *Biochem. Biophys. Res. Commun.* **46**, 1639 (1972).
5. D. H. STRUMEYER, W. WHITE, AND D. E. KOSHLAND, JR., *Proc. Nat. Acad. Sci. USA* **50**, 931 (1963).
6. D. E. FAHRNEY AND A. M. GOLD, *J. Amer. Chem. Soc.* **85**, 997 (1963).
7. J. L. MARINI AND M. CAPLOW, *J. Amer. chem. Soc.* **93**, 5560 (1971).
8. R. L. PETERSON, K. W. HUBELE, AND C. NIEMAN, *Biochemistry* **2**, 942 (1963).
9. K. A. KOEHLER AND G. E. LIENHARD, *Biochemistry* **10**, 2477 (1971).
10. D. L. YABROFF, G. E. BRANCH, AND B. BETTMAN, *J. Amer. Chem. Soc.* **56**, 1850 (1934).
11. A. N. KURTZ AND C. NIEMANN, *Biochemistry* **1**, 238 (1962).
12. M. S. MORGAN AND L. H. CRETCHER, *J. Amer. Chem. Soc.* **70**, 375 (1948).
13. J. STEINHARDT, *J. Biol. Chem.* **123**, 543 (1938).

14. S. A. BERNHARD, B. F. LEE, AND Z. H. TASHJIAN, *J. Mol. Biol.* **18**, 405 (1966).
15. A. K. COVINGTON, M. PAABO, R. A. ROBINSON, AND R. G. BATES, *Anal. Chem.* **40**, 700 (1968).
16. K.R HANSON, R. LING, AND E. HAVIR, *Biophys. Res. Commun.* **29**, 194 (1967).
17. H. WEINER AND D. E. KOSHLAND, JR., *J. Biol. Chem.* **240**, 2764 (1965).
18. K. G. BRANDT, A. HIMOE, AND G. P. HESS, *J. Biol. Chem.* **242**, 3973 (1967).
19. G. W. SCHWERT AND S. KAUFMAN, *J. Biol. Chem.* **190**, 807 (1951).
20. M. CAPLOW AND W. P. JENCKS, *J. Biol. Chem.* **239**, 1640 (1964).
21. N. A. RADOMSKY, M. J. GIBIAN, AND D. L. ELLIOT, *J. Amer. Chem. Soc.* **95**, 8717 (1973).
22. M. CAPLOW AND C. HARPER, *J. Amer. Chem. Soc.* **94**, 6508 (1972).
23. (a) C. J. COLLINS AND N. S. BOWMAN, "Isotope Effects in Chemical Reaction," ACS Monograph, p. 325. Van Nostrand Reinhold, New York, 1970; (b) R. L. SCHOWEN, "Isotope Effects on Enzyme-Catalyzed Reactions" (W. W. Cleland, M. H. O'Leary, and D. B. Northrop, Eds.), p. 64. University Park Press, Baltimore, 1977.
24. W. P. JENCKS, *Cold Spring Harbor Symp. Quant. Biol.* **36**, 1 (1971).
25. C. S. WRIGHT, G. P. HESS, AND D. M. BLOW, *J. Mol. Biol.* **63**, 295 (1972).
26. C. D. HUBBARD AND J. F. KIRSCH, *Biochemistry* **11**, 2483 (1972).
27. F. J. KÉZDY, A. THOMSON, AND M. L. BENDER, *J. Amer. Chem. Soc.* **89**, 1004 (1967).
28. J. E. C. HUTCHINS AND T. H. FIFE, *J. Amer. Chem. Soc.* **94**, 8848 (1972).
29. E. POLLOCK, J. L. HOGG, AND R. L. SCHOWEN, *J. Amer Chem. Soc.* **95**, 968 (1973).
30. E. C. LUCAS, M. CAPLOW, AND K. J. BUSH, *J. Amer. Chem. Soc.* **95**, 2670 (1975).
31. B. ZEEBERG, M. CAPLOW, AND M. CASWELL, *J. Amer. Chem. Soc.* **95**, 7346 (1975).
32. A. K. BALLS AND C. A. RYAN, *J. Biol. Chem.* **238**, 2976 (1963).
33. H. AKO, R. J. FOSTER, AND C. A. RYAN, *Biochemistry* **13**, 132 (1974).
34. J. L. WEBB, "Enzyme and Metabolic Inhibitors," Vol. 3, p. 372. Academic Press, New York, 1963.
35. (a) J. D. RAWN AND G. E. LIENHARD, *Biochemistry* **13**, 3124 (1974); (b) R. M. SCHULTZ AND A. C. CHEERVA, *FEBS Lett.* **50**, 47 (1975).
