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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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

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

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

reacting with a minimum number of groups—optimally one single group—in order to get specific chemical information and to avoid any important change in the tertiary structure of the enzyme. Chemical modification of a single enzymatic amino acid may result in abolition, modification, or no change of enzymatic activity. Assuming no modification of tertiary structure of the enzyme an unperturbed reactivity means that the modified group is not necessary to enzymatic activity, whereas a complete loss of activity means that the group is essential to the catalytic process and hence constitutes part of the active site of the enzyme. A most interesting result, however, arises when a chemical change in one group of the enzyme only partially modifies the catalytic activity. The best examples of this phenomenon, for the enzyme chymotrypsin, are modifications of one of the two methionines which occupy the position 180 and 192 in the sequence of chymotrypsinogen.[3-9] These derivatives, obtained either by oxidation or alkylation of the sulfur atom of the methionine group, are chemically well defined and homogeneous enzyme preparations except for the possible diastereoisomers formed at the new asymmetric sulfur atom.

The oxidation of methionine-192 of α-chymotrypsin leaves all hydrolytic rate constants and the K_m of nonspecific substrates practically unchanged whereas the K_m of specific substrates is increased by a factor of two–four.[2,10] On the other hand, alkylation of the same methionine results in an increase in both k_{cat} and K_m of the hydrolysis of tyrosine esters.[7-9] The cause of this phenomenon has been attributed to steric hindrance by the newly introduced alkyl group on the binding site of the aromatic residue of the substrate.[10]

Previously we have formulated the hypothesis that more than one productive binding can occur with nonspecific substrates of chymotrypsin, *e.g.*, with methyl hippurate.[11] This hypothesis could be tested by the use of a methionine-192-modified chymotrypsin if the cause of the K_m modification is, indeed, a steric hindrance of binding, and if only one of the two binding sites is selectively inhibited by the alkyl group introduced into the enzyme. It is the purpose of the present paper to investigate further the interaction of substrates with a modifying group of methionine-192, and to consider the implications of the resulting data on the specificity of α-chymotrypsin. For our study we chose the alkylated chymotrypsin described by Lawson and Schramm[7,9] hoping that the greater bulkiness of the alkyl group introduced here would magnify the steric hindrance caused by the alkyl group. With this alkylated enzyme we have used a variety of specific and nonspecific substrates in order to investigate the relation between specificity and the changes in the kinetic parameters produced by the alkylation of methionine-192.

Experimental Section

Materials. Methyl hippurate (Mann Research Lab. lot E1125) was recrystallized from hot water, mp 82–83° (lit.[12] mp 81.5–82.5°). *p*-Nitrophenyl hippurate was synthesized from *p*-nitrophenol and hippuric acid using dicyclohexylcarbodiimide. The ester was recrystallized twice from chloroform–hexane, mp 171–172° (lit.[13] mp 170–171°). Hippuramide (Mann Research Lab.) was recrystallized from water, mp 186–188°. *p*-Nitrophenyl hydrocinnamate was prepared from *p*-nitrophenol and hydrocinnamoyl chloride. The ester was recrystallized from chloroform–hexane, mp 99–100° (lit.[13] mp 97–98°). N-trans-Cinnamoylimidazole (Aldrich Chemical Co.) was recrystallized from dry *n*-hexane, mp 134°. *p*-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate,[14] *p*-nitrophenyl N-acetyl-DL-tryptophanate,[15] N-acetyl-L-tryptophanamide,[16] and methyl N-acetyl-L-tryptophanate[15] have been described before. Ethyl N-acetyl-L-tyrosinate (Mann Research Lab. lot A-4585, chromatographically pure) was used without further purification. Ethyl N-acetyl-D-tyrosinate (Cyclo Chemicals Corp. lot R-3669) had mp 70°, $\alpha^{25}D$ 24° (*c* 7, ethanol). N-Acetyl-L-tyrosinamide (Mann Research Lab.) had mp 226–227°. Benzamide (Fischer reagent) was recrystallized several times from water–ethanol, mp 128–129°. Indole (Litton Chemicals) was a zone-refined material used without further purification. *p*-Nitrophenyl N-bromoacetyl-α-aminoisobutyrate was a gift of Dr. W. P. Lawson, mp 149–150°. Spectral grade solvents, double-distilled water, and analytical grade buffer materials were used.

Kinetic Measurements. Kinetic measurements were carried out on the Cary 14 recording spectrophotometer or using a Radiometer recording pH-Stat titrator (for the hydrolysis of methyl hippurate). Experimental details have been described in earlier papers for N-*trans*-cinnamoylimidazole,[17] *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate,[18] *p*-nitrophenyl N-acetyl-DL-tryptophanate,[15] methyl N-acetyl-L-tryptophanate,[15] and ethyl N-acetyl-L-tyrosinate.[19] The hydrolyses of *p*-nitrophenylhippurate and *p*-nitrophenyl hydrocinnamate were measured by spectrophotometric determination of the *p*-nitrophenol liberated at 340 mμ at low pH's (pH < pK_a of *p*-nitrophenol) or 400 mμ at high pH's (pH > pK_a of *p*-nitrophenol).

