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Fast Hydrolysis of an Aliphatic Amide at Neutral pH and Ambient Temperature. A Peptidase Model

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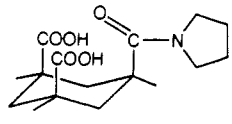
Abstract: An intramolecular-catalyzed cleavage of an aliphatic amide under biological conditions (ambient temperature, neutral pH, absence of alien transition metals) was found to occur with the fastest rate yet recorded for such a reaction: $t_{1/2}$ = 8 min (pD = 7.05, 21.5 °C) and an effective molarity EM > 10^{14} M. The peptidase "model" has a carboxyl oxygen perched above the plane of the amide carbonyl at a van der Waals contact distance of 2.8 Å. The carboxyl is poised for synchronous nucleophilic attack and proton delivery. Evidence (based on the observation that the amide actually cleaves much faster than the corresponding methyl ester) suggests that proton transfer plays a key role in the rate-determining step. The results show that an enzyme need not employ esoteric mechanisms to cleave an unreactive entity such as an amide. If the enzyme merely positions a carboxyl adjacent to an amide substrate with the geometry established in the "model", most of the necessary catalytic power would be achieved.

Human beings admire speed whether it be animal, mechanical, or chemical in origin. Within the chemistry arena, fast reactions signify milder conditions and reduced energy consumption. But the desire to achieve speed is motivated by more than economics. Chemists are challenged by a rival, the enzyme, that outpaces us with a perplexing regularity. α-Chymotrypsin, for example, hydrolyzes amides rapidly at neutral pH and ambient temperature.[1] In contrast, a typical chemical procedure for hydrolyzing amides[2] calls for a 10-h reflux in 8 N HCl. Although "models" attempting to duplicate α-chymotrypsin-like rates have been successful with *p*-nitrophenyl esters, rate enhancements often vanish when less reactive ("natural") carboxylic acid derivatives are employed.[3] In the present article we describe cleavage of an aliphatic amide I under biological conditions free from transition metals. Neither a substituent (such as a *p*-nitrophenyl group on the nitrogen) nor ring-strain (as in a β-lactam) nor amide-twisting (as in a bridgehead amide) artificially activate the substrate. To our knowledge, the reaction constitutes the fastest peptidase "model" at pH 7 on record.[4-9]



I

Experimental Section

General Procedures. Melting points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 983 IR spectrophotometer. ^{1}H NMR spectra were obtained with a General Electric QE-300 spectrometer. NMR data are reported as follows: chemical shift (δ) downfield from internal tetramethylsilane, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad signal), coupling constant (Hz), integration, and assignment. Precise mass was determined with the aid of a VG Analytical MM 7070S high-resolution mass spectrometer.

Anhydride Acid III. The precursor *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid[10] was prepared following the published procedure.[11] It was sublimed at 190–195 °C (0.20 mm) to give white crystals of the corresponding anhydride acid III, mp 205–208 °C, in 85% yield. IR (KBr): 1796, 1766, 1702, 1001 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.72 (d, J = 14.1 Hz, 2 H, equat CH), 2.05 (d, J = 13.8 Hz, 1 H, equat CH), 1.35 (s, 9 H, Me), 1.30–1.50 (m, 3 H, axial CH).

Anhydride Acid Chloride. A suspension of anhydride acid III (414 mg; 1.72 mmol) in dry benzene (18 mL) was cooled to 0 °C and oxalyl chloride (2.2 mL; 25.2 mmol) was added followed by 1 drop on *N,N*-dimethylformamide. The resulting mixture was stirred at room temperature for 16 h. The residue obtained upon evaporation of solvent was recrystallized from dry dichloromethane to give 355 mg (80%) of anhydride acid chloride as pale-yellow crystals; mp 253–255 °C (lit.[10] mp 255–260 °C). IR (KBr): 1793, 1770, 1005 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.80 (d, J = 13.8 Hz, 2 H, equat CH), 2.04 (d, J = 13.5 Hz, 1 H, equat CH), 1.39 (d, 1 H, axial CH), 1.35 (s, 3 H, Me–C–COCl), 1.34 (s, 6 H, Me–C–COO), 1.30–1.40 (2 H, axial CH).

