Anal. Calcd for $C_{14}H_{16}N_2O_4$: C, 60.9; H, 5.8; N, 10.14; O, 23.2; ONP, 0. Found: C, 58.8; H, 6.68; N, 10.13, 10.5; O, 23.9; ONP, 0.2.

The nmr spectrum (TFA) showed: 473 (17 cps at half-height, partly resolved), 1.9 (NH); 443 (3 cps), 4.3 (Bl-C_6H_5); 316 (4 cps), 1.7 (Bl-CH_2); 290 (13 cps), 1.3 (CH); 255 (13 cps), 2.2 (Gly-CH_2); 158, 140, 3.9 (2Glu-CH_2).

Poly Glu(OH)-Gly (10). A solution of 1.4 g of poly Glu(OBl)-Gly in 40 ml of trifluoroacetic acid was treated with gaseous hydrogen bromide for 30 min and then was allowed to stand. Evaporation of the solvent gave a residue which was extracted with ether and then dissolved in 100 ml of sodium bicarbonate and dialyzed for 48 hr against three 2500-ml portions of water. The solution in the bag was filtered, and the water was removed on the vacuum train at room temperature to give a glossy solid, 0.7 g (55%).

Anal. Calcd for the sodium salt, $C_7H_9N_2O_4Na$: C, 40.4; H, 4.4; N, 13.5; Na, 11.1. Found: C, 33.1; H, 5.1; N, 11.45; Na, 10.9. Calcd for 15% water: C, 34.3; H, 5.4; N, 11.5; Na, 9.4.

The nmr spectrum (TFA) showed: 483 (23 cps width at half-height), 2 (NH); 298 (23 cps), 1.2 (CH); 263 (18 cps), 2.3 (Gly-CH_2); 163 (17 cps), 143 (25 cps), 4.2 (Glu-CH_2).

In another experiment, 600 mg of poly Glu(OBl)-Gly was debenzylated, and the residue after ether extraction was dialyzed directly to give 100 mg (32%) of free acid.

Anal. Calcd for $C_7H_{10}N_2O_4$: mol wt, 186; N, 15.0. Found: mol wt (neut equiv), 206; N, 13.9.

Poly Glu(OBl)-Ser(H)-Gly (11). A solution of 14.6 g of TFA-H-Glu(OBl)-Ser(H)-Gly-ONP in 25 ml of dimethyl sulfoxide was treated with 2.1 ml of triethylamine (added dropwise over 2 min). Within 15 min the mixture set to a jelly. This was diluted with 25 ml of dimethyl sulfoxide and stirred for 22 hr. The sticky material was thoroughly mixed with a mixture of 200 ml of chloroform and 800 ml of methanol, kept at −10° for 1 hr, and filtered. The polymer was successively resuspended and filtered using 200 ml of methanol plus 800 ml of ether, then 500 ml of ether, then 150 ml of water. After rinsing with methanol and ether, the polymer was dried to constant weight over P_2O_5 at 77° and a few millimeters pressure to give 4.8 g of yellow powder (56%), mp 240–243° dec in bath at 225°.

Anal. Calcd for $C_{17}H_{21}N_3O_6$: C, 56.2; H, 5.8; N, 11.56; O, 26.4; ONP, 0. Found: C, 52.8, 52.5; H, 5.4, 5.2; N, 11.40, 11.57; O, 27.5, 28.1; ONP, 2.4, 0.1.

The nmr spectrum (TFA) showed: 477 (broad, partly resolved), 3.1 (NH); 441 (6 cps at half-height), 4.5 (Bl-CH_2); 315 (6 cps), 2.3 (Bl-CH_2); 290 b, 2.3 (CH); 253 (18 cps), 3.9 (CH_2); 168, 148, 4.1 (2Glu-CH_2).

Poly Glu(OH)-Ser(H)-Gly (12). Hydrogen bromide was bubbled for 30 min through a solution of 3.3 g of poly Glu(OBl)-Ser(H)-Gly in 70 ml of trifluoroacetic acid, and the solution was allowed to stand for 1 hr at room temperature. The solvent was evaporated; the residue was extracted with ether to give a pale yellow powder. This was dissolved in 70 ml of 5% aqueous sodium bicarbonate solution and dialyzed against four 1200-ml portions of water over a period of 44 hr. The solution from the dialysis bag was concentrated and then taken to dryness of the vacuum train to give 1.4 g (45%) of glassy material.

Anal. Calcd for $C_{10}H_{14}N_3O_6Na$: C, 40.6; H, 4.7; N, 14.23; Na, 7.89. Found: C, 35.9; H, 5.0; N, 12.12; Na, 7.1. Calcd for 12% water: C, 35.7; H, 5.4; N, 12.5; Na, 6.94.

The infrared spectrum (137, oil) showed: 1700 sh, 1650, 1580, 1535, 1460 cm^{-1}. The nmr spectrum (TFA) showed: 485 (23 cps at half height, some resolution), 2.8 (NH); 285 (18 cps), 2.4 (2CH); 252, 3.8 (Gly and Ser-CH_2); 169, 153, 3.9 (2Glu-CH_2).

