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Dynamics of the Aromatic Amino Acid Residues in the Globular Conformation of the Basic Pancreatic Trypsin Inhibitor (BPTI)

I. ^{1}H NMR Studies

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Summary. The basic pancreatic trypsin inhibitor (BPTI) was investigated by high resolution ^{1}H NMR techniques at 360 MHz. Observation of the amide proton resonances of the polypeptide backbone showed that the globular conformation of BPTI determined by X-ray studies in single crystals is maintained in aqueous solution over the temperature range from 4° to 87°. NMR studies over this temperature range of the aromatic amino acid residues of BPTI. i.e. 4 tyrosines and 4 phenylalanines, led to complete assignments of all the aromatic spin systems in the protein. From this, information was obtained on the rotational motions about the C^β–C^γ bond axis of the aromatic rings in the globular form of BPTI. At 25°, two tyrosine rings and one phenylalanine ring are rotating rapidly on the NMR time scale. For the other rings the transitions from slow to rapid rotational motions were investigated at variable temperatures and energy barriers for these intramolecular rate processes determined. The studies of the tyrosine resonances had been described in detail in a previous publication. The present paper describes the identification of the phenylalanine resonances and comments on some technical aspects which might be of quite general interest for the analysis of highly resolved ^{1}H NMR spectra of proteins. Data for the tyrosines and the phenylalanines are compiled in three tables, i.e. the pK_a-values for the tyrosines, the NMR parameters for all eight aromatics, and the parameters $\Delta G^{\neq}$, and, where available, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the rotational motions of the rings.

Key words: NMR–Protein conformation–Molecular dynamics–Proteinase inhibitor.

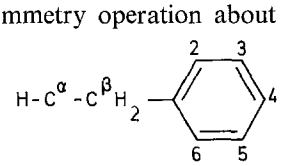
Introduction

For quite some time it has been recognized that the combination of single crystal X-ray crystallography and high resolution NMR in solution is a promising approach for studies of the molecular conformation of globular proteins (Wüthrich, 1976). As a particular asset, NMR spectroscopy has the potential to complete the average spatial structures obtained from the X-ray data with information on dynamic as-

pects of protein conformation. Over the last few years studies of the longitudinal spin relaxation times T_1 of ^{13}C (Allerhand et al., 1971) and ^1H NMR measurements of the rates of exchange of labile protons with deuterium of the solvent (Masson and Wüthrich, 1973) have been particularly emphasized in this context. While the hydrogen-deuterium exchange studies bear mainly on the dynamics of the polypeptide backbone (Hvidt and Nielsen, 1966; Masson and Wüthrich, 1973; Wüthrich, 1976), ^{13}C relaxation times can also provide information on intramolacular motions of amino acid side chains in the time range from approximately 10^{-11} s to 10^{-8} s. Following recent advances in NMR instrumentation and digital data processing, the resolution in certain regions of the ^1H NMR spectra of proteins with molecular weights up to approximately 20 000 can be quite comparable to that obtained hitherto only for low molecular weight compounds. It has thus become possible to obtain complete resonance assignments for the spin systems of individual amino acid residues in proteins, and the well known high resolution NMR techniques for studies of rate processes with characteristic times from approximately 10^{-5} s to 1 s (Pople et al., 1959; Wüthrich, 1976) can now be employed for studies of proteins and possibly other classes of natural and synthetic polymers. The present paper describes an investigation of the basic pancreatic trypsin inhibitor (BPTI) in which a 360 MHz spectrometer was used to delineate the dynamic behaviour of the aromatic rings in the globular form of the protein.

BPTI from bovine pancreas has a molecular weight of 6500, and consists of one polypeptide chain with 58 amino acid residues. The amino acid sequence includes eight aromatic residues, i.e. four tyrosines in the positions 10, 21, 23 and 35, and four phenylalanines in the positions 4, 22, 33 and 45 (Tschesche, 1974). The single crystal X-ray structure of BPTI is known (Huber et al., 1971) and the atomic coordinates were refined at 1.5 Å resolution (Deisenhofer and Steigemann, 1975). The conformation of the polypeptide backbone in BPTI is characterized by the occurrence of a twisted antiparallel β-sheet encompassing the residues 16 to 36, which extends through the full length of the molecule, and a short α-helix formed by the residues 47 to 56 (Huber et al., 1971). The globular conformation of BPTI in aqueous solution is unusually stable towards denaturation by chemicals and by heat (Vincent et al., 1971; Masson and Wüthrich, 1973; Karplus et al., 1973). There is direct evidence from NMR studies, which will be presented in this paper, that the backbone conformation observed in BPTI crystals is maintained in aqueous solution over the entire temperature range from 4° to 87°. The outstanding heat stability made it possible to investigate the proton resonances of the aromatic rings in globular BPTI over a large temperature range, and from this the energy barriers opposing intramolecular rotations of the rings could be characterized.

In the covalent structure of the aromatic rings of tyrosine and phenylalanine, two pairs of protons in positions 2 and 6, and 3 and 5, respectively, are related by a C_2-symmetry operation about the C^β–C^γ bond axis.



Therefore, when these rings are in an isotropic medium, AA′BB′-type NMR spectra for aromatic protons of tyrosine and AA′BB′C-type spectra for phenylalanine are

usually observed (Wüthrich, 1976). In the interior of globular proteins, however, the environment of the aromatic rings is characterized by the non-periodic distribution of structural elements. As a consequence, the individual ring protons may experience different microenvironments. In a static situation where the rings would be rigidly fixed in space in the interior of the protein, one thus has that $\Delta\delta_{2,6} = |\delta_2 - \delta_6|$ and $\Delta\delta_{3,5} = |\delta_3 - \delta_5|$ could be different from 0. Therefore, if the aromatic rings in the interior of the protein are assumed to rotate about the C^{β}–C^{γ} bond, different spectral features may result for each pair of symmetry-related protons. Three limiting cases have been observed in BPTI (Wüthrich and Wagner, 1975; Wagner et al., 1975). (i) If the rotation frequency ν_e is small compared to both $|\nu_2 - \nu_6|$ and $|\nu_3 - \nu_5|$, ABCD type spectra for tyrosine and ABCDE type spectra for phenylalanine are observed. (ii) If ν_e is fast compared to the relative shifts of both pairs of protons, AA′BB′ spectra for tyrosine and AA′BB′C spectra for phenylalanine are observed. (iii) If the conformation dependent shifts $\Delta\delta_{2,6}$ and $\Delta\delta_{3,5}$ are sizeably different, one may at a given rotation rate ν_e have a rapid exchange situation for one of the two pairs of protons, and not for the other one. AA′BC type spectra for tyrosine and AA′BCD type spectra for phenylalanine may thus be encountered. From these general considerations it is quite obvious that investigations of the dynamics of the aromatic rings depend primarily on the identification of the four-spin and five-spin systems of the individual tyrosines and phenylalanines. In previous publications, the identification of the four tyrosine four-spin systems in BPTI at ambient temperature was reported (Wagner and Wüthrich, 1975; Wagner et al., 1975; Snyder et al., 1975); from studies of chemically modified BPTI, these resonances were also assigned to specific residues in the amino acid sequence (Snyder et al., 1975). In the present paper, these earlier studies of the tyrosines are completed by investigations at higher temperatures, and the identification of the four phenylalanine five-spin systems at different temperatures is described. The data on all eight aromatic residues obtained from the experiments reported in this and the previous papers are compiled in three tables, i.e. the NMR parameters, the parameters which characterize the dynamics of the aromatic rings and the pK_a-values for the tyrosines.

