A solution of one g. of *p*-dimethylaminobenzaldehyde in 100 ml. of absolute ethanol was prepared. The aldehyde was Matheson and Co. reagent grade, recrystallized from aqueous methanol, m.p. 75.5–75.9°. One milliliter of this solution in a 10-ml. flask corresponds to a final aldehyde concentration of $0.671 \times 10^{-2} M$.

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[CONTRIBUTION NO. 2250 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Catalytic Activity of Dimeric α-Chymotrypsin[1]

BY R. BRUCE MARTIN AND CARL NIEMANN[2]

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The kinetic consequences of the dimerization of α-chymotrypsin in aqueous solutions at 25° and *p*H 7.0 and 1.0 *M* and 0.30 *M* in sodium chloride have been investigated. Of the seven possible representations that were examined, one was definitely disproved, an additional three were rejected with considerable confidence and of the remaining three, preference has been indicated for two based upon the supposition that ES can combine with E, and E_2 with S, to give E_2S and that E_2S can combine with S to give E_2S_2. However, of the three intermediate enzyme-substrate complexes, *i.e.*, ES, E_2S and E_2S_2 only the first appears to be able to decompose to give reaction products at a substantial rate.

The anomalous behavior observed in sedimentation and light scattering studies with α-chymotrypsin in aqueous media have been interpreted in terms of an equilibrium between monomeric and dimeric species.[3-7] In particular, it has been suggested that the equilibrium is reversible and that above *p*H 5 the association of monomer increases with decreasing *p*H and increasing ionic strength. It is believed that polymeric species larger than dimer[8,9] are not likely to occur except at very low ionic strengths.

Since it has been shown[10,11] that the association of α-chymotrypsin may have a significant influence on the rates of certain reactions catalyzed by this enzyme it appeared desirable to determine the catalytic properties of the dimeric species insofar as they could be inferred from the kinetic behavior of systems involving α-chymotrypsin in aqueous solutions at 25° and *p*H 7.0 and 1.0 *M* in sodium chloride.

In principle it should be possible to distinguish between various alternative formulations involving monomeric and dimeric enzyme and monomeric specific substrate. In this study we have considered the situations summarized in Fig. 1 and Table I. The corresponding equations for K_D, the dissociation constant for the dimer, are given in Table II.

Case 1 is the simplest mechanism that can account for dimerization of the enzyme. In this case, which is free of special simplifying assumptions, it is postulated that the dimer is incapable of combining with the specific substrate.

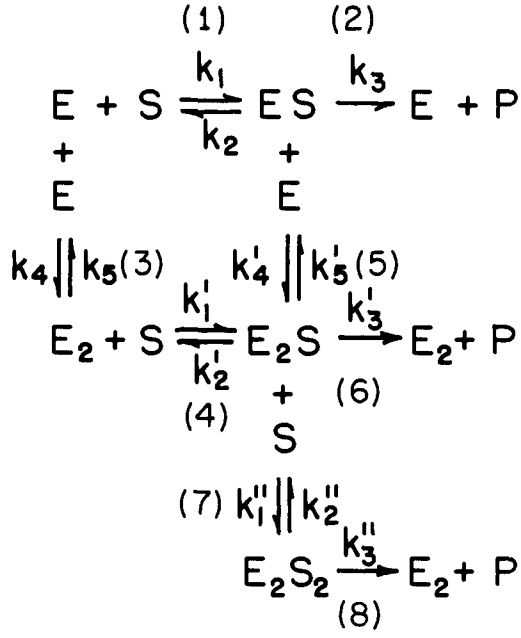

Fig. 1.—Probable equilibria involving enzyme and specific substrate.

