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Individual Assignments of the Methyl Resonances in the ^1H Nuclear Magnetic Resonance Spectrum of the Basic Pancreatic Trypsin Inhibitor†

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ABSTRACT: In earlier work the resonances of the 20 methyl groups in the basic pancreatic trypsin inhibitor (BPTI) had been identified in the 360-MHz ^1H nuclear magnetic resonance (NMR) spectra and most of the methyl lines had from spin-decoupling experiments been assigned to the different types of amino acid residues. The assignments to the different amino acid types were now completed by studies of the saturation transfer between the denatured and the globular forms of the inhibitor and by spin-decoupling experiments in nuclear Overhauser enhancement (NOE) difference spectra. These distinguished between the methyl resonances of Ala and Thr. Furthermore, for most of the methyl resonances, individual assignments to specific residues in the amino acid sequence were obtained from measurements of intramolecular proton–proton NOE's, use of lanthanide NMR shift and relaxation probes, and comparative studies of various chemically modified forms of BPTI. These data provide the basis for individual assignments of the methyl ^{13}C NMR lines in BPTI and for detailed investigations of the relations between the spatial structure of the protein and the chemical shifts of the methyl groups. The methyl groups in BPTI are of particular interest since they are located almost exclusively on the surface of the protein and thus represent potential natural NMR probes for studies of the protein–protein interactions in the complexes formed between BPTI and a variety of proteases.

The basic pancreatic trypsin inhibitor (BPTI) is a small protein which regulates the function of proteases (Tschesche, 1974a,b). In its biological role, BPTI[1] forms quite outstandingly stable complexes with trypsin and other proteolytic enzymes (Tschesche, 1974a,b; Laskowski & Sealock, 1971). Studies of the intermolecular contacts in these complexes are of interest both to investigate the structural basis of the biological function of BPTI and more generally to gain further insight into the nature of intermolecular protein–protein interactions. High resolution nuclear magnetic resonance (NMR) is a suitable technique to complement the single crystal x-ray data on the trypsin–BPTI complex (Rühlmann et al., 1973; Huber et al., 1974) by measurements in solution (Wüthrich, 1976). In this context it is then of interest that the methyl groups in BPTI are located almost exclusively on the surface of the protein (Deisenhofer & Steigemann, 1975) and are thus potential natural NMR probes for studies of intermolecular interactions of BPTI. To provide a basis for such experiments, the present paper describes a ^1H NMR investigation of the methyl groups in the isolated form of BPTI.

BPTI has a molecular weight of 6500 and consists of one polypeptide chain with 58 amino acid residues (Tschesche, 1974a,b). The amino acid sequence includes 15 amino acid residues which contain 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 (Kassell & Laskowski, 1965). Previously, the resonances of the 20 methyl groups were identified in the ^1H NMR spectrum at 360 MHz (De Marco et al., 1977). To relate the NMR parameters with aspects of the protein structure, these resonances had to be assigned to specific protons in the polypeptide chain. Since in the primary structure of BPTI most of the different types of amino acid residues are contained more than once, two levels of resonance identification had to be considered (Wüthrich, 1976). First, in the previous paper (De Marco et al., 1977) the spin systems of most of the different types of amino acids had been distinguished by spin-decoupling experiments. The assignments on this level were now completed by additional double irradiation techniques which resulted in the distinction between the methyl

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[1] Abbreviations used: BPTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor, Trasylol, Bayer Leverkusen, West Germany); BPTI*, modified form of BPTI in which the peptide bond between Lys-15 and Ala-16 had been cleaved; des-(A,R)-BPTI, modified form of BPTI in which the polypeptide chain had been cleaved by removal of the amino acid residues Ala-16 and Arg-17; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt; FT, Fourier transform; NOE, nuclear Overhauser enhancement.

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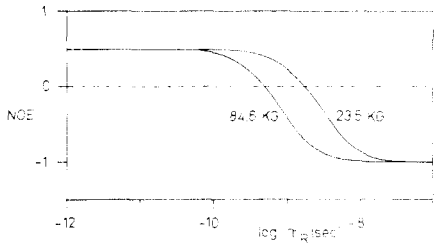

FIGURE 1: Plot of the NOE vs. the correlation time for isotropic reorientation for a pair of protons. The curves are given for two field strengths corresponding to $\omega = 6.28 \times 10^8\ s^{-1}$ and $\omega = 2.26 \times 10^9\ s^{-1}$, respectively (eq 1-6).

resonances of Ala and Thr. Second, the individual methyl resonances had to be assigned to specific positions in the amino acid sequence. It is the main purpose of the present article to describe how the second level assignments for most of the methyl lines in BPTI were achieved by a combination of different NMR techniques. For these experiments it was essential that the spin systems of the eight aromatic residues in BPTI, i.e., Phe-4, -22, -33, and -45, and Tyr-10, -21, -23, and -35, had previously been identified (Wagner et al., 1975, 1976), the resonances of the four Tyr had also been individually assigned (Snyder et al., 1975), and a highly refined crystal structure was available (Deisenhofer & Steigemann, 1975).

Materials and Methods

(a) *Preparation of NMR Samples.* The basic pancreatic trypsin inhibitor BPTI (Trasylol Bayer Leverhusen) was obtained as a gift from the Farbenfabriken Bayer AG. Two chemically modified inhibitors, BPTI* and des-(A,R)-BPTI, for which the preparation was previously described (Jering & Tschesche, 1976), were obtained from Professor H. Tschesche, Technical University Munich. In BPTI*, the peptide bond Lys-15–Ala-16 had been cleaved and in des-(A,R)-BPTI the polypeptide chain had been cleaved by removal of the amino acid residues Ala-16 and Arg-17. The NMR spectra of these modified forms of BPTI were previously investigated in detail (De Marco et al., 1977; Wagner, 1977).

For the NMR studies, 0.01 M to 0.001 M solutions of the inhibitor in D_2O were used. Solutions with different pD values were prepared by the addition of minute amounts of NaOD or DCl. The pD values reported in the paper are pH-meter readings uncorrected for isotope effects.

For the experiments with lanthanides, stock solutions with 0.05 M concentration of Gd(III) and 0.5 M concentration of the other metal ions were prepared by dissolving the oxides with heating in D_2O using 35% DCl (Perkins, 1977). The final solutions used for the experiments reported here contained 0.005 M BPTI and 0.08 M lanthanide, with the exception of the measurements with Gd(III) reported in Figure 8. NMR experiments with Gd(III) employed NMR tubes treated with Repelcote (Hopkin and Williams Ltd.) to minimize the problems of Gd(III) binding to the glassware (Jones et al., 1974). In experiments at constant pD, piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Sigma Chemical Co. Ltd.) was used as the buffer.

^1H NMR spectra were recorded on a Bruker HXS 360 spectrometer. The spectral resolution was improved by digital filtering with the sine bell routine (De Marco & Wüthrich, 1976). The sample temperature in the probe was measured using an ethylene glycol standard. Chemical shifts are in ppm relative to internal DSS.

