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Steric Course and Specificity of α-Chymotrypsin-catalyzed Reactions. II[1]

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A general theory of the steric and structural specificity of α-chymotrypsin has been developed by considering the significance of all possible interactions of a given asymmetric substrate with an asymmetric active site of singular conformation. The behavior of substrates, and certain inhibitors, can be accounted for by invoking eight postulates pertaining to details of the enzyme–substrate interaction. The most significant postulate involves consideration of non-productive as well as productive binding modes for certain substrates. The theory leads to a definition of the term "specific substrates" which can be used to distinguish experimentally between these and other substrates. The theory further specifies the different roles performed by structural components of the substrate or inhibitor molecule, and predicts that interaction between a potentially reactive center of a molecule and its complementary locus at the active site need not lead to substrate activity. Finally, it provides a reasonable explanation of the relative stereospecificity of α-chymotrypsin and for a recently observed inversion of antipodal specificity.

Introduction

An adequate explanation of the steric course and specificity of α-chymotrypsin-catalyzed reactions must accommodate all available data and afford at least semi-quantitative estimates of the relative reactivities of substrates of varying molecular structure. A "complete" description of the structural and stereospecificity of α-chymotrypsin-catalyzed reactions would necessarily include elucidation of the reaction mechanism as well. Unfortunately, the present state of knowledge does not lead to such combined information.

Mechanistic studies, which employ the techniques of physical organic chemistry, aim to elucidate the details of the bond-making and breaking steps, but provide relatively little information concerning specificity. Bender has recently concluded,[4] "At the present time, the efficient and specific catalysis of α-chymotrypsin appears to be brought about by a general base, or possibly a combination of functionalities such as a general base and a general acid or a general acid and a nucleophile. The facile reaction is due to precise stereochemical requirements including the correct fit and rigidity of the substrate at the active site of the enzyme." This statement is instructive in its indication of the small amount of concrete information available about the detailed mechanism of α-chymotrypsin catalyzed reactions. It also emphasizes the necessity of an approach with its primary emphasis on elucidation of the structural and stereospecificity of the reactions in question to guide purely mechanistic studies. Investigations of the former kind have been pursued in these laboratories for the past decade and have now progressed to the point where it is possible to offer a provisional theory of the steric course and specificity of α-chymotrypsin-catalyzed reactions. This theory appears to be consistent with the known facts, has predictive value and is capable of development both in a descriptive and quantitative sense.

Discussion

Attempts to discuss enzyme specificity, based on a lock and key analogy,[5] or more generalized template theories, must reconcile the assumption of enzyme rigidity inherent in all such theories with the growing evidence for enzyme flexibility.[6-8] However, the fact that α-chymotrypsin *does* show marked structural and stereospecificity both for classes of compounds and members within classes requires that the structural indeterminacy of the enzyme be of a limited nature.

The flexibility encountered, which would favor particular structures over others in a given interaction with a portion of the active site or surface, would probably result from increased binding energy derived from "tying down" an indeterminate portion of a peptide chain rather than from rearrangement of portions of chains in thermodynamically stable structures. Conformational changes of the active site, while affecting quantitative predictions concerning interactions between structural elements of the substrate and the active site, would not make correlations impossible. They would result instead in less than one-to-one correspondence between changes in the structure of the substrate molecule and changes in binding energy. Experimentally, they would require that near linear correlations be sought only in closely graded series of substrates.

The nature of the "best" substrates of α-chymotrypsin, *i.e.*, proteins and α-N-acylated aromatic α-amino acid esters, makes it reasonable to identify the specificity of enzyme action with a three point, or more, interaction between the substrate and active site of the enzyme, and further to associate the three structural elements of the substrate involved in this process with the functional groups attached to the asymmetric α-carbon atom. Because of the limited potential of the single α-hydrogen atom, the groups chosen are the α-acylamino group, the α-amino acid side chain and the carboxylate function. There are no *a priori* grounds for this choice: the requirements of a "three point interaction"[9-10] are met equally well by its geometric equivalent—a point and a

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(4) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

(5) E. Fischer, *Ber.*, **27**, 2985 (1894).

(6) D. E. Koshland, Jr., *Proc. Natl. Acad. Sci.*, **44**, 98 (1958).

(7) D. E. Koshland, Jr., *Adv. Enzymology*, **22**, 45 (1960).

(8) B. H. Havsteen and G. P. Hess, *J. Am. Chem. Soc.*, **84**, 491 (1962).

(9) A. G. Ogston, *Nature*, **162**, 963 (1948).

(10) G. W. Wolstenholme and C. M. O'Connor, "Steric Course of Microbiological Reactions," Ciba Foundation Study Group No. 2, Little, Brown and Co., Boston, Mass., 1959.

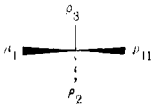

Fig. 1.

plane. In fact, an interaction of the latter kind may be determining in some cases. However, consideration of the effect of alteration of any one of the groups mentioned above on the observed kinetic constants of the appropriate rate equation

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]) \quad (1)$$

generally suggests that each may make important and largely independent contributions to binding of the substrate to the active site of the enzyme.[11]

Consideration of three groups as determining specificity of the enzyme catalysis does not mean that only point interactions are envisioned. There is considerable evidence[11] to indicate that the interactions with each group are extensive, involving relatively large areas and perhaps multiple types of combination with complementary loci. For example, combined nucleophilic and electrophilic attacks on the potentially reactive carbonyl group are considered as components of only one of the binding modes.

In accord with nomenclature proposed earlier,[11] the three groups involved in positive interactions with the surface of the active site are designated R_1, R_2 and COR_3, where for a typical α-N-acylamino acid type substrate, $R_1CHR_2COR_3$, R_1 = $R_1'CONH$, R_2 the side chain and COR_3 the potentially reactive carbonyl function. The corresponding loci of the active site of the enzyme are designated as ρ_1, ρ_2 and ρ_3. In addition, the fourth group on the asymmetric α-carbon atom, R_h = H can, in principle, occupy a fourth site ρ_H. A Fischer projection of the disposition of the four loci at the active site might appear as in Fig. 1.