Anhydride Amide II. A solution of anhydride acid chloride (300 mg; 1.16 mmol) in dry acetonitrile (40 mL) was purged with nitrogen, and dry pyridine (3.0 mL; 37 mmol) was added by means of a syringe. This mixture was cooled to −40 °C and pyrrolidine (101 μL; 1.21 mmol) was added, resulting in the immediate development of an intense yellow color. The homogeneous mixture was stirred at room temperature for 3 h. Evaporation of solvent gave 495 mg of yellow crystals which were flash-chromatographed on 7 g of aluminum oxide, using dichloromethane as eluent, to yield 214 mg (63%) of anhydride amide as white crystals; mp 215–217 °C. Precise mass for $C_{16}H_{23}NO_4$: 293.1627 (calcd), 293.1623 (found). IR (KBr): 1790, 1760, 1611, 1008 cm^{-1}. ^{1}H NMR (CDCl3) δ 3.46 (t, J = 6.6 Hz, 4 H, N–CH_2), 2.91 (d, J = 13.5 Hz, 2 H, equat CH), 2.00 (d, J = 13.5 Hz, 1 H, equat CH), 1.87 (br s, 2 H, N–CH_2–CH_2), 1.77 (br s, 2 H, N–CH_2–CH_2), 1.35 (d, 1 H, axial CH), 1.34 (s, 6 H, Me–C–COO), 1.22 (s, 3 H, Me–C–CON), 1.16 (d, J = 13.5 Hz, 1 H, axial CH).

Kinetic Measurements. Rate of anhydride opening in anhydride amide II (k_1 in eq 1) was studied between pD 2 and 11. In a typial experiment,

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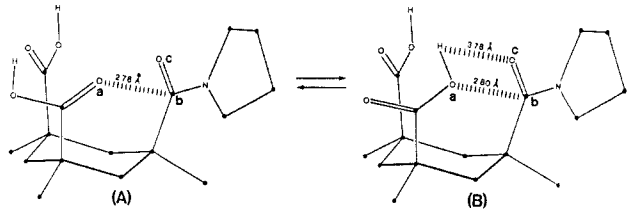


Figure 1. MM2-derived conformations of "diacid amide" I. The O_a/C_b separation of 2.8 Å lies within the "critical distance" (i.e., less than the 3-Å diameter of a water molecule).[17]

anhydride amide (1.0 mg) was dissolved in CD_3CN (20 μL), and a buffer in D_2O (1.0 mL) of the desired pD was added. Samples for measurements below pD 8 were prepared in 50 mM phosphate buffer, whereas for measurements above pD 8 samples were prepared in 50 mM borate buffer. The progress of reaction was monitored by the disappearance of the anhydride amide ^1H NMR signal at δ 3.50 (t, 2 H, CO–N–CH_2) and by the appearance of the dicarboxylate amide NMR signal at δ 3.65 (t, 2 H) for reactions in basic pD, or the pyrrolidine NMR signal at δ 3.23 (t, 4 H) for reactions in acidic media. Spectral changes in all other regions of NMR spectra were fully consistent with the cited reactions, and they gave the same values of the k_1 (within the experimental error estimated at ±15%).

In order to measure rates of amide cleavage (k_2 in eq 1) the anhydride amide was dissolved in a buffer adjusted to pD 12. At this pD the anhydride opening (k_1 step) is very fast, but the k_2 step is insignificant. After a few minutes the pD was abruptly lowered to values between 1 and 10, and the rate of amide cleavage was monitored by the disappearance of the amide NMR signal at δ 3.65 (t, 2 H) and by the appearance of the pyrrolidine NMR signal at δ 3.23 (t, 4 H). Multiple integrations of these signals after consecutive time intervals enabled calculation of percentages of the unreacted amide and free pyrrolidine product.

The rate constant for amide cleavage in amide acid ester was calculated by using a procedure similar to the above except that unbuffered deuterium oxide was used. The anhydride methyl ester[12] (1.0 mg) was mixed with CD_3CN (20 μL), deuterium oxide (1.0 mL), and pyrrolidine (2.5 μL). After 15 min the anhydride opening was completed, whereupon the product, the amide acid methyl ester (eq 3), showed an amide NMR signal at δ 3.65 (t, 2 H). This sample was quickly acidified to pD 6.3, and the amide cleavage was followed by disappearance of the amide NMR signal and appearance of pyrrolidine NMR signal as before. Multiple integrations of these two signals enabled calculation of relative ratios of the remaining amide acid ester and the pyrrolidine. Rate constants for all the above reactions were calculated from ln (100/(% unreacted amide)] = kt.

