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The Nonenzymatic Decomposition of Guanidines and Amidines

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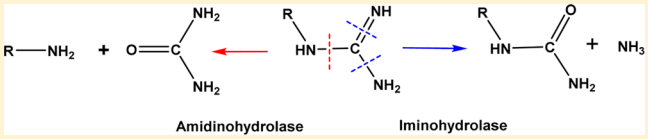
Supporting Information

ABSTRACT: To establish the rates and mechanisms of decomposition of guanidine and amidine derivatives in aqueous solution and the rate enhancements produced by the corresponding enzymes, we examined their rates of reaction at elevated temperatures and used the Arrhenius equation to extrapolate the results to room temperature. The similar reactivities of methylguanidine and 1,1,3,3-tetramethylguanidine and their negative entropies of activation imply that their decomposition proceeds by hydrolysis rather than elimination. The influence of changing pH on the rate of decomposition is consistent with attack by hydroxide ion on the methylguanidinium ion ($k_2 = 5 \times 10^{-6}$ M^{-1} s^{-1} at 25 °C) or with the kinetically equivalent attack by water on uncharged methylguanidine. At 25 °C and pH 7, *N*-methylguanidine is several orders of magnitude more stable than acetamidine, urea, or acetamide. Under the same conditions, the enzymes arginase and agmatinase accelerate substrate hydrolysis 4×10^{14}-fold and 6×10^{12}-fold, respectively, by mechanisms that appear to involve metal-mediated water attack. Arginine deiminase accelerates substrate hydrolysis 6×10^{12}-fold by a mechanism that (in contrast to the mechanisms employed by arginase and agmatinase) is believed to involve attack by an active-site cysteine residue.



INTRODUCTION

To evaluate the power of an enzyme as a catalyst and its potential sensitivity to inhibition by transition state analogues, it is necessary to establish the rate constant of the reaction in water in the absence of a catalyst. That information is available for the cleavage of amides, peptides,[1] and urea[2] but not for the cleavage of guanidines, another group of C−N derivatives that is widely distributed in nature.

For guanidines with the general formula RHN−C(=NH)−NH_2, the products of enzymatic decomposition may include (a) urea and an amine or (b) a substituted urea and ammonia (Scheme 1A). Enzymes of the first type, which are classified as amidinohydrolases, include arginase (EC 3.5.3.1), creatinase (EC 3.5.3.3), and agmatinase (EC 3.5.3.11). Enzymes of the second type are termed iminohydrolases and include arginine deiminase (EC 3.5.3.6), agmatine deiminase (EC 3.5.3.12), dimethylarginase (EC 3.5.3.18), and peptidyl/protein-arginine deiminases (or "PADs") (EC 3.5.3.15), which participate in the post-translational modification of histones.[3,4]

In the work described here, we set out to determine the reactivities of guanidines and amidines in neutral aqueous solution in the absence of a catalyst. Because the spontaneous decomposition of these molecules is very slow at ordinary temperatures, we examined their rates of decomposition at elevated temperatures and used the Arrhenius relationship to estimate their rate constants for decomposition at ordinary temperatures.

In principle, these reactions might proceed by nucleophilic attack at the central carbon atom or by elimination to yield a carbodiimide as the immediate product (Scheme 1B). We sought to obtain an indication of the actual mechanism from the thermodynamics of activation by comparing the behavior of methylguanidine with that of 1,1,3,3-tetramethylguanidine, in which methyl substitution precludes elimination. For comparative purposes, we conducted similar experiments on pivalamidine (2,2,2-trimethylacetamidine) and pivalamide (2,2,2-trimethylacetamide). The results permit quantitative comparison of the rates and mechanisms of decomposition of guanidines, amidines, amides, and urea in neutral solution and the extent to which these reactions are enhanced by hydrolytic enzymes.[5]

Scheme 1. Possible Sites (A) and Mechanisms (B) of Guanidine Cleavage

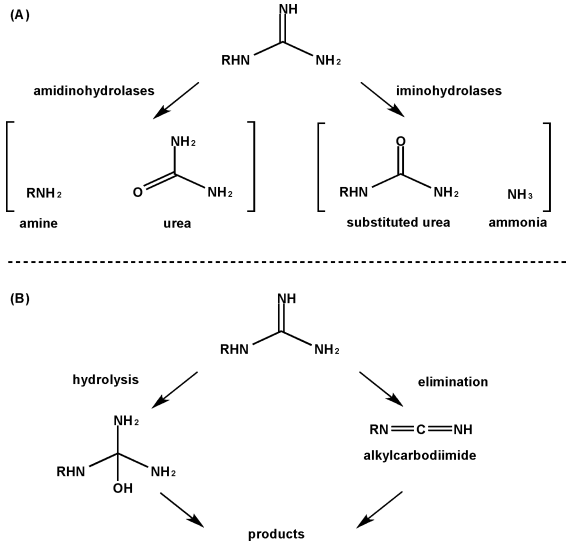


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Table 1. Kinetic and Thermodynamic Constants for the Decomposition of Guanidines, Amidines, Ureas, and Amides (Observed Values Are Not Italicized, While Calculated Values Are Italicized)

