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Acid Dissociation Kinetics of the Deprotonated Nickel(II)–Tetraglycinamide Complex

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The values of the pseudo-first-order rate constants for the solvent and acid dissociation of nickel(II) tetraglycinamide, $Ni(H_{-3}G_4a)^-$, increase from 8×10^{-5} s^{-1} at pH 10.7 to 490 s^{-1} at pH 1.08. As many as three protons can assist in the acid dissociation, and at least two form the outside-protonated species $Ni(H_{-3}G_4a)\cdot H$ and $Ni(H_{-3}G_4a)\cdot 2H^+$, with equilibrium constants of $10^{2.4}$ M^{-1} and $10^{1.3}$ M^{-1}, respectively, prior to the loss of the square-planar nickel complex.

Introduction

The ionization of peptide protons from polypeptide ligands is promoted by complexation to metal ions.[1-7] When nickel(II) is the metal ion, the resulting complexes are square planar, diamagnetic, and yellow. The reactions of a variety of metal–peptide complexes, $M(H_{-n}L)$ (where n indicates the number of peptide protons ionized), with acids and with nucleophiles have been studied.[5-10] These reactions display both general-acid and specific-acid catalysis. A simplified scheme for the reaction of a metal–peptide complex with acid (with the formation of a single outside-protonated species) is given in eq 1 and 2. General-acid catalysis is observed when the

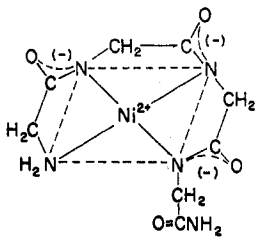
$$M(H_{-n}L) + HX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M(H_{-n}L)\cdot H + X \qquad (1)$$

$$M(H_{-n}L)\cdot H \xrightarrow{k_2} \text{products} \qquad (2)$$

protonation step (eq 1) is rate determining. If the outside-protonated species, $M(H_{-n}L)\cdot H$, formed in eq 1 is slow to dissociate ($k_2 \ll k_{-1}[X^-]$), k_2 becomes rate determining and specific-acid catalysis is observed. The mechanism in eq 1 and 2 is simplified in that k_1 can represent H^+ transfer to either the peptide oxygen or the peptide nitrogen. The peptide oxygen is protonated in the outside-protonated species,[11,12] whereas, when k_1 is the rate-determining step and general-acid catalysis is observed, the proton transfer occurs directly to the peptide nitrogen.

More than one deprotonated group may be present in the peptide $M(H_{-n}L)$ complex; consequently, more than one outside-protonated species may be formed in the course of the acid dissociation reaction. This has been observed in several instances;[7,8,12] however, in previous cases it has not been possible to account individually for these proton steps.

The kinetics and mechanism of the acid decomposition of the nickel(II)–tetraglycinamide complex, $Ni(H_{-3}G_4a)^-$ (structure I), are now presented. From pH 7 to 2, the acid dissociation rate for the $Ni(H_{-3}G_4a)^-$ complex is substantially slower than that observed for its tetraglycine (G_4^-) and triglycinamide (G_3a) analogues, and this permits the decomposition rates of $Ni(H_{-3}G_4a)^-$ to be extended to 0.1 M $HClO_4$. Examination of the observed acid dissociation rate constants indicates the addition of one proton for each of the three deprotonated peptide groups. The first and third protonations cause the most dramatic changes in reactivity.



I, $Ni(H_{-3}G_4a)^-$

Experimental Section

Chromatographically pure tetraglycinamide hydrochloride was obtained commercially (Cyclo Chemical Co.). Stock $Ni(ClO_4)_2$ solutions were standardized against EDTA with a murexide indicator,[13] and free-base triethylenetetramine was prepared as described elsewhere.[7]

The $Ni(H_{-3}G_4a)^-$ solutions were prepared by the slow addition of NaOH to a solution of $Ni(ClO_4)_2$ and $G_4a\cdot HCl$ (1:1.1 mole ratio) until a pH of 10–10.5 was attained. These solutions were freshly prepared for each series of experiments in order to avoid ligand autoxidation.[14,15]

The ionic strength of all solutions was maintained at 1.0 with $NaClO_4$ and the pH measurements were made with an electrode pair (Sargent S 30080-15C and S 30050-15C) having saturated NaCl as the electrolyte in the calomel reference electrode. The pH meter and electrodes were initially adjusted with standard buffers (pH 4.01 and 6.86). The pH readings obtained for several standard $HClO_4$ solutions (0.001–0.2 M) at an ionic strength of 1.0 ($NaClO_4$) were then used to yield the relationship $-\log [H^+] = pH + 0.29$. This relationship was used to determine the H^+ concentrations above pH 2.