In case 2, the dimer is considered capable of combining with the specific substrate to give the ternary complex E_2S, which also can arise by combination of the monomeric enzyme with the complex ES

TABLE I

SITUATIONS CONSIDERED[a]

Case no.	Reactions	Assumptions
1	1-3	
2	1-5	$K_S^b = K_S'^c$; $K_D^d = K_D'^e$
3	1-6	$K_S = K_S'$; $K_D = K_D'$; $k_3 = k'_3$
3a	1-6	$K_S = \frac{1}{2}K_S'$; $K_D = \frac{1}{2}K_D'$; $k_3 = k_3'$
4	1-5,7	$K_S = K_S' = K_S''^f$; $K_D = K_D'$
4a	1-5,7	$K_S = K_S' = \frac{1}{2}K_S''$; $K_D = K_D'$
5	1-8	$K_S = K_S' = K_S''$; $K_D = K_D'$; $k_3 = k_3' = k_3''$

[a] Based upon equilibria given in Fig. 1. [b] $K_S = [E][S]/[ES]$. [c] $K_S' = [E_2][S]/[E_2S]$. [d] $K_D = [E]^2/[E_2]$. [e] $K_D' = [ES][E]/[E_2S]$. [f] $K_S'' = [E_2S][S]/[E_2S_2]$.

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) G. W. Schwert, *J. Biol. Chem.*, **179**, 655 (1949).
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(6) R. F. Steiner, *Arch. Biochem. Biophys.*, **53**, 457 (1954).
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(8) V. Massey, W. F. Harington and B. S. Hartley, *Disc. Faraday Soc.*, **20**, 24 (1955).
(9) I. Tinoco, Jr., *Arch. Biochem. Biophys.*, **68**, 367 (1957).
(10) K. A. Booman, Ph.D. Thesis, Calif. Inst. of Tech., Pasadena, Calif., 1956.
(11) K. A. Booman and C. Niemann, *Biochem. Biophys. Acta*, in press.

TABLE II

EQUATIONS FOR K_D[a,b]

Case no.	K_D
1	$\frac{2v_0^2K_S^2}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}}$
2	$\frac{2v_0^2K_S(K_S + [S]_0)}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}}$
3	$\frac{K_S\{2v_0(K_S + [S]_0) - k_3[S]_0[E]\}^2}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}(K_S + [S]_0)}$
3a	$\frac{K_S\{2v_0(2K_S + [S]_0) - k_3[S]_0[E]\}^2}{2k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}(3K_S + [S]_0)}$
4	$\frac{2v_0^2(K_S^2 + K_S[S]_0 + [S]_0^2)}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}}$
4a	$\frac{v_0^2(2K_S^2 + 2K_S[S]_0 + [S]_0^2)}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}}$
5	$\frac{\{2v_0(K_S^2 + K_S[S]_0 + [S]_0^2) - k_3[S]_0[E](K_S + [S]_0)\}^2}{k_3[S]_0\{k_3[S]_0[E] - v_0(K_S + [S]_0)\}(K_S^2 + [S]_0^2)}$

[a] Based upon assumptions given in Table I. [b] Values of K_D in units of mg. protein-nitrogen per ml. when values of K_S are in units of M, k_3 in units of M/min./mg. protein-nitrogen per ml., v_0 in units of M/min., $[S]_0$ in units of M and [E], the total enzyme concentration, in units of mg. protein-nitrogen per ml.

but that the ternary complex is incapable of giving reaction products. In the special solution of this case it has been assumed that $K_S = K_S'$ and $K_D = K_D'$, *cf.*, Tables I and II. A general solution of case 2 leads to equation 1. It is seen that equation 1 requires that a plot of the reciprocal of K_D, evaluated for case 1, *vs.* $[S]_0$ be linear.

$$(1/K_D + [S]_0/K_D'K_S) = \{k_3[S]_0(k_3[S]_0[E] - v_0(K_S + [S]_0))\}/2v_0^2K_S^2 \quad (1)$$

In case 3 it is postulated that E_2S, formed *via* either E_2 or ES, decomposes to give products at the same rate as ES, *i.e.*, $k_3' = k_3$. In the special solution, *cf.*, Tables I and II, it is assumed that $K_S = K_S'$ and $K_D = K_D'$. This case has been considered previously.[10]

