TABLE I

RATE CONSTANTS OF THE DEACYLATION OF SOME ACYL-α-CHYMOTRYPSINS AND ACYL-TRYPSINS[a,f,i]

Acyl group	pH	k_3, sec.$^{-1}$ α-Chymotrypsin	k_3, sec.$^{-1}$ Trypsin	k(α-chymotrypsin)/k(trypsin)
Indoleacryloyl-[b]	8.8	0.0019	0.0036	0.53
Furylacryloyl-[b]	8.8	0.0025	0.0019	1.3
Acetyl-[b,g]	8.8	0.0068	0.0099	0.70
Cinnamoyl-[b]	8.8	0.0125	0.0169	0.74
N-Acetyl-L-leucyl-[d]	4.46	0.0918	0.123	0.75
	5.02	0.256	0.192	1.3
	6.10	2.26	1.47	1.5
N-Acetyl-L-tryptophanyl-[d]	3.46	0.0438	0.068[e]	0.55
	7.00	30.5	31	0.99
N-Benzyloxycarbonyl-L-tyrosyl-[h]	3.21	0.0453	0.0470	0.97
	3.21		0.0354[e]	
	5.02	1.47	0.95	1.5
	5.02		0.65	
	6.10	13.32	9.0	1.5
	6.50	23.54	19.4	1.2
	6.50		16.5	1.4
	6.50		16.4[e]	1.4
	7.10	59.6	64.0	0.93
	7.10		28[e]	

[a] 25.0°; 0.8–1.6% (v./v.) acetonitrile–water; citrate, phosphate, Tris, or borate buffers; μ = 0.025 to 0.10. [b] These values correspond to k(lim.), the plateau of the sigmoid curve dependent on a group of pK_a 6.9 to 7.4. [c] Porcine trypsin. [d] The corresponding DL-p-nitrophenyl ester was used as substrate. [e] The trypsin used in this experiment was prepared by Dr. F. H. Carpenter by treatment of trypsin with the α-chymotrypsin inhibitor, L-1-tosylamido-2-phenylethyl chloromethyl ketone,[6] (shown as the square in Fig. 1). [f] Preliminary evidence indicates that a difference exists in the deacylations of the cationic benzoyl-L-arginyl enzymes. [g] Other data on the deacylation of acetyl-α-chymotrypsin and acetyl-trypsin show similar results.[8] [h] The k_{cat} of the α-chymotrypsin- and trypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester is reported to be 7-fold different.[7] [i] The relative order of reactivity of p-nitrophenyl substrates is reported to be similar for thrombin, trypsin, and α-chymotrypsin.[9]

constants, k_3, is shown in Table I.[7-9] The determination of k_3 for the first four acyl-enzymes was carried out by preparing the acyl-enzyme and following the discrete first-order deacylation process. The rate of deacylation of the last three compounds was determined from the steady-state (zero-order) production of p-nitrophenol from the p-nitrophenyl ester. The deacylations of Table I, both slow[8] and fast (Fig. 1), depend on a group with a pK_a very close to 7 in its basic form. On this basis, the data of Table I were extrapolated to limiting rate constants (above pH 8), and it was found that the range of kinetic specificities in both the α-chymotrypsin and trypsin reactions, from an indoleacryloyl- to an N-benzyloxycarbonyl-L-tyrosyl reaction, is 10^5-fold.

The startling conclusion of Table I is that, from the slowest to the fastest deacylation, the rates of deacylation for these (nonionic) α-chymotrypsin and trypsin compounds are essentially identical (within 50%). Certainly, the 10^5-fold range of rate constants means that the enzyme is determining the kinetic specificity of this series of reactions. Electronic differences between the various acyl groups could account for perhaps

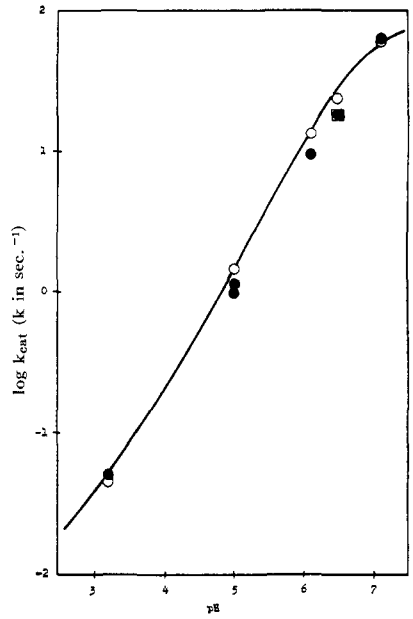

Fig. 1.—The pH dependence of the catalytic rate constant (deacylation) of the α-chymotrypsin-catalyzed hydrolysis of N-benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester, O, and of the trypsin-catalyzed hydrolysis of this ester, ●. The solid line is the theoretical line for the α-chymotrypsin reaction using pH − log $\alpha/(1-\alpha)$ = pK_{int} − 0.868ωZ.

