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# High-Field $^{13}C$ Nuclear Magnetic Resonance Studies at 90.5 MHz of the Basic Pancreatic Trypsin Inhibitor†

René Richarz and Kurt Wüthrich*

ABSTRACT: The carbon-13 NMR spectra of the basic pancreatic trypsin inhibitor (BPTI) were investigated at 90.5 MHz. The identification of the resonances of the 20 methyl groups in this protein is described. Corresponding methyl proton and carbon-13 resonances were assigned by heteronuclear double resonance experiments. Since the proton resonances had previously been individually assigned, assignments to individual amino acid residues were obtained for most of the methyl carbon resonances. Carbon-13 chemical shifts could thus be related to the corresponding proton chemical shifts and to the molecular conformation of BPTI. In the spectral regions of the aliphatic carbons and the carbonyl and carboxylic acid carbons, resonances of the ionizable amino acids were identified. The p$K_a$ values for the two Asp side chains were found to be 3.1 and 3.4, those for the two Glu 3.6 and 3.7. With this, microscopic ionization constants have now been determined for all the groups in BPTI with p$K_a$ values in the range 0–12, which provides a basis for detailed investigations of pH-dependent variations of the protein conformation. From the spectroscopist's viewpoint, these experiments illustrate essential advantages of using high-field carbon-13 NMR for studies of macromolecules, in particular for resolving and assigning resonances of protonated carbons.

While principal aspects of high-field $^{13}C$ NMR[1] of biological macromolecules have been much discussed (Doddrell et al., 1972; Anet, 1974), relatively few experiments with peptides or proteins have so far been reported (Komorowski et al., 1975; Deslauriers et al., 1975, 1976; Wilbur et al., 1976; Shindo & Cohen, 1976; Norton et al., 1977; Richarz & Wüthrich, 1977; Wilbur & Allerhand, 1977a; Dill & Allerhand, 1977; Markley et al., 1977). Much emphasis has actually been on work with extra large sample sizes at the lowest field strength commonly used in high resolution NMR (Allerhand et al., 1973, 1977; Oldfield et al., 1975a,b; Wilbur & Allerhand, 1977b). This paper reports on $^{13}C$ NMR studies at 90.5 MHz of the globular protein basic pancreatic trypsin inhibitor (BPTI). Previously we presented the identification of the 20 methyl carbon resonances in BPTI (Richarz & Wüthrich, 1977; Wüthrich et al., 1977). Here, individual assignments for most of the methyl lines are described. In addition, resonances of the ionizable side chains of Asp, Glu, and Lys and the chain terminal amino acid residues were identified and used to investigate the pH titration of these groups.

BPTI is a small globular protein with molecular weight 6500, which in its biological function regulates protease activity (Tschesche, 1974). The sequence of the 58 amino acid residues (Kassell & Laskowski, 1965) and the x-ray structure (Deisenhofer & Steigemann, 1975) are known, and the protein was extensively studied by NMR (Masson & Wüthrich, 1973; Snyder et al., 1976; Wagner et al., 1976; Wüthrich & Baumann, 1976). BPTI contains a total of 20 methyl groups, i.e., those of 6 Ala in the positions 16, 25, 27, 40, 48, and 58, Val-34, Leu-6, Leu-29, Ile-18, Ile-19, 3 Thr in positions 11, 32, and 54, and Met-52 (Kassell & Laskowski, 1965). The proton resonances of all 20 methyls were identified (De Marco et al., 1977) and most of them individually assigned (Wüthrich et al., 1978). On this basis individual assignments for most of the methyl $^{13}C$ lines could be obtained by the identification of the corresponding $^{1}H$ and $^{13}C$ resonances.

Earlier investigations indicated the occurrence of pH-dependent conformation changes in solutions of BPTI (Vincent et al., 1971; Brown et al., 1976; Wagner et al., 1976; Wagner, 1977). For more detailed studies of these conformational transitions it was essential to know the microscopic dissociation constants for all the ionizable groups in the protein. Previously, $^{1}H$ NMR measurements provided the p$K_a$ values for the four Tyr (Wagner & Wüthrich, 1975; Wagner et al., 1976; Snyder et al., 1976), the four Lys (Brown et al., 1976) and the N- and

† From the Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, CH-8093 Zürich-Hönggerberg, Switzerland. This work was supported by the Swiss National Science Foundation (Project 3.0040.76).

[1] Abbreviations used: BPTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor, Trasylol, Bayer Leverhusen, West Germany); NMR, nuclear magnetic resonance; ppm, parts per million; $Me_4Si$, tetramethylsilane; TSP, 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate; FT, Fourier transform.