Materials and Methods

The basic pancreatic trypsin inhibitor (BPTI, Trasylol®, Bayer Leverkusen, Germany) was obtained from the Farbenfabriken Bayer AG. Ca. 0.01 M solutions of BPTI in ^2H_2O were used for the NMR studies. Solutions with different p^{2}H values between 7.0 and 12.5 were prepared by the addition of minute amounts of 1 M KO^{2}H solution in ^2H_2O. The p^{2}H values reported in the figures and tables are pH meter readings uncorrected for isotope effects.

NMR spectra were recorded on the Bruker instruments HXS-270 and HXS-360. Chemical shifts, δ, are quoted in ppm relative to $\delta_{3,5}$ (Tyr 23) = 6.300 ppm (see footnote to Table 2).

To further improve the resolution in the spectra recorded at 360 MHz, convolution difference techniques (Ernst, 1966; Campbell et al., 1973) were employed. In the convolution difference spectra, the line shapes are given by the difference between two Lorentzian lines L_A and L_B

$$L_{\text{conv. diff.}} = L_A - KL_B \tag{1}$$

where K is a scaling factor. L_A and L_B represent the result of the application of two different line broadening functions with characteristic parameters LW_A and LW_B to the experimental Lorentzian lines L_0

$$L_0 = \int_0^\infty \exp(-t/T_2^*) \cos(\Delta\omega t)\, dt \tag{2}$$

$$L_A = \int_0^\infty \exp(-t/T_2^*) \exp(-t\pi LW_A) \cos(\Delta\omega t)\, dt \tag{3}$$

where $\Delta\omega$ is the difference between the applied frequency and the resonance frequency and T_2^* is a decay constant which includes the natural transverse relaxation time T_2 and the influence of the magnetic field inhomogeneities. Replacement of LW_A by LW_B in Equation (3) gives the corresponding expression for L_B. In all the convolution difference spectra presented in this paper, the following parameters were used:

$$K = 0.94; \quad LW_A = 0.5 \text{ Hz}; \quad LW_B = 2.5 \text{ Hz}$$

The identification of some phenylalanine resonance lines was dependent on a reliable integration of the convolution difference spectra. This was achieved by a simulation in which the spectrum was first arbitrarily divided into a convenient number of readily distinguishable groups of resonances. Each individual group of resonances was then simulated as an envelope of convolution difference line shapes (1) until a satisfactory fit with the experiment was obtained. From this fitting procedure the relative intensities of the individual groups of resonances were evaluated. Since for some resonance lines the number of protons corresponding to their intensities had independently been established, the absolute integrals for all the groups of resonances could thus be evaluated (see Fig. 6 for an illustration of this procedure).

To ascertain the resonance assignments, spectra were simulated by using the parameters of Table 2 in the non-iterative part of the LAOCN3 program (Castellano and Bothner-By, 1964). A conventional plot routine was slightly modified for reproducing convolution difference spectra (see Fig. 9 for an illustration).

Results and Discussion

The significance of the data on the dynamics of the aromatic amino acid residues in BPTI depends critically on the stability of the overall globular molecular conformation over a large range of temperature and pH. Therefore the first part of this section describes experimental evidence concerning the stability of the globular conformation under variable conditions. This is followed by a brief recapitulation and completion of earlier studies of the tyrosine rings, a detailed description of the identification of the phenylalanine resonance lines and a discussion of the dynamics of the aromatic rings.

BPTI Conformation at Different Temperatures and pH-Values

The hydrogen bonded amide protons of the β-sheet involving the residues 16 to 36 and the α-helix formed by the residues 47 to 56 (Huber et al., 1971) can under suitable conditions readily be observed in the ^1H NMR spectra of BPTI. In neutral or slightly acidic solutions the exchange of most of the hydrogen bonded amide proton is very slow, indicating that the backbone conformation is quite outstandingly rigid (Masson and Wüthrich, 1973; Karplus et al., 1973). At temperatures above approximately 60° or at pH values above 10, however, the exchange rates for most of these hydrogen bonded protons are considerably faster so that they cannot readily be studied by measurements of the isotope exchange (4) (Masson and Wüthrich, 1973).

$$N{-}^1H + {}^2H{-}O{-}^2H \rightarrow N{-}^2H + {}^1H{-}O{-}^2H. \quad (4)$$

Under these extreme conditions, BPTI solutions were therefore also examined in H_2O. The proton exchange (5) between the peptide groups and the solvent was thus found to be in the slow exchange limit on the NMR time scale (e.g. Wüthrich, 1976) both at high temperature and high pH.

$$N{-}^1H + {}^1H^*{-}O{-}^1H \rightleftarrows N{-}^1H^* + {}^1H{-}O{-}^1H. \quad (5)$$

This is illustrated in Figure 1 with two ^1H NMR spectra of BPTI which were recorded at 87°. At this temperature the proton exchange (4) was so rapid that the resonances of the amide protons were not observed in the spectrum obtained immediately after dissolving BPTI in ^2H_2O (Fig. 1A). The spectrum in H_2O, on the other hand, contained seven well resolved doublet resonances between 8.5 and 11.0 ppm (Fig. 1B). These lines correspond to seven of the eleven slowly exchanging amide

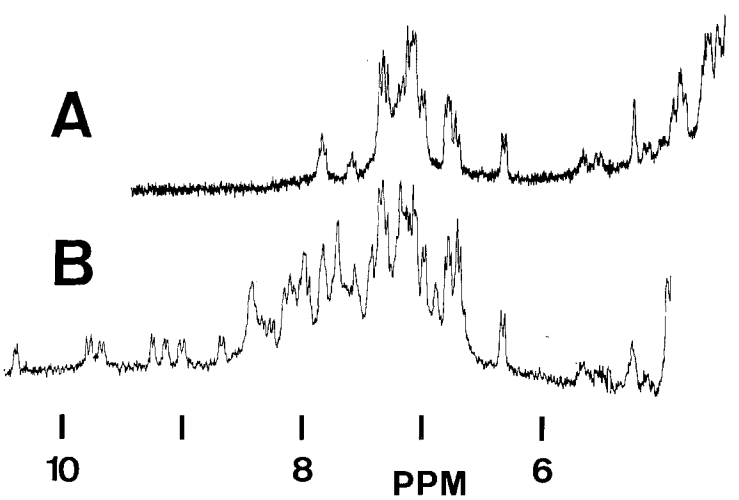


Fig. 1. Spectral region from 6 to 11 ppm of the CW ^1H NMR spectrum at 270 MHz of BPTI, $T = 87°$. **A.** 0.01-M solution in ^2H_2O, $p^2H = 7.0$; **B.** 0.01-M solution in H_2O, pH = 7.0

proton resonances observed in the same spectral region at 25° (Masson and Wüthrich, 1973), indicating that both the β-sheet and the α-helix in the protein were intact at temperatures up to 87°. Comparison of the line widths of the low field doublet resonances in Figure 1B with the widths of the corresponding lines at lower temperatures showed that for most of the hydrogen bonded protons the life time with respect to the exchange (5) was $\gg 0.1$ s at 87°.