	pK_a	k_{25} (s^{-1})	$\Delta H^{\ddagger}$ (kcal/mol)	$T\Delta S^{\ddagger}$ (kcal/mol)
		Methylguanidines		
MG (pH 7)		5.3×10^{-13}	34.7	0.6
MG (uncharged)	*13.4*	*1.1×10^{-6}*	*20.1*	*−5.3*
M4G (pH 7)		9.2×10^{-12}	33.1	0.7
M4G (uncharged)	*12.8*	*1.3×10^{-6}*	*14.7*	*−9.8*
		Acetamidines		
acetamidine (pH 7)		4.3×10^{-9}	28.6	−0.2
acetamidine (uncharged)	*12.5*	*1.4×10^{-3}*	*13.2*	*−8.0*
pivalamidine (pH 7)		1.1×10^{-9}	28.6	−1.0
pivalamidine (uncharged)	*12.5*	*3.7×10^{-4}*	*13.2*	*−8.9*
		Ureas		
urea		2.3×10^{-13}	29.7	−4.9
M4U[6]		4.2×10^{-12}	22.0	−10.6
		Acetamides		
acetamide		7.0×10^{-11}	23.0	−8.2
pivalamide		5.4×10^{-12}	25.7	−7.0

MATERIALS AND METHODS

1,1-Dimethylurea, *N*-methylguanidine hydrochloride, tetramethylurea, and urea were purchased from ACROS Organics. Pivalamide (2,2,2-trimethylacetamide), pivalamidine (2,2,2-trimethylacetamidine) hydrochloride, and 1,1,3,3-tetramethylguanidine were purchased from TCI America. Guanidine hydrochloride and acetamidine hydrochloride were purchased from Spectrum Chemical Manufacturing Corp.

Kinetic experiments were conducted using solutions of guanidines, amidines, and amides (0.02 M) in potassium acetate, phosphate, borate, and carbonate buffers (0.1 M), as indicated in the Results. Solutions were flushed with argon, sealed under vacuum in quartz tubes, heated for various time intervals in Thermolyne 47900 and 48000 furnaces, and cooled with ice. Samples of the product mixtures (0.025 mL) were transferred to 5 mm NMR tubes and diluted with D_2O (0.5 mL) containing added pyrazine to serve as an internal reference for calibrating chemical shifts (pyrazine = 8.60 ppm) and integrated signal intensities. ^{1}H NMR spectra were acquired using a Bruker AVANCE 500 MHz spectrometer equipped with a cryoprobe, usually with four transients. At each temperature, triplicate samples were heated long enough to allow the reaction to proceed to between 10% and 90% completion. The integrated intensities of the reactants and products were used to calculate their percentages in each product mixture. The chemical shifts observed for the reactants and products are shown in Table S1 in the Supporting Information.

In each of these reactions, the disappearance of starting material followed first-order kinetics to at least 90% completion, and the results were used to calculate the rate constant. The logarithms of the observed rate constants were then plotted as a function of the reciprocal of absolute temperature. Linear regression was used to obtain the slope and intercept of the resulting Arrhenius plot and the associated correlation coefficient. To determine the pH dependence of the decomposition of methylguanidine, initial rates were determined at 140 °C in potassium acetate-d_3, potassium phosphate, sodium borate, and sodium carbonate buffers (0.1 M) after the pH of each reaction mixture was determined at 25 °C with a glass electrode. The corresponding pH values at 140 °C were calculated from the values determined at 25 °C on the basis of the known heats of ionization of these buffers (see the Results and the Supporting Information).

RESULTS

Rate Constants and Thermodynamics of Activation for Decomposition of Methylguanidine, Acetamidine, Acetamide, and Their Methyl-Substituted Derivatives at pH 7. At early stages in the decomposition of methylguanidine (MG) (0.02 M) in potassium phosphate buffer (0.1 M, pH 7.0) over the temperature range from 100 to 200 °C, traces of *N*-methylurea (<2%) made a transitory appearance and then disappeared, consistent with the somewhat more rapid decomposition of ureas by elimination under these conditions (Table 1 and Figure S4 in the Supporting Information). An Arrhenius plot of the rate constants observed for methylguanidine decomposition was linear (Figure 1), and extrapolation to 25 °C yielded an estimated rate constant of k_{25} = (5.3 ± 0.5) × 10^{-13} s^{-1} at pH 7 with $\Delta H^{\ddagger}$ = 34.7 ± 1.0 kcal/mol (R^2 = 0.997). In these experiments, as in the others described below, the coefficients of variation of the logarithms of the rate constants at each temperature were less than 5%.

