# HIGH FIELD $^{13}C$ NMR STUDIES AT 90.5 MHz OF THE METHYL GROUPS IN THE BASIC PANCREATIC TRYPSIN INHIBITOR

René RICHARZ and Kurt WÜTHRICH

*Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, 8093 Zürich-Hönggerberg, Switzerland*

Received 16 April 1977

## 1. Introduction

The basic pancreatic trypsin inhibitor (BPTI) is a well characterized [1,2] small globular protein which functions as an inhibitor of trypsin and other proteases [3]. BPTI was extensively investigated by nuclear magnetic resonance (NMR) techniques [4–9]. In addition to the interest for a better understanding of the structure–function relations in protease-inhibitor systems, these data also provided new insights into general aspects of protein structure and relations between NMR spectral properties and protein conformation. To further pursue these different areas of interest we have started additional investigations using high field $^{13}C$ NMR at 90.5 MHz. The present paper illustrates the advantages of the high field for the identification of individual resonance lines of protonated carbon atoms, in particular methyl carbons, and reports conformation dependent chemical shifts for numerous methyl $^{13}C$ resonances in BPTI.

While principal aspects of high field $^{13}C$ nuclear magnetic resonance (NMR) of biological molecules have been much discussed [10,11], relatively few experiments with proteins have so far been reported [12–14]. Much emphasis has actually been on work with extra large sample sizes at the lowest field strength commonly used in high resolution NMR, which were shown to be favorable conditions for studies of non-protonated carbons [15–17]. The high field $^{13}C$ NMR experiments so far mainly included investigations of the field dependence of the spin relaxation times [12,14] and a study of the pH titration of carboxyl groups in lysozyme [13]. In the present experiments with BPTI the main emphasis is on using the potential of high field $^{13}C$ NMR for studies of protonated carbons, which also depend critically on the improved resolution of the $^{1}H$ NMR spectra of proteins at higher fields [18].

## 2. Materials and methods

The basic pancreatic trypsin inhibitor (BPTI, Trasylol®, Bayer Leverkusen, Germany) was obtained from the Farbenfabriken Bayer AG. For the NMR studies 0.025–0.05 M solutions of the protein in $D_2O$ were prepared. Different p*D*-values were obtained by the addition of minute amounts of 1 M DCl or NaOD. The p*D*-values reported in the figures and the table are pH meter readings uncorrected for isotope effects. Dioxane was used as an internal reference.

$^{13}C$ NMR spectra at 90.5 MHz were recorded on a Bruker HXS-360 spectrometer. The $^{13}C$ NMR spectrum at 25.2 MHz was obtained on a Varian XL-100 spectrometer. Additional experimental details are given in the figure captions.

## 3. Results

Figure 1 shows the natural abundance $^{13}C$ NMR spectra of BPTI at 25.2 MHz and 90.5 MHz. The improved spectral resolution attained at high field, in particular for the protonated carbon atoms observed in the regions 0–80 ppm and 110–140 ppm, was essential for the experiments described below.