NMR studies in H_2O solutions of BPTI also showed that the backbone conformation was maintained over the entire pH range from 0.8 to at least 12.0. Measurements of the conformation dependent chemical shifts (Wüthrich, 1976) of the nonlabile protons in BPTI gave independent evidence that not only the polypeptide backbone but also the amino acid side chain conformations were essentially maintained over the range of temperatures and pH considered. Overall the NMR spectra thus showed that the average spatial locations of the atoms in the globular conformation of BPTI in aqueous solutions are maintained over a considerable range of temperatures and pH-values. Concerning the practical requirements of the following studies of the aromatic amino acid residues in BPTI we have that over a pH range from at least 3 to 8, BPTI solutions are stable at temperatures between 1° and $\gtrsim 85°$. At temperatures between 1° and $\lesssim 40°$ BPTI solutions are stable over the pH range from ~ 0.5 to $\gtrsim 12.0$.

Tyrosyl Residues: pH-Titration and Resonance Identification

After replacement of all the labile protons with ^2H, the spectral region from 6.0 ppm to 9.0 ppm contains only the ring proton resonances of the eight aromatic amino acid residues (Fig. 1A), i.e. a total of 36 protons. As a first step in the identification of individual lines, the pH dependence of this spectral region was investigated between pH 8.0 and 12.5 (Karplus et al., 1973; Wagner and Wüthrich, 1975); in the absence of pH-dependent conformational changes, only the tyrosine resonances should shift with pH. Figure 2 shows that a number of pH-dependent lines could indeed be singled out in the ^1H NMR spectra. From the analysis of the pH-dependent chemical shifts, pK_a-values for the individual tyrosyl residues were obtained (Table 1). In all, the intensity of the resolved pH-dependent resonances (Fig. 2) corresponded to 11 protons. On the basis of the identical pK_a-values, two pairs of two-proton resonances were assigned to two of the four rings. These assignments were confirmed by double resonance experiments; double resonance techniques also led to the identification of the four-spin systems of the other two tyrosines (Wagner and Wüthrich, 1975; Wagner et al., 1975). The resulting resonance identification at 22° (Table 1 of Wagner and Wüthrich, 1975) agree with those obtained by Snyder et al. (1975) from studies of chemically modified BPTI. These latter experiments led also to the assignment of the individual spin systems to specific positions in the amino acid sequence (Tables 1—3). Earlier tentative assignments of the tyrosine lines (Karplus et al., 1973; Wüthrich and Wagner, 1975) had to be somewhat modified in the light of new evidence.

The temperature dependence of the tyrosine resonances was now investigated in detail and the spin systems were identified at variable temperatures with double resonance technique. The NMR parameters at 61° are included in Table 2. These

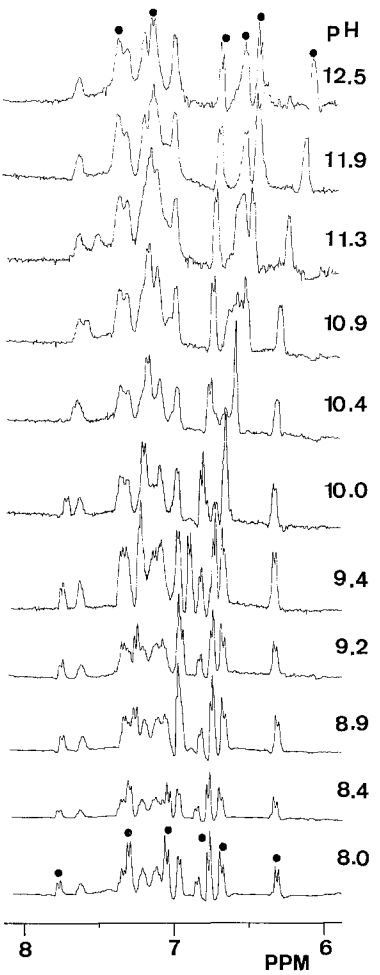


Fig. 2. Aromatic region of the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M solution of BPTI in ^2H_2O at the different p^{2}H-values indicated in the figure, $T = 26°$. The resonance lines which had been used to determine the pK_a-values of the tyrosines (Wagner and Wüthrich, 1975) are indicated by ●

temperature studies confirmed the identifications of the tyrosine spin systems at ambient temperature (Snyder et al., 1975; Wagner and Wüthrich, 1975). Furthermore they provided the data needed for the quantitative analysis of the dynamics of the tyrosine rings described below. In Table 3, the classification of the tyrosine spin systems at the resonance frequency 360 MHz is given in the usual notation (Wüthrich, 1976). For Tyr 10 and Tyr 21, a symmetrical spectrum prevails over the entire temperature range from 4° to 72°; for Tyr 23 and Tyr 35, the asymmetric low temperature spectra go over into more symmetric spectral types at higher temperatures.

The apparent pK_a-values in Table 1 were obtained by linear least squares fits of the data in Figure 2. The pK_a-values for Tyr 23 and Tyr 35 agree with those of Snyder et al. (1975); the pK_a-values reported by these authors for Tyr 10 and Tyr 21 are approximately 0.6 pH units higher than those in Table 1. A systematic deviation between the two sets of data might arise from the different ionic strengths used; Snyder et al. (1975) studied solutions which contained 0.1 M KCL whereas no

salt was added in the experiments which yielded the results of Table 1. From our experience it appears more likely, however, that the pK_a-values in Table 1, which were derived from the 360 MHz spectra (Fig. 2), are more reliable simply because of the improved spectral resolution.

Table 1. pH titration parameters for the four tyrosyl residues in BPTI

Residue[a]	pK_a[b]	slope[c]
Tyr-10	9.9	0.86 0.80
Tyr-21	10.4	1.11 0.93
Tyr-23	11.5	1.00[d]
Tyr-35	11.1	0.77[d]

[a] The assignment of the tyrosine resonances to specific residues is from Snyder et al. (1975)
[b] pK_a values from Wagner and Wüthrich (1975)
[c] $\log (\delta_A - \delta)/(\delta - \delta_B)$ has been plotted vs. pH. δ_A and δ_B are the chemical shifts of the protonated and deprotonated tyrosine rings, respectively. Slope = 1 corresponds to a simple one-proton-titration curve
[d] For these residues only one resonance line was observed during the pH-titration

Table 2. NMR parameters used for the simulation of the aromatic resonances in the ^1H NMR spectrum at 360 MHz of a BPTI solution in ^2H_2O at $p^2H = 7.8$ and $T = 61°$ (Fig. 9)[a]