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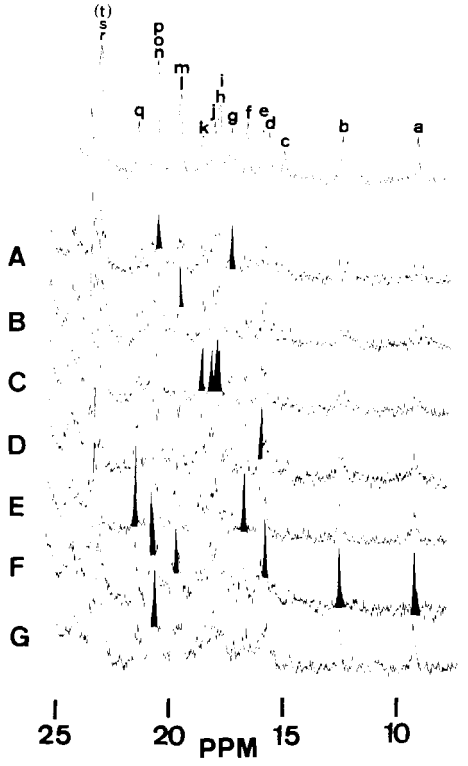


FIGURE 1: (Top trace) Spectral region from 0 to 25 ppm of the $^1H$ noise-decoupled FT $^{13}C$ NMR spectrum at 90.5 MHz of a 0.025 M solution of BPTI in $^2H_2O$, p$^2$H 6.5, $T$ = 35 °C. The letters indicate the positions of individual methyl carbon resonances. (A-G) Same as top trace, with selective $^1H$ irradiation of 0.8 W at: (A) 1.619 ppm; (B) 1.464 ppm; (C) 1.311 ppm; (D) 1.133 ppm; (E) 0.958 ppm; (F) 0.758 ppm; (G) 0.597 ppm (see Figure 2). The collapsed multiplets in the individual spectra are shadowed. For each spectrum 56 000 transients were accumulated with a recycle time of 0.5 s.

C-terminal amino acid residues (Wagner, 1977). To determine the dissociation constants for the remaining four groups which titrate in the pH range 0-12, i.e., Asp-3, Asp-50, Glu-7, and Glu-49, $^{13}C$ NMR had to be used, since the side chain methylene resonances could so far not be identified in the $^1H$ NMR spectrum.

## Materials and Methods

The basic pancreatic trypsin inhibitor (BPTI, Trasylol, Bayer Leverkusen, West 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 pD values were obtained by the addition of minute amounts of 1 M DCl or NaOD. No extra salt was added to the samples, so the ionic strength was determined by the protein and the DCl or NaOD used for the titration. The pD values reported in the figures and the table are pH meter readings uncorrected for isotope effects. As an internal reference, a trace of TSP was added for the $^1H$ NMR studies and a trace of dioxane for $^{13}C$ NMR.

$^1H$ NMR spectra at 360 MHz and $^{13}C$ NMR spectra at 90.5 MHz were recorded on a Bruker HXS-360 spectrometer. For $^{13}C$ NMR sample tubes with an outer diameter of 10 mm were used; the sample volume was 1 mL. Additional experimental details are given in the figure captions.

## Results

(a) *Assignment of Corresponding Methyl $^1H$ and $^{13}C$ Resonances.* The entire proton noise-decoupled $^{13}C$ NMR spectrum of BPTI at 90.5 MHz was previously presented

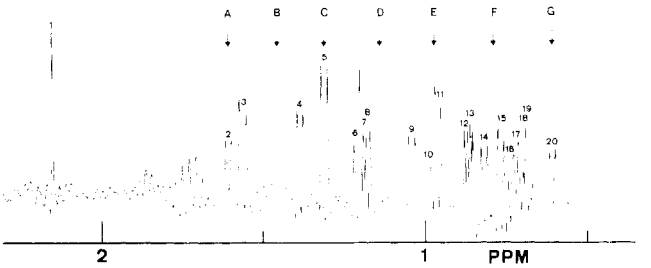


FIGURE 2: Spectral region from 0 to 2.5 ppm of the FT $^1H$ NMR spectrum at 360 MHz of BPTI in $^2H_2O$, p$^2$H 6.5, $T$ = 35 °C. The spectral resolution was improved with the sine bell routine (De Marco & Wüthrich, 1976). The numbers indicate the resonances of the 20 methyl groups in the protein. The letters and arrows indicate the proton irradiation frequencies used in the selective decoupling experiments of Figure 1.

(Richarz & Wüthrich, 1977; Wüthrich et al., 1977). By comparison with the corresponding 25 MHz spectrum it was shown that the spectral resolution was quite dramatically improved at the higher field, in particular in the spectral regions containing the resonances of protonated carbon atoms. Selected regions of $^1H$ noise decoupled 90.5 MHz spectra of BPTI are shown on expanded scales in the top trace of Figure 1 and in Figures 5 and 7. Previously (Richarz & Wüthrich, 1977; Wüthrich et al., 1977), it was also demonstrated how the 20 lines numbered a to t in the top trace of Figure 1 were from their quartet fine structures in an off-resonance $^{13}C\{^1H\}$ double irradiation experiment identified as methyl resonances. In the following, experiments are described which resulted in the identification of corresponding methyl $^{13}C$ and $^1H$ resonances. Since the $^1H$ NMR lines had previously been individually assigned (Wüthrich et al., 1978), these data provide assignments for most of the methyl carbon resonances to individual residues in the amino acid sequence.

