extremely mild set of conditions required for this rearrangement, suggesting that analogous processes might occur in biological systems.

Acknowledgment. We wish to thank Dr. Koshiro Toriumi of the Institute for Molecular Science and Dr. Kiyotaka Asakura of the Faculty of Science, University of Tokyo, for their technical assistance and helpful suggestions during the course of EXAFS measurements and analysis and Dr. Nobuyuki Watanabe for detecting ketoses in HPLC analysis. This work was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan (63107002, 63470035, 63612002), and by a grant-in-aid from the Mitsubishi Foundation.

Supplementary Material Available: Plots of background-subtracted raw EXAFS data ($\mathbf{k}^3[\chi(\mathbf{k})]$) and the results of curve fitting (observed vs calculated EXAFS oscillations) (4 pages). Ordering information is given on any current masthead page.

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Structural and Conformational Study of the Aluminum-Thymulin Complex Using 1-D and 2-D NMR Techniques

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Received December 15, 1987

The interaction between aluminum and thymulin, a linear nonapeptide of thymic origin isolated from serum, was investigated by means of one- and two-dimensional NMR experiments. These experiments were performed in dimethyl-d_6 sulfoxide solution at different metal:peptide ratios. The results lead the following conclusions: (i) the Al(III) complexation corresponds to a fast exchange on the NMR time scale; (ii) the evolution of ^1H and ^{13}C NMR chemical shifts indicates the existence of one type of complex with a 1:2 stoichiometry, associating two peptide molecules and one Al(III) ion; (iii) analysis of the spectra suggests that Al(III) has a specific binding site involving the Asn^9COO^- terminal group and the hydroxyl group of the Ser^4 residue; (iv) from the NOESY data a conformation has been proposed and compared to the biologically active Zn(II)-thymulin complex.

Introduction

Thymulin, formerly called FTS (for facteur thymique sérique), is a thymic hormone isolated from serum,[1,2] which has been characterized by its capacity to induce T-cell markers and functions on immature cells.[3] Its amino acid sequence was determined to be <Glu^1-Ala^2-Lys^3-Ser^4-Gln^5-Gly^6-Gly^7-Ser^8-Asn^9, and the synthetic hormone proved to be biologically active.[2]

The biological activity of this peptide was shown to be zinc-dependent, and the binding of this metal to thymulin was demonstrated by using labeled molecules ($Kd = (5 \pm 2) \times 10^{-7}$ M).[4] More recently, 1-D and 2-D NMR determinations carried out in DMSO-d_6 led to the conclusion that zinc forms two complexes with the nonapeptide, associating one Zn^{2+} with one (1:1 complex) or two (1:2 complex) peptide molecules. The association constants ($K_1 = 3540$ M^{-1} and $K_2 = 5.0 \times 10^6$ M^{-2} for the 1:1 and 1:2 complexes respectively) are not indicative of a strong interaction and the $Ser^4O\gamma H$, $Ser^8O\gamma H$ and $Asn^9CO_2^-$ sites are coordinated to the metal ion in the 1:1 complex, while in the 1:2 complex the $Ser^8O\gamma H$ site probably no longer interacts with the metal ion.[5]

The importance of metal ions (other than Zn^{2+}) in the biological activity of thymulin has recently been demonstrated.[6]

Among the series of 16 metals investigated in this study,[6] only aluminum and gallium proved to be as active as zinc. However, monoclonal antibodies have been shown to recognize the nonapeptide molecule specifically when complexed with zinc, but neither the free molecule nor the complexes it forms with other metals, notably aluminum.[7]

NMR data indicate the existence on the thymulin molecule of two zinc-specific conformations with a unique structure; it will be of great interest to compare these conformations with those obtained with other metals, and more specifically with aluminum. Such a comparison will be considered very important for the structure-activity relationship, since the thymic hormone is virtually devoid of biological activity.

To this end, we have attempted to obtain information on the conformational states of the nonapeptide-aluminum complex in dimethyl sulfoxide solution by means of the ^1H, ^{13}C, ^{27}Al one- and two-dimensional (^1H, ^1H NOESY) NMR spectroscopy.

From analyses of the spectral data, it is concluded that Al^{3+} interacts with free thymulin to give one kind of complex where the $Ser^4O\gamma H$ and $Asn^9CO_2^-$ sites are bonded in a 1:2 (metal:peptide) complex.

Experimental Section

Materials. Synthetic serum thymic factor was provided by Institut Choay, Paris.[8] $Al(NO_3)_3 \cdot 9H_2O$ was purchased from Sigma. Dimethyl-d_6 sulfoxide (99.95% D) from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) was used as solvent.

