The nonapeptide <Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn (formerly called serum thymic factor) is a factor produced by the thymic epithelium, which needs a zinc ion to express its immunoregulatory properties. We report here on 1H and 13C NMR investigation of the conformational properties of the free peptide in aqueous medium and in dimethyl sulfoxide-d6 solution, by a combination of homo- and heteronuclear one- and two-dimensional experiments. The various resonances have been assigned in a straightforward manner on the basis of (i) 1H,1H COSY spectroscopy for the recognition of the proton spin systems; (ii) two-dimensional NOESY spectra with the correlation peaks across amide bonds and for the amino acid sequence assignment; (iii) 13C,1H COSY experiments using selective polarization transfer from 1H to 13C-nucleus via the 13C,1H long-range couplings for the attribution of the carboxyl and carbonyl groups; and (iv) 13C,1H COSY experiments with selective polarization transfer via the 13C,1H direct couplings for the assignment of all the aliphatic carbons.

Other experiments such as (i) pH-dependent chemical shifts, (ii) combined use of multiple and selective proton-decoupled 1H and 13C NMR spectra, (iii) the temperature and the concentration dependence of the proton shifts of the amide resonances, (iv) the solvent dependences of peptide carbonyl carbon resonances, and (v) comparison of the spectra with three different analogues were performed. In aqueous solution, the data are compatible with the assumption of a highly mobile dynamic equilibrium among different conformations, whereas in dimethyl sulfoxide-d6, a more rigid structure is found involving three internal hydrogen bonds. These observations provide an insight into the conformational tendencies of this peptidic hormone in two different media.

Thymulin, formerly called serum thymic factor, is a metalloepitopic hormone, selectively produced by thymic epithelial cells, able to induce T-cell markers, and functions on immature cells (1). It is a nonapeptide which was first isolated from porcine serum by Bach and co-workers (2, 3) and ultimately shown to be present in calf thymus extract. Its amino acid sequence was determined (<Glu-Ala-Lys-Ser-Gly-Gly-Ser-Asn), and the synthetic peptide was shown to be fully biologically active (3) in the presence of a zinc ion (4). Nevertheless, a comparative study of the conformational tendencies of free and complexed peptide is of interest to determine the conformation-activity relationships between the two important species.

In this work, we have attempted to obtain information on the conformational states of the nonapeptide in aqueous medium and in dimethyl sulfoxide solution by means of one- and two-dimensional 1H,1H and 13C,1H NMR and CD spectroscopy. The spectra of several analogues with Ala4, Ala5, and Nva5 substitution were also recorded for comparison with thymulin. Complete assignments of the spectra were made by 1H,1H and 13C,1H COSY and 1H,1H NOESY experiments. A conformational analysis of the nonapeptide has been achieved by NMR through measurements of the concentration, temperature, and solvent dependence of chemical shifts.

The combined use of NMR and CD spectroscopy indicates strongly that the nonapeptide in aqueous solution is flexible and is in rapid equilibrium between multiple conformations. In dimethyl sulfoxide-d6, the peptide adopts a partially folded structure stabilized by three intramolecular hydrogen bonds.

NMR Study of Thymulin, a Lymphocyte Differentiating Thymic Nonapeptide

CONFORMATIONAL STATES OF FREE PEPTIDE IN SOLUTION*

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Jean-Pierre Laussac§, Manh-Thong Cung¶, Maurice Pasdeloup‡, Raymond Haran‖, Michel Marraud¶, Pierre Lefrancier‖, Mireille Dardenne§§, and Jean-François Bach‡‡

From the §Laboratoire de Chimie de Coordination, 31400 Toulouse, the ¶Laboratoire de Chimie-Physique Macromoléculaire, ENSIC-INPL, Centre National de la Recherche Scientifique UA 494, 54042 Nancy, Cedex, the ‡Institut Choay, 75016 Paris, and the ‡‡Institut National de la Santé et de la Recherche Médicale U 25, Centre National de la Recherche Scientifique UA 122, Hôpital Necker, 75730 Cedex 15 Paris, France

