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Metal-catalyzed hydroxylaminolysis of unactivated amide and peptide bonds

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Kinetics of the hydroxylaminolysis of acetamide, glycinamide, glycylglycine and triglycine have been studied in the range of temperatures 37–60 °C as a function of pH and hydroxylamine concentration. Rate constants for specific acid, general-acid and general-base catalyzed pathways have been determined for all substrates (for glycine derivatives rate constants for different protonation forms were obtained). Testing different metal ions as possible reaction catalysts revealed a significant catalytic effect of Zn(II) on the hydroxylaminolysis of glycine substrates, but not acetamide. On the basis of the kinetic results, a mechanism of Zn(II) catalysis is proposed, which involves the coordination of the metal ion to the α-amino group of the substrate and the base-assisted nucleophilic attack of hydroxylamine on the bound substrate. The product analysis by proton NMR shows that the primary reaction product in the catalytic reaction is glycine hydroxamic acid, which undergoes further Zn(II)-catalyzed hydrolysis to glycine. Thus the final result of the Zn(II)-catalyzed treatment of peptides by hydroxylamine is hydrolytic cleavage.

Introduction

Reagents, which can afford the cleavage of peptides and proteins under mild conditions find important applications in biochemistry.[1] Much attention has been paid to the catalytic peptide hydrolysis in this connection.[2] The uncatalyzed peptide hydrolysis is a very slow reaction: estimated half-lives for short peptides range from 7 to 600 years at pH 7 and 25 °C.[3] In earlier studies cupric ions were found to catalyze the hydrolysis of glycinamide (GNH_2)† and dipeptides; lower activities were reported also for Ni(II), Co(II) and Zn(II).[4] A much higher reactivity was observed for the cleavage of peptides coordinated to Co(III) tetraamino complexes [5] and this reaction was used for the determination of *N*-terminal amino acids of peptides and proteins.[5b] Recently a macrocyclic Cu(II) complex,[6] a series of Pd(II) aquo complexes,[7,2f] and the Ce(IV) hydroxide gel,[8,2d] as well as the peptide-attached iron(II) complexes in a combination with H_2O_2 and ascorbic acid [9,2e] were proposed for the efficient peptide hydrolytic cleavage.

In spite of significant progress made in this area further efforts are necessary to develop practically useful systems for the peptide cleavage in aqueous neutral solutions. This paper explores a possibility to achieve this by the metal-catalyzed hydroxylaminolysis instead of hydrolysis. Hydroxylamine is a strong nucleophile towards amide bonds.[10,11] A detailed kinetic study of the hydroxylaminolysis of formamide revealed that the reaction is second-order in total hydroxylamine and the reaction rate shows a maximum near pH 6 due to the general-acid catalysis of the reaction by NH_3OH^+.[11] The observed first-order rate constant 0.04 min^{-1} at 39 °C for the reaction of formamide with 1 M hydroxylamine at optimum pH is only 10 times smaller than that for the alkaline hydrolysis in 1 M NaOH.[11] Thus, one may consider hydroxylamine as a convenient nucleophile for amide cleavage in neutral solution, which could require only modest catalysis to achieve acceptable rates of the peptide cleavage.

The hydroxylaminolysis of *N*-substituted amides including peptides proceeds much slower than that of simple amides.[10a] The only exception is the relatively fast hydroxylaminolysis at the Asn–Gly bonds of peptides and proteins, which is employed as a selective method of protein cleavage.[12] The reaction mechanism involves intramolecular nucleophilic catalysis by the asparagine amide group.[12a] To our knowledge, metal ion catalysis has not been reported for the hydroxylaminolysis of amides or peptides. Attempts to observe metal ion catalysis in the hydroxylaminolysis of more reactive carboxylic acid anhydride [13] and ester [14] bonds were unsuccessful, although the hydrolysis of both substrates was strongly accelerated by the same metal ions. The reason for this different behavior is that the catalytic hydrolysis involves the nucleophilic attack of the substrate by the metal-bound hydroxide anion and such a mechanism is impossible for the hydroxylaminolysis because the nitrogen of NH_2OH possesses only one pair of unshared electrons, which it may use either for coordination to the metal or for the nucleophilic attack on the substrate. The alternative mechanism of catalysis involves the Lewis acid activation of the substrate by the metal cation and an attack of free nucleophile on the activated substrate. Due to the poor donor ability of esters and anhydrides such a mechanism would be inefficient with these substrates, but amides are stronger donors and they can at least weakly coordinate metal ions even in aqueous solutions.[15] Thus, we chose to study the metal ion catalysis of hydroxylaminolysis of amides rather than reactive esters.

This paper reports a first example of the metal ion catalysis in the hydroxylaminolysis of unactivated amides and peptides. Preliminary testing of a series of divalent cations revealed a significant catalytic effect of Zn(II) in the cleavage of glycine derivatives, which was studied in detail. A tentative mechanism of the Zn(II)-catalyzed reaction is proposed.

Results and discussion

Kinetics of the non-catalytic hydroxylaminolysis

Scheme 1a illustrates the mechanism of amide hydroxylaminolysis established by Jencks and Gilchrist [11] with formamide as a substrate.

The rate-determining step is changed from the formation to the decomposition of the tetrahedral addition intermediate on increase in the concentration of hydroxylammonium cations. This is manifested in the non-linear dependence of the observed second-order rate constant of the formamide hydroxylaminolysis on the total concentration of hydroxylamine at a fixed pH value. The rate equation, which follows from Scheme 1a has the form: [11]

† Abbreviations used in the paper are: G - glycine, GNH_2 - glycinamide, GH - glycine hydroxamic acid, GG - glycylglycine, GGG - triglycine, DKP - diketopiperazine, GGH - glycylglycine hydroxamic acid.

