NMR INVESTIGATIONS OF THE DYNAMICS OF THE AROMATIC AMINO ACID RESIDUES IN THE BASIC PANCREATIC TRYPSIN INHIBITOR

K. WÜTHRICH and G. WAGNER

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, 8049 Zürich, Switzerland

Received 5 December 1974

1. Introduction

For quite some time it has been recognized that the combination of single crystal X-ray data with high resolution NMR studies is a promising approach for investigations of the molecular conformations of globular proteins in solution [1]. It is a particular asset of the NMR method that the data on the static average spatial structure obtained from the X-ray measurements can be complemented with information on the dynamic aspects of the protein conformations. Recently, studies of the longitudinal spin relaxation times T_1 of ^{13}C [2], and ^1H NMR investigations of the kinetics of the exchange of labile protons with ^2D of the solvent [3] have been particularly emphasized in this context. Following the most recent advances of the instrumentation for high field ^1H NMR measurements, these studies can now be extended to include investigations of the intramolecular rotational motions of the aromatic amino acid side chains in globular proteins. In the present report this will be illustrated with a proton NMR study at 360 MHz of the basic pancreatic trypsin inhibitor (BPTI).

BPTI from bovine pancreas has a molecular weight of 6500, and consists of one polypeptide chain with 58 amino acid residues. The amino acid sequence includes 4 tyrosines and 4 phenylalanines as the only aromatic residues [4]. The molecular conformation in single crystals is known [5], and it was found that the solution conformation of BPTI is unusually stable towards denaturation by chemicals and by heat [3,6,7]. This outstanding heat stability made it possible in the present experiments to observe the NMR of the aromatic protons in the globular protein conformation over the temperature range from 4°C to 85°C, and from this to characterize the dynamic states of most of the aromatic rings in the protein at variable temperatures.

2. Materials and methods

The basic pancreatic trypsin inhibitor (BPTI, Trasylol ® Registered trade mark Bayer Leverkusen, Germany) was obtained from the Farbenfabriken Bayer AG. A 0.01 M solution of BPTI in D_2O, pD = 1.8, was used for the NMR studies. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was added as an internal reference. Prior to the experiments reported in this paper, the labile protons had been replaced with deuterium of the solvent by heating the solution to 85°C for 5 min [3].

^1H NMR spectra at 360 MHz were obtained on a Bruker HXS-360 spectrometer equipped with a standard temperature unit. The probe temperature was measured with an ethylene glycol standard sample.

3. Results and discussion

Fig.1 shows the ^1H NMR spectrum at 360 MHz and 34°C of the non-labile protons between 5 and 8 ppm in the basic pancreatic trypsin inhibitor. The intensity of the resonance lines between 6.0 and 8.0 ppm was found to correspond to 34±2 protons. As will be shown below, these lines account for all the 36 aromatic protons of the 4 tyrosyl and the 4 phenylalanyl residues in BPTI [8]. The previously described identification of the tyrosine resonances with double resonance techniques [8] indicated that each of the

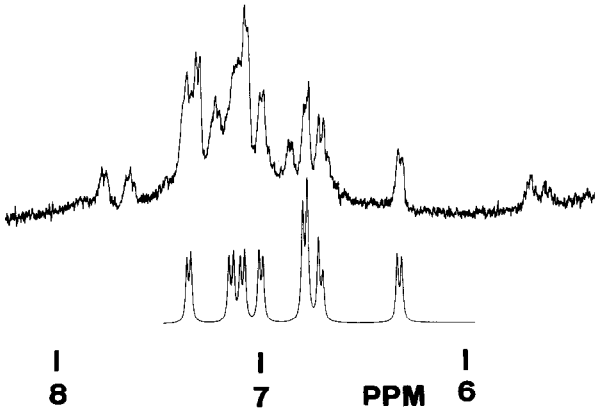


Fig.1. ^{1}H NMR spectrum at 360 MHz between 5 and 8 ppm of the basic pancreatic trypsin inhibitor (BPTI) at 34°C. The spectrum corresponds to a 0.01 M solution of BPTI in D_2O, pD = 1.8, where the labile protons had previously been replaced by deuterium by heating the solution to 85° C for 5 min. The lower trace shows the contributions to the spectrum from the 4 tyrosyl residues, which have been computed from the previously reported resonance assignments [8].

four tyrosines gives rise to an AA'BB' type ^{1}H NMR spectrum. The lower trace in fig.1 corresponds to the sum of the resonances of the 4 tyrosyl residues computed from the data in ref. [8]. Subtracting the tyrosine resonances from the experimental spectrum then leaves one with the resonances corresponding to the 4 phenylalanines in BPTI. At 34°C these are thus found to cover the spectral range from 6.6 to 7.8 ppm, and to contain at least 5 lines with the intensity of one proton. This in turn indicates that on the NMR time scale the rotational motions of at least one of the phenylalanine rings in BPTI are essentially restricted to the motions of the entire molecule. This will in the following be confirmed by investigating the temperature dependence of the ^{1}H NMR spectrum, which will also explain why a rather small overall intensity is found for the aromatic resonances observed at ambient temperature (fig.1).