One-Dimensional NMR Instrumentation. ^1H NMR spectra at 250 MHz and ^{13}C NMR spectra at 62.8 MHz were recorded on a Bruker WM 250 spectrometer equipped with an Aspect 3000 computer system. Field stabilization was provided by an internal deuterium lock-signal. Samples were examined at 33 ± 1 °C. The usual ^1H spectrometer conditions were 2800-Hz sweep width, 16K data points and 250 scans. ^{13}C NMR spectra were recorded with quadrature detection and broad-band proton decoupling. Spectra were obtained by accumulating 60 000-90 000 transients for a $1.5-10 \times 10^{-2}$ M solution of nonapeptide with 16K data points. A radio-frequency pulse of 90° was used, with a spectral width of 15 000 Hz without repetition time. Both ^1H and ^{13}C

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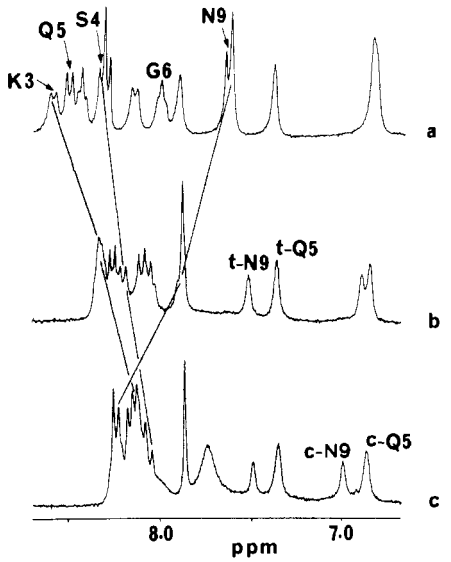

Figure 1. Effect of increasing Al(III):nonapeptide ratios (a, free peptide; b, R = 0.22; c, R = 0.5) on the 250-MHz ^{1}H NMR spectrum of the nonapeptide in DMSO-d_6 solution. Part corresponding to the downfield region is shown.

chemical shifts were measured in parts per million (ppm) with tetramethylsilane as reference.

^{27}Al NMR spectra were generated at 25 °C on a Bruker WM250 spectrometer at 65.18 MHz. Spectra were obtained with a spectral window of 30 kHz, line broadening of 3 Hz, and 45° pulse width of 20 μs. Typically, 8000–10000 transients were collected. It was found that, under the conditions stated, a broad asymmetric resonance some 7 kHz in width occurred at about 70 ppm downfield from the $[Al(H_2O)_6]^{3+}$ reference. This is believed to be attributable to aluminum metal in the probe head. It was therefore necessary to run a DMSO blank and subtract this from each spectrum, which in turn required us to use standard conditions for all spectra.

Two-Dimensional ^{1}H NMR Instrumentation. Two-dimensional experiments were performed on a Bruker AM 400 apparatus at 400 MHz by means of the standard Bruker microprograms and quadrature detection in both dimensions. The 2-D NMR experimental conditions (^{1}H, ^{1}H and ^{1}H, ^{13}C COSY measurements) used for the assignments of the ^{1}H and ^{13}C resonances of the free thymulin (FTS) peptide in DMSO, are described in our preceding paper.[9]

The assignments of the aluminum–thymulin complex were performed by ^{1}H, ^{1}H COSY (not shown) and ^{1}H, ^{1}H NOESY techniques on a 0.005 M solution in DMSO of the 0.5 Al(III):peptide ratio. The sample was degassed by several freeze–thaw cycles on a high-vacuum line to remove dissolved oxygen. Classical NOESY spectroscopy in the absolute mode gave weak correlation peaks, and we used the NOESY experiments in the phase-sensitive mode.[10] The sequence 90°–t_1–90°–τ_m–90°–acquisition (t_2) was performed with a mixing time of 350 ms. A 10% random variation of mixing time was applied to cancel scalar correlation effects and to minimize the t_1 noise. Other conditions were spectral width in F_1 and F_2 of 4000 Hz, 512 experiments with 96 scans of 2048 points, and data points in t_1 zero-filled to give a 2048 × 2048 data matrix; only the 1.024 × 1.024 real part is plotted, and sine bell shifted $\pi/8$ multiplication in t_2 and Gaussian multiplication in t_1 (LB = −2.0; GB = 0.2).

Results

One-Dimensional ^{1}H NMR Studies. Important changes in the positions, widths, and intensities of the lines as well as in the spin–spin coupling constants are observed when the nitrate salt of Al^{3+} is added to the peptide in DMSO-d_6 (Figure 1). The magnitudes of the chemical shift perturbations induced by Al(III) increase with increasing Al(III):peptide ratios R, indicating that the nonapeptide is in fast exchange between its free and complexed states on the ^{1}H NMR time scale.

In order to obtain the stoichiometry of our complex(es), we followed the variations of amide N^1H signals as a function of R.

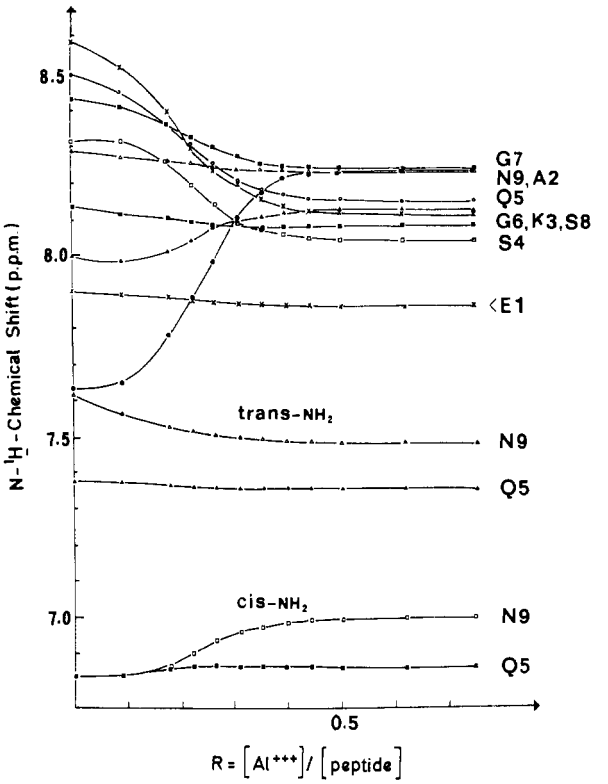

Figure 2. Effect of the Al(III):peptide ratio (R) on the N ^{1}H NMR chemical shifts. Standard single-letter amino acid abbreviations are used.

