Tetrahedron. Vol 24, pp 3453 to 3459 Pergamon Press 1968. Printed in Great Britain

ON THE GENERAL BASE–GENERAL ACID MECHANISM FOR α-CHYMOTRYPSIN ACTION

F. M. MENGER and H. T. BROCK[1]
Department of Chemistry, Emory University, Atlanta, Georgia 30322

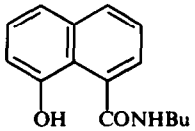
(*Received in USA* 9 *September* 1967; *accepted for publication* 17 *November* 1967)

Abstract—The hydrolysis of the conjugate base of N-n-butyl-8-hydroxy-1-naphthoamide (compound A in text) has been shown to proceed many orders of magnitude more slowly than the hydrolysis of amides bound to the active site of α-chymotrypsin. In the model compound, a naphtholate anion (which is a stronger base and a better nucleophile than imidazole) is held rigidly in close proximity to the p-orbital of an amide CO, an ideal geometry for anchimeric assistance. Yet intramolecular catalysis is very slow. The naphtholate anion is unable to accelerate amide hydrolysis *via* removal of a proton from the nucleophile (water) and subsequent delivery of the proton to the departing amine, although just such a mechanism has been proposed for the imidazole-catalyzed hydrolysis of enzyme-bound substrates. Therefore, we conclude that a currently advanced mechanism for α-chymotrypsin action, based on an imidazole ring acting as the sole catalytic entity, cannot adequately explain the efficiency of the enzyme.

THE rate determining step in the α-chymotrypsin-catalyzed hydrolysis of peptides is believed to involve nucleophilic attack on the substrates by a serine OH group.[2] An imidazole ring of a histidine assists the formation of the tetrahedral intermediate by acting as a general base. Collapse of the intermediate is facilitated by transfer of a proton from imidazolium ion to the departing amine.* The presence of only a single catalytic group at the active site has been neither proved nor disproved. Certainly, catalysis by a lone imidazole ring is still considered a possibility. This point is made clear in a recent and thorough discussion of α-chymotrypsin.[3]

This paper deals with a fundamental question concerning the above speculative but important mechanism. Is an imidazole ring a powerful enough catalyst to explain the rapid hydrolyses of amides at the active site, or are other factors necessary (such as one or more additional catalytic groups or an enzyme-induced strain)? Thus, the purpose of the present work is to establish whether or not the general base–general acid mechanism for α-chymotrypsin is at least chemically reasonable if not biologically correct.

We have studied the hydrolytic properties of N-n-butyl-8-hydroxy-1-naphthoamide (A) under conditions where the naphthol is largely ionized. In this model, a general base (naphtholate anion) is rigidly held in close proximity to the amide

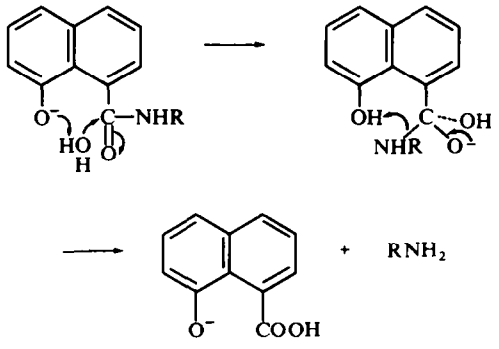


A

* It is also possible that imidazole attacks directly the carbonyl of a bound substrate, but at the present time such a mechanism is in disfavor because evidence for an acylimidazole intermediate is lacking.

functionality. Since naphtholate anion is a stronger base (and better nucleophile) than imidazole, the amide should hydrolyze rapidly, as does an enzyme-bound peptide, if indeed monofunctional catalysis by the enzyme is operative. Chart 1 shows a possible

CHART 1

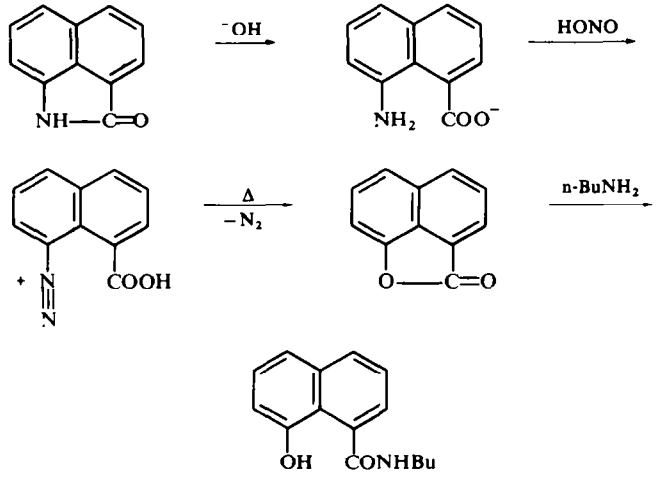


mechanism for catalyzed hydrolysis of *A* which is completely analogous to the monofunctional general base–general acid mechanism for α-chymotrypsin action.[4]