Molecular Mechanics Calculations. Calculations were performed with MACROMODEL[13] with use of an MM2 force field and VAX 11/785 computer. All energy minimizations were carried out with MACROMODEL's default values for the dielectric constant and the nonbonded cutoff distances. Structures were minimized until the root mean square of the gradient vectors was less than 0.01 kcal/Å.

Results and Discussion

Enzyme model I, hereafter called the "diacid amide", was synthesized via cis, *cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid,[10–12] the remarkable Kemp triacid whose carboxyls are known by X-ray analysis to reside in an all-axial orientation.[14] Molecular mechanics (MM2) calculations on the diacid amide show a global minimum having an acid carbonyl oxygen only 2.78 Å from the amide carbonyl carbon (Figure 1A). A second minimum, 2.2 kcal/mol higher in energy, has an acid hydroxyl 2.80 Å from the amide carbonyl carbon (Figure 1B). In both conformers, the cited oxygens lie in the Π*-region of the amide carbonyl with a nearly ideal Dunitz "attack angle" ($O_a/C_b/O_c$)[15,16]

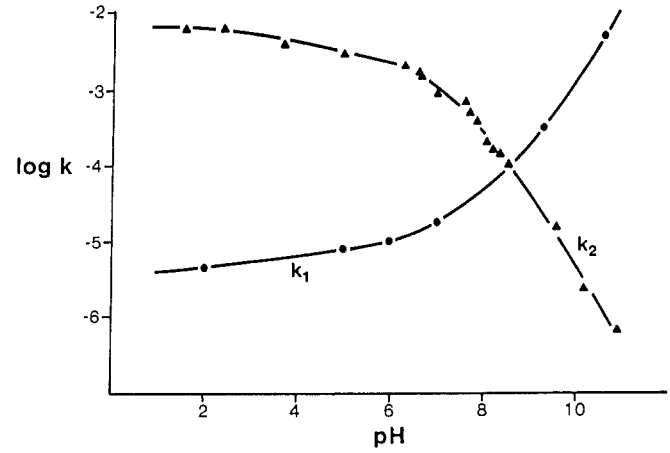
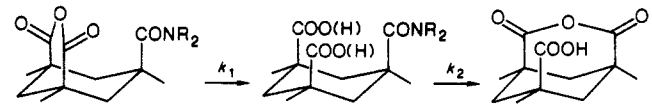


Figure 2. Log k_1 and log k_2 in eq 1 (where k's are in s^{-1} at 21.5 ± 0.5 °C) vs pH meter reading (add 0.4 to give pD in D_2O solutions).

of 105°. Moreover, as seen in Figure 1B, the carboxyl is poised for synchronous nucleophilic attack and proton delivery. These geometric considerations, especially the short sustained distances as invoked in the "spatiotemporal hypothesis",[17] led us to anticipate amide cleavage with an enzyme-like rate.

Diacid amide turned out in fact to be too reactive to isolate. Consequently, it was prepared in situ from the corresponding anhydride amide (II in eq 1). Thus, when anhydride amide was dissolved in a pD = 12 buffer, the compound opened up immediately to dicarboxylate amide as manifested, for example, by a shift in the δ 3.50 ^1H NMR signal of the pyrrolidine ring to δ 3.65 (see Experimental Section). Since the amide signal at δ 3.65 persisted at pH 12, dicarboxylate amide must hydrolyze only slowly in the basic buffer. The system was then treated with aliquots of concentrated HCl to abruptly lower the pH values between 1 and 10. Rate constants at 21.5 °C for amide cleavage (k_2 in eq 1) were determined from time-dependent amide peaks. The pH–rate profile is given in the k_2 plot of Figure 2 along with the pH–rate profile for anhydride hydrolysis (k_1 plot) obtained by adding anhydride amide to buffers without "pretreatment" in base. Figure 2 indicates that at pD = 7.05 (21.5 °C) amide cleavage is actually much faster than anhydride hydrolysis (k_2/k_1 = 150). Note that since the final product in eq 1 is an anhydride,



(1)

and since anhydride hydrolysis is rate limiting below neutrality, we are phenomenologically performing an "acyl transfer" as opposed to an amide "hydrolysis" in the short time scale of the assisted reaction. Often, however, the term "hydrolysis" is applied to nucleophilic-catalyzed cleavages of carboxylic acid derivatives whether or not the acyl intermediate persists.