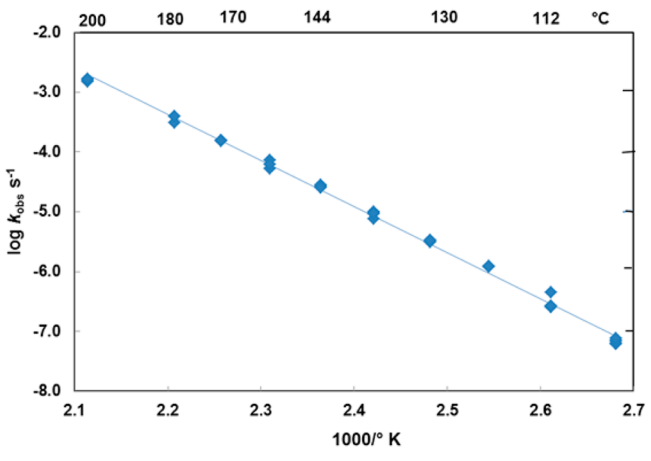

Figure 1. Rate constants for decomposition of methylguanidine (0.02 M) in potassium phosphate buffer (0.1 M, pH 7) plotted logarithmically as a function of the reciprocal of absolute temperature over the range from 100 to 200 °C. Linear regression yielded (5.3 ± 0.5) × 10^{-13} s^{-1} and $\Delta H^{\ddagger}$ = 34.7 ± 1.0 kcal/mol (R^2 = 0.997).

Under the same conditions, 1,1,3,3-tetramethylguanidine (M4G) decomposed to give mainly dimethylamine along with a small amount (<10%) of 1,1,3,3-tetramethylurea. The resulting rate constants yielded a linear Arrhenius plot over the temperature range from 100 to 180 °C with k_{25} = (9.2 ± 1.0) × 10^{-12} s^{-1} and $\Delta H^{\ddagger}$ = 33.1 ± 1.0 kcal/mol (R^2 = 0.994) (Figure S1 in the Supporting Information).

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Acetamidine under the same conditions decomposed several orders of magnitude more rapidly than methylguanidine, yielding acetamide, which then decomposed more slowly to acetate (Figure 2). Rate constants for the disappearance of

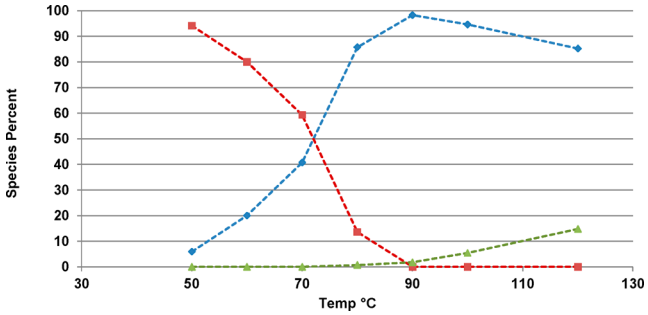


Figure 2. Disappearance of acetamidine (red) and formation of acetamide (blue) and finally acetate (green) after heating for 70 h from 50 to 120 °C in potassium phosphate buffer (0.1 M, pH 7).

acetamidine, monitored from 50 to 100 °C, yielded a linear Arrhenius plot and an extrapolated rate constant of 4.3×10^{-9} s^{-1} at 25 °C with $\Delta H^{\ddagger}$ = 28.6 kcal/mol (R^2 = 0.991). Similar experiments yielded a linear Arrhenius plot for acetamide decomposition over the temperature range from 80 to 180 °C with an extrapolated rate constant of 7.0×10^{-11} s^{-1} at 25 °C and $\Delta H^{\ddagger}$ = 28.6 kcal/mol (R^2 = 0.986) (Figure S2 in the Supporting Information)

Under the same conditions, pivalamidine (2,2,2-trimethylacetamidine) decomposed to pivalamide over the temperature range from 70 to 130 °C. The Arrhenius plot, which was linear, yielded k_{25} = 1.1×10^{-9} s^{-1} at 25 °C (R^2 = 0.994). The decomposition of pivalamide to pivalate, observed over the temperature range from 120 to 200 °C, yielded a linear Arrhenius plot and an extrapolated rate constant at 25 °C of 5.4×10^{-12} s^{-1} at 25 °C (Figure S3 in the Supporting Information).

Influence of pH on the Decomposition of Methylguanidine at 140 °C. The logarithm of the rate constant observed for the decomposition of methylguanidine was found to increase linearly over the range from pH 3.6 to pH 10 at 140 °C based on the pH values measured at 25 °C (blue data in Figure 3). After those pH values had been corrected to 140 °C using the heats of ionization of the conjugate acids of the corresponding buffers (see the Supporting Information), these results yielded a line with a steeper slope (red data in Figure 3).