10-fold of this 10^5-fold range. But the interaction of the various acyl groups with the two enzymes must explain the other 10^4-fold range. This interaction is obviously the same for both α-chymotrypsin and trypsin.

Acknowledgment.—The authors gratefully acknowledge the assistance of Mr. C. R. Gunter and Dr. J. Feder.

DEPARTMENT OF CHEMISTRY
NORTHWESTERN UNIVERSITY
EVANSTON, ILLINOIS

MYRON L. BENDER
JOHN V. KILLHEFFER, JR.
FERENC J. KÉZDY

RECEIVED AUGUST 31, 1964

The Active Site of Chymotrypsin[1]

Sir:

The usual approach to the elucidation of the active site of an enzyme has been to probe the individual groups of the site using specific reagents[2] or to probe the topography of the site by the collection of a large amount of data which can be analyzed as a template reflection of the site.[3] The present report presents a different approach to this problem, based on a comparison between two related enzymes.

The previous communication[4] indicated a very broad identity in deacylation rate constants of nonionic acyl-α-chymotrypsins and acyl-trypsins over a 10^5-fold range in kinetic specificity. These identical deacylation rate constants demand identical gross mechanisms, such as identical serine hydroxyl and imidazole groups. *In addition, this kinetic identity demands an identical interaction of the various acyl groups with identical specificity sites of both enzymes,* since the 10^5-fold range of rates reflects to a large measure the specificity imposed by the

(7) T. Inagami and J. M. Sturtevant, *J. Biol. Chem.*, **235**, 1019 (1960).
(8) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959); H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).
(9) L. Lorand, W. T. Brannen, and N. G. Rule, *Arch. Biochem. Biophys.*, **96**, 147 (1962).

(1) This research was supported by grants from the National Institutes of Health. Presented in part at the Sixth International Congress of Biochemistry, New York, N. Y., July, 1964.
(2) D. E. Koshland, Jr., D. H. Strumeyer, and W. J. Ray, Jr., *Brookhaven Symp. Biol.*, **15**, 101 (1962).
(3) G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, 4487, 4495 (1962).
(4) M. L. Bender, J. V. Killheffer, and F. J. Kézdy, *ibid.*, **86**, 5330 (1964).

SCHEME I

α-Chymotrypsin:	His·Phe·Cys·Gly·Gly·Ser·Leu·Ile·Asn·Glu·Asn·Try·Val·Val·Thr·Ala·Ala·His·Cys·	
	40	58
Trypsin:	His·Phe·Cys·Gly·Gly·Ser·Leu·Ile·Asn·Ser·Gln·Try·Val·Val·Ser·Ala·Ala·His·Cys·	
	29	47
α-Chymotrypsin:	Cys·Met·Gly·Asp·Ser·Gly·Gly·Pro·Leu·Val·Cys·	
	191	201
Trypsin:	Cys·Gln·Gly·Asp·Ser·Gly·Gly·Pro·Val·Val·Cys·	
	179	189

enzymes. Specificity in deacylation may be interpreted in terms of noncovalent interactions between the active site of the enzyme and the acyl group.[5] Identical specificities of α-chymotrypsin and trypsin must therefore mean identical environments. This requirement may be met only if the two enzymes contain a portion of their structural sequence in common.

The sequences of both enzymes have recently become available.[6-10] By equating the N-terminal isoleucine group of trypsin to the N-terminal isoleucine group of the B chain of α-chymotrypsin, identities in the catalytic components of the active site are discernible[9]: (1) two of the three histidines of trypsin parallel the two histidines of α-chymotrypsin; (2) the sequential distances between the histidines and the serine are identical. In addition, two major *regions* of identical sequence appear,[9] as shown in Scheme I using numbering related to the zymogens.[9] Finally, there exist numerous minor regions of identical sequences, if displacements of the sequence of both enzymes are performed.[9] Of the sequence of 19 amino acids including histidine 57 of α-chymotrypsin (alkylated by the "specific" reagent L-1-tosylamido-α-phenylethyl chloromethyl ketone[6]), 16 correspond to the sequence in trypsin. Two of the three differences are merely differences in a CH_2 group. Of the sequence of 11 amino acids, including serine 195 of α-chymotrypsin (the site of phosphorylation and acylation[6]), 9 correspond to the sequence of trypsin. One of the two differences is merely a difference in a CH_2 group.

