Biophys. Struct. Mechanism 3, 303–315 (1977)

Biophysics of Structure and Mechanism

^{1}H NMR Studies at 360 MHz of the Methyl Groups in Native and Chemically Modified Basic Pancreatic Trypsin Inhibitor (BPTI)

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Abstract. In the ^{1}H NMR spectra obtained at 360 MHz after digital resolution enhancement, the multiplet resonances of the methyl groups in the basic pancreatic trypsin inhibitor (BPTI) were resolved. With suitable double irradiation techniques the individual methyl resonances were assigned to the different types of aliphatic amino acid residues. Furthermore, from pH titration and comparison of the native protein with chemically modified BPTI, the resonance lines of Ala 16 in the active site and Ala 58 at the C-terminus were identified. Potential applications of the resolved methyl resonances as natural NMR probes for studies of the molecular conformations are discussed.

Key words: NMR – Proteinase inhibitor – Protein modification – Protein structure – Basic pancreatic trypsin inhibitor.

Introduction

From the spectroscopists viewpoint the methyl groups in proteins have long been of interest as natural "NMR probes", since they can give quite prominent narrow resonance lines even in relatively large molecules. However, most of the methyl resonances fall into a spectral region which is crowded by a large number of mutually overlapping resonance lines (Wüthrich, 1976). Therefore, the NMR studies so far usually concentrated mainly on a small number of methyl resonances with outstanding high field chemical shifts caused by interactions with the local ring current fields of the aromatic amino acid residues (McDonald and Phillips, 1967; Sternlicht and Wilson, 1967) or of prosthetic groups (Wüthrich, 1970). With advanced experimental techniques which combine the use of high magnetic fields with digital resolution enhancement techniques (Ernst, 1966; Campbell et al., 1973; De Marco and Wüthrich, 1976b) and double resonance difference spectroscopy (Gibbons et al., 1975), studies of the methyl resonances can now be extended to the entire ^{1}H NMR

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spectrum of small proteins (see e.g. Campbell et al., 1975). This is in the following illustrated with some studies of the bovine basic pancreatic trypsin inhibitor (BPTI).

BPTI from bovine pancreas has a molecular weight of 6500, and consists of one polypeptide chain with 58 amino acid residues. The amino acid sequence includes 15 residues with methyl groups, i.e. 6 Ala in the positions 16, 25, 27, 40, 48, and 58, Val 34, 2 Leu in the positions 6 and 29, 2 Ile in the positions 18 and 19, 3 Thr in the positions 11, 32, and 54, and Met 52 (Tschesche, 1974). Ala 16 is of particular interest since it is located in the active site of the protein. The X-ray atomic coordinates of BPTI were refined at 1.5 Å resolution (Deisenhofer and Steigemann, 1975). The molecular conformation in aqueous solution was shown to be closely related to the crystal structure (Wagner et al., 1976; Brown et al., 1976) and to be outstandingly stable towards denaturation by chemicals or by heat (Vincent et al., 1971; Masson and Wüthrich, 1973; Wagner et al., 1976). In the present paper, the resonances of the methyl groups are analysed in the ^{1}H NMR spectra of BPTI and two chemically modified analogs, i.e. BPTI*, where the active site peptide bond Lys 15-Ala 16 is open, and des(Ala 16-Arg 17)BPTI, where the two residues 16 and 17 are removed and the peptide bond Lys 15-Ile 18 is open (Jering and Tschesche, 1976). Since in the X-ray structure of BPTI (Deisenhofer and Steigemann, 1975) essentially all the methyl groups are located at or near the surface of the molecule, their NMR observation is of particular interest in view of investigations of the solution structure and dynamics of the proteinase-BPTI complexes (De Marco and Wüthrich, 1976a).

Materials and Methods

The basic pancreatic trypsin inhibitor (BPTI, Trasylol®, Bayer Leverkusen, Germany) was obtained from the Farbenfabriken Bayer AG. BPTI* was prepared via reduction of the disulfide bond Cys 14-Cys 38, enzymatic cleavage of the active site peptide bond Lys 15-Ala 16 in the reduced protein and autoxidation of the Cys 14-Cys 38 disulfide bridge in the active site-cleaved inhibitor. Des(Ala 16-Arg 17)BPTI was obtained by digestion of BPTI* with aminopeptidase K. A detailed description of the preparation and the characterization of BPTI* and des(Ala 16-Arg 17)BPTI was published elsewhere (Jering and Tschesche, 1976).

