^{1}H NMR TITRATION SHIFTS OF AMIDE PROTON RESONANCES IN POLYPEPTIDE CHAINS

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Received 14 February 1977

1. Introduction

Observation of amide proton resonances has a prominent role in ^{1}H NMR studies of peptide and protein structure and conformation [1]. Primarily, structural information was so far derived from measurements of the rate of exchange of amide protons with the solvent and the temperature and solvent dependence of the amide proton chemical shifts [1–6]. Since modern instrumentation enables one to obtain quite well resolved resonance lines of amide protons in aqueous solutions of peptides [4,7] and proteins [5,6,8], additional information might be obtained from studies of conformation-dependent pH titration-shifts of labile protons. Sizeable titration-shifts were indeed observed for a number of amide proton resonances in the basic pancreatic trypsin inhibitor [9], and it is quite likely that this will be found to be a general phenomenon in globular proteins.

This paper presents amide proton titration-shifts of selected model peptides. It is suggested that these data serve as a basis for the interpretation of amide proton titration-shifts in terms of the molecular conformations of peptides and proteins.

2. Materials and methods

The protected linear tetrapeptides CF_3CO–Gly–Gly–Gly–L-Ala–OCH_3 and CF_3CO–Gly–Gly–L-Asp–L-Ala–OCH_3 were obtained from Bachem AG, Liestal. H–Gly–Gly–Gly–L-Ala–OH was prepared by hydrolysis of the protected peptide with $Ba(OH)_2$. The purity and composition of the compounds were checked by thin layer chromatography with *n*-butanol/pyridine/acetic acid/water, 50:12:12:25 and by ^{13}C NMR [10]. For the ^{1}H NMR studies, 0.05 M solutions of the peptides in a mixed solvent of 90% H_2O and 10% ^2H_2O were prepared and the pH-value adjusted by the addition of HCl or NaOH.

^{1}H NMR Spectra were recorded with the Fourier transform technique on a Bruker HXS 360 spectrometer. The system was locked on the internal ^2H_2O, and the intensity of the H_2O resonance was reduced by double resonance irradiation. Chemical-shifts are relative to internal sodium-2,2,3,3-*tetra-deutero*-3-trimethylsilyl propionate (TSP).

3. Results and discussion

To obtain a survey of the sign and extent of amide proton titration-shifts in random-coil polypeptide chains, the selection of the peptides used for this study was based on the following considerations. First, amide proton titration-shifts caused by interactions through covalent bonds may arise from protonation–deprotonation reactions of the chain terminal-groups. To investigate these effects without interference with ionizable amino acid side-chains, the tetrapeptide H–Gly–Gly–Gly–L-Ala–OH was used. Second, interactions through covalent bonds with the ionizeable groups of the side chains of Asp, Glu, His, Lys, Tyr and Arg had to be considered. Among these amino acids, the ionizable group of Asp is closest to the backbone, i.e., it is separated by four and five covalent bonds, respectively, from the two nearest amide protons, whereas for all the other amino acids the corresponding distances are five and

six bonds, or more. Therefore, Asp peptides should provide a generally valid upper limit for through-bond side-chain ionization effects. To eliminate interference with the titration shifts from the backbone termini, the protected tetrapeptide CF_3CO–Gly–Gly–L-Asp–L-Ala–OCH_3 was used. Third, the choice of the Asp peptide for studies of amino acid side-chain effects was also indicated for practical reasons. Because of rapid proton exchange with the solvent, amide proton chemical-shifts can not readily be measured for oligopeptides in neutral or basic H_2O solution [1,4], which would make titration studies with His, Lys, Tyr or Arg very difficult, if not impossible.

Some representative ^1H NMR spectra of H–Gly–Gly–Gly–L-Ala–OH are shown in fig.1. The three amide proton resonances were identified with double resonance experiments based on the C_α-proton chemical-shifts known from studies in ^2H_2O. From high to low field one has the lines corresponding to Ala 4, Gly 3 and Gly 2. The terminal amino group is not observed because of rapid proton exchange with H_2O [1]. The titration curves obtained from the pH-variation of the chemical-shifts in the spectra of fig.1 are shown in fig.2. Figure 1 reveals further some quite striking differences between the line intensities of the individual amide proton resonances. The different intensities arise through saturation transfer from the double resonance irradiation of the water resonance and are related to the rate of the proton exchange with the solvent [1]. It is seen that at a given pH the proton exchange rates decrease in the order

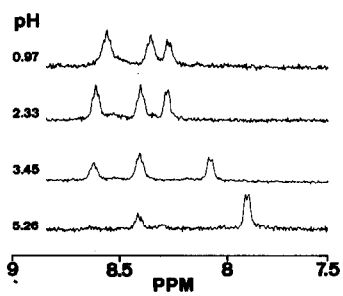

Fig.1. Amide proton resonances in the ^1H NMR spectra at 360 MHz of a 0.05 M solution of H–Gly–Gly–Gly–L-Ala–OH in a mixed solvent of 90% H_2O and 10% ^2H_2O at different pH-values, T = 25°C. The chemical-shift scale is relative to the internal reference TSP.