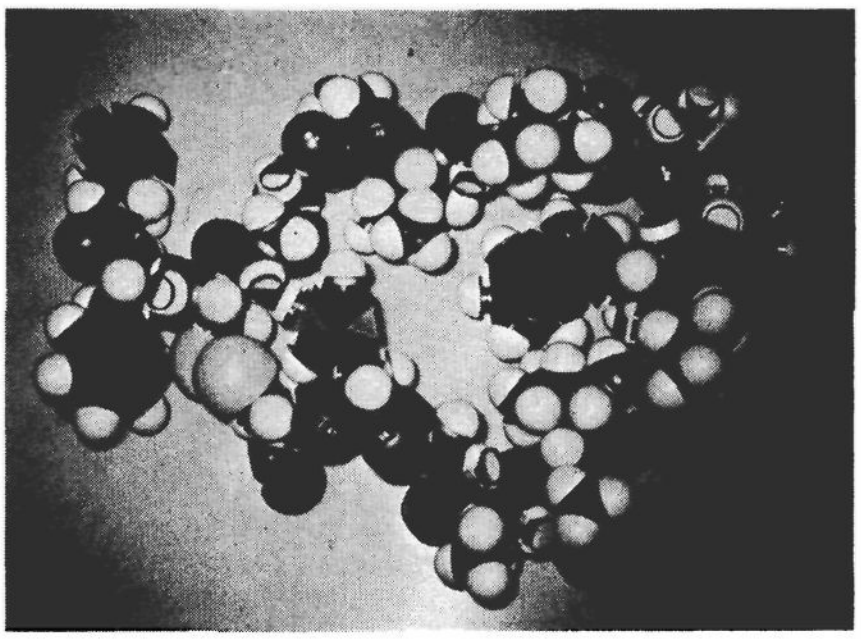

Fig. 1.—Courtauld's model of a portion of the active site of α-chymotrypsin.

Since the sequences above are the major identical portions of these enzymes, and since these sequences contain the mechanistically important serine hydroxyl and imidazole groups, these sequences may be postulated to constitute the common portion of the active sites of these enzymes, which produce identical deacylation rate constants for nonionic substrates.