For the NMR studies, ca. 0.005 M solutions of the protein in ^2H_2O were used. Solutions with different p^{2}H values were prepared by the addition of minute amounts of 1 M ^{2}HCl or KO^{2}H solution. The p^{2}H values reported in the figures and tables are pH meter readings uncorrected for isotope effect.

Fourier transform (FT) ^{1}H NMR spectra were recorded on a Bruker HXS-360 MHz spectrometer. Typically, 250–500 transients were accumulated. Chemical shifts are measured relative to internal sodium 2,2,3,3-tetradeutero-3-trimethylsilyl propionate (TSP). To improve the spectral resolution, digital filtering techniques (Ernst, 1966) were applied to the free induction decays, i.e. either the convolution difference method (Campbell et al., 1973) or the sine bell routine (De Marco and Wüthrich, 1976b).

Assignment of the individual methyl resonances to the spin systems of the different types of aliphatic amino acid residues was based mainly on double resonance experiments. To obtain suitable spectral resolution in the crowded spectral regions containing the protons which are coupled to the methyl protons, double resonance difference spectra (Gibbons et al., 1975) were recorded. To eliminate field drifts and possible other instabilities of the spectrometer system, direct accumulation of the difference spectra was used and the digital resolution enhancement routines applied to the free induction decays thus obtained. To minimize spurious signal due to Bloch-Siegert shifts (Hoffmann and Forsén, 1966), the off-resonance frequency was set very close to the decoupling frequency and low power in the decoupling channel was employed. Consequences of these experimental conditions will be further discussed in the following section.

Computer simulation (Castellano and Bothner-By, 1964) of the difference spectra was used to verify the spectral interpretations. A conventional plot routine was modified for reproducing the non-Lorentzian line shapes resulting from the resolution enhancement routines.

Results and Discussion

The ^1H NMR spectra of BPTI in Figures 1 and 2 illustrate the spectral resolution which can be obtained by suitable digital filtering of the free induction decays at 360 MHz. In the spectrum of Figure 1A the region from 0—6.0 ppm contains the resonances of all the C_α-protons and the non-aromatic amino acid side chains. The residual water protons give rise to the sharp strong line at 4.6 ppm. The region from 6.0—7.8 ppm contains the resonances of the aromatic side chains, which have previously been studied extensively (Wüthrich and Wagner, 1975; Wagner et al., 1975; Snyder et al., 1976; Wagner et al., 1976). Between 7.0 and 11.0 ppm there are the resonances of hydrogen bonded amide protons of the α-helix and the β-sheet in BPTI. These exchange slowly with deuterium of ^2H_2O (Masson and Wüthrich, 1973; Karplus et al., 1973; Sehr, 1974; Richarz, 1975; Wagner et al., 1976), as is illustrated in Figure 1 where the spectra B and C were recorded at different times after the protein had been dissolved in ^2H_2O. For the experiments discussed in this paper it was essential that the fine structure arising from proton-proton spin-spin coupling was well resolved for the methyl resonances (Fig. 2) and the amide proton resonances (Fig. 1B and C). With double resonance difference spectroscopy, well resolved multiplet patterns could also be obtained for selected additional resonances between 1.0 and 5.0 ppm, as will be illustrated in Figure 3.

Figure 1D shows a spin decoupling experiment which revealed that the C_α-proton at 4.85 ppm and the amide proton at 9.4 ppm come from the same amino acid residue. Similar techniques can be used to find the resonances of side chain protons coupled to the C_α-protons, and thus the complete spin systems of individual amino acid residues can in principle be identified. In the following we first discuss the assignment of the 20 methyl resonances in BPTI to the different types of amino acid residues and then describe some experiments which resulted in the identification of the NMR lines of two particular residues in the amino acid sequence.