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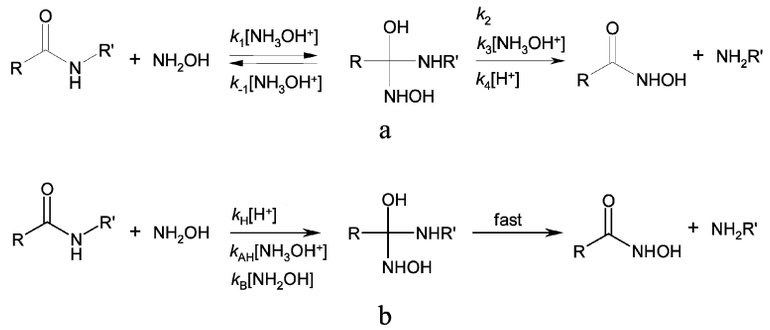


Scheme 1

$$k_{obs}/[NH_2OH] = k_1[HA](k_2 + k_3[HA] + k_4[H^+])/(k_2 + k_3[HA] + k_4[H^+] + k_{-1}[HA]) \quad (1)$$

where k_{obs} is the observed first-order rate constant of the reaction and HA is NH_3OH^+.

Since formamide is to some extent an activated substrate we have studied first the kinetics of the hydroxylaminolysis of much less reactive acetamide, which is closer by its intrinsic reactivity to peptides. Figs. 1 and 2 show the pH profiles for the observed rate constants at two temperatures and the concentration profile, respectively.

Like in the case of formamide a pH-optimum is observed at pH ≈ pK_a=6 and the reaction order in hydroxylamine is higher than unity. The plot of $k_{obs}/[NH_2OH]$ (open squares in Fig. 2) follows the equation (1) with k_1 = 6.4 × 10^{-4} M^{-2} s^{-1}, (k_2 + $k_4[H^+]$)/k_{-1} = 0.17 M and k_3/k_{-1} = 0.16 at 50 °C and pH 6.0. Fitting of the pH profile to the expression for k_{obs} which follows from the equation (1) is shown by the dash line in Fig. 1. It is satisfactory only around the pH-optimum, but both at higher and lower pH values the equation requires k_{obs} to approach zero. Apparently, the general-acid catalyzed addition of hydroxylamine in accordance with Scheme 1a is the predominant pathway only at a pH close to pK_a of hydroxylammonium when the product $[NH_3OH^+][NH_2OH]$ reaches its maximum value, but at more acidic and more basic solutions the specific-acid and general-base catalysis respectively become the predominant pathways. Fitting the results between pH 5 and 7 to equation (1)gives the following parameters: k_1 = 2.2 × 10^{-4} M^{-2} s^{-1}, k_2/k_{-1} = 0.08 M, k_4/k_{-1} = 6.2 × 10^4 and k_3/k_{-1} = 0.36 at 37 °C. In comparison, formamide as a substrate has the parameters k_1 = 8.5 × 10^{-3} M^{-2} s^{-1}, k_2/k_{-1} = 0.18 M, k_4/k_{-1} = 4 × 10^4 and k_3/k_{-1} = 0.09 at 39 °C.[11] Thus, formamide is *ca*. 40 times more reactive than acetamide due to the faster formation of the addition intermediate, as one would expect for a substrate with the activated acyl part.

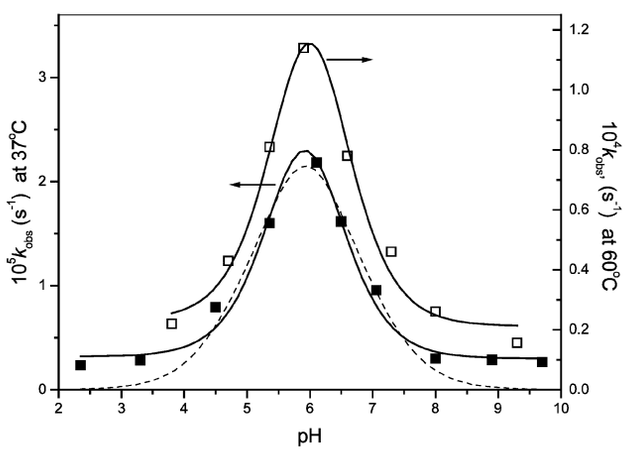


Fig. 1 Observed first-order rate constants for the cleavage of acetamide by 1 M hydroxylamine as a function of pH at 37 °C and 60 °C. Solid lines are theoretical profiles in accordance with eqn. (2) with parameters given in Table 1; dash line is the fitting curve to eqn. (1).

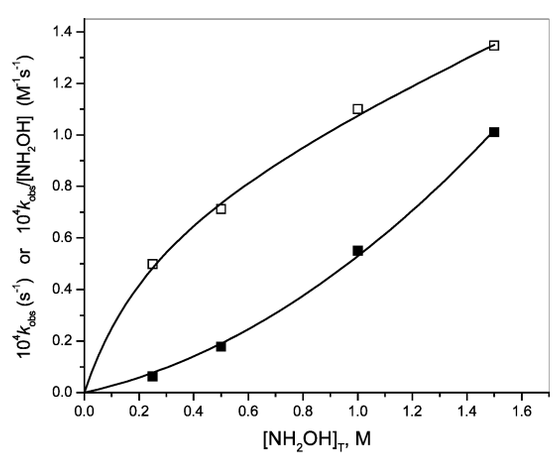


Fig. 2 Observed first-order (■) and second-order ($k_{obs}/[NH_2OH]$) (□) rate constants for the hydroxylaminolysis of acetamide at pH 6 and 50 °C as a function of total hydroxylamine concentration.

