Received: 20 April 2018 | Accepted: 15 May 2018

DOI: 10.1002/kin.21194

ARTICLE

WILEY

On the hydrolysis mechanisms of amides and peptides

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Abstract

Here the possibility is raised that peptide hydrolysis, in the absence of catalysis by proteases or buffers, may still have a self-catalyzing mechanism that differs from ordinary amide hydrolysis. Second, an attempt is made to clarify the ongoing confusion in the computational chemistry literature regarding the rate-limiting step in ordinary amide hydrolysis. Third, Gibbs activation energies (free-energy barriers) for formamide hydrolysis are derived from rate constants and presented under different concentration conventions, for ease of comparison to values from computational chemistry predictions past and future.

KEYWORDS

amide, formamide, hydrolysis rate law, mechanism, peptide, square root reaction order

1 | INTRODUCTION

Amides (A) are subject to slow hydrolysis ($RCONHR' + H_2O \rightarrow RCOOH + R'NH_2$) at moderate pH conditions at which kineticists apply a rate law with base-, water-, and acid-catalysed terms[1–5]:

$$v_A = -k_{obs}[A], \tag{1}$$

$$k_{obs} = k_{OH}[OH^-] + k'_w + k_H[H^+] \tag{2a}$$

$$= k_{OH}[OH^-] + k_w[H_2O] + k_H[H^+] \tag{2b}$$

Normally the symbol k_w is used for k'_w in Equation 2a, but for free-energy calculation (see Section 4) it is desireable here to have the definition of k_w parallel that of k_{OH} and k_H. The underlying chemical mechanism for each catalyst channel ($X = OH^-$, H_2O, or H^+) is expressed here as a set of three elementary steps:

$$A + X \underset{k^X_{-1}}{\overset{k^X_1}{\rightleftharpoons}} I_X \xrightarrow{k^X_2} P$$

The degree of protonation of the intermediate I_X (always a "tetrahedral" complex) and the products P ("acid + amine") varies with pH. Variations of the second step are needed to explain (i) cases of rate dependence upon buffer concentration (buffer catalysis)[6,7] or (ii) $[OH^-]^2$ dependence for some amides at pH > 11,[8,9] cases not considered here. The mechanism described above generates Equations 1 and 2 at steady-state conditions, with each channel having

$$k_X = k^X_1 k^X_2 / \left(k^X_{-1} + k^X_2\right) \tag{3}$$

For many amides at particular pH values and temperatures, one of the three catalytic channels is dominant, and this has been demonstrated with plots of log k_{obs} versus pH (Figure 1). There are large pH regions for which the hydrolysis is dominated by acid catalysis ($0 < pH < 5$, slope $= -1$) or base catalysis ($6 < pH < 10$, slope $= +1$ or $+1/2$).

2 | DISCUSSION 1: AMIDES VERSUS PEPTIDES

The first point to be raised here is the curious case of slope +1/2 in Figure 1, for hydrolysis of the capped dipeptide PAGVH in the pH range 6-11. This peculiar slope escaped the attention of the original scientists, Smith and Hansen,[3] who instead assumed that this slope was flat and concluded that the hydrolysis was water catalyzed at pH 7. Here it is counterproposed that the slope of +1/2 may be real, and that the underlying mechanism complexity could be due to the acid end of this capped dipeptide. Others have noted that nearby carboxylate groups (intramolecular,[10] buffer,[7,11] or enzymatic[12]) can affect peptide-bond hydrolysis rates. A generic mechanism that could account for a $[OH^-]^{1/2}$ rate dependence is a

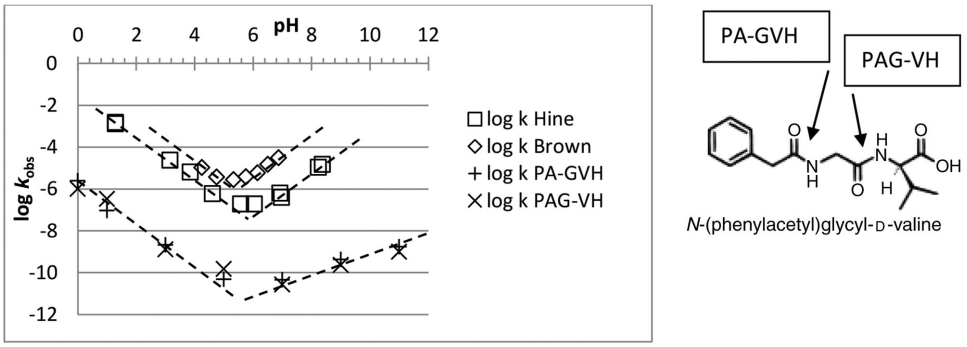

FIGURE 1 Plot of experimental values of log k_{obs} versus pH, showing regions where one mechanism becomes dominant. Diamonds: formamide at 120°C, from Brown et al's Table 4.[4] Squares: formamide at 80°C, from Hine et al's Table II.[2] The PA-GVH and PAG-VH data (+ and x) are for two different amide bonds within the small fragment dipeptide *N*-(phenylacetyl)glycyl-D-valine (shown in the figure), at 37°C, from Smith and Hansen's Table 3.[3] Dashed lines indicate slopes of –1 (low pH), +1/2 (PAGVH, moderate pH), or +1 (formamide, moderate pH)