Residue	Tyr-10	Tyr-21	Tyr-23	Tyr-35	Phe-I	Phe-II	Phe-III	Phe-IV	
$\delta_{2,6}$	7.279	6.670	7.144	not observed	6.952	7.339	7.096	$\delta_2 =$ 7.236	$\delta_6 =$ 7.076
$\delta_{3,5}$	7.036	6.744	6.300	6.769	7.329	7.823	7.170	$\delta_3 =$ 7.030	$\delta_5 =$ 6.940
δ_4					7.017	7.559	7.294	7.270	
$^3J_{23}$	8.0	8.5	8.0	8.0	7.4	7.0	7.0	7.0	
$^3J_{56}$	8.0	8.5	8.0	8.0	7.4	7.0	7.0	7.0	
$^3J_{34}$					7.4	7.0	7.0	7.0	
$^3J_{45}$					7.4	7.0	7.0	7.0	

[a] In this and the previous papers (Wüthrich and Wagner, 1975; Wagner and Wüthrich, 1975; Wagner et al., 1975) the chemical shifts δ in ppm were referred to $\delta_{3,5}$ (Tyr 23) = 6.300 ppm at pH 7.8. With respect to two different internal standards, TSP = sodium 2,2,3,3-tetradeutero-3-trimethyl-silylpropionate and DSS = sodium 2,2-dimethyl-2-silapentane-5-sulfonate, $\delta_{3,5}$ (Tyr 23) was actually found to be 6.339 ppm at pH 7.8 over the temperature range 5° to 72°. The spin-spin coupling constants ^3J are given in Hz. The assignment of the tyrosine resonances to specific residues is from Snyder et al. (1975). The numeration of the phenylalanines is arbitrary

The logarithmic fitting procedures used to analyse the titration data give meaningful pK_a-values only for groups which have a simple one-proton titration behavior. The slopes of the resulting plots (Table 1) indicate that Tyr 21 and Tyr 23 give rise to unperturbed titration curves. The perturbation of Tyr 10 has previously been shown to arise from a specific intramolecular interaction of this residue with Lys 41 (Brown et al., 1976). For Tyr 35, the apparent deviation from simple one-proton titration behavior (Table 1) can not be traced at the present stage to a particular structural feature of BPTI; it could also not be excluded that this phenomenon might be the consequence of a local pH-dependent conformational change.

Phenylalanyl Residues: Resonance Identification

From the previously discussed amino acid composition of BPTI it was obvious that all the resonances in the spectra of Figure 2 which were not assigned to protons of the tyrosines originate from the four phenylalanines. Among these resonances there is a two-proton doublet at 6.95 ppm (Fig. 3A), which was found to be coupled with a multiplet at 7.37 ppm (Fig. 3B and C). In the spin decoupling experiments of Figures 3B and 3C, spectral changes were also observed at around 7.1 ppm. Irradiation at 7.06 ppm produced a four line spectrum of the AA'XX' type, with the two doublets at 6.95 and 7.37 ppm (Fig. 3D). With the experiments of Figure 3 the five-spin systems of one of the phenylalanines, arbitrarily denoted "Phe I", was thus identified. When inspecting Figure 3 and the subsequent spin decoupling experiments, one should keep in mind that because of the mutual overlap of resonance lines from different rings, double resonance irradiation may also cause perturbations in other spectral features than those arising from the particular ring considered. For example in the spectrum Figure 3B, irradiation at 7.37 ppm also cause the collapse of a doublet at 7.07 ppm which had previously been assigned to Tyr 10 (Snyder et al., 1975; Wagner et al., 1975).

The spectrum recorded at 26° (Fig. 2, Fig. 3A) contained no resolved two-proton resonance which had not been assigned to one of the tyrosines or to Phe I. At 72°, however, a well resolved multiplet of intensity corresponding to two protons was observed at 7.82 ppm (Fig. 4). The identification of the five-spin system of Phe II, which includes this multiplet, was achieved with the double resonance experiment described in Figure 5. Additional temperature-dependent spectral features in Figure 4 will be discussed below in connection with the dynamics of the aromatic rings.

Since even at 72° there were no well-separated resonance lines left for which the resonance intensity could unambiguously have been determined and which could then have been used as starting points for additional resonance assignments, a suitable basis for the identification of the two remaining phenylalanines had to be found in the crowded spectral region between 6.9 and 7.4 ppm. For this, the resonance intensities in the aromatic region were evaluated with the procedures described in "*Materials and Methods*" and in the figure caption 6. Subtraction of the intensities of the resonances which had been assigned to the four tyrosines, Phe I and Phe II (Table 2) from the total intensities of the spectral regions to which they belong (Fig. 6B) then indicated the following distribution of the resonances corresponding to the remaining two phenylalanines: Each of the groups of resonances centered at 6.96 and 7.03 ppm (Fig. 6) contains one of these protons, the quite well-resolved doublet

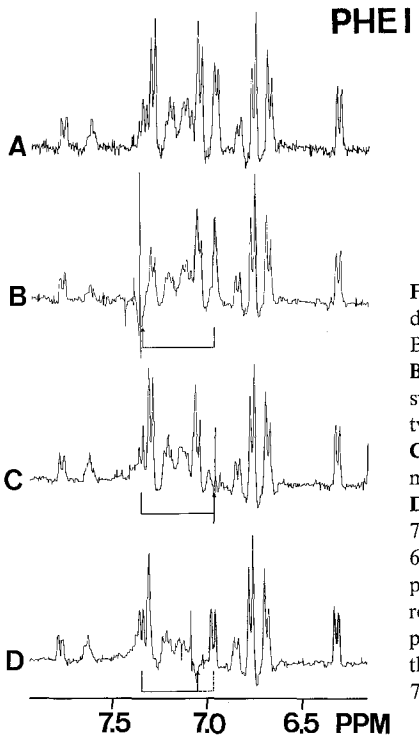


Fig. 3. A. Aromatic region of the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M BPTI solution in ^2H_2O, $p^2H = 7.8$, $T = 26°$. **B.** Double resonance irradiation of a multiplet structure at 7.37 ppm causes the collapse of a two-proton doublet at 6.95 ppm. **C.** Irradiation of the doublet at 6.95 ppm causes marked changes of the multiplet at 7.37 ppm. **D.** Irradiation of a one-proton multiplet at 7.06 ppm causes a sharpening of the doublet at 6.95 ppm, and produces a two-proton doublet in the place of the multiplet at 7.37 ppm. These three resonances constitute the AA′BXX′ spectrum of phenylalanine I, where the 2,6-protons are at 6.95 ppm, the 4-proton at 7.06 ppm, and the 3,5-protons at 7.37 ppm