These variations are depicted in Figure 2 and clearly indicate that the salt-induced changes in the chemical shifts are different for different protons. Both downfield and upfield shifts are observed. Addition of aluminum results in strong shifting of some of the NH signals. The change in chemical shift is linear up to a ratio of ~0.5, where it seems to plateau. These variations differ from those observed after the addition of zinc, which shows rapid variations of amide N^{1}H and ^{13}CO signals with R up to a ratio of 0.5. Beyond this value, the evolution of signals continues, but is slackened. This observation indicates the existence of two types of zinc–peptide complexes (1:2 and 1:1 species).[5,11] Similarly, from the chemical shift change that occurs with aluminum titration, we infer that the stoichiometry of our complex is 1:2. Contrary to the case of thymulin–zinc complexation, which involves two complex states, the variations of the NMR amide proton signals are quite compatible with the equilibrium

$$Al(III) + 2\,\text{nonapeptide} \rightleftharpoons \text{1:2 complex } (K_2)$$

A complete consideration of the metal complexation process has been reported previously for the Zn(II)–peptide system.[11] An optimization of Rosenbrock's method affords the ^{1}H contributions of free and complexed peptides, together with the association constant K_2, which has been adjusted to $K_2 = (3.0 \pm 0.5) \times 10^6$ M^{-2}. Although in the 1:2 Al(III)–thymulin complex the K_2 association constant is lower than those of the zinc–thymulin system, its concentration remains higher than that of the 1:2 Zn(II)–thymulin complex, which decreases owing to the formation of the 1:1 Zn(II)–thymulin species. Considering the concentration of free and 1:2 complexed thymulin, it appears that, for a peptide concentration of 0.01 M, 87% of the 1:2 complex was predicted for R = 0.5 (Figure 3). The concentration of free peptide steeply decreases upon metal addition, and a concentration of ca. 5% of free thymulin remains at a ratio of R = 1.

While the <Glu^{1}NH, Ala^{2}NH, and Ser^{8}NH proton signals remain practically unshifted upon addition of the aluminum salt, Asn^{9}NH and Gly^{6}NH move downfield, while the remaining NH resonances move upfield. In the carboxamide proton region, only the Asn^9 cis and trans $CONH_2$ signals move notably downfield

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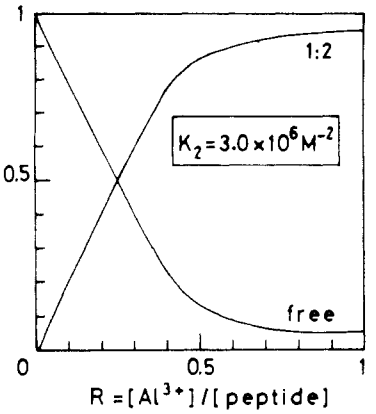

Figure 3. Calculated variations as a function of the Al(III):peptide ratio (R) of the concentration of free and 1:2 complexed thymulin for a peptide concentration of 0.01 M.

and upfield, respectively. It must be noted that all NH resonances remain observable upon addition of Al(III). Thus, none of these nitrogens atoms can serve as ligand where Al(III) could have replaced a proton in the neutral species. All the signal attributions have been confirmed by ^1H, ^1H COSY experiments.

The behavior of the $Ser^{4,8}OH$ and $Lys^3NH_3^+$ resonances is interesting. The free peptide shows a broad hydroxyl resonance at 5.56 ppm while the $Lys^3NH_3^+$ signal can be detected at ~7.8 ppm. The addition of aluminum ions to the free form gradually establishes a process of slow exchange between these protons and the solvent water. As the OH and NH_3^+ resonances sharpen, so does the water absorption near 3.43 ppm. At a ratio of 0.50, the $Lys^3NH_3^+$ peak is observed at 7.74 ppm while the two $Ser^{4,8}OH$ groups resonate at 5.15 and 5.01 ppm. At this ratio, the upfield resonance (Ser^8OH) still remains broad while the downfield one (Ser^4OH) gives a well-resolved triplet ($^3J \approx 2.75$ Hz).

The examination of the aliphatic region also shows some interesting changes, particularly in the 3.6–3.9 ppm region, where the $Gly^{6,7}CH_2$, and the $Ser^{4,8}C\beta H_2$ resonances occur. The free form exhibits a typical four-spin ABX crowded pattern in this region, but aluminum ions yield a degenerate resonance for Gly^6, Gly^7, and Ser^8 while the Ser^4 protons keep an ABXY structure. At the same time, the $Asn^9C\alpha H$ signal shifts downfield by 0.40 ppm while the $Asn^9C\beta H_2$ protons shift downfield by 0.044 and 0.122 ppm, respectively. These protons give rise to a three-spin ABX type from which the observed vicinal coupling constants J_{AX} and J_{BX} may be extracted (J_{AX} = 5.20 Hz and J_{BX} = 7.10 Hz for the free peptide; J_{AX} = 6.05 Hz and J_{BX} = 6.50 Hz for the aluminum complex).