The values shown in blue are based on buffer pH values measured at 25 °C and are related by the line $\log_{10}(k/s^{-1})$ = 0.69(pH) − 9.85 with R^2 = 0.99. The values shown in red represent the same results after the pH values measured at 25 °C were corrected by taking into account the heats of ionization of the buffer acids as described in the Supporting Information. The equation for the red line is $\log_{10}(k/s^{-1})$ = 0.81(pH) −10.39 with R^2 = 0.99. Figure S5 in the Supporting Information shows the equations and R^2 values for rate constants observed for each buffer system.

DISCUSSION

Methylguanidine Decomposition: Elimination or Hydrolysis? In earlier work, urea was shown to decompose mainly by elimination rather than hydrolysis.[2] In principle, methylguanidine might decompose by either elimination (A or B in Scheme 2) or hydrolysis (C or D in Scheme 2). In an

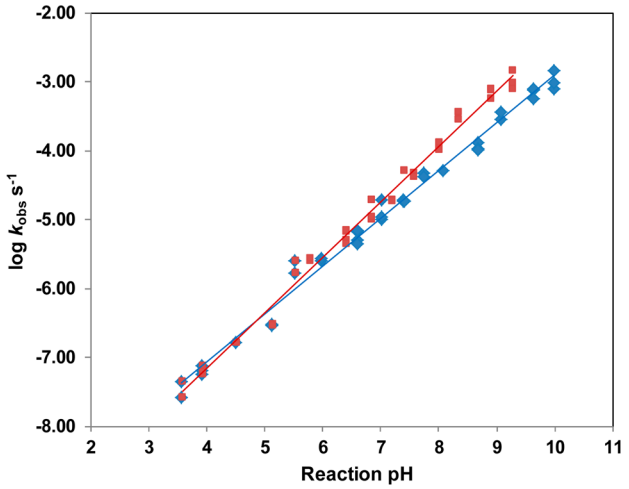
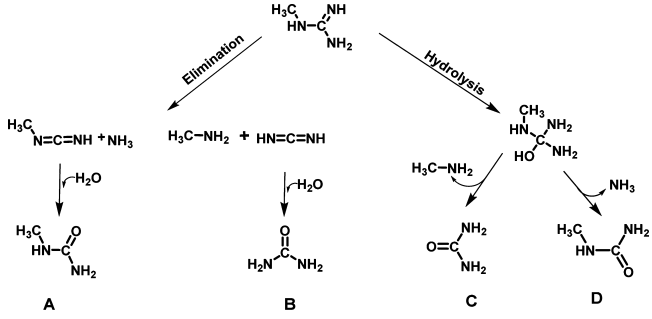


Figure 3. Observed logarithm of the rate constant for methylguanidine decomposition at 140 °C plotted as a function of pH. The 0.1 M buffers used in this experiment were 0.1 M potassium acetate-d_3, potassium phosphate, sodium borate, and sodium carbonate.

Scheme 2. Alternative Pathways of Decomposition of Methylguanidine



effort to distinguish between these alternatives, we examined the thermodynamics of activation for this reaction and the effects of replacing the exchangeable hydrogen atoms with methyl groups.

The negative entropy of activation observed for the decomposition of methylguanidine ($T\Delta S^{\ddagger}$ = −5.3 kcal/mol) falls toward the lower end of a range of values that have been reported for conventional hydrolytic processes (−4 to −11 kcal/mol).[6] However, its value is considerably more negative than the near-zero or positive values that have been reported for most elimination reactions (e.g., $T\Delta S^{\ddagger}$ = +2.0 kcal/mol for the elimination of ammonia from urea).[2]

A further indication of the probable mechanism arises from the observed effects of methyl substitution. When the exchangeable protons of methylguanidine were replaced by methyl groups in 1,1,3,3-tetramethylguanidine, the enthalpies and entropies of activation for decomposition were very similar to those observed for methylguanidine (Scheme 3 and Table 1). Since elimination is precluded in the case of 1,1,3,3-tetramethylguanidine, that similarity suggests once again that both methylguanidine and 1,1,3,3-tetramethylguanidine decompose by hydrolysis rather than elimination.