If, as in case 3, the formation of the dimer results in the loss of one catalytically active site,[12] it might be argued that a specific substrate molecule would have one-half the chance of combining with the remaining site, present in E_2, than it would of combining with the site of E. If the rates of dissociation of ES and E_2S remain the same the value of K_S' will be twice that of K_S. A similar argument may be applied to reaction 5 as compared to reaction 3 whence $K_D' = 2K_D$. From the free energy requirements of a cyclic system $K_S'K_D = K_SK_D'$. This is consistent with applying the above argument to reactions 3 and 5 if a similar argument has been previously applied to reactions 1 and 4. The elements of this discussion are included in the assumptions used in obtaining an expression for K_D for case 3a.

In case 4 both E_2S and E_2S_2 are considered to be formed, but neither to be active in the sense of giving reaction products. In the special solution, *cf.*, Tables I and II, it is assumed that $K_S = K_S' = K_S''$ and $K_D = K_D'$. A general solution of case 4 gives equation 2. The use of this equation requires a reasonably accurate solution of simultaneous equations.

$$K_D = \{1 + [S]_0/K_S' + [S]_0^2/K_S'K_S''\}/\{(2v_0^2K_S^2)/(k_3[S]_0(k_3[S]_0[E] - v_0(K_S + [S]_0)))\} \quad (2)$$

An argument analogous to that used in case 3a may be used to construct case 4a, *i.e.*, the value of k_1'' may be considered to be one-half of that of k_1 or k_1' so that $K_S'' = 2K_S' = 2K_S$. The identical argument of case 3a cannot be invoked with regard to k_1 and k_1' because the occurrence of reaction 7 to form E_2S_2 implies that an active site is not involved in dimerization. Thus k_1 is assumed to be equal to k_1' and hence $K_S = K_S'$.

In case 5 it is postulated that ES, E_2S and E_2S_2 are formed and that all give rise to reaction products at the same rate, *i.e.*, $k_3 = k_3' = k_3''$.

All of the expressions given for K_D in Table II contain the factor $k_3[S]_0[E] - v_0(K_S + [S]_0)$ in the denominator. If this factor were equal to zero, the usual expression for an enzyme-specific substrate equilibria involving only the monomeric species would result and the value of K_D would approach infinity. Thus, the departure of the above factor from zero is a measure of the variance of the system from ideality.

In order to determine which of the seven expressions for K_D, *cf.*, Table II, were capable of describing the behavior of α-chymotrypsin under a specified set of conditions, it was decided to examine the α-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester and of methyl hippurate in aqueous solutions at 25° and *p*H 7.00 ± 0.01 and 1.0 M in sodium chloride when both [E], the total enzyme concentration and $[S]_0$ were varied. The above specific substrates were chosen because it was anticipated that their K_S values would be markedly different[13,14] and their hydrolysis could be followed with a *p*H-Stat.[15,16] This latter device eliminates the need for conventional buffers, thus simplifying the reaction systems and also affords the opportunity for relatively precise and convenient observations.[13,14]

The constants K_S and k_3[17] for acetyl-L-valine methyl ester, in 1.0 and 0.3 M sodium chloride and for methyl hippurate, in 1.0 M sodium chloride, were evaluated at an enzyme concentration of 0.031 mg. protein-nitrogen per ml., *i.e.*, at 8.8×10^{-6} M,[19] where dimerization of the enzyme was assumed to be negligible. The values of K_S and k_3 given in Table III were obtained by first evaluating v_0 by the orthogonal polynomial procedure of Booman and Niemann,[20] from observations of the

(12) The term catalytically active site is herein defined as that region of the enzyme that can be recognized by its ability to combine with a specific substrate and to cause its transformation into reaction products.

(13) T. H. Applewhite, H. Walte and C. Niemann, THIS JOURNAL, **80**, 1465 (1958).