An essential element for the methyl resonance assignments was the good spectral resolution obtained in the high-field $^1H$ NMR spectra of BPTI. Figure 2 shows that, after suitable digital filtering, all the 20 methyl $^1H$ NMR multiplets in BPTI were resolved (De Marco et al., 1977). Previously, the methyl proton resonances of the different types of aliphatic amino acid residues had been identified by homonuclear double irradiation experiments (De Marco et al., 1977; Wüthrich et al., 1978) and by a combination of various different techniques most of the resonances were subsequently assigned to specific residues in the amino acid sequence (Wüthrich et al., 1978). As will be seen in the following, the methyl $^1H$ NMR lines in the spectra at 360 MHz (Figure 2) were sufficiently well separated so that the corresponding $^{13}C$ NMR lines could be identified by selective $^1H$-$^{13}C$ heteronuclear double resonance experiments.

The identification of corresponding [$^1H$]- and [$^{13}C$]methyl resonances was based on the linear dependence on the proton irradiation frequency of the residual $^1H$-$^{13}C$ spin-spin couplings in the $^{13}C$ NMR spectra obtained with selective $^1H$ off-resonance irradiation (Ernst, 1966; Birdsall et al., 1972). Figure 1 shows the $^{13}C$ NMR spectra obtained with selective proton irradiation at the positions indicated by the arrows in Figure 2. For the ten well-resolved one-carbon resonances a to g, j, k, and q, the multiplet components could unambiguously be identified from inspection of the spectra. The individual resonances were by least-squares fits of the $^1H$ frequency dependence of the residual spin-spin couplings related to individual $^1H$ resonances (Table I). Between 16 and 22 ppm there are three groups of overlapping resonance lines, i.e., h and i, l and m, and n to p. The analysis of this more crowded region was confirmed by the spectral simulations in Figure 3. Even

TABLE I: $^{13}C$ NMR Chemical Shifts, $\delta(^{13}C)$, and Assignments of the Methyl Carbon Resonances in BPTI.[a]

| $^{13}C$ NMR | | Double resonance experiments | | $^1H$ NMR[e] | |
|---|---|---|---|---|---|
| Resonance[f] | $\delta(^{13}C)$[b] (ppm) | $^1H$ decoupling[c] (ppm) | Resonance assignment[d] | Resonance[f] | $\delta(^1H)$ (ppm) |
| a | 9.13 | 0.68 ± 0.04 | Ile-$\delta$ | 18 | 0.688 |
| b | 12.37 | 0.70 ± 0.02 | Ile-$\delta$ | 19 | 0.680 |
| c | 14.97 | 2.23 ± 0.07 | Met-52$\epsilon$ | 1 | 2.164 |
| d | 15.70 | 0.76 ± 0.03 | Ile-$\gamma^2$ or Leu-29$\delta^1$, $\delta^2$ | 15 or 16 | 0.759 0.728 |
| e | 16.02 | 1.06 ± 0.02 | Ala-48$\beta$ | 9 | 1.040 |
| f | 16.67 | 1.00 ± 0.04 | Ile-$\gamma^2$ | 10 | 0.970 |
| g | 17.32 | 1.61 ± 0.07 | Ala-25$\beta$ | 3 | 1.568 |
| h | 17.91 | 1.19 ± 0.10 | Ala-16$\beta$ | 7 | 1.193 |
| i | 18.04 | 1.19 ± 0.10 | Ala-27$\beta$ | 8 | 1.183 |
| j | 19.25 | 1.33 ± 0.02 | Ala-58$\beta$ | 5 | 1.312 |
| k | 18.69 | 1.24 ± 0.04 | Ala-40$\beta$ | 6 | 1.214 |
| l | 19.69 | 0.85 ± 0.04 | Leu-6$\delta^1$, $\delta^2$ or Leu-29$\delta^1$, $\delta^2$ or Val-34$\gamma^2$, $\gamma^2$ | 12 or 13 or 14 | 0.863 0.859 0.811 |
| m | 19.69 | 1.41 ± 0.04 | Thr-11$\gamma$ | 4 | 1.388 |
| n | 20.63 | 1.63 ± 0.06 | Thr-54$\gamma$ | 2 | 1.609 |
| o | 20.70 | 0.62 ± 0.03 | Thr-32$\gamma$ | 20 | 0.592 |
| p | 20.73 | 0.85 ± 0.03 | Leu-6$\delta^1$, $\delta^2$ or Leu-29$\delta^1$, $\delta^2$ | 12 or 13 | 0.863 0.859 |
| q | 21.49 | 0.94 ± 0.02 | Leu-6$\delta^1$, $\delta^2$ | 11 | 0.953 |
| r | 23.3 | | Val$\gamma^1$, $\gamma^2$ or Leu-$\delta^1$, $\delta^2$ or Ile-$\gamma^2$ | 3 out of 12 to 17 | 0.863 to 0.712 |
| s | 23.3 | | | | |
| t | ≥23.3 | | | | |