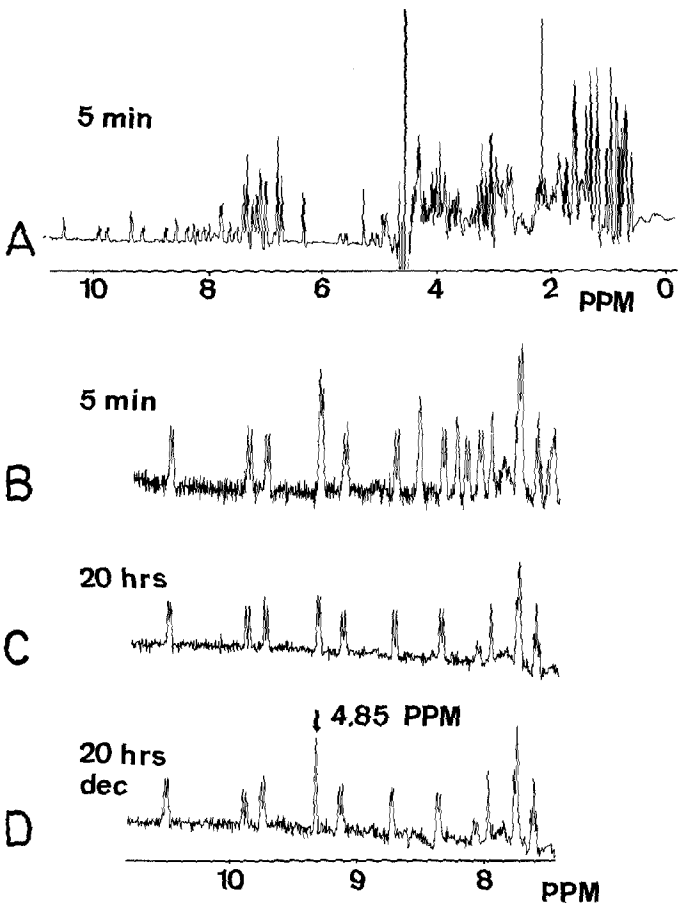

Fig. 1. A. FT ^1H NMR spectrum at 360 MHz of a 0.005 *M* solution of BPTI in ^2H_2O, $p^2H = 4.5$, $T \approx 45°$, after resolution enhancement with the convolution difference method. The spectrum was recorded 5 min after the protein had been dissolved in ^2H_2O. **B.** Expanded representation of the region from 7.6–11.0 ppm in spectrum A, which contains the resonances of hydrogen bonded amide protons of the α-helix and the β-sheet in BPTI. **C.** Same as B, recorded 20 h after the protein had been dissolved in ^2H_2O. **D.** Same as C, except that a double resonance irradiation field was applied to the C_α-proton resonance at 4.85 ppm. It is seen that the amide proton resonance at 9.4 ppm was thus decoupled

Fig. 2. Spectral region between 0.5 and 2.2 ppm of the FT ^1H NMR spectrum at 360 MHz of a 0.005 M solution of BPTI in ^2H_2O, $p^2H = 7.0$, $T = 45°$, after resolution enhancement with the sine bell routine. The resonances of the 20 methyl groups in this protein are numbered in order of increasing magnetic field

Fig. 3. Assignment of three methyl resonances in BPTI by double resonance difference spectroscopy. **A.** Region from 0.5–4.5 ppm in the FT ^1H NMR spectrum at 360 MHz of a 0.005 M solution of BPTI in ^2H_2O in which all the amide protons had been exchanged with deuterium, $p^2H = 7.0$, $T = 45°$, after resolution enhancement with the sine bell routine. **B–E.** Difference spectra obtained by subtracting from a spectrum recorded with double resonance irradiation at the position of the filled arrow a spectrum obtained with off-resonance irradiation at the position of the empty arrow. The difference patterns of interest, which are to be compared with the simulated difference multiplets B′–E′ (see text), have been shadowed. Additional multiplets appear in the spectra D and E, since the double resonance irradiation had to be applied in a very crowded region of the spectrum

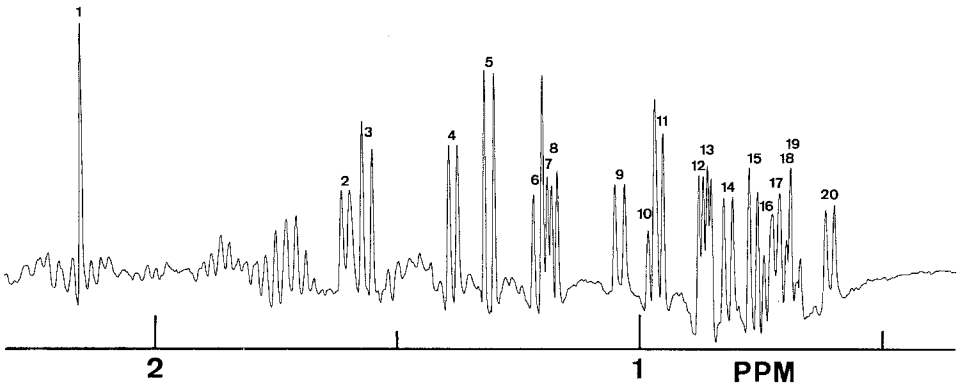

Fig. 2. (Legend see page 306).