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§ To whom correspondence should be addressed.
saturation between scans. $^{13}$C NMR spectra were recorded with quadrature detection and broad-band proton decoupling. 30,000–40,000 transients are necessary for a 0.05 M solution of peptide. Other conditions were: spectra width, 15,000 Hz; size, 16,384 data points; repetition time, 0.5 s. Both $^1$H and $^{13}$C chemical shifts were measured in parts/million with 4,4-dimethyl-4-silapentane-1-sulfonate or tetramethylsilane as internal reference. Several proton homodecoupling experiments were performed automatically by a microprogram edited with one frequency list, similar to those of NOE$^1$ difference experiments.

Two-dimensional NMR Instrumentation—All two-dimensional experiments were performed on a Bruker AM-400 apparatus ($^{1}$H 400 MHz, $^{13}$C 100.6 MHz) by using the standard Bruker microprograms and quadrature detection in both dimensions. A $^{1}$H,$^{1}$H COSY 45° was used for minimization of diagonal peaks. For $^1$H,$^1$H COSY 45° experiments, the sequence 90°-t$_1$-45° acquisition was performed; relaxation delay was 2 s, and a 90° pulse for 8.6 $\mu$s was used. For a dimethyl sulfoxide concentration of 0.005 M, the acquisition time was 256 ms; spectral width in $F_1$ and $F_2$ was 4000 Hz; 512 experiments with 64 scans of 2048 points were performed; data points in $t_1$ were zero-filled to give a (1024 x 1024) data matrix; and sine-bell apodization was performed in both dimensions. For a D$_2$O concentration of 0.01 M, the acquisition time was 0.520 ms; spectral widths in both dimensions were 512 points with 32 scans of 1024 points were performed.

For $^1$H,$^1$H NOESY experiments, a 0.005 M solution of the nonapeptide was prepared in dimethyl sulfoxide-d$_6$ (CEA 100%, isotopic purity). The sample was degassed by several freeze-thaw cycles on a high vacuum line to remove dissolved oxygen. The sequence 90°-t$_1$-90°-$\tau_{\text{mix}}$-$\phi$ acquisition was performed with three mixing times (0.2, 0.4, and 0.6 s). A 15% random variation of mixing time was applied to cancel correlation effects. A relaxation delay of 5 s was inserted between scans to ensure quantitative correlation peak intensities. Other conditions were: 90° pulse for 8.6 $\mu$s; acquisition time of 256 ms; spectral width in $F_1$ and $F_2$ of 4000 Hz; 256 experiments with 64 scans of 2048 points; data points in $t_1$ were zero-filled to give a (1024 x 1024) data matrix; and sine-bell multiplication in $t_1$ and sine-bell shifted ($\pi$/4) multiplication in $t_2$.

For $^1$H,$^1$C heteronuclear shift-correlated two-dimensional NMR experiments, the sequence 90°($^1$H)-t$_1$-210°($^{13}$C)-t$_2$-2$\Delta_{\text{1}}$-90°($^{13}$C)-$\Delta_2$ acquisition, proposed by Bax and Morris (8) was applied on a 0.100 M solution of serum thymic factor in D$_2$O. The 90° pulse is 33 $\mu$s for $^1$H and 19 $\mu$s for $^{13}$C.

For aliphatic carbon assignment, the relaxation time was 1.2 s; $\Delta_1$ = 3.7 ms; $\Delta_2$ = 1.85 ms (correlation established for selective polarization transfer via the 135-Hz average direct coupling); the spectral width was 5000 Hz (17–67 ppm) in $F_2$ and 1600 Hz in $F_1$ (1.2–5.2 ppm); 128 experiments with 320 scans and four dummy scans of 2048 points were performed; the acquisition time was 204.8 ms; sine-bell multiplication was performed in both dimensions, and zero filling in $t_1$ (512 points).