(14) T. H. Applewhite, R. B. Martin and C. Niemann, *ibid.*, **80**, 1457 (1958).

(15) C. F. Jacobsen and L. Leonis, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **27**, 333 (1951).

(16) J. B. Neilands and M. D. Cannon, *Anal. Chem.*, **27**, 29 (1955).

(17) The constants may be actually K_S' and k_3' in the sense used by Huang and Niemann.[18] For the purposes of this study, this distinction is immaterial.

(18) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952).

(19) Based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin.

(20) K. A. Booman and C. Niemann, THIS JOURNAL, **78**, 3612 (1956).

TABLE III

KINETIC CONSTANTS FOR ACETYL-L-VALINE METHYL ESTER AND METHYL HIPPURATE[a]

NaCl, M	[E][b]	[S]$_0$[c]	No.[d] expts.	K_S[e]	k_3[f]	[E_S'][g]	[Ss']
			Acetyl-L-valine methyl ester				
1.0	0.031	10–50	13	51 ± 3	1.61 ± 0.08	0.017	0.19–0.98
0.30	.031	10–50	9	70 ± 5	1.33 ± .08	.013	.14– .71
			Methyl hippurate				
1.0	0.031	5–30	10	1[h]	2.12 ± 0.07	...	.....

[a] In aqueous solutions at 25.0° and *p*H 7.00 ± 0.01 and 1.0 *M* and 0.30 *M* in sodium chloride. [b] In units of mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] In each experiment observations of extent of reaction for the period from $t = 0$ to 8 min. were made at intervals of 1 min., the extents of reaction corrected for the enzyme and specific substrate blank[14] and the initial velocities evaluated by the orthogonal polynomial procedure.[20] [e] In units of 10^{-3} *M*. [f] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [g] In units of 10^{-2}. [h] See text.

TABLE IV

VALUES FOR K_D[a]

[E][b]	[S]$_0$[c]	v_0[d]	Case 1	2	3	3a	4	4a	5
				Acetyl-L-valine methyl ester					
0.309	15.54	0.913	1.03	1.35	0.36	0.68	1.45	1.40	0.185
.309	25.46	1.33	0.90	1.35	.38	.63	1.58	1.47	.176
.309	35.89	1.68	.74	1.30	.39	.57	1.69	1.50	.182
.617	15.55	1.46	.77	1.00	.083	.37	1.07	1.04	.002
.617	25.43	2.18	.70	1.05	.122	.38	1.22	1.13	.002
.617	35.94	2.78	.60	1.03	.139	.34	1.32	1.17	.002
3.07	16.45	3.74	.49	0.65	.48	.005	0.70	0.67	.172
3.07	31.61	5.88	.30	0.49	.43	.001	0.65	0.59	2.30
				Methyl hippurate					
0.309	8.97	4.81	0.002	0.225	0.068	0.045	2.05 (1.26)[e]	1.14 (0.63)[e]	0.49
.309	16.48	5.69	.001	.215	.077	.090	6.25 (3.54)	3.31 (1.77)	1.27
.309	32.62	5.69	.003	.108	.039	.028	4.43 (3.54)	2.28 (1.77)	1.27
.620	8.91	6.50	.009	.084	.001	.003	0.76 (0.60)	0.42 (0.30)	0.001
.620	16.57	9.13	.007	.118	.019	.019	2.42 (1.96)	1.27 (0.98)	0.308
.620	32.92	10.8	.004	.144	.044	.031	5.67 (4.73)	2.92 (2.37)	1.46

[a] In units of mg. protein-nitrogen per ml. and for aqueous solutions at 25.0° and *p*H 7.00 ± 0.01 and 1.0 *M* in sodium chloride. [b] In units of mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] In units of 10^{-4} *M*/min., each value the mean of three separate determinations evaluated as specified in footnote *d* of Table III. [e] Values of this group calculated for the limit where $[S]_0 \gg K_S$.

extent of reaction during the total time interval from $t = 0$ to 8 min. and then fitting the values of v_0 and $[S]_0$ to the relation $([S]_0[E]/v_0) = (K_S/k_3) + ([S]_0/k_3)$ by the method of least squares.