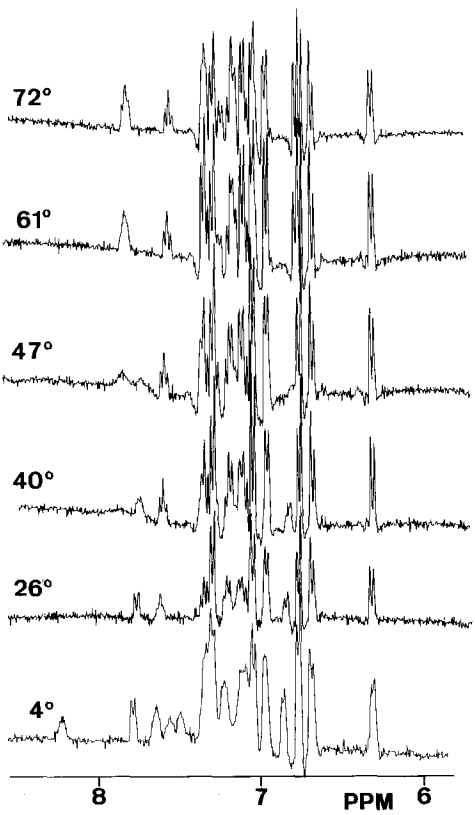


Fig. 4. Temperature dependence of the aromatic region from 6 to 9 ppm in the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M solution of BPTI in ^2H_2O, $p^2H = 7.8$

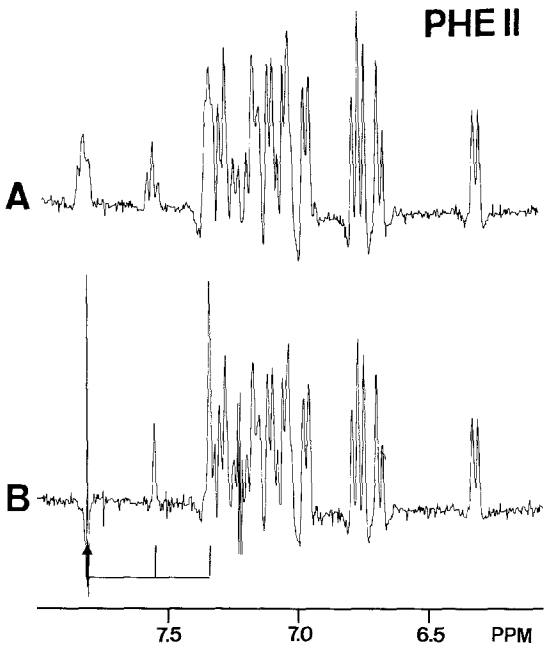


Fig. 5. A. Aromatic region of the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M BPTI solution in ^2H_2O, $p^2H = 7.8$, $T = 72°$.
B. Double resonance irradiation of the two-proton multiplet at 7.8 ppm causes the collapse of the one-proton multiplet at 7.5 ppm, and a two-proton multiplet at 7.3 ppm. These three resonances constitute the AA'MXX' spectrum of phenylalanine II, where the 2,6-protons are at 7.3 ppm, the 4-proton at 7.5 ppm, and the 3,5-protons at 7.8 ppm

at 7.23 ppm corresponds to one proton, each of the regions centered about 7.16 and 7.28 ppm contains two of these protons, and the group of resonances centered about 7.10 ppm has all its intensity of three protons from the two remaining phenylalanines. These results were by themselves quite conclusive in that they contained two independent pieces of evidence that at 72° at least one of the phenylalanine rings III and IV did not give rise to a symmetrical spectrum of the AA'BB'C type, i.e. the appearence of three one-proton lines and the observation that the one-proton resonance at 7.23 ppm was a doublet.

With the approximate locations of the ten protons of Phe III and Phe IV thus determined, double resonance techniques were used to identify the two five-spin systems. The overall shape of the group of resonances of intensity three protons at approximately 7.10 ppm (Fig. 6) indicated that it might contain the two-proton doublet of the 2,6-protons of one of the phenylalanines, say Phe III. If this were indeed so, this doublet would most likely be coupled with one of the regions centered about 7.16 and 7.28 ppm, both of which could contain a two-proton multiplet of Phe III (Fig. 6). This is born out by the experiments of Figure 7, which showed that the resonances at 7.10 ppm consist indeed of a two-proton doublet superimposed on a one-proton multiplet, and that the two-proton doublet is coupled with the two-proton resonance at 7.17 ppm (Fig. 7B). Figure 7C confirmed that the doublet at 7.10 was

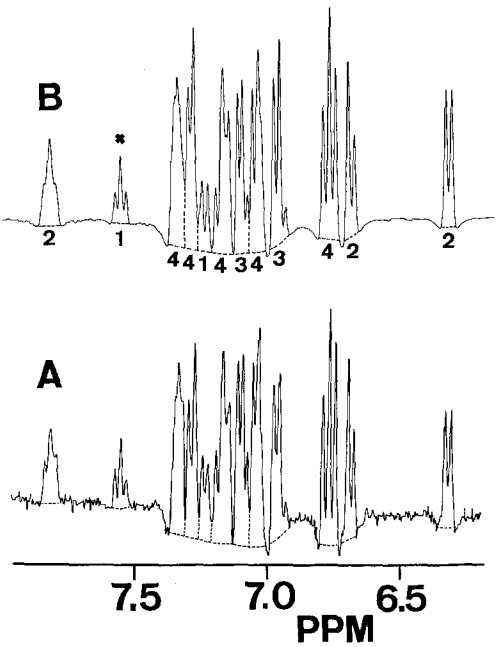


Fig. 6. Evaluation of the resonance intensities in the aromatic region of the convolution difference ^1H NMR spectrum of a 0.01-M solution of BPTI in ^2H_2O, $p^2H = 7.8$, $T = 72°$. A Experimental spectrum. The integral of this spectrum was obtained on the basis of the close fit with the computed spectrum B. The latter corresponds to the envelope of a sum of resonances with convolution difference line shapes, which had been selected by trial and error until a satisfactory fit with spectrum A had been obtained. The numbers indicate the number of protons corresponding to the areas of the 12 groups of resonances distinguished in the spectra. The resonance intensities were normalized by using that the triplet marked with a cross corresponds to a single proton, and rounded to the closest integer value. Note that the total number of protons is only 34, since at 72° the lines of two protons of Tyr-35 are too broad to be observed

not directly coupled with the resonances of intensity two protons at around 7.29 ppm. Furthermore, in combination with the spin decoupling experiments in Figure 8, Figure 7C showed that the most likely assignment of the 4-proton resonance of Phe III was to a line located at 7.29 ppm.

From the resonance intensities in Figure 6 and the chemical shifts and resonance intensities of the seven rings identified so far (Table 2) it was apparent that at 72° Phe IV gave rise to a spectrum consisting of five one-proton resonances. The approximate chemical shifts were known from Figure 6. More accurate values for the chemical shifts and information on the spin-spin couplings in this system were obtained from the double resonance experiments in Figure 8. The chemical shifts for Phe IV thus obtained had to be only slightly adjusted to obtain a satisfactory fit between the experimental spectra and the spectrum simulated with the parameters of Table 2 (Fig. 9). When inspecting Figure 8 one should recall that because of the mutual overlap of resonance lines from the different rings, most of the double resonance irradiations caused modifications not only in the one-proton resonances of Phe IV, but also in multiplets which had previously been assigned to the other aromatics in BPTI.

