Complex Formation of Aliphatic Dipeptides with Zinc(II) and Manganese(II)

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Received March 18, 1982

Formation constants for complexes between zinc (as well as manganese) and a number of aliphatic dipeptides consisting of glycine, alanine, leucine and proline have been determined by potentiometric titration. NMR and ESR spectra have been recorded in order to support the potentiometric results and to get some information about the structure of the detected species.

Introduction

Interaction between proteins or peptides and transition metals plays an important role in biochemistry and biology [1–6]. Traces of Mn(II) and Zn(II) participate widely in biological systems, forming enzyme–metal complexes or combining with proteins [7–12]. Most of the naturally occurring ligands are molecules of extremely complicated structure and composition and thus model substances are frequently used to mimic the metal binding site. For less specific but still highly informative studies of the metal binding ability of these ligands, even studies of dipeptides can supply much information.

Relatively little information is available concerning the complex formation of zinc(II) with aliphatic dipeptides [13–16], but many authors have detected the existence of a simple 1:1 complex $ZnLH^+$ (LH_2 = ^+H_3N–CHR–CO–NH–CHR′–COO^-). A structure similar to $CuLH^+$ was proposed on the basis of IR measurements [13, 14]. Brunetti *et al.* considered an additional species (ZnL_2H_2) and found a reasonable formation constant. There is no information so far about the structure of this 1:2 complex, about possible side chain influences and the existence of further species, which could be detected in the case of copper and nickel. Thus a careful study on a series of dipeptides consisting of glycine, alanine, leucine and proline was performed in this work by means of potentiometric titrations and ^{1}H-NMR spectroscopy.

Manganese(II) forms only weak complexes with aliphatic dipeptides. Datta and Rabin [17] calculated stability constants for gly–gly and pro–gly using potentiometric titrations. Only $MnLH^+$ could be detected. Some invesitgations were carried out by means of NMR, ESR and IR spectroscopy in order to obtain information about the structure of this species [18–22]. An investigation including a larger number of dipeptides was expected to improve knowledge about the species being formed. Thus stability constants were evaluated by ESR spectroscopy as well as potentiometric titrations for the same peptides as in the case of Zn^{2+}.

Experimental

Materials

Metal stock solutions were prepared by dissolving $Zn(NO_3)_2 \cdot 4H_2O$ and $MnCl_2 \cdot 4H_2O$ in CO_2-free water. The concentrations were examined by complexometric titrations. The dipeptides gly–gly, gly–d,l-ala*, gly–d,l-leu*, gly–l-pro, d,l-ala–gly*, d,l-ala–d,l-ala*, d,l-ala–d,l-leu*, l-ala–l-pro, d,l-leu–gly*, d,l-leu–d,l-leu*, l-pro–gly, l-pro–l-ala and l-pro–l-leu were obtained from Sigma Chemical Co., generally of Sigma analytical grade.

Physical Measurements

Formation constants were calculated from potentiometric titrations as described previously [23]. Solutions for ESR spectroscopy were prepared by potentiometric titrations: during titration 0.2 ml were taken as ESR probe and the spectra were recorded on a Varian E-104 A spectrometer. The concentration of manganese chloride was 0.00201 *M* in all experiments. The metal/ligand ratio was varied from 1:4 to 1:5.5. All measurements were carried out at 20 °C and ionic strength of 0.20 *M* KCl. At least 10 ESR spectra were recorded in order to evaluate one manganese dipeptide system.

*These dipeptides were used in the d,l-form. The calculated formation constants are therefore mean values for all present stereoisomers.

0020-1693/82/0000-0000/$02.75

TABLE I. Complex Formation Constants of Zinc and Manganese with Aliphatic Dipeptides.

Dipeptides	$lgK^{Zn^{2+}}_{ZnLH^+}$	Δ	$lgK^{Zn^{2+}}_{ZnL_2H_2}$	Δ	$lgK^{Mn^{2+}}_{MnLH^+}$	Δ[e]	$lgK^{Mn^{2+}}_{MnLH^+}$[f]
gly–gly	3.25[a]	0.08	6.20	0.09	1.90	0.38	1.83
	3.45[b]		6.31[b]		2.19[c]		
gly–ala	3.47	0.06	6.51	0.04	2.22	0.16	1.79
gly–leu	3.50	0.08	6.55	0.07	1.93	0.25	1.94
gly–pro	4.03	0.14	7.63	0.10	2.34	0.11	2.27
					2.29[c]		
ala–gly	2.71	0.11	5.52	0.09	1.93	0.11	1.85
ala–ala	2.97	0.10	5.74	0.10	1.86	0.12	1.95
ala–leu	2.93	0.09	5.67	0.10	1.83	0.19	1.89
	3.20[d]						
ala–pro	3.28	0.10	6.21	0.09	1.91	0.19	1.96
	3.68[d]						
leu–gly	2.77	0.16	5.61	0.13	2.01	0.20	1.91
leu–leu	2.76	0.04	5.27	0.07	2.15	0.10	1.96
	3.00[d]						
pro–gly	3.58	0.08	7.00	0.07	2.39	0.21	2.34
pro–ala	3.69	0.13	7.32	0.08	2.46	0.25	2.57
pro–leu	3.61	0.13	7.59	0.08	2.47	0.14	2.40