For $^{13}$CO signal assignment, the relaxation time was 1.5 s; $\Delta_1$ = 100 ms; $\Delta_2$ = 50 ms (correlation established for selective polarization transfer via the 5-Hz average long-range coupling); the $^J$ and $^J$ long-range coupling are in the 4 to 7.5-Hz frequency interval); spectral width was 1400 Hz (172–186 ppm) in $F_2$ and 1600 Hz in $F_1$ (1.2–5.2 ppm); 256 experiments with 260 scans and eight dummy scans of 1024 points were performed; the acquisition time was 731.2 ms; zero filling in both dimensions; exponential apodization was with LB = 2 Hz in $t_2$ and sine-bell multiplication in $t_1$.

Circular Dichroism—CD spectra were recorded on the Jobin-Yvon Dichrograph III model using fused quartz cells of 0.5-mm path lengths for the peptide spectral region. Sample concentrations were 10$^{-3}$ M. Results are expressed in molar ellipticity [θ] = 3300 $\Delta$ degrees cm$^2$/dnl.

RESULTS

$^1$H NMR Assignments in Aqueous Medium—Due to the fast exchange of amide protons in D$_2$O, aqueous solution of the nonapeptide was investigated only in the 1–5-ppm chemical shifts range. The spin systems of the nonapeptide were determined by $^1$H,$^1$H COSY experiments (Fig. 1). The attribution of Ala$^5$, Lys$^3$, and Asn$^9$ spin systems was immediate. The only ambiguity lies in the distinction of Ser$^l$/Ser$^d$, Gly$^l$/Gly$^d$, and <Glu$^l$/Gln$^d$ spin systems, and this problem was solved by the $^1$H proton and $^{13}$C-carbonyl group two-dimensional NMR correlation.

The correlation peaks of the Lys$^3$ spin systems in the COSY map draw some remarks: (i) a small intensity of cross-correlated peaks between $\alpha$- and one $\beta$-protons indicative of a small value of the corresponding coupling constant and (ii) lack of $\gamma$/$\gamma$ and $\gamma$/$\beta$ cross-correlation peaks probably due to small coupling constants.

The spin decoupling and the pH-dependent chemical shift experiments were consistent with the assignment indicated in Fig. 1. For example, the titration of the Asn$^9$ spin system was observed at pH $\approx$ 3.1 (doublet of doublet centered at 4.522 ppm corresponding to the proton H$^\beta$, multiplet structure at 2.803 and 2.674 ppm for the two protons H$^3$). The four methylene protons of the Lys$^3$ side chain are also pH-dependent at pH $\approx$ 10.8. The remaining resonances of the spectrum are essentially pH-insensitive.

$^{13}$C NMR Assignments in Aqueous Medium—Spin system assignment and sequence determination of peptide residues are a challenge to NMR spectra analysis. Several combined two-dimensional homo- and heteronuclear shift correlations are helpful in solving this problem. To this end, we have used the two-dimensional shift correlation technique developed by Bax and Morris (8) which was also reported elsewhere (9) as a convenient method of assigning CO signals. Two separate heteronuclear shift-correlated two-dimensional experiments using the selective polarization transfer from the proton to the carbon $^{13}$C-nucleus via the scalar couplings were performed on the nonapeptide aqueous solution for assignment of the carbonyl and carboxyl region. In the peptide sequence, each carbonyl $^{13}$CO is coupled with three kinds of protons: two NH, two $\alpha$-protons, and one or two $\beta$-protons (Fig. 2).

The long-range scalar couplings (4 to +7.5 Hz) can be used to assign the carbonyl groups and to obtain the connectivity of the peptide residues. The two-dimensional heteronuclear shift correlation between $^{13}$CO carbonyls and protons was performed by the selective polarization transfer technique via the $\pm 5$-Hz average long-range coupling. In aqueous solution, all amide groups are exchanged; each carbonyl group is only

$^1$ The abbreviation used is: NOE, nuclear Overhauser effect.
NMR Study of Free Thymulin

FIG. 2. $^3J\text{C-H}$ and $^3J\text{C-C}$ scalar coupling of the carbon $^{13}\text{C}_\text{O}$ with several neighboring protons (10).