RESULTS AND DISCUSSION

We find that hydrolysis of anionic A is in fact very slow. A substance with the same m.p. and IR as A can be isolated in high yield by acidifying a solution of the substrate in aqueous 0·1N NaOH. Virtually no UV spectral change is observed after thermostating at 25·0° a dilute solution of A ($1{\cdot}23 \times 10^{-4}$ M) for 48 hr at pH = 11·48. (Substrate A, pK_a = 10·42, is more than 90% ionized under these conditions). A *maximum* possible value of the rate constant for hydrolysis of *A via* intramolecular

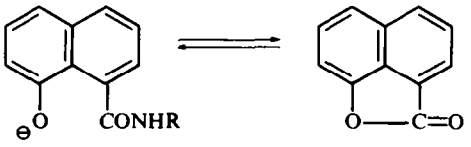
CHART 2



A

catalysis can be obtained by comparing carefully the reactant and product (8-hydroxy-1-naphthoic acid) UV spectra. We calculate that there is less than 5% (the experimental error) hydrolysis after 48 hr at pH = 11·48, which means that the rate constant for neighboring group participation in **A** at 25·0° must be less than 3×10^{-7} sec.$^{-1}$. The rate constant for hydrolysis of N-acetyl-L-tryptophan amide by α-chymotrypsin[5] (pH = 8, 25°) is $4{\cdot}4 \times 10^{-2}$ sec^{-1}. Therefore, an amide bound to the enzyme is many orders of magnitude more labile than the amide group of the conjugate base of **A**.

The very slow hydrolysis of N-n-butyl-8-hydroxy-1-naphthoamide (**A**) relative to that of an enzyme-bound amide is highly significant since steric and kinetic considerations, described below, demand that hydrolysis of **A** be very fast if imidazole is the sole catalytic group in α-chymotrypsin. Hydrolysis of **A** could conceivably have occurred by either of two mechanisms, nucleophilic catalysis (Eq. 1) or general base–general acid catalysis (Chart 1). We are primarily interested in the latter because of its analogy to the proposed enzymatic mechanism, but it is interesting to consider the lack of nucleophilic participation in **A** as well.



(1)

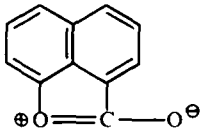
B

With regard to nucleophilic catalysis, the observed absence of reaction after exposure of **A** to basic conditions for 48 hr means one of the following: (a) Intramolecular naphtholate anion attack on the amide group is too slow and/or partitioning of the resulting tetrahedral intermediate towards product too unfavorable when the amine moiety is unprotonated. (b) Ring closure to form lactone is inhibited by strain in the transition state leading to cyclized product; this is a possibility since the *peri* positions of naphthalenes are 2·44 Å apart.[6] (c) Lactone formation does indeed occur, but the equilibrium between amide and lactone/amine favors the starting material.

The evidence points strongly towards the first rationale. Clearly, lactone never succeeds in forming, because under the reaction conditions most of the lactone molecules would be *irreversibly* hydrolyzed to 8-hydroxy-1-naphthoate rather than be aminolyzed back to starting material. The concentration of substrate used in the experiment was very low, $1{\cdot}23 \times 10^{-4}$ M. The n-butylamine concentration could never exceed this value. The concentration of hydroxide ion (at pH = 11·48) was $3{\cdot}0 \times 10^{-3}$ M. Since the more concentrated hydroxide ion is a better nucleophile than the amine towards esters,[7] the rate of lactone hydrolysis would far exceed its rate of aminolysis under our experimental conditions. Thus, anionic **A** is a non-reactive molecule.