The value of K_S for methyl hippurate was very small and could not be determined accurately. The value of 1×10^{-3} *M* given in Table III is a maximum value and may be too large by a factor of five. A lower value of K_S would give lower values of K_D for cases 1 through 4a and would have little influence on the values of case 5. As will be seen later, in cases 4 and 4a values of K_D were calculated for the limit where $[S]_0 >> K_S$. These latter values are more accurate than those based upon $K_S = 1 \times 10^{-3}$ *M*, at least for higher values of $[S]_0$.

The experiments conducted with acetyl-L-valine methyl ester and methyl hippurate in aqueous solutions at 25° and *p*H 7.00 ± 0.01 and 1.0 *M* in sodium chloride under conditions where [E] was varied from 8.77×10^{-5} *M* to 8.72×10^{-4} *M* with the former specific substrate and from 8.77×10^{-5} *M* to 1.76×10^{-4} *M* with the latter, are summarized in Table IV. In all but one instance three different values of $[S]_0$ were employed at each of the values of [E]. The values of v_0 that are given in Table IV are the mean of three values determined under the specified conditions. It is believed that their accuracy is better than ±2%. The accuracy of the values of K_D is difficult to estimate, but it should be better than ±20%. Values of K_D are extremely sensitive to variation in values of K_S and k_3. However, the larger the value of $[S]_0$ and the smaller the value of K_S, the less the importance of K_S.

Inspection of the values of K_D given in Table IV reveals that one of the cases can be rejected forthwith.

Values of K_D for case 1 vary more than a hundred-fold and since the expression for K_D for this case involves no special assumptions, we can dismiss the possibility that the kinetic consequences of dimerization can be expressed in terms of reactions 1, 2 and 3, *cf.*, Fig. 1.

Values of K_D for case 2 are more constant than those for case 1. However, it was noted previously that a general solution for case 2, involving no special assumptions, requires that a plot of the reciprocal of K_D, evaluated for case 1, *vs.* $[S]_0$ be linear. Since this relationship is not observed, it appears that we can reject case 2 and the possibility that the interaction of a specific substrate with an equilibrium mixture of monomer and dimer can be represented by reactions 1 through 5.

While the values of K_D for cases 3a and 5 are based upon a number of special assumptions, *cf.*,

Table I, the more than a ten thousand-fold variation in the values of K_D for these two cases suggests that neither are close to a possible representation of the kinetic behavior of an equilibrium mixture of monomeric and dimeric α-chymotrypsin.

At one time[10] case 3 was accepted as a reasonable representation. However, this was before cases 4 and 4a were considered. Inspection of Table IV reveals that the variation in values of K_D is more than twenty-fold for case 3, less than ten-fold for case 4 and less than sixfold for case 4a. The degree of variability of K_D for cases 4 and 4a can be lessened to a modest degree by basing the calculation of K_D on the further assumption that K_S is very small relative to $[S]_0$ when methyl hippurate is the specific substrate.

Case 4a, involving reactions 1 through 5 and 7, with only ES decomposing to give reaction products and with $K_S'' = 2K_S'$, has given values of K_D with the least variability and consequently could be taken as the best representation of the seven that have been considered. However, it is impossible to exclude cases 3 and 4 as likely possibilities if several of the assumptions were modified. Many adjustments are possible to produce more nearly constant values of K_D. Relationships but slightly different than those assumed for K_S, K_S' and K_S'' could markedly affect the results. In addition, the assumption, in cases 4 and 4a, that both E_2S and E_2S_2 are completely incapable of yielding reaction products may not be wholly true. In fact the simultaneous solution of the general equation for case 4a, *i.e.*, equation 2 with the assumption that $K_S'' = 2K_S' = 2K_S$, gave, with the data obtained with acetyl-L-valine methyl ester, a value of $k_3 = 2.0 \pm 0.1$.[21,22] The fact that this value is greater than that taken for the computation of the values of K_D given in Table IV, *i.e.*, 1.6,[21] suggests that a path to reaction products other than *via* ES may be operative, *e.g.*, through E_2S and/or E_2S_2.