Preparation of the Alkylated Enzyme. All enzyme alkylations were carried out at pH 5 using a tenfold molar excess of *p*-nitrophenyl N-bromoacetyl-α-aminoisobutyrate according to the method of Lawson and Schramm.[7,9] In preliminary experiments modification of the ethanol content of the incubation mixture from 2 to 15% ethanol, changes of the organic solvent from ethanol to methanol or acetonitrile, change of the pH of the dialysis from pH 2 to pH 5, and lyophilization after dialysis were shown not to change the amount or the kinetic characteristics of the enzyme species obtained, as measured either by N-*trans*-cinnamoylimidazole titration[20] or by the kinetic constants of the hydrolysis of methyl N-acetyl-L-tryptophanate. In agreement with previous results[7] the number of active sites does not change on alkylation of methionine-192, as measured by the N-*trans*-cinnamoylimidazole titration. The native enzyme (Worthington 3 × crystallized, lyophilized batch CDI 6110-1) gave on titration a purity by weight of 77 ± 1% (assuming a molecular weight of 24,800) while the alkylated enzyme after dialysis and lyophilization gave 75 ± 1%. Nevertheless, N-*trans*-cinnamoylimidazole titrations were used on all enzyme preparations for the determination of the actual enzyme concentration.

For kinetic measurements necessitating very low enzyme con-

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Table I. The Kinetics of the Hydrolysis of Methyl N-Acetyl-L-tryptophanate by Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin[a]

Buffer	pH	$E \times 10^7$, M	k_{cat}, sec^{-1}	K_m(app) $\times 10^4$, M	k_{cat}/K_m-(app) $\times 10^{-4}$, M^{-1} sec^{-1}
Acetate	5.02	28.6	...	...	0.234
Phosphate	6.46	1.92	110	23.6	4.67
	6.73	1.92	133	17.4	7.65
	7.16	1.92	144	10.6	13.6
Tris-HCl	7.91	1.92	154	8.2[b]	18.8[b]
	8.01	5.60	162	7.8[c]	20.8[c]
	8.57	1.92	158	7.58	20.8
	8.87	19.2[d]	173	9.5	18.1
Carbonate	9.42	19.2[d]	161	8.9	20.2
	10.03	19.2[d]	149	14.0	10.6

[a] 25°, 2.4% (v/v) acetonitrile-water, 300 mμ, $S = 2.1 \times 10^{-5}$ M, $I = 0.1$. [b] Experiment carried out in 3.4% acetonitrile-water; K_m-(app) converted to 2.4% acetonitrile by using the data of G. E. Clement and M. L. Bender, *Biochemistry*, 2, 836 (1963). [c] In 1.6% acetonitrile-water; K_m(app) corrected to 2.4% acetonitrile. [d] At these high pH's the modified enzyme denatures relatively fast; in order to accelerate the hydrolysis and thus make the denaturation negligible the enzyme concentration was increased. Since the reaction was very fast, the k_{cat} and K_m(app) values are less accurate.

centrations (of the order of 10^{-8} M), alkylated enzyme solutions were used directly after reaction with the alkylating agent. The presence of 10^{-7} M N-bromoacetyl-α-aminoisobutyrate and *p*-nitrophenol had no observable effect on the kinetic behavior of chymotrypsin. Unless otherwise stated all rate constants were determined by using Lineweaver-Burk plots of single experiments.

The alkylated enzyme was very stable at low pH's: at pH 6.25 a 10^{-5} M alkylated enzyme in 0.067 M phosphate buffer and 2% (v/v) methanol-water loses less than 2% of its activity at 25° in one day, when measured with a *p*-nitrophenyl-N-benzyloxycarbonyl-L-tyrosinate rate assay. However, its resistance to loss of activity at higher pH's (>7) is considerably lower than that of native chymotrypsin.

Results

The pH dependence of the rate of the alkylated enzyme-catalyzed hydrolysis of methyl N-acetyl-L-tryptophanate was determined from pH 5 to 10. The data shown in Table I indicate that k_{cat} increases from pH 6.46 to 8.01 and then remains sensibly constant. The value of K_m(app) increases very fast at pH's lower than 6 and thus we were unable to determine accurate values for k_{cat} and K_m(app) in this region. In the high pH region (>8) the fast denaturation of the enzyme limits the accuracy of the results. From the k_{cat} data the approximate pK_a of a base governing k_{cat} was determined graphically to be 6.20 and k_{cat}(lim) was found to be 165 sec^{-1}. The values of k_{cat}/K_m(app) probably define a bell-shaped curve as shown in Figure 1. Using values of pH_{max} 8.43 the ionization constants of the acid and base controlling this bell-shaped curve were found by a graphical method[16] to be: $pK_1 = 6.96$ and $pK_2 \cong 9.90$.

The effect of methanol on the rate of deacylation of N-benzyloxy-L-tyrosyl-α-chymotrypsin (alkylated on methionine-192) was measured at pH 5 by determining the acceleration of the turnover reaction of the corresponding *p*-nitrophenyl ester in 10% methanol–water. From these data (Table II) and by using the equation $k_{obsd} = k_3[H_2O] + k_4[CH_3OH]$,[21] we can

(21) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3697 (1964).