FIG. 3. Two-dimensional $^{13}\text{C}$,$^1\text{H}$ shift correlation spectrum of the thymulin carboxyl and carbonyl $^{13}\text{C}$ signals in $\text{D}_2\text{O}$. Cross-peaks yield the complete $^{13}\text{C}_\text{O}$ assignment.

coupled to the $\alpha$- and $\beta$-protons of its own residue and to the
$\alpha$-proton of the neighboring peptide residue. All cross-correlated peaks are observed and allowed the attribution of the
carbonyl groups (Fig. 3).

The <Glu$^1$ proton spin system is characterized by the cross-
correlated peak at 184.7 ppm between the <Glu$^1$ $\alpha$-proton and
the C$^\text{O}$ carbonyl group. The cross-peak affords a distinction
between the two C$^\text{O}$ carbonyl groups of <Glu$^1$ and Gln$^6$. Moreover, the <Glu$^1$ and Gln$^6$-CO groups at 177.2 and 176.2
ppm are correlated to the Ala$^2$ and Gly$^6$-$^7$-$^8$ protons, respecti-
vely.

The recognition of the Ser$^4$, Ser$^8$, Gly$^6$, and Gly$^7$ residue
spin systems is straightforward, thanks to the presence of cross-peaks relative to the Lys$^5$-Ser$^4$, Ser$^4$-Gln$^6$, Gln$^6$-Gly$^8$, Gly$^7$-Ser$^8$ and Ser$^8$-Asn$^9$ dyads. All $\beta$-protons are also assigned,
and the conclusion is consistent with the $^1\text{H}$,$^1\text{H}$ COSY map.

The $\text{pH}$ dependence of $^{13}\text{C}$ resonances (Fig. 4) was studied
to obtain eventual long range interactions resulting from
titrations of the different charged groups. The COOH-terminal
Asn$^9$-COO$^-$ carboxylate group and the Lys$^5$-NH$_2$ ammonium
group define two titration domains at $\text{pH} \approx 3.0$ and
11.0. Three CO groups titrate at acidic medium. The Asn$^9$-
COO$^-$ peak at 176.5 ppm undergoes a downfield shift of 2.8
ppm; similarly the Asn$^5$-C$^\text{O}$ at 177.3 ppm titrates downfield
(0.9 ppm), whereas the Ser$^8$-CO is shifted upfield by 0.5 ppm
by the deprotonation of the Asn$^9$-COO$^-$ group. The only
resonance affected at basic pH corresponds to Lys$^5$-CO at
176.5 ppm. The eight remaining resonances are pratrically $\text{pH}$-
insensitive.

For assignment of the aliphatic $^{13}\text{C}$ region, the value of 135
Hz was used as the average $^3J\text{C-H}$ direct coupling in the het-
eronuclear shift-correlated experiment. Cross-correlation
peaks in the $^{13}\text{C}$,$^1\text{H}$ COSY map (Fig. 5) allow the complete
assignment of side chain aliphatic carbons.

The Ser-$^{13}\text{C}$ signal which undergoes a small downfield shift
of 0.1 ppm in acidic medium was identified as Ser$^8$-$^{13}\text{C}$ on the
basis of the proximity effect of the Asn$^9$-COO$^-$ terminal group.
The $\text{pH}$ dependence for aliphatic carbons of the nonapeptide
is similar to that of the carbonyl groups: the Asn$^9$-$^{13}\text{C}$ signals
titrate downfield at $\text{pH}$ 3.1, and all the Lys$^5$-$^{13}\text{C}$ signals

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is similar to that of the carbonyl groups: the Asn$^9$-$^{13}\text{C}$ signals
titrate downfield at $\text{pH}$ 3.1, and all the Lys$^5$-$^{13}\text{C}$ signals
One of the purposes of peptide conformational analyses is the elucidation of the intramolecular hydrogen bonds, which is of great help in gaining knowledge about the different conformers. The most widely used procedure is to measure the temperature dependence of the chemical shifts of amide proton resonances, which shift downfield by 0.10 and 0.12 ppm, respectively. This observation indicates the possibility of the intermolecular associations. Consequently, all our spectra were obtained in dilute solution (10 mM) in order to minimize peptide aggregation.