(b) *Nuclear Overhauser Enhancement (NOE) Studies.* The NOE for a pair of protons was long ago calculated for the situation that dipole-dipole coupling between the two protons was the only relaxation mechanism and that the dipolar interaction was modulated by isotropic rotational motions (Solomon, 1955).

$$\mathrm{NOE} = \sigma/\rho \tag{1}$$

with

$$\sigma = W_2 - W_0 \tag{2}$$

$$\rho = W_0 + 2W_1 + W_2 \tag{3}$$

W_0, W_1, and W_2 are the transition probabilities between the eigenstates of the longitudinal components of the spin operators given by

$$W_0 = \frac{\tau_R}{8\hbar^2}\frac{4}{5}k^2 \tag{4}$$

$$W_1 = \frac{\tau_R}{2\hbar^2}\frac{3}{10}k^2\frac{1}{1+\omega^2\tau_R{}^2} \tag{5}$$

$$W_2 = \frac{2\tau_R}{\hbar^2}\frac{3}{10}k^2\frac{1}{1+4\omega^2\tau_R{}^2} \tag{6}$$

where $k = \hbar^2\gamma^2/r^3$, τ_R is the correlation time for isotropic rotational motion, ω the proton resonance frequency, γ the gyromagnetic ratio, and r the distance between the two spins. In Figure 1 the NOE for a pair of protons is plotted vs. τ_R. It is seen that for very short τ_R, i.e., for the situation of extreme motional narrowing (Abragam, 1962), the NOE is +0.5 and is independent of the field strength H_0. In the τ_R range between 10^{-10} and 10^{-8} s, the NOE goes from +0.5 to −1. For these intermediate tumbling rates, the NOE is markedly dependent on the field strength H_0. For τ_R longer than 10^{-8} s the NOE is −1 and is independent of H_0.

In the interior of a globular protein, each individual proton is generally surrounded by several nearby protons which all contribute to the spin relaxation. However, if the mutual dipole-dipole coupling between two protons is much larger than the dipolar coupling with any of the other protons in the protein molecule, Figure 1 may be used to estimate the NOE expected between the two nearby spins. Since rotational correlation times for globular proteins are typically of the order 10^{-9} to 10^{-7} s, negative NOE's can result. This expectation was borne out by earlier experiments (Balaram et al., 1972; Campbell et al., 1974). Obviously, since in real proteins additional interactions may contribute to the proton relaxation, the curves in Figure 1 correspond to upper limits for the size of the NOE to be expected. On the other hand, Figure 1 clearly illustrates the importance of using high field strengths for NOE experiments with small and medium sized proteins, where correlation times of 10^{-9} to 10^{-8} s prevail and hence the NOE's at lower fields may be very small.

In the present paper NOE measurements were used to identify nearby methyl and tyrosine ring protons in the interior of the BPTI molecule. Computation of the values $r_{ij}{}^{-6}$, where r_{ij} is the proton–proton distance between the methyl group i and the Tyr ring j, in the refined crystal structure (Deisenhofer & Steigemann, 1975) had shown that outstandingly strong dipole-dipole coupling occurred for a small number of combinations of methyl and tyrosine protons. Since the tyrosine resonances had previously been identified (Wagner et al., 1975; Snyder et al., 1975), several methyl groups could thus be assigned.

The NOE was also employed to obtain improved spectral resolution in "NOE difference spectra" (Richarz & Wüthrich, 1978a). With the use of suitable gating sequences, which are described in detail in the above reference, the difference be-

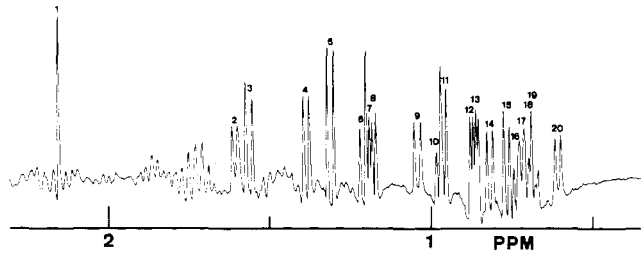

FIGURE 2: The 360-MHz FT ^{1}H NMR spectrum between 0.5 and 2.2 ppm of a 0.005 M BPTI solution in neutral D_2O at 45 °C. The spectral resolution was improved with the sine bell routine (De Marco & Wüthrich, 1976). The numbers indicate the multiplets of the 20 methyl groups in BPTI.

tween a spectrum acquired with selective presaturation and a spectrum recorded without double resonance irradiation was obtained. Spin-decoupled NOE difference spectra were obtained when a double resonance irradiation frequency was applied during data acquisition.

(c) *Use of Lanthanide Probes.* On the basis of previous experience (Dwek, 1973; Nieboer, 1975; Reuben, 1975; Dobson & Levine, 1976; Wüthrich, 1976), it can readily be argued that contact shifts are negligibly small in the experiments described here and hence only pseudocontact shifts need to be considered. The pseudocontact shift at a nucleus i, δ_{pi}, is given by

$$\delta_{pi} = D'\left(\frac{3\cos^2\theta_i - 1}{r_i^3}\right) - D''\left(\frac{\sin^2\theta_i \cos 2\phi_i}{r_i^3}\right) \quad (7)$$

where D' and D'' are ligand field parameters, and r_i, θ_i, and ϕ_i are the spherical coordinates of the ith proton, where the lanthanide is at the origin. θ defines the angle between the principal axis z and the vector joining the metal ion with proton i, and ϕ_i is the angular position in the plane perpendicular to z. Equation 7 has been found empirically in many cases (Dobson & Levine, 1976) to reduce to the form for axial symmetry, either because D'' is zero as a consequence of true axial symmetry or because cos 2ϕ becomes effectively zero by a dynamic averaging in solution. In such cases, only the first term of eq 7 is retained and the geometrical term relating the lanthanide-induced shifts with the molecular structure is simply $(3\cos^2\theta_i - 1)r_i^{-3}$.

Besides producing pseudocontact shifts (eq 7), certain lanthanides affect also the spin relaxation times (Solomon, 1955; Bloembergen, 1957; Dwek, 1973; Wüthrich, 1976). Here, Gd(III) was used as a relaxation probe. From the Solomon–Bloembergen equation, the paramagnetic contribution to the transverse relaxation time, T_{2p}, is given by

$$\frac{1}{T_{2pi}} = \frac{1}{r_i^6} f(\tau_c) \quad (8)$$

where only dipolar relaxation is considered. r_i is the distance between the metal ion and the ith proton, τ_c the effective correlation time for the dipolar interaction, and $f(\tau_c)$ is a complex function of τ_c. In eq 8, the geometrical term relating T_{2pi} to the molecular structure is r_i^{-6}. As the width of a Lorenztian line at half-height in Hz is given by $1/\pi T_2$, line widths increase upon binding of Gd(III). These line broadening effects were detected by means of conventional NMR difference spectra, where the difference between a normal and a broadened spectrum was recorded (Dwek, 1973; Wüthrich, 1976).

The analysis of the lanthanide shift and relaxation data was based on the refined crystal coordinates of BPTI (Deisenhofer & Steigemann, 1975) and the energy-refined coordinates of BPTI (Hetzel et al., 1976). It was assumed that lanthanide binding occurred at the carboxyl groups of BPTI. The lanthanide coordinates at the carboxyl groups were generated using a metal-to-oxygen distance of 2.3 Å and assuming bidentate ligation. The r_{ij}^{-6} and r_{ij}^{-3} terms from each proton i to the metal position j were computed in Å^{-6} and Å^{-3}. In the calculation of the geometric terms corresponding to $(3\cos^2\theta_i - 1)$ (eq 7), it was assumed that the principal symmetry axis of the lanthanide susceptibility tensor coincided with the direction of the C–C bond between the carboxyl carbon and the penultimate carbon atom (Dobson & Levine, 1976).