The free acid was prepared by dissolving 160 mg of the sodium salt in water, adding 10 ml of 0.1 *N* hydrochloric acid, and dialyzing to remove salt.

DNP Molecular Weight Determinations.[40] The polymer was dissolved in aqueous sodium bicarbonate and treated with an alcoholic solution of dinitrofluorobenzene for 3 hr at room temperature. The solvents were removed under vacuum in a desiccator over P_2O_5 at room temperature; the residue was rinsed with ether, and then taken up in 15 ml of 6 *N* hydrochloric acid. After 22 hr at 110° in a sealed tube, the solution was diluted with three volumes of water and extracted with ether, and the ether solution evaporated. The residue was taken up in acetone, diluted to volume, and chromatographed on several sheets of Whatman No. 1 paper, ascending, using benzene:1% acetic aicd. The only DNP–amino acid spot was DNP-Glu(OH)-OH; as expected, neither DNP-Gly-OH nor, with the tripeptide, DNP-Ser(H)-OH was present. The DNP-Glu(OH)-OH spots were eluted with water and the absorption measured at 360 mμ. Blanks and controls were run.

(40) Sanger procedure patterned after the description in R. J. Block, E. L. Durrum, and G. Zweig, "Paper Chromatography," 2nd ed, Academic Press Inc., New York, N. Y., 1958, p 154.

Studies on the Reaction of Chymotrypsin and L-1-Chloro-3-tosylamido-4-phenyl-2-butanone[1]

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Abstract: The second-order rate constant for the reaction of α-chymotrypsin with L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK) is dependent on a basic group of pK = 6.8 and an acidic group of pK = 8.9. The ionization of the tosylamido group of TPCK at 9.9 is not observed kinetically except to a minor extent. Essentially, no deuterium oxide kinetic solvent isotope effect is seen in this reaction. Benzamide competitively inhibits the reaction of TPCK and chymotrypsin with an inhibition constant identical with that observed in the enzyme-catalyzed hydrolytic reactions. The basic group of pK = 6.8 is identified as the imidazole group of histidine 57 of the enzyme previously shown to be the site of reaction. The acidic group of pK = 8.9 is presumably the same group as the one controlling the second-order rate constant in α-chymotrypsin-catalyzed hydrolytic reactions and the binding of certain compounds to the enzyme.

Recently the irreversible inactivation of α-chymotrypsin by L-1-chloro-3-tosylamido-4-phenyl-2-butanone (the chloromethyl ketone derivative of N-tosyl-L-phenylalanine, TPCK) was demonstrated.[2] The inactivation was found to occur by the selective modification of one of the two histidine residues on the enzyme molecule[3] later identified as histidine 57.[3-5]

(1) This research was supported by grants from the National Institutes of Health.

(2) G. Schoellmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).

(3) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Am. Chem. Soc.*, **86**, 1271 (1964).

The mechanism of this inhibition reaction was postulated to involve a stereospecific adsorption of the inhibitor molecule at the active site of the enzyme, followed by a bimolecular nucleophilic substitution of chloride by the imidazole side chain of the histidine moiety. Evidence for the formation of an enzyme–inhibitor complex prior to the covalent reaction was provided by the fact that the substrate specificity of the inhibition reaction was qualitatively identical with the specificity of the hydrolytic reactions. In addition, the rate of the inhibition reaction was decreased by β-phenylpropionate ion.[2] The mechanism of the actual covalent change was modeled after analogous nonenzymatic systems which predict a dependence of the rate on a basic group of $pK \sim 7$ since the imidazole must be in its basic form in order to act as a nucleophile. However, in the enzymatic reaction a bell-shaped pH–rate profile was observed,[2] formally necessitating the presence of a base of $pK_1 \sim 7$ and an acid of $pK_2 \sim 7.9$. The latter was tentatively identified as the amide group of the inhibitor itself.[2]

During a study of the trypsin-catalyzed hydrolysis of methyl α-N-tosyl-L-argininate the ionization constant of the tosylamino group was needed. For this ionization constant we obtained the pK of 9.9, considerably different from the postulated pK mentioned above. Thus the kinetic observation of $pK_2 \sim 7.9$ appears not to be an ionization of the substrate but rather must be the ionization of a group on the enzyme. Since in the hydrolytic reactions of chymotrypsin an acidic group on the enzyme with a pK of ~8.6 is observed in pH *vs.* k_{cat}/K_m(app) curves, and since the mechanistic role of this acid has not been clearly defined as yet,[6,7] it appeared to be of interest to investigate the mechanism of the reaction of α-chymotrypsin with TPCK and to use these data in the interpretation of the pH dependence of the hydrolytic reactions of the enzyme.