We have determined the rate constant for hydrolysis of lactone **B** in order to test the possibility that ring closure in **A** is greatly inhibited by strain in the transition state leading to cyclized product. If there is such strain, then the rate of hydrolysis of the lactone (i.e. the rate of ring opening) should be unusually fast. Ring strain should enhance the rate limiting attack of hydroxide ion on the lactone carbonyl by making

resonance contributor C less important. Ring strain should also enhance partitioning of the tetrahedral intermediate towards product. However, the second-order rate constant for basic hydrolysis of lactone **B** (71 M^{-1} sec^{-1}, 25·0°, I = 0·5) is only 263

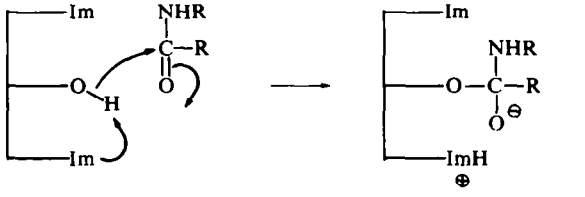


C

times larger than that for the hydrolysis of phenyl 1-naphthoate at the same temperature. This ratio is *less* than that for the hydrolysis of γ-butyrolactone relative to ethyl acetate (461), a value attributed to the activating *s-cis* arrangement of the cyclic ester.[8] Therefore, strain does not appear to be a significant factor in the slow rate of intramolecular nucleophilic catalysis of the amide hydrolysis.

Thus, the conjugate base of compound **A** demonstrates the well known inertness of unactivated amides to intramolecular nucleophilic catalysis.[9] It is a particularly striking example, because the p-orbital of the amide carbonyl carbon and an oxygen orbital containing an unshared pair of electrons are held rigidly in close proximity to each other. Note that the rate determining step (Eq. 2 and 3) of two of the most important α-chymotrypsin mechanisms proposed at this time[4,10] involves a general base catalyzed nucleophilic attack of a poor nucleophile, serine OH, on an *unactivated* amide.

(2)

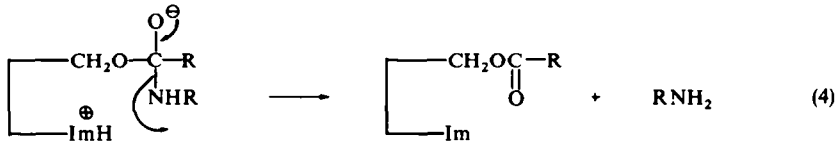


(3)

Equations 2 and 3 seem particularly improbable since imidazole is in fact a *poor* catalyst for all but the most reactive carboxylic acid derivatives (such as esters of dichloroacetic acid, aryl esters, thiolesters and acylimidazoles). There is not a single known example of an imidazole ring, in its free base form, enhancing the hydrolysis rate of a peptide by either general base or nucleophilic catalysis in either intermolecular or intramolecular systems.

We now turn our attention to the key finding that anionic N-n-butyl-8-hydroxy-1-naphthoamide (**A**) is inert to general base catalysis as well as nucleophilic catalysis. Since naphtholate anion (pK_a = 10·42) is a much stronger base than imidazole

(pK_a = 6·95), general base-catalyzed attack of the amide group of A through the solvation shell of the naphtholate anion (first step, Chart 1) would be expected to be roughly 10^3 faster than the corresponding step in the proposed enzymatic reactions (Eq. 2 and 3). On the other hand, naphthol is a poorer acid than imidazolium ion, so that general acid-catalyzed decomposition of the tetrahedral intermediate of the enzyme by imidazolium ion (Eq. 4) would be roughly 10^3 faster than the corresponding process in the model system (second step, Chart 1). At the optimal pH for α-chymotrypsin (pH 8), there is 10% of the imidazole in the protonated form. At the pH of the model studies in this paper, there is also about 10% of the compound in the naphthol form. We conclude from these comparisons that the overall rate of hydrolysis of N-n-butyl-8-hydroxy-1-naphthoamide (A) should be of the same order of magnitude as that of the enzyme-substrate complex. But the model amide hydrolyzes a minimum of 10^5 times slower than the enzyme-bound amide. Therefore, the proposed enzymatic mechanisms are incorrect.



(4)

If the *peri*-naphthalene system (A) were a reasonably accurate model of the active site of α-chymotrypsin, as the general base–general acid mechanism suggests it should be, then the amide would hydrolyze rapidly. Since this is not the case, there must be at least one critically important feature of the active site which is absent in the model. There are, of course, obvious small differences between the model and enzyme systems. For example, the model is an aromatic amide, whereas a peptide is not. But steric or electronic factors of this sort are trivial in light of the minimum 10^5 rate difference. The general base-general acid mechanism is fundamentally inadequate.

Obviously, understanding of enzyme mechanisms is too meager for anyone to have claimed that the general base-general acid mechanism (or any other mechanism) of α-chymotrypsin *is* correct. Nevertheless, the idea is prevalent that the monofunctional general base–general acid mechanism, outlined above, *may* be correct.[11] The important conclusion of this paper is that the mechanism *is not* correct in the sense that it adequately explains the catalytic efficiency of the enzyme.