It must be recognized that the configuration given in Fig. 1 is only one of the twelve possible representations of an asymmetric active site which cannot change its absolute configuration. However, if the active site is considered to be essentially rigid in the sense that no significant transposition of loci is possible, then any one of the twelve may be arbitrarily selected for a discussion of the specificity problem.

There is no reason to assume that the conformation of a substrate of the type $R_1CHR_2COR_3$ is fixed. Therefore, one must consider all possible interactions of a given substrate with the active site. Again, there are twelve different combinations for a given enantiomer, depending upon which of the substrate groups, R_1, R_2 COR_3 or R_h, interact with each of the enzyme loci, ρ_1, ρ_2, ρ_3 and ρ_H. Figure 2 illustrates the possible interactions.

Basic Postulates.—Certain postulates must be developed in order to choose among the possible orientations accessible to a substrate during the act of combination with the active site. The significance of the following discussion depends not so much on the truth of a particular postulate, but on

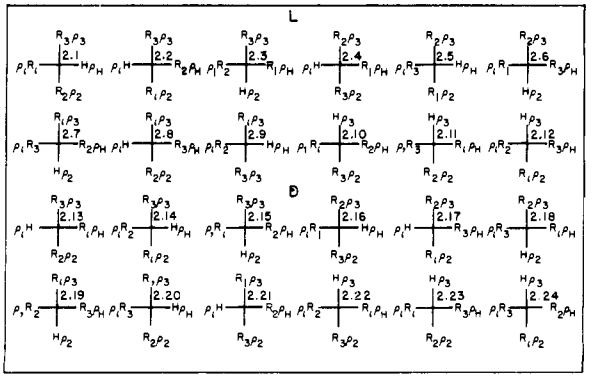

Fig. 2.—Substrate–site interactions.

the type of requirements needed for a successful interpretation. The basic postulates are: 1. *Only orientations including COR_3–ρ_3 interactions can lead to substrate activity, to successful binding leading to reaction products.* Many studies[12] have suggested that a complex of chemical functionalities is required for catalytic hydrolysis. It is unlikely that such a complex would be available in proper orientation at more than one locus.

The above requirement can be used to define a "specific substrate." Some controversy in recent years has involved the problem of similarity of mechanism for various substrates of α-chymotrypsin. It could be argued that only peptides and certain α-N-acylaminoacid esters are "specific substrates." Bender[4] has recently assumed that all methyl esters, regardless of structure, are specific substrates. It is possible, but not certain, that both our "specific substrates" and those of Bender[4] involve COR_3–ρ_3 interactions. However, there is merit in a more precise definition. We propose that the term "specific substrate" be reserved for those substrates whose interaction with the active site of α-chymotrypsin clearly involves a COR_3–ρ_3 interaction. A further useful distinction can be made between classes of "specific substrates" in the manner described by Hein and Niemann[11] by specifying the potentially reactive group as well as those interactions which appear to determine the magnitude of the enzyme–substrate dissociation constant and indirectly the rate of formation of products from the enzyme–substrate complex.

It is conceivable that the α-chymotrypsin catalyzed hydrolysis of a given compound may proceed without a COR_3–ρ_3 interaction in the sense used above. Such reactions, which would be expected to proceed at very slow rates, should be considered as involving "non-specific substrates." A significant feature of this definition is the ability, at least in principle, of distinguishing experimentally between specific and non-specific substrates. If an inhibitor can be found which will combine only with the ρ_3 site, it could be used to distinguish between substrates which require this site for hydrolysis and those, if any, which do not. Diisopropyl phosphorofluoridate may be considered by some to be an inhibitor of this kind. Unfortunately, the kinetic properties of one-to-one stoichiometrically diisopropyl phosphorylated α-chymotrypsin have not been studied extensively.

(11) G. E. Hein and C. Niemann, *Proc. Natl. Acad. Sci.*, **47**, 1341 (1961).

(12) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960).

2. *Interactions of R_1, R_2 or COR_3 with ρ_H do not contribute positively to either binding energy or orientation.* This is essentially equivalent to stating that no specific ρ_H binding locus exists. An R group in this position does not interact with the active site in a positive manner.

3. *Interactions of COR_3 with ρ_3 are more effective for $R_3 = OR'$ than for $R_3 = NHR''$ when R' and R'' are of comparable size.* This point emphasizes that α-N-acylaminoacid esters are better models for the α-chymotrypsin-catalyzed hydrolysis of proteins than are simple amides. Although the α-acylaminoacid esters lack the extended R_3 group, which in peptides and proteins probably provides efficient interactions with the ρ_3 locus, esters compensate for this deficiency by possessing a COR_3 group which interacts more effectively and specifically with the ρ_3 locus at the active site.

4. *The R_2–ρ_2 interaction becomes a dominant feature in determining the magnitude of the constant K_0 of eq. 1 as the size of R_2 increases.* For example, among the α-acylaminoacid esters values of the constant K_0 of eq. 1 of *ca.* 30 mM or less will be observed only when R_2 is greater than ethyl. However, with a less effective COR_3–ρ_3 interaction, such as that obtaining in the case of the simple α-acylaminoacid amides, R_2 may have to be substantially greater than ethyl (for example, benzyl) to lead to values of K_0 of *ca.* 30 mM.

Unlike the first two postulates given above, the third and fourth are empirical generalizations derived from considering the effect on the kinetic constants of altering one group while holding all others constant.[11]

5. *All other factors being equal, a conformation which provides a larger number of interactions than another will result in greater binding energy and, therefore, lower K_0 values of eq. 1.* This postulate is worth discussing for its differential effects on the constants K_0 and k_0 of eq. 1.