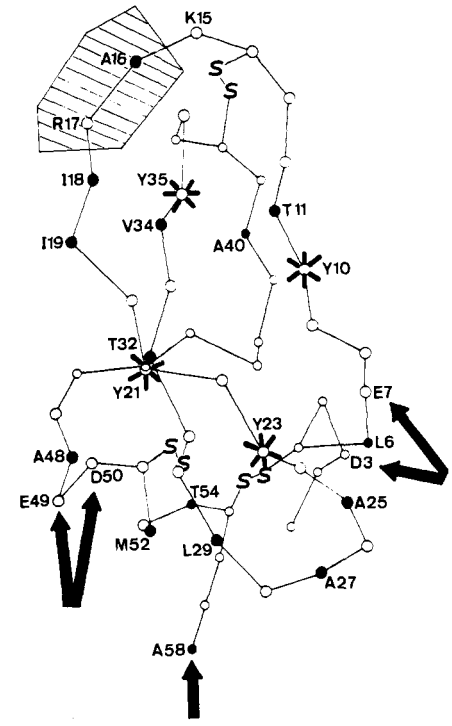

FIGURE 3: α-carbon diagram of the BPTI molecule obtained from a single crystal x-ray analysis (Huber et al., 1971). The three disulfide bonds are also indicated. The figure illustrates structural features which were important for the resonance assignments described in this paper. Amino acid residues are identified with one-letter symbols: A = Ala, V = Val, T = Thr, L = Leu, I = Ile, E = Asp, D = Glu, Y = Tyr, K = Lys. Furthermore, filled circles (●) indicate the residues bearing methyl groups and the four tyrosines are denoted by ☼. The arrows point to the five carboxyl groups in BPTI, which are potential binding sites for lanthanides. The lysyl residue 15 in the reactive site is next to the shaded area which covers the locations of the chemical modifications in BPTI* and des(A,R)-BPTI.

Results

The first part of this section describes the experiments used to resolve the remaining ambiguities in the assignments of the 20 methyl resonances in BPTI (Figure 2) to the different types of amino acid residues (De Marco et al., 1977). Three additional parts, b–d, treat the procedures used for individual assignments of methyl multiplets to specific residues. These studies were based on the features of the primary structure (Kassell & Laskowski, 1965; Jering & Tschesche, 1976) and the molecular conformation in single crystals (Huber et al., 1971; Deisenhofer & Steigemann, 1975) which are outlined in Figure 3. Reference points for locating individual methyl groups were the four tyrosyl residues, the resonances of which were previously studied in detail and individually assigned (Wagner & Wüthrich, 1975; Wagner et al., 1975; Snyder et al., 1975), the five carboxyl groups as potential binding sites for lanthanides, and the sites of the chemical modifications of the protein in BPTI* and des-(A,R)-BPTI (Jering & Tschesche, 1976). A survey of the results is given in Table I.

TABLE I: Methyl Resonance Assignments in the ^{1}H NMR Spectrum of the Basic Pancreatic Trypsin Inhibitor: Results Obtained with the Different Techniques Described in This Paper (for Details See Text).

Resonance[a]	Identification of spin systems[c]	New identifications of spin systems: By spin decoupling in NOE difference spectra	New identifications of spin systems: By saturation transfer between globular and denatured protein	Individual resonance assignments: By NOE (aromatics related to the methyls by NOE)	Individual resonance assignments: Using lanthanide probes (metal binding sites)	Individual resonance assignments: From chemical modifications	Individual assignments from combined data
1	Met-52						Met-52
2	Ala or Thr	Thr	Thr + Ala				Thr-54
3	Ala or Thr	Ala or Thr		Ala-25 (Tyr-23)			Ala-25
4	Thr	Thr	Thr			Thr-11 or Thr-32	Thr-11
5	Ala-58	Ala or Thr			Ala-58 (C terminus)		Ala-58
6	Ala-16	Ala or Thr		Ala-40 (Tyr-35)		6-8 contain Ala-16 and Ala-40	Ala-40
7	Ala or Thr	Ala or Thr					Ala-27[a]
8	Ala or Thr	Ala or Thr			Ala-48		Ala-16[a]
9	Ala or Thr	Ala or Thr		Ala-48 (Tyr-21)	(Glu-49, Asp-50)		Ala-48
10	Ile-γ^2						Ile-γ^2 18 or 19
11, 12	Val or Leu				Leu-6 (Asp-3, Glu-7)		Leu-6
13, 15	Val or Leu					Leu-29	Leu-29
14, 17	Val or Leu					Val-34	Val-34
16	Ile-γ^2						Ile-γ^2 18 or 19
18	Ile-δ						Ile-δ 18 and 19
19	Ile-δ						
20	Ala or Thr	Ala or Thr	Thr	Thr-32 (Tyr-21)		Thr-11 or Thr-32	Thr-32

[a] Same notation as in Figure 2. [b] Both the methyl and C_α proton chemical shifts of Ala-27 and Ala-16 are nearly identical and could therefore not be distinguished. [c] From De Marco et al., 1977.

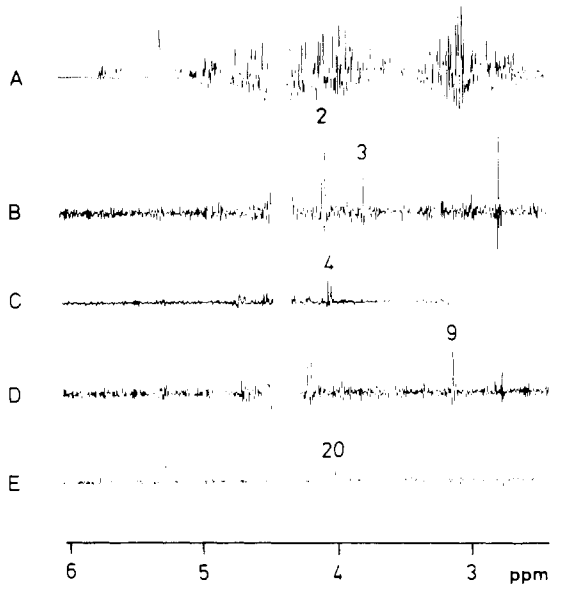

FIGURE 4: The 360-MHz ^{1}H NMR spectra of BPTI between 2.5 and 6.0 ppm, solvent D_2O, pD = 7.0, T = 35 °C. The spectral resolution was improved with the sine bell routine (De Marco & Wüthrich, 1976). (A) Normal FT spectrum. (B-E) Spin-decoupled NOE difference spectra where the same frequency was selected for presaturation and spin decoupling. The double resonance irradiation was applied at the following locations in the spectrum: (B) methyl resonances 2 and 3 at 1.58 ppm; (C) methyl 4 at 1.39 ppm; (D) methyl 9 at 1.04 ppm; (E) methyl 20 at 0.59 ppm.

(a) *Identification of the Ala and Thr Spin Systems.* The methyl resonance assignments to different types of amino acid residues obtained previously with spin decoupling difference spectra (De Marco et al., 1977) are given in the second column of Table I. With this technique, the distinction between Ala and Thr depends mainly on recognizing in the difference spectra the multiplet structure for the protons attached to the carbon atoms next to the methyl groups. One limitation of the method is a consequence of the limited selectivity of this type of difference spectroscopy in cases where the spin-decoupling must be applied in a crowded spectral region. Improved selectivity was now obtained using spin-decoupled NOE difference spectra (Richarz & Wüthrich, 1978a). All the spin systems assigned previously to Ala or Thr (Table I) were reinvestigated with this technique. Figure 4 illustrates that quite selective difference spectra were obtained in the crowded region from 3 to 5 ppm (Figure 4A). A negative NOE was built up by presaturation of individual methyl groups, and the same methyl resonance was then decoupled during data acquisition. The spectra 4B-E correspond to the differences between decoupled spectra obtained with and without presaturation of the methyl multiplets. In spectrum 4B the decoupling frequency was centered on the nearly overlapping methyl resonances 2 and 3 (Figure 2). This resulted in a doublet at 3.95 ppm and a singlet at 3.74 ppm. As a control, irradiation at 3.95 ppm caused the collapse of the multiplet of methyl 2 and irradiation at 3.74 ppm decoupled methyl 3. From this, the resonance at 3.95 ppm was assigned to β-CH of Thr and the corresponding methyl 2 to γ-CH_3 of Thr. From the singlet structure in spectrum 4B, the resonance at 3.74 ppm and the corresponding methyl 3 can come either from Ala or Thr, as is further discussed below. The experiment 4C showed that the methyl resonance 4 (Figure 2) corresponds to Thr. For the methyls 5–9 and 20, singlets were observed in the spin-decoupled NOE difference spectra (Figure 4, D and E) (Table I).

