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^{1}H-NMR studies on thymopoietin-type oligopeptides — assignment of the proton resonances and investigation of conformational preferences

WEI GUO,† BÉLA NOSZÁL,*‡ ISTVÁN SCHÖN§ and OLGA NYÉKI§

† *University of California, Chemistry Department, Riverside, CA 92521, USA*
‡ *Eötvös University, Department of Inorganic and Analytical Chemistry, 1518 Budapest 112, P.O.B. 32, Hungary*
§ *Chemical Works of Gedeon Richter, 1475 Budapest 10, P.O.B. 27, Hungary*

Abstract: The thymopoietin-type tripeptides TP3 (HArg-Lys-AspOH), TP(D-Asp)3(HArg-Lys-D-AspOH) and tetrapeptide TP4 (HArg-Lys-Asp-ValOH) were studied by one- and two-dimensional, 500 MHz ^{1}H-NMR spectroscopy in H_2O and D_2O solutions at four different pH values. All proton resonances of the three oligopeptides were assigned by two-dimensional phase-sensitive TOCSY experiments at pH 12.2, 9.1, 5.9 and 3.6. At these pH-values well-defined stages of protonation and concomitant molecular charges exist, allowing different possibilities for intra-molecular and inter-residual orientations. Conformation-sensitive rotating frame nuclear Overhauser enhancement (ROESY) two-dimensional experiments were also performed at the above pH values. These experiments indicated no definite solution conformation of any of the molecules at any pH. Standard one-dimensional experiments were also carried out and three-bond coupling constants were measured for the NH—CH and the Asp CH—CH moieties. The coupling constants provided evidence that non-statistical orientations of the functional groups exist which are changed upon protonation of the basic sites.

Keywords: *Thymopoietin; ^{1}H-NMR spectroscopy; chemical shift assignments; TOCSY; ROESY; coupling constants; conformation.*

Introduction

Thymopoietins are polypeptide hormones indispensable to the immune system [1, 2]. The native, species-specific thymopoietins consist of 49 amino acids [3]. Shorter synthetic segments including thymopentin (TP5, HArg-Lys-Asp-Val-TyrOH, the sequence in the 32–36 region of the natural compound), thymocartin (TP4, HArg-Lys-Asp-ValOH) and thymotrinan (TP3, HArg-Lys-AspOH) also have thymopoietin-like effects [4–6]. The synthetic and pharmacological aspects of thymopoietin fragments were discussed earlier [5], and their acid–base properties have been characterized at molecular and submolecular levels [7], in terms of protonation macroconstants and microconstants, respectively. The conformational properties of TP5 have been studied by empirical potential function calculations and ^{13}C NMR spin-lattice relaxation time measurements [8] and by (one-dimensional) ^{1}H-NMR methods [9]. No data were reported, however, on the NMR properties of TP3 and TP4, nor high field (two-dimensional) NMR studies.

Recent two-dimensional and high field one-dimensional NMR techniques are powerful aids to the evaluation of solution conformation [10]. In addition, NMR is a useful analytical tool for identification of synthetic products and side-products, for monitoring pH- and association-dependent equilibrium processes [11] and for the determination of kinetic parameters [12]. The fundamental information for any further NMR studies is assignment of the proton resonances. One- and two-dimensional ^{1}H-NMR experiments are reported here for assignment of the proton chemical shifts of TP3 (HArg-Lys-AspOH), TP(D-Asp)3(HArg-Lys-D-AspOH) and TP4 (HArg-Lys-Asp-ValOH) at several solution pH values. In order to establish unambiguous chemical shift assignments and possibly conformational information, high field two-dimensional TOCSY (Total Correlation Spectroscopy [13, 14]), ROESY (rotating frame nuclear Overhauser enhancement spectroscopy [15, 16]) and stan-

* Author to whom correspondence should be addressed.

dard one-dimensional experiments were carried out. To provide circumstances similar to *in vivo* solutions, H_2O and D_2O were used as solvent. The use of H_2O as NMR solvent allowed the observation of nitrogen-bound protons in acidic solutions, which would have been NMR-invisible in D_2O.

