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Reductive Nitrosation of Peptides Ligated to High-Valent Metal Cations

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NO reacts with M^{III}–(glycylglycylglycine), M = Fe; Ni and Cu, to form the terminal *N*-nitrosated peptide complex. This reaction proceeds by a radical mechanism.

The role of NO in a variety of physiological processes renders its reaction mechanisms with species present in biological media of importance. Of special interest are nitrosation processes that are involved in signaling[1–2] and pathological[2] processes as well as in buffering the concentration of NO in the cell.[1,3–6] Two transition-metal-initiated reductive nitrosation mechanisms were reported:

(1) Ligation of NO to the central cation, which induces a partial positive charge on the nitrogen atom and thus enables attack of a nucleophile at the nitrogen atom[7–10] [Reaction (1)].

$$M^{n+1}L_n \xrightarrow[-L]{NO} L_{n-1}M^{n+1}\text{–}NO \xrightarrow[L]{:B} M^nL_n + BNO^- \quad (1)$$

In water: B is usually H_2O or OH^- and the product is NO_2^-.

(2) Reaction of NO with a ligand bound to the central cation with a lone electron pair on the atom ligated to the cation. This process often requires proton loss to form the lone pair. Recently,[11] it was suggested that this is a radical process as outlined in Reactions (2)–(4).

$$M^{n+1}\text{–}E(H)R_m \rightleftharpoons M^{n+1}\text{–}\ddot{E}R_m + H^+ \quad (2)$$

$$M^{n+1}\text{–}\ddot{E}R_m \rightleftharpoons M^n\text{–}\dot{E}R_m \quad (3)$$

$$M^n\text{–}\dot{E}R_m + NO \longrightarrow M^n + R_mENO \quad (4)$$

Where E = N, S and plausibly O.

Reactions of this type were previously attributed to attack of NO on the lone pair followed by electron transfer.[7–10] However, as NO does not react with strong nucleophiles, for example, OH^-, it is reasonable that the radical mechanism is the occurring one. As the mechanism described by Reactions (2)–(4) was observed for $E(H)R_m$ = CH_3NH_2, it seemed of interest to check whether it took place also for $E(H)R_m$ = peptides. The present study proves that indeed peptide *N*-nitrosations of the terminal amine occur by this mechanism.

The first peptide investigated in this study was glycylglycylglycine (glyglygly). The complexes Ni^{III}–(glyglygly) and Cu^{III}–(glyglygly) were prepared by a known procedure,[12,13] and the complex Fe^{III}–(glyglygly) was prepared by slow addition of 0.3 M tris buffer to a solution containing $Fe(ClO_4)_3$ and 10–20% excess of tripeptide. The solutions containing the complexes were saturated with Ar and mixed with NO saturated aqueous solutions at the same pH. By using stopped flow, the kinetic results, Figure 1, confirm that these complexes react with NO in reactions obeying pseudo-first-order rate laws. The observed rates depend linearly on the concentrations of NO and OH^-, Figures 2 and 3, respectively. The observed rate constants at pH 6.0 are summed up in Table 1. The pH dependence of the rate con-

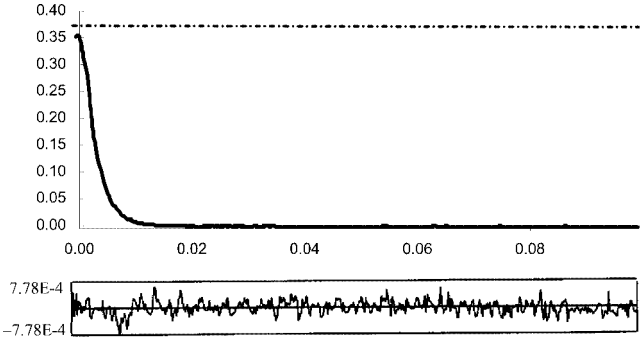

Figure 1. Kinetics of reaction of Ni^{III}–(glyglygly) at pH 7.0 with NO measured at 332 nm. Solution composition: (——) Ni^{III} 0.0005 M, glyglygly 0.0011 M, (50% saturated NO); (-·-·-·) Ni^{III} 0.0005 M, glyglygly 0.0011 M (saturated Ar).

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stants, Figure 3, suggests that these reactions occur with an alkaline form of the complexes [the results suggest a p$K_a \geq 7$ for Cu^{III}–(glyglygly); ≥ 9 for Ni^{III}–(glyglygly) and ca. 10 for Fe^{III}–(glyglygly)] (for the Cu and Ni complexes, measurements could not be performed at higher pH values, as they are faster than the mixing time of the stopped flow). The pK_a values of the amide nitrogen atoms of these complexes are ≤ 6.0.[14–17] Thus, the results suggest that the reactions observed require the loss of a proton from the terminal amine of the peptide.