Even with the improved selectivity of the NOE differenee spectra, only two of the three methyls of Thr in BPTI could be identified on the basis of the β-CH multiplet structures (Table I). This is a consequence of an inherent limitation of this approach: The doublet structure of the β-CH resonance of Thr expected upon decoupling of the methyl line may not be resolved when either the dihedral angle χ is fixed near a gauche configuration for the α- and β-methine protons and hence $J_{\alpha\beta}$

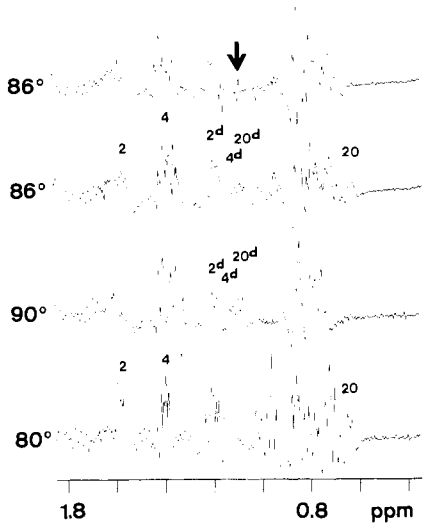

FIGURE 5: Saturation transfer experiments used to distinguish between methyl resonances of Ala and Thr. The spectra were obtained at 360 MHz from a BPTI solution in D_2O, pD = 1.0, after resolution enhancement with the sine bell routine. The bottom trace shows the spectrum of the native inhibitor at 80 °C. Three methyl resonances are identified by their numbers in Figure 3. At 90 °C the inhibitor was denatured. At 86 °C the spectrum contained two sets of resonances originating from the native and denatured forms of BPTI. The top trace illustrates how the chemical shifts of corresponding resonances in the two forms of BPTI were identified. Double resonance irradiation of resonance 20^d (arrow) lead via saturation transfer to the disappearance of resonance 20. The positions of the resonances 2d, 4d, and 20d, which were determined by this technique, are also indicated.

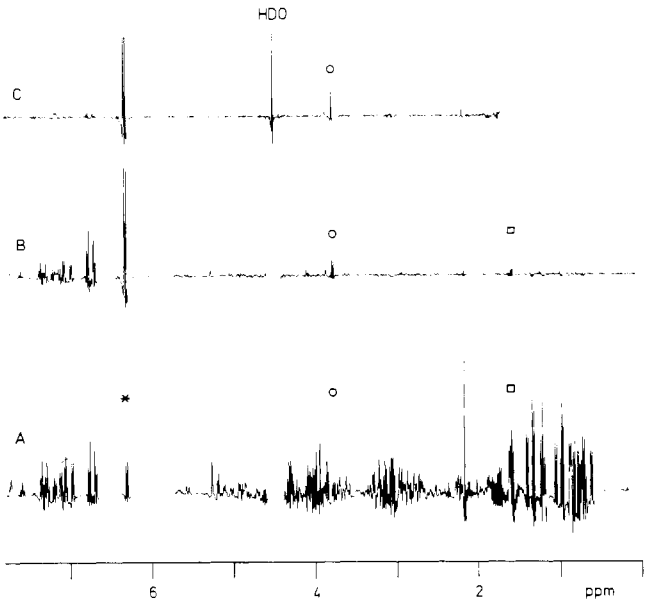

FIGURE 6: Assignment of methyl resonance 3 (Figure 3) to Ala-25 by NOE experiments. The spectra were obtained at 360 MHz from a solution of BPTI in D_2O, pD = 7.0, T = 35 °C, after resolution enhancement with the sine bell routine (De Marco & Wüthrich, 1976). (A) Normal FT ^{1}H NMR spectrum. (B) NOE difference spectrum obtained with presaturation of the doublet resonance of the ϵ protons of Tyr-23 at 6.30 ppm (*). (C) Spin-decoupled NOE difference spectrum obtained with presaturation at 6.30 ppm (*) and spin decoupling during the data acquisition at the position of the methyl resonance 3 (□).

≈ 2 Hz (Wüthrich, 1976), 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. For BPTI, inspection of the refined crystal structure (Deisenhofer & Steigemann, 1975) showed that $\chi^1 = -77°$, +53°, and −60° for Thr-11, Thr-32 and Thr-54, respectively. The corresponding coupling constants $^3J_{\alpha\beta}$ would be (Wüthrich, 1976) 10.1 Hz, 2.0 Hz, and 11.3 Hz for Thr-11, -34, and -54, respectively. To distinguish between methyl groups of Ala and Thr, different criteria must thus in certain cases be employed. In the following experiments the identification of the third Thr methyl resonance in BPTI was based on the different random coil chemical shifts for Ala and Thr (Wüthrich, 1976; Bundi, 1977).

The globular form of BPTI is outstandingly stable with respect to thermal denaturation (Vincent et al., 1971) and under suitable conditions the denaturation is reversible (Masson & Wüthrich, 1973). Comparison of spectra recorded between 80 and 90 °C in acidic solutions of BPTI (Figure 5) with spectra taken at ambient temperature showed that the globular form of BPTI prevailed at 80 °C, whereas the spectrum at 90 °C was typical for the denatured protein (Wüthrich, 1976). Because of the different random coil chemical shifts for the methyl protons of Ala and Thr (Wüthrich, 1976; Bundi, 1977), the two types of residues could be distinguished in the spectrum of the denatured BPTI at 90 °C. The three doublet resonances between 1.1 and 1.2 ppm (Figure 5, 2d, 4d, and 20d) correspond to the three threonines; five methyl resonances of Ala were at around 1.4 ppm and the C-terminal Ala-58 was expected to be at around 1.6 ppm at pD = 1.0 (Wüthrich, 1976). At intermediate temperatures between 80 and 90 °C, BPTI was only partially denatured and the NMR spectra contained two sets of resonances corresponding to the globular and the denatured forms. At 86 °C and pD = 1.0, 50% of the inhibitor was in the native form and 50% was denatured (Figure 5). By small variations of the temperature, the relative populations of the native and denatured forms could be changed. While the rate of exchange of BPTI molecules between the native and denatured forms was obviously slow on the time scale of the conformation dependent chemical shifts of the threonine methyl resonances at 360 MHz (Figure 5), it was fast compared with the methyl proton spin relaxation rates T_1^{-1}. Hence, saturation transfer experiments (Wüthrich, 1976) could be employed to relate the threonine methyl resonances in native and denatured BPTI. This is illustrated in the top trace of Figure 5, where irradiation of the doublet 20d at 1.1 ppm, which comes from the denatured BPTI, caused saturation of the doublet 20 in the native protein. Similarly, 1:1 correspondences were established between the resonances 2 or 3, which were completely overlapped in the spectrum at 80 °C (Figure 5), and 2d, and 4 and 4d, respectively (Table I, column 4). Combining the results of columns 3 and 4 in Table I, the methyl resonances 2, 4 and 20 correspond to the three threonines in BPTI, the resonances 3, 5 and 6–9 to the six alanines.