*North-Holland Publishing Company – Amsterdam*

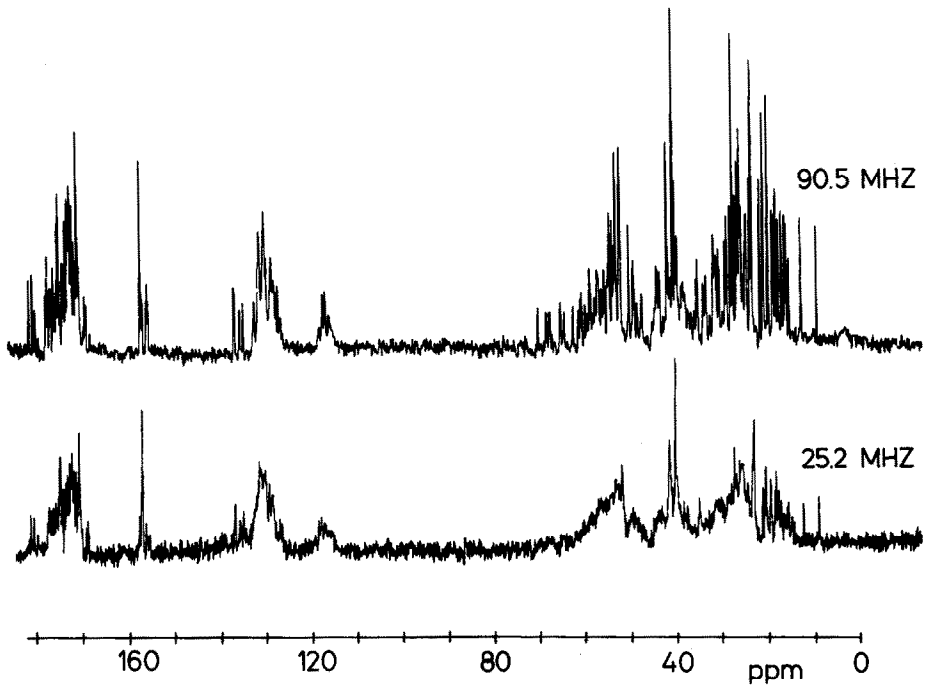


Fig.1. Natural abundance $^{1}H$ noise-decoupled FT $^{13}C$ NMR spectra at 25.2 MHz and 90.5 MHz of a 0.025 M solution of the basic pancreatic trypsin inhibitor (BPTI) in $D_2O$, p$D$ = 8.2, $T$ = 35°C, accumulation time 12 h. At 25.2 MHz, the sample diameter was 12 mm, 54 000 transients were accumulated with a recycle time of 0.8 s, the digital resolution is 1.25 Hz/point. At 90.5 MHz, the sample size was 10 mm, 86 000 transients were accumulated with a recycle time of 0.5 s, the digital resolution is 2.5 Hz/point. At both frequencies, a digital broadening of 1 s was applied.

Here the discussion is limited to the methyl carbon resonances between 0 ppm and 25 ppm. A more detailed discussion of the entire spectrum will appear elsewhere (R. Richarz and K. Wüthrich, manuscript in preparation).

The individual methyl $^{13}C$ NMR lines were related to the corresponding proton resonances by heteronuclear off-resonance double irradiation experiments. Figure 2 illustrates the quality of the

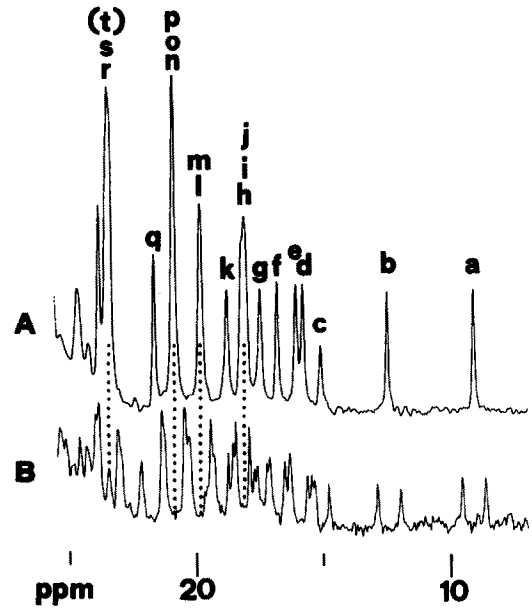


Fig.2. Spectral region from 0–25 ppm of the FT $^{13}C$ NMR spectrum at 90.5 MHz of a 0.05 M solution of BPTI in $D_2O$, p$D$ = 4.2, $T$ = 35°C. (A) $^{1}H$ Noise-decoupled spectrum. The methyl resonances are indicated by the letters a–t. (B) Off-resonance selective $^{1}H$ irradiation of 1.0 W at 4.0 ppm. For each of the spectra A and B, 100 000 transients were accumulated with a recycle time of 0.5 s. The resonance intensities indicated by the letters in spectrum A were confirmed by experiments with different selective proton irradiation frequencies.