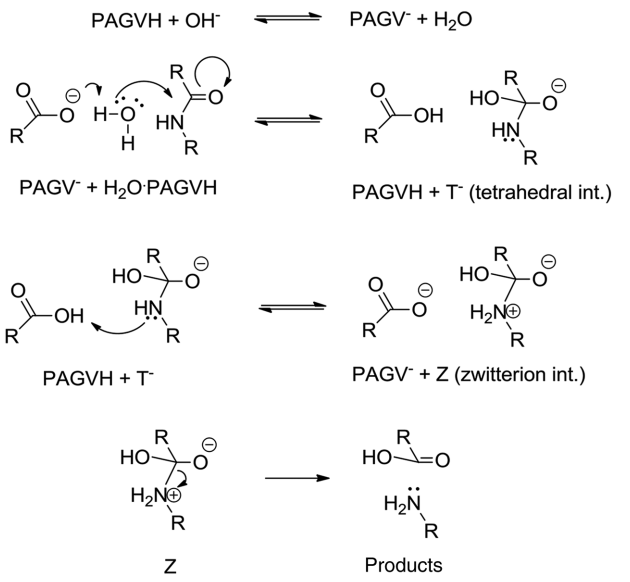

FIGURE 2 Proposed mechanism for the Smith and Hansen[3] hydrolysis of capped dipeptide PAGVH in the pH range 6-11, to explain its $[OH^-]^{1/2}$ rate dependence

preequilibrium of $2A + OH^- \rightleftharpoons C + D$, with a slow $C \rightarrow P$ step: at early times when $[D] \approx [C]$ and C is in a steady state, the rate of production of P is $k_2K^{1/2}$ [A] $[OH^-]^{1/2}$. A more detailed proposal is offered in Figure 2, where D is the deprotonated dipeptide ($PAGV^-$) and C is the zwitterion intermediate Z (brought to light in a valuable quantum-chemical simulation by Zahn[13]). This proposal may have ramifications for the choice of baseline for defining protease efficiency.[14]

3 | DISCUSSION 2: RATE-DETERMINING STEP

In 2009, Khan[5] commented in this journal that some contemporary computational chemistry research papers were mistakenly claiming that, for amide hydrolysis in dilute alkaline conditions (pH 7-11), the first step is "usually" rate determining. Khan pointed out several instances (including formamide) where the empirical hydrolysis rate law has $k_{OH}[A][OH^-]$ and $k_{OH}''[A][OH^-]^2$ terms, and the third-order term almost surely requires that the rate-determining step (RDS) come during or after a second step involving a second hydroxide. The misunderstandings unfortunately continue in the computational chemistry literature (see below), possibly due to RDS dependence upon pH and enzyme catalysts. Further clarity is offered below, in hope of curing ongoing misconceptions.

First, let us summarize emperimental results for amides. For pH range of 6-13, all results of which we are aware are ordinary base-catalyzed second-order hydrolyses, with X = hydroxide only, $k_{obs} = k_{OH}$ $[OH^-]$, and $k_{OH} = k_1k_2/(k_{-1} + k_2)$. By this last equation, the RDS is controlled by the relative magnitudes of k_{-1} and k_2 (ie, the rates of going backward vs forward from the intermediate). Absolute magnitudes of k_{-1} and k_2 are quite difficult to obtain, but the ratio k_{-1}/k_2 (sometimes reported as $k_{exchange}/k_{hydrolysis}$, which is $k_{-1}/2k_2$ in most cases[6]) has been determined for some amides via isotope labeling experiments. For formamide[4] and secondary toluamides[15] (toluamides of secondary amines), Brown et al's k_{-1}/k_2 ratios would be 0.95 and 0.8–1.4 respectively, that is *neither* step is rate determining on its own. For substituted anilides, Bender and Thomas[16] found ratios between 4 and 15, implicating the *second* step (not the first) was rate determining. For amides of tertiary amines, Brown and co-workers first found ratios of 0.02–0.04 (*first* step is rate determining),[15] but later[17] found a ratio of 67 (*second* step is rate determing) by placing a $–CH_2CF_3$ withdrawing group on the N atom. All of these experiments were run near pH 13, in the interests of speed. The *only* case in which the first step was the RDS was for tertiary amides.