(b) *Individual Assignments of Methyl Resonances with NOE Studies.* Negative NOE's between the previously assigned Tyr spin systems (Wagner & Wüthrich, 1975; Wagner et al., 1975; Snyder et al., 1975) and the methyl resonances were investigated. Again the NOE difference technique (Richarz & Wüthrich, 1978a) was used as illustrated in Figure 6. The doublet resonance at 6.30 ppm (Figure 6A, *), which was previously assigned to the ϵ protons of Tyr-23 (Snyder et al., 1975), was presaturated. The NOE difference spectrum (Figure 6B) contains a quartet at 3.74 ppm (○) and a doublet at 1.57 (□). In the spin-decoupled NOE difference spectrum obtained with presaturation at 6.30 ppm (Figure 6A, *) and spin decoupling at 1.57 ppm (Figure 6A, □), the resonance at 3.74 (○) appears as a singlet (Figure 6C). These experiments thus manifest a through-space interaction between the ϵ pro-

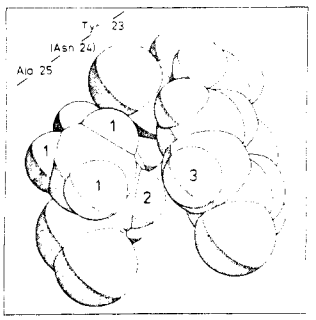

FIGURE 7: Computer drawing of the peptide fragment from Tyr-23 to Ala-25 in the refined crystal structure of BPTI (Deisenhofer & Steigemann, 1975); the side chain of Asn-24 was omitted. The size of the individual atoms corresponds to the van der Waals radii. The following atoms are identified by numbers: (1) methyl protons of Ala-25; (2) α-proton of Ala-25; (3) ϵ-proton of Tyr-23.

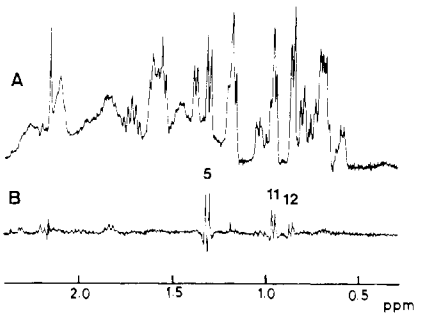

FIGURE 8: Resonance assignments with the use of Gd(III) as a relaxation probe. The figure shows 360-MHz ^{1}H NMR spectra of the methyl region of BPTI after resolution enhancement with a shifted sine bell (De Marco & Wüthrich, 1976; Wagner, 1977). (A) D_2O solution of 5×10^{-3} M BPTI and 4×10^{-4} M La(III), pD = 5.4, T = 25 °C. (B) Difference spectrum of A from a spectrum of the same solution of BPTI in which there was 3.8 $\times 10^{-4}$ M La(III) and 2×10^{-5} M Gd(III). The methyl signals 5, 11, and 12 (Figure 3) are identified in spectrum B.

tons of Tyr-23 and the alanine spin system 3 (Figure 2). As a control, presaturation of methyl resonance 3 produced a NOE in the Tyr resonance at 6.30 ppm.

In similar experiments, evidence for through space interactions between Tyr-21 and Tyr-35 and individual methyl groups was obtained. These studies were somewhat less straightforward, since the resonances of the ϵ protons of Tyr-21 and -35 are superimposed (Snyder et al., 1975; Wagner et al., 1976). Hence, irradiation at 6.75 ppm saturated the ϵ protons of both, Tyr-21 and Tyr-35, producing NOE's on the methyl resonances 6, 9, and 20. Since the δ proton resonances of Tyr-21 and Tyr-35 have largely different chemical shifts (Snyder et al., 1975; Wagner et al., 1976), more specific correlations were obtained from the spin-decoupled NOE difference spectra obtained with presaturation of one of the methyls 6, 9, or 20 and spin decoupling of the δ proton lines of either Tyr-21 and Tyr-35 during acquisition. It was thus found that methyl 6 interacts with Tyr-35, whereas the methyls 9 and 20 were correlated with Tyr-21.

Inspection of the refined crystal structure of BPTI (Deisenhofer & Steigemann, 1975) showed that only a very limited number of close contacts between aromatic protons of Tyr and methyl groups occur. Figure 7 illustrates the local structure including Tyr-23 and Ala-25. In Table II, values of r^{-6} are given for all possible combinations of the tyrosines-21, -23, and -35 with the 20 methyl groups. Combining Table II with the experimental NOE data, in particular considering the assignments of the methyl lines to the different types of amino

TABLE II: Relative Values for $10^7 \times r_{ij}^{-6}$.[a]

Methyl group	Tyr-21		Tyr-23		Tyr-35	
	δ-H	ϵ-H	δ-H	ϵ-H	δ-H	ϵ-H
Met-52	18	11	576	3400	0	0
Ala-16	1	1	0	0	135	93
Ala-25	2	1	3320	8700	0	0
Ala-27	10	6	82	118	0	0
Ala-40	5	1	1	0	800	6400
Ala-48	3850	10800	32	14	1	0
Ala-58	1	0	7	15	0	0
Thr-11	2	1	0	0	660	132
Thr-32	3430	9100	5	2	7	2
Thr-54	6	2	57	23	1	1
Ile-18γ^2	39	24	0	0	12400	737
Ile-18δ	5	3	0	0	4000	2140
Ile-19γ^2	650	1330	1	0	33	8
Ile-19δ	32	43	0	0	34	8
Leu-6	0	0	68	96	0	0
Leu-6	1	1	1811	2800	0	0
Leu-29	88	50	147	137	0	0
Leu-29	20	19	40	75	0	0
Val-34	5	4	0	0	130	28
Val-34	11	10	0	0	32	8

[a] r_{ij} is the average distance between ring protons of the tyrosines and methyl protons of the aliphatic amino acid side chains in the refined crystal structure of BPTI (Deisenhofer & Steigemann, 1975). Large numbers indicate that sizable NOE's might be produced between the protons i and j. In the computation of r_{ij} the 180° flips of the Tyr rings (Wagner et al., 1976; Hetzel et al., 1976) and the rotational motions of the methyl groups were considered. Hence, the average was taken over two equally populated rotation states of the aromatic rings and 12 equally populated and equally spaced rotation states of the methyl groups. Very similar numbers were obtained when instead of the refined crystal structure the energy refined structure of BPTI (Hetzel et al., 1976) was used.

acid residues (Table I, columns 2–4) leads to the individual assignments given in the fifth column of Table I.

(c) *Individual Assignments of Methyl Resonances with the Use of Lanthanide Probes.* The methyl resonances in the ^{1}H NMR spectrum of BPTI were investigated after the addition of diamagnetic La(III), the relaxation probe Gd(III) and the shift probes Pr(III) and Yb(III). A 0.005 M solution of BPTI at pD 5.4 gave essentially identical methyl resonances with and without the addition of 0.08 M La(III), which indicated that the protein conformation was at most very little affected by lanthanide binding (see also Marinetti et al., 1976). Combined with the observation that the paramagnetic probes affected the methyl resonances selectively, this provided the basis for using the lanthanides to obtain a number of individual methyl resonance assignments (Table I).

The use of Gd(III) as a relaxation probe is illustrated in Figure 8. Measurements were performed at variable pD and variable ionic strength. The difference spectra (Figure 8B) contained only those resonances which were broadened by Gd(III). In acidic solutions, there was no evidence for line broadening in the spectrum. At higher pD and low ionic strength, the methyls 5, 11, and 12 appeared in the difference spectrum against a clean background (Figure 8B).

The chemical shifts of the methyl resonances produced by the addition of Yb(III) and Pr(III) are shown in Figure 9. The extent of the lanthanide induced shifts depended markedly on pD. In the acidic solutions at pD around 1.0, the spectrum was essentially unaffected by the methyl ions. For most of the methyl lines, maximum shifts occurred at pD around 5.0 or higher. Sizeable lanthanide induced perturbations were observed for the methyl resonances 5, 9, 11, and 12, which were

previously assigned to Ala-58, another alanine, and a leucine or valine (Table I, columns 2–4). The assignment of the methyls 11 and 12 to one Leu or Val residue was confirmed at selected points in the titration curves of Figure 9 by the spin decoupling criteria used for the assignment in the absence of lanthanides (De Marco et al., 1977); e.g., in a solution of 0.005 M BPTI and 0.08 M Yb(III) at pD 5.2 and 45 °C, irradiation at 1.96 ppm decoupled methyls 11 and 12 at 1.50 and 1.20 ppm without affecting any of the other methyl signals. The Yb(III) induced shift of the coupled proton was thus 0.23 ppm downfield, compared with downfield shifts of 0.55 and 0.34 ppm for the methyls 11 and 12 in the same spectrum. The data in Figure 9 provide also evidence for the pseudocontact origin of the lanthanide induced shifts, in that the opposite directions of the Pr(III) and Yb(III) induced shifts concur with the theoretical pseudocontact shifts of 11.0 and −22 ppm for Pr(III) and Yb(III), relative to Dy(III) at 100 ppm (Dwek, 1973; Wüthrich, 1976).