Exactly as for the aqueous solution, the connectivity within the spin systems of each amino acid residue in the thymulin were obtained by a two-dimensional COSY spectrum (Fig. 6) giving a direct assignment for the proton resonances of <Glu', Ala2, Lys3, Gln5, and Asn9. The absence of cross-peaks between the Lys3-@/-y- and y/-b-proton couples is indicative of small average scalar coupling values.

The Ser4/Ser9 and Gly6/Gly7 spin systems were identified on the basis of NOE experiments which involved the observation of the NOE effects between coupled and uncoupled spectra (Fig. 7). Additional selective homodecoupling experiments complete this attribution. The difference spectrum between coupled and uncoupled spectra (Fig. 8) was used to calculate all coupling constant values in the AXB spin systems of Gly6 and Gly7 (Fig. 8, a and b) and Ser4 and Ser9 (Fig. 8, c and d). 1H NMR data and the rotamer distribution of the Ca-Ca bond in the Ser1, Ser4, and Asn9 residues are given in Table I.

One of the purposes of peptide conformational analyses is to provide no evidence for a change in aggregation of the peptide over the concentration range 0.7-12 mM. The concentration dependence of the chemical shifts of the different NH protons in dimethyl sulfoxide has been examined. The small variations of the chemical shifts observed (upfield of 0.07 ppm for Lys3-NH and 0.05 ppm for Gln5-NH) at concentrations ranging from 0.7 to 12 mM showed that only very few and negligible intermolecular associations. As the concentration is raised, however, concentration dependence of chemical shifts is observed for the Asn9-NH and Gly6-NH resonances, which shift downfield by 0.10 and 0.12 ppm, respectively. This observation indicates the possibility of the intermolecular associations. Consequently, all our spectra were obtained in dilute solution (10 mM) in order to minimize peptide aggregation.
protons. Exposed protons exhibit larger temperature coefficients than do hydrogen-bonded protons (17, 18). The temperature coefficients (dδ/dT) for all amide protons of the nonapeptide are given in Table II.

The one- and two-dimensional nuclear Overhauser effect experiments were also performed with several mixing times. Excepting the short-range NOE effects used for the Ser\(^{\alpha}\)/Ser\(^{\beta}\) and Gly\(^{\alpha}\)/Gly\(^{\beta}\) assignment (Fig. 7), no long-range NOE effect was observed in any of the cases considered. This result seems to indicate the absence of cross-relaxation due to the dipole-dipole interaction between two protons. In this case, the average interproton distances are probably more than 3.0 Å (19).

\(^{13}\)C NMR Spectra in Dimethyl Sulfoxide-d\(_6\).—To avoid an eventual aggregation in dimethyl sulfoxide, the concentration of the peptide solution must be quite low. In these conditions, two-dimensional heteronuclear shift correlations would be exceedingly time-consuming.

Nevertheless, \(^{13}\)C NMR was used to delineate the internal hydrogen bonds in peptides (20, 21). It has been shown that solvent titrations using the solvent pair dimethyl sulfoxide-d\(_6\)/D\(_2\)O can discriminate between solvent-exposed and solvent-shielded peptide carbonyls. Indeed, D\(_2\)O is a good proton donor, whereas dimethyl sulfoxide-d\(_6\) is a proton acceptor, and the resonances of solvent-exposed carbonyls shift downfield on addition of D\(_2\)O to dimethyl sulfoxide-d\(_6\) solutions to a greater extent than do those of solvent-shielded carbonyls. The attribution of the \(^{13}\)C resonances in dimethyl sulfoxide was then achieved by the progressive addition of dimethyl sulfoxide-d\(_6\) to D\(_2\)O. The results are listed in Table III. All resonances shift downfield in the range of 4 ppm to more than 7 ppm. Gly\(^{\alpha}\)-CO is seen to shift least, and is taken as the internal reference. Lys\(^{\alpha}\)-CO, Glu\(^{\alpha}\)-CO, and Ser\(^{\alpha}\)-CO exhibit a small shift relative to Gly\(^{\alpha}\)-CO, whereas all the remaining resonances show substantial shift.