Since monofunctional catalysis by an imidazole ring cannot adequately explain the facility of enzyme-catalyzed peptide hydrolyses, other factors such as one or more catalytic groups must be involved. In light of the points brought forth in this article, it appears very likely that the active site contains a general acid which promotes nucleophilic attack on substrate CO group. Bender and Kézdy[12] have proposed that an imidazole acts simultaneously as a general acid and base. As these authors point out, the mechanism is probably incorrect since the stereochemistry is awkward, but the mechanism does embody a feature which may be essential to facile hydrolysis, namely acid catalysis of the initial nucleophilic attack on the CO group. Possibly, a second imidazole ring serves as a general acid.[12, 13] In any event, it still remains to be shown rigorously that even the combined action of a weak general base and a weak

general acid is sufficient to explain the rapid rates of enzymatic hydrolyses of amide linkages.[18]

EXPERIMENTAL

The model compound, N-n-butyl-8-hydroxy-1-naphthoamide (A), was prepared as shown in Chart 2.

8-Hydroxy-1-naphthoic acid lactone. The lactam of 8-amino-1-naphthoic acid was obtained from Sapon Laboratories and recrystallized once from aqueous EtOH, m.p. 179–180° (lit.[14] m.p. 180–181°). After the purified lactam (5·0 g, 0·029 mole) had been boiled under reflux in 60 ml 1N NaOH for 2 hr, only a small amount of material remained undissolved, and this was removed by filtration and discarded. The filtrate, following partial neutralization with 2N HCl to a pH = 8–9, was added without delay to a flask containing 4 g $NaNO_2$ in 100 ml 6N HCl which was cooled in ice and vigorously stirred. The addition was performed with the aid of a funnel whose stem extended below the surface of the HNO_2 soln. Solid material tended to plug the funnel and had to be removed with a glass rod. The addition was completed in less than 5 min. Stirring was continued for 1 hr at 25°, whereupon the flask was placed on a steam bath until evolution of N_2 ceased (about 10 min). The reaction mixture was cooled and filtered. Purification of the collected solid (in particular removal of azo dye) was affected by two sublimations, yielding 2·8 g (56%) pale yellow crystals, m.p. 105–107° (lit.[15] m.p. for 8-hydroxy-1-naphthoic acid lactone 108°). Beautiful white needles, m.p. 106–107°, were obtained after two recrystallizations from MeOH. The CO stretching band of the lactone appears at 5·62 μ.

N-n-*Butyl-8-hydroxy-1-naphthoamide* (A). This compound was prepared by dissolving 0·44 g (2·6 mmole) 8-hydroxy-1-naphthoic acid lactone in 10 ml reagent-grade n-butylamine at 0°. The soln was allowed to warm up to room temp and remain there for 25 min. The amine was then removed, first by means of a stream of N_2 and next with the aid of a vacuum desiccator. A pale yellow powder (0·60 g, 95%) was obtained after one recrystallization of the residue from aqueous MeOH. Two more recrystallizations from aqueous MeOH (using activated charcoal) and a final one from heptane produced nearly white crystals. The product melted at 107–108° and had an intense amide II band at 6·54 μ. (Found: C, 74·18; H, 7·12; N, 5·80. Calc. for $C_{15}H_{17}NO_2$: C, 74·04; H, 7·04; N, 5·76%).

Phenyl 1-naphthoate. The ester was prepared by warming a mixture of 1-naphthoic acid (0·85 g, 4·9 mmole), phenol (0·50 g, 5·3 mmole) and $POCl_3$ (2 ml) on a steam bath for 1 hr and then pouring the thick liquid onto ice. The resujting solid was recrystallized 3 times from aqueous MeOH (with considerable loss of material) to give white needles, m.p. 94–96° (lit.[16] m.p. 95·5–96°). A final recrystallization from anhyd MeOH (cooled in a Dry Ice-acetone bath) raised the m.p. of the product to 96–97·5°; $\lambda_{max}^{CHCl_3}$ 5·79 μ.

Kinetic measurements. The basis hydrolysis of 8-hydroxy-1-naphthoic acid lactone was followed by measuring the decrease in absorbance at 360·0 mμ as a function of time using a Cary 14 spectrophotometer thermostated at 25·0 ± 0·1°. Reactions were initiated by adding 50 μl of a soln of the lactone in MeCN (0·0204 M) to a cuvette containing 3·00 ml of a borate buffer (pH 9·98 or 10·50). The reactions were followed to completion, and the first-order plots were linear to greater than 80% reaction. The calculated second-order rate constants for reaction between lactone and hydroxide ion in the two buffers agreed with each other to better than 6%.