To be able to use the spectral perturbations by Gd(III), Pr(III), and Yb(III) for the assignment of individual resonances, the lanthanide binding sites had to be localized. The primary structure of BPTI contains five carboxyl groups as potential ligands for lanthanides (Figure 3). The titration curves of Figure 9 are compatible with carboxyl groups as weak lanthanide binding sites. From the observation of a second titration endpoint in the neutral pD range for the previously assigned (De Marco et al., 1977) C-terminal Ala resonance 5 (Figure 9), one binding site was shown to be at the C terminus. A second titration endpoint of resonance 5 was previously also observed in the absence of lanthanides; from comparison with BPTI chemically modified at the N terminus, it was attributed to a salt bridge interaction between the N terminus and the C terminus of the polypeptide chain (Brown et al., 1978). At least two additional binding sites were inferred from the locations of the independently assigned residue types (Table I) relative to the carboxyl groups in the crystal structure of BPTI. Figure 3 shows that the set of five carboxyl groups of BPTI may be divided into the three subsets of the C terminus, Asp-3 and Glu-7, and Glu-49 and Asp-50 on the grounds of proximity in the protein structure. Of the three leucine and valine residues in BPTI, only one can be near to any of the five carboxyl groups. This is Leu-6, which is close to Asp-3 and Glu-7. Likewise, of the five nonterminal alanine residues in BPTI, only Ala-48 can be close to any of the five carboxyl groups, i.e., Glu-49 and Asp-50. These findings suggest that methyls 11 and 12 are from Leu-6, and that methyl 9 is that of Ala-48, and at the same time showing that the lanthanides bind at all three of the subsets of carboxyl groups.

These assignments were confirmed by predictions of the lanthanide induced shifts and line broadenings using the refined atomic coordinates of BPTI (Deisenhofer & Steigemann, 1975). A satisfactory analysis resulted with the assumptions that only one binding site in each of the three subsets was occupied under the conditions of our experiments and the positions and directions of the three lanthanide symmetry axes were those determined by the C terminus and the side chain carboxyls of Asp-3 and Asp-50 (Table III; for procedures used, see Materials and Methods). For the line broadening data with Gd(III), the theoretical data of Table III combined with the previous assignments to types of amino acid residues show that methyl 5 corresponds to Ala-58 and methyls 11 and 12 to Leu-6. With similar argumentation, the lanthanide induced shifts confirm the assignments of resonances 5, 9, and 11 and 12 to Ala-58, Ala-48, and Leu-6, respectively. The Ala-48 and Ala-58 methyls have the largest shifts of the six alanine methyls both in the experiments (Figure 9) and in the predictions (Table III). Of the three leucines and valines in BPTI, only the leucine-6 methyls have shifts opposite in sign to those predicted for Ala-58 and Ala-48. The agreement between predicted and experimental shifts is quite satisfactory, bearing in mind that the possible binding of lanthanides at Glu-7 and Glu-49 has been neglected, and that the calculations are first order. A more detailed account of weak binding of lanthanides to BPTI, which will also include a more thorough assessment of the role of Glu-7 and Glu-49 in the protein–lanthanide interactions, is given elsewhere (Perkins & Wüthrich, 1978a).

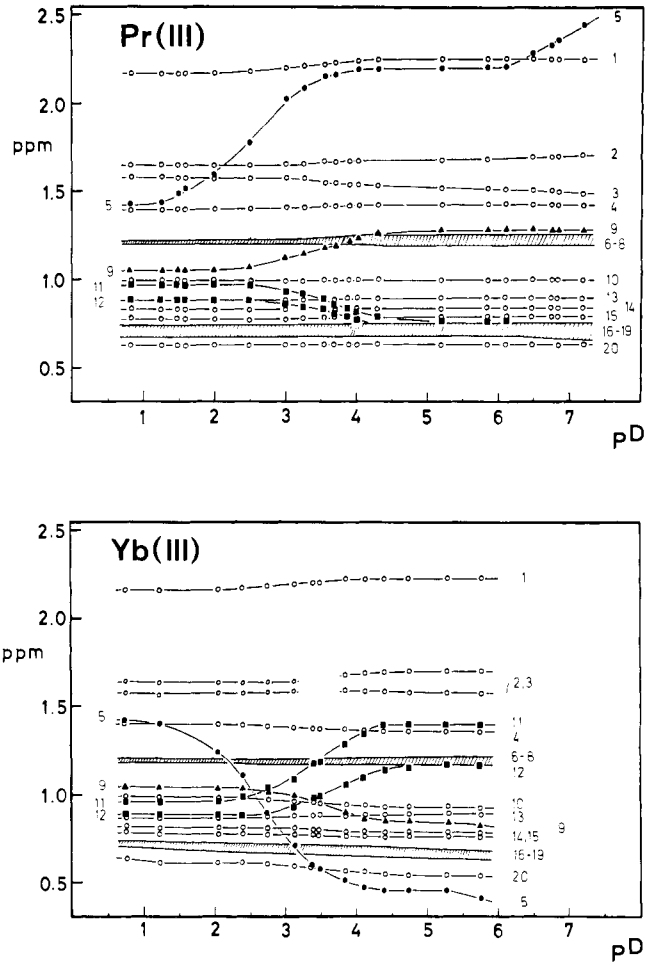

FIGURE 9: Plots vs. pD of the chemical shifts of the 20 methyl ^{1}H NMR signals of BPTI in solutions containing 5×10^{-3} M BPTI and 8×10^{-2} M Yb(III), or 8×10^{-2} M Pr(III), respectively, at 25 °C. The titration curves of individual methyl multiplets are identified by the respective numbers (Figure 3). Shaded areas contain overlapping resonances which could not be individually resolved over the entire pD range. It is readily seen that the methyls 5, 9, 11, and 12 (●, ▲, and ■) have the largest titration shifts.

(d) *Comparative Studies of Chemically Modified BPTI Species.* Native BPTI was compared with two species which had been modified at the active site (Figure 3), i.e., BPTI*, where the peptide bond Lys-15–Ala-16 was cleaved, and des(A,R)-BPTI, which was obtained from BPTI* by removal of the two amino acid residues Ala-16 and Arg-17. Inspection of the ^{1}H NMR spectra of the three proteins, in particular the resonances of the amide protons and the aromatic rings, showed that the overall features of the molecular conformations of BPTI were maintained after the chemical modifications, which caused local perturbations near the modification site (Figure 3; De Marco et al., 1977; Wagner, 1977). On this basis, comparison of BPTI, BPTI*, and des(A,R)-BPTI was now used to identify those among the 20 methyl resonances in

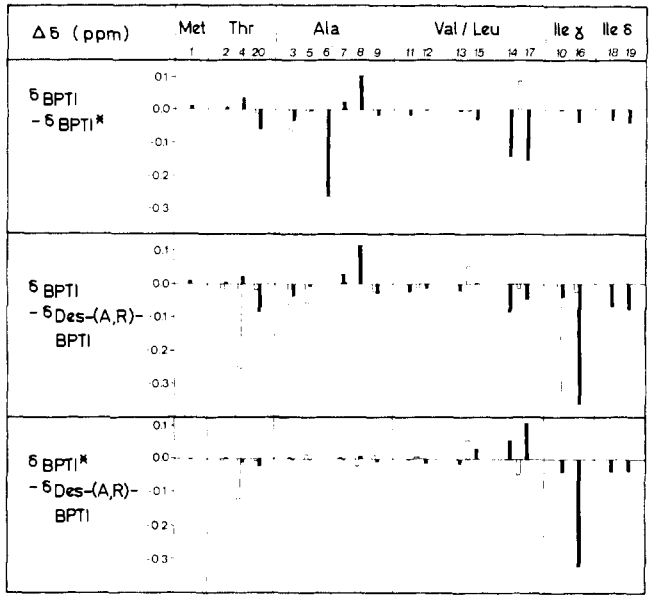

FIGURE 10: Chemical-shift differences between BPTI, BPTI*, and des-(A,R)-BPTI for the 20 methyl proton resonances (filled bars) and the protons attached to the carbon atom to which the methyl groups are bonded (open bars). The top line gives the relative chemical shifts for BPTI and BPTI*, the middle line those for BPTI and des-(A,R)-BPTI, and the bottom line those for the two chemically modified species. The data were obtained at 360 MHz in D_2O solutions of the proteins, pD = 4.6, T = 45 °C (Wagner, 1977).