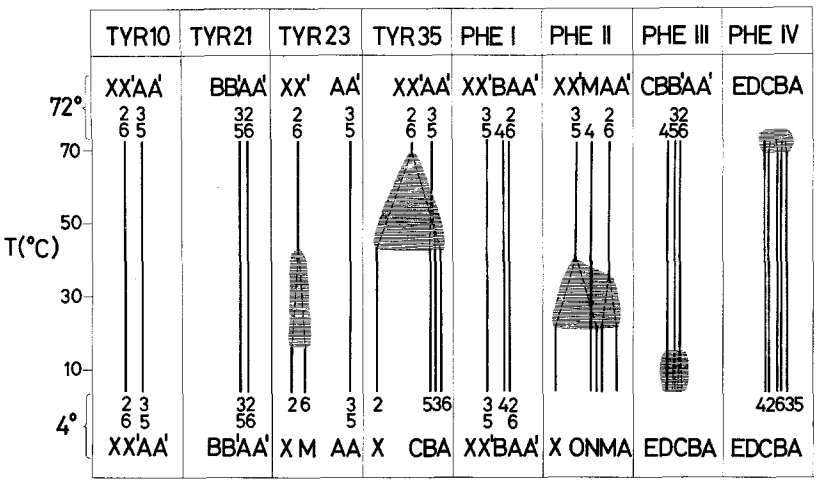


Table 3. Spectral types (in the common notation, see e.g. Wüthrich, 1976) of the aromatic protons of the eight aromatic residues in BPTI at 4° and 72°. In as far as they were determined, the resonance assignments are also indicated. The table further describes the temperature dependence of the NMR spectra between 4° and 72°. The hatched areas indicate the temperature ranges where the transitions from slow to rapid rotation on the NMR time scale occur for the individual rings

Considering the complexity of the spectrum of BPTI, the agreement between experimental and simulated spectrum (Fig. 9C and D) is quite remarkable. The following comments on the parameters used for the spectral simulations may be added: (i) Natural line widths of $\delta\nu_{1/2} = 6.5$ Hz, where $\delta\nu_{1/2}$ is the full line width at half height of the lines, were used for all the resonances except the low field multiplets of Tyr-23, and Phe II where some residual exchange broadening at 61° (see Wagner et al., 1975) was accounted for with line widths $\delta\nu_{1/2}$ of 9.0 Hz and 11.0 Hz, respectively. (ii) Somewhat different spin-spin couplings ^3J were used for the individual rings (Table 2); the spectra were found to be quite sensitive to these small variations of the coupling constants. (iii) The spectral simulations confirmed an experimental observation made in the BPTI spectra and also in model peptides (Cohen, 1971). Due to long range coupling with the 4-proton, the component lines of the doublet of the 2,6-proton of phenylalanine are in general noticeably broader than those of the tyrosine doublets. This spectral feature might possibly be useful as a complementary criterium for resonance assignments in the aromatic regions of peptides and proteins.

Overall, with the above described combination of double resonance techniques, studies at variable temperature, quantitative determination of resonance intensities and simulations of the spectra at different temperatures, the identifications of the phenylalanine five-spin systems needed for investigations of the dynamics of the aromatic rings were obtained. In Table 3, the phenylalanine spin systems at 360 MHz are characterized in the common notation (Wüthrich, 1976). For Phe I, a symmetrical spectrum prevails over entire temperature range from 4° to 72°; for Phe IV, the spin system is asymmetric throughout; for Phe II and Phe III, the asymmet-

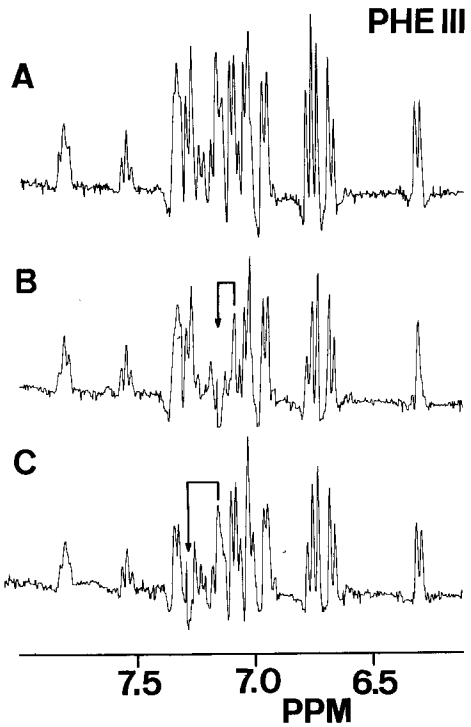


Fig. 7. A. Aromatic region of the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M solution of BPTI in ^2H_2O, $p^2H = 7.8$, $T = 72°$.
B. Double resonance irradiation of a two-proton multiplet at 7.17 ppm causes the two-proton doublet at 7.10 ppm to collapse.
C. Irradiation at 7.29 ppm produces a perturbation of the multiplet at 7.17 ppm, while the doublet at 7.10 ppm is only slightly affected (note that in this experiment the resonance at 7.04 ppm, which was previously assigned to Tyr 10, is also decoupled). These three resonances constitute the AA'BB'C spectrum of phenylalanine III. where the 2,6-protons are at 7.10 ppm, the 3,5-protons at 7.17 ppm, and the 4-proton at 7.30 ppm

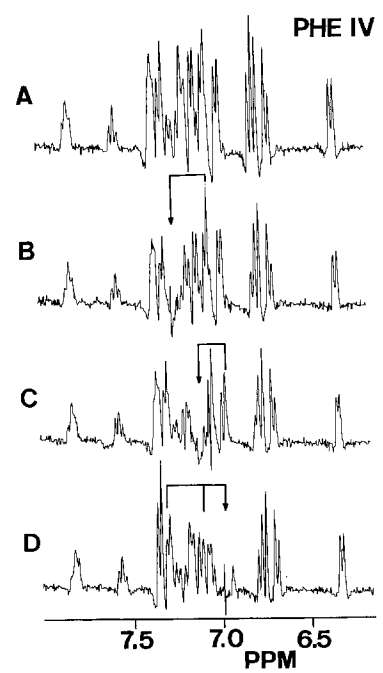


Fig. 8. A. Same as Figure 7A.
B. Double resonance irradiation of the one-proton doublet at 7.24 ppm produces a spectral change on the right of the multiplet at 7.03 ppm.
C. Irradiation of the one-proton doublet at 7.08 ppm (only the right component is visible) decouples the one-proton triplet at 6.94 ppm (only the right component is visible).
D. Irradiation of the one-proton triplet at 6.94 ppm causes the collapse of the one-proton doublet at 7.08 ppm (confirms experiment C) and perturbs the multiplet at 7.27 ppm. The spectrum is therefore of the ABCDE type, with the 4-proton at 7.27 ppm, the 3,5-protons at 6.94 and 7.24 ppm, and the 2,6-protons at 7.08 and 7.03 ppm