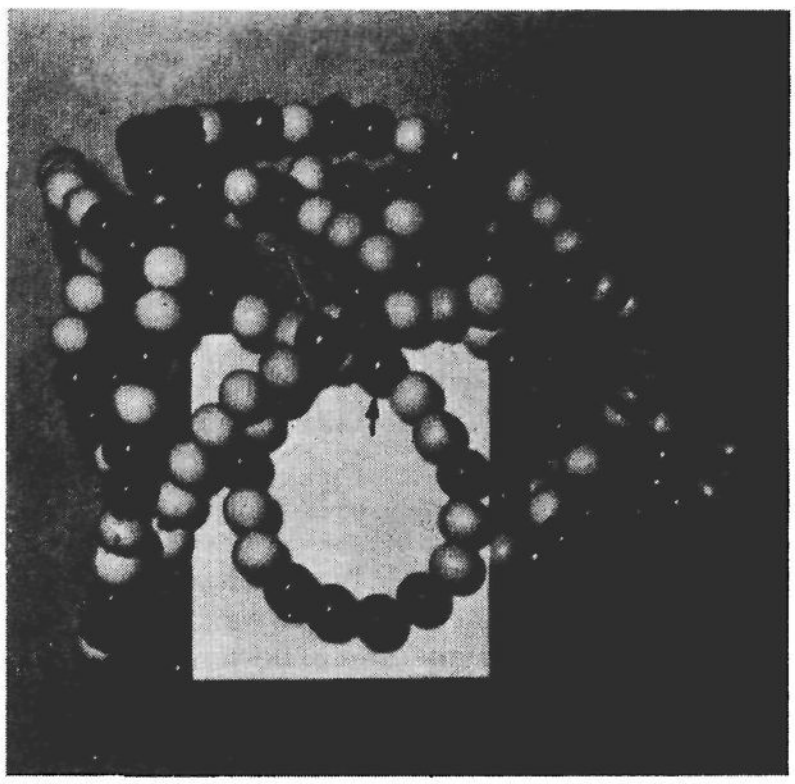

Fig. 2.—A model of α-chymotrypsin. The constraints used to build this model were: (1) the sequence of amino acids; (2) the position of the disulfide linkages; (3) no helical structures; (4) the cycle 42–58 must be external; (5) serine 195 must be adjacent to histidine 57; (6) serine 195 must be external to this cycle so that a peptide bond of a protein may be transferred to serine 195 while the side chain of the peptide interacts with the cycle; (7) charged groups must be external; (8) polar groups will tend to be external; (9) nonpolar groups will tend to be internal; (10) the activation peptides removed from chymotrypsinogen must be external; (11) no empty space exists within the molecule; (12) the external dimensions of the molecule are 40 × 40 × 50 Å.; (13) the majority of cationic groups are far from the active site; (14) leucine 13 which may participate in head to head acyl-enzyme dimer formation is near the active site.

In α-chymotrypsin, Cys 42 is linked to Cys 58 forming a cycle which can serve as the major binding site, while the sequence from Cys 191 to Cys 201 can serve as a subsidiary binding site. A Courtauld model of the α-chymotrypsin cycle (Fig. 1) shows the hydrophobic acid chains within and the hydrophilic and ionic side chains outside the cycle. If this hypothesis is correct, the cavity of this cycle will be a hydrophobic

(5) M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Am. Chem. Soc.*, **86**, 3714 (1964).

(6) B. S. Hartley, *Nature*, **201**, 1284 (1964); Abstracts, Sixth International Congress of Biochemistry. New York, N. Y., July, 1964, p. 253.

(7) B. Keil, *ibid.*, p. 103.

(8) K. A. Walsh, D. L. Kauffmann, K. S. V. Sampath Kumar, and H. Neurath, *Proc. Natl. Acad. Sci., U. S.*, **51**, 301 (1964); K. A. Walsh, *et al.*, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July, 1964, p. 186; H. Neurath, *ibid.*, p. 249.

(9) K. Walsh and H. Neurath, *Proc. Natl. Acad. Sci., U. S.*, **52**, 884 (1964).

(10) O. Mikeš, V. Holeyšovský, V. Tomášek, and F. Šorm, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July, 1964, p. 169.

cavity of proper size to interact, either on the surface of the cycle or within the cavity, with aromatic groups of chymotrypsin substrates. This hypothesis receives support from the cyclodextrins,[11-13] cyclic substances which bind numerous aromatic compounds including the α-chymotrypsin substrate, N-acetyl-L-tyrosine ethyl ester,[13] *via* inclusion compounds. The cyclodextrins are thought to be doughnut-shaped molecules whose cavity is largely hydrophobic,[12] as is Fig. 1. Certainly Fig. 1 will explain the hydrophobic bonding which occurs in both α-chymotrypsin and trypsin. But in trypsin, some additional feature of the site must explain the ionic interaction between substrate and enzyme.

The bead model of α-chymotrypsin, shown in Fig. 2, was constructed on the basis of its amino acid sequence. In front of the white card are seen both components of the active site, the cycle bounded by Cys 42 to Cys 58 and the strand from Cys 191 to Cys 201. Serine 195 (↓), is almost directly above histidine 57 (↑). The remaining amino acids of the site presumably determine binding and specificity. The acyl group of the substrate may bind longitudinally across the cycle while the chemistry occurs at the top portion of the cycle. Figures 1 and 2 make many predictions concerning the structure of the active site, the mode of binding, and the mechanism of action of the enzyme which may be tested.

Acknowledgment.—The authors thank Mr. Lewis Brubacher for assistance and Professor H. Neurath for the pre-publication manuscript on α-chymotrypsin and trypsin sequences.

(11) F. D. Cramer, *Rev. Pure Appl. Chem.*, **5**, 143 (1955).
(12) D. French, *Advan. Carbohydrate Chem.*, **12**, 189 (1957).
(13) M. Laskowski, Jr., and T. L. Warrington, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July, 1964, p. 163.

DEPARTMENT OF CHEMISTRY
NORTHWESTERN UNIVERSITY
EVANSTON, ILLINOIS

MYRON L. BENDER
JOHN V. KILLHEFFER, JR.
FERENC J. KÉZDY

RECEIVED AUGUST 31, 1964

The Acyl-enzyme Intermediate, *trans*-Cinnamoylpapain[1]

Sir:

α-Chymotrypsin, which contains a reactive alcoholic group, catalyzes the hydrolysis of cinnamate derivatives through the intermediate formation of *trans*-cinnamoyl-α-chymotrypsin, as has been shown spectrophotometrically.[2] In order to investigate the occurrence of acyl-enzyme intermediates in reactions catalyzed by enzymes presumed to contain a reactive thiol group,[3] we have investigated the catalysis of the hydrolysis of N-*trans*-cinnamoylimidazole by papain. We report our findings now, since the spectral observation of a dithioacylpapain intermediate in the papain-catalyzed hydrolysis of methyl thionohippurate was recently reported.[4]