[a] The corresponding $^1H$ NMR chemical shifts, $\delta(^1H)$, and the results of the off-resonance $^1H$ double resonance irradiation experiments used for the resonance assignments are also indicated. [b] $^{13}C$ chemical shifts are relative to external $Me_4Si$, where internal dioxane was taken to be at 67.8 ppm. $T$ = 35 °C, $p^2H$ 6.5. [c] $^1H$ decoupling frequency in ppm from internal TSP determined by a linear least-squares fit of the $^1H$ frequency dependence of the residual spin–spin couplings in the $^{13}C$ NMR spectra (Figure 2). [d] The resonance assignments are based on comparison of the observed $^1H$ decoupling frequencies with the methyl $^1H$ chemical shifts in the last column. [e] From DeMarco et al., 1977; Wüthrich et al., 1978. The $^1H$ chemical shifts are relative to internal TSP. [f] See Figures 1 and 2.

though the computed spectra were found to be very sensitive to variations of the chemical shifts and the residual coupling constants, close agreement between simulated and experimental spectra was obtained (Figures 1 and 3). For 17 methyl carbon resonances, the chemical shifts, the parameters obtained from the linear fit of the off-resonance $^1H$ irradiation data and the chemical shifts of the corresponding $^1H$ resonances are collected in Table I. Two or quite likely all three of the remaining methyl carbon resonances are contained in the intense line at 23.3 ppm (Richarz & Wüthrich, 1977). These lowest field methyl resonances r–t must correspond to Val, Leu, or Ile residues, since all the resonances of other amino acid residues had previously been identified (Table I). Compared with the previously described identification of the methyl carbon resonances (Richarz & Wüthrich, 1977; Wüthrich et al., 1977), all the correspondences between $^1H$ and $^{13}C$ chemical shifts (Table I) are the same. However, as a consequence of the more detailed assignments obtained in the meantime for the $^1H$ NMR lines (De Marco et al., 1977; Wüthrich et al., 1978), definite distinctions between the methyl resonances of the alanyl and the threonyl residues could now be given.

Most of the assignments to individual residues in the amino acid sequence (Table I) were based on the earlier individual assignments of the $^1H$ resonances (Wüthrich et al., 1978). For Ala-58, the assignment of the methyl carbon resonance could independently be established from the pH dependence of the chemical shift (Figure 4).

(b) *Identification of the Carboxyl Carbon Resonances.* BPTI contains 61 carbonyl carbons and 5 carboxyl carbons, all of which are observed in the spectral region between 168 and 182 ppm (Figure 5). In the high field spectrum at 90.5 MHz recorded with the experimental conditions indicated in Figure 5, essentially all of these 66 carbon resonances were resolved as individual lines. Among these the resonance lines of the two terminal amino acid residues Arg-1 and Ala-58, and

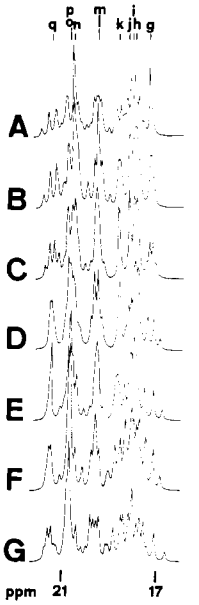


FIGURE 3: Spectral simulation of the region from 16 to 22 ppm in the selectively $^1H$-decoupled $^{13}C$ NMR spectra of Figure 1A–G. In the computation, the $^{13}C$ chemical shifts obtained from the $^1H$ noise-decoupled spectrum (Table I) and a Lorentzian line shape with a half-width of half-height of 2.5 Hz were used. The residual spin–spin couplings were adjusted for an optimal fit with the experiment.

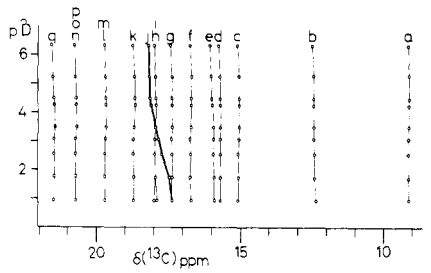


FIGURE 4: Plot of the chemical shifts of the methyl carbons in the $^1H$ noise decoupled $^{13}C$ NMR spectra of BPTI as a function of $p^2H$, $T$ = 35 °C. From the $p^2H$ dependence, which corresponds to a one-proton titration with $pK_a = 2.8 \pm 0.1$, resonance $j$ was assigned to the methyl carbon of the C-terminal Ala-58.