The above results allow one to estimate the ratio of rates of decomposition of the tetrahedral intermediate in the forward direction to final products (r_+) and in the back direction to starting molecules (r_-): r_+/r_- = (k_2 + k_3[HA] + $k_4[H^+]$)/(k_{-1}[HA]). At pH 6 and 37 °C r_+/r_- = 0.65 (from the pH-dependence) and at 50 °C r_+/r_- = 0.44 (from the concentration dependence). Thus at pH 6 the rates of decomposition in both directions are similar. It is easy to see that both an increase and decrease in pH will lead to an increase in r_+/r_- due to decrease in [HA] and increase in $[H^+]$ respectively.[16] Values of r_+/r_- considerably larger than unity indicate that the rate-determining step is the formation of the addition intermediate. Therefore, the whole pH-dependence of the rate of hydroxylaminolysis of acetamide was analyzed in terms of a simplified scheme, which assumes the rate-determining formation of the intermediate *via* three pathways: specific-acid, general-acid and general-base catalyzed attack by hydroxylamine, Scheme 1b. The respective rate equation has the form:

$$k_{obs} = k_H[H^+][NH_2OH]_T/(1+[H^+]/K_a) + (k_{AH}[H^+]/K_a + k_B)[NH_2OH]_T^2/(1+[H^+]/K_a)^2 \quad (2)$$

where $[NH_2OH]_T$ is the total concentration of hydroxylamine and K_a is the acid dissociation constant of NH_3OH^+.[17] Solid lines in Fig. 1 are the fitting curves in accordance with the equation (2) and the respective rate constants at three temperatures together with the activation energies are collected in Table 1.[18]

The reactivity of GNH_2 at the optimum pH value is close to that of acetamide, but the optimum range of pH is wider and is shifted to 7. In addition, in acid solutions the reaction rate goes to zero on decrease in pH, Fig. 3.

The plot of $k_{obs}/[NH_2OH]$ *vs*. total hydroxylamine concentration shown in Fig. 4 (open squares) is linear up to 1.2 M hydroxylamine. As follows from equation (1), such linearity can

Table 1 Rate parameters for the uncatalyzed hydroxylaminolysis of acetamide and some glycine derivatives in differently protonated forms

Substrate species	T/°C	k_H/M^{-2} s^{-1}	k_{AH}/M^{-2} s^{-1}	k_B/M^{-2} s^{-1}
CH_3CONH_2	37	2.9 ± 0.5	$(8.3 \pm 0.7)\times10^{-5}$ [a]	$(2.9 \pm 0.6)\times10^{-6}$
	45	7.1 ± 3.3	$(1.4 \pm 0.2)\times10^{-4}$ [a]	$(8.3 \pm 3.0)\times10^{-6}$
	60	23 ± 4	$(4.0 \pm 0.2)\times10^{-4}$ [a]	$(2.1 \pm 0.3)\times10^{-5}$
Ea/kJ mol^{-1} =		76.4 ± 5.8	54.8 ± 0.5	71.4 ± 14.1
$^+H_3NCH_2CONH_2$	50	0	$(8.7 \pm 0.5)\times10^{-5}$	0
$H_2NCH_2CONH_2$	50	0	$(4.6 \pm 0.1)\times10^{-4}$	$(1.6 \pm 0.1)\times10^{-5}$
$^+H_3NCH_2CONHCH_2COO^-$	60	2.1 ± 0.4	$(6.7 \pm 1.6)\times10^{-6}$	0
$H_2NCH_2CONHCH_2COO^-$	60	0	$(2.9 \pm 0.2)\times10^{-4}$	$(1.6 \pm 0.1)\times10^{-6}$
$^+H_3NCH_2CONHCH_2CONHCH_2COO^-$	60	3.2 ± 0.2	$(1.5 \pm 0.2)\times10^{-5}$	0
$H_2NCH_2CONHCH_2CONHCH_2COO^-$	60	0	$(2.3 \pm 0.3)\times10^{-4}$	0

[a] $k_1 = 2.3 \times 10^{-4}$, 3.7×10^{-4} and 1.05×10^{-3} M^{-2} s^{-1} at 37, 45 and 60 °C respectively, as estimated by using the equation (1) from the results around pH 6 only.

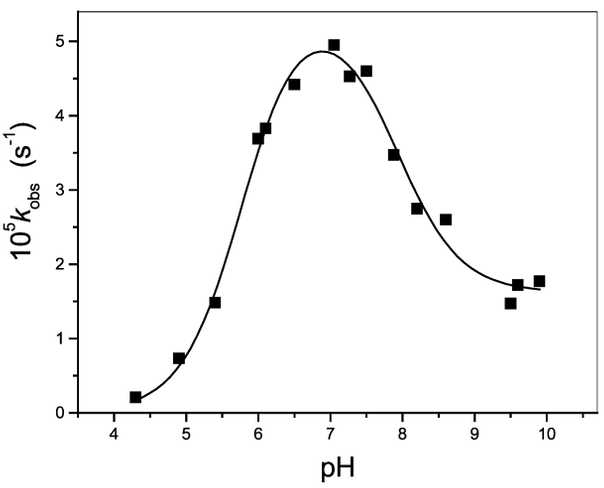


Fig. 3 Observed first-order rate constants for the cleavage of glycinamide by 1 M hydroxylamine as a function of pH at 50 °C. The solid line is the theoretical profile in accordance with Scheme 2 with parameters given in Table 1.

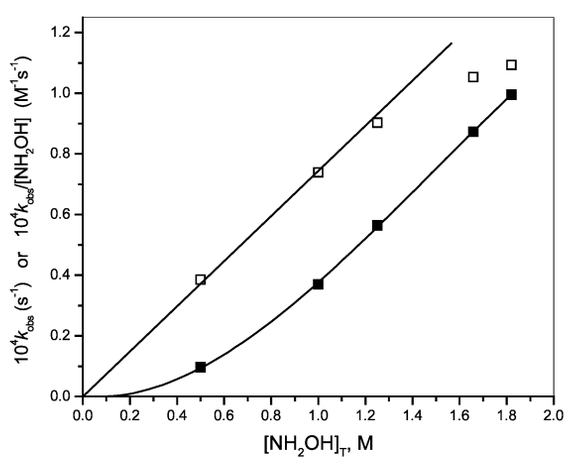
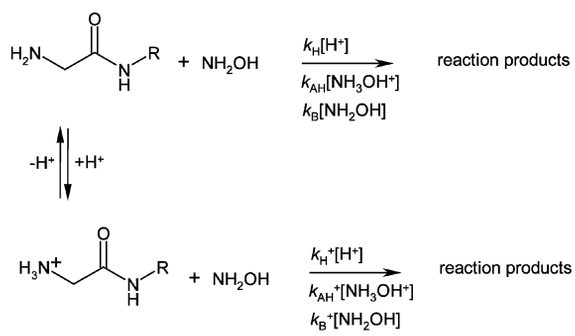