The log k_2 vs pH profile in Figure 2 conforms to a *single* carboxyl participating in the amide cleavage. Thus, the rate data within the pH 5–8 range were analyzed according to eq 2 where K_a is the carboxyl ionization constant and k_{lim} is the rate at 100%

$$\frac{1}{k_2} = \frac{K_a}{k_{lim}[H^+]} + \frac{1}{k_{lim}} \qquad (2)$$

conjugate acid. A plot of $1/k_2$ vs $1/[H^+]$ in Figure 3 provides a pK_a = 6.9 ± 0.4 and a k_{lim} = (3.5 ± 0.8) × 10^{-3} s^{-1}. Rather large uncertainties in these values arise from the NMR method used to secure the rates. Moreover, protonation of the second carboxylate at lower pH values would be expected to perturb an otherwise simple pH–rate profile. Although not directly participating in the amide cleavage, the second carboxylate does seem

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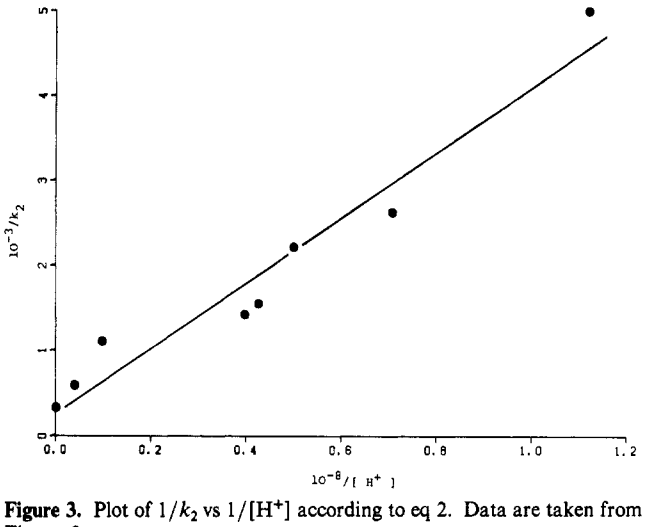
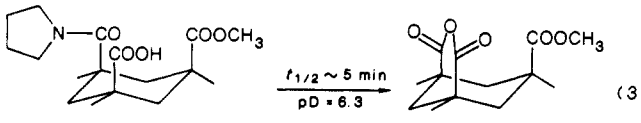


Figure 3. Plot of $1/k_2$ vs $1/[H^+]$ according to eq 2. Data are taken from Figure 2.

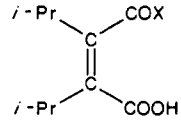
to induce a minor acceleration when it protonates (causing the k_2 at pH 2 to be 2-fold larger than the value estimated for k_{lim} by the plot in Figure 3).

Additional evidence was accrued to prove that only one of the two carboxyls is necessary to accelerate the hydrolysis. Thus, the acid-amide-methyl ester in eq 3 (prepared in situ from the anhydride methyl ester) reacts with a $t_{1/2}$ = 5 min at pD = 6.3 (21.5 °C), a value comparable to the $t_{1/2}$ of the diacid amide. NMR spectra of the compound produced from the acid-amide-methyl ester leave no doubt that amide cleavage predominates over ester cleavage. This striking reversal from customary behavior suggests that proton transfer to the amide (10^4 more basic than the ester),[18] or to an intermediate derived therefrom, plays a key role in the



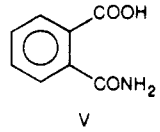
(3)

mechanism. It is relevant here that Kirby published, in separate articles,[19,20] kinetic data on the hydrolysis of *N-n*-propyldiisopropylmaleamic acid IV and the corresponding acid methyl ester; one can calculate from the numbers that the Kirby amide likewise reacts faster than the ester.