Equation 1 may be taken as a satisfactory empirical rate equation describing the dependence of the rates of a relatively large number of α-chymotrypsin-catalyzed hydrolyses upon enzyme and substrate concentrations in terms of two observed constants K_0 and k_0. If, as a first approximation, these two constants are now interpreted in terms of the reactions represented by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \qquad (2)$$

$$E + S \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} ES' \xrightarrow{k_4} E + P \qquad (3)$$

where the substration constants $K_{S.1} = (k_{-1} + k_2)/k_1$ and $K_{S.2} = (k_{-3} + k_4)/k_3$, the existence of two or more binding modes leads to the relation

$$1/K_0 = 1/K_{S.1} + 1/K_{S.2} \qquad (4)$$

or for the more general case of n binding modes

$$1/K_0 = \sum_j^n 1/K_{S.j} \qquad (5)$$

Thus, values of K_0 will vary between that approximating the lowest $K_{S.j}$ value if one binding mode is dominant to that given by $\sum_j^n K_{S.j/n}$ if each of the n binding modes leads to equivalent substration constants. In most cases where more than one binding mode is involved, only one will be capable of yielding products according to postulate 1, but this need not be the conformation associated with the lowest value of $K_{S.j}$.

For the case of two binding modes, one productive and one non-productive, *i.e.*, $k_4 = 0$

$$k_0 = K_{S.2}\, k_2/(K_{S.1} + K_{S.2}) \qquad (6)$$

and, generally

$$k_0 = k_2/\left(1 + K_{S.1} \sum_{j+1}^{n} 1/K_{S.j+1}\right) \qquad (7)$$

If $k_4 \neq 0$

$$k_0 = (K_{S.2}\, k_2 + K_{S.1}\, k_4)/(K_{S.1} + K_{S.2}) \qquad (8)$$

and, generally

$$k_0 = \left(\sum_j^n k_{(2j)}/K_{S.j}\right)\Big/\left(\sum_j^n 1/K_{S.j}\right) \qquad (9)$$

The effect of multiple binding modes on k_0 will be to decrease k_0 significantly whenever a non-productive binding mode competes successfully with one which leads to products. This suggests that for any series an unusually low k_0 value associated with a low K_0 value implies the existence of a significant unproductive binding mode. From these considerations it is apparent why no general correlation between observed magnitudes of k_0 and K_0 exists.[11]

If the principles outlined above are applied to acetyl-L-phenylalanine methyl ester, whose kinetic constants are given in Table I, it is apparent that only one configuration, 2.1 of Fig. 2, provides for three positive interactions with the proper loci at the active site. This configuration involves CH_3CONH–ρ_1, $C_6H_5CH_2$–ρ_2 and CO_2CH_3–ρ_3 interaction. The two other conformations which maintain the important $C_6H_5CH_2$–ρ_2 interaction no longer have the CO_2CH_3–ρ_3 interaction and, therefore, would be expected to be both non-productive and to possess higher K_S values. On the other hand, if the CO_2CH_3–ρ_3 interaction is maintained, simultaneous CH_3CONH–ρ_1 and $C_6H_5CH_2$–ρ_2 interactions are no longer possible. The conclusion is reached, therefore, that acetyl-L-phenylalanine methyl ester has a single preferred orientation associated with a low K_0 and a high k_0 value. The above conclusion is, of course, not a prediction, but a description of the known reactivity of this substrate in terms of the model being developed.

For acetyl-D-phenylalanine methyl ester there are no conformations which allow interaction of each of the R groups with their corresponding ρ-loci. If the $C_6H_5CH_2$–ρ_2 interaction is taken as dominant, there is one conformation, 2.20 of Fig. 2, with three positive interactions and two, 2.13 and 2.23 of Fig. 2, with two positive interactions each. Of these three conformations only one maintains a carbomethoxy group in the ρ_3-position and so this mode, according to postulates 3 and 4, should be the most significant binding mode.

The conclusion that acetyl - D - phenylalanine methyl ester is preferentially bound to the active site of α-chymotrypsin in a conformation involving

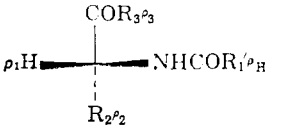

Fig. 3.

a COR_3–ρ_3 interaction immediately raises a question concerning the reactivity of this compound. If a COR_3–ρ_3 interaction were a sufficient as well as necessary condition for substrate activity, acetyl-D-phenylalanine methyl ester should be a substrate of α-chymotrypsin. In fact, its k_0 value is at least 10^{-7} that of the L-isomer[11,13] and operationally

TABLE I

KINETIC CONSTANTS FOR SOME REPRESENTATIVE SUBSTRATES OF α-CHYMOTRYPSIN[a]

Substrate	K_0, mM	k_0,[b] sec.$^{-1}$	Ref.
Acetyl-L-phenylalanine methyl ester[c]	1.8	63.1[d]	[e]
Acetyl-D-phenylalanine methyl ester	2.3[f]		15
Formyl-L-phenylalanine methyl ester	..[g]	[g]	13
Formyl-D-phenylalanine methyl ester[h]	0.25	0.0034	13
DL-α-Benzyllevulinic acid methyl ester[i]	0.49	0.063	18
Acetyl-L-alanine methyl ester[j]	611	1.29	[k]
Acetyl-L-valine methyl ester[l]	112	0.151	[m]
Benzoyl-L-alanine methyl ester[h]	9.75	.261	13
Benzoyl-L-valine methyl ester[l]	4.6	.045	[m]
Benzoylglycine methyl ester[n]	6.6	.205	29
Acetyl-L-norvaline methyl ester[l]	10.2	2.70	25
Acetyl-L-leucine methyl ester[h]	2.9	4.61	[o]
D-3-Carbomethoxydihydroisocarbostyril[h]	0.53	22.7	13
L-3-Carbomethoxydihydroisocarbostyril[h]	11.7	0.124	13