[a]This work 0.20 *M* KCl 20 °C. [b]Ref. 15 0.10 *M* KNO_3 25 °C. [c]Ref. 17 ? 25 °C. [d]Ref. 16 0.5 *M* KNO_3 20 °C. [e]Δ: measure for significance of the constants. Changing the constant by Δ leads to an increase of $\sum_i (v_i^{theoret.} - v_i^{calc.})^2$ by a factor 2 [23, 24]. [f]Calculated by means of ESR spectroscopy (±0.25).

The NMR spectra were obtained at a Varian EM 3602 NMR spectrometer. The dipeptide concentration was kept constant at 0.100 *M*, while the zinc/dipeptide ratio was varied from 1:1 to 1:5. t-Butanol (0.1%) was taken as internal standard. All NMR investigations were carried out at 32 °C and ionic strength of 0.10 *M* KCl. A special FORTRAN program was developed to evaluate the recorded spectra.

Results

Zinc Complexes

Potentiometric Titrations

In the following, LH_2 denotes the zwitterionic dipeptide, $^+H_3N–CHR–CO–NH–CHR'–COO^-$. The following reactions have been considered:

K_1^*: $LH_2 + H^+ \rightarrow LH_3^+$

K_2^*: $LH_2 \rightarrow LH^- + H^+$

K_3: $Zn^{2+} + LH_2 \rightarrow ZnLH_2^{2+}$

K_4: $Zn^{2+} + LH^- \rightarrow ZnLH^+$

K_5: $Zn^{2+} + LH^- \rightarrow ZnL + H^+$

K_6: $Zn^{2+} + 2LH^- \rightarrow ZnL_2H_2$

K_7: $Zn^{2+} + 3LH^- \rightarrow ZnL_3H_3^-$

The investigations of all systems prove that only the species $ZnLH^+$ and ZnL_2H_2 are formed in detectable amounts. Although the complexes ML and $ML_3H_3^-$ are clearly defined in copper and nickel systems respectively, these species cannot be detected in the case of zinc. The reason for this may be found in the decreasing complex stability in the series Cu > Ni > Zn, as proposed by Irving and Williams [25], which holds for the species MLH^+ and ML_2H_2 [23, 24]. Estimation of formation constants for the species ZnL and $ZnL_3H_3^-$ according to the Irving–Williams series predict very low concentrations for these species regarding the pH range until 8. Above pH 8 precipitation of $Zn(OH)_2$ occurs. The formation constants of $ZnLH^+$ and ZnL_2H_2 are listed in Table I.

NMR Measurements

NMR spectra of the dipeptides gly–ala, ala–gly and ala–ala have been recorded in the absence and presence of zinc ions in order to obtain information about the structure of the complexes. The chemical shifts of the methyl groups have been calculated using the following method:

*The protonation and dissociation constants of the dipeptides were taken from our previous work [24].

TABLE II. Specific Chemical Shifts of the Dipeptides: Glycylalanine, Alanylglycine and Alanylalanine.

Species	Dipept.	δ_{A_1} [a]	δ_{A_2} [a]	J_A [b]	δ_{B_1} [a]	δ_{B_2} [a]	J_B [b]	δ_{C_1} [a]	δ_{C_2} [a]	J_C [b]	pK
LH_3^+	gly–ala							1.7	0.9	0.8	−3.19
	ala–gly	2.0	1.4	0.6							−3.18
	ala–ala	2.7	1.1	0.6	2.0	1.4	0.6	1.7	0.9	0.8	−3.19
LH_2	gly–ala							1.1	0.3	0.8	
	ala–gly	2.3	1.5	0.8							
	ala–ala	2.4	1.6	0.8	2.2	1.4	0.8	1.1	0.3	0.8	
LH^-	gly–ala							1.1	0.3	0.8	[c]
	ala–gly	0.6	−0.2	0.8							8.28
	ala–ala	0.4	−0.3	0.5	0.5	−0.2	0.7	1.0	0.2	0.8	8.31
$ZnLH^+$	gly–ala							1.0	0.3	0.8	[c]
	ala–gly	2.0	1.3	0.7							[c]
	ala–ala	1.8	1.1	0.7	1.5	1.0	0.5	1.1	0.4	0.7	[c]
ZnL_2H_2	gly–ala							1.0	0.3	0.7	[c]
	ala–gly	1.4	0.8	0.6							[c]
	ala–ala	1.6	0.8	0.8	1.6	0.8	0.8	1.1	0.3	0.8	[c]