BPTI (Figure 2) which correspond to methyl groups located near the modification sites.

The ^1H NMR spectra of BPTI* and des-(A,R)-BPTI were presented in detail elsewhere (De Marco et al., 1977; Wagner, 1977; Wagner et al., 1978). In Figure 10, the chemical shift data on the methyl groups in native BPTI and the two modified proteins are collected. In the three rows, the figure presents the chemical shift differences for corresponding methyl groups in BPTI and BPTI*, BPTI and des(A,R)-BPTI, and BPTI* and des(A,R)-BPTI, respectively. For both modified species, the methyl resonances were separated into the subgroups of Met, Ile, Val or Leu, and Ala or Thr by double resonance difference spectroscopy (Wagner, 1977). Furthermore, from the chemical shifts of the spin systems attributed to Ala or Thr, the Thr resonances in BPTI* and des(A,R)-BPTI could be directly related to those in BPTI. For the investigation of their individual assignments, the methyl resonances in the three species could therefore be compared within the small groups indicated in Figure 10.

Met-52 contains the only singlet methyl resonance in the protein, which could thus unambiguously be assigned (De Marco et al., 1977). In the present context, the Met resonance contributed to the evidence that the protein conformation outside the immediate environment of the fragment Lys-15 to Ile-18 (Figure 3) was only little affected by the chemical modifications.

Among the three threonines, those containing the methyl resonances 4 and 20 are more extensively affected by the protein modifications than resonances 2. This comes out most clearly when the two derivatives are compared, where the conformational differences should be even more strictly localized than between the native molecule and any one of the modified species. In the crystal structure, Thr-32 and Thr-11 are closer to the modification site than Thr-54, which is at the opposite end of the molecule (Figure 3). Therefore, the chemical shift differences in Figure 10 indicate that methyl

TABLE III: Relative Values for $\Sigma_j r_{ij}^{-6}$ and $\Sigma_j r_{ij}^{-3}(3\cos^2\theta_{ij} - 1)$ for the 20 Methyl Groups i in BPTI.[a]

Methyl group	$\Sigma_j r_{ij}^{-6}$	$\Sigma_j r_{ij}^{-3}(3\cos^2\theta_{ij} - 1)$[b]
Met-52	4	122
Ala-16	0	4
Ala-25	5	11
Ala-27	1	37
Ala-40	1	24
Ala-48	6	117
Ala-58	673	1117
Thr-11	0	18
Thr-32	1	53
Thr-54	13	86
Ile-18γ^2	1	8
Ile-18δ	0	1
Ile-19γ^2	1	35
Ile-19δ	0	20
Leu-6	175	−176
Leu-6	25	−34
Leu-29	1	54
Leu-29	1	48
Val-34	0	17
Val-34	0	20

[a] The summation is over the three lanthanide binding sites at the C terminus and the side chain carboxyls of Asp-3 and Asp-50. The refined crystal structure coordinates (Deisenhofer & Steigemann, 1975) were used. The lanthanide coordinates at the three carboxyl groups were generated as described in the Experimental Section. The methyl proton coordinates correspond to the average of 12 equally spaced and equally populated rotation states. Very similar numbers were obtained when instead of the refined crystal structure the energy refined coordinates (Hetzel et al., 1976) were used. [b] Negative numbers correspond to upfield shifts in the presence of Pr(III).

2 corresponds to Thr-54, and the methyls 4 and 20 to Thr-11 and Thr-32.

For three of the six methyl doublets in BPTI which were assigned to Ala residues (Table I), the corresponding resonances in the modified species could readily be identified. These are resonance 5 of the C-terminal Ala, which was in all three species identified from its pH dependence (De Marco et al., 1977), and resonances 3 and 9, which were in all three species well separated from the other four Ala lines and experienced only small shifts due to the modifications (Figures 2 and 10). The remaining three Ala methyl resonances 6 to 8 in BPTI have very similar shifts at 1.214, 1.194, and 1.183 ppm, and the α-proton multiplets coupled to 7 and 8 have also almost identical chemical shifts at 4.300 and 4.290 ppm (De Marco et al., 1977). Therefore, these resonances were not individually related to specific lines in the corresponding group of three resonances 6′ to 8′ at 1.457, 1.183 and 1.111 ppm in BPTI*. The effect of the protein modification on the resonances 6′ to 8′ in BPTI*, $\Delta\delta$, could be determined to within 0.031 ppm, which is the chemical shift spread of the lines 6 to 8 in BPTI, i.e., -0.274 ppm $\leq \Delta\delta(6') \leq -0.243$ ppm, 0.000 ppm $\leq \Delta\delta(7') \leq 0.031$ ppm, and 0.072 ppm $\leq \Delta\delta(8') \leq 0.103$ ppm. Resonance 6′ in BPTI* was assigned to Ala-16 since it titrated with a pK_a corresponding to that of a terminal α-amino group and since it disappeared in des(A,R)-BPTI (De Marco et al., 1977). The correspondence between resonances 7′ and 8′ in BPTI* and des(A,R)-BPTI could readily be established since these resonances were essentially unaffected by the removal of Ala-16 and Arg-17 (Figure 10). Overall the data for the alanines in Figure 10 thus showed that two of the three

resonances 6 to 8 in BPTI experience the largest shifts in the modified proteins. Besides Ala-16, only Ala-40 is near the modification site (Figure 3). Hence we concluded that two of the resonances 6 to 8 correspond to Ala-16 and Ala-40. In BPTI*, resonance 6′ was assigned to Ala-16, and in both modified proteins, resonance 8′ corresponds to Ala-40.

Of the three pairs of Leu or Val methyls 11 and 12, 13 and 15, and 14 and 17, the latter were most strongly affected by the chemical modifications (Figure 10). Since Val-34 is the only Val/Leu type residue near the modification site, resonances 14 and 17 were assigned to this position. In des-(A,R)-BPTI, and in particular in the comparison between BPTI* and des(A,R)-BPTI, small chemical shifts were also observed for the resonances 13 and 15. Even though both Leu residues are well separated from the modification site (Figure 10), the latter is related to Leu-29 in that both are located in the β sheet (Deisenhofer & Steigemann, 1975). It seems quite conceivable that removal of two amino acid residues from the β sheet in des-(A,R)-BPTI might cause a slight rearrangement of this structural entity. On this basis, resonances 13 and 15 were assigned to Leu-29.

Quite sizeable chemical shifts were observed for the resonances of Ile-18 and -19. This was not unexpected, since both residues are near the modification site (Figure 3). These resonances were not further distinguished, since the chemical shifts for the δ methyls of Ile-18 and Ile-19 were essentially identical in all three species (De Marco et al., 1977; Figure 10), and since 1:1 correspondences between the Ile γ^2-methyl resonances in BPTI and the modified proteins could not unambiguously be established.