When approximately equimolar papain and N-*trans*-cinnamoylimidazole were mixed at pH 5.2, the substrate absorption disappeared while a small maximum at ~325 mμ appeared; this absorption eventually disappeared leaving only the spectrum of cinnamate ion. Clearly a species involving the cinnamoyl group with λ_{max} near 325 mμ was present during the reaction. Since the activity of a *trans*-cinnamoylpapain solution toward α-N-benzoyl-L-arginine ethyl ester reappears at the same rate as *trans*-cinnamoylpapain deacylates, the cinnamoyl group must be attached to the active site. The cinnamoyl group is probably covalently attached to the enzyme rather than physically adsorbed because the species is formed slowly and survives Sephadex G-25 filtration.

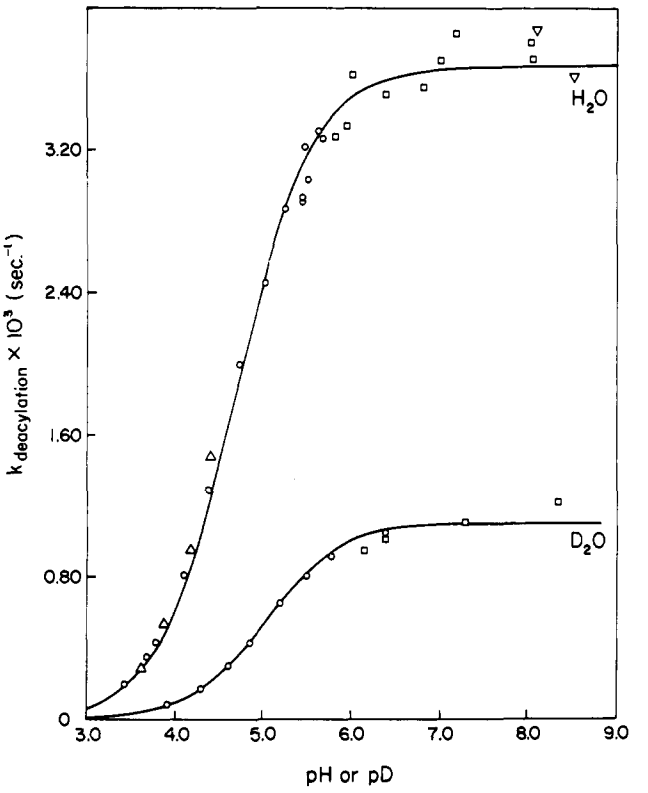

Fig. 1.—The effect of pH (or pD) on the deacylation of cinnamoylpapain. △ 0.05*M* formate; ○, 0.05 *M* acetate; □, 0.033 *M* phosphate; ▽, 0.0125 *M* borate; μ = 0.100, T = 25.0°. The solid lines are theoretical curves assuming dependence of rate constant on a single basic group; H_2O, pK_a = 4.69 and k^{lim} = 3.68 × 10^{-3} sec.$^{-1}$; D_2O, pK_a = 5.03, k^{lim} = 1.10 × 10^{-3} sec.$^{-1}$.

To prepare a solution of *trans*-cinnamoylpapain, a solution of papain (1.47 × 10^{-5} *M*)[5] was mixed with an excess of N-*trans*-cinnamoylimidazole (2.9 × 10^{-4} *M*) at pH 3.43 at 25°; after 300 sec., the excess substrate was removed by Sephadex filtration, prewashing the column with the desired buffer. The deacylation reactions, observed at 330 mμ, followed first-order kinetics over a range of enzyme (17-fold) and substrate (3-fold) concentrations. The rate constants were independent of buffer concentration and of ionic strength greater than μ = 0.10. The deacylation is dependent on a basic group of pK_a = 4.69 (Fig. 1). The deacylation of *trans*-cinnamoylpapain is affected by D_2O in the same manner as is the deacylation of cinnamoyl-α-chymotrypsin.[6] The pK_a of the basic group

(1) This research was supported by grants from the National Institutes of Health.
(2) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).
(3) E. L. Smith and J. R. Kimmel in "The Enzymes," Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, Inc., New York, N. Y., 1960, p. 133.
(4) G. Lowe and A. Williams, *Proc. Chem. Soc.*, 140 (1964).
(5) Papain solutions were titrated using N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.
(6) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).