At the present time we can only admit a preference for case 4a, or a slight modification thereof in terms of the simplifying assumptions, but cannot reject cases 3 and 4. Provisionally accepting case 4a as the best available representation of the kinetic consequences of the reversible dimerization of α-chymotrypsin, the value of K_D, for aqueous solutions at 25° and *p*H 7.00 ± 0.01 and 1.0 *M* in sodium chloride, may be assigned a value of 1.2 mg. protein-nitrogen per ml., when K_D is defined as in Table II and based on the assumptions given in Table I.

It was assumed in the evaluation of K_S and k_3 for acetyl-L-valine methyl ester and methyl hyppurate, *cf.*, Table III, that at an enzyme concentration of 8.8×10^{-6} *M*, *i.e.*, 0.031 mg. protein-nitrogen per ml., dimerization would be negligible. From the preceding value of K_D it may be calculated that in aqueous solutions at 25° and *p*H 7.00 ± 0.01 and 1.0 *M* in sodium chloride more than 95% of the added enzyme is present as the monomer.

The experiments summarized in Table V were performed in order to determine the effect produced by a decrease in ionic strength with all other factors being constant. It will be seen from the values of K_D, which were calculated for case 4a, that a decrease in the sodium chloride concentration from 1.0 to 0.30 *M* led to an increase in the value of K_D from 1.2 to 1.9 mg. protein-nitrogen per ml. The observed direction of this effect is consistent with the observations of Steiner,[6] which were based upon light-scattering studies. The value of K_A given by Steiner[6] for a system at *p*H 5.2 and 0.1 *M* in sodium acetate and 0.2 *M* in potassium chloride yields a value of K_D = 1.0 mg. protein-nitrogen per ml. Since it is known that a value of K_D at *p*H 5.2 would be considerably smaller than one at *p*H 7.0,[6,7] the two sets of independent observations are qualitatively in agreement.

TABLE V

VALUES FOR K_D[a]

[E][b]	$[S]_0$[c]	v_0[d]	K_D for case 4a
0.309	15.95	0.609	1.59
.309	25.50	0.905	1.81
.309	36.22	1.21	2.33
.620	16.09	1.06	1.51
.620	25.50	1.63	1.98
.620	36.08	2.11	2.00

[a] In units of mg. protein-nitrogen per ml. and for aqueous solutions at 25.0° and *p*H 7.00 ± 0.01 and 0.30 *M* in sodium chloride. [b] In units of mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] In units of 10^{-4} *M*/min., each value the mean of three separate determinations evaluated as specified in footnote *d* of Table III with acetyl-L-valine methyl ester as the specific substrate.

The question as to whether the catalytically active site of monomeric α-chymotrypsin[23] is involved in dimer formation rests in large part on how the term catalytically active site is defined. If this term is used in its most general sense,[12] *i.e.*, as those regions of the enzyme that are instrumental in promoting the hydrolysis, or synthesis, of α-amino acid and acylated α-amino acid alkyl esters, hydroxamides, amides and hydrazides and of peptides,[25] it follows that if the dimerization process involved the active sites of both monomers, the consequences of case 1 should have been observed. Since case 1 was rejected with certainty, the question then arises as to the likelihood of the correlative situation, *i.e.*, the dimer contains the active sites of both monomers, neither having been involved in the dimerization process. This is case 5 which also was rejected. The remaining possibility, as long as the above definition is maintained, is that dimerization involves the active site of but one monomer. This is the situation considered in cases 3 and 3a. While case 3a was rejected with some confidence, this was not true for case 3 even though it was the poorest of the three cases considered to be possible representations.