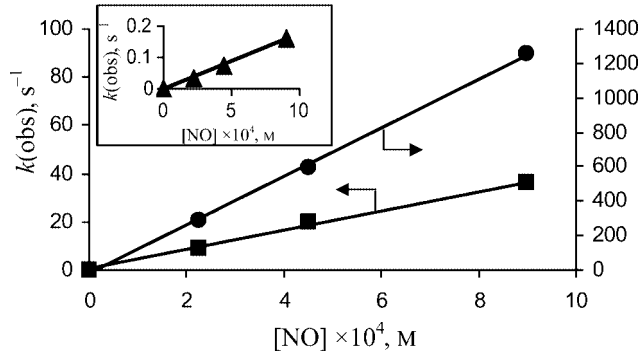


Figure 2. Dependence of the observed rate constants on [NO]. Solution composition: A: k(obs.) vs. [NO] pH 6.0, [bis tris buffer] = 5×10^{-5} M, [M^{III}] = 0.005 M, [glyglygly] = 1.1×10^{-3} M; (●) = Cu^{III}; (▲) = Fe^{III}; (■) = Ni^{III}.

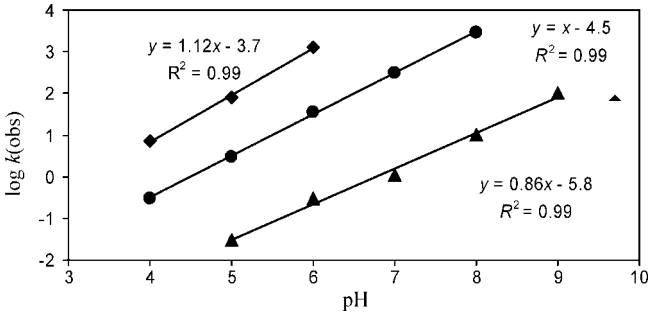


Figure 3. Dependence of the observed rate constants on pH. Solution composition: (●) Ni^{III} 0.0005 M, glyglygly 0.0011 M, (■) Cu^{III} 0.0005 M, glyglygly 0.0011 M, (▲) Fe^{III} 0.0005 M, GGG 0.0011 M, NO 50% saturated.

Table 1. Observed rate constants at pH 6.0.

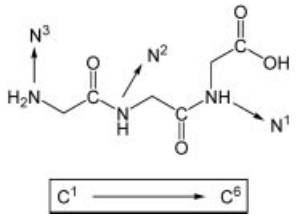
M^{III}	$K_{obs.}$, $M^{-1} s^{-1}$ (alaglygly)	$k_{obs.}$, $M^{-1} s^{-1}$ (glyglygly)
Cu^{III}	880	1260
Ni^{III}	21	36
Fe^{III}	0.090	0.16

The pH dependence of the observed rate constants of the reactions (Figure 3) suggests that the site of the nitrosation of the ligand is the terminal amine of the peptide. The observed rate constants (Table 1 and Figure 3) decrease along the series $Cu^{III} > Ni^{III} > Fe^{III}$. This result is in accord with expectations as the redox potentials vs. NHE of $M^{III/II}$–(glyglygly) complexes are 0.92,[18,19] 0.83[19,20] and –0.21 V [this study] for M = Cu, Ni and Fe, respectively. Though the rate constants decrease with the decrease in the oxidation potential of the complexes, one would expect a larger difference between the Ni^{III} and Fe^{III} complexes. The source of this observation is not clear although it might be due to a higher pK_a of the bound terminal amine in the Ni^{III} complex. Analysis of the final products was performed by the following techniques: (1) Ion chromatography, which proved that NO_2^- is not a product of the reactions studied. (2) ESI MS (Supporting Information, Figure S1), which detected a product with m/z = 219 in solutions that contained M^{III}–(glyglygly) and NO (Ar saturated and acidified to pH 3.0 after the nitrosation). This peak is absent in a blank experiment that contained Ar-saturated solutions of M^{II}–(glyglygly), which were then acidified. A mass of 219 fits the adduct glyglyglyH-NO, namely, the reductive nitrosation product, which as expected is obtained by adding NO to solutions containing the three M^{III}–(glyglygly) complexes. (3) Determination of pH − the pH dependence of the rate constants suggests that the nitrosation occurs on the terminal amine. In order to verify this assumption the ^{13}C NMR spectra of the peptides before and after the nitrosation reactions were measured [deaerated D_2O solutions of M^{II}–(glyglygly) and of M^{III}–(glyglygly) were treated with NO then Ar saturate in order to inhibit nitrite formation, and acidified to pH 3.0 in order to release the peptide from the paramagnetic cation].