spectra obtained with this technique. In this particular experiment, the proton irradiation was applied at 4.0 ppm. As all the 20 methyl proton resonances in BPTI are between 0.6 ppm and 2.2 ppm [9], this resulted in relatively large residual spin–spin couplings. Since only aliphatic methyl and methylene carbon resonances are expected to occur at higher field than 23 ppm [18], fig.2B shows readily that all the 17 resonances a–q must come from methyl groups, and that the resonance line marked r, s (t) contains at least two, more likely 3 methyl resonances. In all, at least 19 and quite possibly all the 20 methyl resonances in BPTI had thus been observed.

In additional off-resonance experiments, where selective proton irradiation was applied at various frequencies between 0.60 ppm and 2.15 ppm, the methyl carbon resonances a–t were assigned to the different types of amino acid residues in BPTI (table 1). These experiments were based on the

Table 1
$^{13}C$ NMR chemical shifts, $\delta(^{13}C)$, and assignments of the methyl carbon resonances in BPTI

| $^{13}C$ NMR | | Double resonance experiments | |
|---|---|---|---|
| Resonance (see fig.2) | $\delta(^{13}C)$[a] (ppm) | $^{1}H$ Decoupling[b] (ppm) | Resonance assignment[c] |
| a | 9.13 | 0.68 ± 0.04 | Ile-$\delta$ |
| b | 12.37 | 0.70 ± 0.02 | Ile-$\delta$ |
| c | 14.97 | 2.23 ± 0.07 | Met 52-$\epsilon$ |
| d | 15.70 | 0.76 ± 0.03 | Ile-$\gamma^2$ or Val-$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ |
| e | 16.02 | 1.06 ± 0.02 | Ala-$\beta$ |
| f | 16.67 | 1.00 ± 0.04 | Ile-$\gamma^2$ |
| g | 17.32 | 1.61 ± 0.07 | Ala-$\beta$ |
| h | 17.91 | 1.19 ± 0.10 | Thr-$\gamma$ |
| i | 18.04 | 1.19 ± 0.10 | Thr-$\gamma$ |
| j | 18.25 | 1.33 ± 0.02 | Ala 58-$\beta$ |
| k | 18.69 | 1.24 ± 0.04 | Ala 16-$\beta$ |
| l | 19.69 | 0.85 ± 0.04 | Val-$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ or Ile-$\gamma^2$ |
| m | 19.69 | 1.41 ± 0.04 | Thr-$\gamma$ |
| n | 20.63 | 1.63 ± 0.06 | Ala-$\beta$ |
| o | 20.70 | 0.62 ± 0.03 | Ala-$\beta$ |
| p | 20.73 | 0.85 ± 0.03 | Val-$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ |
| q | 21.49 | 0.94 ± 0.02 | Val-$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ |
| r | 23.3 | | Val-$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ or Ile-$\gamma^2$ |
| s | 23.3 | | |
| t | > 23.3 | | |

[a] $^{13}C$ Chemical shifts are relative to external TMS, where internal dioxane was taken to be at 67.8 ppm. $T$ = 35°C, p$D$ = 6.5.

[b] $^{1}H$ Decoupling frequency in ppm from internal TSP determined by a linear least squares fit of the $^{1}H$ frequency-dependence of the residual spin–spin couplings. (see text).

[c] The resonance assignments are based on comparison of the $^{1}H$ decoupling frequencies determined by the $^{1}H$–$^{13}C$ double resonance experiments[b] with the previously established $^{1}H$ chemical shifts [9].

good resolution of the methyl resonances in the high field $^1$H NMR spectrum of BPTI at 360 MHz [9,19] and on the previous assignments of the methyl $^1$H NMR lines to the different types of amino acid residues [8,9]. Furthermore, they used that over a certain range the residual $^1$H–$^{13}$C spin–spin coupling varies linearly with the proton irradiation frequency [20,21]. Individual $^{13}$C resonances could thus by least squares fits of the $^1$H frequency dependence of the residual spin–spin couplings be related to individual $^1$H resonances (table 1). A more detailed documentation of these experiments will be given elsewhere (R. Richarz and K. Wüthrich, manuscript in preparation).