Experimental Section

Materials. Worthington 3× crystallized lyophilized α-chymotrypsin was used, mainly batch CDI-6114-5. A sample of the same batch purified by gel filtration on Sephadex G-25 at pH 3 was also used.[8,9] Enzyme solutions were routinely titrated using N-*trans*-cinnamoylimidazole at 335 mμ.[10]

L-1-Chloro-3-tosylamido-4-phenyl-2-butanone (Cyclo Chemical Co. lot F1199) was recrystallized twice from 95% ethanol,[11] mp 105–106.5° (lit.[2] mp 102–103°). *Anal.* Calcd for $C_{17}H_{18}O_2NSCl$: C, 58.03; H, 5.61; N, 3.98; Cl, 10.08. Found: C, 58.28; H, 5.27; N, 3.96; Cl, 9.70.[12] The nmr spectrum in $CDCl_3$ yields the following five resonances (ppm upfield from $CHCl_3$ (in $CDCl_3$)) In addition to the complex aromatic hydrogen peak: a doublet at 1.9 (1), a triplet at 2.9 (1), a singlet at 3.2 (2), a doublet at 4.4 (2), and a singlet at 4.9 (3). The numbers in parentheses are the relative areas under the peaks. These five peaks can be assigned to NH, CH, CH_2Cl, CH_2, and CH_3, respectively. When the compound was twice equilibrated in 1 ml of deuteriochloroform with 0.3 ml of deuterium oxide at 40° for 1 hr and evaporated to dryness, its nmr spectrum in deuteriochloroform showed a considerable decrease of the 1.9- and 2.9-ppm peaks. Thus the nmr spectrum is in agreement with the postulated structure of TPCK. The infrared spectra of TPCK and deuterated TPCK were determined in potassium bromide pellets. A sharp N–H stretching band was observed at 3320 cm^{-1} and the deuterated compound showed a new band at 2420 cm^{-1}. Thus both the nmr and infrared spectra indicate the absence of a methyl group on nitrogen. This demonstration was necessary because the melting point of the N-methyl compound is 102–103°.[2]

Methyl α-tosyl-L-argininate (Mann Research Lab., lot B3765) was used without further purification. *p*-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate (Mann Research Lab., lot H1693) was recrystallized twice from chloroform–hexane, mp 157–158°. Benzamide (Fischer reagent) was crystallized several times in water–ethanol, mp 128–129°.

Kinetic Measurements. The concentration of active enzyme in a partially inhibited reaction mixture was determined by rate assay using *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate as substrate in pH 5.00 acetate buffer (0.2 *M*) and 0.65% (v/v) acetonitrile–water at 25°. A 20-μl aliquot of 2.3×10^{-3} *M* *p*-nitrophenyl ester in acetonitrile solution was added to 3 ml of pH 5.00 acetate buffer in a 1-cm quartz cuvette. At a given moment 50 μl of the enzyme solution to be analyzed was added to this mixture and the rate of liberation of *p*-nitrophenol was measured at 340 mμ using a Cary 14 recording spectrophotometer equipped with a cell compartment thermostated at 25° and 0.0–0.2 absorbance unit slide wire. Using the independently determined values of $k_{cat} = 1.17$ sec^{-1}, $K_m(app) \simeq 0.3 \times 10^{-6}$ *M*, and $\Delta\epsilon_{340} = 6014$ for this reaction, the initial zero-order rate of liberation of *p*-nitrophenol allowed us to calculate the enzyme concentration in the assay mixture and thus in the original enzyme solution.

Ionization Constants. The increase of pH from 6 to 10 of a solution of tosylamido derivatives, including both TPCK and methyl α-N-tosyl-L-argininate, is accompanied by a reversible spectral change in the region of 250–255 mμ. This spectral change, assigned to the ionization of the amide group, has been used for the spectrophotometric determination of the ionization constants of these compounds. The two compounds are stable for at least several minutes at 25° in aqueous solution over the entire pH range studied as determined by the fact that the rate of reaction of these substrates with α-chymotrypsin and trypsin was independent of the time of preincubation at a given pH. The absorbance of a given concentration of substrate was measured as a function of the pH at a given wavelength. If the measured absorbance at an intermediate pH is designated by A and the absorbance of the acid and basic forms by A_A and A_B, respectively, the ratio of the concentration of the acid and basic forms (C_A and C_B) is given by

$$C_B/C_A = (A - A_A)/(A_B - A) = K_a/[H^+] \qquad (1)$$

Since the absorbance of a strongly basic solution is not measurable accurately owing to the instability of the compounds, eq 1 was transformed into

$$A = A_B - (1/K_a)[H^+](A - A_A) \qquad (2)$$

Thus by plotting A *vs.* $[H^+](A - A_A)$ one obtains K_a from the slope of the resultant straight line.

Results

Determination of the Ionization Constant of Tosylamide Groups. Spectrophotometric determinations of the ionization constants of L-1-chloro-3-tosylamido-4-phenyl-2-butanone and methyl α-N-tosyl-L-argininate were carried out at 255 and 250 mμ, respectively. The data plotted according to eq 2 are shown in Figure 1. It is seen that the slopes of both lines, the inverse of the ionization constants, are essentially identical with one another. From the slopes of Figure 1 the pK_a of TPCK is 9.9 and that of the arginine derivative is also 9.9. The identity of the ionization constants of these two tosylamido derivatives confirms that the spectral changes are due to the ionization of the same group.