[a] In aqueous solutions at 25° and *p*H 7.9 ± 0.1 unless otherwised noted. [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for the enzyme. [c] Reaction system *ca.* 0.04 M in sodium chloride. [d] Bender and Glasson give a value of 48.2 sec.$^{-1}$. The difference in the two values arises from use of different conversion factors in converting mg. protein-nitrogen per ml. to molality. [e] M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960). [f] Value of K_I. [g] Estimated value of $k_0/K_0 \cong 10^5\ M^{-1}$ sec.$^{-1}$. [h] Reaction system 0.2 M in sodium chloride. [i] Reaction system 0.1 M in sodium chloride and 5% in acetone. [j] Reaction system 0.5 M in sodium chloride. [k] J. P. Wolf III, Ph.D. Thesis, California Institute of Technology, Pasadena, 1959. [l] Reaction system 0.1 M in sodium chloride. [m] H. R. Waite and C. Niemann, *Biochem.*, **1**, 250 (1962). [n] Reaction system 0.02 M in sodium chloride. [o] Unpublished results by Dr. G. E. Hein.

it is a fully competitive inhibitor of the hydrolysis of representative trifunctional substrates of this enzyme[14,15] and inferentially of that of its L-antipode. Examination of the projection formula given in Fig. 2.13 immediately suggests an answer: the difference between the D- and L-enantiomers is that when bound in their preferred conformations the positions of R_1 and H are interchanged, the D-antipode has a R_1 group in ρ_H whereas the L-antipode has its α-H in this position. If either nucleophilic or electrophilic attack on the adjacent carbonyl group of COR_3 requires that this region be sterically accessible, it is conceivable that bulky groups replacing the α-hydrogen atom might interfere with this attack and subsequent hydrolysis. This argument leads to the additional postulate.

6. *Bulky groups occupying the ρ_H-locus lead to a dramatic decrease in values of k_0 without necessarily causing a substantial change in corresponding values of K_0.* Ample support for this postulate is provided by two independent sets of experimental observations. If the α-hydrogen atom in α-N-acetyl-L-aromatic α-amino acid methyl esters is replaced by a methyl group, the values of K_0 are changed by less than an order of magnitude, whereas the values of k_0 are decreased by a factor of at least 10^4.[16] This is strong evidence that a COR_3–ρ_3 interaction is possible *without* product formation at measurable rates. Another supporting experiment involves formyl-L-phenylalanine methyl ester and its D-antipode.[13] In this case the D-enantiomer is hydrolyzed at a slow but measurable rate. Similar, but somewhat more ambiguous, results have been obtained with the α-chloro- and α-hydroxydihydrocinnamic acid methyl esters. With these substrates the stereospecificity is markedly decreased and both isomers are hydrolyzed at relatively slow rates.[17] These results are consistent with the conclusion that at least certain of the α-N-acylated D-aromatic α-amino acid esters are preferably bound to the active site in the conformation depicted in Fig. 3, which is identical with 2.13 of Fig. 2.

The evidence cited above suggests the order $CH_3CONH > CH_3 > HCONH > OH, Cl$ for effectiveness of R_1 groups in the ρ_H-position in causing a decrease in the value of k_0 of eq. 1. Several experiments immediately suggest themselves. On one extreme esters of α-hydroxydihydrocinnamic acid should possess higher k_0 values than the α-methyl analogs. On the other, since the above sequence suggests that the effect may be subject to a geometric factor, compounds such as 2-carbomethoxy-3-phenylpropene-1 should be investigated. Also, the presence of an electronic factor is not excluded.

Even if the conformation pictured above is dominant in determining the magnitude of the constant K_0 of eq. 1, there remain two conformations of the α-N-acylated D-aromatic α-amino acid esters which could lead to products, *i.e.*, those given in 2.14 and 2.15 of Fig. 2.

The second of these is easily dismissed on the grounds discussed above since it places a very bulky R_2 group in the ρ_H-locus. The first requires more careful examination. Part of the lack of substrate activity, as determined by a low value of k_0, can be attributed to the decreased significance of this binding mode relative to the preferred orientation. Replacement of the $C_6H_5CH_2$–ρ_2 interaction by a RCONH–ρ_2 interaction can be expected to increase the value of K_0 of eq. 1 by a factor of approximately 10^2 for the formyl and acetyl deriva-

(13) G. E. Hein and C. Niemann, accompanying article.

(14) H. T. Huang, R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **74**, 105 (1952).

(15) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).

(16) H. R. Almond, Jr., D. T. Manning and C. Niemann, *Biochem.*, **1**, 243 (1962).

(17) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

tives so that the successful binding mode, *i.e.*, the one leading to products, would be only 1% as frequent as the preferred, unreactive orientation. However, since the D-antipode is only $\sim 10^{-7}$ times as reactive as the L-antipode, this factor cannot be the only significant one. From these considerations it becomes apparent that the binding mode under discussion must be considered to be substantially unproductive when compared to the preferred orientation of, for example, acetyl-L-phenylalanine methyl ester. Some new postulate must be introduced to explain this. Fortunately, there exists a reasonable explanation with independent evidence in support. It was stated previously that with α-N-acylated aromatic α-amino acid esters the observed constant K_0 of eq. 1 was determined primarily by interactions of COR_3 with ρ_3 and R_2 with ρ_2. A function for R_1 now becomes apparent. This group serves an orienting function: it helps position the COR_3 group at the ρ_3-locus for maximum reaction. Replacement of CH_3CONH by $C_6H_5CH_2$ at the ρ_1-locus and $C_6H_5CH_2$ by CH_3CONH at the ρ_2-locus apparently reduces the value of k_0 by at least 10^4. The same conclusion is reached when one compares α-N-acetyl-L-phenylalanine methyl ester and DL-α-benzyllevulinic acid methyl ester.[18] The values of K_0 of eq. 1 for these two substrates are similar, but the k_0 values differ by 10^3 in favor of the amino acid derivative. Either the absence of a proton donor or a decrease in steric orientation in substituting a methylene group for an amido NH group has resulted in a remarkable drop in substrate reactivity.

The preceding argument permits the formulation of a new postulate:

7. *An α-acylamino group is required in the ρ_1-position for high values of k_0. Its removal or replacement by less polar or less constrained groups can decrease k_0 by factors up to 10^4.* Again, experiments suggest themselves to distinguish between polar and steric requirements of the R_1 group. For example, how would $CH_3C(NH)NHCH(CH_2C_6H_5)CO_2CH_3$, $CH_3C(S)NHCH(CH_2C_6H_5)CO_2CH_3$ and $CH_3CONCH_3CH(CH_2C_6H_5)CO_2CH_3$ compare with $CH_3CONHCH(CH_2C_6H_5)CO_2CH_3$?