Experimental

The peptide concentration was 0.2 M or 50 mM in the TP3, TP(D-Asp)3 and TP4 solutions for the two-dimensional TOCSY, ROESY and standard one-dimensional experiments. Solutions of pH* near 12.2 were prepared by using D_2O as solvent, where pH* is the pH-meter reading in D_2O solutions. Solutions of pH at 9.1, 5.9 and 3.6 were prepared by using 90% H_2O and 10% D_2O as solvent. For further details of the solution compositions see the footnotes of Tables 1–3. The pH values were measured by a Fisher AccupHast combined glass–calomel electrode and standardized by using pH 4.000 and pH 9.000 Fisher buffer solutions at 25°C. The electrode-pH equilibrium was attained within a few seconds.

All the NMR experiments were performed on a Varian VXR 500S spectrometer, operating at 500 MHz. The probe temperature was 25°C. The ^{1}H-NMR chemical shifts were measured against internal standard *tert*-butyl alcohol (618.1 Hz or 1.236 ppm). The phase-sensitive two-dimensional TOCSY experiments [13, 14] were performed on TP3, TP(D-Asp)3 and TP4 samples at every reported pH value (Tables 1–3), using a Varian-provided pulse sequence. The H_2O and HDO signals were presaturated through the decoupler. The mixing time was 75 ms and the two trim pulses were 1 ms each. Eight transients were collected for every value of t_1. Typically, a 1K × 128 data matrix was obtained and then zero-filled to a 2K × 1K matrix. Gaussian broadening was used in both dimensions prior to Fourier transformation.

Rotating frame NOE (ROESY) [15, 16] experiments were also performed on TP3, TP(D-Asp)3 and TP4 under the same solution circumstances and pH values as for the TOCSY experiments. The mixing time varied from 0.3 to 0.5 s.

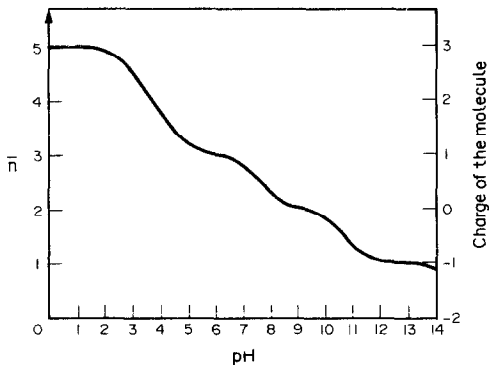

Figure 1
The average number of protons bound to the backbone of a TP3 molecule and the charge of a typical species in solution as a function of pH. TP3 was selected as an example. The analogous diagrams of TP(D-Asp)3 and TP4 are similar to this.

Results and Discussion

Earlier, the protonatation macro- and microconstants of 21 thymopoietin fragments and derivatives, including several diastereomers were reported [7]. These studies revealed that configurational changes of the component amino acids are not accompanied by significantly different basicities. For TP3 and TP(D-Asp)3, the site-specific protonation microconstants are near 10.7, 7.6, 4.2 and 3.2, in log K units. Previous works on the component amino aicds [17–19] demonstrated that constants 10.7 and 7.6 belong essentially to the lysine-ϵ amino and arginine-α amino groups, whereas the constants 4.2 and 3.2 predominantly reflect the aspartic acid β- and α-carboxylate basicities, respectively. The analogous log K values for TP4 are 10.6, 7.6, 4.0 and 3.2, which can be assigned to the lysine ϵ-amino, arginine-α amino, aspartic acid β-carboxylate and valine C-terminal carboxylate site, respectively. The arginine guanidinium group remains positively charged throughout the entire pH range [19]. Figure 1 shows the number of bound protons per peptide molecule and the charge of a typical species in solution as a function of pH. Due to the sufficiently different group-basicities, there are conveniently wide pH ranges (around pH 12.2, 9.1 and 5.9) where the protonation state of the molecules and basic sites is well-defined, and the vast majority of the molecules in solution exist in one particular stage of protonation. The definite charge of basic sites and residues is a precondition of specific intramolecular and inter-residual orientations. Conformational information, therefore can be expected from solutions of such pH values only. Tri- and