Two-Dimensional ^1H NMR Studies. Nuclear Overhauser effects between nonbonded protons within the range of mutual dipolar relaxations have been a major source of information on the conformations of proteins and peptides. The NOE intensity depends on the character of the motional process, and we have to distinguish between the slow motion limit ($\omega_0\tau_c \gg 1$) corresponding to proteins studied at high field and the fast motion limit ($\omega_0\tau_c \ll 1$) corresponding to small molecules. Generally, linear medium-sized peptides, i.e., molecules of masses between 600 and 1200 Da, have correlation times τ_c of the order of 10^{-9} s at 295 K, which lead to values of $\omega_0\tau_c$ close to unity. This condition often corresponds to a minimum of spin–lattice relaxation time and a minimum of NOE cross-peaks intensities. The τ_m mixing time dependence of the cross-peak intensities in the NOESY experiment is characterized by magnetization-built-up rates. In the first phase, the NOE intensities are proportional to the mixing time τ_m. Nevertheless, additional cross-peaks can occur at short mixing times due to J coupling. These transitions reach through a maximum and undergo subsequent magnetization decay due to spin diffusion.

In order to increase the NOE effects, in small- and medium-sized-peptide studies, the current tendency is to select great values for the τ_m parameter. For example, we have collected the observations of NOE cross-peaks with a value of τ_m = 800 ms in

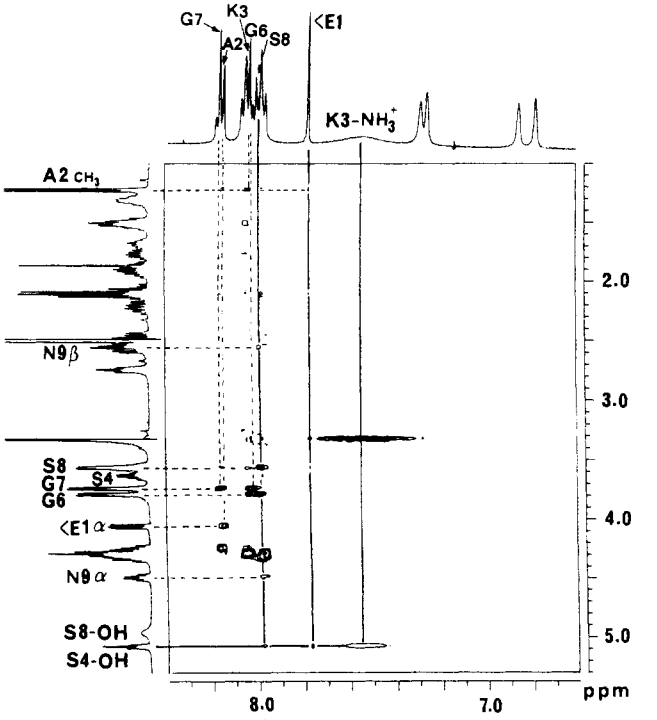

Figure 4. Contour plot of the ^1H NOESY spectrum at 400 MHz of the aluminum complex in DMSO-d_6. Aliphatic chemical shifts appear along the F_1 axis, while the amide chemical shifts appear along the F_2 axis. See the text for the connectivities.

the case of the Gly-Gly-Arg-Ala tetrapeptide in H_2O solution for the observation of the $H_\alpha(i)$–$NH(i+1)$ connectivities[12] and a value of τ_m = 500 ms in the NOESY experiment with the DMSO solution of the linear octapeptide T as proof of the β-turn presence.[13] In the study of the DMSO solution of an antibiotic cyclodecapeptide, griselimycin, the dependence of NOE cross-peak intensities on the mixing time (50–800 ms) indicates a maximum of magnetization at 500 ms.[14] The same results are observed for the DMSO solution of thymulin[9] and Zn(II)–thymulin complexes.[11] For small- and medium-sized peptides, this value of τ_m (500 ms) may be considered as an optimum value of a qualitative dipole–dipole correlation by NOE connectivities with moderate perturbation by spin-diffusion phenomena.

The built-up rates of the NOE's are related to the inverse sixth power of the interproton distances. The fast exchange, occurring between the free peptide and its complexed form, could suggest that the observed off-diagonal peaks come from a weighted average population of the various conformational states.

In previous studies with the free nonapeptide[9] and the zinc complexes,[11] the 2-D NOESY experiments were performed for different mixing times τ_m and for different metal ratios R. The evolution of NOE cross-peaks corresponding to the free conformation and, respectively, to the major population of the 1:2 and 1:1 zinc–peptide species may be observed.

In relation to this behavior, the 2-D NOE spectra of the Al(III)–thymulin complex were recorded in DMSO solution (Figure 4) with the metal ratio R = 0.5 where a population of the 1:2 Al(III)–thymulin species of >85% is present and where a mixing time of 350 ms is used in order to avoid the perturbation of the spin-diffusion phenomena. Furthermore, observation of the behavior of the free-peptide 2-D spectra[9] would immediately suggest that the complexation of thymulin with aluminum is of quite a different nature compared to that of the nonapeptide.