Fig. 4 Observed first-order (■) and second-order (k_{obs}/[NH_2OH]) (□) rate constants for the hydroxylaminolysis of glycinamide at pH 6 and 50 °C as a function of total hydroxylamine concentration.

be observed only when the k_{-1}[HA] term in the denominator is negligible, that is when k_{-1}[HA] << (k_2+k_3[HA]+k_4[H^+]). This inequality means that the rate of decomposition of the addition intermediate in the back direction is much smaller than that in the forward direction and the addition step is the rate-determining. Shift and widening of the pH-optimum can be explained by different reactivities of neutral and protonated forms of GNH_2 (pK_a=7.93),[19] Scheme 2. Fitting the results in Fig. 3 to the equation (2) modified in accordance with Scheme 2 allowed us to determine the rate constants for different reaction pathways



Scheme 2

with both neutral and cationic forms of the substrate collected in Table 1. The specific acid catalysis does not operate in this case apparently because of repulsion of hydroxonium cations from the protonated substrate. For the same reason the rate constant for the general acid catalysis by hydroxylammonium cation (k_{AH}) is smaller for the protonated than for neutral substrate, although the protonated substrate should be more reactive due to the inductive effect of the ammonium group, as is observed in the alkaline hydrolysis of glycine ethyl ester.[4b] The specific acid catalysis should be efficient for the reaction with the neutral form of GNH_2 also, but we were unable to estimate the respective rate constant probably because of too small fraction of the neutral form in acid solutions.

The hydroxylaminolysis of peptides GG and GGG proceeds as expected much slower than that of GNH_2 and the reaction kinetics were studied at a higher temperature, Fig. 5.

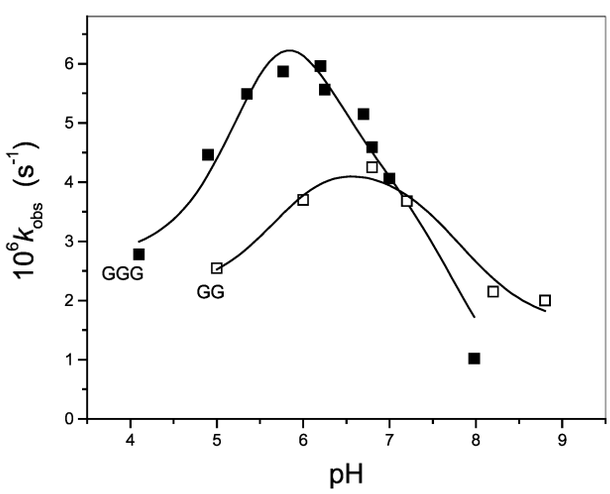


Fig. 5 Observed first-order rate constants for the cleavage of glycylglycine (GG) and triglycine (GGG) by 1 M hydroxylamine as a function of pH at 60 °C. Solid lines are theoretical profile in accordance with Scheme 2 with parameters given in Table 1.

Table 2 Representative first-order rate constants for the hydroxylaminolysis of glycinamide and glycine peptides in the presence of metal cations (1 M hydroxylamine)

	Substrate	Metal ion	T/°C	pH	k/s^{-1}
1	GNH_2	None	50	7.2	4.5×10^{-5}
2		Ni(II), 0.03 M	—	—	8.6×10^{-5}
3		Ni(II), 0.06 M	—	—	8.0×10^{-5}
4		Zn(II), 0.01 M	—	—	1.0×10^{-4}
5		Zn(II), 0.03 M	—	—	2.1×10^{-4}
6		Cd(II), 0.025 M	—	—	7.3×10^{-5}
7	Asn	None	65	6.3	6.6×10^{-5}
8		Zn(II), 0.03 M	—	—	6.0×10^{-5}
9	N–AcGly	None	50	7.0	4.5×10^{-6}
10		Zn(II), 0.03 M	—	—	8.5×10^{-6}
11	GG	None	60	6.8	4.2×10^{-6}
12		Zn(II), 0.03 M	—	—	1.5×10^{-5}
13		Uncatalyzed hydrolysis[a]	60	7.0	3.6×10^{-9}
14		Hydrolysis by $Zn_2L(OH)$[b]	70	*ca*. 8.5	4.6×10^{-7}
15		Hydrolysis by CuL[c]	50	8.1	2.6×10^{-7}
16	GGG	None	60	6.8	5.2×10^{-6}
17		Zn(II), 0.03 M	—	—	2.1×10^{-5}

[a] Ref. 3*a*. [b] Ref. 23; L=OBISDIEN. [c] Ref. 6; L=[9]aneN_3, k estimated from the yield of GG hydrolysis after 7 days.

The optimum pH for GG is about 7 like in the case of glycinamide, but for GGG it is closer to 6 like in the case of acetamide. For the tripeptide two peptide bonds are cleaved, one of which is more distant from the terminal amino group and its cleavage may be insensitive to the protonation. For both peptides the reaction order in hydroxylamine is two. The rate constants found from the fitting of these pH-dependences are given in Table 1. The most essential difference from glycinamide is a considerable contribution of the specific acid catalysis for the hydroxylaminolysis of both peptides in protonated forms. This is explicable by the fact that the protonated peptides are zwitterions rather than cations, as in the case of GNH_2. The general-acid catalyzed reaction is faster for deprotonated anionic peptides than for zwitterions. This can be due to the electrostatic attraction of the hydroxylammonium cation to the anionic substrate.