[a] δ: Chemical shift in cps in comparison with t-butanol, which was used as internal standard (1 cps = 0.0167 ppm). A, B indicate the N-terminal methyl group, C the C-terminal one. [b] J: coupling constant for the coupling with the αH in cps. [c] For these calculations the formation constants have been taken from potentiometric titrations (Table I).

If the exchange rate of the species in solution is very high, the measured NMR signals $PPM_{i,j}$ (i indicates the different peaks in one dipeptide spectrum, j indicates different spectra) consist of the contributions $x_{j,k}$ (k indicates different species) of all existing species [26]:

$$PPM_{i,j} = \sum_k x_{j,k} \cdot \delta_{i,k} \qquad \sum_k x_{j,k} = 1$$

$\delta_{i,k}$ are the specific chemical shifts of the species existing in the solution. Almost 100% of the dipeptide exist in the zwitterionic form within the pH range 5.5–6.0. Thus, δ_{i,LH_2} could be obtained directly from such solutions. Decreasing the pH leads to protonation of the dipeptide. The constant K_1 and δ_{i,LH_3^+} were calculated evaluating the pH range 3 to 6 by means of an iteration method. $\log K_1$ and δ_{i,LH_3^+} have been varied until the error F reached a minimum, F being defined by

$$F = \left[\sum_i \sum_j \left(\sum_k (x_{j,k} \cdot \delta_{i,k}) - PPM_{i,j}\right)\right]^2$$

K_2 and δ_{i,LH^-} were obtained in a similar way at pH values larger than 6. If zinc(II) is added to solutions containing dipeptides, complexes are formed. The formation constants and chemical shifts of the complexes can be computed again taking into account the data of the dipeptide (except for such cases, where changes of the chemical shifts $\delta_{i,k}$ and variations of the constants could replace each other). This condition was not fulfilled, however, and it was impossible therefore to get significant formation constants for $ZnLH^+$ and ZnL_2H_2. Using the formation constants obtained by potentiometric titrations, however, the specific chemical shifts $\delta_{i,ZnLH^+}$ and δ_{i,ZnL_2H_2} could be calculated. The data are listed in Table II.

Structure of $ZnLH^+$ and ZnL_2H_2

The formation of $ZnLH^+$ and ZnL_2H_2 depends on the basicity of the dipeptides and on the N-terminal side chain similar to copper and nickel complexes [23, 24]. A plot of logK *versus* pK_2 according to Rabin [27] shows this dependence (Fig. 1). As complex formation increases with increasing pK_2 and decreases when the N-terminal side chain is enlarged, the amino group and the carbonyl oxygen are expected to coordinate to the zinc ion forming a five-membered chelate ring. The C-terminal side chain apparently causes no significant steric hindrance, indicating that the carboxyl group is not involved in the ion binding. An analysis of the NMR spectra confirms this assumption. Figure 2 shows the dependence of the chemical shifts of ala–ala on the pH for two systems. The dotted line was obtained from pure dipeptide solutions, the full one from zinc dipeptide mixtures. The chemical shift of the C-terminal methyl group does not change signifi-