In light of these arguments, it would seem that the observed off-diagonal peaks in the NOESY map (Figure 4) correspond

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Table I. ^{13}C NMR Internal Chemical Shifts between FTS Bound to Al(III) and Its Free Form in DMSO-d_6

resonance	Δ,[a] ppm	resonance	Δ,[a] ppm	resonance	Δ,[a] ppm
<Glu^{1}CδO	+0.29	Ser^{4,8}Cβ	−0.05	Asn^{9}Cα	−2.34
Asn^{9}COO$^-$	−1.51		−0.26	Asn^{9}Cβ	−1.79
Gln^{5}CγO	+0.38	Ser^{4,8}Cα	−0.31	Lys^{3}Cβ	+0.24
Asn^{9}CγO	−1.12		−0.05	Gln^{5}Cβ	+0.57
Lys^{3}CO	+0.26	<Glu^{1}Cα	−0.25	Lys^{3}Cδ	−0.20
Ser^{4}CO	+0.50				

[a] Δ (ppm) = δ(bound) − δ(not bound); (+) downfield shift; (−) upfield shift.

exclusively to the aluminum complex and may be interpreted in terms of structural features.

From Figure 4, representative lines are drawn, connecting spins related by an NOE. A large number of cross-peaks can be distinguished in this spectrum, and connectivities between the labile amide protons from 6.5 to 8.5 ppm and the aliphatic and hydroxyl protons from 1.0 to 5.5 ppm can readily by observed.

Usually, two kinds of information should be obtained from a NOESY map: (i) long-range NOE effects and (ii) either intraresidue effects or nearest-neighbor residue effects. In the latter case, the observed NOE data furnish structural information in terms of the short-range distances d_1 (from NH_{i+1} to $C\alpha H_i$), d_2 (from NH_{i+1} to either NH_{i+2} or NH_i), and d_3 (from NH_{i+1} to $C\beta H_i$).[15] Without attempting to quantify these distances, we can view them as providing a set of constraints on the structure.

Figure 4 shows some of these connectivities as dashed lines. Thus intraresidue effects are observed between Ala^{2}NH/Ala^{2}CβH$_3$, Gly^{7}NH/Gly^{7}CH$_2$, Gly^{6}NH/Gly^{6}CH$_2$, and Ser^{8}NH/Ser^{8}CβH$_2$. In addition to the expected intraresidue NOE effects, a number of interresidue effects are clearly observed between nearest neighbors in the nonapeptide. Thus NOE's between the Ser^8 and Ala^2 amide protons and aliphatic protons of Asn^{9}CβH$_2$A, Asn^{9}CαH, and <Glu^{1}CαH, respectively, give rise to strong cross-peaks, while medium or weak cross-peaks exist between Lys^{3}NH/Ala^{2}CβH$_3$ and <Glu^{1}NH/Ala^{2}CβH$_3$. However, more interesting from the point of view of revealing structure in this complex are the NOE's between nonadjacent residues or long-range NOE effects. For example, it can easily be seen in Figure 4 (solid lines) that there are nuclear Overhauser enhancement connectivities linking the following protons: Ser^{8}NH/Ser^{4}OH, <Glu^{1}NH/Ser^{4}OH, and the ammonium group of the lysine residue with Ser^{4}OH, which gives a very strong cross-peak. It is interesting to note that no NOE effects are found between Ser^{8}OH and any residue of the nonapeptide sequence, contrary to the case of zinc addition, which produces a cross-peak between Ser^{8}OH and the <Glu^{1}NH.[11]

^{13}C NMR Studies. The major influence on the position on the position of a carbon resonance line appears to be the distance of the carbon atom from the metal center. It is a fact that carbon atoms far from the metal-binding site remain unshifted or only undergo a small shift upon metal complexation due, for example, to changes in molecular conformation. In either case, the first change in magnetic environment will be in the immediate vicinity of the metal.

When Al(III) is added to the nonapeptide solution, there is a significant perturbation in the frequencies arising from several groups in this peptide and both downfield and upfield shifts are observed. As with proton resonance, metal titration studies show that the magnitude of the chemical shift perturbations induced by Al(III) increases monotonically in increasing metal ion concentration to $R \approx 0.50$. Beyond this ratio the shift remains practically unaffected; i.e., our system is in a fast-exchange process. Table I summarizes the Δ (ppm) values that are affected by complexation. Observation of the low-field resonances shows that the largest shift occurs for the Asn^{9}COO$^-$ group (Δ = −1.51 ppm), while Asn^{9}CγO is also strongly affected (Δ = −1.12 ppm). It is to be noted that the Ser^{4}CO resonance moves downfield by 0.50 ppm. The remaining resonances are less, or not at all, affected.