tetrapeptides, in general, are too small molecules to exist in specific solution conformations, and they occur in random states. TP3, TP(D-Asp)3 and TP4 are, however, extremely rich in charged groups, their specific inter-residual orientations and the concomitant preferred conformation are more likely than for other peptides. For example, at pH 5.9 TP3, TP(D-Asp)3 and TP4 bear three positive charges (Arg-guanidinium, Lys ϵ-ammonium and Arg α-ammonium groups) and two negative charges (aspartic acid β-carboxylate and C-terminal carboxylate groups). Solutions of pH values (12.2, 9.1, 5.9 and 3.6) were therefore prepared where the protonation states are definite and possible conformational evidence (if any) is most likely to be extracted from the NMR experiments.

As an example, Fig. 2 shows the TOCSY map and the corresponding one-dimensional spectrum of TP4 and pH 3.61. Tables 1–3 contain assigned chemical shifts of TP3, TP(D-Asp)3 and TP4 at four pH values. The assignments were based upon literature values [20] and were verified and completed by the through-bond connectivities of the phase-sensitive two-dimensional TOCSY maps. Earlier acid–base chemistry studies [7] allowed prediction of the deprotonation shift for each residue from which assignments could be confirmed. This was especially important in the 1–2 ppm chemical shift and high pH basicity regions. The H_α of Asp and Lys residues in TP3 have similar chemical shifts at pH above 3.6. In fact, from the two-dimensional TOCSY map it is difficult to differentiate the two signals due to the limited digital resolution which is a feature of most two-dimensional techniques. The same is true for TP(D-Asp)3. The assignments of the Asp and Lys H_α