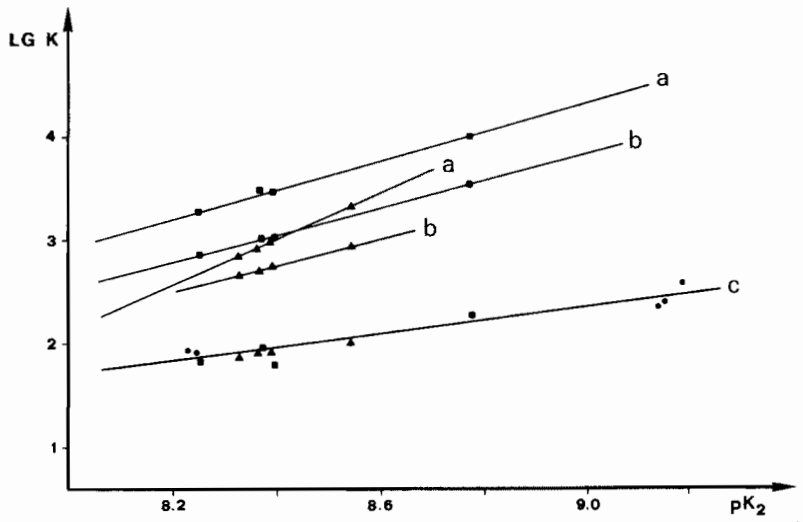

Fig. 1. A plot of formation constants *versus* pK_2: a: log $K^{Zn^{2+}}_{ZnLH^+}$; b: log $K^{ZnLH^+}_{ZnL_2H_2}$; c: log $K^{Mn^{2+}}_{MnLH^+}$. ■: glycine–X dipeptides. ▲: alanine–X dipeptides; ●: leucine– and proline–X dipeptides.

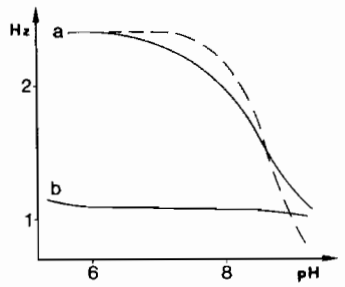

Fig. 2. The dependence of the chemical shift on the pH: a: chemical shift of N-terminal methyl group (lowest field peak); b: chemical shift of the C-terminal methyl group (lowest field peak). —— [ala–ala] = 0.100 *M*, $[Zn^{2+}]$ = 0.00200 *M*; – – – [ala–ala] = 0.100 *M*.

cantly, if metal ion is added (Fig. 2b). The NMR signal of the N-terminal methyl group, however, shows a distinct shift for both systems starting at pH = 5, where complex formation also begins. The calculated chemical shifts of all species of the dipeptides gly–ala, ala–gly and ala–ala support this result (Table II). The values of $\delta_{i,ZnLH^+}$, δ_{i,ZnL_2H_2} and δ_{i,LH^-} are close together for the C-terminal methyl group (1.1–1.0 and 0.4–0.3), whereas significant changes are found for the N-terminal one (2.0–0.6 and –0.2–1.3).

Species Distribution

Complex formation starts at pH = 5 (Fig. 3). The first species is $ZnLH^+$, ZnL_2H_2 is formed at pH > 6. The concentration of the 1:1 complex reaches a maximum at pH 7–7.5, whereas the concentration of ZnL_2H_2 increases continuously. Its maximum cannot be detected because of the beginning precipitation of zinc hydroxide at pH > 8. Increasing dipeptide concentrations shift the begin of the complex formation to lower pH values, and the formation of ZnL_2H_2 is favoured. At physiological pH (7.4) the following species distribution is to be expected: regarding a 2:5 metal/ligand ratio the concentration of the free zinc ion is between 30 and 60 percent of the total zinc concentration, depending on the kind of dipeptide being used. The concentration of $ZnLH^+$ (20–40%) exceeds the amount of ZnL_2H_2 (5–20%). Increase of the metal/ligand ratio to 1:5 leads to a higher concentration of ZnL_2H_2 (20–40%) and a slight decrease of free metal ion in solution (10–50%). At twenty-fold dipeptide excess still 1% Zn^{2+} ions exist in their hydrated form.

Manganese Complexes

All titration curves could be simulated satisfactorily taking into account only the complex species $MnLH^+$. No evidence was found for the existence of $MnLH_2^{2+}$ or MnL_2H_2. A small amount of $MnLH_2^{2+}$ cannot be excluded, however, as this species is hardly detectable by means of potentiometric titrations. The reason for the detection of only one manganese complex can be attributed to a decrease of all stability constants according to the Irving–Williams series [25] and to the restricted pH range, as precipitation occurs at pH > 7.5.