Before leaving the α-N-acylated aromatic α-amino acid esters, it is interesting to compare these esters with the corresponding amides according to the principles outlined above. The outstanding feature of the α-N-acylated-L-aromatic α-amino acid amides when compared with the analogous esters[19] is the drastic decrease in values of k_0 of *ca.* 10^3 and the relatively slight increase in values of K_0 of *ca.* 10. For the D-antipodes, which again are not substrates but are fully competitive inhibitors,[20-22] there is an even more modest increase in values of $K_I = k_{-5}/k_5$ associated with the reaction represented by eq. 10

$$E + I \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EI \qquad (10)$$

For the esters the ratio K_{0L}/K_{ID} is consistently less than unity: for the amides the same ratio may be less than, equal to or greater than unity.[21,22] However, in the absence of explicable perturbations[11] the ratio for the amides is greater than unity. The reasons for a ratio of less than unity for the esters has already been discussed and for reasons summarized elsewhere[11,23] both constants determining the ratio may be taken as apparent dissociation constants. When the ester group is replaced by the carboxamide group, K_0 increases. This evidence is the basis for postulate 4. For the D-series, the increase in K_I is almost negligible, reflecting the poorer binding originally, with other binding modes not involving a COR_3–ρ_3 interaction becoming competitive. One reasonable mode, which preserves both R_1–ρ_1 and R_2–ρ_- interactions, *cf.* 2.23 Fig. 2, would not be available to the L-antipode. The model can, therefore, readily accommodate the data that for esters $K_{ID} > K_{0L}$ and that $K_{ID\ amide}/K_{ID\ ester} < K_{0L\ amid}/K_{0L\ ester}$.

The rationalization of the kinetic constants for derivatives of the α-N-acylated aromatic α-amino acids leads directly to application of the same principles to comparable derivatives of the α-N-acylated aliphatic α-amino acids. For acetyl-L-alanine and acetyl-L-valine methyl ester there are evidently no binding modes strictly comparable to those available to the α-N-acetyl-L-aromatic α-amino acid methyl esters, as indicated by their relatively high K_0 values, 611 and 110 mM, respectively. There is every reason to believe that in these cases $K_0 \cong K_S$ and, therefore, $k_0 \cong k_2$ where the four constants are those of eq. 1 and 2.

For benzoyl-L-alanine methyl ester and benzoyl-L-valine methyl ester both the K_0 values 9.8 and 4.3 mM, respectively, and the k_0 values, 0.261 and 0.045 sec.$^{-1}$, respectively, have decreased considerably. The concurrent decrease in both kinetic constants on introducing the larger R_1' group strongly suggests that for the benzoyl compounds one or more non-productive alternative binding modes have become dominant. The application of postulate 4, which makes interactions with ρ_2 dominant in some cases, immediately suggests the possible nature of the alternative binding modes: they involve R_1–ρ_2 interactions. As a working hypothesis, the original postulate can be modified to give:

4′. *For trifunctional substrates containing only one bulky R group, interaction of this group with ρ_2 becomes a dominant feature in determining the magnitude of the constant K_0 of eq. 1.*

Although R_1–ρ_2 interaction can, in terms of eq. 2, 3 and 5, account for a decrease in K_0 values, the concurrent decrease in values of k_0 would only occur if conformations involving such interactions are indeed non-productive. Examination of the relevant structures shows that this is the case. The only conformation which combines both R_1–ρ_2 and COR_3–ρ_3 interactions places R_2 in the ρ_H-position.

(18) Dr. Neil Isaacs, unpublished experiments conducted in these laboratories.

(19) R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 1886 (1955).

(20) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(21) D. T. Manning and C. Niemann, *ibid.*, **80**, 1478 (1958).

(22) D. T. Manning and C. Niemann, *ibid.*, **81**, 747 (1959).

(23) H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54**, (Suppl. 1), 179 (1959).

According to postulate 6 such an interaction decreases k_0, actually k_2, by a factor of 10^3 to 10^4, sufficient to make the conformation non-productive even when compared with others of higher K_0 values.

The inclusion of alternative binding modes, as indicated in postulate 5, not only offers a qualitative explanation of the observed kinetic constants, but also suggests methods for the quantitative evaluation of these effects. By assuming reasonable numbers for the K_S values for the benzoyl compounds, that is, values for the less favored but productive binding modes, it is possible to calculate k_2 values, the actual hydrolysis constants, for the productive modes. Starting with the acetyl derivatives as models which exhibit "pure" K_S and k_2 values in the sense of eq. 2, it is reasonable to assume that the comparable configuration of the benzoyl derivative would have a smaller but not greatly diminished, K_S value. If K_S values for the benzoyl derivatives are taken as 60–100 mM, then the calculated k_2 values are 1.34–2.39 sec.$^{-1}$ for benzoyl-L-alanine methyl ester and 0.63–1.04 sec.$^{-1}$ for benzoyl-L-valine methyl ester. These values are of the same order of magnitude as the k_0 values for the acetyl esters. The benzoyl derivatives are slightly more reactive as might be expected for the more rigid benzoyl group.

The same type of argument can be applied to the bifunctional glycine derivatives. These will be discussed more fully later, but it is interesting to compare methyl hippurate, K_0 = 6.6 mM, k_0 = 0.199 sec.$^{-1}$, to benzoyl-L-alanine methyl ester. The two compounds have similar kinetic constants, which suggests the relative lack of importance of the α-methyl group in the alanine derivative in positively influencing the kinetic constants.

In comparing the alanine and valine derivatives one feature stands out: the lower values of k_0 for the latter substrate. Even calculated k_2-values remain depressed assuming the two benzoyl derivatives have reasonably similar substration constants. There appears to be no way of rationalizing this situation on the basis of any of the previous postulates. A new *ad hoc* postulate must be introduced.