The Reaction of α-Chymotrypsin with TPCK. In preliminary experiments the loss of activity of 10^{-5}

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(11) The authors thank Dr. R. L. Blakeley for this compound.
(12) Analysis by Micro-Tech Lab., Inc., Skokie, Ill. Dr. J. F. Sebastian kindly supplied both the nmr and infrared spectra.

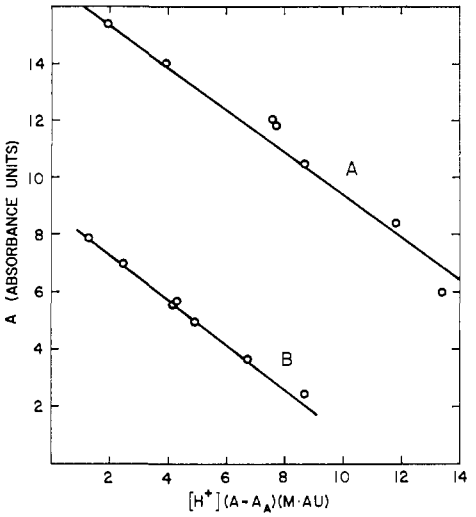

Figure 1. The spectrophotometric determination of the ionization constants at 25° and $I = 0.2 \pm 0.05$ of TPCK, A, 0.66% (v/v) methanol–water, [TPCK] = 4.96×10^{-5} *M*, abscissa × 10^{-14}, ordinate × 10^{4}; and of ethyl α-tosyl-L-argininate, B, 2.15×10^{-5} *M*, abscissa × 10^{-13}, ordinate × 10^{3}.

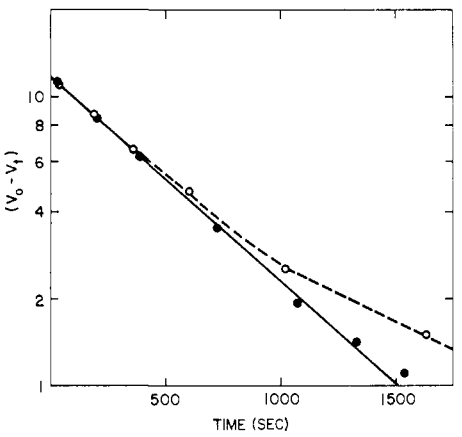

Figure 2. The reaction of α-chymotrypsin with TPCK at pH 7.74, 25°, 2.3% (v/v) methanol–water; ○, untreated Worthington chymotrypsin, 6114-5; ●, Worthington chymotrypsin, 6114-5, gel filtered on Sephadex G-25.

M α-chymotrypsin in pH 8.3, 8.9, 9.5, and 9.9 solutions for 1 hr was found to be less than the experimental error in the rate assays (~2%). The rate of reaction of α-chymotrypsin with TPCK was then determined by incubating these two materials at 25° and a given pH. At determined times a 50-μl sample was withdrawn from the incubation mixture and was immediately assayed with *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate at pH 5, where the rate of reaction of the enzyme with TPCK was negligible with respect to the time required for the rate assay (~50 sec). The TPCK concentration was always at least 15 times that of the enzyme. However, even under such conditions the loss of activity of the enzyme still did not follow pure pseudo-first-order kinetics under certain conditions as it should. Figure 2 shows a semilogarithmic plot of activity *vs.* time which shows a significant concave curvature at large times. Moreover, after reaction for more than 20 apparent half-lives at pH 7.7, about 4% of the initial activity still remained. Addition of a fresh amount of TPCK, however, rapidly caused the disappearance of most of this residual activity. Thus, much more than the stoichiometric amount of TPCK

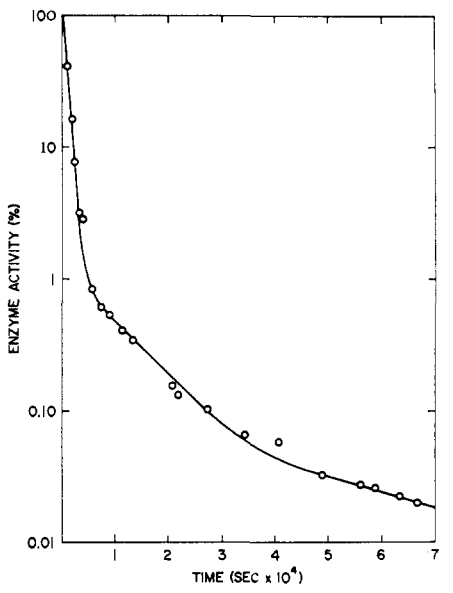

Figure 3. The reaction of α-chymotrypsin with TPCK at pH 7.7, Tris-HCl buffer, 25°, 0.95% (v/v) methanol–water; E_0 (gel filtered on Sephadex G-25) = 8.55×10^{-6} *M*; TPCK = 1.24×10^{-4} *M*. Activity determined by rate assay using methyl N-furylacryloyl-L-tryptophanate at pH 7.76.