At pH 14, however, the situation is different. For Bender and Thomas's anilides,[16] and for formamide (first by Marlier et al,[18] followed up by Brown and co-workers[9]), the second step switched to a faster one catalyzed by a second OH^-

moiety, and the first step became the RDS. For formamide, Marlier et al found k_{back}/k_{fwd} ratios of 0.15–0.25 for pH near 14 (with k_{fwd} dependent on $[OH^-]$).[18]

Hence, to date, for amides, the first step (OH^- association) has been rate limiting only for tertiary amides or amides at pH > 13. Therefore, with regard to the apparently popular case of secondary amides (peptide bonds) at moderate pH, it is likely that the second step (C–N bond dissociation) is contributing to, if not controlling, overall rate.

Second, let us clarify the statements in the computational chemistry literature. The 1974 paper of Guthrie[19] showed free energy plots for tertiary amides at pH 14, showing the first barrier higher than the second. This does not apply to secondary amides at milder pH, as reviewed above. In 1986, Madura and Jorgensen[20] stated, in their introduction to nucleophilic addition to a carbonyl group, that the "formation of the tetrahedral intermediate is normally the rate-determining step," after citing Guthrie[19] and others. This may be true for several additions to carbonyl groups in several conditions, but not for mild-pH secondary-amide hydrolysis; their paper went on to explore hydroxide addition to formaldehyde. In 1999, Bakowies and Kollman,[21] via simulation with approximations, obtained a second barrier higher than a first one for formamide hydrolysis, correctly revealing the importance of the second barrier. A year 2000 paper by Warshel and co-workers[22] studied methanolysis (not hydrolysis) of an amide computationally, attempting to mimic enzymatic hydrolysis, and with approximate modeling found its first step to have a somewhat higher barrier than the second; this result is not terribly relevant to nonenzymatic hydrolysis.

Three papers appeared in 2004 that each confused the RDS issue, starting the problem lamented by Khan.[5] The base-catalyzed study of Zahn[13] via simulation studied all steps of formamide hydrolysis, but the paper misconcluded that the first step is the RDS; his own data, if spliced together for a common intermediate energy, show the second barrier being higher than the first. The studies of Carloni et al[23] (simulation) and Pliego[24] (molecule optimization) studied only the first step, misassuming that it was the RDS: Carloni's introduction cited the works of Bakowies and Kollman,[21] Madura and Jorgensen,[20] and Warshel et al[22] (but gave no particular comment on the RDS) whereas Pliego's introduction regrettably stated that first step "is usually the rate determining" in amide hydrolysis and mentioned Guthrie,[19] Madura and Jorgensen,[20] and other works. Others that were also misled into studying only the first step of base-catalyzed hydrolysis were Klein and co-workers,[25,26] Xiong and Zhan,[27] and Gräter et al,[28] citing many of the above works. Boulatov and co-workers[29] also mistakenly stated the first step to be the RDS; they studied both steps but did not report energies.

A more awkward issue in the computational literature is the occasional use of unactivated H_2O, rather than OH^- or base-activated H_2O, as the attacking species X. While not a good model for amide hydrolysis, it may have relevance if trying to mimic certain enzyme-catalyzed hydrolyses of peptides. In 2005, Gorb et al[30] simulated formamide hydrolysis via unassisted H_2O attack, referencing the capped-peptide experimental paper of Smith and Hansen,[3] and similar work by Radzicka and Wolfenden.[31,32] In 2011, Trout et al[33] followed suit, simulating *N*-methylacetamide hydrolysis at neutral pH via unassisted H_2O attack, and mentioning Smith and Hansen.[3] In 2013, Makshakova and Ermakova,[34] in a molecule optimization paper, studied uncapped-dipeptide hydrolysis via "assisted" H_2O attack, citing Gorb et al.[30] Enzyme mechanisms aside, the reader is reminded that the idea of unassisted H_2O attack in the Smith/Hansen experiment is gently questioned (Discussion 1, vide supra).

In contrast, two other computational chemistry papers reveal better understanding of the amide RDS issue. A 2009 molecule-optimization paper by Galabov and co-workers[35] cited "conflicting reports regarding the RDS," studied base-catalyzed hydrolysis of three amides, and found the second energy barrier to be higher than the first. Also, a 2013 molecule-optimization paper by Yamabe et al[36] noted the varying RDS in Brown et al's toluamide data,[15] and thus studied all steps in base-catalyzed hydrolysis of *N*-ethylbenzamide.

To summarize, hydrolysis of secondary amide at moderate pH (6 < pH < 13), in the absence of enzymes or buffers, is likely occurring by traditional hydroxide attack, with a second step (C–N bond rupture) that contributes to, if not controls, the rate of hydrolysis. For singly capped or uncapped peptides (eg, Smith and Hansen[3]) there may be complications.