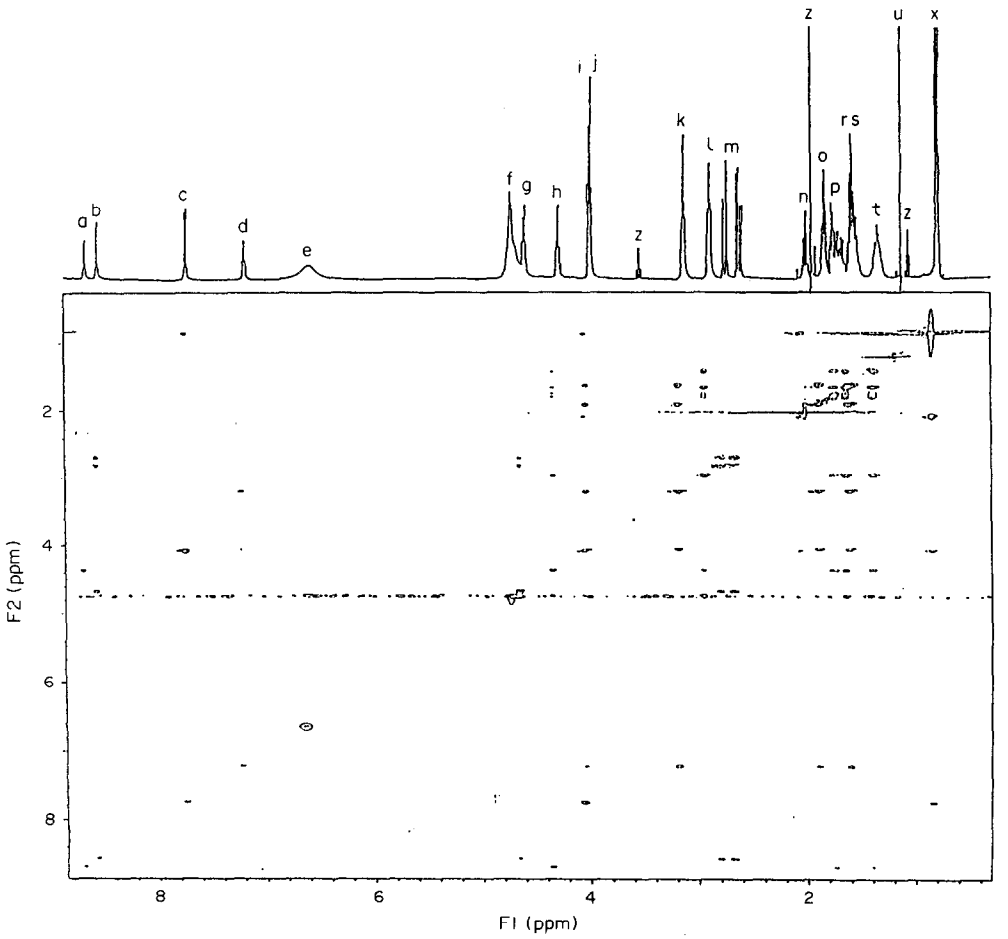

Figure 2
TOCSY map and the standard one-dimensional ^{1}H-NMR spectrum of TP4 at pH 3.61. Assignments and abbreviations are as follows: (a) Lys peptide NH; (b) Asp peptide NH; (c) Val peptide NH; (d) Arg δ-NH; (e) Arg ϵ-NH; (f) residual peak of H_2O; (g) Asp $C_\alpha H$; (h) Lys $C_\alpha H$; (i) Val $C_\alpha H$; (j) Arg $C_\alpha H$; (k) Arg $C_\delta H$; (l) Lys $C_\epsilon H$; (m) Asp $C_\beta H$; (n) Val $C_\beta H$; (o) Arg$C_\beta H$; (p) Lys $C_\beta H$; (r) Lys $C_\delta H$; (s) Arg $C_\gamma H$; (t) Lys $C_\gamma H$; (u) *tert*-butanol (reference); (x) Val $C_\gamma H$; (z) contaminant.

Table 1
^{1}H-NMR chemical shifts of TP3 at 25°C*

Residue	pH†	H_α	H_β	H_γ	H_δ	H_ϵ	NH‡	NH_2‡
Arg	3.66	4.092	1.952	1.654	3.235		7.272§	6.698‖
	5.90	4.071	1.946	1.646	3.226			
	9.10	3.468	(1.68)	1.570	3.182			
	12.20	3.451	(1.67)	1.573	3.180			
Lys	3.66	4.395	1.784	1.464	1.698	3.006	8.767¶	
			1.863					
	5.90	4.413	1.766	1.458	1.693	3.004		
			1.865					
	9.10	4.402	(1.75)	1.444	(1.68)	2.993		
			(1.87)					
	12.20	4.382	(1.70)	(1.38)	(1.45)	2.602		
			1.850					
Asp	3.66	4.502	2.767				8.241¶	
			2.824					
	5.90	4.377	2.564					
			2.667					
	9.10	4.370	2.553					
			2.658					
	12.20	4.366	2.563					
			2.642					

*Values are in ppm relative to resonance of methyl protons of internal standard *tert*-butyl alcohol (1.236 ppm or 618.1 Hz). Values in parentheses are less precise due to overlapping or complexity of spin system.
†Direct pH meter reading.
‡0.2 M TP3 solutions of pH 3.66, 5.90 and 9.10 were made in H_2O–D_2O (90:10). 50 mM TP3 solution of pH 12.20 was made in D_2O. NH and NH_2 resonances were observed at pH 3.66 only.
§Arg-NH_ϵ.
‖Arg-guanidino NH_2.
¶Backbone NH.