Discussion

As a complement to the ^{1}H NMR studies of the amide protons (Masson & Wüthrich, 1973; Karplus et al., 1973; Wagner, 1977) and the aromatic side chains (Snyder et al., 1975; Wagner & Wüthrich, 1975; Wagner et al., 1975, 1976), which provided information on static and dynamic features of the interior parts of the solution structure of BPTI, investigations of the methyl resonances are of interest for characterizing the protein surface both in the isolated molecule and in complexes formed between BPTI and the proteases. The main theme of the present paper was the assignment of the methyl resonances. Resonance assignments in a protein, which can in certain cases be a real challenge for the spectroscopist, present the groundwork needed for detailed studies of various aspects of the molecular structure in solution. This section presents some comments on the experimental techniques used to obtain the data in Table I, describes structural information which resulted directly from the present experiments, and gives a brief outline of potential uses of the assigned methyl resonances for further structural studies of BPTI.

The identification of the methyl resonances from the different types of amino acid residues in this and a previous paper (De Marco et al., 1977) provided the distinctions between the subgroups 1 Met, 6 Ala, 3 Thr, 3 Val + Leu, 2 Ile γ^2, and 2 Ile δ (Table I, columns 2–4), where the γ^2- and δ-methyl multiplets of the two isoleucines were not individually correlated. These data depended for most of the amino acid residues entirely on recognizing the characteristic spin systems in the double resonance difference spectra. The distinction between Ala and Thr methyls by the saturation transfer experiments was further based on the different random coil chemical shifts for the two types of residues (Bundi, 1977). In no case reference was made to the crystal structure of the protein. It should be emphasized that the distinction between the different types of amino acid residues was essential for the unambiguous interpretation of the experiments used for the individual resonance assignments.

Three techniques, NOE studies, use of lanthanide probes, and comparison of different chemically modified proteins, were applied for individual methyl resonance assignments (Table I, columns 5–7), and Met and Ala-58 were previously independently assigned (De Marco et al., 1977). In all, 14 of the 20 methyls in BPTI were thus assigned. For the remaining 6 methyls, i.e., those of the two alanines-16 and -27 and the two isoleucines-18 and -19 (Table I, column 8), a further distinction was not achieved. However, since the chemical shifts of the methyl groups and the α protons of the two alanines-16 and -27 coincide to within 0.01 ppm and the δ-methyl chemical shifts of the two isoleucines agree to within <0.01 ppm (De Marco et al., 1977), the NMR parameters of these 4 methyls were accurately determined even without individual assignments. For the two Ile γ^2-methyls, individual assignments could so far not be obtained, in spite of the apparent favorable situation for distinguishing the two resonances by NOE studies (Table II).

The three techniques used for the individual methyl resonance assignments represent a quite ideal combination for BPTI since they cover different regions of the molecular structure. Figure 3 shows that, while the chemical modifications in BPTI* and des-(A,R)-BPTI are near the reactive site, the lanthanide binding sites are near the chain termini at the opposite end of the molecule. The NOE studies are related to three of the four tyrosines, which are located in interior parts of the molecular structure. As a consequence of this fortunate situation, almost all the methyl resonance assignments could be based on positive evidence, and the assignments of several lines could independently be assured by two different techniques (Table I).

In contrast to the experiments which led to the distinction of methyl resonances from different types of amino acid residues, the studies leading to the individual assignments depended to some extent on the crystal structure atomic coordinates (Deisenhofer & Steigemann, 1975). The three techniques used may hence also be distinguished by the use made of the crystal structure and by the perturbations imposed on the native protein structure. By the NOE experiments, the native protein was not perturbed. It was assumed that the molecular structure of BPTI observed in single crystals is maintained in aqueous solution, which seems well justified by earlier observations (Vincent et al., 1971; Masson & Wüthrich, 1973; Wagner, 1977). They did not, however, rely on the accurate preservation of the atomic coordinates but rather on the qualitative nearest neighbor relations between Tyr rings and methyl groups manifested in the numbers of Table II. All four interactions between tyrosines and methyl groups of Ala and Thr which were likely to cause NOE's (Table II) were actually observed by the technique used. The above-mentioned lack of success with the γ^2-methyls of Ile (Table II) might be due to dominance of proton–proton dipolar coupling within the Ile side chains which might quench the NOE between individual Ile protons and protons of neighboring side chains.

In principle, binding of lanthanides may cause conformational changes in proteins. In BPTI, the comparison of those NMR spectral features which were not affected by the lanthanides clearly showed that at most small local changes occurred. In contrast to earlier studies with nitrotyrosine-BPTI (Marinetti et al., 1976), only weak lanthanide binding to the carboxyl groups had to be considered, so that the two sets of experiments cannot readily be compared. Similar to the analysis of the NOE experiments, the use of lanthanide probes was based on the assumption that the crystal structure of BPTI

was essentially maintained in solution, but it did not have to rely on accurate preservation of the crystal structure atomic coordinates in the solution conformation. A detailed account of metal binding to native BPTI is given elsewhere (Perkins & Wüthrich, 1978a).

Cleavage of the polypeptide chain and removal of amino acid residues will generally affect the protein conformation. It was all the more essential that earlier studies of the amide protons and the aromatic residues had shown that the solution conformations of BPTI* and des-(A,R)-BPTI are very similar to that of native BPTI (De Marco et al., 1977; Wagner, 1977). In contrast to the NOE and lanthanide experiments, not all the effects of the chemical modifications used for methyl assignments were strictly short range perturbations. As was argued in the results section, some long range perturbations on methyl groups appeared to be propagated through the β sheet in the BPTI structure (Figure 3; Huber et al., 1971).

The combination of the data collected in Table I reflects an overall consistency of the results obtained from the different techniques. These results provide the most complete set of evidence to date that the molecular conformations of BPTI in single crystals and in solution are very similar. The coincidence of crystal and solution conformations manifested, e.g., in the NOE experiments based on the local structure illustrated in Figure 7, is all the more remarkable since most of the methyl groups are located near the protein surface (Deisenhofer & Steigemann, 1975) where they might be affected by different intermolecular interactions in the crystal and in solution. The three Thr residues 11, 32, and 54 are unique in that the spatial orientation of the side chains could also be characterized by measurement of the spin–spin coupling constants $^3J_{\alpha\beta}$. The experimental coupling constants agreed for all three Thr to within ± 1 Hz with the values expected from the crystal structure on the basis of the dependence of $^3J_{\alpha\beta}$ on the dihedral angle χ^1 (Wüthrich, 1976).

In addition to the interest for studies of the protease–BPTI complexes discussed in the introductory section, individual resonance assignments open the way for systematic investigations of the relations between NMR parameters and protein conformation. BPTI is a particularly suitable "model protein" for research in this area, since the crystal structure was highly refined (Deisenhofer & Steigemann, 1975), the globular conformation is outstandingly stable in aqueous solution (Vincent et al., 1971; Masson & Wüthrich, 1973; Wagner, 1977), and it was recently the subject of theoretical investigations on the dynamics of the globular structure (Gelin & Karplus, 1975; Hetzel et al., 1976; McCammon et al., 1977; Berendsen, 1977), which must also be considered in the interpretation of the spectral properties. Since 1:1 correspondences between ^{1}H and ^{13}C resonances of the methyl groups in BPTI were previously established (Richarz & Wüthrich, 1977), individual assignments are now available for the ^{1}H and ^{13}C lines of most of the methyl groups (Table I; Richarz & Wüthrich, 1978b). The ^{1}H and ^{13}C chemical shifts of individual methyl groups can hence be related to their local environment in the protein (Perkins & Wüthrich, 1978b). It is to be hoped that projects of this type will eventually lead to more direct correlations between protein structure and NMR chemical shifts, which is undoubtedly one of the essential gaps to be filled in order to further enhance the potential of NMR techniques for structural studies of proteins.

Acknowledgments

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

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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 pK_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 pK_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 pK_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

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[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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