If we abandon the above definition in the sense of being concerned only with those features of the active site of the monomer that are responsible for

(21) In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml.

(22) The nature of the equation does not permit the accurate computation of K_S or K_D.

(23) There is considerable evidence to support the view that monomeric α-chymotrypsin contains but one active site, *cf.*, ref. 24 for pertinent ref.

(24) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

(25) Substrates of the "anhydride type," *e.g.*, diisopropylfluorophosphate and *p*-nitrophenyl acetate, have not been included in this group because of the possibility that their behavior may be substantially different than that of the specific substrates listed.

the combination of the specific substrates previously enumerated, without regard for the subsequent formation of reaction products, the results of this investigation support the proposition that none of the features so defined are involved in the dimerization process. Cases 4 and 4a are based upon the supposition that both E_2S and E_2S_2 are formed but do not decompose to give reaction products. These two cases, particularly the latter, provide the best representation of all those considered. While the correctness of the above proposition cannot be regarded as being established beyond reasonable doubt, it should be noted that if it is correct it follows that the K_I values for competitive inhibitors of α-chymotrypsin should be independent of the extent of dimerization with all other factors being constant.[26] Experiments designed to test this prediction are now in progress.

When concern is limited to those features of the active site of the monomer that are responsible for the decomposition of the primary enzyme–specific substrate complex to reaction products, assuming that they do not participate in the primary combination process, the results of this study support the correlative proposition that such features are involved in the dimerization process, partially if case 3 is accepted and totally if cases 4 and 4a are preferred.

The similar sedimentation behavior of α-chymotrypsin and diisopropylphosphorylated α-chymotrypsin led Schwert and Kaufman[4,27] and Smith and Brown[5] to conclude that the "active center" of α-chymotrypsin is not responsible for association, *i.e.*, the "active centers" of the monomers are not involved in the dimerization process. In contrast Egan, *et al.*,[7] from sedimentation studies of α-chymotrypsin and photooxidized α-chymotrypsin, which is enzymatically inactive,[28] concluded that dimerization involved the same imidazole group of the monomer that was associated with the loss of activity, *i.e.*, dimerization involved the "active centers" of both monomers.

If the term "active center" is regarded as synonymous with the term catalytically active site in its most general sense,[12] the above conclusions appear to be in violent disagreement. Furthermore, the results of this study lead to the further conclusion that both are wrong if total availability or loss of activity is implied. If this is not the case, then all three require modification to accommodate the consequences of case 3. However, if it is assumed that diisopropylphosphorylation of monomeric α-chymotrypsin effects those features of the catalytically active site of monomeric α-chymotrypsin that are involved in the primary combination process involving the specific substrate and photooxidation effects those features that are responsible for the decomposition of the primary enzyme–specific substrate complex to reaction products, then the disagreement between the two conclusions derived from sedimentation studies disappears and both are in agreement with the results of this investigation, if cases 4 and 4a are accepted as possible representations of the kinetic consequences of the dimerization of α-chymotrypsin.

Experimental

General Procedure.—The procedure employed in this study has been described previously.[14] Pertinent details of the individual experiments are given in Tables III, IV and V.

Specific Substrates.—Methyl hippurate, m.p. 82.8–84.1, and acetyl-L-valine methyl ester, m.p. 61.3–62.5°, were prepared as before.[13,14]

Enzyme Preparations.—All experiments were performed with crystalline salt free α-chymotrypsin, Armour preparation no. 283.

(26) Snoke[27] has observed that the sedimentation behavior of α-chymotrypsin is not influenced by the presence of β-phenylpropionic acid. While the proposition advanced above would predict this behavior, we regret we cannot agree with Schwert and Kaufman[4] that β-phenylpropionic acid is a powerful competitive inhibitor of α-chymotrypsin and consequently cannot regard the observation of Snoke as a significant confirmation of the prediction because of the negative character of the evidence.

(27) J. E. Snoke, ref. 4, p. 811, footnote 1.

(28) L. Weil, S. James and A. R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).

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