must have disappeared from the solution during the initial reaction. The decomposition of TPCK at alkaline pH's occurs very slowly and thus the decrease in the rate of the reaction in the presence of α-chymotrypsin cannot be explained by the spontaneous decomposition of TPCK. The only reasonable postulate to explain this result is that the enzyme preparation must have contained more than 1 equiv amount of other groups which also can react with TPCK. These groups could either be other groups on the enzyme or impurities in the enzyme. The latter was found to be the case when an α-chymotrypsin sample which had been previously purified by gel filtration on Sephadex G-25[8] was used. The loss of activity in the presence of excess TPCK using this purified enzyme preparation followed good first-order kinetics to at least 90% of inactivation and the initial rate of inactivation was identical with the initial rate of inactivation of the untreated enzyme (Figure 2). Nonpurified commercial α-chymotrypsin has been shown to contain ninhydrin-positive, small molecular weight impurities.[8] Thus it is reasonable to assume that these impurities, probably amino acids and oligopeptides, are responsible for the time-dependent nonenzymatic disappearance of TPCK. Consequently, the first-order rate constants of the reaction of α-chymotrypsin with TPCK were determined by using experimental points only in the first 50% of the reaction where the difference between the purified enzyme and the unpurified enzyme was negligible.

In one series of experiments, the reaction of TPCK with α-chymotrypsin, purified by gel filtration, was observed at pH 7.7 for as long as 4 days, with the object of discovering whether TPCK-chymotrypsin has any residual activity. The enzyme activity was determined by rate assay using *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate at pH 5 or 6, or using methyl N-furylacryloyl-L-tryptophanate at pH 7.76; spontaneous hydrolysis of the latter substrate is undetectable at pH 7.76. As shown in Figure 3, the reaction of TPCK with α-chymotrypsin gave a good first-

Table I. The Kinetics of the Reaction of α-Chymotrypsin and L-1-Chloro-3-tosylamido-4-phenyl-2-butanone[a]

Expt	pH or pD	$E_0 \times 10^6$, M	(TPCK) $\times 10^4$, M	Buffer[b]	$k_{exp} \times 10^4$, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}
1	6.25	7.42	1.50	P	3.56	2.34
2	6.49	9.51	1.69	P	7.94	4.70
3	7.02	9.51	1.69	P	11.03	6.53
4	7.39	6.90	1.74	P	19.7	8.45
5[c]	7.39	6.90	1.74	P	12.3	(7.07)
6	7.73	6.90	1.74	P	13.98	8.03
7	7.74	9.51	0.895	P	8.32	9.85
8[d]	7.74	10.4	1.69	P	16.3	9.64
9[e]	7.79	10.2	1.69	P	16.3	9.64
10	8.35	6.90	1.74	T	12.4	7.13
11	8.88	9.51	1.69	T	8.40	5.15
12[f]	8.90	9.51	1.69	T	7.88	(4.66)
13	9.32	9.51	1.69	C	3.80	2.25
14	9.53	9.51	1.69	C	2.37	1.40
15[g]	9.53	9.51	1.69	C	2.39	(1.41)
16	9.96	9.51	1.69	C	~0.6	~0.36
17[h]	6.25	6.11	1.50	P	0.107	0.071
18[h]	7.35	5.80	1.49	P	0.545	0.33
19[i]	8.37	9.66	1.69	T	12.9	7.63
20[j]	7.94	9.15	1.69	P	3.87	2.29

[a] At 25° in 2.3% (v/v) methanol-water. [b] P = 0.067 M phosphate; T = 0.05 M Tris-HCl; C = 0.1 M carbonate. [c] Aged TPCK: 3 hr. [d] Untreated chymotrypsin. [e] Sephadexed chymotrypsin. [f] Aged TPCK: 1580 sec^{-1}. [g] Aged TPCK: 1100 sec. [h] Derivatized enzyme according to Lawson and Schramm.[15] [i] D_2O solvent. [j] 2.25×10^{-2} M benzamide.