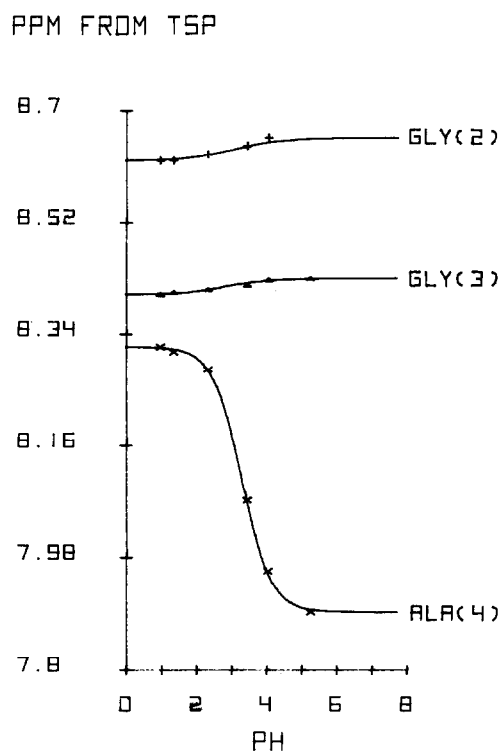

Fig.2. pH Titration-shifts of the amide proton resonances in H–Gly–Gly–Gly–L-Ala–OH at 25°C. The experimental data were corrected for the pH-dependence of the internal reference TSP [11]. The solid lines correspond to least squares fits of one-proton titration curves to the experimental data, pK_a (Ala 4) = 3.33 ± 0.02, $\Delta\delta$(Ala 4) = −0.423 ppm; pK_a (Gly 3) = 2.8 ± 0.5, $\Delta\delta$(Gly 3) = 0.025 ppm; pK_a (Gly 2) = 3.0 ± 0.3, $\Delta\delta$(Gly 2) = 0.037 ppm.

Gly 2, Gly 3, Ala 4. With increasing pH the proton exchange rates increase, so that at pH-values above 5.0 only the amide proton resonance of the C-terminal Ala is observed with the full intensity. These exchange phenomena coincide with earlier observations in related oligopeptides, where the pH-dependence of the proton exchange rates was used for amino acid sequence determination in linear oligopeptides [4].

The pK_a-value of 3.33 for the amide proton titration of Ala 4 coincides with the pK_a-value of the terminal carboxylic acid group determined by ^{13}C NMR. This supports that the titration shift of −0.423 ppm for the amide proton of Ala 4 is a direct consequence of the deprotonation of the C-terminus and can be taken as a characteristic value for C-terminal

amino acid residues in random-coil polypeptide chains. This conclusion is further corroborated by the pH-dependence of C-terminal amide resonances observed by others for different tetrapeptides in the pH-region from approx. 3.1–7.5 (figs.2,3 and 5 of [4]).

Small downfield-shifts with increasing pH were observed for Gly 2 and Gly 3 in H–Gly–Gly–Gly–L-Ala–OH (figs 1 and 2). Since the pK_a-values obtained from these titration-shifts appear to be different from that of the terminal-carboxylate (fig.2), and since Gly 2 shows a somewhat larger shift than Gly 3, we exclude that these shifts come from through-bond interactions with the C-terminus. They are quite possibly a consequence of through-space interactions as they would occur, e.g., in a γ-turn arrangement of the peptide chain. This conformation was previously suggested for the linear tripeptide H–Gly–Gly–Gly–NH_2 [12].