8. *Branching of the side chain beta to the potentially reactive carbonyl group results in a steric hindrance to binding and hydrolysis of the carbonyl group.*

Although there is no previous requirement for this postulate in the discussion presented here, there is ample precedent for it as well as other supporting experimental evidence. Steric interference of hydrolysis by β-substituents has been postulated previously and is commonly referred to as Newman's "rule of six."[24]

The proper compounds for testing a steric effect of this type are obviously analogous esters with lower "6 numbers" or those with equal "6 numbers" because of branching at another position. Such compounds behave exactly as predicted; acetyl-L-norvaline methyl ester exhibits a K_0 value of 10.2 mM, much lower than the valine derivative,[25] suggesting that not only is the steric factor impeding interaction at ρ_3 diminished but the R_2–ρ_2 interaction is also enhanced. The k_0-value for the norvaline derivative is 2.695 sec.$^{-1}$, a value expected from that obtained for acetyl-L-alanine methyl ester. If one wished to predict that k_2 for acetyl-L-norvaline methyl ester would be the same as k_2 for acetyl-L-alanine methyl ester, calculated values of $K_{S1} = K_{S2} = 20$ mM are obtained. This suggests a beautiful gradation in values of K_S for the methyl esters of acetyl-L-alanine, -L-valine, -L-norvaline and -L-leucine of 610, 110, 20, 3, respectively. Note the 5- to 6-fold decrease in each adjacent value.

Even a semi-quantitative estimate of the degree to which postulate 8 is operative is instructive. If one assumes, as a first approximation, that the only significant difference between the k_0-values of the L-alanine and L-valine derivatives results from steric interference, the following ratios are significant. For the acetyl methyl esters $k_{0_{ala}}/k_{0_{val}}$ = 8.4. for the benzoyl methyl esters $k_{0_{ala}}/k_{0_{val}}$ = 5.8 and for the latter substrates the calculated $k_{2_{ala}}/k_{2_{val}} \cong 2.0 - 2.4$. Unfortunately, comparison of k_0-values with kinetic constants for similar reactions in solution is complicated since the latter involve second-order rate constants. However, comparison of ratios is a legitimate procedure and may be fruitful here. For the base-catalyzed hydrolysis of the ethyl esters at 25°, $k_{B_{acetyl}}/k_{B_{isovaleryl}} = 14.5$,[26] while for the acid-catalyzed methanolysis of the same esters the ratio is 9.1.[27] If these values are taken as representative it is apparent that the observed ratio of enzymatic hydrolysis constants is approximately the same for the acetyl esters, somewhat less for the benzoyl esters and significantly less for the latter when the calculated values are used.

It is not surprising that the acetyl derivatives, which may be regarded as being poorly bound to the enzyme, should be subject to strong steric effects similar to those found in solution. The high substration constants could mean low affinity, which can be interpreted to mean little change in conformation from solution. The observed or calculated ratio for the benzoyl compounds implies that this particular effect is less important for these compounds and, granted the validity of the theory under discussion here, the calculated values confirm this.

The mechanistic implications of this observation involve two possibilities. Either optimal or enhanced binding of substrate at the active site decreases steric interference to nucleophilic or electrophilic attack, or in the enzymatic reaction nucleophilic or electrophilic attack is less important in the rate-determining step than it is for the reactions in solution. It is also possible that both of these effects are operative.

Although the conclusions concerning mechanism from this experiment are not very detailed or particularly new, they suggest the possibility of

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(25) Dr. J. B. Jones, unpublished experiments conducted in these laboratories.

(26) D. P. Evans, J. J. Gordon and H. B. Watson, *J. Chem. Soc.*, 1439 (1938).

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deriving mechanistic information from essentially specificity data. The increasing importance of the steric factor with decreasing binding energy, *i.e.*, with poor substrates, suggests that the enzyme contribution is more in orienting substrate rather than in altering the importance of nucleophilic or electrophilic attack in the rate-determining step.

A more important conclusion from this analysis concerns the use of particular substrates for studies on mechanism. The degree to which an enzyme can exert its specific catalytic function may depend on the particular substrate used. A given factor can easily be overlooked or an invalid analogy to non-enzyme-catalyzed reactions may be made using poor substrates of the enzyme. It is unfortunate that because of certain experimental advantages almost all mechanistic studies on α-chymotrypsin have involved some of its poorest substrates, such as *p*-nitrophenyl acetate,[23] methyl cinnamate[4] and methyl hippurate.[28] For example, if the analysis presented above is correct, then the constancy of K_0 for methyl hippurate for both hydrolysis and hydroxylaminolysis[28] may simply reflect the invariant binding energy of the preferred, but unproductive, binding mode and need not reflect a relation between K_S and the second substrate concentration.

The discussion presented so far correlates most of the data available for both aromatic and aliphatic α-amino acid derivatives with the aid of eight postulates. The data were obtained from approximately 100 compounds, and K_0- or k_0-values for each of these can be predicted within an order of magnitude by considering the above postulates. The discussion has been illustrative rather than thorough. Similar arguments cover other cases, some of which are mentioned below.

The remarkably low K_0-value of N-carbethoxy-L-tyrosinamide has already been attributed to "wrong-way" binding featuring R_1–p_3 interaction.[11] If R_2–p_2 interaction remains important in this compound it is readily seen why the "wrong" interaction is indeed non-productive: it places R_1 in the p_H-position.

In previous discussions, the strikingly low k_0-value of α-N-acetyl-L-tryptophanamide[19] and the high K_I-value observed for α-N-acetyl-L-tryptophanmethylamide when evaluated against methyl hippurate[29] were considered "anomalous." Both of these results can be correlated with other data if postulate 5 is applied to the interpretation of kinetic data involving tryptophan and other indole derivatives.