order plot as far as 98% reaction. At this point the reaction apparently becomes much slower. After 96 hr, the activity was less than 0.001% that of the starting material. The same type of curve was obtained using both assay substrates. Figure 3 shows a multiphasic first-order plot which can be interpreted most easily by saying that several reactions are being observed, the faster one accounting for *ca.* 98% of the original chymotrypsin and the slower ones accounting for the other *ca.* 2%. Several explanations may account for this result. (1) A second enzyme may contaminate the α-chymotrypsin. Contaminants such as β, γ, and δ-chymotrypsin may be ruled out since their second-order rate constants with TPCK are 7.28, 6.68, and 8.47 M^{-1} sec^{-1}, respectively, at pH 7.71 and are thus quite similar to the rate constant of α-chymotrypsin (see Table I). Trypsin may be ruled out since addition of TLCK does not decrease the activity of a sample of the enzyme which has been treated with TPCK for 24 hr and, furthermore, the value of K_m of a concentrated re-Sephadexed solution after 24 hr of TPCK treatment *vs.* methyl N-furylacryloyl-L-tryptophanate was 3–6 × 10^{-7} M, very close to that of chymotrypsin (4–8 × 10^{-7} M) and very much lower than that expected for trypsin. (2) Some alkylation takes place on methionine, yielding an alkylated enzyme which is further susceptible to alkylation on histidine, but at a lower rate. This suggestion is unlikely since it would predict that the K_m of the enzyme after 24 hr of TPCK treatment would be much higher than that of the native enzyme, contrary to experiment. (3) Even chymotrypsin purified by gel filtration can autolyze to a small extent yielding a small contaminant with lowered activity toward TPCK. Although none of these explanations is satisfactory, the phenomenon probably does not affect the kinetics given later on the basis of the first 50% of reaction.

The first-order rate constants, determined under various conditions, are summarized in Table I. Experiments 5, 12, and 15 show that TPCK is stable at pH's 7.4, 8.9, and 10.0 for the duration of our experiments (≤2000 sec). The reaction is first order with respect to the enzyme. Changing the concentration of TPCK by a factor of two changes the first-order experimental rate constant by a factor of two (experiments 7 and 8) thus showing that the order of the reaction is one for TPCK also. In the last column of Table I the calculated second-order rate constants of this reaction are shown. Reproducibility of the rate constant is estimated to be ±10%. Saturation of the enzyme by TPCK under our conditions was impossible due to the limited solubility of the substrate.

The presence of 2.25×10^{-2} M benzamide reduces the rate of the inhibition reaction considerably. The ratio of the uninhibited to the inhibited rate constant is 3.93 at pH 7.94. If the TPCK reaction occurs with an adsorption step then the rate constant of the uninhibited reaction is given by $k_{exp} = k_2/K_s$. Then if benzamide (I) acts as a competitive inhibitor with a binding constant K_i, then $k_{exp}^{inh} = k_2/(K_s)(1 + (I/K_i))$. Thus from the ratio of the experimental rate constants we can calculate $K_i = 7.7 \times 10^{-3}$ M. This inhibition constant is identical within experimental error with the one determined in reactions where benzamide competitively inhibits the α-chymotrypsin-catalyzed hydrolysis of specific substrates: 8.8×10^{-3} M[13] and $10 \pm 2 \times 10^{-3}$ M.[14] Previously it had been shown on a qualitative basis that competitive inhibitors would slow down the TPCK reaction. The present results prove on a quantitative basis that the TPCK reaction necessitates an unoccupied active site.

Inhibition of an α-chymotrypsin whose methionine 192 has been modified by alkylation[15] occurs at a highly reduced rate with respect to native α-chymotrypsin: at pH 6.25 the rate constant is decreased by a factor of 33 and at pH 7.4 by a factor of 26.

Changing the solvent from water to deuterium oxide (pH 7.8 *vs.* pD 8.4) does not change the value of the second-order rate constant by more than 20%.

The pH dependence of the second-order rate constant was analyzed by using the theoretical equation for a reaction involving both a base of pK_1 and an acid of pK_2 ($k_{exp} = k_{lim}/(1 + (H/K_i) + (K_2/H))$) by a graphical method described earlier.[16] Using the above equation in the form of a plot of $1/k_{exp}$ *vs.* $(H + H^2_{max}/H)$[16] experimental points below pH 8.5 yield a good straight line when H_{max} is taken as 1.48×10^{-8} and $pH_{max} = 7.83$, yielding $pK_1 = 6.85$ and $pK_2 = 8.73$. However, the higher pH points deviate strongly from the theoretical line and when k_{exp} is calculated on the basis of these data, the experimental points at high pH's are lower than the theoretical line. This deviation reaches ~50% at pH 10 suggesting that the ionized TPCK is unable to react with chymotrypsin.[17] On this basis, the experimental points have been corrected for the ionization of the substrate before applying this treatment. Using this procedure and again using $pH_{max} =$

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(16) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).
(17) The pK_a of methyl α-N-*p*-toluenesulfonyl-L-argininate has been determined to be approximately 10: J. J. Bechet, *J. Chim. Phys.*, **62**, 1096 (1965). Further, it was found that the trypsin-catalyzed hydrolysis of this substrate fell off with a pK of 9.65 indicating that the anionic form of the substrate is resistant to enzymatic hydrolysis.