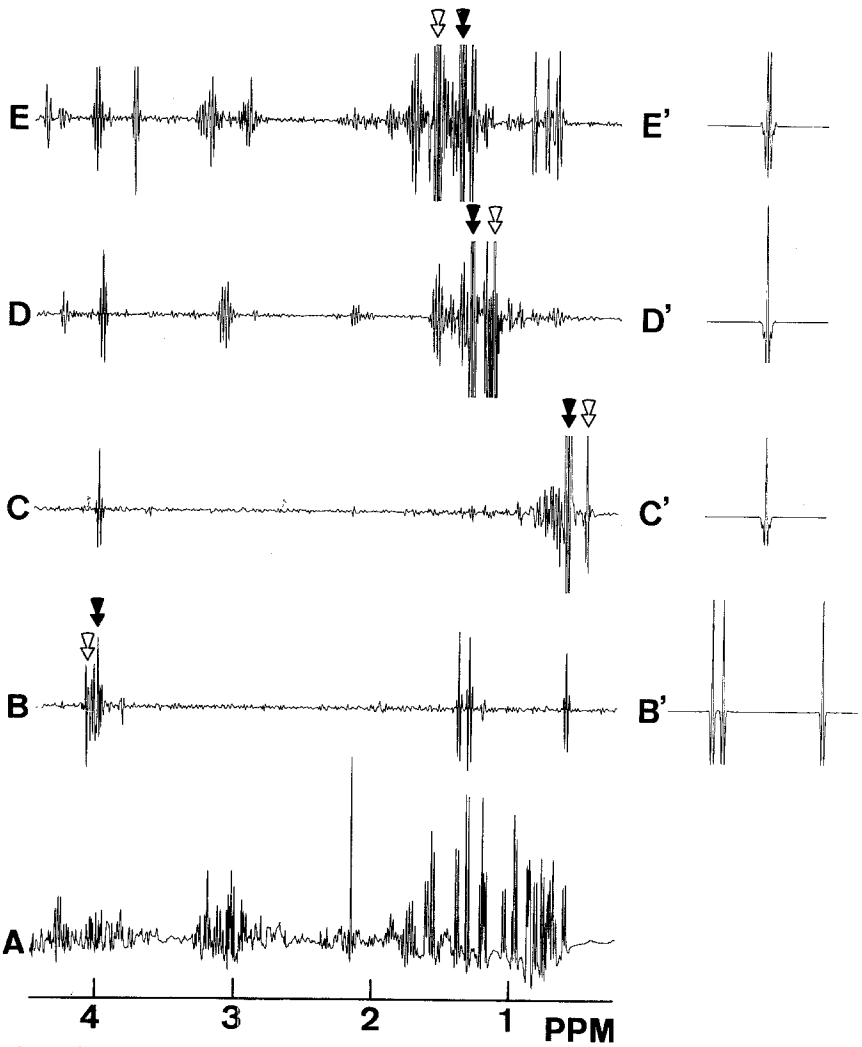

Fig. 3. (Legend see page 306).

Assignment of the Methyl Proton Resonances by Double Irradiation Techniques

Among the 20 methyl groups in BPTI (Tschesche, 1974), that of Met 52 is unique in that it gives a singlet resonance at approximately 2.1 ppm (Wüthrich, 1976). From inspection of Figure 2, the resonance at 2.164 ppm could therefore readily be assigned.

Double resonance irradiation at positions between 3.0 and 4.3 ppm caused the collapse of a total of 9 methyl doublet resonances. Since inspection of the X-ray structure (Deisenhofer and Steigemann, 1975) showed that with the exception of Pro 9 (Wüthrich et al., 1976) extensive ring current shifts of the order of 1 ppm or more were not to be expected for methylene or methine protons of the amino acid side chains, these 9 methyls were on the basis of the chemical shifts (Wüthrich, 1976) assigned to the 6 Ala and 3 Thr in BPTI. By double resonance difference spectroscopy, these assignments were then confirmed and the methyl resonances of Ala and Thr could be distinguished[c]. These results are presented in Table 1, together with all the other assignments described in this section. The technique used is illustrated in Figure 3. In the spectrum of Figure 3B, which corresponds to the difference between

Table 1. Methyl proton resonances in BPTI at $p^2H = 7.0$ and $T = 45°$. The table summarizes the chemical shifts, multiplet structures and spin decoupling experiments which resulted in the assignments of the individual methyl lines. The spin-spin couplings J were found to be 0 Hz for resonance 1, 7.8 Hz for resonance 8, 5.4 Hz for resonance 10 and 7.0 ± 0.5 Hz for all the other methyl resonances