Three of the methyl $^{13}$C resonances were further assigned to specific residues in the amino acid sequence of BPTI (table 1). For the $^{13}$C resonances k and j the assignments to Ala 16 in the active site of BPTI and the C-terminal Ala 58, respectively, resulted from the previous identification of the corresponding $^1$H NMR lines. The assignment of the $^{13}$C resonance j to Ala 58 could also be directly confirmed from its pH titration, with a $pK_a \approx 2.9$ and a titration shift of 0.8 ppm. The assignment of resonance c to Met 52 was straightforward on the basis of the amino acid composition.

## 4. Discussion

Reliable identification of the spin systems of the different types of amino acid residues and assignment of resonances to specific positions in the amino acid sequence are the key to meaningful applications of NMR for structural studies of proteins. The present experiments demonstrate the advantage of using high fields for studies of protonated carbon atoms. Table 1 shows that while at the highest presently available field strength of 84 kG unambiguous identification of corresponding $^1$H and $^{13}$C resonances was possible for the methyl groups of Ala, Thr, Met and Ile-$\delta$ in BPTI, establishing 1 : 1 correspondences for the $^1$H and $^{13}$C resonances of Val, Leu and Ile-$\gamma^2$ was still limited mainly by the small separations of the individual $^1$H NMR lines [9].

The potential interest of NMR relaxation times of protonated carbons for studies of dynamic aspects of protein structure is well recognized [7,10–12,14,18]. Since the methyl carbons give quite prominent lines in the $^{13}$C NMR spectra of proteins, they are attractive as spectroscopic probes also from the point of view of ease of observation. It is, e.g., quite conceivable that certain methyl resonances of BPTI will also be observable in the complexes with proteases. The data in table 1 provide a basis for more meaningful structural interpretation of methyl carbon relaxation times and spectral changes in BPTI, which may, e.g., arise from the interactions with proteases or from the addition of paramagnetic NMR probes to systems including BPTI. Experiments currently in progress showed that the assignments of Ala-16 in the active site and the C-terminal Ala-58 are of particular interest for studies of structure–function relations in BPTI.

It is an important aspect of the present experiments that they yield data on conformation-dependent $^{13}$C chemical shifts of the methyl groups, which may hopefully contribute to establish additional empirical relations between $^{13}$C chemical shifts and polypeptide conformation [18]. Meaningful values for dispersions of $^{13}$C chemical shifts for methyl groups in BPTI are 4.7 ppm for the six Ala, 3.3 ppm for the two Ile $C_\delta$ and 1.8 ppm for the three Thr. The 'random coil' chemical shifts obtained from model peptides are 17.7 ppm for Ala, 11.3 ppm for Ile $C_\delta$ and 20.0 ppm for Thr [18]. Table 1 shows that in BPTI both high and low field shifts of the methyl resonances arise as a consequence of the folding of the peptide chain. The extent of the shifts is comparable to the conformation-dependent shifts of quaternary aromatic carbons reported for lysozyme [15]. For Met 52 the methyl $^{13}$C chemical shift coincides with the random coil value [18], and the Ile $C_\gamma$ resonance f is shifted downfield by 1.0 ppm. The remaining seven methyls of Val, Leu and Ile $C_\gamma$ are in the range 15.7– $\geqslant$ 23.3 ppm, while the corresponding random coil values are between 15.7 ppm and 23.1 ppm [18]. The distribution of the chemical shifts for these resonances (table 1) indicates again the occurrence of conformation-dependent shifts of the order of one to several ppm for individual lines. Comparison of corresponding methyl $^{13}$C and $^1$H [9] chemical shifts shows that the $^{13}$C resonances experience more extensive conformation-dependent chemical shifts. For example, the total range covered by the six