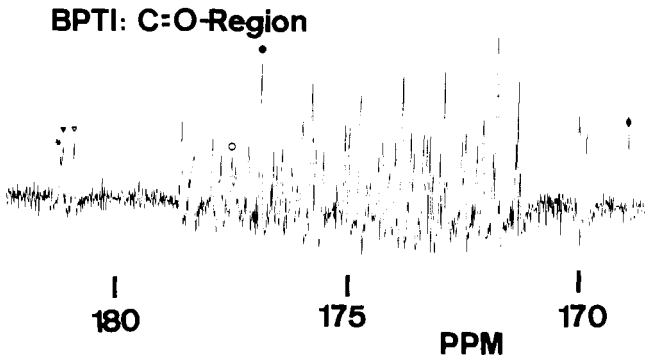


FIGURE 5: Spectral region from 168 to 182 ppm of the $^1H$ noise-decoupled $^{13}C$ FT NMR spectrum of BPTI, $T$ = 40 °C, pD = 4.2. Within approximately 48 h, 292 000 transients were accumulated with an acquisition time of 0.44 s. To improve the spectral resolution, the free induction decay was multiplied with the window function $\sin \pi t/t_s$, with $t_s$ = 0.44 s (De Marco & Wüthrich, 1976). Resonance identifications (see text) are indicated by (✱) Ala-58 -COOH; (▼, ▽) Glu -COOH; (●, ○) Asp -COOH; (♦) Arg-1 >C=O.

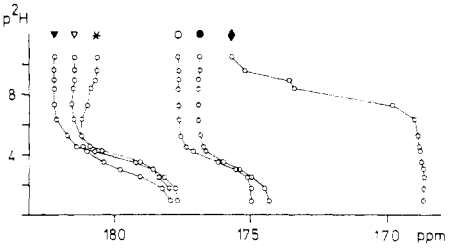


FIGURE 6: Plot vs. $p^2H$ of the chemical shifts of the carbon resonances in Figure 5, which were assigned to Ala-58 -COOH (✱); Glu -COOH (▼, ▽); Asp -COOH (●, ○) and Arg-1 >C= (♦).

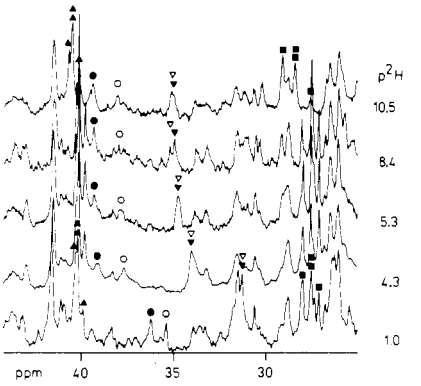


FIGURE 7: Spectral region from 25 to 45 ppm of the $^1H$ noise-decoupled $^{13}C$ FT NMR spectrum of BPTI at various $p^2H$ values, $T$ = 35 °C. Resonance identifications (see text) are indicated by (▲) Lys ε-$CH_2$; (●, ○) Asp β-$CH_2$; (▼, ▽) Glu γ-$CH_2$; (■) Lys δ-$CH_2$.

the four side chain carboxyl carbons of Asp-3, Asp-50, Glu-7, and Glu-49 were identified from the $p^2H$ dependence of the chemical shifts in the range from $p^2H$ 0.5 to 10.5. In Figure 5, the titrating resonances are indicated by ✱, ▼, ▽, ○, ●, and ♦, and the chemical shifts of these resonances at different $p^2H$ values are plotted in Figure 6. Individual ones of these six lines were then further assigned to specific locations in the polypeptide chain.

Since it titrates between $p^2H$ 7 and 9 with a titration shift of approximately 7 ppm, resonance ♦ had to come from the carbonyl carbon of Arg 1 (Christl & Roberts, 1972; Wüthrich, 1976). The two resonances ● and ○, which are at 177.0 and 177.7 ppm at neutral $p^2H$ (Figure 6), were assigned to the two Asp side chain carboxyls, since the corresponding chemical shifts for Asp in model oligopeptides are very similar, i.e., 178.5 ppm at neutral $p^2H$ (Christl & Roberts, 1972; Wüthrich, 1976; Richarz & Wüthrich, 1978). The resonance ✱ was assigned to the chain terminal carboxyl group of Ala-58, since its $pK_a$ coincides with the $pK_a$ value previously determined for Ala-58 from $^1H$ NMR studies (De Marco et al., 1977) and from $^{13}C$ NMR of the methyl group (Figure 4), and since the small upfield titration shift between pH 7 and 9 parallels similar phenomena in the $^1H$ NMR and $^{13}C$ NMR of the α-methine group and the β-methyl group of Ala-58 (Brown et al., 1978). From comparison with model peptides, where the Glu side chain carboxyl is at 182.8 ppm at neutral $p^2H$ (Wüthrich, 1976), the two lowest field resonances ▽ and ▼, which are at 181.6 and 182.3 ppm at neutral $p^2H$ (Figure 6), were assigned to the two Glu side chain carboxyls. The chemical shift of the resonance ✱ is 181.4 ppm at $p^2H$ 6.0, whereas the carboxyl chemical shift of C-terminal Ala in model peptides is 180.4 ppm at neutral $p^2H$ (Richarz & Wüthrich, 1978).