Resonance (see Fig. 2)	Assignment	Chemical shift (ppm)	Multiplicity[a]	Double resonance irradiation[b] (ppm)
1	Met 52	2.164	S	–
2	Ala[c]	1.609	D	3.950
3	Ala[c]	1.568	D	3.740
4	Thr	1.388	D	4.040
5	Ala	1.312	D	3.990
6	Ala[c]	1.214	D	4.090
7	Thr[c]	1.193	D	4.300
8	Thr[c]	1.183	D	4.290
9	Ala[c]	1.040	D	3.140
10	Ile-γ^2	0.970	D	1.874
11, 12	Val-γ^1, γ^2 or Leu-δ^1, δ^2	0.953 0.863	D D	1.731
13, 15	Val-γ^1, γ^2 or Leu-δ^1, δ^2	0.859 0.759	D D	1.470
14, 16, 17	Val-γ^1, γ^2 or Leu-δ^1, δ^2 and Ile-γ^2	0.811 0.728 0.712	D D D	1.961
18	Ile-δ	0.688	T	1.080
19	Ile-δ	0.680	T	1.470
20	Ala[c]	0.592	D	4.010

[a] S = singlet, D = doublet, T = triplet

[b] Double resonance irradiation at the position indicated caused the collapse of the multiplet structure of the methyl resonance

[c] See note added in the proofs

the decoupled and the off-resonance decoupled spectrum, it is seen that three methyl resonances were simultaneously decoupled by irradiation at 4.02 ppm. The difference multiplet patterns expected under conditions of complete decoupling were simulated in Figure 3B. It is seen that for each methyl group one has a negative doublet for the undecoupled resonance and a positive singlet for the decoupled resonance. These features are also readily recognized in the experimental spectrum of Figure 3B. However, since very low power was used in the decoupling channel to obtain the desired selective irradiation of individual resonances in crowded spectral regions, incomplete decoupling resulted in the appearance of additional weak lines symmetrical with respect to the principal resonance in the decoupled spectrum (Pople et al., 1959). As expected, the positions and intensities of these additional lines could be monitored by variation of the amplitude of the decoupling field. Corresponding "wings" are also observed in the spectra C to E of Figure 3. The difference multiplets at 4.01 and 3.99 ppm in Figure 3C and D, respectively, correspond otherwise to what one expects for the A_3X spin system (for the notation used see e.g. Wüthrich, 1976) of Ala (Fig. 3C′ and D′). The multiplet at 4.04 ppm in Figure 3E corresponds to the A_3MX spin system of Thr (Fig. 3E′), where the coupling constants $^3J_{AM}$ and $^3J_{MX}$ appear to be essentially identical.

Additional methyl resonances had to come from the single Val, two Leu and two Ile in BPTI (Tschesche, 1974). Each Val or Leu gives rise to a pair of methyl doublet resonances which should simultaneously collapse when the neighboring methine proton is irradiated. Each Ile gives rise to one methyl doublet and one methyl triplet (Wüthrich, 1976). On this basis, the remaining assignments in Table 1 were made by additional double resonance experiments. Irradiation at 1.87 ppm caused the collapse of a single doublet, which thus had to come from Ile-γ^2. Irradiation at 1.73 ppm resulted in the collapse of two doublets which were therefore assigned to Leu or Val. Similarly, irradiation at 1.47 ppm led to the assignment of another pair of methyls from either Leu or Val. Irradiations at 1.47 and 1.08 ppm caused the collapse of one methyl triplet each, which were assigned to Ile-δ. Finally, irradiation at 1.96 ppm resulted in the simultaneous collapse of three methyl doublets, which were in view of the other results assigned to Ile-γ^2 and a pair of methyls from either Val or Leu.

Table 1 shows that the chemical shifts of the six Ala methyl resonances cover a range of approximately 1.0 ppm, whereas the chemical shifts observed for the methyls of Val, Leu, Ile and Thr differ only slightly, i.e. less than $\pm$ 0.2 ppm from the corresponding random coil values (Wüthrich, 1976). It would thus appear that the methyl groups of Ala, which are directly attached to the polypeptide backbone, experience particularly large conformation dependent chemical shifts. The observed sizeable chemical shift differences among the Ala methyls came all the more rather as a surprise, since in the crystal structure they are all located on the protein surface.

More detailed resonance assignments than those in Table 1 were not obtained, mainly because successive decouplings to search for the resonances of the additional protons along the side chains produced so many simultaneous perturbations that no unambiguous interpretation seemed possible. The C_α-proton resonances were therefore assigned only for the six alanyl residues. Experiments of the type of Figure 1D showed that none of the amide proton resonances between 8.0 and 11.0 ppm which

exchange with deuterium of ^2H_2O with a half time longer than 10 h at 22° and neutral p^{2}H (Masson and Wüthrich, 1973), belong to an alanyl residue. This is of particular interest for Ala 16 and Ala 27, where the amide protons are involved in the first and the last, respectively, of a total of 10 hydrogen bonds in the β-sheet of the crystal structure of BPTI (Deisenhofer and Steigemann, 1975).