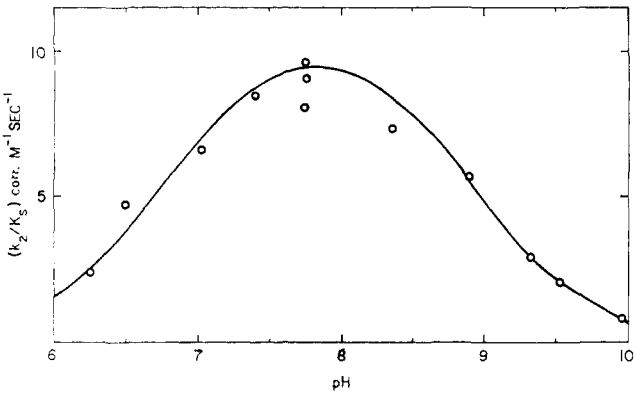

Figure 4. The reaction of α-chymotrypsin with TPCK at 25° in 2.3% (v/v) methanol–water. The line is a theoretical line calculated according to the data in the text.

7.83, a plot of $1/k_{exp}$ *vs.* $(H + H^2_{max}/H)$ yields a straight line with a lesser dispersion, from which we obtain: $pK_1 = 6.79$, $pK_2 = 8.87$, and $k_{lim} = 11.2\ M^{-1}\ sec^{-1}$. On the basis of these data a theoretical curve has been calculated which gives an excellent fit of the experimental points corrected for the ionization constant of TPCK (Figure 4).

Discussion

In the inhibition reaction described here, the chloro ketone must at least partially overlap the active site of the enzyme. This conclusion is based on the following facts. (1) The reaction product of TPCK and α-chymotrypsin is enzymatically inactive. (2) The reaction was shown in a qualitative manner to be inhibited by β-phenylpropionate ion[2] and was shown here in a quantitative manner to be competitively inhibited by benzamide. Furthermore, the inhibition constant obtained for the benzamide inhibition of the TPCK reaction is identical with the inhibition constant of benzamide when the latter acts as a competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of specific substrates. (3) Alkylation of methionine 192 of chymotrypsin very strongly decreases the rate of the TPCK reaction.

Reaction of TPCK with α-chymotrypsin under the conditions used in the present experiment exhibits second-order kinetics: it is first order in TPCK and first order in α-chymotrypsin. Thus the reaction can theoretically be either a pure second-order process in which the TPCK and enzyme react in a collision process or alternatively the transition state can be preceded by an adsorption step. Alkylation of trypsin by the chloro ketone derived from lysine exhibits prior binding before reaction.[18] The TPCK reaction may also.[19] Furthermore, there is evidence that not only is there an adsorption step but in addition the adsorption site is the same as the one operating in the hydrolysis reaction of the enzyme. The most important argument to this point is that the specificity of the TPCK–chymotrypsin reaction has the same structural requirements as is seen in the enzyme-catalyzed hydrolytic reactions. For example, it has been reported that N-methyl-TPCK is not an inhibitor[2] nor is the D isomer of TPCK.[2,20] The greater rate of reaction of TPCK with α-chymotrypsin than of chloroacetone with α-chymotrypsin directly demonstrates the specificity of this reaction.[19] Even on a quantitative basis the specificity of the enzyme in inhibition by TPCK and in hydrolytic reactions seems to be identical. The effect of a side chain on the alkylation reaction may be seen by comparison of the rates of phenoxychloromethyl ketone (PMCK)[20] and TPCK. This comparison indicates that the introduction of the tosylamido side chain accelerates the reaction 850-fold (if the structural role of the ether oxygen is assumed to be equivalent to a methylene group). The corresponding hydrolytic comparison includes methyl hydrocinnamate[21] *vs.* N-acetyl-L-phenylalanine methyl ester.[22] The ratio of the second-order hydrolytic rate constants for these two substrates is 880, in very good agreement with the ratio of the second-order rate constant of the inhibition reactions, thus showing that the same side-chain specificity mechanism must be operating in the two reactions.

If the existence of an adsorption step is accepted, then the experimental second-order rate constant must represent the value of the first-order rate constant of the decomposition of enzyme-inhibited complex, divided by the dissociation constant of that complex. Unfortunately, the latter quantity cannot be determined directly under the conditions of the present experiments.

The alkylation by TPCK takes place on the 3 position of histidine 57 as shown by the isolation of 3-carboxymethylhistidine from the hydrolysate of the TPCK–chymotrypsin reaction product which had been subjected to performic acid oxidation. That this product originates from an alkylation followed by a subsequent rearrangement in the presence of performic acid is shown by the isolation of an alkylated histidine derivative from the reaction of trypsin and L-1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) in which the alkyl group on nitrogen-3 of the histidine remains intact.[23]

The deuterium oxide kinetic solvent isotope effect measured by comparing the rate of the reaction in pD 8.4 and pH 7.8 showed a difference of only 20%. Since we postulate that the experimental rate constant is a complex constant involving a rate constant and a dissociation constant, the absence of any isotope effect can be interpreted either as a true absence or as an identical isotope effect on both constants. The former possibility is supported by extensive data which indicates no measurable kinetic solvent isotope effect on the value of K_m in the hydrolysis of acetyl-L-tryptophanamide by α-chymotrypsin.[16,24] On this basis we conclude that in all likelihood the rate constant of the inhibition reaction is insensitive to deuterium oxide isotope effects, thus indicating the absence of proton transfers in the rate-limiting step. This result is in agreement with results in other reactions where imidazole acts solely as a nucleophile.[25]