All reactions were run under pseudo-first-order conditions in well-buffered solutions at 25.0 °C. A Cary 16 or a computer-interfaced[16] Durrum stopped-flow spectrophotometer was used to monitor the reactions at 255 nm [$\epsilon \approx 7300$ M^{-1} cm^{-1} for $Ni(H_{-3}G_4a)^-$]. In several cases, the disappearance of the $Ni(H_{-3}G_4a)^-$ complex also was followed at 410 nm ($\epsilon = 170$ M^{-1} cm^{-1}). The value of the rate constants obtained were independent of the monitoring wavelength.

The observed pseudo-first-order rate constant, k_{obsd}, is defined by the rate expression

$$-d[Ni(H_{-3}G_4a)]_T/dt = k_{obsd}[Ni(H_{-3}G_4a)]_T$$

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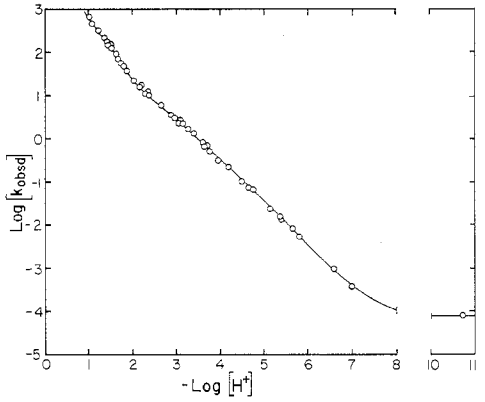

Figure 1. pH dependence of the observed rate constant for the reaction of $Ni(H_{-3}G_4a)^-$ with acid. The solid line is calculated from eq 10 by using the constants given in Table II.

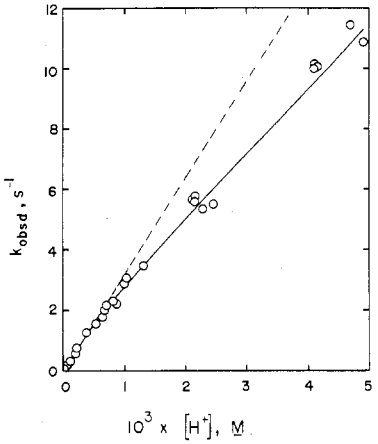

Figure 2. Hydrogen ion dependence for the reaction of $Ni(H_{-3}G_4a)^-$ with acid at intermediate acid concentrations. The solid line is calculated from eq 10 by using the constants given in Table II. The dashed line is extrapolated from the data at low $[H^+]$ by asssuming only a first-order $[H^+]$ dependence.

where $[Ni(H_{-3}G_4a)]_T = [Ni(H_{-3}G_4a)^-] + [Ni(H_{-3}G_4a)\cdot H] + [Ni(H_{-3}G_4a)\cdot 2H^+]$ (T = total). The reported k_{obsd} values are the average of four to seven replicates. At the highest acidity used (0.10 M H^+), the k_{obsd} value becomes very large (490 s^{-1}) and approaches the limit of the stopped-flow instrument.

Results

The acid decomposition of the $Ni(H_{-3}G_4a)^-$ complex was investigated from −log $[H^+]$ 1.08 to 7.02 in the presence and absence of buffers (Table I, Figure 1). The identity of the buffer did not affect the decomposition rate, indicating that the reaction is specific acid catalyzed to at least $[H^+] = 0.02$ M. Although a plot of log k_{obsd} vs. −log $[H^+]$ appears to be almost linear, deviations from a simple first-order behavior in $[H^+]$ are obvious in the plot of k_{obsd} vs. $[H^+]$ (Figures 2 and 3).