Kinetics of the hydroxylaminolysis in the presence of metal cations

At the first step several divalent cations were tested for possible catalysis in the hydroxylaminolysis of more reactive GNH_2. Additions of 0.025 M Cu(II), Co(II), Fe(II) and Pb(II) to 1 M hydroxylamine solution at pH 6–7 and 50 °C produced precipitates and Mn(II) did not affect the reaction rate. Positive results were observed with Ni(II), Zn(II) and Cd(II). Representative rate constants for these cations are given in Table 2 (lines 1–6). The largest (*ca*. five-fold) acceleration is observed with Zn(II). Additions of Ni(II) at higher concentrations lead to *ca*. two-fold acceleration, which is independent of the metal concentration. The "saturation" of the reaction rate is observed also with Zn(II) (see below), but for Ni(II) it occurs at lower metal concentration apparently due to much higher stability of GNH_2 complexes with Ni(II) as compared with Zn(II) (log$K$=4.20 and 3.28 for Ni(II) and Zn(II) respectively).[19] The catalytic effect of Cd(II) is lower than that of Ni(II). The reaction in the presence of Zn(II) was studied in more detail.

Catalysis by Zn(II) was not observed in the hydroxylaminolysis of acetamide. It is absent also for asparagine (Table 2, lines 7 and 8). The reaction with *N*-acetylglycine shows a modest catalytic effect (Table 2, lines 9 and 10). Thus the presence of the α-amino group is essential for the efficient catalysis.

Fig. 6a shows the observed first-order rate constant for the hydroxylaminolysis of GNH_2 as a function of Zn(II) concentration at three pH values. The variation in concentration at pH 7.5 was limited by precipitation of Zn(II) hydroxide above 0.03 M metal. The profiles at lower pH values fit to a Michaelis–Menten type equation (3)

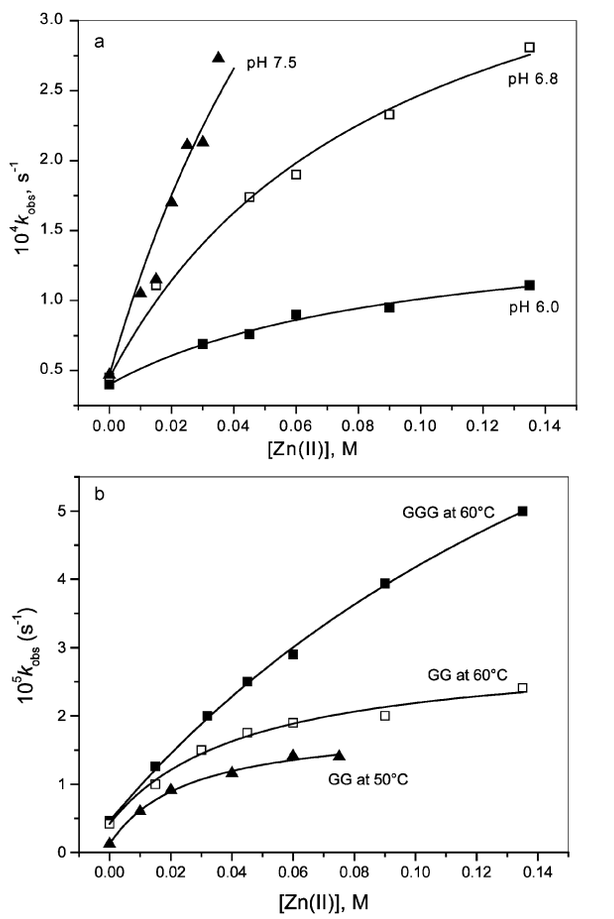


Fig. 6 **a** Observed first-order rate constants for the cleavage of glycinamide by 1 M hydroxylamine at 50 °C *vs*. Zn(II) concentration at pH 6.0 (■), 6.8 (□) and 7.5 (▲); **b** Observed first-order rate constants for the cleavage of glycine peptides by 1 M hydroxylamine at pH 6.8 *vs*. Zn(II)concentration: GGG at 60 °C (■), GG at 60 °C (□) and GG at 50 °C (▲).

$$k_{obs} = (k_0 + k_C K[Zn(II)])/(1 + K[Zn(II)]) \qquad (3)$$

where k_0 and k_C are the rate constants for the hydroxylaminolysis of free and bound to Zn(II) GNH_2 and K is the stability constant for the metal–substrate complex. The values of parameters of equation (3) are given in Table 3.

The values of K are practically pH-independent, but the acceleration effect of Zn(II) expressed as k_C/k_0 increases from 4

Table 3 Rate constants of the uncatalyzed (k_0) and catalytic (k_C) hydroxylaminolysis of different substrates in the presence of Zn(II) and stability constants (K) of their complexes with Zn(II), Scheme 3, equation (3)

Substrate	T/°C	pH	k_0/s^{-1}	k_C/s^{-1}	K/M^{-1}	k_C/k_0
GNH_2	50	6.0	4.0×10^{-5}	1.6×10^{-4}	10.4	4.0
—	50	6.8	4.5×10^{-5}	4.3×10^{-4}	10.9	9.5
—	50	7.5	4.7×10^{-5}	7.8×10^{-4}	10.6[a]	16.6
GG	50	6.8	1.2×10^{-6}	1.9×10^{-5}	38.5	15.8
—	60	6.8	4.2×10^{-6}	3.0×10^{-5}	22.2	7.1
GGG	60	6.8	4.6×10^{-6}	1.3×10^{-4}	4.4	28.3

[a] Mean value between binding constants at pH 6.0 and 6.8.

to 9 on going from pH 6.0 to pH 6.8. The results at pH 7.5 cover only the initial linear part of the concentration dependence, but using the K value found at lower pH one can obtain from this results the ratio k_C/k_0=16. Thus the efficiency of Zn(II) catalysis strongly increases on going to more basic solutions. A plot of logk_{obs} corrected for the rate of uncatalyzed reaction at fixed Zn(II) concentration *vs*. pH is linear (Fig. 7) with the slope 0.86 close to unity. Measurements of k_{obs} at different NH_2OH concentrations showed that in contrast to the uncatalyzed reaction, the catalytic reaction is first-order in hydroxylamine.