4 | DISCUSSION 3: FREE ENERGY BARRIERS, FORMAMIDE

Crude overall free-energy barriers for each catalyst channel (X = OH^-, H_2O, or H^+) are here derived from previously measured k_X values,[4] using the rearrangment of the Eyring equation $k_X \approx (k_B T/h) Q_X^{TS} e^{-\Delta G^{TS}/RT}$:

$$\Delta G_X^{TS} \approx RT \ln\left(\frac{k_B T}{k_X h} Q_X^{TS}\right), \quad Q_X^{TS} = \frac{[\mathrm{TS}]}{[\mathrm{amide}][\mathrm{X}]}. \quad (4)$$

Here Q is the ratio of standard-state concentrations assumed for the ΔG^{TS} value; k_X is independent of choice of standard-state concentrations. Table 1 lists such derived ΔG^{TS} values for the case of formamide at 56°C, for three different sets of concentration choices. This table may serve two useful purposes, described below.

The values under the 1 M convention ("conventional," sixth column) may be the most appropriate for comparison to values from quantum chemistry computations, which often consider the same concentration of two reactants (often just

TABLE 1 Free-energy barriers for kinetics of solvolysis of formamide, moderate pH, 56°C[a]

X	k_X [Ref. 4]	[X] (conventional)	[X] (pH 5)	[X] (pH 7)	ΔG_X^{TS} (conventional)[b]	ΔG_X^{TS} (pH 5)[b]	ΔG_X^{TS} (pH 7)[b]
H^+ (acid catalysis)	3.03×10^{-3}	1	1.00×10^{-5}	1.00×10^{-7}	23.1	**30.7**	33.7
H_2O	6.50×10^{-11}[c]	1	5.54×10^{1}	5.54×10^{1}	34.7	32.0	32.0
OH^- (base catalysis)	3.20×10^{-2}	1	7.94×10^{-9}[d]	7.94×10^{-7}[d]	21.6	33.8	**30.8**

[a]Units: $M^{-1}\ s^{-1}$ for k_X, M for [X]; kcal mol^{-1} for ΔG.
[b]Denotes choice of concentrations for Q in Equation 4: [TS] = [amide] = 1 M but [X] from three columns previous.
[c]Derived from expt. k'_w ($3.60 \times 10^{-9}\ s^{-1}$) by dividing by $[H_2O] = 55.4$ M.
[d]Derived from $[H^+][OH^-] = 10^{-13.1}$ at this elevated temperature.[4,5]

one molecule of each). Consider the value 21.6 kcal mol^{-1} from this convention for X=OH^-. Bakowies and Kollman[21] from simulations for formamide obtained 27 kcal mol^{-1}, and he mentions that their prediction would be somewhat larger had they accounted for the work done to confine the two reactant molecules for collision in their algorithm. Also noteworthy is the disagreement of two other computed values for a different amide (*N*-methylacetamide): Galabov et al[35] obtained 33 from molecule optimization, a poor value they attributed to gas-phase entropy calculation, whereas Zahn's simulation data[13] would give (52 + 72 – 28)/4.184 = 23. Improvements in computational predictions are anticipated.

The ΔG^{TS} values from the 1 M convention are not in themselves appropriate for comparing relative weights of each catalyst channel, because they omit concentration effects; in particular, the high intrinsic energy barrier (34.7 kcal mol^{-1}) for the H_2O-catalyzed channel is strongly counterbalanced by the far greater frequency of collisions with H_2O molecules than with hydronium or hydroxide ions. To compare relative channel weights at a particular pH, one would normally use the k_X value to compute channel rate: $v_x = k_X$ [A][X]. At pH 7, using column 2 for k_X, column 5 for [X], and [A] = 1 M, one finds $\{v_H, v_{H_2O}, v_{OH}\}$ to be $\{3 \times 10^{-10}, 4 \times 10^{-9}, 3 \times 10^{-8}\}$ M s^{-1}; the biggest contributor to v_{total} is thus v_{OH} (base catalyzed) in this case (formamide, pH 7, 56°C). However, a different way to compare relative channel weights is to absorb the concentration effect into ΔG^{TS} by changing the standard-state convention, that is use Equation 4 but insert actual concentrations (instead of 1 M) into the standard-state ratio Q. Table 1 does this for pH 5 and pH 7 (last two columns). Now the ΔG^{TS} values for each channel X are appropriate for the actual concentrations present and can be directly compared without needing to compute rate. These correctly demonstrate the known (eg, Figure 1) dominance of the acid-catalysis channel at pH 5 and the base-catalysis channel at pH 7. These free energy values may have relevance for comparison to free energy values from simulations.

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How to cite this article: East ALL. On the hydrolysis mechanisms of amides and peptides. *Int J Chem Kinet*. 2018;50:705–709. https://doi.org/10.1002/kin.21194