**DISCUSSION**

Nonapeptide in Aqueous Solution.—The nonapeptide has only two titratable protons in the pH range studied (1.70–12.20); they are attributed to the terminal \(\alpha\)-carboxyl group and to the \(\epsilon\)-amino group of the lysine residue. Dissociation shifts of these ionizable groups produce characteristic changes in the chemical shifts of resonances emanating from proton and carbon atoms in the proximity of the titratable group. These parameters, as a function of pH, due to the direct influence of changes in electron density about the individual atoms or indirectly from an altered conformation of the peptide, are able to yield rich information about intramolecular interactions and through-bond and through-space influences (22). One of these indications is obtained from the dissociation constants (pK\(_a\)). Thus, the two pK\(_a\) values obtained are close to that found in small peptides or amino acids (23), whereas the chemical shifts of many of the assigned resonances are very similar to the corresponding chemical shifts observed in these peptides. These findings indicate that the Asn\(^{\alpha}\) carboxyl group and the Lys\(^{\alpha}\) \(\epsilon\)-amino group are not involved in salt-bridge formation or hydrogen bonds with positively or negatively charged groups.

However, the small downfield shift observed for the <Glu1-C-H and Ala4-C-H resonances near pK\(_a\) = 10.6 could be due to spatial closeness of the \(\epsilon\)-amino group and may reflect through-space effects. These observations agree well with the interesting behavior encountered in basic medium. At pH > 11, some of the resonances corresponding to the -Ala\(^{\alpha}\)-Lys\(^{\alpha}\)

**TABLE I**

| Residue | Ser\(^{\alpha}\) | Ser\(^{\beta}\) | Asn\(^{\alpha}\) |
|---------|--------------|--------------|-------------|
| δH\(^{\alpha}\) | 3.59 | 3.53 | 2.36 |
| δH\(^{\beta}\) | 3.69 | 3.63 | 2.51 |
| 3J\(_{\alpha\beta}\)| 4.6 | 5.2 | 6.6 |
| 3J\(_{\alpha\gamma}\)| 5.8 | 4.5 | 5.5 |
| % [rot I] (χ) \approx -60° | 10 | 16 | 30 |
| % [rot II] (χ) \approx 180° | 22 | 10 | 19 |
| % [rot III] (χ) \approx 60° | 68 | 74 | 51 |

* Tentative assignment on the basis of the general attribution by selective \(\beta\)-deuteriation (13–15).

* Percentages of the C\(^{\alpha}\)-C\(^{\gamma}\) rotamers I, II, and III estimated from Ref. 16.

**TABLE II**

| Resonance | b (ppm) | dδ/dT (ppm/°C) x 10\(^{3}\) | 3J\(_{\alpha\gamma\gamma}\) (Hz) |
|-----------|--------|-----------------|-----------------|
| Lys\(^{\alpha}\)-NH | 8.451 | 6.50 | 7.0 |
| Glu\(^{\alpha}\)-NH | 8.340 | 2.66 | 6.8 |
| Gly\(^{\alpha}\)-NH | 8.332 | 3.43 | 5.2, 6.9* |
| Ala\(^{\alpha}\)-NH | 8.200 | 2.98 | 7.5 |
| Ser\(^{\alpha}\)-NH | 8.236 | 3.35 | 7.2 |
| Ser\(^{\beta}\)-NH | 8.035 | 3.15 | 7.6 |
| Gly\(^{\beta}\)-NH | 8.782 | 0.87 | 6.2, 6.1* |
| <Glu1-NH | 7.816 | 3.35 | |
| Asn\(^{\alpha}\)-NH | 7.595 | 1.75 | 7.5 |
| trans-Asn\(^{\alpha}\)-NH2 | 7.445 | 0.53 | |
| trans-Gln\(^{\alpha}\)-NH2 | 7.298 | 3.42 | |
| cis-Asn\(^{\alpha}\)-NH2 | 6.785 | 4.52 | |
| cis-Gln\(^{\alpha}\)-NH2 | 6.785 | 4.52 | |