The assignments of the resonances ♦ and ✱ to Arg-1 and Ala-58 on the basis of the pH dependence of the chemical shifts are unambiguous. The differentiation between the carboxyl resonances of Asp (● and ○) and Glu (▼ and ▽) from comparison of the chemical shifts with those in model peptides should also be a valid approach. All these four carboxyl groups are located near the protein surface in BPTI (Deisenhofer & Steigemann, 1975), and the relative chemical shifts between the carboxyls of Asp and Glu, i.e., ca. 5 ppm at neutral $p^2H$, are large compared with the extent of most conformation dependent $^{13}C$ chemical shifts in globular proteins (Wüthrich, 1975). With the resonance assignments in Figures 5 and 6, the pH titration shifts for the two Glu carboxyl resonances in BPTI are markedly larger than those of the two Asp carboxyls, which was also observed in model peptides of Glu and Asp (Richarz & Wüthrich, 1978).

(c) *Identification of Methylene Carbon Resonances of Ionizable Amino Acid Side Chains.* In the 90.5-MHz $^{13}C$ NMR spectrum, the region from 25 to 45 ppm was sufficiently well resolved so that $p^2H$ dependences of individual resonance lines could be observed (Figure 7). Four groups of resonances in this region were found to titrate in the acid $p^2H$ range, i.e., the lines ▽, ▼, ○, and ● in Figure 7. From comparison of the chemical shifts with those in model peptides (Wüthrich, 1978), the two lines ▽ and ▼, which are at approximately 35.0 ppm at neutral $p^2H$, were assigned to γ-$CH_2$ of Glu, the resonances ○ and ● at around 39.0 ppm to β-$CH_2$ of Asp. Eight resonances were found to be shifted downfield at $p^2H$ values higher than 9.0 (Figure 7, ▲ and ■). From comparison with the chemical shifts in model peptides (Richarz & Wüthrich, 1978), the lines at around 40 ppm (▲ in Figure 7) were assigned to

TABLE II: $^{13}C$ Chemical Shifts $\delta^a$ and $pK_a$ Values $^b$of the 5 Carboxylic Acid Groups in BPTI.

| Residue | Resonance[e] | | $pK_a$ | $\delta_{HA}$ | $\delta_{A^-}$ | $\Delta\delta^c$ |
|---|---|---|---|---|---|---|
| Glu-7 | δ-COOH | (▼) | 3.6 | 177.7 | 182.3 | +4.6 |
| | δ-COOH | (▽) | 3.7 | 178.0 | 181.6 | +3.6 |
| Glu-49 | γ-$CH_2$ | (▼) | 3.7 | 31.2 | 35.0 | +3.8 |
| | γ-$CH_2$ | (▽) | 3.8 | 31.2 | 35.2 | +4.0 |
| Asp-3 | γ-COOH | (●) | 3.1 | 174.3 | 177.0 | +2.7 |
| | γ-COOH | (○) | 3.4 | 174.9 | 177.7 | +2.8 |
| Asp-50 | β-$CH_2$ | (●) | 3.0 | 36.3 | 39.6 | +3.3 |
| | β-$CH_2$ | (○) | 3.4 | 35.4 | 38.2 | +2.8 |
| Ala-58 | COOH | (✱) | 3.0 | 178.3 | 181.4[d] | +3.1 |
| | α-CH | | 2.7 | 49.9 | 52.5[d] | +2.6 |
| | β-$CH_3$ | | 2.8 | 17.3 | 18.1[d] | +0.8 |

[a] $^{13}C$ chemical shifts are in ppm relative to external $Me_4Si$, where internal dioxane was taken to be at 67.8 ppm. $T$ = 35 °C. [b] $pK_a$ values were determined by nonlinear least-squares fits of the observed chemical shifts to one-proton titration curves (Figure 9). [c] $\Delta\delta = \delta_{A^-} - \delta_{HA}$. [d] For Ala-58, only the $p^2H$ dependence in the range from $p^2H$ 1.0 to $p^2H$ 6.0 (Figures 4 and 6) was included in the present least-squares analysis. A more detailed interpretation including the $p^2H$ dependence of the chemical shifts between $p^2H$ 6.0 and 10.5 (Figure 6) will be given elsewhere (Brown et al., 1978). [e] See Figures 5-9.

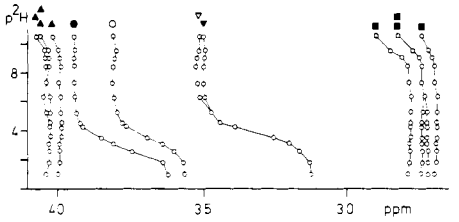


FIGURE 8: Plot vs. $p^2H$ of the chemical shifts of the methylene carbon resonances in Figure 7 which were assigned to Lys ε-$CH_2$ (▲); Asp β-$CH_2$ (●, ○); Glu γ-$CH_2$ (▼, ▽); and Lys δ-$CH_2$ (■).

the ε-$CH_2$'s and the lines at around 27 ppm (■ in Figure 7) to the δ-$CH_2$'s of the four lysines in BPTI.