Depending on which C−N bond is cleaved, hydrolysis of 1,1,3,3-tetramethylguanidine would be expected to yield one of two alternative sets of reaction products: tetramethylurea + NH_3 (route 1 in Scheme 4) or 1,1-dimethylurea + dimethylamine (route 2 in Scheme 4). Because the two dimethylamine

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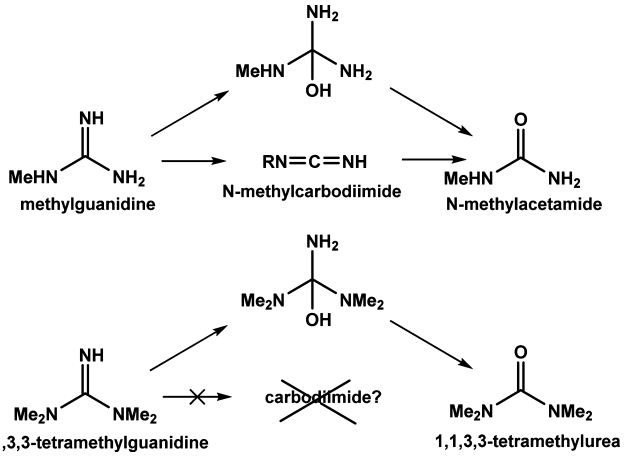
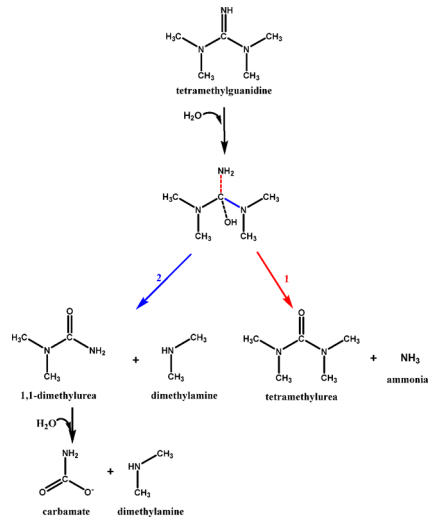
Scheme 3. Effect of Methyl Substitution on Guanidine Decomposition



Scheme 4. Alternative Pathways of Hydrolysis of 1,1,3,3-Tetramethylguanidine

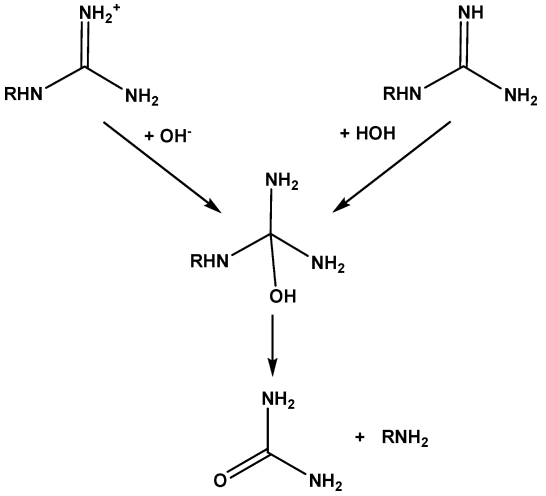


moieties are equally likely be removed in route 2, that route would be expected to be preferred over route 1 by a factor of 2 for statistical reasons alone. In fact, dimethylamine was generated as the major product (~80%) along with a minor fraction (10%) of tetramethylurea and ammonia. 1,1-Dimethylurea decomposes relatively rapidly to dimethylamine and carbamate under the conditions of these experiments (Figure S4 in the Supporting Information) and was therefore not expected to accumulate.

Influence of pH on Guanidine Hydrolysis. Of the ionizable groups that are present in biological molecules, guanidine derivatives are distinguished by the high pK_a values of their conjugate acids (e.g., 13.6 for unsubstituted guanidinium ion). However, because the heats of ionization of guanidinium ions are much larger than those of other functional groups in biological molecules (e.g., 18.2 kcal/mol for guanidinium ion[7] compared with 13.2 kcal/mol for methylammonium ion and 8.8 kcal/mol for imidazolium ion), their pK_a values are extremely sensitive to temperature (e.g., pK_a for unsubstituted guanidinium ion decreases from 13.6 at 25 °C to 9.7 at 140 °C). Accordingly, the fraction of methylguanidine that is present in the reactive uncharged form at pH 7 is 1.9×10^{-3} at 140 °C, which is much larger than the fraction that is present in uncharged form at 25 °C (4.0×10^{-7}).

When the decomposition of methylguanidine was examined at 140 °C at various pH values, the logarithm of the rate constant was found to increase linearly with increasing pH over the pH range from 3.6 to 10 with a slope of 0.81 after correction for the effect of temperature on the buffer pK_a values (Figure 3). In view of the approximations involved in estimating pH values at 140 °C from values measured at 25 °C based on heats of buffer ionization, it seems reasonable to suppose that this slope is actually unity.[8] That behavior would be consistent with OH^- attack on methylguanidinium ion, or with the kinetically indistinguishable decomposition of uncharged methylguanidine, via the same tetrahedral intermediate as shown in Scheme 5. The second-order rate constant for the

Scheme 5. Decomposition of Methylguanidine



reaction of OH^- with methylguanidinium ion is 5.3×10^{-6} M^{-1} s^{-1} at 25 °C, whereas the first-order rate constant for decomposition of the uncharged species is 1.1×10^{-6} s^{-1} (see the Supporting Information for calculations).