The basic hydrolysis of phenyl 1-naphthoate at 25·0 ± 0·1° was followed in a similar manner except that the decrease in absorbance at 310·0 mμ was utilized (0·1 slidewire). Initial substrate and hydroxide ion concentrations in the cuvettes were $1·11 \times 10^{-5}$ M and 0·020 M respectively.

The rate constant for hydrolysis of N-n-butyl-8-hydroxy-1-naphthoamide (A) in basic solutions at 25° is too small to measure. The stability of A in solutions of high pH was demonstrated by an experiment in which A (30·0 mg) was dissolved in 25% MeCN–75% 0·1N NaOH. Acidification with 0·1N HCl after 10 min resulted in a ppt which was collected by filtration. The dried solid, m.p. 105–108°, weighed 25·9 mg (86%) and had an IR spectrum identical to that of A.

In order to obtain an upper limit to the value of the rate constant for hydrolysis of N-n-butyl-8-hydroxy-1-naphthoamide (A) *via* intramolecular catalysis, the following experiment was performed. A $1·23 \times 10^{-2}$ M soln of the substrate in MeCN was prepared and a portion of it (250 μl) added to 25·0 ml phosphate buffer of pH 11·48. (At this pH, greater than 90% of A is ionized). The buffer, containing 2% Na_2SO_3, had been thoroughly purged with N_2 in order to protect the substrate against oxidation. An UV spectrum of the aqueous substrate soln was secured. The soln was then thermostated for 48 hr at 25·0 ± 0·1°. After this period, another UV spectrum was taken and compared carefully with the first one as well as with a spectrum of a soln of the hydrolysis product (8-hydroxy-1-naphthoic acid) in the same phosphate buffer. The spectra

showed that there was less than 5% hydrolysis of the amide during the 48 hr period. An attempt to repeat this experiment at 60° failed because of the susceptibility of the small amounts of substrate to oxidation during the prolonged exposure to basic conditions and elevated temps.

pK_a Determination. The pK_a of **A** (10·42 at 25·0°, I = 0·5) was determined spectrophotometrically.[17]

Acknowledgement—We are grateful to the McCandless Fund of Emory University for sole support of this work.

REFERENCES

[1] Ford Undergraduate Career Scholar.
[2] T. C. Bruice and S. J. Benkovic, *Bioorganic Mechanisms* Vol. 1, Chap. 2. Benjamin, New York, N.Y. (1966).
[3] Ref. 2, p. 247.
[4] M. L. Bender, *J. Am. Chem. Soc.* **84**, 2582 (1962).
[5] M. L. Bender, F. J. Kézdy, and C. R. Gunter, *J. Am. Chem. Soc.* **86**, 3714 (1964).
[6] V. Balasubramaniyan, *Chem. Revs.* **66**, 567 (1966).
[7] T. C. Bruice, A. Donzel, R. W. Huffman, A. R. Butler, *J. Am. Chem. Soc.* **89**, 2106 (1967).
[8] M. L. Bender, *Chem. Revs.* **60**, 53 (1960).
[9] T. C. Bruice and J. M. Sturtevant, *J. Am. Chem. Soc.* **81**, 2860 (1959).
[10] Ref. 2, p. 250.
[11] Ref. 2 and refs cited therein.
[12] M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.* **86**, 3704 (1964).
[13] K. A. Walsh, D. L. Kauffman, D. S. V. Sampth Kumar, and H. Neurath, *Proc. Natl. Acad. Sci. U.S.* **51**, 301 (1964).
[14] A. G. Ekstrand, *Chem. Ber.* **18**, 2881 (1885).
[15] A. G. Ekstrand, *Ibid.* **19**, 1131 (1886).
[16] Belg. Patent 634,563; *Chem. Abstr.* **61**, P8239b (1964).
[17] A. Albert and E. P. Serjeant, *Ionization Constants of Acids and Bases*, Chap. 4. Methuen, London (1962).
[18] Note added in proof. An X-ray analysis of tosyl-α-chymotrypsin shows only one imidazole ring near the active site: B. W. Matthews, P. B. Sigler, R. Henderson and D. M. Blow, *Nature, Lond.* **214**, 652 (1967). This fact, coupled with the assertions made in the present paper, suggests that the enzyme must derive much of its rate enhancement from substrate distortion. We elaborate on this point in another paper in which the reactivity of a distorted (twisted) amide is examined.