Figure 8 shows a plot vs. $p^2H$ of the observed chemical shifts for the methylene carbons identified in Figure 7. From the $p^2H$ variations of the $^{13}C$ chemical shifts observed in model peptides (Christl & Roberts, 1972; Keim et al., 1973; Richarz & Wüthrich, 1978), no further resonance lines of BPTI in the spectral region from 25 to 45 ppm were expected to have titration shifts of 1.0 ppm or more between $p^2H$ 1.0 and 10.5, unless such shifts were caused by $p^2H$ dependent conformational changes.

(d) *Ionization Constants of the Carboxyl Groups.* The pH-dependent chemical shifts of the carboxyls and the peripheral methylene carbons assigned to the Asp and Glu side chains were used to determine the microscopic ionization constants for these residues. With a nonlinear least-squares procedure the experimental points were fitted to a one-proton titration curve

$$\delta(\mathrm{pH}) = \frac{\delta_{HA} + \delta_{A^-}10^{(\mathrm{pH}-pK_a)}}{1 + 10^{(\mathrm{pH}-pK_a)}} \qquad (1)$$

The results of this fitting procedure are shown in Figure 9, where the titration curves were obtained with the parameters in Table II. The chemical shifts for the protonated and the deprotonated species, $\delta_{HA}$ and $\delta_{A^-}$, and the $pK_a$ value were all optimized by these fits.

Figure 9 shows that the experimental pH dependences of the chemical shifts correspond very closely to one-proton titration curves. Inspection of the $pK_a$ values obtained (Table II) also confirms the correspondences between the carboxyl and methylene carbon resonances of Asp and Glu, which were derived from the chemical shifts (Figure 7).

In Table II, the results of a least-squares fit analysis of the

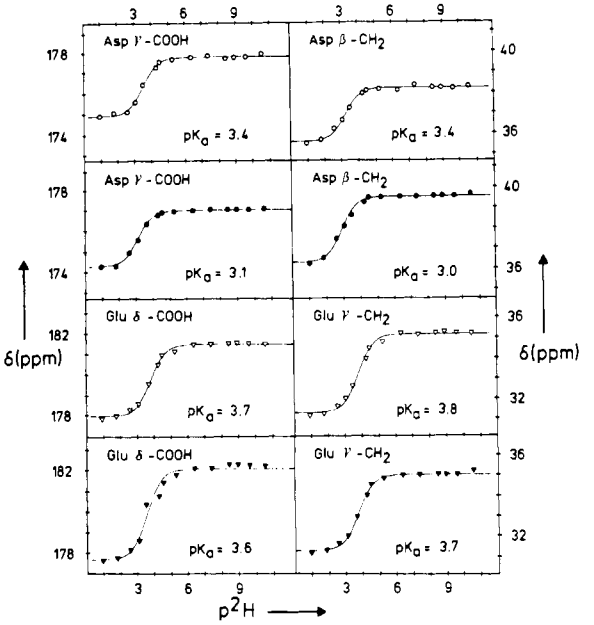


FIGURE 9: Nonlinear least-squares fits to one-proton titration curves (eq 1) of the $p^2H$ dependent resonances in Figures 5-8 which were assigned to the two Asp and the two Glu residues in BPTI. The fit parameters are listed in Table II.

pH dependence in the range from $p^2H$ 1.0 to 6.0 of the $^{13}C$ NMR lines of Ala-58 are also included. The $pK_a$ value of 2.9 ± 0.2 agrees with the data obtained from $^1H$ NMR studies (De Marco et al., 1977).

## Discussion

If one considers the technical aspects of obtaining the $^{13}C$ NMR data collected in Tables I and II, it must be emphasized that the studies of the methyl and methylene carbon resonances depended critically on the use of a high-field spectrometer. Typically, the protonated carbons give rise to unresolved bands in the low-field $^{13}C$ NMR spectra of proteins (Wüthrich, 1976). Since the dipolar line widths of the individual resonances tend to rather decrease at higher fields (Doddrell et al., 1972; Anet, 1974), markedly improved spectral resolution is obtained in the high field $^{13}C$ NMR spectra, which in the present investigation made it possible to resolve even some methylene carbon lines (Figure 7). For the experiments with

the methyl resonances, it was further of particular importance that the $^1H$ NMR spectrum was well resolved at 360 MHz (Figure 2). The important role of the high field for the assignment of corresponding $^1H$ and $^{13}C$ resonances in macromolecules by heteronuclear double resonance experiments is underlined by the observation in Table I that the identification of corresponding $^1H$ and $^{13}C$ chemical shifts in BPTI was mainly limited by the separation of the individual $^1H$ NMR lines. In some cases the $^1H$ chemical shift differences were too small to establish one-to-one correspondences. Even for studies in the carbonyl carbon region (Figure 5), where one expects in principle an increase of the resonance line widths at higher fields to arise from chemical shift anisotropy (Doddrell et al., 1972; Anet, 1974), the present experiments and data presented by others (Shindo & Cohen, 1976) indicate that use of high fields results in improved spectral resolution.