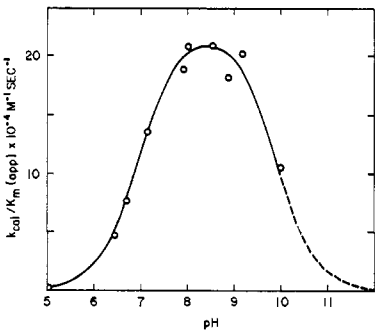


Figure 1. The pH dependence of the hydrolysis of methyl N-acetyl-L-tryptophanate by α-chymotrypsin (alkylated on methionine-192) at 25°. See conditions of Table I. Curve calculated with the equation: $k_{cat}/K_m(app) = 22.2 \times 10^4/[1 + H/(1.1 \times 10^{-7}) + (1.25 \times 10^{-10})/H]$.

calculate the second-order rate constant of the methanolysis of the acyl-enzyme, k_4. By comparing the value of k_4 with the second-order hydrolysis constant, k_3, we find $k_4/k_3 = 80.5$ while for native α-chymotrypsin $k_4/k_3 = 109$ under identical conditions. Both of these values compare favorably with the k_4/k_3 ratios obtained earlier with a variety of acyl-chymotrypsins.[21]

Table II. Catalysis of the Methanolysis of *p*-Nitrophenyl N-Benzyloxycarbonyl-L-tyrosinate by Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin[a]

Enzyme	% MeOH, v/v	k_{cat}, sec^{-1}	$K_m \times 10^7$, M	k_4, M^{-1} sec^{-1}	k_3, M^{-1} sec^{-1}
α-Chymotrypsin	0	1.17	3.3	...	2.11×10^{-2}
	10	6.78	17.7	2.31	...
Alkylated on methionine-192	0	6.80	29.1	...	12.3×10^{-2}
	10	30.7	288	9.90	

[a] 25°, 1.6% (v/v) acetonitrile–water, pH 5.0, 0.2 M acetate buffer, 340 mμ, $S_0 = 1.53 \times 10^{-5}$ M, $E_0 = 5.11 \times 10^{-8}$ M for α-chymotrypsin and $E_0 = 5.60 \times 10^{-8}$ M for alkylated enzyme.

The rate of reaction of L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK) with both α-chymotrypsin and the methionine-192 alkylated enzyme was determined by measuring the decrease of activity of the enzyme using a *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate rate assay at pH 5.[22] Plots of the logarithm of the remaining activity, as a function of time, gave good straight lines from which apparent first-order rate constants were calculated. By dividing these constants by the TPCK concentration second-order alkylation constants were obtained. The results are summarized in Table III.

The initial rate of liberation of *p*-nitrophenol from *p*-nitrophenyl 2-(bromoacetamido)isobutyrate in the presence of both native α-chymotrypsin and the alkylated enzyme were measured (pH 5.00, 0.2 M acetate buffer, 25°, 1.52% (v/v) methanol–water). The amount of *p*-nitrophenol liberated was much less than 1 mole per mole of enzyme. Under these conditions the initial rate was proportional to both the enzyme and the isobutyrate concentration, indicating that the enzyme was not saturated. The results of these investigations are given in Table IV.

Table IV also summarizes extensive kinetic data on the hydrolysis of a number of specific and nonspecific

(22) F. J. Kézdy, A. Thompson, and M. L. Bender, *ibid.*, **89**, 1004 (1967).

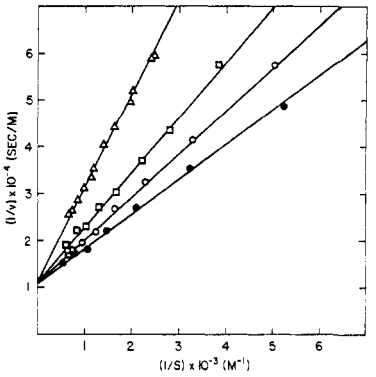


Figure 2. The hydrolysis of methyl N-acetyl-L-tryptophanate with α-chymotrypsin (alkylated on methionine-192) at pH 8.01, 0.1 *M* Tris–HCl buffer, 25°, 1.6% (v/v) acetonitrile–water, $E_0 = 5.6 \times 10^{-7}$ *M*, ester = 2.16×10^{-3} *M*; ●, no inhibitor; ○, 1.65×10^{-2} *M* ethyl N-acetyl-D-tyrosinate; □, 5.56×10^{-2} *M* N-acetyl-L-tyrosinamide; △, 2.14×10^{-2} *M* hippuramide.

substrates catalyzed by both α-chymotrypsin and the methionine-192 alkylated enzyme.

Inhibition constants for a variety of inhibitors were determined by using either N-acetyl-L-tryptophan methyl ester or methyl hippurate as substrate (Table V). As shown in Figure 2 for some inhibitions of the alkylated enzyme, all the inhibitions observed were of a purely competitive nature within experimental error, *i.e.*, the V_{max} remained unchanged. Values of K_i were calculated by comparing the slopes of Lineweaver–Burk plots of individual experiments with and without an inhibitor.

Table III. Reaction of L-1-Chloro-3-tosylamido-4-phenyl-2-butanone with α-Chymotrypsin and with Methionine-S-(N-2-carboxyisopropyl)carbamylsulfonium Bromide-192-α-chymotrypsin[a]

Enzyme	pH	[TPCK] × 10^4, *M*	$E_0 \times 10^6$, *M*	$k \times 10^4$, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}
α-Chymotrypsin	6.25	1.50	7.42	3.56	2.37
	7.39	1.74	6.90	14.7	8.45
	7.39	1.74	6.90	12.3	7.07
Alkylated on methionine-192	6.25	1.50	6.11	0.107	0.071
	7.35	1.74	5.80	0.575	0.33

[a] 25°; 0.067 *M* phosphate buffers, 2.3% (v/v) methanol–water.