A bell-shaped, pH–rate profile has been obtained for the second-order rate constant of the TPCK–chymotrypsin reaction. The base of $pK = 6.8$ which is kineti-

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(19) D. E. Koshland, Jr., and D. M. Glick, private communication.
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(22) J. B. Jones and C. Niemann, *Biochemistry*, **2**, 498 (1963).
(23) P. H. Petra, W. Cohen, and E. N. Shaw, *Biochem. Biophys. Res. Commun.*, **21**, 612 (1965).
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cally required for the reaction may be readily identified as the imidazole group of the histidine moiety since imidazole can act only as a nucleophile in its basic form. Presumably the same base of $pK \simeq 6.2$ was observed in the reaction of PMCK with chymotrypsin[20] and in the reaction of iodoacetamide with trypsin.[26]

On the contrary, the identity of the role of the acid of $pK_a = 8.9$, necessary for the reaction, is far from clear. This group cannot be an acid group on the substrates since the same acid (of $pK \simeq 8.2$) has been observed in the reaction of PMCK with α-chymotrypsin.[19] Moreover, general acid catalysis by an enzymatic group is ruled out because of the absence of deuterium oxide solvent isotope effect. An acidic group of similar pK is observed in the second-order acylation reaction in the α-chymotrypsin-catalyzed hydrolysis of all substrates,[16] and is also observed in the binding of acetyl-L-tryptophanamide,[7,27,28] and indole[27,28] to α-chymotrypsin. The most obvious interpretation of these results is to postulate that the acidic form of this group is necessary for the binding (or productive binding) of any substrate. Since the requirement for the acidic group appears to be independent of the structure of the bound molecule it cannot participate in the binding process but rather it must act indirectly, possibly by a pH-dependent intramolecular competitive inhibition.[27] This point is being pursued further.

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(28) R. Lumry, personal communication.

The reaction of TPCK with α-chymotrypsin has previously been shown to be an alkylation of the imidazole group of histidine 57.[2-5] The present results indicate that the reaction proceeds by an adsorptive step showing the full specificity of α-chymotrypsin reactions. The alkylation reaction may be preceded by the equilibrium formation of a tetrahedral intermediate in which the serine hydroxyl group of the active site adds to the carbonyl group of TPCK, by analogy with the mechanism of the hydrolytic reactions of the enzyme. Such a reaction would be followed by the displacement of the chloride ion by the imidazole group, and finally collapse of the tetrahedral intermediate. This suggestion is consistent with the observation that the lack of the serine hydroxyl group in "anhydro-chymotrypsin" precludes the reaction of TPCK with histidine 57.[29]

The presence of three distinct nucleophiles at the active site of α-chymotrypsin has been shown so far including serine, histidine,[2] and methionine.[30] Three possible causes of selective reaction of a given reagent with one of the three nucleophiles include: (1) structure of the reagent, (2) the relative orientation of the reagent and the enzymatic nucleophile, and (3) modification of the nucleophilicity by pH. Up to the present time these three effects have not been separated, but a study of model systems and specificity studies could shed light on this interesting aspect of enzyme specificity.

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The Catalytic Activity of Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin[1]

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Abstract: The altered reactivity of chymotrypsin bearing an alkyl group on methionine-192 has been investigated. The alkyl group is the $CH_2CONHC(CH_3)_2CO_2^-$ group.[7] The pH dependence of k_{cat} and k_{cat}/K_m(app) of reactions catalyzed by this enzyme is altered only slightly. Alkylation of the enzyme increases k_3 from three- to eightfold (with the exception of the deacylation of cinnamoylchymotrypsin) whereas it does not appear to affect k_2. The binding of some substances such as hippuramide and benzamide is not modified on alkylation of the enzyme whereas that of others such as indole and acetyl-L-tryptophanamide is decreased three- to fivefold. The hypothesis that the decrease in the binding ability of the alkylated enzyme is due to an intramolecular competitive inhibition by the alkyl group is in accord with the known data. The independence of the $k_{methanolysis}/k_{hydrolysis}$ ratio on alkylation rules out an interaction of the alkyl group with the leaving group site. The independence of the binding of benzamide on alkylation rules out an interaction of the alkyl group with the hydrophobic binding site. It is therefore suggested that the alkyl group must reside at the site of interaction of the enzyme with the acylamino group of the substrate. The acceleration of k_3 by the alkyl group must be due to an independent phenomenon such as steric strain introduced by the bulky alkyl group.

The study of the reactivity of chemically modified enzymes provides a potentially powerful method for the investigation of enzymatic mechanism and specificity. For α-chymotrypsin several chemically modified forms have been prepared in the past decade.[2] In enzyme modifications preference is given to reagents

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(2) See D. E. Koshland, Jr., D. H. Strumeyer, and W. J. Ray, Jr., *Brookhaven Symp. Biol.*, **15**, 101 (1962) for a review.