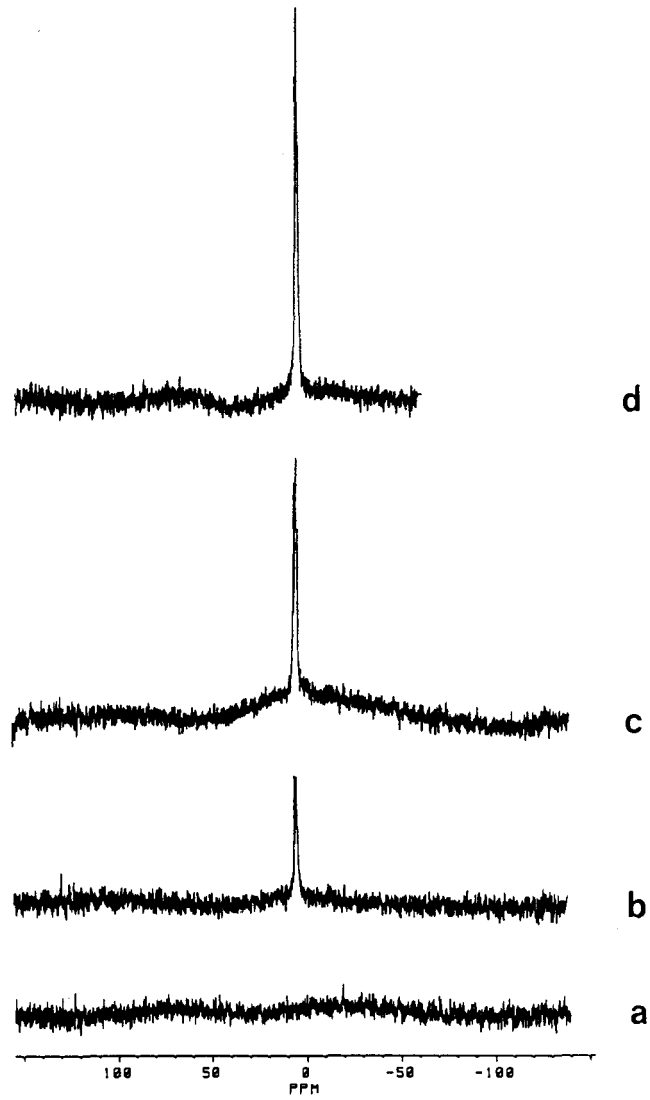

Figure 5. ^{27}Al NMR spectra at 65.18 MHz and 24 °C for several Al(III):nonapeptide ratios (R): a, 0.5; b, 0.58_9; c, 0.67_8; d, 0.76_7.

The carbon resonances belonging to the aliphatic residues show an interesting behavior. As observed for the CO region, peaks corresponding to the asparagine residue (Asn^{9}Cα, Δ = −2.34 ppm; Asn^{9}Cβ, Δ = −1.79 ppm) are more affected. It is interesting to note that the peak corresponding to Gln^{5}Cβ shifts notably downfield by 0.57 ppm while, at the same time, <Glu^{1}CδO and <Glu^{1}Cα move downfield and upfield, respectively. Both Ser^{4,8}Cβ carbons move upfield; nevertheless, the most important shift is that of Ser^{4}Cβ.

^{27}Al NMR Studies. Aluminum-27 is an attractive nucleus on account of its high sensitivity and of a 100% isotopic abundance. However, its spin number, $I = {}^5/_2$, results in a nuclear quadrupolar moment (0.149×10^{-24} cm^2) so that it is sensitive both to the symmetry of its local environment and to molecular dynamics in solution. As a consequence, only the resonances of aluminum in a highly symmetrical environment are sharp enough to be detected. For this reason the ^{27}Al NMR results reported here are confined to the detection of the excess of aluminum up to the stoichiometry of our complex. An example of this effect is shown in Figure 5. As can be seen from this figure, no peaks are observed with R up to a ratio of 0.5. Beyond this ratio, the progressive addition of aluminum salt is accompanied by an increase in intensity resonance due to the excess of metal. These results confirm the presence of a 1:2 complex.

Discussion

NMR Perturbation Induced by Al(III) Complexation. Aluminum, a diamagnetic ion, does not cause line broadening or other

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NMR phenomena usually associated with ligand–paramagnetic ion interactions.[16,17] Hence, well-resolved spectra can be obtained. The chemical shifts resulting from metal binding to the peptide may be due to the direct influence of changes in electron density or may result indirectly from an altered conformation of the peptide. In either case, the first change in magnetic environment will be in the immediate vicinity of the metal. Consequently, from the analysis of metal-induced changes in the NMR spectrum, information about the structure of the metal binding site can be obtained.

The results of metal titration on the ^1H and ^{13}C NMR spectra clearly show that the Asn^9 residue is the most affected, which provides strong evidence that this residue is involved in metal ligation through the C-terminal carboxylate group in a 1:2 complex. The behavior of the ^{13}C chemical shift of COO^- groups is not easily predictable owing to the high number of factors that determined this parameter. This conclusion has been recently supported by Bertini et al.,[18] who reported the contributions to the ^{13}C chemical shifts of different carboxylate groups interacting with diamagnetic cations. Thus, contrary to the observation made with the thymulin–zinc complex,[5,11] the Asn^9 $^{13}COO^-$ shifts upfield. Shifts of similar magnitude and sign have been found by the complexation study of [Leu^5]-enkephalin with Al(III).[19] Furthermore, the decrease in the intensity of the Asn^9 $^{13}COO^-$ peak, due to a modification of the relaxation time, agrees fairly well with its implication as a ligand. These results seem to indicate the key role played by the C-terminal group in the coordination mode, which is consistent with the loss of activity for thymulin analogues with a deleted or substituted Asn^9 residue.

Let us now consider the NMR data depicted in Figure 2 and those reproduced in Table I. Comparison of these values clearly shows that, besides the Asn^9 residue, the -Lys^3-Ser^4-Gln^5-fragment is the most affected upon complexation. As is the case in some conformational changes, these results could be interpreted by the proximity of the aluminum ions in this peptide fragment region.

The behavior of the two serine hydroxyl protons is of interest. The addition of aluminum ions to thymulin provokes the appearance of the two hydroxyl groups due to the slowing down of their exchange with residual water. However, the most downfield peak corresponding to Ser^{4}OH is very well resolved as a triplet, while that for Ser^{8}OH is broad. We tentatively explain this phenomenon by considering a more protected position of the Ser^{4}OH proton, due to o a buried position of this proton inside a folded structure owing to complexation.