The pH dependence of the observed rate constant (k_{obsd}) is complex. As the pH decreases from 11 to 1, the slope of the plot of log k_{obsd} against −log $[H^+]$ (Figure 1) goes through a number of significant changes. Proceeding from high to low pH, the following changes are observed: (1) The slope changes from zero to −1 in the range of −log $[H^+] = 7$–4. (2) It then becomes less negative, reaching a value of −0.8 at a −log $[H^+] = 3$. (3) As the acid concentration increases, the slope once again tends toward a more negative value until, at −log $[H^+] = 1.5$, it is −1.5. (4) At still lower values of −log $[H^+]$, the slope becomes less negative, approaching a value of −1 once again.

Table I. Observed Protonation Rate Constants for the Reaction of $Ni(H_{-3}G_4a)^-$ with Acid[a]

−log $[H^+]$	buffer[b]	k_{obsd}, s^{-1} [c]	−log $[H^+]$	buffer[b]	k_{obsd}, s^{-1} [c]
1.08		490[d]	3.06	G	2.18 ± 0.07
1.22		316 ± 4	3.09	G	2.27 ± 0.04
1.34		218 ± 8	3.15	C	2.14 ± 0.04
1.35		217 ± 6	3.17	A	2.0 ± 0.1
1.40		176 ± 5	3.19	C	1.71 ± 0.01
1.40		184 ± 4	3.28	C	1.64 ± 0.03
1.40		177 ± 5	3.29	C	1.56 ± 0.04
1.45		157 ± 2	3.41	C	1.26 ± 0.03
1.48		138 ± 2	3.62	C	(7.9 ± 0.6) × 10^{-1}
1.51		124 ± 1	3.63	C	(7.61 ± 0.06) × 10^{-1}
1.61		93 ± 2	3.67	G	(6.3 ± 0.2) × 10^{-1}
1.62	C	85 ± 3	3.73	G	(4.8 ± 0.1) × 10^{-1}
1.62		90 ± 9	3.76	A	(4.98 ± 0.06) × 10^{-1}
1.69		69.8 ± 0.6	3.78	G	(4.9 ± 0.2) × 10^{-1}
1.75		54.1 ± 0.6	3.98	G	(2.89 ± 0.01) × 10^{-1}
1.80		47.7 ± 0.5	4.20	C	(2.09 ± 0.05) × 10^{-1}
1.87	G	36.5 ± 0.4	4.21	C	(2.14 ± 0.03) × 10^{-1}
2.04	G	21.5 ± 0.2	4.51	A	(9.60 ± 0.02) × 10^{-2}
2.17	G	15.1 ± 0.4	4.67	A	(6.88 ± 0.01) × 10^{-2}
2.18	G	15.7 ± 0.1	4.68	A	(6.72 ± 0.01) × 10^{-2}
2.31	G	10.9 ± 0.3	4.77	C	(5.95 ± 0.07) × 10^{-2}
2.33	G	11.5 ± 0.1	4.77	C	(6.12 ± 0.07) × 10^{-2}
2.38	C	10.1 ± 0.2	5.14	A	(2.21 ± 0.03) × 10^{-2}
2.39	C	10.0 ± 0.2	5.37	C	(1.47 ± 0.02) × 10^{-2}
2.39	C	10.2 ± 0.1	5.37	C	(1.57 ± 0.02) × 10^{-2}
2.61	G	5.52 ± 0.06	5.42	C	(1.27 ± 0.02) × 10^{-2}
2.64	G	5.3 ± 0.1	5.67	A	(7.7 ± 0.2) × 10^{-3}
2.65	G	5.6 ± 0.1	5.81	A	(5.23 ± 0.05) × 10^{-3}
2.67	C	5.8 ± 0.2	6.59	Mes	9.0 × 10^{-4} [e]
2.68	C	5.59 ± 0.02	6.61	Mes	9.4 × 10^{-4} [e]
2.88	G	3.46 ± 0.02	7.02	Pipes	3.57 × 10^{-4} [e]
2.98	C	3.06 ± 0.05	7.02	Pipes	3.61 × 10^{-4} [e]
3.00	C	2.89 ± 0.02			