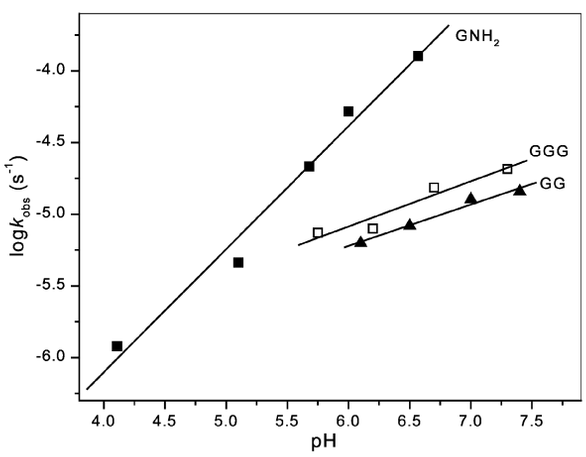


Fig. 7 Logarithms of k_{obs} for Zn(II)-catalyzed cleavage of GNH_2 (at 50 °C), GG and GGG (at 60 °C) by 1 M hydroxylamine *vs*. pH.

The catalytic effect of Zn(II) on the hydroxylaminolysis of peptides GG and GGG is similar to that for GNH_2 (Table 2, lines 11 and 12; 16 and 17). Rate *vs*. Zn(II) concentration profiles for the cleavage of GG and GGG (Fig. 6b) also follow equation (3) with rate parameters given in Table 3. Rates of catalytic reactions for both peptides increase on increase in pH, although plots of logk_{obs} *vs*. pH are flater than in the case of GNH_2 (Fig. 7). Like in the case of GNH_2 the reaction order in hydroxylamine is reduced to one in the presence of Zn(II) for both peptides. Thus, the catalytic hydoxylaminolysis of GNH_2 and peptides generally have similar features.

The reported stability constants for Zn(II) complexes with GNH_2 and both peptides are similar and are *ca*. 2×10^3 M^{-1} at 25 °C.[20] "Kinetic" stability constants given in Table 3 are much smaller. This is due partly to a significant degree of protonation of the amino group of substrates at about pH 7. The pK_a values of the protonated forms of all substrates are close to 8.[19] Therefore the expected stability constant at pH 7 can be estimated as 200 M^{-1} at 25 °C, which is still one order of magnitude larger than the "kinetic" values. A possible reason of this disagreement is the enhanced temperature. Indeed, measurements with GG at a lower temperature give a larger value of K, Table 3.

The above results allow one to propose a tentative reaction mechanism, which should take into account the coordination of Zn(II) to the α-amino group of the substrate, the first-order kinetics in NH_2OH and the first-order dependence of k_{obs} on OH^-. Such a mechanism is illustrated in Scheme 3. It is proposed that the Zn(II) cation serves as a Lewis acid catalyst, that makes unnecessary the participation of NH_3OH^+ and the reaction becomes first-order in total hydroxylamine. The role of hydroxide ion is most probably a general-base assistance to the nucleophile, as illustrated in structure **1**, Scheme 3.

Fig. 8 shows the reaction course for the hydroxylaminolysis of GNH_2 in the presence of Zn(II) followed by the proton NMR. The signal of GNH_2 reduces to *ca*. 7% of its initial intensity after 2.7 h and disappears after 9.5 h. Surprisingly, together with glycine hydroxamic acid (GH), an equal amount of glycine (G) appears after 2.7 h, and after 9.5 h *ca*. 95% of the starting amount of GNH_2 is converted into G and only *ca*. 5% remains as GH.

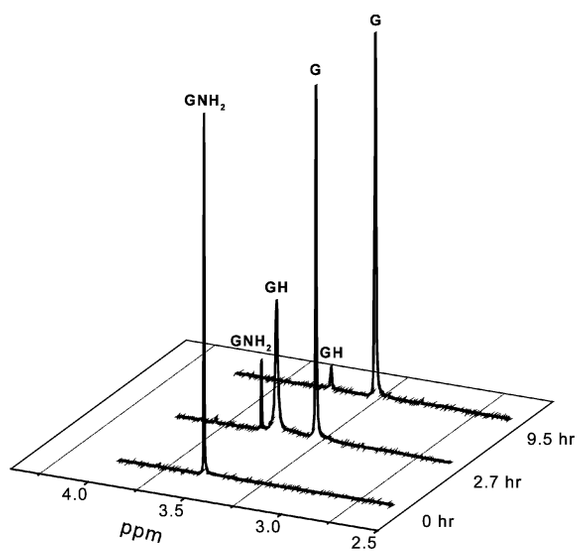
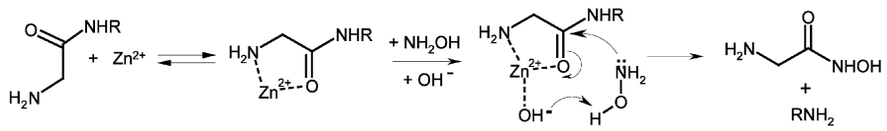


Fig. 8 Proton NMR spectra of the reaction mixture containing 0.05 M GNH_2, 0.1 M Zn(II) and 1.0 M hydroxylamine in D_2O recorded after 0, 2.7 and 9.5 h of incubation at pH 7 and 65 °C.

These results indicate that under experimental conditions GH is further hydrolyzed to G and hydroxylamine. The hydrolysis of hydroxamic acids is a slow process, which requires strongly acidic or basic conditions.[11,21] Metal ion catalysis by Fe(III) and to a much lesser degree by divalent cations (Cu(II), Mn(II), Ni(II) and Cd(II)) was reported for the hydrolysis of desferal hydroxamic acid in 1.5 M sulfuric acid at 70 °C.[22] Apparently, the hydrolysis of GH is more sensitive to the metal ion catalysis. In a separate experiment the first-order rate constant for the hydrolysis of GH was found to be 6.5×10^{-5} s^{-1} (half-life 3 h) at 65 °C, pH 7.0 in the presence of 0.1 M Zn(II) and 1 M hydroxylamine.