Identification of the C-Terminal Ala 58 Resonances by pH Titration

Figure 4 shows several ^{1}H NMR spectra of BPTI which were recorded during a p^{2}H-titration in the acidic range. With the exception of the doublet marked by an asterisk, which corresponds to the resonance 5 in Figure 2, the resonances which had been assigned to methyl protons (Table 1) were essentially independent of p^{2}H. The resonance 5, which was previously assigned to an alanyl residue (Table 1), shows between p^{2}H 1.0 and 7.9 a titration shift which is quite similar to that ob-

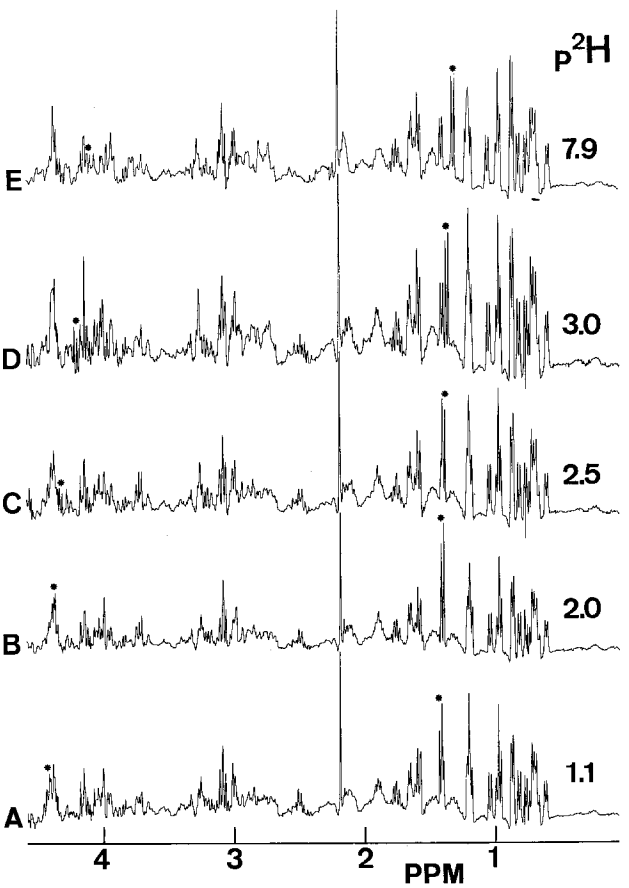

Fig. 4. p^{2}H dependence of the region from 0.5—4.5 ppm in the FT ^{1}H NMR spectrum at 360 MHz of a 0.005-M solution of BPTI in ^2H_2O, $T = 25°$. The spectral resolution was improved with the convolution difference method. The two resonances at around 1.3 and 4.2 ppm which are marked by *, were from their pH dependence assigned to the methyl protons and the C_α-proton of the C-terminal Ala 58

served otherwise for β-CH_3 of alanine (Wüthrich, 1976). The pK_a was approximately 3.0. With double irradiation experiments of the type shown in Figure 3, the resonance which is coupled with the methyl group 5 was identified in the spectra recorded at different p^2H-values. It was found to experience a titration shift between p^2H 1.0 and 7.9 which is typical for C_α-protons in amino acids (Wüthrich, 1976). The resonances 5 in Table 1 could thus be identified as those of Ala 58, which was subsequently an essential factor for comparative investigations of the protein surface structure in single crystals and in solutions of BPTI (L. R. Brown, A. De Marco, R. Richarz, G. Wagner, and K. Wüthrich, to be published).

Identification of the Active Site Ala 16 Resonances by Comparison of BPTI *with Chemically Modified Inhibitors*

The preparation of the two modified proteins used for these experiments (Fig. 5), BPTI* and des(Ala 16-Arg 17)BPTI, was described in the "Materials and Methods" section. In BPTI*, double irradiation experiments of the type presented in Figure 3 showed that five Ala methyl resonances had essentially identical chemical shifts to those of the methyl resonances 2, 3, 5, 9, and 20 in BPTI (Table 1). In place of resonance 6 in BPTI a new Ala methyl doublet was found at ca. 1.45 ppm (Fig. 5). Since in BPTI* the peptide bond between Lys 15 and Ala 16 is cleaved, Ala 16 becomes an N-terminal residue and its ^1H NMR lines would therefore be expected to be shifted downfield by this modification (Wüthrich, 1976). The identification of the new doublet resonance at 1.45 ppm as the methyl line of Ala 16 could subsequently