[a] $[Ni(H_{-3}G_4a)^-]_T = 5.0 \times 10^{-4}$, $\mu = 1.0$ ($NaClO_4$), 25.0 °C, λ = 255 nm. [b] Key: A, acetic acid (0.025 M); C, citric acid (0.025 M); G, glycine (0.025 M); Mes, 2-(*N*-morpholino)ethanesulfonic acid (0.02 M); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (0.02 M). [c] Error limit is 1σ for four to seven replicates. [d] Corrected from the experimentally obtained value of 450 s^{-1} to account for the effect of mixing on fast reactions. See: Owens, G. D.; Taylor, R. W.; Ridley, T. Y.; Margerum, D. W., submitted for publication. [e] Single runs.

The reaction mechanism proposed to account for the observed acid dependence is given in eq 3–9. The observed

$$Ni(H_{-3}G_4a)^- \xrightarrow{k_{0d}} \text{products} \quad (3)$$

$$Ni(H_{-3}G_4a)^- + H^+ \underset{}{\overset{K_{1H}}{\rightleftharpoons}} Ni(H_{-3}G_4a)\cdot H \quad (4)$$

$$Ni(H_{-3}G_4a)\cdot H \xrightarrow{k_{1d}} \text{products} \quad (5)$$

$$Ni(H_{-3}G_4a)\cdot H + H^+ \overset{K_{2H}}{\rightleftharpoons} Ni(H_{-3}G_4a)\cdot 2H^+ \quad (6)$$

$$Ni(H_{-3}G_4a)\cdot 2H^+ \xrightarrow{k_{2d}} \text{products} \quad (7)$$

$$Ni(H_{-3}G_4a)\cdot 2H^+ + H^+ \overset{K_{3H}}{\rightleftharpoons} Ni(H_{-3}G_4a)\cdot 3H^{2+} \quad (8)$$

$$Ni(H_{-3}G_4a)\cdot 3H^{2+} \xrightarrow{k_{3d}} \text{products} \quad (9)$$

pseudo-first-order rate constant (k_{obsd}) is given by eq 10.

$$k_{obsd} = \frac{k_{0d} + K_{1H}[H^+](k_{1d} + K_{2H}k_{2d}[H^+] + K_{2H}K_{3H}k_{3d}[H^+]^2)}{1 + K_{1H}[H^+] + K_{1H}K_{2H}[H^+]^2} \quad (10)$$

The first-order hydrogen ion dependence observed from pH 7 to 4 is accounted for by the reactions given in eq 4 and 5.

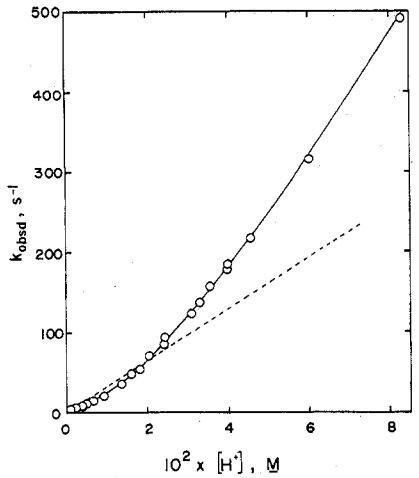

Figure 3. Hydrogen ion dependence for the reaction of $Ni(H_{-3}G_4a)^-$ with acid at the highest acid concentrations. The solid line is calculated from eq 10. The dashed line is extrapolated from the data at low $[H^+]$ by assuming only a first-order $[H^+]$ dependence.

The increasing (less negative) slope observed in the vicinity of $-\log [H^+] = 3$ (Figures 1 and 2) is attributable to the formation of appreciable concentrations of the mono-outside-protonated species $Ni(H_{-3}G_4a)\cdot H$. That is, below $-\log [H^+] = 3$, the reactant changes from $Ni(H_{-3}G_4a)^-$ to the $Ni(H_{-3}G_4a)\cdot H$ species.