Discussion

Theoretically, the alkylation of methionine-192 could lead to enantiomorphic products. However, the kinetic data do not show any indication of the presence of a mixture of two enzymes having different K_m(app) values for any of the substrates studied.[23] Thus either there is only one enantiomorph present, or the two enantiomers have identical catalytic properties, or the newly formed asymmetric sulfonium ion racemizes faster than any of the kinetic processes observed. The first of these possibilities seems to be the most probable. Certainly the kinetic data strongly indicate a homogeneous enzyme preparation, as far as catalytic properties are concerned.

The alkylated chymotrypsin-catalyzed hydrolysis of methyl N-acetyl-L-tryptophanate has a second-order constant [k_{cat}/K_m(app)] which showed a characteristic bell-shaped pH–rate constant profile with $pK_1 = 6.96$ and $pK_2 \simeq 9.9$, the latter being quite inaccurate. These data are quite comparable to the pH dependence of k_{cat}/K_m(app) of the α-chymotrypsin-catalyzed hydrolysis of ethyl N-acetyl-L-tryptophanate: $pK_1 = 6.77$ and $pK_2 = 9.2$.[16] This comparison indicates that the ionization constant of the catalytically important base is not modified in an important way by the alkylation of methionine-192. This result suggests a negligible electrostatic interaction between the catalytic base and the internal salt formed on alkylation: either the catalytic base must be widely separated from the electric charges introduced or the positively charged sulfur atom must be in close proximity to the carboxylate ion of the alkylating agent through formation of an internal salt.

Although data on the pH dependence of k_{cat} of the alkylated enzyme is severely limited by inability to saturate the enzyme at low pH's, the data do indicate that k_{cat} of the hydrolysis of methyl N-acetyl-L-tryptophanate (presumably the deacylation rate constant) is dependent on a basic group of $pK_a = 6.2$. This value is significantly lower than the pK_a of the base-controlling k_{cat} in chymotrypsin-catalyzed hydrolysis of comparable substrates, which range from 6.74 to 6.86.[16] The value of K_m(app) of the alkylated enzyme is unusual: whereas K_m(app) is pH independent in the pH 4–7 region in the α-chymotrypsin-catalyzed hydrolysis of ethyl N-acetyl-L-tryptophanate,[16] K_m(app) of the alkylated enzyme increases strongly with decreasing pH in the same pH region (Table I). The K_m(app) of the alkylated enzyme levels off, however, probably at pH >5, since the *p*-nitrophenyl ester has nearly an identical K_m(app) at pH 2.7 and 5 (Table IV). Since K_m(app) is a complex constant $\{= K_s/[1 + (k_2/k_3)]\}$, this behavior can be interpreted in two ways: (1) k_2 and k_3 have nearly identical p*K*'s and K_s is pH dependent, or (2) K_s is pH independent but the pK_a of k_2 is higher than that of k_3.

The methanolysis data in Table II indicate that the k_4/k_3 ratio is identical within experimental error for α-chymotrypsin and the alkylated enzyme. Thus it appears that the alkyl group introduced on methionine-192 does not interact with methanol (acting as a nucleophile) and hence, on the basis of the symmetry of the enzyme mechanism,[24] the alkyl group on methionine-192 does not interact with the leaving group of the substrate.

The presence of 15% ethanol in the alkylating solution does not appear to have any effect on the catalytic properties of the alkylated enzyme. The mechanism proposed for the alkylation process[7] consists of the following sequence of steps: acylation, alkylation, and deacylation. Thus in the presence of 15% ethanol a large part of the deacylation should occur *via* ethanolysis,[21] producing an ethyl ester of the alkylating agent. Since 15% ethanol does not modify the catalytic properties of the alkylated enzyme, either the ethyl ester thus formed is rapidly hydrolyzed by the enzyme or alternatively the carboxylate ion of the alkyl moiety does not play any role in the modification of the catalytic properties of the enzyme.

(23) J. M. Reiner, "Behavior of Enzyme Systems," Burgess Publishing Co., Minneapolis, Minn., 1959, p 98.

(24) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).

Table IV. Kinetics of Hydrolysis of Substrates by α-Chymotrypsin and by Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin[a]