The examination of the two Ser^{4,8}CβH$_2$ protons corroborates these findings. Indeed, as observed in Figure 1, the Ser^{4}CβH$_2$ side chain group appears as an ABX spin system, while Ser^{8}CβH$_2$ does not retain its ABX appearance. Experimentally, the ABX pattern of the Ser^4 residue is consistent with a more rigid and constrained conformation of its side chain, due to aluminum binding, while a more flexible environment is expected for the Ser^8 side chain,[20] giving a broad peak for the Ser^{8}OH resonance.

Another point of view seems to be very decisive in favor of the implication of the Ser^{4}OH group in the binding process. This point comes from the comparison of the effects of the Al(III):nonapeptide ratios on the NH NMR chemical shifts with those obtained with two thymulin analogues, [Ala^4]-nonapeptide and [Ala^8]-nonapeptide, complexed with zinc.[11] From the confrontation of the curves corresponding to the aluminum complex and to the [Ala^8] analogue with zinc, it is obvious that both the sign and the magnitude of the NH chemical shift perturbations are very similar. Furthermore, with the [Ala^8] analogue, zinc is coordinated to Asn^{9}COO$^-$ and Ser^{4}OH, as expected with aluminum. However, with the [Ala^4] analogue, where the Ser^{4}OH residue is remote, zinc addition produces a different titration curve.

Both Gly^6 and Gly^7 residues experience moderate perturbation upon complexation. However, gradual addition of Al(III) to thymulin results in the disappearance of the ABX spin system structure observed for the free ligand.[9] This result is indicative of a great mobility of both methylene protons[20] due in part to the breakdown of the β-turn structure encountered in thymulin.[9] These groups require some flexibility in order to fold properly around the metal.

Conformational Interpretation. NOE data are the most significant spectral data for deducing the three-dimensional overall shape of the molecule. The conformational constraints imposed by the metal complexation place the thymulin in a restricted conformation, which is particularly suitable for a 2-D NOE study.

As already observed, long-range NOE effects give interesting information about the molecular conformation. The main features of interest seem to be the long-range NOE effects between <Glu^{1}NH and the Ser^{4}OH and between the ammonium group of Lys^3 and the hydroxyl group of Ser^4. Thus, we may assume a conformation where the <Glu^{1}NH proton brings the amide proton of the pyroglutamic amino acid close to the hydroxyl group of the serine in the fourth position. This proximity may explain the chemical shifts observed for the <Glu^{1}CδO and <Glu^{1}Cα resonances (vide supra). At the same time, the lysine side chain, which is more mobile than that of <Glu^1, approaches the Ser^{4}OH group.

It is interesting to note that, contrary to the zinc complex with thymulin, no measurable NOE's were found between Ser^{8}OH and any other groups.[11]

As has already been observed, the ammonium group of Lys^3 is not essential for the binding but it comes close to the environment of the metal. Abiko et al. have shown that the positive charge of the lysine residue in the third position is a requisite for the expression of the biological activity.[21] Such an effect is of relative importance, as the lysine side chain could be involved in the recognition process. This result agrees with the conclusion obtained from binding studies of glycylhistidyllysine, a growth-modulating tripeptide from plasma, where the lysyl residue is not required for the copper binding but is necessary for the recognition of a cell surface receptor.[22]

On the basis of these observations, it is suggested that the NOE observed between <Glu^{1}NH and Lys^{3}NH$_3^+$ and Ser^{4}OH could be explained by two different forms of the complex conformation: one corresponding to the mobile lysine side chain outside the molecule and the other corresponding to one inside the metal cavity. It is therefore believed that a dynamic equilibrium must exist between these two different conformations. This equilibrium should be fast enough to yield a unique observable NOE. In any case, it becomes evident from these NMR data that the complex possesses a compact structure in DMSO-d_6.

Conclusion

Thymulin is a naturally occurring zinc metallopeptide. Reaction of this peptide with monoclonal antibodies shows that the hormone is only recognized if zinc is present in the molecule, but that is not the case with aluminum. However, the importance of Al(III) ions in the biological activity of thymulin has been demonstrated.

The addition of the Al(III) ions to a DMSO solution of thymulin results in obvious modifications of the ^1H and ^{13}C NMR data. These variations are compatible with the existence of a single type of species having a 1:2 stoichiometry with an association constant $K_2 = 3.0 \times 10^6$ M^{-2}. In this complex, coordination occurs via the Asn^{9}COO$^-$ terminal function and the hydroxyl group of the Ser^4 residue.

From the NOESY spectra it can be concluded that the N-terminal pyroglutamic acid and the ammonium group of the lysine side chain are located near the metal cavity, resulting in a very

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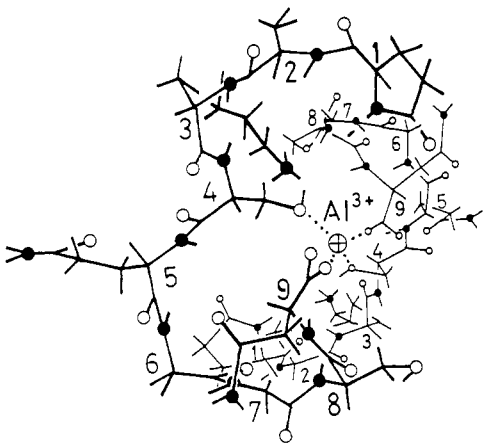

Figure 6. Schematic Dreiding model representation of the 1:2 aluminum:nonapeptide complex in which the long-range NOE effects (<-Glu[1]NH/Ser[4]OH and $Lys^3NH_3^+$/Ser[4]OH) were taken into account.

compact structure. On the basis of our NMR results, the 1:2 complex can be represented as in Figure 6.