At $-\log [H^+] = 1.7$, the slope in Figure 1 is -1.5. Since the slope is significantly more negative than -1, more than one proton is assisting the decomposition of the $Ni(H_{-3}G_4a)\cdot H$ species in this pH region. The absence of a full $[H^+]^2$ dependence in the range of $[H^+] = (0.5\text{–}10) \times 10^{-2}$ M (Figure 2) indicates that both first-order (eq 6 and 7) and second-order (eq 6–9) hydrogen ion terms contribute to the observed rate constant. The change in the slope of the log k_{obsd} vs. $-\log [H^+]$ plot from -1.5 toward a value of -1 at the highest acid conditions investigated indicates the formation of appreciable concentration of a second protonated species, $Ni(H_{-3}G_4a)\cdot 2H^+$ (eq 7). Since the slope does not become more positive than -1, appreciable concentrations of the $Ni(H_{-3}G_4a)\cdot 3H^{2+}$ species (eq 8) are not observed. Hence, we conclude that K_{3H} is less than 1 M^{-1}.

As a reliable estimate for the solvent dissociation rate constant (k_{0d}) for the $Ni(H_{-3}G_4a)^-$ species could not be obtained from the reactions with acid, it was determined at high pH in the presence of triethylenetetramine (trien). The reaction of $Ni(H_{-3}G_4a)^-$ with trien to produce $Ni(trien)^{2+}$ and G_4a conforms to the rate expression $-d[Ni(H_{-3}G_4a)^-]/dt = (k_{0d} + k_T[trien]_T)[Ni(H_{-3}G_4a)^-]$. At $-\log [H^+] = 10.74$ (μ = 1.0 ($NaClO_4$)), the rate constants k_{0d} and k_T are $(8 \pm 4) \times 10^{-5}$ s^{-1} and $(2.14 \pm 0.04) \times 10^{-2}$ M^{-1} s^{-1}, respectively.

The remaining equilibria and rate constants were evaluated by using a nonlinear least-squares computer program.[17] A weighting factor of $1/(0.1k_{obsd})^2$ was used. The resolved values for K_{1H}, k_{1d}, K_{2H}, k_{2d}, and $K_{3H}k_{3d}$ are 261 ± 1 M^{-1}, 12.3 ± 0.5 s^{-1}, 21.4 ± 0.3 M^{-1}, 42.7 ± 0.5 s^{-1}, and $(8.92 \pm 0.01) \times 10^3$ M^{-1} s^{-1}, respectively. The solid lines in Figures 1–3 are calculated from eq 10 by using these values, and Table II provides a comparison of these constants with those previously obtained for the analogous nickel complexes of tetraglycine (G_4^-) and triglycinamide.

Discussion

Square-planar deprotonated nickel(II)–peptide complexes having four strong donor groups generally display specific-acid catalysis in their reactions with acid.[5,10] The formation of one or more outside-protonated species is expected and has been observed in a number of cases.[6,12]

Table II. Comparison of Protonation Constants and Dissociation Rate Constants for Nickel(II)–Peptide Complexes Containing Three Deprotonated Nitrogens

	ligand		
	G_4a[a]	G_4^-[b]	G_3a[c]
	$Ni(H_{-3}L)$		
k_{0d}, s^{-1}	8×10^{-5}	1.6×10^{-5}	2×10^{-4}
	$Ni(H_{-3}L)\cdot H$		
K_{1H}, M^{-1}	$10^{2.4}$	$10^{4.2}$	$10^{2.6}$
k_{1d}, s^{-1}	12	5.6	208
$K_{1H}k_{1d}$, M^{-1} s^{-1}	3.2×10^3	7.1×10^4	7.5×10^4
	$Ni(H_{-3}L)\cdot 2H$		
K_{2H}, M^{-1}	$10^{1.3}$	$\sim 10^{1.5}$[d]	
k_{2d}, s^{-1}	43		
$K_{2H}k_{2d}$, M^{-1} s^{-1}	9.1×10^2		
	$Ni(H_{-3}L)\cdot 3H$		
K_{3H}, M^{-1}	(<1)[e]		
k_{3d}, s^{-1}	(>8.9×10^3)[e]		
$K_{3H}k_{3d}$, M^{-1} s^{-1}	8.9×10^3		

[a] μ = 1.0 ($NaClO_4$). [b] Reference 12; μ = 0.1 ($NaClO_4$). [c] Kupchunos, B.; Margerum, D. W., unpublished results; μ = 2.0 ($NaClO_4$). [d] Estimated from data in ref 12. [e] See text.