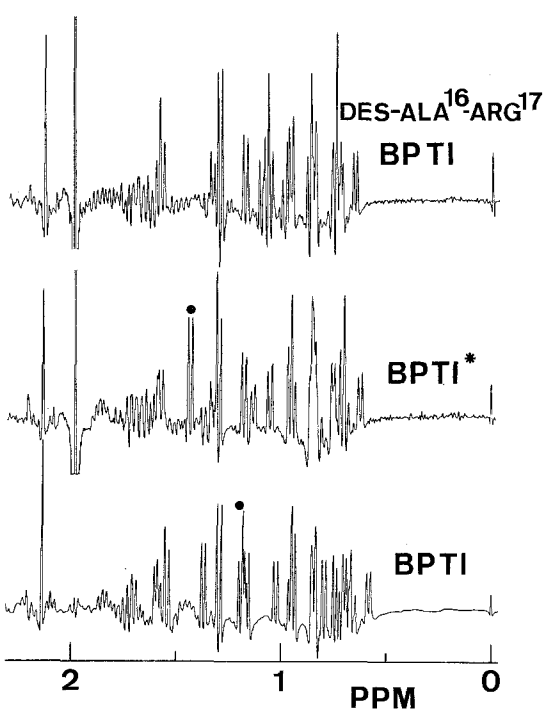

Fig. 5. Comparison of the region between 0 and 2.5 ppm in the FT ^1H NMR spectra at 360 MHz of BPTI and two chemically modified analogs. For these experiments approx. 0.003 M solutions of the modified proteins in ^2H_2O were prepared, $T = 36°$. The resonance at 0 ppm corresponds to the internal reference TSP. The spectral resolution was improved with the sine bell routine.
A. BPTI, $p^2H = 6.3$.
B. BPTI*, where the peptide bond Lys 15-Ala 16 is open, $p^2H = 4.7$.
C. des(Ala 16-Arg 17)BPTI, where Ala 16 and Arg 17 were removed and the peptide bond Lys 15-Ile 18 is open, $p^2H = 4.7$. The methyl resonance of the active site Ala 16, which was identified from these experiments, is marked by ●. The strong singlet resonance at 1.98 ppm in the spectra of the modified proteins comes from acetate

be confirmed by two additional independent lines of evidence. First, in the pH region 7.0—12.5 the doublet resonance at 1.45 ppm in BPTI* (Fig. 5) shows a titration shift which is typical for the methyl resonance of alanine (Wüthrich, 1976). Second, in des(Ala 16-Arg 17)BPTI, five methyl resonances of Ala could be shown by double irradiation experiments to have nearly identical chemical shifts to those of the resonances 2, 3, 5, 9, and 20 in BPTI, whereas the resonance which had been assigned to Ala 16 in BPTI* was missing (Fig. 5).

The methyl resonance of the active site Ala 16 may quite possibly become a useful NMR probe for future studies of systems including BPTI. Experiments of the type of Figure 1D indicated that the resonance of the amide proton of Ala 16 is probably at 7.4 ppm and that its exchange with deuterium of ^2H_2O is sufficiently slow so that the line can be observed immediately after dissolving BPTI in ^2H_2O at p^2H 4.6. Thus, even though in the crystal structure it is involved in the first hydrogen bond of the β-sheet of BPTI (Deisenhofer and Steigemann, 1975), the amide proton of the active site peptide group, which is cleaved by the protease in the complexes formed with BPTI (Tschesche, 1974), is not among the very slowly exchanging protons between 8.0 and 11.0 ppm (Masson and Wüthrich, 1973). In as far as the structural aspects of the modified proteins are concerned, the ^1H NMR spectra of Figure 5 provide unequivocal support for the previously described covalent structures of BPTI* and des(Ala 16-Arg 17)BPTI (Jering and Tschesche, 1976). Furthermore, as will be discussed in more detail elsewhere, the NMR data also indicate that the overall features of the molecular conformations of the three species of Figure 5 are quite similar.