Mechanisms of Decomposition of Acetamidine and Acetamide. In neutral solution at temperatures between 50 and 120°, acetamidine was found to decompose rapidly to acetamide, which then underwent relatively slow hydrolysis (Figure 2). Table 1 shows that pivalamidine, in which elimination is precluded by methylation, decomposes with a heat and entropy of activation very similar to those of acetamidine. That behavior suggests that acetamidines—like guanidines but unlike ureas—decompose mainly by hydrolysis rather than elimination. Scheme 6 illustrates the blocking of elimination reactions in amidines and amides. Likewise, the rate and temperature dependence of the decomposition of pivalamide are similar to those of acetamide (Table 1). That accords with the generally accepted view that amides decompose in water by hydrolysis rather than elimination, as implied by the results of the ^{18}O exchange experiments on benzamide by Bender and Ginger.[9]

Effects of Neutral versus Charged Species. Figure 4 compares the rates of decomposition of amides, amidines, guanidines, and urea in dilute aqueous solution at 25 °C. In the case of amidines and guanidines, rate constants are shown for the uncharged species of the substrate and also for the substrate in all forms that are present at pH 7 (for details, see the

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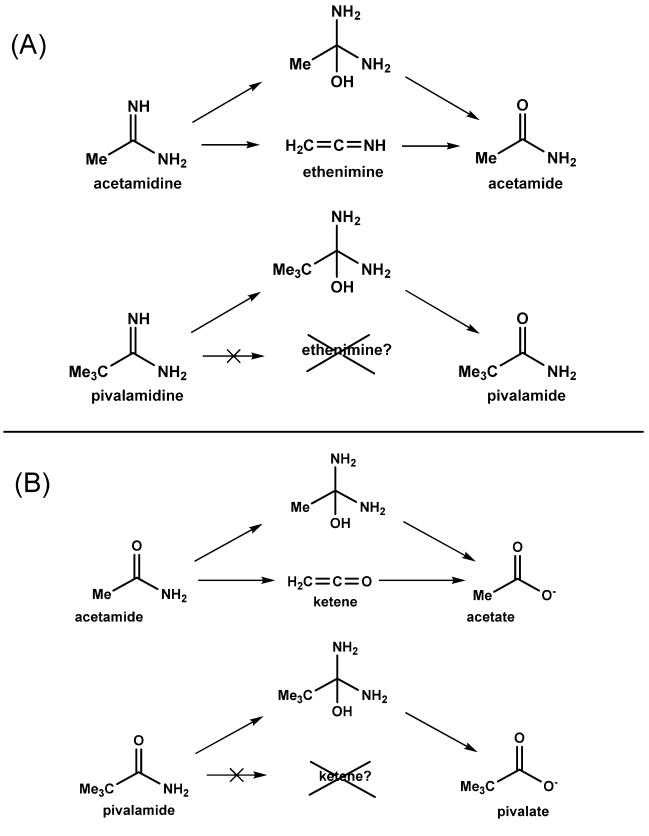
Scheme 6. Effects of Methyl Substitution on the Decomposition of Acetamidine (A) and Acetamide (B)



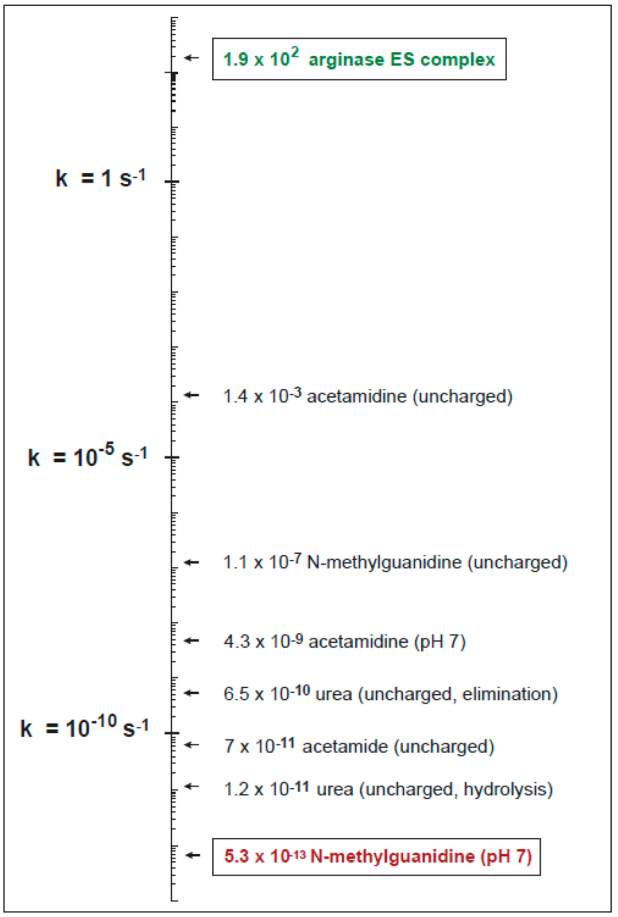


Figure 4. Scale of rate constants for C–N cleavage.