Low-spin, deprotonated nickel(II)–peptide complexes remain square planar when outside-protonated species are formed. The rate-determining step corresponds to the cleavage of one Ni–deprotonated-N(peptide) bond with the concurrent disappearance of the square-planar species.[12] This was shown to be the case for the $Ni(H_{-3}G_4a)^-$ complex by observing the disappearance of the 410-nm absorption band when a solution containing $Ni(H_{-3}G_4a)^-$ was mixed with acid (final $-\log [H^+] \approx 1.3$). Under these conditions, the observed first-order reaction was too fast to allow a precise determination of the initial absorbance; however, extrapolation to $t = 0$ yielded an apparent molar absorptivity at 410 nm of 200 ± 50 M^{-1} cm^{-1} for the reactant. This is in reasonable agreement with the value of 170 M^{-1} cm^{-1} for the $Ni(H_{-3}G_4a)^-$ species and indicates that the outside-protonated species, which are formed to a substantial degree under these conditions, are still low-spin square-planar complexes.

For the $Ni(H_{-3}G_4a)^-$ complex there are three potential protonation sites (the three deprotonated peptide groups, structure I), and a change in reactivity is observed for the reaction of three protons. The equilibrium constants for the first two protonations are $10^{2.4}$ and $10^{1.3}$ M^{-1}, while the third protonation constant is less than 1 M^{-1} (Table II). The value obtained for the first protonation constant ($K_{1H} = 10^{2.4}$) is similar in magnitude to those obtained for the protonations of $Ni(H_{-3}G_3a)^-$ (log $K_H = 2.3$)[7,12] and of $Pd(H_{-2}G_3)^-$ (log $K_H = 2.2$).[9]

The solvent dissociation rate constants k_{0d} and k_{1d} for the $Ni(H_{-3}G_4a)^-$ species are intermediate between the values obtained for the analogous G_4^- and G_3a complexes of nickel(II). For $Ni(H_{-3}G_3a)^-$, k_{1d} is a factor of 20 larger than k_{1d} for either $Ni(H_{-3}G_4a)^-$ or $Ni(H_{-3}G_4)^{2-}$. The ligands in the latter two complexes have the potential to form internal hydrogen-bonding associations,[12,18] and this may be the source of the observed differences in k_{1d} values.

For $Ni(H_{-3}G_4)^{2-}$, the first outside-protonation constant is $10^{4.1}$ M^{-1}. The increased K_{1H} value for this complex can be attributed to intramolecular hydrogen bonding between the protonated peptide oxygen and the adjacent carboxylate group

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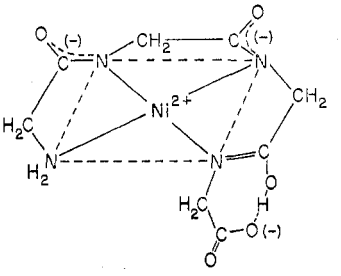
of the tetraglycine molecule (structure II).[12] Similar internal



II, $Ni(H_{-3}G_4)\cdot H$

hydrogen-bonding effects also have been observed for a series of copper(II) peptides which contain L-histidine as the third amino acid residue.[7,8] In addition to increasing the value of the outside-protonation constant, the presence of intramolecular hydrogen bonding shows that the terminal deprotonated peptide group is the first site to be outside protonated in the $Ni(H_{-3}G_4)^{2-}$ complex (structure II).[12] The order in which the three deprotonated peptide groups of the $Ni(H_{-3}G_4a)^-$ complex are outside protonated is not readily apparent as no one site is obviously more basic than any other.