* Determined on ABX part of H\(^{3}\)-glycine protons.

**TABLE III**

| Peptide residue | D\(_2\)O | Dimethyl sulfoxide-d\(_6\) | Δppm\(^{a}\) | ΔΔppm\(^{b}\) |
|----------------|-------|-----------------------------|----------|----------|
| <Glu1-CO | 184.79 | 177.52 | 7.27 | 3.14 |
| Glu\(^{\alpha}\)-CO | 180.58 | 173.87 | 6.51 | 3.28 |
| Asn\(^{\alpha}\)-COO- | 179.28 | 174.28 | 5.00 | 0.87 |
| Asn\(^{\alpha}\)-CO | 178.20 | 172.90 | 5.30 | 1.17 |
| Ala\(^{\alpha}\)-CO | 177.58 | 172.31 | 5.27 | 1.14 |
| <Glu\(^{\alpha}\)-CO | 177.26 | 171.78 | 5.48 | 1.35 |
| Lys\(^{\alpha}\)-CO | 176.52 | 172.17 | 4.35 | 0.22 |
| Glu\(^{\alpha}\)-CO | 176.19 | 171.78 | 4.41 | 0.28 |
| Ser\(^{\alpha}\)-CO | 174.38 | 169.28 | 5.10 | 0.97 |
| Gly\(^{\alpha}\)-CO | 173.90 | 169.14 | 4.76 | 0.69 |
| Gly\(^{\alpha}\)-CO | 174.38 | 170.25 | 4.13 | 0 |
| Ser\(^{\alpha}\)-CO | 173.27 | 169.02 | 4.95 | 0.12 |

* Δppm = Δppm of D\(_2\)O – Δppm dimethyl sulfoxide-d\(_6\).

* ΔΔppm = relative to Gly\(^{\alpha}\)-CO.
Ser-Gln-fragment are split into two unequal peaks. This demonstrates the existence of two unequally populated states in slow equilibrium on the NMR time scale and confirms the role of the lysine side chain in the conformational dependence in basic condition.

CD spectra have been measured at three different pH values. At pH 2.14, there is a strong negative maximum at 205 nm, $\theta = -3.10^4$ degrees-cm$^2$-dmol$^{-1}$, a very weak positive maximum at 217 nm, $\theta = +0.23 \times 10^4$ degrees-cm$^2$-dmol$^{-1}$, and a negative maximum at 234 nm, $\theta = -0.71 \times 10^4$ degrees-cm$^2$-dmol$^{-1}$. At neutral and basic pH (6.25 and 9.56), two negative bands at 205 nm, $\theta < -3.10^4$ degrees-cm$^2$-dmol$^{-1}$, and 238 nm, $\theta < -0.67 \times 10^4$ degrees-cm$^2$-dmol$^{-1}$, occur, whereas a marked intensity decrease of the positive maximum at 217 nm is observed (Fig. 9).

All the observations made (a very small proximity titration effect upon pH variation, absence of NOE effects through-space, and strong negative peak at 205 nm in the CD spectrum) converge to the conclusion that the nonapeptide probably does not adopt any highly preferred folded conformation in D$_2$O. This nonapeptide is probably flexible and assumes multiple conformations in rapid equilibrium with no substantial contribution of structural features such as $\beta$ turns, internal hydrogen bonds, or hydrophobic interactions.

The unexpected titration deviation at basic pH could suggest that the lysine chain may be giving privileged conformation in this extreme pH domain.