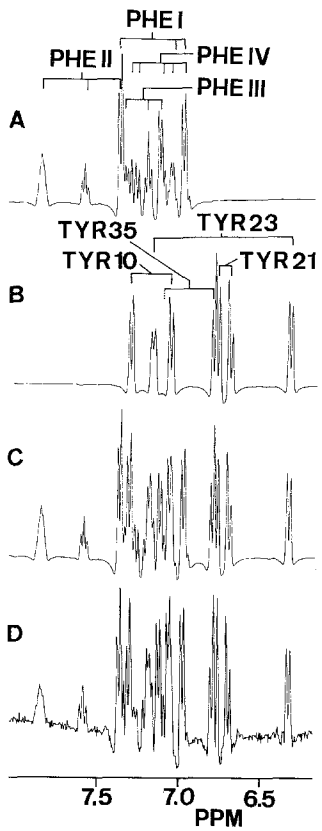


Fig. 9. Aromatic region of the convolution difference ^1H NMR spectrum at 360 MHz of a 0.01-M solution of BPTI in ^2H_2O, $p^2H = 7.8$, $T = 61°$.
A. Resonances of the 4 phenylalanines simulated with the parameters of Table 2.
B. Resonances of the 4 tyrosines simulated with the parameters of Table 2.
C. A + B, which is to be compared with the experimental spectrum D. The natural line width used in the simulations was $\delta\nu_{1/2} = 6.5$ Hz for all the resonances except for the low field multiplets of Tyr 35 and Phe II, where full line widths at half height $\delta\nu_{1/2} = 9.0$ Hz and $\delta\nu_{1/2} = 11.0$ Hz, respectively, were used

ric low temperature spectra go over into more symmetrical spin systems at higher temperatures. In contrast to the tyrosines, the individual phenylalanine spin systems have not yet been assigned to specific positions in the amino acid sequence. These assignments should in the near future at least in part be obtained from comparative studies of homologous inhibitor molecules (Tschesche, 1974; Wüthrich et al., 1976).

Dynamics of the Aromatic Rings

The dynamics of the aromatic rings are primarily manifested in the symmetry of the spin systems (Table 3) and also in the line widths (Wüthrich, 1976). As an illustration, let us consider the resonances of Phe II and Tyr 35 in Figure 4. The AA′ and XX′ parts of the spectrum of Phe II at 72° (Fig. 5) became broader when the temperature was lowered and were too broad to be observed at 40° and 26°. At 4°, four new one-proton lines appeared at 8.2, 7.6, 7.5 and ca. 7.2 ppm. The 4-proton line of Phe could on the other hand be observed throughout the entire temperature range from 72° to 4°; it moved downfield from 7.56 ppm at 72° to 7.67 ppm at 4°. This temperature dependence of the phenylalanine resonances is typical for a transition from rapid rotation at 72° to slow rotation at 4°, with the intermediate dynamic situations being characterized by the symmetry of the spectrum and the line widths.

The spectrum of Tyr 35 at 4° consists of four one-proton doublets at 7.75, 6.80, 6.76 and 6.69 ppm (Snyder, et al., 1975; Wagner and Wüthrich, 1975). In Figure 4, the resonances at 7.75 and 6.80 ppm are well resolved and it is seen that these lines begin to broaden at 40°. In the spectra at 61° and 72°, the resonances at 6.69 and 6.80 ppm had merged into a sharp two-proton doublet at 6.76 ppm, whereas the two-proton resonance corresponding to the low temperature lines at 7.75 and 6.76 ppm was still very broad. It is thus seen that as a consequence of the different relative chemical shifts, the "NMR time scale" for the two pairs of symmetry related resonances in Tyr 35 was quite different. The temperature dependence of the rate of ring rotation was also manifested in the exchange broadening of the resonance lines of Tyr 23 between approximately 50° and 10°, of Phe III at temperatures below 25° and of Phe IV at temperatures above 70° (Table 3). For Tyr 10, Tyr 21 and Phe I the limiting situation of rapid exchange prevailed over the entire temperature range studied (Table 3).

The observation in Figure 4 that the chemical shifts of the exchange-averaged 2,6- and 3,5-proton resonances of Tyr 35 and Phe II correspond very nearly to the average of the chemical shifts for the individual protons provide evidence that the rotational motions of the aromatic rings consist essentially of "180° flips" about the C^{β}–C^{γ} bond. Each ring would thus for most of the time be in an equilibrium rotation state characterized by a torsion angle χ^2 which is probably quite similar to that in the X-ray structure, and each flipping motion would start and end in this equilibrium situation. The temperature variation of the chemical shift of the 4-proton of Phe II is on the other hand an indication that the spatial orientation of the rotation axis C^{β}–C^{γ}–C^{4} varies somewhat during the rotational motions. These qualitative mechanistic aspects of ring rotation coincide with what one would anticipate from conformational energy calculations based on the X-ray conformation of BPTI (Hetzel et al., 1976).

From a quantitative analysis of the exchange broadening of the resonance lines in the different spin systems (see e.g. Bovey, 1969; Wüthrich, 1976), the rotation rates at different temperatures were evaluated. In Table 4 the rotation rates for Tyr 23, Tyr 35 and Phe II at 4°, 40° and 80° are given in the form of the life times τ of the rings with respect to the rotational motions. For these three rings the free energies of activation $\Delta G^{\neq}$ could correspondingly be determined for different temperatures between 4° and 80°, and the enthalpies of activation $\Delta H^{\neq}$ and entropies of activation $\Delta S^{\neq}$ were obtained from a least squares fit of $R \ln \frac{\tau k T}{h} = \frac{\Delta G^{\neq}}{T}$ vs. the reciprocal of temperature (Fig. 10). These parameters are contained in Table 4. It is seen that the dynamic behavior of Tyr 23, Tyr 35 and Phe II is markedly different at low temperatures, while almost identical values of τ and $\Delta G^{\neq}$ for the three rings were obtained at temperatures between 70° and 80°. For Phe IV, τ and $\Delta G^{\neq}$ at 80° could be evaluated from the line broadening in the ABCDE spectrum. The activation energies in Table 4 agree quite closely with those obtained from semiempirical energy calculations (Gelin and Karplus, 1975; Hetzel et al., 1976). It appears particularly worth noting that from Figure 10 significantly different entropies of activation were obtained for the individual rings.