Substrate	Enzyme[b]	$S \times 10^6$, M	$E \times 10^7$, M	pH	Buffer[c]	Ionic strength, M	Organic solvent,[d] % v/v	k_{cat}, sec^{-1}	K_m(app) $\times 10^6$, M	k_{cat}/K_m(app) $\times 10^{-8}$, M^{-1} sec^{-1}
p-Nitrophenyl hydrocinnamate	A	66	18	7.90	P	0.1	15 AC	0.46	46	10
	N	70	26	8.11	P	0.1	15 AC	0.178	2.3	76
	N	70	24	8.30	P	0.1	15 AC	0.21	3.6	58
p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate	N	9.2	0.97	5.00	A	0.2	1.6 AN	1.07	0.23	4620
	A	9.2	0.93	5.00	A	0.2	1.6 AN	3.85	1.76	2190
	N	9.2	0.65	5.04	A	0.2	1.6 AN	0.97	0.17	5750
	A	9.2	0.63	5.04	A	0.2	1.6 AN	3.8	1.99	1900
	A	14	0.80	5.04	A	0.2	1.6 AN	3.3	1.81	1810
	N	15	0.51	5.00	A	0.2	1.6 AN	1.17	0.33	3550
	A	15	56	5.00	A	0.1	1.6 AN	6.8	2.9	2340
Ethyl N-acetyl-L-tyrosinate	N	12,700	20.2	8.05	T	0.1	1.6 AN	233	1,690	138
	A	12,700	19.3	8.05	T	0.1	1.6 AN	864	15,400	56
Methyl N-acetyl-L-tryptophanate	N	2,060	2.06	7.91	T	0.1	3.4 AN	47.1	126	374
	N	2,170	4.95	8.01	T	0.1	1.6 AN	51.0	107	476
	A	2,060	1.92	7.91	T	0.1	3.4 AN	154	1,010	152
	A	2,170	5.60	8.01	T	0.1	1.6 AN	162	680	238
p-Nitrophenyl N-acetyl-DL-tryptophanate	A	56	19.3	2.67	C	0.05	1.6 AN	0.091	24.5	3.71
	N	56	19.2	2.67	C	0.05	1.6 AN	0.013	...	...
	N	78	3.0	5.04	A	0.1	1.6 AN	0.78	1.61	485
	A	78	2.9	5.04	A	0.1	1.6 AN	6.6	34	196
p-Nitrophenyl hippurate	N	183	94.7	5.32	A	0.2	15 AC	0.012	27	0.443
	A	183	90.7	5.32	A	0.2	15 AC	0.044	380	0.117
	N	180	28.0	6.64	P	0.1	15 AC	0.34	40	8.42
	A	180	21.9	6.64	P	0.1	15 AC	1.52	890	1.71
Methyl hippurate	N	...	...	8.28	T	0.2	...	0.25	4,060	0.062
	N	...	...	8.28	T	0.2	...	0.25	4,060	0.062
	A	...	...	8.28	T	0.2	...	0.29	6,600	0.043
	A	...	...	8.28	T	0.2	...	0.30	9,300	0.032
trans-Cinnamoylimidazole	N	10	26.0	6.87	P	0.1	0.32 AN	...	...	24
	A	10	20.8	6.87	P	0.1	0.32 AN	...	...	4.7
	N	100	26.0	6.87	P	0.1	3.4 AN	0.0038	...	...
	A	100	20.8	6.87	P	0.1	3.4 AN	0.0048	...	...
p-Nitrophenyl 2-(bromoacetamido)isobutyrate	N	132	112.3	5.0	A	0.2	1.52 MeOH	...	...	0.0028
	A	132	94.5	5.0	A	0.2	1.52 MeOH	...	...	$<10^{-4}$

[a] 25.0°. [b] A, α-chymotrypsin alkylated on methionine-192; N, native α-chymotrypsin. [c] P, 0.067 M phosphate; A, 0.1 M acetate; T, 0.05 M Tris–HCl; C, 0.05 M citrate; ionic strength adjusted with KCl. [d] AN, acetonitrile; AC, acetone; MeOH, methanol.

Table V. Inhibition Constants of Some Substances toward α-Chymotrypsin and Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin[a]

Enzyme	% v/v, acetonitrile–water	pH	Inhibitor	[I] $\times 10^2$, M	$K_i \times 10^3$ M	$K_i \times 10^3$ M, lit.
Native	3.4	7.91	Indole	0.22	0.97	0.8 ± 0.2[c]
Alkylated	3.4	7.91	Indole	0.22	2.78	
Native	3.4	7.91	Benzamide	2.59	8.86	10 ± 2[c]
Alkylated	3.4	7.91	Benzamide	2.59	9.7	
Native	1.6	8.01	Ethyl N-acetyl-D-tyrosinate	1.69	7.21	5.0 ± 0.5[c]
Alkylated	1.6	8.01		1.69	71	
Native	1.6	8.01	Hippuramide	2.14	14.9	12.5 ± 2[c]
Alkylated	1.6	8.01	Hippuramide	2.14	12.9	
Native	1.6	8.01	N-Acetyl-L-tryptophanamide	2.34	7.5	5.0[d]
Alkylated				2.34	40.3	
Native	1.6	8.01	N-Acetyl-L-tyrosinamide	5.56	37.6	32[d]
Alkylated				5.56	99.5	
Native	0	8.28	Hippurate[b]	...	4.76	
Alkylated	0	8.28	Hippurate[b]	...	9.28	

[a] 25°, 0.1 M Tris–HCl, 2×10^{-3} M methyl N-acetyl-L-tryptophanate substrate, α-chymotrypsin = $2\text{–}5 \times 10^{-7}$ M, alkylated enzyme = $2\text{–}6 \times 10^{-7}$ M. [b] 0.2 M Tris–HCl, methyl hippurate as substrate. [c] R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3370 (1955). [d] R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

A comparison of the kinetic and binding properties of α-chymotrypsin and alkylated enzyme is summarized in Table VI. Setting aside the hippurate ion whose carboxylate ion group must electrostatically perturb the binding, the ratios of the inhibition constants of the native and alkylated enzymes reported in Table VI

Table VI. A Comparison of Inhibitors and Kinetic Constants for α-Chymotrypsin and Methionine-S-(N-2-carboxyisopropyl)carbamylmethylsulfonium Bromide-192-α-chymotrypsin