IV: **a**, X = NHPr; **b**, X = OCH_3

At pD = 7.05 (21.5 °C) the $t_{1/2}$ of our diacid amide is only 7.7 min, a value that compares favorably with previously reported intramolecular carboxyl-catalyzed amide hydrolyses. For example, Bender's phthalamic acid[4] V has a $t_{1/2}$ of 49 min and 17.5 h at



V

pH 3.0 and 5.0 (47.5 °C), respectively (reaction at pH 7 being too slow to measure conveniently). Kirby's *N-n*-propyldiisopropylmaleamic acid[19] IVa reacts 37 times faster than our diacid amide at pH 1.0, but the $t_{1/2}$ becomes a sluggish 16.3 h (15.5°C) at a pH of 6.6. Thus, despite the steric compression offered by the two isopropyl groups in IVa, and despite the conjugation of the amide in IVa to an electron-withdrawing group, the Kirby system is comparatively slow at biologically realistic conditions.

An effective molarity (EM = k_{intra}/k_{inter}) cannot be accurately determined for our diacid-amide at pD = 7.05 because no corresponding intermolecular reaction is detectable near neutrality. Suffice it to point out that Kirby estimated an EM = 3×10^{13} M at pH 1.0 for his maleamic acid[20] and, therefore, our diacid amide has an EM = 8×10^{11} M at the same pH. But since the reactivity of the diacid amide at pD = 7 is only 4-fold slower than at pD = 1 (Figure 1), and since any reference intermolecular reaction should decrease several orders of magnitude when moving 6 pH units toward neutrality, the EM of the diacid amide at pD = 7 substantially surpasses Kirby's EM = 3×10^{13} M which, until now, was the highest recorded value for such systems.

There are at least two reasons for the remarkable velocity of the diacid amide I relative to the maleamic acid IVa. (a) The diacid amide has a carboxyl with a pK_a greater than that of IVa (6.9 vs 4.2, respectively). Thus, at a neutral pH the diacid amide, but not the maleamic acid, possesses significant levels of protonated, and kinetically active, carboxyl groups. (b) As already mentioned, Figure 1 shows our substrate having a nearly ideal geometric disposition between amide and carboxyl; the carboxyl OH resides at van der Waals contact distance above the plane of the amide carbonyl. Such a relationship is more difficult to achieve in the maleamic acid and phthalamic acid systems (IVa and V).

Relief of internal compression during anhydride formation doubtlessly contributes to the observed enzyme-like acceleration in the diacid amide. However, we do not feel that this is the major source of catalysis because nonbonded interactions between two 1,3-diaxial methyls (worth 3.7 kcal/mol or 10^2–10^3 in rate when totally relieved in a transition state)[21] must be *greater* than interactions between two 1,3-diaxial carbonyls. If this were not the case, then the substrate would have its three methyls, as opposed to its three carbonyls, in an all-axial configuration. A factor of $\leq 10^2$–10^3 is small relative to the observed EM of $>10^{14}$ M. Hence, the extremely fast amide cleavage must stem primarily from the sustained proximity at van der Waals contact distances. Energetically costly desolvation processes are thereby alleviated or averted (as we described in detail elsewhere[17]). *Geometry here is critical to the remarkable amide cleavage under conditions typical for biological processes.* Indeed, favorable MM2-derived geometric parameters (Figure 1) prompted the experimental testing of the diacid amide in the first place. Recent claims by Houk[22] that "there is no relationship" between rate and distance are intuitively peculiar and, in the face of experimental results such as in the present article, difficult to take seriously.

Note finally that enzymes need not summon esoteric mechanisms to split amides rapidly. If, for example, an enzyme positioned one of its carboxyls adjacent to an amide substrate with the geometry given in Figure 1, little additional catalytic power would be required.

Acknowledgment. This work was supported by the National Science Foundation. We thank Professor J. Rebek for synthetic procedures for the triacid prior to publication.

Supplementary Material Available: Table of assigned ^1H NMR spectra in D_2O of all compounds mentioned in the text (triacid, anhydride acid, anhydride amide, dicarboxylate amide, anhydride methyl ester, diacid methyl ester, and carboxylate amide methyl ester) (1 page). Ordering information is given on any current masthead page.

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