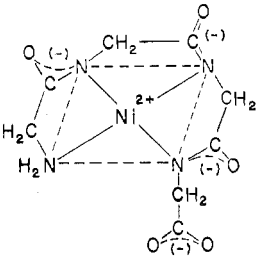
As has been observed for $Ni(H_{-3}G_4)^{2-}$ and $Ni(H_{-3}G_3a)$, the addition of a single H^+ to the $Ni(H_{-3}G_4a)^-$ species results in an enormous ($>10^5$) increase in the dissociation rate. The second protonation has little additional effect, while the third proton addition again increases the dissociation rate by an additional factor of at least 10^2 (Table II). This behavior could be accounted for in one of several ways: (1) Both the first and third protonations greatly diminish the cfse of the Ni^{II} complexes, while the second protonation has less effect. (2) Only a relatively small percentage of the terminal (third from the amine end) peptide group is affected by the first two protonations, while the third proton, which is then of necessity added to the terminal peptide group, greatly destabilizes the complex, causing its rapid decomposition. (3) The third proton reacts by direct proton transfer to the peptide nitrogen and not by outside protonation.

Earlier it was shown that the $Ni(H_{-3}G_4a)\cdot H$ and $Ni(H_{-3}G_4a)\cdot 2H^+$ species were low-spin square-planar complexes; thus, the first explanation is unlikely. This conclusion also is supported by the observation that the stepwise outside-protonation constants (K_{1H}, K_{2H}, and K_{3H}) follow the trend predicted by both electrostatic and statistical effects. If outside protonation greatly diminished the cfse of the Ni(II) complex, one might expect that it would be easier, rather than more difficult, to add the second and third protons.

The second explanation requires the basicity of the terminal deprotonated peptide group to be several orders of magnitude less than that of the other two groups. This too seems unlikely, and, in fact, reasons might be given why it would be easier to add a proton to the terminal group.

The third explanation means that the rate constant, which we have written as a product ($K_{3H}k_{3d}$) of an outside-protonation constant and a first-order dissociation rate constant, is actually a single-step second-order rate constant for the direct proton transfer to the peptide nitrogen. If this is the case, no inferences concerning protonation site preferences can be made. If direct proton transfer is occurring, general-acid catalysis of the third-proton addition would be anticipated. Unfortunately, the presence or absence of general-acid catalysis cannot be confirmed because under the experimental conditions needed the rate constants become too large. Furthermore, the only suitable general acids are carboxylic acids, which at higher concentrations are known to have special effects in the presence of outside-protonated species.[19] In addition, a valid estimate of the value of K_{3H} cannot be made if the third proton reacts without formation of an additional outside-protonated species.

The $Ni(H_{-3}G_4)^{2-}$ complex has four potential protonation sites (structure III). Three deprotonated peptide groups and



III, $Ni(H_{-3}G_4)^{2-}$

one ligand carboxylate group may be protonated. Below pH 2 the acid-decomposition rate constant–pH profile[12] for $Ni(H_{-3}G_4)^{2-}$ is very similar to that observed for the $Ni(H_{-3}G_4a)^-$ complex. This is indicative of the addition of two protons to the $[Ni(H_{-3}G_4)\cdot H]^-$ species. Therefore, over the pH range investigated (12.5–1.4), the influence of three of the four possible protonations was kinetically detected. The fourth protonation may not have been observed either because it had little kinetic influence or because it occurred outside the investigable range of rate constants at low pH.

In summary, the first two outside-protonation constants for a nickel(II)–peptide complex having three deprotonated peptide groups are $10^{2.4}$ and $10^{1.3}$ M^{-1}. If the third protonation results in the formation of outside-protonated species, this constant must be less than 1 M^{-1}, and it may be argued that the terminal deprotonated peptide nitrogen is the last site to be protonated. However, it is more likely that the last protonation results in the direct proton transfer to a peptide nitrogen as the rate-determining step, and, if so, no decision as to the order in which the deprotonated peptide groups are protonated may be made.

Intramolecular hydrogen bonding will increase the outside-protonation constant and may also reorient the usual outside-protonation site preferences.

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