Substrate	pH	$\frac{(k_{cat})\text{ alkylated enzyme}}{(k_{cat})\text{ chymotrypsin}}$	$\frac{[K_m(app)]\text{ alkylated enzyme}}{[K_m(app)]\text{ chymotrypsin}}$	$\frac{[k_{cat}/K_m(app)]\text{ chymotrypsin}}{[k_{cat}/K_m(app)]\text{ alkylated enzyme}}$	$\frac{(K_i)\text{alkylated enzyme}}{(K_i)\text{chymotrypsin}}$
Methyl hippurate	8.28	1.14	1.62	1.42	
p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate	5.0	4.35	9.25	2.24	
Ethyl N-acetyl-L-tyrosinate	8.05	3.70	9.23	2.47	
Methyl N-acetyl-L-tryptophanate	7.91	3.27	8.02	2.46	
	8.01	3.17	6.36	2.0	
p-Nitrophenyl N-acetyl-DL-tryptophanate	2.67	6.93	...	...	
	5.04	8.5	21	2.47	
p-Nitrophenyl hippurate	5.32	3.77	14.3	3.79	
	6.64	4.47	22.1	4.91	
N-*trans*-Cinnamoylimidazole	6.87	1.25	...	5.13	
p-Nitrophenyl hydrocinnamate	8.0	2.17–2.58	13–20	5.8–7.6	
p-Nitrophenyl 2-(bromoacetamido)isobutyrate	5.0	...	...	$\geqslant 28$	
TPCK	6.25	...	...	33.4	
	7.35	...	...	23.7	
Hippuramide	8.01				0.87
Benzamide	7.91				1.09
(Hippurate ion)	8.28				(1.95)
N-Acetyl-L-tyrosinamide	8.01				2.64
Indole	7.91				2.86
N-Acetyl-L-tryptophanamide	8.01				5.38
Ethyl N-acetyl-D-tyrosinate	8.01				9.85

can be divided into three groups: (1) the bindings of hippuramide and benzamide are not modified; (2) bindings of indole, N-acetyl-L-tyrosinamide, and N-acetyl-L-tryptophanamide are decreased by a factor of 2.6–5.4; and (3) the binding of ethyl N-acetyl-D-tyrosinate is modified tenfold. It is of interest to note that for N-acetyl-L-tyrosine derivatives the D-ethyl ester is five times better bound to native α-chymotrypsin than is the amide, whereas the D ester and the L amide have nearly identical binding constants with the alkylated enzyme.

Analysis of the kinetic data is more complicated. The k_{cat} ratios listed in Table VI indicate that all rate constants are higher for the alkylated enzyme than for α-chymotrypsin, with the exception of methyl hippurate and the deacylation of the cinnamoyl-enzyme. Although k_{cat} of methyl hippurate is not accelerated by alkylating the enzyme, the k_{cat} of *p*-nitrophenyl hippurate is accelerated fourfold. Since acylation is largely rate limiting in the hydrolysis of the methyl ester while deacylation is rate limiting in the hydrolysis of the *p*-nitrophenyl ester,[25] it appears that the alkylation of the enzyme does not influence k_2 but accelerates k_3. The only exception to this generalization appears to be the deacylation of the cinnamoyl-enzyme which is insensitive to the alkylation of the enzyme.

The view that k_2 is unchanged while k_3 is accelerated in the alkylated enzyme is further supported by inspection of the K_m(app) and k_{cat}/K_m(app) ratios in Table VI. With the exception of methyl hippurate, all the substrates in Table VI are known to possess a rate-limiting deacylation step in their enzymatic hydrolysis.[24] Thus the complex nature of the K_m(app) constants leads to the ratio of K_m(app)'s of the alkylated and native enzymes which must be expressed as [K_s(alkylated enzyme)/K_s(chymotrypsin)]{[1 + (k_2/k_3)](α-chymotrypsin)/[1 + (k_2/k_3)](alkylated enzyme)}. If k_2 and k_3 were accelerated identically on alkylation of the enzyme, then the [1 + (k_2/k_3)] ratio for the two enzymes should be unity and the over-all ratio would be equal to the ratio of inhibition constants of an inhibitor which is structurally related to the substrate under consideration. This is not the case. For example, the K_m(app) ratio for ethyl N-acetyl-L-tyrosinate is 9.2 while the K_i ratio for N-acetyl-L-tyrosineamide is only 2.64. Similarly, the K_i ratio for hippuramide is 0.87 while the K_m(app) ratio is 1.62 for methyl hippurate and ~18 for *p*-nitrophenyl hippurate (at pH 5.3 and 6.66). Finally, the K_i ratio for N-acetyl-L-tryptophanamide is 5.4 whereas the K_m(app) ratio for methyl N-acetyl-L-tryptophanate is 6.7 and for *p*-nitrophenyl N-acetyl-DL-tryptophanate (at pH 5) is 21. Thus the ratio [1 + (k_2/k_3)](chymotrypsin)/[1 + (k_2/k_3)](alkylated enzyme) is greater than 1 for every set investigated, indicating that alkylation of the enzyme selectively accelerates k_3.

The k_{cat}/K_m(app) ratios for the native and alkylated enzymes are similarly composed of [k_2(chymotrypsin)/k_2(alkylated enzyme)][K_s(alkylated enzyme)/K_s(chymotrypsin)]. This ratio for specific substrates is not greater than the K_i ratio of comparable inhibitors, thus indicating that the ratio k_2(chymotrypsin)/k_2(alkylated enzyme) must be close to unity.