Over the entire temperature range studied there is no manifestation of exchange phenomena in the resonance lines of Tyr 10, Tyr 21 and Phe I (Table 3). As a

Table 4. Parameters of the rotational motions of the aromatic rings in the globular form of BPTI at $p^2H = 7.8$

Residue	τ (s)[a]			$\Delta G^{\neq}$ (kcal mole^{-1})			$\Delta H^{\neq}$ (kcal mole^{-1})	$\Delta S^{\neq}$ (e.u.)
	4°	40°	80°	4°	40°	80°		
Tyr 10	only AA'BB' spectrum observed							
Tyr 21	only AA'BB' spectrum observed							
Tyr 23	$>2 \cdot 10^{-1}$	$3 \cdot 10^{-3}$	$2 \cdot 10^{-5}$	>15.3	14.7	13.3	26	35
Tyr 35	>1	$2 \cdot 10^{-2}$	$2 \cdot 10^{-5}$	>16.3	15.8	13.0	37	68
Phe I	only AA'BB'C spectrum observed							
Phe II	$3 \cdot 10^{-2}$	$6 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	14.2	13.7	13.2	17	11
Phe III	rotating rapidly at temperatures above 26°							
Phe IV			$2 \cdot 10^{-1}$			19.7		

[a] τ is the life time of the aromatic ring i in the equilibrium rotation state χ_i^2 with respect to 180° flips about the C^{β}–C^{γ} bond (see text)

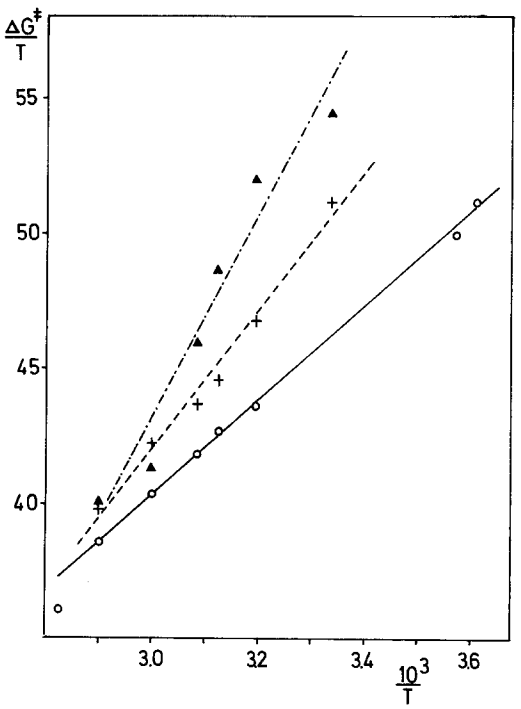


Fig. 10 Eyring plots for intramolecular rotational motions of the aromatic rings of Tyr 23 (+- - - -+), Tyr 35 (▲·····▲) and Phe II (○——○) of BPTI in aqueous solution at $p^2H = 7.8$. $\frac{\Delta G^{\neq}}{T} = R \ln \frac{\tau k T}{h}$ is plotted vs. $\frac{10^3}{T}$. The straight lines correspond to linear least squares fits of the experimental data. A complete set of the parameters obtained is given in Table 4

consequence, the dynamic parameters could not be evaluated. From comparison with the other rings, it is very probable that the limiting situation of rapid rotation prevails at all temperatures above 4°, with this assumption one would estimate from Table 4 that τ (4°) $\lesssim 2 \times 10^{-5}$ s and $\Delta G^{\neq}$ (4°) $\lesssim$ 13 kcal mole^{-1} for these three rings. It must be pointed out, however, that the ^1H NMR spectra give no direct evidence for ring rotation of Tyr 10, Tyr 21 and Phe I. Even though this seems rather unlikely

in the light of the data on the other rings in BPTI, it could in principle be that $|\delta_2 - \delta_6| = |\delta_3 - \delta_5| = 0$ for a phenylalanine or tyrosine ring rigidly fixed in the interior of a protein; and AA'BB'-type spectrum would in this case be observed for all possible dynamic sitations. For Phe III, line broadening of the 2,6- and 3,5-proton multiplets in the AA'BB'C spectrum was noticeable at temperatures below approximately 30° (Fig. 4, Table 3). This is clear evidence that the symmetric spectrum of this ring arises as a consequence of rapid rotational motions. However, since the chemical shifts of the individual ring protons in the ABCDE type spectrum of the immobilized ring were not known, the NMR time scale for Phe III was not fixed and therefore the dynamic parameters could not be evaluated. From comparison with the data on the other rings in Table 4, we estimated that $\tau\ (25°) \gtrsim 2 \times 10^{-5}$ s an $\Delta G^{\neq} \gtrsim 13.0$ kcal mole^{-1} for Phe III.

Independently, information on the dynamics of the aromatic rings was also obtained from studies of the ^{13}C NMR relaxation times in BPTI (Wüthrich and Baumann, 1976). These ^{13}C NMR experiments showed that the effective correlation time τ_c for intramolecular rotational tumbling was $\gg 1 \times 10^{-8}$ s for all eight aromatic rings. For Tyr 23, Tyr 35, Phe II, Phe III and Phe IV this is certainly compatible with the ^1H NMR data in Table 4. Tyr 10, Tyr 21 and Phe I the ^{13}C NMR studies complete the ^1H NMR results in that they allow to estimate an upper limit for the rotation rates. For these three rings we thus have that the life times with respect to the 180° flips about C^β—C^γ are most probably within the range 2×10^{-5} s $\gtrsim \tau \gg 1 \times 10^{-8}$ s.

Conclusions

Despite its small size, BPTI is a typical globular protein (Huber, et al., 1971). It thus appears that the following NMR spectral feature, which could be ascertained because of the outstanding temperature stability of BPTI, might be common for globular proteins in general: Aromatic rings of phenylalanine and tyrosine which are rigidly fixed in space in the interior of globular protein molecules give quite generally rise to asymmetric ^1H NMR spectra of the AA'BB'C-type, and the AA'BB'-type, respectively. Therefore, even though they do not in themselves provide direct evidence for ring rotation, the symmetric spectra observed for aromatic rings in various medium-sized globular protein (Campbell et al., 1975; Dobson et al., 1975; Wüthrich, 1975; Keller and Wüthrich, 1975) can on the basis of the detailed studies with BPTI be taken as conclusive evidence for rapid intramolecular ring rotation about the C^β—C^γ bond. This empirical rule should also be useful for future studies of other proteins.

In view of the well documented rigidity of the polypeptide backbone conformation in BPTI (Masson and Wüthrich, 1973; Karplus et al., 1973), it appears that rapid rotation of individual aromatic rings should be a structural feature of most globular proteins. This may quite conceivably add some new aspects to the functional interpretations of single crystal X-ray structures of proteins. Aromatic rings in their X-ray positions, and in particular groups of several aromatics in specific spatial configurations, have frequently been implicated as functionally relevant structural features. The dynamics of the rings will now also have to be considered in such

structure-function relations. More generally, the ring rotations give direct evidence that the overall dynamics of protein structures include "breathing motions" of sizeable amplitudes, which might affect the functional properties of the molecules. A detailed investigation of the effects of ring rotations on the average molecular structure of BPTI observed by the X-ray method will be presented in a companion paper (Hetzel et al., 1976).

In the past few years, ^{13}C NMR relaxation measurements were propagated as a powerful technique for studies of the dynamics of biopolymers (Doddrell et al., 1972). Comparison of the results obtained from ^{1}H NMR and ^{13}C NMR (Wüthrich and Baumann, 1976) of BPTI shows that for studies of the aromatics, the ^{1}H NMR approach is potentially more interesting, since it can provide more detailed information in the time range which is typical for intramolecular motions of aromatic rings in proteins. It appears quite likely that relatively slow intramolecular motions are characteristic also for certain other amino acid residues in proteins, which might thus accordingly be more readily accessible for studies with high resolution ^{1}H NMR, and perhaps high resolution ^{13}C NMR, than with spin relaxation measurements.

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