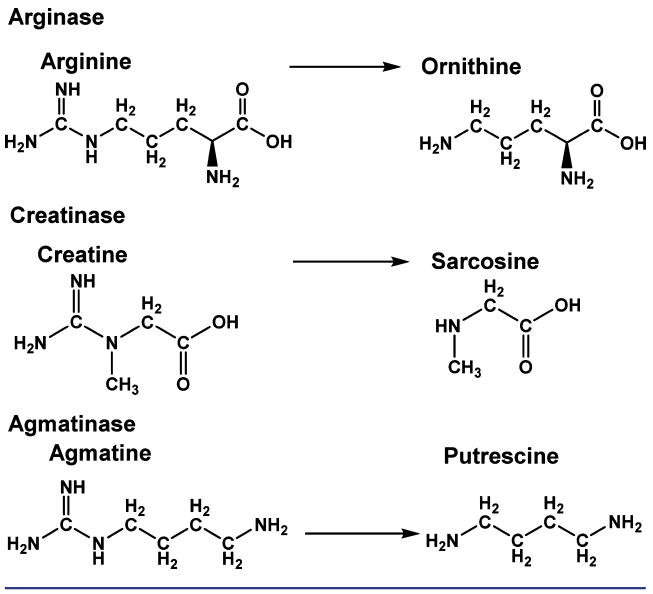
Supporting Information). For both methylguanidine and acetamidine, protonation is seen to reduce the rate of hydrolysis by more than 5 orders of magnitude.

Comparisons with Enzymatic Reactions. Schemes 7 and 8 summarize the variety of reactions that are known to be catalyzed by amidinohydrolases and iminohydrolases. Notably, a single substrate, arginine, decomposes in the presence of different enzymes to yield different products (ornithine and citrulline, respectively).

In principle, these reactions might by proceed by direct water attack or a double displacement mechanism. Amidinohydrolases (Scheme 7) are believed to catalyze direct water attack on their substrates. Thus, arginase and agmatinase use two Mn^{2+} ions to activate a water molecule for direct attack on the guanidino group,[10,11] whereas creatinase uses an active-site histidine to activate the attacking water molecule.[12] In that respect, they resemble the mechanisms employed by cytidine deaminase[13] and the carboxypeptidases,[14] which are also metalloenzymes. In such cases, the enzyme's formal affinity for the altered substrate in the transition state is given by the reciprocal of $(k_{cat}/K_m)/k_{non}$.[6] For human arginase, the strongest known reversible inhibitor, 2-amino-6-boronohexanoic acid (ABH), has been shown to displace a water molecule from its position between the two Mn^{2+} atoms.[10,11] The reported K_i value of ABH for human arginase (5×10^{-9} M)[15] is much larger than the value expected for an ideal transition state analogue [5×10^{-18} M, the reciprocal of the catalytic proficiency $(k_{cat}/K_m)/k_{non}$ in Table 2], suggesting that there is considerable room for improvement.

In contrast with the enzymes mentioned above, the iminohydrolases (Scheme 8), which include arginine deiminase,[16] agmatine deiminase,[17] dimethylarginine dimethylaminohydrolase (DDAH),[18] and protein-arginine deiminase (PAD)[19] are believed to act by double displacement mechanisms. The crystal structures of arginine deiminase[20] and agmatine deiminase are consistent with the intervention of a reaction intermediate in which ammonia or dimethylamine is displaced by the thiol group of an active-site cysteine residue to

Scheme 7. Amidinohydrolases: Water Attack



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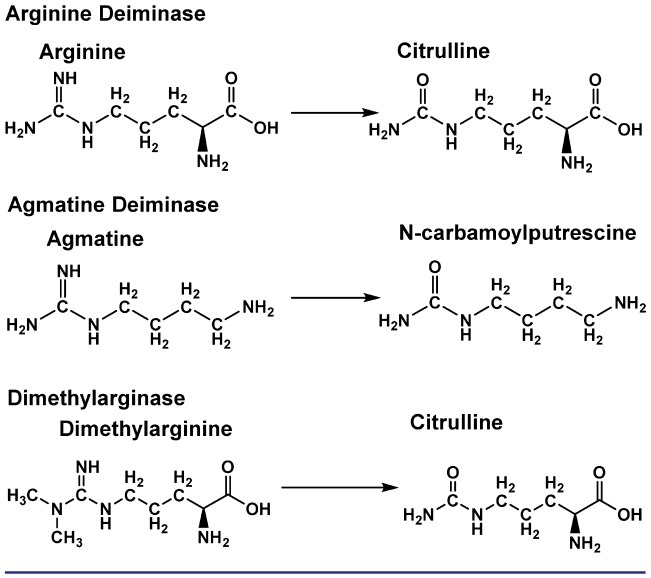
Scheme 8. Iminohydrolases: Double Displacement