36. R. WOLFENDEN, *Accounts Chem. Res.* **5**, 10 (1972).
37. G. E. LIENHARD, *Science* **180**, 149 (1973).
38. K. E. NEET, K. M. SACKRISON, G. R. AINSLIE, AND L. C. BARRITT, *Arch. Biochem. Biophys.* **160**, 569 (1974).
39. I. M. KLOTZ AND J. S. FRANZEN, *J. Amer. Chem. Soc.* **84**, 3461 (1962).
40. V. K. ANTONOV AND T. I. VOROTYNTSEVA, *FEBS Lett.* **23**, 361 (1972).
41. H. WEINER, W. N. WHITE, D. G. HOARE, AND D. E. KOSHLAND, JR., *J. Amer. Chem. Soc.* **88**, 3851 (1966).
42. R. HENDERSON, C. S. WRIGHT, G. P. HESS, AND D. M. BLOW, *Cold Spring Harbor Symp. Quant. Biol.* **36**, 63 (1971).
43. V. N. DOROVSKA, S. D. VARFOLOMEYEV, N. F. KAZANSKAYA, A. A. KLYOSOV, AND K. MARTINEK, *FEBS Lett.* **23**, 122 (1972).
44. M. L. BENDER, F. J. KÉZDY, AND G. R. GUNTER, *J. Amer. Chem. Soc.* **86**, 3714 (1964).
45. D. W. INGLES AND J. R. KOWLES, *Biochem. J.* **104**, 369 (1967).
46. T. C. BRUICE, T. H. FIFE, J. J. BRUNO, AND N. E. BRANDON, *Biochemistry* **1**, 7 (1962).
47. E. C. LUCAS AND M. CAPLOW, *J. Amer. Chem. Soc.* **94**, 960 (1972).
48. E. A. BARNARD AND W. D. STEIN, *Advan. Enzymol.* **20**, 51 (1958).
49. M. W. HUNKAPILLER, S. H. SMALLCOMBE, D. R. WHITAKER, AND J. H. RICHARDS, *Biochemistry* **12**, 4732 (1973).
50. G. ROBILLARD AND R. G. SHULMAN, *J. Mol. Biol.* **71**, 507 (1972).
51. (a) J. DRENTH, J. N. JANSONIUS, R. KOEBOEK, H. M. SWEN, AND B. G. WOLTHERS, *Nature (London)*, **218**, 929 (1968) (b) M. SHIPTON, T. STUCHBURY, AND K. BROCKLEHURST, *Biochem. J.* **159**, 235 (1976).
52. W. P. JENKS AND J. REGENSTEIN, "Handbook of Biochemistry and Selected Data for Molecular Biology" (H. A. Sober, Ed.), p. j-152. Chemical Rubber Co., Cleveland, Ohio, 1968.
53. S. L. JOHNSON, *Advan. Phys. Org. Chem.* **5**, 237 (1967).
54. A. ITO, K. TOKAWA, AND B. SHIMIZA, *Biochem. Biophys. Res. Commun.* **49**, 343 (1972).
55. M. S. SILVER, M. STODDARD, T. STONE, AND M. S. MATTA, *J. Amer. Chem. Soc.* **92**, 3151 (1970).
56. J. P. GUTHRIE, *J. Amer. Chem. Soc.* **95**, 6999 (1973).

57. J. FASTREZ AND A. R. FERSHT, *Biochemistry* **12**, 1067 (1973).
58. S. W. BENSON, *J. Chem. Phys.* **20**, 1605 (1952).
59. J. FRANKFATER AND F. J. KÉZDY, *J. Amer. Chem. Soc.* **93**, 4039 (1971).
60. H. HIROHARA, M. L. DEUDER, AND R. S. STARK, *Proc. Nat. Acad. Sci. USA* **71**, 1643 (1974).
61. C. B. SAWYER AND J. F. KIRSCH, *J. Amer. Chem. Soc.* **97**, 1963 (1975).
62. A. L. FINK, *Biochemistry* **15**, 1580 (1976).
63. C. B. SAWYER AND J. F. KIRSCH, *J. Amer. Chem. Soc.* **95**, 7375 (1973).
64. J. FASTREZ AND A. R. FERSHT, *Biochemistry* **12**, 2025 (1973).
65. D. M. GLICK, *Biochemistry* **7**, 3391 (1968).
66. D. S. RYAN AND R. E. FEENEY, *J. Biol. Chem.* **250**, 843 (1975).