**Peptide in Dimethyl Sulfoxide Solution**—As shown in Table II, when the temperature was raised from 20 to 60 °C, the signal assigned to the trans-carboxamide proton of Asn$^9$-NH$_2$ (24) and the signal of the Gly$^8$-NH proton were little affected. Similarly, the Asn$^9$-NH resonance shows small variation, whereas the Lys$^6$-NH move upfield by 6.5 × 10$^{-3}$ ppm/°C. The lack of effect of the temperature variation on the three trans-Asn$^9$-NH$_2$, Gly$^8$-NH, and Asn$^9$-NH establishes that they are solvent-protected and strongly suggests that they are at least partly involved in intramolecular interactions.

From the solvent titration of peptide CO chemical shifts, it appears that the Lys$^6$-CO, Gly$^8$-CO, and Gln$^5$-CO moieties are solvent-shielded and become candidates for intramolecular hydrogen bonding to be appropriately paired with the delineated peptide NH moieties of the above $^1$H studies.

The temperature dependence and solvent perturbation studies of the peptide NH protons and CO carbons suggest that a possible folded structure (Fig. 10) for the monomeric nonapeptide could contain a 10-membered H bond encompassing the Ser$^4$-Gln$^5$ sequence ($\beta$-turn), an 11-membered H bond between the trans-Asn$^9$-NH$_2$ side chain carboxamide and the Gly$^8$-CO, plus a 13-membered H bond between the Asn$^9$-NH and the Gln$^5$-CO. The small value of the Ser$^4$-CO perturbation can be explained by a rather weak shielding effect exerted by the Ser$^4$-O$^\ominus$H bond on it. However, the absence of cross-relaxation between proton groups in the one- and two-dimensional NOE experiments indicates that this folded structure is probably not predominant or is in rapid equilibrium with other random or open structures. The medium coupling constants observed ($J_{	ext{HN-C}^\beta}$ ranging from 5.2 to 7.5 Hz, Table II) are effectively consistent with fluctuating conformations in rapid equilibrium (25).

The vicinal coupling constants $J_{\text{COH}}$ and $J_{\text{COH}}$ in Ser$^4$, Ser$^5$, and Asn$^9$ indicate a high percentage of the rotamer III ($\chi_1 \neq 60^\circ$) for the three C$^\alpha$-C$^\beta$ bonds (Table I). In the case of the Ser$^4$ residue, this observation is compatible with the $\beta$ turn of the folded structure (26).

Fig. 10 reveals two regions in the peptide structure: (i) a COOH-terminal Lys$^4$-Ser$^4$-Gln$^5$-Gly$^8$-Ser$^5$-Asn$^9$ sequence showing $\beta$-folding tendency and (ii) an exposed solvent NH$_2$-terminal <Glu$^1$-Ala$^2$ sequence.

This result can be related to the partial retention of the radioimmunoassay activity of des-$<$Glu$^1$,Ala$^2$>thymulin analogue (1, 27, 28) which shows the persistence of moderate biological activity.

**CONCLUSION**

The NMR and CD data presented here strongly indicate that the nonapeptide in aqueous solution is flexible and assumes multiple conformations in rapid equilibrium. Temperature dependence and solvent perturbation studies in dimethyl sulfoxide-$d_6$ solution suggest the existence of more defined conformational properties with some amount of a folded structure stabilized by three intramolecular hydrogen bonds. The 3-9 COOH-terminal part of the nonapeptide is solvent-shielded, whereas the <Glu$^1$-Ala$^2$ NH$_2$-terminal part is solvent-exposed. Although the free nonapeptide hormone is not active in vivo, the present studies could help in understanding how the complexation by zinc can modify the ternary structure of the thymulin-zinc complex, making it highly active. Such a comparison was considered very important for the structure-activity relationship since the thymic hormone is virtually devoid of biological activity. Work is now in progress to determine more accurately the exact secondary structure of the folding conformation and the conformational influence of Zn$^{2+}$ complexation.

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