methyls of Ala is 4.7 ppm for $^{13}C$ and 1.0 ppm for $^{1}H$, and for the three methyls of Thr the corresponding numbers are 1.8 ppm and 0.2 ppm. The most striking observation is that the δ-methyl $^{13}C$ resonances of the two Ile differ by 3.3 ppm, while the corresponding $^{1}H$ lines differ by $< 0.01$ ppm [9]. The δ-carbon chemical shifts of Ile thus appear to be particularly sensitive to certain aspects of the protein conformation, which might be of potential practical interest. Experiments to relate the conformation-dependent $^{13}C$ chemical shifts with other paramaters, e.g., the relaxation times, are currently in progress.

## Acknowledgements

We thank Dr R. Schmidt-Kastner, Farbenfabriken Bayer AG, for a generous gift of BPTI (Trasylol®). Financial support by the Schweizerischer Nationalfonds (project 3.1510.73) is gratefully acknowledged.

## References

[1] Kassell, B. and Laskowski, M., jr. (1965) Biochem. Biophys. Res. Commun. 20, 463.
[2] Deisenhofer, J. and Steigemann, W. (1975) Acta Cryst. 31, 238–250.
[3] Tschesche, H. (1974) Angew. Chemie 86, 21–40.
[4] Masson, A. and Wüthrich, K. (1973) FEBS Lett. 31, 114–118.
[5] Snyder, G., Rowan, R., Karplus, S. and Sykes, B. D. (1975) Biochemistry 14, 3765–3777.
[6] Wagner, G., DeMarco, A. and Wüthrich, K. (1976) Biophys. Struct. Mech. 2, 139–158.
[7] Wüthrich, K. and Baumann, R. (1976) Organic Magnetic Resonance 8, 532–535.
[8] DeMarco, A. and Wüthrich, K. (1976) 7th Int. Conf. Magnetic Resonance in Biological Systems, St. Jovite, Québec, poster.
[9] DeMarco, A., Tschesche, H., Wagner, G. and Wüthrich, K. (1977) Biophys. Struct. Mech. in press.
[10] Doddrell, D., Glushko, V. and Allerhand, A. (1972) J. Chem. Phys. 56, 3683–3689.
[11] Anet, F. A. L. (1974) in: Topics in Carbon-13 NMR Spectroscopy (Levy, G. ed) Vol. 1, pp. 209–227, Wiley-Interscience, New York.
[12] Wilbur, D. J., Norton, R. S., Clouse, A. O., Addleman, R. and Allerhand, A. (1976) J. Amer. Chem. Soc. 98, 8250–8254.
[13] Shindo, H. and Cohen, J. (1976) Proc. Natl. Acad. Sci. USA 73, 1979–1983.
[14] Norton, R. S., Clouse, A. O., Addleman, R. and Allerhand, A. (1977) J. Amer. Chem. Soc. 99, 79–83.
[15] Allerhand, A., Childers, R. and Oldfield, E. (1973) Biochemistry 12, 1335–1341.
[16] Oldfield, E., Norton, R. and Allerhand, A. (1975) J. Biol. Chem. 250, 6368–6380.
[17] Oldfield, E., Norton, R. and Allerhand, A. (1975) J. Biol. Chem. 250, 6381–6402.
[18] Wüthrich, K. (1976) in: NMR in Biological Research: Peptides and Proteins, North Holland, Amsterdam.
[19] DeMarco, A. and Wüthrich, K. (1976) J. Magn. Resonance 24, 201–204.
[20] Ernst, R. (1966) J. Chem. Phys. 45, 3845–3861.
[21] Birdsall, B., Birdsall, N. J. M. and Feeney, J. (1972) J. C. S. Chem. Commun. 316, 316–317.