Finally, the mixed inhibition observed for indole against methyl hippurate, but not against trifunctional substrates,[30] can again be simply interpreted by postulate 5. If two binding modes are considered, one, ES leading to products and the other, ES′, competitive with the first and nonproductive, then applying the usual conditions [S] >> [E], and initial stages of reaction, it follows from eq. 2–10 that

$$-d[S]/dt = d[P]dt = k_2[E][S]/\{K_{S.1} + [S](1 + K_{S.1}/K_{S.2})\} \quad (11)$$

Equation 11 can be transformed into a Michaelis–Menten type equation where

$$k_0 = k_2K_{S.2}/(K_{S.1} + K_{S.2}) \quad (12)$$

and

$$K_0 = K_{S.1}K_{S.2}/(K_{S.1} + K_{S.2}) \quad (13)$$

If inhibition involving both binding modes is considered as well as conventional fully competitive inhibition, that is permitting the formation of EI, ESI and ESI′, eq. 14 expresses the inhibited

$$-d[S]/dt = d[P]/dt = k_2[E][S]/\{K_{S.1}(1 + [I]/K_I) + [S]\{(1 + [I]/K_{S.11}) + K_{S.1}/K_{S.2}(1 + [I]/K_{S.21})\}\} \quad (14)$$

rate if *none* of the complexes containing inhibitor can give product. It is apparent that on rearrangement to a Michaelis–Menten form eq. 14 predicts mixed type of inhibition. Even if one ternary complex were assumed to lead to products at a rate identical to that of the corresponding binary complex, the resulting rate expression would, in general, predict mixed type of inhibition.

Although the rate expression 14 is complex, the degree to which terms which deviate from fully competitive behavior enter in depends on the ratio of $K_{S.1}/K_{S.2}$. When $K_{S.1} << K_{S.2}$ eq. 14 reduces to fully competitive inhibition as long as ES′I is not formed. On the other hand, as the ratio $K_{S.1}/K_{S.2}$ increases the perturbation of fully competitive inhibition increases. In the analogous situation where ESI can lead to products, the inhibition is fully non-competitive when $K_{S.1}/K_{S.2} << 1$ and mixed as this ratio increases.

It would be of interest to examine a series of bifunctional substrates and for each evaluate the inhibition by indole. A correlation of percentage competitive inhibition and K_0-values should indicate the relative importance of $K_{S.1}$ and $K_{S.2}$ for each of the preceding values. Alternatively, one could study the *p*H dependence of percentage competitive inhibition for a single substrate in an attempt to determine the separate *p*H dependencies of $K_{S.1}$ and $K_{S.2}$. Finally, the examination of a related series of inhibitors such as variously substituted quinolines or indoles against a bifunctional substrate might provide information from the percentage competitive inhibition observed in each case.

A critical test of any theory concerning the substrate specificity of α-chymotrypsin involves its ability to explain the results obtained with D- and L-3-carbomethoxydihydroisocarbostyril.[13] One might hope that the theory would directly and necessarily predict their respective kinetic constants, expect that the theory can accommodate them, and fear that the results obtained with these compounds were incompatible with the previous postulates or required additional assumptions. Neither the hopes nor fears are realized; the theory can adequately explain the results for these compounds but would not have predicted them.

Predictions may go astray for two types of reasons. First, the result may be different from that expected but of the same class and, second, the result may be of a different type. The first error usually involves a mistaken assumption, while the

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(29) T. H. Applewhite and C. Niemann, *ibid.*, **81**, 2208 (1959).

(30) H. T. Huang and C. Niemann, *ibid.*, **75**, 1395 (1953).

second indicates that the particular result is not analogous to previous ones from which the prediction was made. In the case of D- and L-3-carbomethoxydihydroisocarbostyril, the results are clearly of a different class for two reasons. It should be emphasized that this difference manifests itself independently of the remarkable inversion of antipodal specificity encountered with these compounds. One difference is that, although in terms of the theory, these compounds should be classed as "bifunctional," and, therefore, like all other bifunctional compounds would possess low k_0-values, one of the isomers has, in fact, a k_0-value comparable to those of the better substrates. The other difference is that with one of these compounds fully non-competitive inhibition may be observed, a phenomenon that has been seen for no other substrate of α-chymotrypsin.

There can be no doubt that the results just mentioned are different from any observed previously. Fortunately, they are compatible with each other and with the theory. Both results indicate that these two compounds can achieve substantial binding at the active site while involving only two of the three groups usually associated with specificity. It must be determined whether this more limited interaction can satisfy the demands of any lock and key theory, whether it is compatible with the tenets of this particular theory and whether or not the structures of the compounds can be related to their behavior.

It has been pointed out that the requirements of stereospecific control as envisaged by the Ogston hypothesis[9] can be satisfied by less than "three-point" interaction.[10] In molecular terms, the kind of stereochemistry envisaged requires that one particular bond from a tetrahedral carbon atom be fixed directionally by interaction of two of the other groups attached to that atom. However, if only one group is "fixed" but the rigidity imparted to it includes restriction of rotation about the bond that attaches it to the particular carbon atom in question, then stereochemical specificity can be achieved by invoking no other enzyme–substrate interaction. The rigidity may be a function of the group rather than of the type of binding. If the group is part of a conformationally determined series of bonds, such as unsaturated cyclic functions, amides, etc., then rotation around the bond to the tetragonal carbon is restricted without invoking specific binding of that carbon atom.

Specificity is presumed to arise from binding at ρ_2 and orientation at ρ_1 along with a COR_3–ρ_3 interaction. For the heterocyclic dihydroisocarbostyril compounds the R_2–ρ_2 interaction can fulfill both binding and orienting functions since the compound has available only a limited range of conformations. By specifying the positions of R_1 and R_2 in an α-N-acylated α-amino acid derivative, the position of the carbonyl function is determined to the same extent as by specifying the position of the carbostyril nucleus in the heterocyclic compounds.

The conformation preferred for certain α-N-acylated-L-aromatic α-amino acid esters is shown in 2.1 of Fig. 2. With the aid of models it can be shown that if D-3-carbomethoxydihydroisocarbostyril is positioned to achieve maximum R_2–ρ_2 interaction, its axial conformation will allow an excellent R_3–ρ_3 interaction. Although no R_1–ρ_1 interaction is invoked, the R_3 group is "correctly" positioned without this. Further, the amide function of the substrate is oriented toward the ρ_1-locus and might interact with it.