The k_{cat}/K_m(app) ratio for the native and alkylated enzymes is much greater for some nonspecific substrates than for specific ones, the effect being maximal for *p*-nitrophenyl α-(bromoacetamido)isobutyrate.[26] This result, together with the fact that the K_i ratio is maximal

(25) B. Zerner and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964).

(26) This statement applies to those nonspecific substrates which have a bulky (nitrophenol, imidazole) leaving group. With methyl hippurate which has a small leaving group, no such perturbation is seen, implying that the bulky leaving group interacts with the alkyl group introduced into the enzyme.

for ethyl N-acetyl-D-tyrosinate, appears to indicate that the binding of these substrates occurs in a position which only partially overlaps the binding site of the specific substrates, and that this "nonspecific binding site" must be the one occupied by the alkyl group introduced into the enzyme.

The k_{cat} and K_m(app) ratios are uniformly higher at lower pH's whereas the k_{cat}/K_m(app) ratios are not higher at lower pH's. This result is interpreted as the effect of the decreased pK_a values of deacylation. Thus, of the alternatives presented earlier for the explanation of an increased K_m(app) of the alkylated enzyme at lower pH's, it appears that the best explanation is that the pH dependence of the k_2/k_3 ratio in the alkylated enzyme is the source of the abnormal increase of K_m(app) at low pH's for substrates which have deacylation as the rate-controlling step.

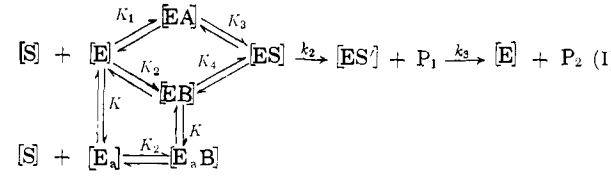
The Interaction of the Alkyl Group with the Substrate. The experimental observations on which any interpretation of the interaction of the alkyl group on methionine-192 with the substrate must be based can be summarized as follows: (1) binding constants are unchanged for hippuramide and benzamide but increased for all other inhibitors when methionine-192 is alkylated; (2) k_2 is not affected and k_3 is increased by alkylation of methionine-192; (3) alkylation of the enzyme does not change the pK_a of the catalytic base on the free enzyme or on the enzyme–substrate complex but decreases the pK_a of the catalytic base on the acyl-enzyme. *A priori* several hypotheses can explain these observations. Any interaction between the alkylated methionine and the catalytic groups can be ruled out since the observed effects are dependent on the nature of the substrate and since substrate binding is altered. Similarly, if the effect of alkylation of the enzyme were the suppression of a nonproductive binding, then the ratio k_{cat}(alkylated enzyme)/k_{cat}(chymotrypsin) should be identical with the ratio K_m(app)(alkylated enzyme)/K_m(app)(chymotrypsin),[11] which is obviously not the case (Table VI). Thus at least part of the interaction between the methionine alkyl group and the substrate must result from a decrease of the binding ability of the true ("productive") binding site or at least part of this binding site. This decrease of binding ability can be caused by: (1) a permanent, rigid deformation of the adsorption site; (2) elimination of a group participating in the binding (*e.g.*, the sulfur atom of methionine); or (3) an intramolecular competitive inhibition by the alkyl group on the methionine.

The close relationship between kinetic specificity and binding rules out both the permanent deformation of the binding site and the permanent loss of the binding group. The former is ruled out since the k_2 is unchanged whereas k_3 and K_s are increased on alkylation of the enzyme. The latter is ruled out simply since kinetic specificity is maintained. Thus, intramolecular competitive inhibition must be the phenomenon responsible for the altered binding constants. This suggestion, of course, presupposes a mobility of the inhibiting alkyl group, and an equilibrium between the adsorbed and desorbed conformations which is at least theoretically possible through the mobility of the methionine side chain. If, indeed, an intramolecular competitive inhibition accounts for the alteration of the adsorption site, then the question arises as to which part of the active site is completely inhibited. Assuming the hypothesis of the rigidly adsorbed substrate molecule,[26] the site of the interaction may be located with respect to the position of the substrate in the following manner.

The invariant k_4/k_3 ratio for methanolysis together with the symmetry of the enzyme mechanism rules out an interaction of the alkyl group of the enzyme with the substrate at the position of the leaving group of the substrate. An interaction of the alkyl group of the enzyme with the carboxyl group of the substrate would necessarily modify both acylation and deacylation rate constants of every substrate; thus, such an interaction is ruled out. Finally, the unaltered binding of benzamide rules out any interaction at the hydrophobic binding site. The latter conclusion is supported by the observations of Schramm and Lawson[8] that the inhibiting action of the methionine alkyl group on the hydrolysis of ethyl L-tyrosinate is independent of the structure of this alkyl group; particularly the presence or absence of an aromatic residue does not greatly modify the inhibitory action of the alkyl group of the modified enzyme. Thus by process of elimination the interaction of the alkyl group of the enzyme with the substrate must occur at the site of the acylamino group of the substrate.