Fig. 9(a,b) shows proton NMR spectra of the reaction mixtures containing GG and GGG respectively after 24 h. In both cases the degree of cleavage is *ca*. 80% and the principal product is G. In the case of GG also small amounts (1.5% of total cleavage products) of diketopiperazine (DKP) and glycylglycine hydroxamic acid (GGH) are formed. The cyclization of GG may occur under conditions employed [3a] and the cleavage of DKP by hydroxylamine explains the formation of GGH. The cyclization of a tripeptide affording DKP should be much more rapid.[3a] Indeed, the signal of DKP is relatively strong for the GGG reaction mixture (Fig. 9b) and accounts for *ca*. 7% of the total amount of the cleavage products. Thus the Zn(II)-catalyzed hydroxylaminolysis does not suppress completely the DKP formation, but favorably competes with the cyclization.

The time course of the peptide cleavage was followed also by recording NMR spectra of the reaction mixtures in D_2O at different time intervals. Fig. 10 shows the time course of



1

Scheme 3

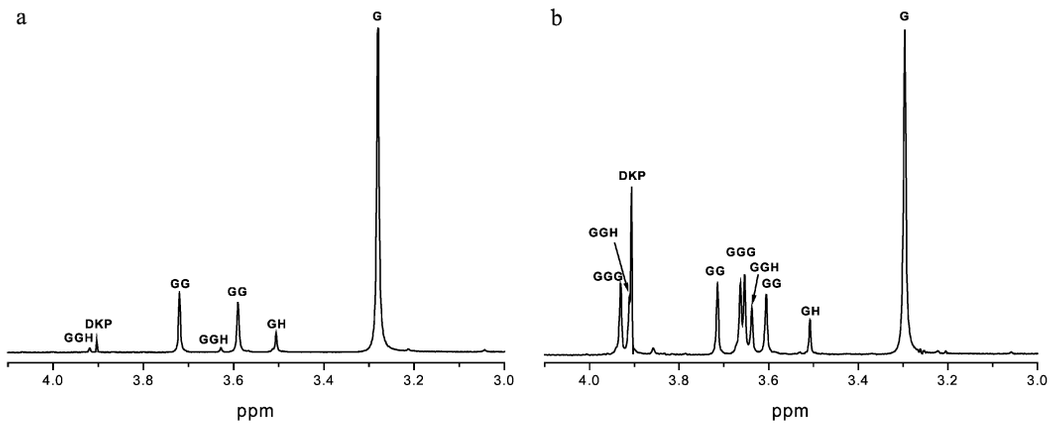


Fig. 9 Proton NMR spectra of the reaction mixtures containing 0.05 M GG (**a**) or GGG (**b**), 0.1 M Zn(II) and 1.0 M hydroxylamine in D_2O recorded after 24 h of incubation at pH 7 and 65 °C.

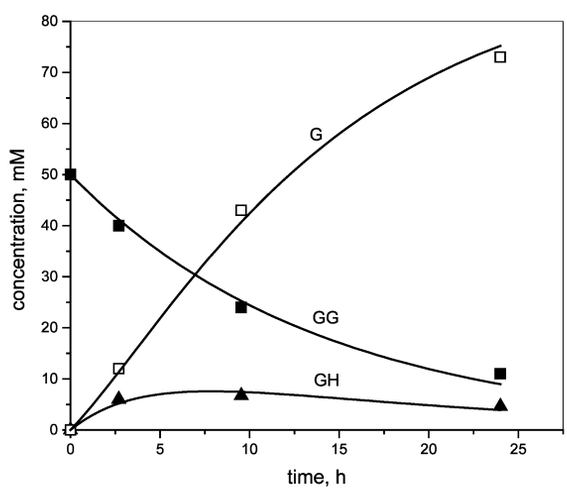


Fig. 10 The time course of the cleavage of glycylglycine in the presence of 0.1 M Zn(II) and 1 M NH_2OH in D_2O at pH 7 determined by proton NMR spectra of the reaction mixture recorded at various times.

GG cleavage in the presence of 0.1 M Zn(II) and 1 M hydroxylamine at 65 °C and pH 7 monitored by NMR. The kinetic curve for GH has a typical form for a reaction intermediate. The maximum concentration of GH is *ca.* 15% of the initial GG concentration indicating that the rate constant of GH decomposition is higher than the rate constant of its formation.

The time profiles for all reaction components may be fitted to a formal scheme of two consecutive reactions:

$$GG \longrightarrow GH + G\ (k_I)$$
$$GH \longrightarrow G\ (k_{II})$$

from which one obtains:

$$[GG] = [GG]_0 e^{-k_I t} \quad (4)$$

$$[GH] = k_I[GG]_0(e^{-k_I t} - e^{-k_{II} t})/(k_{II} - k_I) \quad (5)$$

$$[G] = [GG]_0(2 + (k_I e^{-k_{II} t} + (k_I - 2k_{II})e^{-k_I t})/(k_{II} - k_I)) \quad (6)$$

Solid lines in Fig. 10 are the theoretical profiles calculated by using $k_{II} = 6.5 \times 10^{-5}\ s^{-1}$ as given above and the value of $k_I = 2.2 \times 10^{-5}\ s^{-1}$ determined independently form the initial rate of GH formation measured by the Fe(III) procedure (see Experimental). Obviously there is a good agreement between results obtained by the two different techniques. The NMR spectra recorded at various time intervals for the hydroxylaminolysis of GGG under similar conditions (1 M NH_2OH, 0.1 M Zn(II) at 65 °C) showed that GH also behaves as an intermediate and the maximum concentration of GH reached after *ca.* 7 hours is 12% of the total amount of the peptide. Thus the final result of the Zn(II)-catalyzed treatment of peptides by hydroxylamine is the hydrolytic cleavage.