Figure 3 shows the amide proton titration curves for CF_3CO–Gly–Gly–L-Asp–L-Ala–OCH_3. The pK_a-values coincide with those determined by ^{13}C NMR for the carboxylic acid group of Asp 3, indicating that the observed amide proton titration-shifts of 0.036 ppm for Asp 3 and 0.062 ppm for Ala 4 are a direct consequence of the deprotonation of Asp 3. No measurable titration-shift was observed for Gly 2. We conclude that these amide proton titration-shifts are characteristic for non-terminal fragments *–a–*Asp*–c–* in random-coil polypeptide chains. For the reasons outlined above we propose that these values represent upper limits for amide proton titration-shifts caused by any of the common ionizable amino acid residues in position *b* of non-terminal fragments *–a–b–c–* in random-coil polypeptide chains, where *a* and *c* are amino acid residues which do not titrate in the same pH-range as *b*.

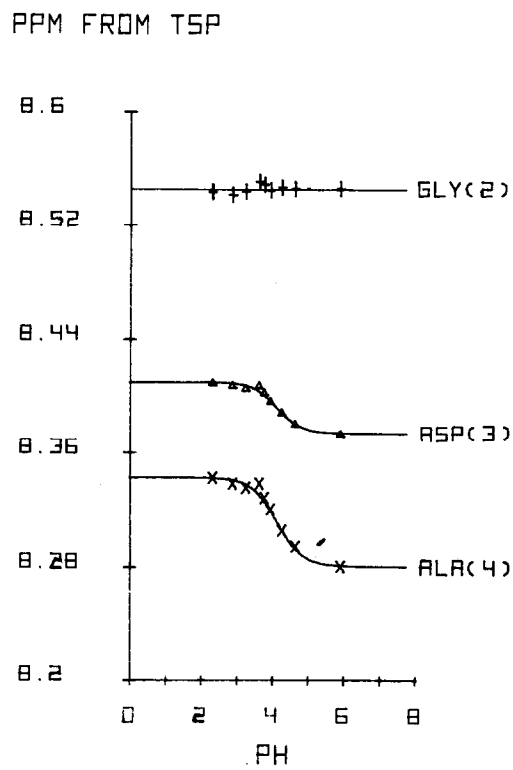

Fig.3. pH Titration-shifts of the amide proton resonances in CF_3CO–Gly–Gly–L-Asp–L-Ala–OCH_3 at 25°C. The experimental data were corrected for the pH-dependence of the internal references TSP [11], the solid lines correspond to least squares fits of one-proton titration curves to the experimental data, pK_a (Ala 4) = pK_a (Asp 3) = 4.20 ± 0.10, $\Delta\delta$(Ala 4) = 0.062 ppm, $\Delta\delta$(Asp 3) = 0.036 ppm; $\Delta\delta$(Gly 2) < 0.01 ppm.

4. Conclusions

The present data indicate that in random-coil polypeptide chain only a very limited number of backbone amide proton resonances should experience measurable pH titration-shifts. In the pH-range 1–5, the amide proton of the C-terminal residue experiences an upfield-shift of approximately –0.4 ppm or possibly somewhat larger when the terminal amino acid is Asp or Glu. This extreme titration-shift is unique in the entire polypeptide chain. In addition, shifts of approximately –0.05 ppm are to be expected for $2n$ amide protons, where n is the number of non-terminal Asp and Glu residues in the polypeptide chain. Between pH 5 and pH 8, upfield titration-shifts of the order –0.05 ppm might arise for $2m$ amide protons, where m is the number of non-terminal His-residues.

In the globular protein BPTI upfield and downfield titration-shifts of more than 0.05 ppm were observed in the pH-range 1–8 for a number of amide proton resonances [9]. On the basis of the results described in this note, all the downfield titration-shifts and most of the upfield titration-shifts must arise from through-

space interactions and are hence related to the three-dimensional arrangement of the polypeptide chain. It is therefore suggested that studies of amide proton titration-shifts are a suitable additional technique for investigations of peptide and protein conformation.

To complete the present data on amide proton titration-shifts in extended flexible polypeptide chains, we plan to further investigate model peptides containing neighboring residues which titrate in the same pH-range. It seems quite conceivable that cumulative side-chain ionization effects exceeding −0.05 ppm might occur for one or several amide protons in peptide fragments such as *–a–*Asp–Asp–*b–*. Because of the rapid proton exchange at high pH [1,4], it appears on the other hand to be of only limited practical interest to try to extend these studies to titrations of the amino-terminus and the basic amino acid side-chains.

Acknowledgements

We thank Drs K. Loth and R. Richarz for communication of the unpublished ^{13}C NMR titration data on the peptides used in this study. Financial support by the Schweizerischer Nationalfonds (project 3.151.73) is gratefully acknowledged.

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