Knowles[10] observed that upon oxidation of methionine-192 the binding constants of specific substrates increased while the binding constants of nonspecific substrates remained unchanged. Since the qualitative aspects of Knowles's observations and those found here are the same, and since both the enzyme used by Knowles and that used here were modified at the same position, it would appear that his results can be best interpreted in the same way as the present results: the modification of the binding ability occurs at the acylamino site, but nonspecific substrates (with small leaving groups) bind mainly at the hydrophobic site and thus do not come in contact with the oxidized methionine. This conclusion is reinforced by the observation that the binding of both proflavin and β-phenylpropionate to chymotrypsin is unaffected by oxidation of methionine-192.[4]

Assuming two independent binding sites[11] the intramolecular competitive inhibition of the acylamino site can be pictured by the following scheme



where $[E_a]$ is the intramolecularly inhibited enzyme species, and [EA] and [EB] are the incomplete enzyme–substrate complexes in which only the acylamino or the hydrophobic group is bound, respectively. The dissociation constants are defined as follows: $K = [E]/[E_a] = [EB]/[E_aB]$; $K_1 = [E][S]/[EA]$; $K_2 = [E][S]/[EB]$; $K_3 = [EA]/[ES]$; $K_4 = [EB]/[ES]$. The apparent inhibition constant must then be given by

$$K_i(\text{alkylated enzyme}) = \frac{[S]([E] + [E_a])}{[EA] + [EB] + [E_aB] + [ES]} \quad (2)$$

From eq 2 we can then derive

$$K_i(\text{alkylated enzyme}) = \frac{K_1K_3[1 + (1/K)]}{1 + K_3 + K_4[1 + (1/K)]} \quad (3)$$

Furthermore, for α-chymotrypsin where K equals infinity we can derive

$$K_i(\text{chymotrypsin}) = K_1K_3/(1 + K_3 + K_4) \quad (4)$$

From eq 3 and 4 we find

$$\frac{K_i(\text{alkylated enzyme})}{K_i(\text{chymotrypsin})} = \frac{1 + (1/K)}{1 + [K_i(\text{chymotrypsin})/K_2K]} \quad (5)$$

Thus if binding of the substrate occurs mainly at the hydrophobic site (as is the case for benzamide), $K_2 = K_i$(chymotrypsin), and thus K_i(alkylated enzyme)/K_i(chymotrypsin) = 1. For all other substrates K_i(alkylated enzyme)/K_i(chymotrypsin) > 1, since for these substrates $K_2 > K_i$(chymotrypsin). The K_i ratio for the alkylated and native enzymes for indole is greater than 1. Since indole is larger than a simple benzene ring it can come in contact with the position normally occupied by the α-nitrogen atom of the amino acid and thus the acylamino site. Thus suggestion is supported by the fact that indole accelerates the deacylation of acetyl-α-chymotrypsin.[27] If the inhibitor is rigidly adsorbed at the site, then the ethoxycarbonyl group of ethyl N-acetyl-D-tyrosinate must occupy the acylamino site of the enzyme.[26] Since in chymotrypsin the K_i of this ester is lower than the K_i of the L-amide, the binding of the former must be stronger than the binding of the latter to the acylamino site. Thus inhibition of the acylamino site should make this difference disappear, which is indeed observed. For substrates reacting according to the above scheme the rate equation takes the form

$$V(\text{alkylated enzyme}) = \frac{\dfrac{k_2E_0[S]}{1 + (k_2/k_3) + K_3 + K_4[1 + (1/K)]}}{[S] + \dfrac{K_1K_3[1 + (1/K)]}{1 + (k_2/K_s) + K_s + K_4[1 + (1/K)]}} \quad (6)$$

(27) R. J. Foster, *J. Biol. Chem.*, 236, 2461 (1961).

Thus for the alkylated enzyme and for α-chymotrypsin the following relations hold

$$[k_{cat}/K_m(\text{app})](\text{alkylated enzyme}) = k_2/K_1K_3[1 + (1/K)] \quad (7)$$

$$[k_{cat}/K_m(\text{app})](\text{chymotrypsin}) = k_2/K_1K_3 \quad (8)$$

$$\frac{[k_{cat}/K_m(\text{app})](\text{chymotrypsin})}{[k_{cat}/K_m(\text{app})](\text{alkylated enzyme})} = 1 + (1/K) \quad (9)$$

Equation 9 indicates that for specific substrates the $[k_{cat}/K_m(\text{app})](\text{chymotrypsin})/[k_{cat}/K_m(\text{app})](\text{alkylated enzyme})$ ratio should be constant and independent of the substrate. This is, in fact, observed. Since nonspecific substrates do not occupy exactly the same position as specific substrates in acylation, this relation should not necessarily hold for the nonspecific substrate. Actually the observed ratios are much higher for the latter (containing large leaving groups), indicating again that the methionine alkyl group preferentially inhibits the nonspecific binding site in agreement with its chemical structure.

Equation 6 is unable to account for the accelerating effect of the alkyl group on k_3; in fact, it actually predicts a decrease of the catalytic rate constant. Thus the acceleration of k_3 must be due to an independent phenomenon not directly related to the binding. This is clearly seen in the hippurate data: the binding constant of hippuramide is not modified on alkylation but the deacylation of hippurylchymotrypsin is modified. The fact that oxidation of methionine-192[10] does not accelerate k_3 whereas alkylation of the methionine does indicates that the acceleration is due to the bulkiness of the substituent on methionine-192. This conclusion points to an acceleration by steric strain.

The present data are barely enough to allow us the formation of a hypothesis concerning the interaction between the alkyl group of the modified enzyme and the substrate. Further experimentation such as changing the structure of the alkyl group will be necessary before the ideas presented here will become more than a working hypothesis.