The experiments presented in this paper are part of a continued study by $^1H$ and $^{13}C$ NMR of BPTI (Masson & Wüthrich, 1973; Wagner & Wüthrich, 1975; Wagner et al., 1976; Wüthrich & Baumann, 1976; Brown et al., 1976; De Marco et al., 1977; Richarz & Wüthrich, 1977; Wagner, 1977; Wüthrich et al., 1977). This project is an attempt to arrive at a detailed description of various aspects of the solution conformation of a globular protein, also in the hope to establish structural features which might be of more general interest for proteins at large. BPTI is a suitable protein for this purpose, since its single crystal structure was extensively refined (Deisenhofer & Steigemann, 1975) and its globular conformation is outstandingly stable in solution (Vincent et al., 1971; Masson & Wüthrich, 1973). Recently, BPTI has also become the subject of theoretical investigations on the dynamics of the solution conformation, which may be of interest for the analysis of the spectroscopic data (Gelin & Karplus, 1975; Hetzel et al., 1976; McCammon et al., 1977). In view of the continued work on this project, the individual assignments for numerous methyl $^{13}C$ lines (Table I) provide an interesting basis for the interpretation of spin relaxation data (Richarz & Wüthrich, to be published). Quite generally the methyl carbons give prominent lines in the $^{13}C$ NMR spectra of proteins, which makes them 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 assignment of individual methyl resonances to specific positions in the polypeptide chain can then provide for more meaningful structural interpretations of spectral changes arising from intermolecular interactions of the protein.

The $pK_a$ values for Asp and Glu (Table II) provided the remaining data needed for a quantitative analysis of the pH dependence of the amide proton exchange rates (G. Wagner & K. Wüthrich, to be published) and were also essential for the analysis of the NMR phenomena arising from complex formation between BPTI and lanthanide shift and line broadening reagents (Wüthrich et al., 1978). One might have expected mutual interactions between the nearby residues Asp-3 and Glu-7, and Glu-49 and Asp-50, respectively, to be manifested in the pH titration curves. Since the experimental data correspond to simple one-proton titration curves (Figure 9), no evidence for through-space interactions between different side chain carboxyl groups was obtained in the present study. It is then interesting to note that, in the x-ray structure in crystals grown from a solution with pH 10.5, the Asp and Glu side chains are oriented so as to evade close mutual contacts (Deisenhofer & Steigemann, 1975).

Apparently, the data in Table I are the first set of $^{13}C$ chemical shifts for protonated carbon atoms in a globular protein, where the resonances could be individually assigned and can thus be related to the corresponding $^1H$ chemical shifts and to the protein conformation. Meaningful values for the dispersion of methyl $^{13}C$ chemical shifts by conformation dependent interactions in BPTI are 2.7 ppm for the five nonterminal alanines, 3.3 ppm for the two isoleucine $C_\delta$'s and 1.0 ppm for the three threonines. The "random coil" chemical shifts obtained from model peptides are 17.7 ppm for Ala, 11.1 ppm for Ile $C_\delta$, and 20.0 ppm for Thr (Wüthrich, 1976; Richarz & Wüthrich, 1978). Table I then 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 (Allerhand et al., 1973). For Met-52 the methyl $^{13}C$ chemical shift coincides with the random coil value, 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 to $\geq$23.3 ppm, while the corresponding random coil values are between 15.7 and 23.1 ppm. The distribution of the chemical shifts for these resonances (Table I) indicates again the occurrence of conformation dependent shifts of the order of one to several ppm for individual lines.

A comparison of the dispersion of corresponding methyl $^1H$ and $^{13}C$ chemical shifts in BPTI (Table I) shows that overall the spread of the $^{13}C$ resonances is markedly larger. Most strikingly, the $^{13}C$ chemical shifts for the $\delta$-methyl groups of Ile-18 and Ile-19 differ by 3.2 ppm, whereas the corresponding $^1H$ chemical shifts coincide to within <0.01 ppm. An attempt to relate the observed methyl $^{13}C$ chemical shifts with the ring current shifts computed from the crystal structure of BPTI (Wüthrich, 1976) clearly showed that more sophisticated models will have to be developed to relate $^{13}C$ shifts with proton conformations; e.g., ring current effects would account only for a dispersion of ca. 0.6 ppm among the five nonterminal alanines and for a relative chemical shift of 0.2 ppm between the two Ile $\delta$-$CH_3$ resonances. Earlier work implied that electric field effects (Buckingham, 1960; Horsley & Sternlicht, 1968; Batchelor et al., 1973; Yonemoto, 1973, 1974; Batchelor, 1975; Seidman & Maciel, 1977) and bond polarization effects (Cheney & Grant, 1967) are important factors governing $^{13}C$ chemical shifts. Work is currently in progress in our laboratory to include these and possibly other interactions in attempts to interpret the methyl $^{13}C$ shifts in BPTI on the basis of the crystal structure of the protein.

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