The conformation of the Al(III)–nonapeptide complex, as revealed by this NMR study in DMSO solution, may be compared with that of the biologically active Zn(II) complex. Indeed, to interpret the conformational data obtained on our complex, it is of primary importance to determine whether the parameters observed can be correlated with those of the Zn(II) complex. From this comparison, the most significant difference is observed for the hydroxyl group of the Ser[8] residue. From NOESY data, it appears that, with zinc, the Ser[8]OH, which serves as ligand for the metal, gets closer to <Glu[1]NH but that this is not the case with aluminum. These results and those obtained for copper[23] and for thymulin analogues show that the Asn^9COO^- and Ser[4]OH groups play a crucial role in contributing activity but are not sufficient to explain the in vivo behavior of thymulin (FTS–Zn-(II)).

Finally, the results of this study show that the Al(III) complex is identical with one of those formed with Zn(II) (complex 1:2). This similarity may account for the lack of biological activity in vivo of the Al(III)–nonapeptide system. Furthermore, comparisons and correlations between the structure of peptide–metal complexes and their biological activity could lead to better insight into the conformational requirements at receptor sites.

Acknowledgment. This research was supported by the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale. We thank Gerard Commenges for his excellent technical assistance and the Secrétariat for their help in typing the manuscript.

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cis- and *trans*-Dihalotetrakis(dimethyl sulfoxide)ruthenium(II) Complexes ($RuX_2(DMSO)_4$; X = Cl, Br): Synthesis, Structure, and Antitumor Activity

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Received January 28, 1988

The chemistry of halogen–dimethyl sulfoxide–ruthenium(II) complexes with the general formula $RuX_2(DMSO)_4$ (X = Cl, Br) is reported. In particular the synthesis and X-ray structure of *trans*-$RuCl_2(DMSO)_4$ and *cis*-$RuBr_2(DMSO)_4$ are described and compared with those of the already known *cis*-$RuCl_2(DMSO)_4$ and *trans*-$RuBr_2(DMSO)_4$. The structure of a new crystal form of *cis*-$RuCl_2(DMSO)_4$ is also reported. Crystal data: for *trans*-$RuCl_2(DMSO)_4$, tetragonal, $I4/m$, a = 9.121 (3) Å, c = 11.167 (4) Å, Z = 2, R = 0.040; for *cis*-$RuBr_2(DMSO)_4$, monoclinic, $P2_1/n$, a = 8.547 (3) Å, b = 27.873 (4) Å, c = 8.637 (2) Å, β = 115.85 (3)°, Z = 4, R = 0.040; for *cis*-$RuCl_2(DMSO)_4$, monoclinic, $P2_1/n$, a = 8.417 (2) Å, b = 27.695 (4) Å, c = 8.598 (2) Å, β = 116.88 (3)°, Z = 4, R = 0.033. While the cis isomers are thermodynamically more stable and form from the trans species, a photochemically driven cis to trans isomerization reaction is observed in dimethyl sulfoxide solution. Kinetic parameters for the thermal trans to cis isomerization reactions for *trans*-$RuCl_2(DMSO)_4$ and *trans*-$RuBr_2(DMSO)_4$, respectively, are $k = 2.58 \times 10^{-6}$ s^{-1} at 25 °C, $\Delta H^{\ddagger} = 128 \pm 2$ kJ·mol^{-1}, and $\Delta S^{\ddagger} = 110 \pm 5$ J·mol^{-1}·K^{-1} and $k = 1.25 \times 10^{-5}$ s^{-1} at 25 °C, $\Delta H^{\ddagger} = 114 \pm 5$ kJ·mol^{-1}, and $\Delta S^{\ddagger} = 79 \pm 18$ J·mol^{-1}·K^{-1}. In chloroform solution the complexes, and in particular the trans isomers, tend to release a dimethyl sulfoxide molecule to give pentacoordinated Ru(II) complexes. However, in aqueous solution, while the cis complexes immediately release one DMSO, the trans ones release two. In both cases, this step is followed by the slow dissociation of a halide ion. For the chloro derivatives the dissociation is completely inhibited at physiological chloride concentrations. Preliminary results from pharmacological tests show that *trans*-$RuCl_2(DMSO)_4$ is more active than the cis isomer against Lewis lung carcinoma, a metastasizing murine tumor. A remarkable dependence of activity on the halogen nature (Cl > Br) is also observed.

Introduction

cis-$PtCl_2(NH_3)_2$ (cisplatin) is, at present, the most widely used drug in anticancer therapy.[1] After the discovery of its antineoplastic activity in the late 1960s,[2] many efforts have been made to understand its mechanism of action and to improve its therapeutic efficacy. In particular, activity against a broader tumor panel and reduced host toxicity has been sought.

Despite the success obtained in understanding the interactions of *cis*-$PtCl_2(NH_3)_2$ with DNA,[3] which are responsible for its cytotoxicity and, probably, for its antitumor activity, the thousands

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