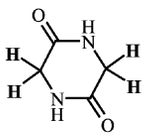
For the purpose of comparison of the efficiency of Zn(II)-catalyzed hydroxylaminolysis of peptides with the metal-catalyzed peptide hydrolysis we also included in Table 2 the literature values of the rate constants of spontaneous hydrolysis and Zn(II) and Cu(II) catalyzed hydrolysis of GG (Table 2, lines 13–15). One can see that in the presence of 0.03 M Zn(II) and 1 M NH_2OH the cleavage of GG proceeds *ca.* 10^4 times faster than the uncatalyzed hydrolysis and *ca.* 10^2 times faster than the Zn(II) and Cu(II) catalyzed hydrolysis. Peptide half-lives at pH 7 in the presence of 0.1 M Zn(II) and 1 M hydroxylamine are about 5 h at 60 °C, similar to those reported for the most reactive hydrolytic systems based on Pd(II) [7] and Ce(IV).[8] The rate *vs.* pH profiles, Fig. 7, indicate that the catalytic activity of Zn(II) may be strongly increased on going to higher pH values, but the precipitation of Zn(II) hydroxide limits the possible pH interval to values below pH 7.5. To prevent the hydroxide precipitation, polyamine ligands (ethylenediamine and diethylenetriamine) or imidazole were added to Zn(II), but caused inhibition of the catalytic activity. Search for suitable stabilizing ligands as well as a study of the reaction selectivity in respect of the amino acid side groups is in progress.

Experimental

Materials

The compounds studied as substrates (acetamide, glycinamide, *N*-acetylglycine, glycylglycine, and triglycine), acetohydroxamic and glycine hydroxamic acids were purchased from Sigma and used without further purification. Glycylglycine hydroxamic

Table 4 Proton NMR chemical shifts in D_2O at pH 7 in the absence and in the presence of 0.085 M $ZnSO_4$

Substance	Chemical shift	
	alone	*with $ZnSO_4$*
$H_2NCH_2CONH_2$	3.652	3.551
$H_2NCH_2CONHCH_2COOH$	3.633	3.654
$H_2NCH_2CONHCH_2COOH$	3.686	3.713
$H_2NCH_2CONHCH_2CONHCH_2COOH$	3.670	3.660
$H_2NCH_2CONHCH_2CONHCH_2COOH$	3.918	3.931
$H_2NCH_2CONHCH_2CONHCH_2COOH$	3.644	3.639
H_2NCH_2COOH	3.320	3.276
$H_2NCH_2CONHOH$	3.506	3.508
$H_2NCH_2CONHCH_2CONHOH$	3.906	3.906
$H_2NCH_2CONHCH_2CONHOH$	3.609	3.609
 Diketopiperazine	4.080	3.909

acid used as a standard for identification of the reaction products by NMR was prepared by reacting glycylglycine ethyl ester with excess hydroxylamine in D_2O. Reagent-grade inorganic salts and hydoxylammonium hydrochloride from Aldrich were used as supplied. All solutions were prepared in purified (Milli-Q Reagent Water System) water or in D_2O (Aldrich) for NMR studies.

Instrumentation

Ultraviolet–Visible spectra were obtained with a Hewlett Packard 8452A spectrophotometer. ^1H NMR spectra were recorded on 300 MHz Varian Unity INOVA spectrometer.

Methodology

Kinetics of the hydroxylaminolysis of glycinamide and peptides were followed by colorimetric reaction of aliquots taken periodically from the reaction mixture with $Fe(NO_3)_3$ in 0.3 M nitric acid.[10,11] The molar absorptivity at 540 nm produced by glycine hydroxamic acid (650 $M^{-1}cm^{-1}$ respectively) was determined by using the standard solutions of glycine hydroxamic acid of known concentrations. The hydroxylaminolysis of acetamide was followed spectrophotometrically by appearance of acetohydroxamic acid at 215 nm (molar absorptivity 744 M^{-1} cm^{-1}).Typically 0.02–0.05 M solutions of amides or peptides, 0.01–0.1 M metal salt and 0.5–1.5 M hydroxylamine were employed. The reaction mixtures were adjusted to desired pH by adding concentrated NaOH or HCl solutions and placed into the thermostat.

Products of the hydroxylaminolysis of glycine derivatives were identified by proton NMR in D_2O. Chemical shifts of starting materials and reaction products in D_2O at pH 7 are given in Table 4.

Acknowledgements

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16 With the given above rate parameters at 37 °C the ratio r_+/r_- equals 1.1 and 6.4 at pH 5 and 4 respectively, and 1.2 and 8.4 at pH 7 and 8 respectively. Thus, in the range of pH 5–7 the ratio r_+/r_- is between 0.5 and 1, but outside this range $r_+/r_- > 1$ both in acidic and basic solutions.
17 Since pH values were always measured at room temperature the rate *vs*. pH profiles were analyzed by using pK_a=6.0 for hydroxylammonium at all temperatures. When pK_a was left as an adjustable parameter in the equation (2), the fitting of the pH-profiles at different temperatures gave the values 5.94, 6.10 and 6.0 at 37, 45 and 60 °C respectively. Thus using uncorrected pK_a at different temperatures seems justified and on this basis we used also uncorrected pK_a values for glycine derivatives employed as substrates.
18 Obviously in the range of pH 5–7 the assumption of the rate-determining addition step for the hydroxylaminolysis of acetamide is rather rough and the calculated value of k_{AH} (see Scheme 1b) should be smaller than the real value of the addition rate constant (k_1 in Scheme 1a) by a factor of 2 or 3. The correct values of k_1 at different temperatures were calculated by using the equation (1) from the results around pH 6 only and they are given as footnotes to Table 1. For glycine derivatives as the substrates the assumption is valid at all pH values.
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