Conclusions

In addition to the information on structural and NMR-spectral features of BPTI and two chemically modified analogs described in the foregoing sections, the experiments presented in this paper lead to some conclusions which may generally be applicable for proteins. They show that it is feasible without perturbation of the molecule, e.g. by extrinsic paramagnetic shift or line broadening reagents (Wüthrich, 1976), to resolve and assign methyl resonances in the crowded region between 0.5 and 2.5 ppm of the ^1H NMR spectra of proteins. This applies also to larger molecules than BPTI; e.g. in the chymotrypsin-BPTI complex, which has a molecular weight of 30,000, a considerable number of methyl multiplet resonances could be resolved (De Marco and Wüthrich, 1976a). Since quite generally numerous methyl groups in proteins are located at or near the surface of the molecule (see e.g. Deisenhofer and Steigemann, 1975), it appears an attractive possibility to use resolved methyl resonances as NMR probes for studies of the surface structure and of intermolecular interactions of proteins.

It seems worth while at this point to reemphasize that well resolved spectra of certain methyl resonances in proteins can be obtained because their natural line widths tend to be small compared to those of the methylene and methine proton resonances encountered in the same spectral region (Bradbury et al., 1971). As was demonstrated previously (De Marco and Wüthrich, 1976b) and can also be seen in a large number of papers published during the last decade, the resolution of CW or FT

^{1}H NMR spectra of proteins recorded without digital resolution enhancement is not sufficient to even recognize most of the methyl resonances as such. Application of a resolution enhancement routine emphasizes the resonances with a small natural line width and essentially eliminates the broad "background components" in the spectrum, resulting in a series of prominent methyl resonances in the high field region of a protein spectrum (Fig. 2). The resolution enhancement routines discriminate at the same time between methyl resonances with different natural line width and emphasize these differences. It can readily be shown (De Marco and Wüthrich, 1976b) that for two lines of equal intensity and with a ratio of the natural line widths of 2 : 1 and hence a ratio of the peak heights of approximately 1 : 2, the peak height ratio after application of the sine bell routine is approximately 1 : 4. It is obvious from inspection of Figure 2 that the natural line widths differ quite markedly for the 19 methyl multiplet resonances in BPTI. The narrowest lines by far are those of Ala 58, which one would have anticipated to have more motional freedom than the methyl groups of the non-terminal amino acid residues. Systematic studies of the line widths of the methyl resonances in proteins might with the use of these techniques become an attractive additional source of information on static and dynamic features of protein conformations.

Acknowledgements. We would like to thank Mrs. S. Kupfer for skilful technical assistance in the preparation of BPTI* and Dr. R. Schmidt-Kastner, Farbenfabriken Bayer AG, for a generous gift of BPTI (Trasylol®). Financial support by the Schweizerischer Nationalfonds (project 3.1510.73), the Italian NRC (fellowship to A. De M.), the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg and the Fonds der Chemischen Industrie, Frankfurt a. M. is gratefully acknowledged.

Note added in proof

Experience gained with our continued investigations of the relations between polypeptide conformation and ^{1}H and ^{13}C NMR chemical shifts in proteins prompts us to add the following comments: (i) It should be reemphasized that the resonance assignments in Table 1 are based solely on the amino acid sequence and the characterization of the spin systems of individual amino acids. No use was made of the single crystal structure determined by X-ray techniques. (ii) With one exception, the criteria used to identify the methyls of the different types of amino acids proved to be reliable in work with different peptides and proteins. The exception concerns the distinction of Ala and Thr from the multiplet structures of the α- and β-proton resonances, respectively (Fig. 3): The doublet expected in the difference spectrum obtained with irradiation of the methyl group of Thr (Fig. 3, E′) may in practice not be resolved when either the dihedral angle χ^1 is fixed near a gauche configuration for the α- and β-methine protons and hence $J_{\alpha\beta} \sim 2.5$ Hz, or when the α- and β-protons are chemical shift equivalent. Both these situations may prevail for individual Thr residues in the interior of globular proteins. Hence, while a quintet-doublet difference spectrum (Fig. 3, E and E′) unambiguously identifies Thr, a quartet-singlet difference spectrum can come either from Ala or in certain cases from Thr. (iii) We are reexaming the assignments of Thr and Ala in BPTI with various different techniques. On the one hand, on the basis of the amino acid sequence and the NMR evidence, there remain at present some ambiguities for the assignments of the seven resonances indicated by ^{c} in Table 1, which correspond to five Ala and two Thr. On the other hand it should also be mentioned that we have long known that with the assumption that the environment of the Ala and Thr in the crystal structure of BPTI (Deisenhofer and Steigemann, 1975) is strictly maintained in solution, one would predict that resonance 20 (Fig. 2, Table 1) corresponds to Thr 32 rather than to one of the Ala (A. Masson, Ph. D. Thesis Nr 5229, p. 83, ETH Zürich 1974).

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Received December 21, 1976/Accepted February 23, 1977