Table 2
^{1}H-NMR chemical shifts of TP(D-Asp)3 at 25°C*

Residue	pH†	H_α	H_β	H_γ	H_δ	H_ϵ	NH‡	NH_2‡
Arg	3.62	4.095	1.942	1.644	3.224		7.196§	6.674‖
	5.94	4.044	1.928	1.638	3.209			
	9.13	3.453	(1.65)	(1.56)	3.177			
	12.48	3.445	(1.65)	(1.57)	3.174			
Lys	3.62	4.416	1.792	1.424	1.685	2.999	8.742¶	
			1.870					
	5.94	4.432	1.753	1.420	1.674	2.996		
			1.819					
	9.13	4.398	1.755	1.423	(1.66)	2.989		
			1.818					
	12.48	4.374	(1.71)	(1.36)	1.440	2.592		
			1.812					
D-Asp	3.62	4.534	2.757				8.302¶	
			2.863					
	5.94	4.434	2.526					
			2.703					
	9.13	4.419	2.531					
			2.679					
	12.48	4.389	2.553					
			2.641					

*See corresponding footnote for Table 1.
†Direct pH-meter reading.
‡50 mM TP(D-Asp)3 solution of pH 3.62 was made in H_2O–D_2O (90:10) with 0.3 M $[NH_3OH]Cl$. 50 mM TP(D-Asp)3 solutions of pH 5.94, 9.13, and 12.48 were made in D_2O. NH and NH_2 resonances were observed at pH 3.62 only.
§Arg–NH_ϵ.
‖Arg-guanidino NH_2.
¶Backbone NH.

Table 3
^{1}H-NMR chemical shifts of TP4 at 25°C*

Residue	pH†	H_α	H_β	H_γ	H_δ	H_ϵ	NH‡	NH_2‡
Arg	3.61	4.087	1.942	(1.67)	3.236		7.284§	6.697‖
	5.93	4.038	1.935	1.667	3.222			
	9.12	3.448	(1.65)	(1.57)	3.180			
	12.51	3.438	(1.65)	(1.57)	3.175			
Lys	3.61	4.393	(1.80)	1.446	1.692	2.997	8.758¶	
			(1.87)					
	5.93	4.430	1.772	1.436	1.690	2.991		
			1.856					
	9.12	4.394	1.757	1.444	(1.68)	2.985		
			1.850					
	12.51	4.364	(1.71)	(1.36)	1.437	2.580		
			1.816					
Asp	3.61	4.703	2.725				8.644¶	
			2.855					
	5.93	4.642	2.587					
			2.736					
	9.12	4.638	2.596					
			2.712					
	12.51	4.654	2.589					
			2.698					
Val	3.61	4.105	2.118	0.890			7.826¶	
				0.910				
	5.93	4.032	2.082	0.873				
				0.897				
	9.12	4.030	2.084	0.867				
				0.891				
	12.51	4.044	2.091	0.862				
				0.888				

*See corresponding footnote for Table 1.
†Direct pH meter reading.
‡A 50 mM TP4 solution of pH 3.61 was made in H_2O–D_2O (90:10) with 0.3 M [NH_3OH]Cl. 50 mM TP4 solutions of pH 5.93, 9.12 and 12.51 were made in D_2O. NH and NH_2 resonances were observed at pH 3.61 only.
§Arg-NH_ϵ.
‖Arg-guanidino NH_2.
¶Backbone NH.

resonances were confirmed by taking into consideration the coupling constants between H_α and H_β of Asp from the one-dimensional spectra, where the digital resolution was much better. The H_α resonances of Asp and Lys cross each other at pH 5.9 in TP3; this is not the case for TP(D-Asp)3. In general, TP3 and TP(D-Asp)3 chemical shifts are similar, but their differences in most cases exceed the experimental error. Accuracy of the chemical shift values in Tables 1–3 is proportional to the number of decimals listed.