The results (Supporting Information, Figures S2, S3) show that the glyglygly peptide has ^{13}C NMR signals at 176.4, 170.8 and 167.7 ppm for the carboxylate and amide carbons and at 43.1, 42.3 and 40.4 ppm for the CH_2 carbons. For the nitrosation product, signals at 173.3, 173.2, 172.8, 50.0, 42.1 and 41.7 ppm and an additional weak signal at 41.0 ppm are observed. These results are in accordance with those obtained by approximate simulation by using the semiempirical simulation − CS ChemDraw Ultra program − for the different plausible nitrosation sites (Table 2). Comparison of the experimental results with the simulations points out that the nitrosations do not occur on the CH_2 groups. However, the results do not enable a decision regarding which nitrogen is nitrosated.

Table 2. Simulations of the ^{13}C NMR data of glyglygly, and the different plausible nitrosation products.

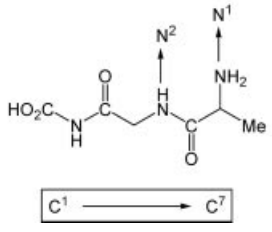
Nitrosation Site	C^1	C^2	C^3	C^4	C^5	C^6
None	43.0	171.1	42.9	171.1	42.7	173.2
N^1	43.4	171.1	42.7	171.0	48.7	173.2
N^2	42.8	171.0	49.1	171.1	42.7	173.2
N^3	48.4	171.2	42.9	171.1	42.7	173.2
N^3 acidic form	50.7	171.1	42.9	171.1	42.7	173.2
C^1	77.5	171.2	42.9	171.1	42.7	173.2



In order to resolve this dilemma, the experiments were repeated with the peptide alaglygly, as the ^{13}C NMR spectroscopic simulation (Table 3) clearly demonstrates that *N*-nitrosation at the terminal amine and the amide N adjacent to the alanine residue will have significantly different ^{13}C NMR spectra. The results (Supporting Information, Figures S4, S5) point out signals for the alaglygly peptide at 176.4, 171.3, 170.6, 49.0, 43.3, 42.3 and 16.2 ppm and for the nitrosation product signals at 178.9, 176.8, 170.7, 57.2, 43.1, 42.2 and 9.8 ppm and additional very weak signals 16.9 and 10.4 ppm. Comparison of the results with the simulation clearly points out that the signal of the CH_3 group is shifted to higher field from 16.2 to 9.8 ppm in perfect accord with the simulation results for nitrosation of the terminal amine group. In contrast, the simulation of the nitrosation product on N^2 predicts that the signal due to the CH_3 group will be shifted to a lower field. The kinetics of the reaction of the M^{III}–(alaglygly) with NO are very similar to those observed for the M^{III}–(glyglygly) complexes. The observed rate constants at pH 6.0 (Table 1) are slightly lower than those for the M^{III}–(glyglygly) complexes. This result is tentatively attributed to the fact that the terminal amine of alaglygly is a slightly stronger base than that of glyglygly. The corresponding pK_a values are 8.16 and 7.95,[21] respectively. The stronger base is expected to stabilize better the M^{III} oxidation state and probably to increase the pK_a value of the terminal amine; both these effects are expected to lower the rate constant of the nitrosation reactions.

Table 3. Simulations of the ^{13}C NMR spectra of alaglygly and the different plausible nitrosation products.

Nitrosation Site	C^1	C^2	C^3	C^4	C^5	C^6	C^7
None	173.2	42.7	171.1	43.2	172.1	49.3	20.5
N^1	173.2	42.7	171.1	43.2	172.1	56.1	15.7
N^2	173.2	42.7	171.1	49.4	176	49.1	21.1



It is of interest to note that nitrosations do not occur at the amide sites although a lone electron pair is also available at these sites even at the lowest pH values used in the this study. This observation is attributed to the higher energy of the orbital of the lone pair of the terminal amine than that of the amide groups.

Finally one should consider whether the ·NO attacks the lone pair of the M^{III}–N̈HR group followed by an electron transfer or the NO attacks the radical isomer of this species:

$$M^{III}-\ddot{N}HR \rightleftharpoons M^{II}-\dot{N}HR$$

As the lone pair in M^{III}–N̈HR is a weaker base than OH^- and NO does not react with the latter, it is suggested that the nitrosations proceed by a radical mechanism. This suggestion is also attractive as it suggests that the nitrosation of E(H)R and the release of NO from ON–ER[7,22,23] proceed through the same mechanism: a radical mechanism. Finally as *N*-nitroso compounds are carcinogenic[2] this mechanism of reductive *N*-nitrosation might be of biological importance.

Supporting Information (see footnote on the first page of this article): Mass spectra and ^{13}C NMR spectra of pertinent solutions studied.

Acknowledgments

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