Applying the same considerations to L-3-carbomethoxydihydroisocarbostyril the same type of fit can be obtained. However, in order to orient the COR_3 group correctly the heterocyclic ring system must be oriented with the amide function toward ρ_H. This difference is sufficient so that this isomer acts more like a typical bifunctional substrate. Its kinetic constants are very similar to those for methyl hippurate.

It is impossible to decide conclusively, on the basis of the evidence available, whether the large difference in reactivity between D- and L-3-carbomethoxydihydroisocarbostyril is related to positive involvement of ρ_1 for the D-isomer or unfavorable consequences of placing the amide function near ρ_H for the L-isomer. However, the evidence available provides no support for the last hypothesis, but does reflect favorably on the former.

One of the significant consequences of the consideration of alternative binding modes presented earlier involves prediction of types of inhibition observed. Equation 14 predicts that (unless the dissociation constants for the ternary complexes are smaller than for binary complexes) deviations from fully competitive inhibition are only observed if ternary complexes can be formed with the inhibitor and the enzyme–substrate complex with the lowest dissociation constant. That is, unless the ternary complex involves the ES complex which determines K_0, its effect on K_0 will be negligible. This is true as long as K_0 is determined mainly by one preferred conformation. If two or more conformations are equally important, deviations from fully competitive inhibition can arise for other reasons.

For compounds such as methyl hippurate, K_0 is determined primarily by a non-productive binding mode involving R_1–ρ_2 interaction. Since the inhibition observed with indole is of the mixed type, a ternary complex must be possible when the substrate occupies the ρ_2-locus. This implies that indole can inhibit by interacting with ρ_1. The same type of argument can be applied to the observed fully non-competitive inhibition of the hydrolysis of L-3-carbomethoxydihydroisocarbostyril by indole. If one assumes that binding at ρ_2 is much more effective than binding at ρ_1, then only the formation of the ternary complex with indole at ρ_1 can result in deviation from fully competitive inhibition. Other suggestions of the anomalous behavior of indole as indicated by the behavior of tryptophan derivatives have been mentioned previously.

Although the binding of D-3-carbomethoxydihydroisocarbostyril was also presumed to involve only ρ_2 and ρ_3 interaction, this isomer exhibits only fully competitive inhibition with indole. This implies that the ρ_1-locus is not available for simultaneous combination with inhibitor

and suggests, instead, that the ρ_1-locus is involved in binding of the substrate.

It is clear that the attempt to incorporate the conformationally constrained molecules into the theory has done more to demonstrate the striking differences between these compounds and the more conventional acylated α-amino acid derivatives than to clarify the theory. These compounds represent a new and challenging tool for elucidating the specificity of α-chymotrypsin action. Only when more compounds like these have been studied and more detailed information is available concerning the effects of reaction parameters other than enzyme and substrate concentration on the kinetic constants obtained in the presence as well as absence of various types of inhibitors can one hope to integrate completely this class of compounds into a general scheme.

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On the Kinetics and Mechanism of Helix Formation: The Two Stranded Poly (A + U) Complex from Polyriboadenylic Acid and Polyribouridylic Acid[1a]

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The kinetics of formation of the poly (A + U) double-stranded helical complex from random coil polyriboadenylic acid and polyribouridylic acid has been studied as a function of ionic strength, temperature and polymer concentration by means of the spectrophotometric stopped-flow technique. The reaction follows complex kinetics, initially of second order form, then deviating toward first order. The exact form followed depends both upon the ionic strength and upon the polymer concentration used. At high salt concentrations, the rate of formation of the poly (A + U) complex is found to pass through a maximum about 40° below the helix melting temperature (65° in 0.25 *M* NaCl). This latter result provides support of the similar theories for the kinetics of helix–coil transformations recently proposed by Flory and by Saunders and Ross. The significance of the parameters appearing in these theories and limitations on their quantitative interpretation are discussed.

Introduction

Equimolar quantities of the synthetic polynucleotides, polyriboadenylic acid (poly A) and polyribouridylic acid (poly U), when combined in aqueous salt solutions at neutral pH react to form an ordered 1:1 complex, poly (A + U).[2-3] X-Ray studies have shown the poly (A + U) fiber to have a two-strand helical structure similar to that of naturally occurring deoxyribonucleic acid (DNA).[2b]

In a previous paper,[4] using the stopped-flow technique[5] to observe the kinetics of the optical density change at 259 mμ accompanying the formation of the poly (A + U) complex, Ross and Sturtevant found the reaction to follow complex kinetics, initially of second order form, then deviating toward first order. The reaction rate decreased with increasing temperature and approached zero rate in the vicinity of the helix "melting" temperature[6] T_m.

Concurrent with this work, two essentially similar theories of the kinetics of helix–coil transformations were proposed: one by Flory[7] who likened the process to a biased one-dimensional random walk to which he applied the solution of the gambler's ruin problem of probability theory, and the other by Saunders and Ross who based their argument on a kinetic model for the reaction.[8] One of the consequences of both of these theories is that at some temperature below the helix melting temperature, the rate of helix formation should pass through a maximum. The present investigation was undertaken in order to obtain evidence which might be used to test these theories.

By increasing the salt concentration, T_m is raised, thus permitting examination of the kinetics of the reaction at temperatures farther below T_m than was possible at the lower salt concentrations previously employed. This investigation, under conditions where the reaction is more rapid, was made possible by the construction of a new stopped-flow apparatus[9] capable of studying reactions in the millisecond range.

Through a detailed study of the kinetics of suitable model systems, it is hoped that an increased understanding of the mechanism of helix-coil transformations may be gained. Such knowledge is of fundamental chemical interest and may have application in the interpretation of similar phenomena occurring in macromolecules of biological interest.

Experimental

The polyribonucleotides, poly A and poly U, were synthesized enzymatically using polynucleotide phosphorylase isolated from *M. lysodeikticus* and the appropriate monomer diphosphates (Sigma Chemical Company).[10] The preparations were deproteinized by successive chloroform emulsi-

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