form a covalently bound intermediate, which then undergoes hydrolysis.[21] In the hands of Dunaway-Mariano and her associates,[22] a detailed mechanism for the action of arginine deiminase has emerged from experiments involving the mutation of residues in the enzyme's catalytic core, which showed that an aspartate residue and a histidine residue participate as general acid/base catalysts in the generation and breakdown of an *S*-alkyluronium ion intermediate. Moreover, agmatine deiminase and PAD share a common backbone fold and catalytic scaffold with arginine deiminase, consistent with a common mechanism for all three enzymes.

In double displacement reactions involving the formation of a covalently bound intermediate, it is not possible to estimate transition state affinity in the usual sense. However, as pointed out by Lienhard,[23] it is possible to estimate an equilibrium constant for "transition state transfer" by comparing the value of k_{cat}/K_m for the enzyme reaction with the second-order rate constant for reaction with a model nucleophile resembling the nucleophilic residue at the enzyme's active site.

Table 2 compares the rates of the present guanidine-cleaving reactions at pH 7 (k_{non}) with the k_{cat} and k_{cat}/K_m values that have been reported for their enzyme-catalyzed reaction counterparts. Arginase, agmatinase, and arginine deiminase are comparable in catalytic proficiency $[(k_{cat}/K_m)/k_{non}]$ with other C−N-cleaving enzymes that include urease, cytidine deaminase, and also carboxypeptidase B, the most proficient proteolytic enzyme for which information is available.

CONCLUSION

The decomposition of guanidines, amidines, and amides in water proceeds by hydrolysis rather than elimination. The influence of changing pH on the rate of methylguanidine hydrolysis is consistent with hydroxide attack on the protonated species or the kinetically indistinguishable hydrolysis of uncharged methylguanidine. Under physiological conditions, guanidines are hydrolyzed 2 orders of magnitude more slowly than amides and peptides and 4 orders of magnitude more slowly than amidines. The rate enhancements produced by enzymes that catalyze these reactions range from 3×10^{10}-fold to 4×10^{14}-fold.

ASSOCIATED CONTENT

Supporting Information

Proton chemical shifts of reactants and products; Arrhenius plots for 1,1,3,3-tetramethylguanidine, acetamidine, acetamide, pivalamidine, pivalamide, and 1,1-dimethylurea decompositions; calculations of the rate constants and thermodynamics of activation for uncharged guanidines and acetamdines; and the detailed pH dependence plot for methylguanidine decomposition. This material is available free of charge via the Internet at http://pubs.acs.org.

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C.A.L. and R.W. contributed equally to the preparation of the paper.

Notes

The authors declare no competing financial interest.

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Table 2. Rate Enhancements by C−N-Cleaving Enzymes in Neutral Solution

enzyme	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{non} (s^{-1})	rate enhancement k_{cat}/k_{non}	catalytic proficiency $(k_{cat}/K_M)/k_{non}$ (M^{-1})
Noncovalent Mechanisms					
arginase[a]	100	1.03×10^5	5.3×10^{-13}	1.9×10^{14}	2×10^{17}
agmatinase[b]	3	2.1×10^3	5.3×10^{-13}	5.7×10^{12}	4×10^{15}
creatinase[c]	25	1.9×10^1	5.3×10^{-13}	4.7×10^{13}	4×10^{13}
cytidine deaminase[d]	300	2.7×10^6	2.7×10^{-10}	1.1×10^{12}	1×10^{16}
carboxypeptidase B[e]	240	6.0×10^6	1.8×10^{-11}	1.3×10^{13}	3×10^{17}
Covalent Mechanisms					
dimethylargininase[f]	0.014	1.3×10^2	5.3×10^{-13}	2.6×10^{10}	3×10^{14}
arginine deiminase[g]	3.2	1.0×10^4	5.3×10^{-13}	6.0×10^{12}	2×10^{16}
agmatine deiminase[h]	0.082	2.5×10^3	5.3×10^{-13}	1.5×10^{11}	5×10^{15}
protein-arginine deiminase (PAD 1)[i]	0.45	4.1×10^3	5.3×10^{-13}	8.5×10^{11}	8×10^{15}

[a]Data from ref 24. [b]Data from ref 25. [c]Data from ref 26. [d]Data from ref 27. [e]Data from ref 28. [f]Data from ref 29. [g]Data from ref 30. [h]Data from ref 31. [i]Data from ref 32.

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