ESR measurements were performed in order to examine the formation constants for $MnLH^+$. Tiezzi *et al.* [19] had derived concentrations of the free metal ion recording ESR spectra and compared these values with published potentiometric data. They could not find good agreement, however,

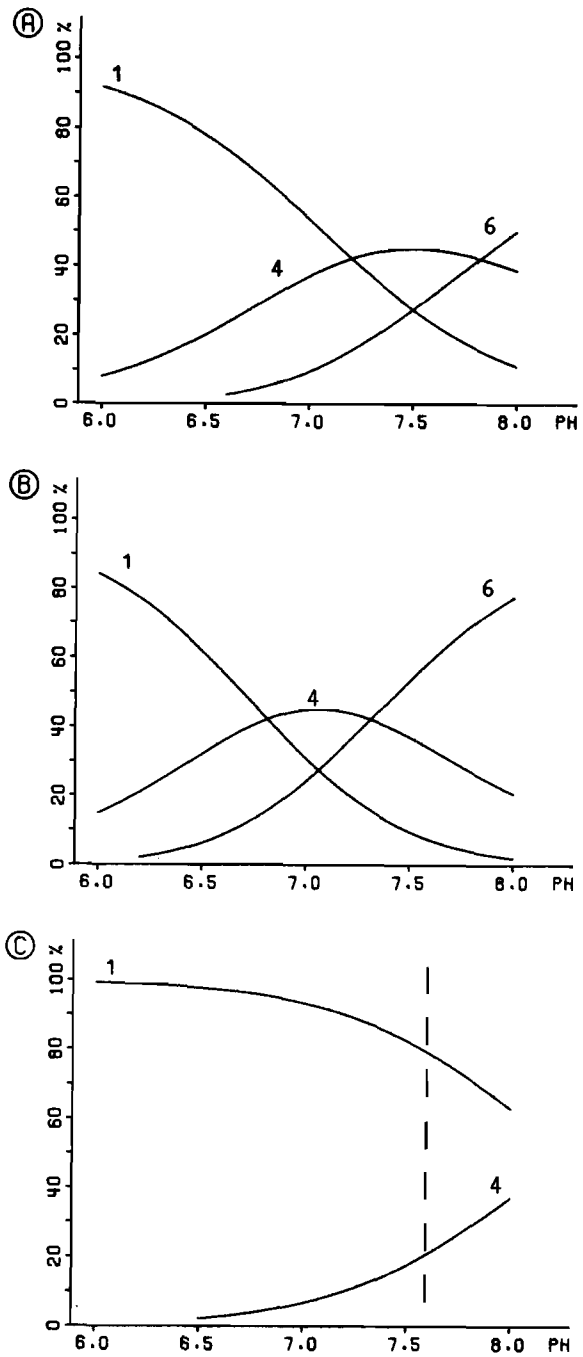

Fig. 3. Species distribution depending on metal/ligand ratio and pH: A: $[Zn^{2+}]$ = 0.00200 *M*, [gly–pro] = 0.00500 *M*; B: $[Zn^{2+}]$ = 0.00200 *M*, [gly–pro] = 0.0100 *M*; C: $[Mn^{2+}]$ = 0.00200 *M*, [gly–pro] = 0.0100 *M*. – – – precipitation occurs. 1: M^{2+}, 4: MLH^+; 6: ML_2H_2.

which may be attributed to different experimental conditions. The following evaluation lead to very consistent results:

Small changes of ligand field symmetry cause a strong line width variation in the case of Mn(II) ESR spectra [28–31]. Thus, only the hexaquo complex can be detected and the height of the signals can be used to measure the concentration of the free manganese ion, if the line width does not change during a series of measurements [19]. As the manganese concentration was kept constant and the excess of the dipeptide was relatively small, no change of the line width could be observed. Thus it was possible to compute the formation constant of $MnLH^+$ by calibrating the height of the signals using an ESR spectrum of a pure manganese solution. The obtained values are very similar to the potentiometric data (Table I). This agreement confirms the existence of only one species ($MnLH^+$). Otherwise the ESR computed constants should be higher indicating the formation of some additional species.

Figure 1c demonstrates the dependence of the formation constant on the pK_2 of the dipeptides. Within experimental error a linear relation can be observed, including all constants. This means that the N-terminal amino group coordinates to the manganese ion. As no steric influence of any side chain is found, the formation of a chelate ring similar to copper, nickel and zinc species may be excluded. NMR measurements support this assumption [20].

The formation of $MnLH^+$ starts at pH 6.5–7.0 and its concentration reaches only a small amount within the studied pH range (Fig. 3c). At physiological pH, the free manganese ion is dominating. Even at relatively high excess of dipeptide a considerable amount of Mn^{2+} remains in uncomplexed form.

Acknowledgement

Financial support by 'Jubiläumsfonds' of the National Bank of Austria (Proj. 1934) and the Federal Ministry of Science and Research (Erl. Zi. 18.854/6- 10/81) is gratefully acknowledged.

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