The two-dimensional rotating frame nuclear Overhauser enhancement experiment, also known as CAMELSPIN [15] or ROESY [16] is of considerable use in conformational NMR studies of molecules in solution [21, 22], in particular, for molecules of intermediate size which give small or zero enhancement in the conventional NOE experiment [23]. ROESY experiments therefore were made on all three peptides at the pH values indicated. These experiments, however, showed through-bond sequential-type connectivities only, and provided no conformational information.

Sensitive, but indirect conformational information can be obtained from three-bond coupling constants of the α- and β-protons of the Asp residue. The Asp carboxyl groups are completely deprotonated above pH 5.9 and no through-bond effects of the lysyl or arginyl ammonium groups can reach the aspartyl moiety, due to their isolation by the several intervening bonds. Hence, any possible alterations of the Asp coupling constants above pH 6 can be attributed to changes in ammonium-carboxylate electrostatic effects upon deprotonation of the arginyl or lysyl ammonium group. Thus, standard one-dimensional experiments were made not only to monitor the pH-

Table 4
$^1H-^1H$ Coupling constants (Hz) of L-Asp in TP3 and D-Asp in TP(D-Asp)3

	pH	$^2J_{\beta-\beta}$	$^3J_{\alpha-\beta(\text{down field})}$	$^3J_{\alpha-\beta(\text{up field})}$	$^3J_{NH-\alpha}$
L-Asp	3.66	−16.36	5.11	7.02	7.54
	5.90	−15.77	4.10	8.72	
	9.10	−15.70	4.18	8.68	
	12.20	−15.75	4.27	8.35	
D-Asp	3.62	−16.40	4.65	7.83	7.71
	5.94	−15.66	3.71	9.83	
	9.13	−15.66	3.92	9.53	
	12.48	−15.80	4.18	8.66	

dependent protonation shifts of the residues, but also to extract the coupling constants (Table 4). Differences were observed (i) between coupling constants of TP3 and TP(D-Asp)3 solutions of the same pH, and (ii) to a lesser extent, between solutions of the same compounds at different pH values. The most significant differences occur between data of the L-Asp and D-Asp containing molecules, indicating that orientation of the C-terminal residue is influenced by the configuration of the Asp moiety.

Comparison of coupling constants belonging to solutions of pH near 5.9, 9.1 and 12.3 allows the conclusion that protonation of the Arg α-NH_2 and Lys ϵ-NH_2 groups influence the rotamer populations of the Asp moieties. This effect is most significant in the case of TP(D-Asp)3 at pH where the Lys ϵ-NH^+_3 group deprotonates.

Table 5 contains three-bond $^1H-^1H$ coupling constants of NH—CH proton–proton vicinal couplings which are also indicators of conformational differences or similarities. Such data can be obtained at low pH only (near 3.6) in H_2O solutions, because the exchange rate of the NH protons is too high at high pH and such protons are 'invisible' in D_2O, due to fast proton/deuterium exchange. The NH—CH coupling constants are difficult to interpret: any such constant may be the result of *cis–trans* amide bond isomerization which can further correspond to four different dihedral angles [24]. No attempt was made to extract structural information from these data. Their differences, however, certainly indicate conformational dissimilarities between the tetrapeptide and tripeptides, and also between the tripeptide diastereomers.

Table 5
Three-bond $^1H-^1H$ coupling constants (Hz) at low pH*: $^3J_{NH-CH_\beta}$ of Arg and $^3J_{NH-CH_\alpha}$ of Lys, Asp and Val in TP3, TP(D-Asp)3 and TP4

	TP3	TP(D-Asp)3	TP4
Arg	5.40	5.32	5.48
Lys	6.90	7.10	6.79
L-Asp	7.54	—	7.18
D-Asp	—	7.71	—
Val	—	—	8.35

*pH 3.66 for TP3, 3.62 for TP(D-Asp)3 and 3.61 for TP4.

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