Fig.2 shows the temperature dependence of the resonances of the aromatic protons in BPTI between 4°C and 81°C. It is readily seen that quite extensive changes occur before the overall denaturation sets in at around 80°C [3,6], and that variations with temperature can be observed throughout the spectral region from 6.5 to 8.5 ppm. In the present preliminary

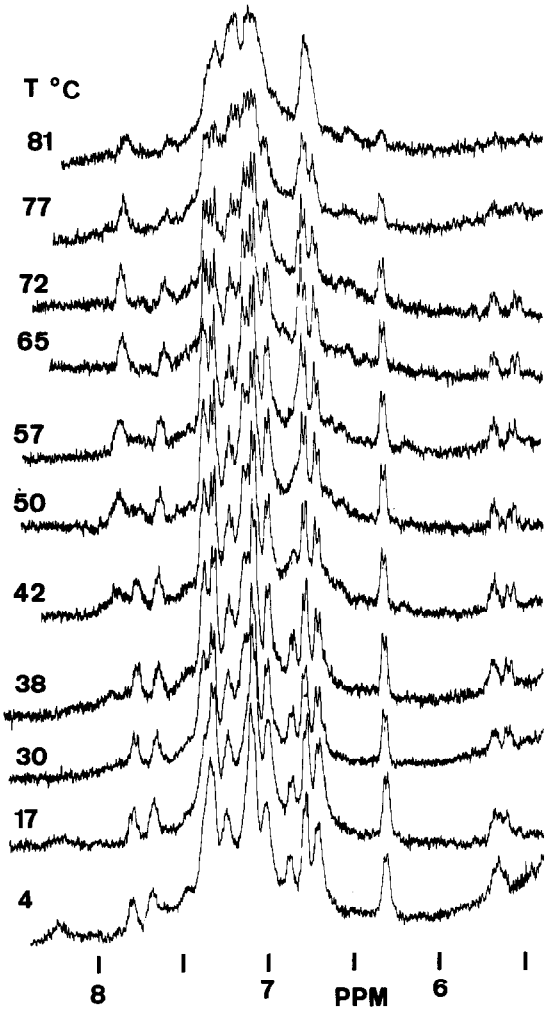
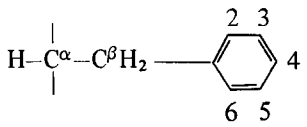


Fig.2. Temperature dependence between 4°C and 81°C of the spectral region from 5 to 9 ppm in the 360 MHz ^{1}H NMR spectrum of a 0.01 M solution of BPTI in D_2O, pD = 1.8. Prior to these experiments, the labile protons had been replaced by deuterium.

discussion of the data of fig.2, we shall, however, concentrate on a few selected temperature dependent features which are particularly well resolved in the spectra, and at the same time pertinent for the description of the solution conformation of BPTI.

The structural information to be derived from the data of fig.2 concerns mainly the mobility of the aromatic rings in BPTI, and is obtained from the following symmetry considerations. Two pairs of

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



In view of the non-periodic distribution of structural elements in the interior of globular proteins, chemical shift equivalence of the protons 2 and 6, and 3 and 5, respectively, will in most cases only be compatible with a dynamic situation where the aromatic rings would flip about the C^β–C^γ axis at a rate which is rapid on the NMR time scale. Observation of an AA′BB′ type ^{1}H NMR spectrum for tyrosine, or an AA′BB′C type spectrum for phenylalanine is therefore indicative of a mobile aromatic ring. On the other hand the appearance of single proton resonance lines for tyrosine, or more than one single proton line for any given phenylalanine implies that the rings are quite rigidly fixed in the protein molecule.

To the extent that they can be recognized as resolved lines, the tyrosine resonances (fig.1) are almost independent of temperature between 4°C and 72°C (fig.2). The only temperature dependent feature of those resonances between 6.0 and 7.0 ppm which had been assigned to tyrosine protons [8] is a small chemical shift between the two two-proton doublet resonances which are both at 6.76 ppm at temperatures below 50°C, and give rise to a 3 line structure at higher temperature. The observations in fig.2 are thus compatible with the earlier assignments of the tyrosine resonances [8], and support the conclusion that the tyrosine rings are rotating about the C^β–C^γ axis at a rate which is rapid on the NMR time scale at ambient temperature.

Following fig.1, the resonances between 7.4 and 8.5 ppm correspond to phenylalanine ring protons. At 4°C, this spectral region contains five one-proton lines at 7.48, 7.58, 7.67, 7.78, and 8.22 ppm, where the resonances at 7.58 and 8.22 ppm are markedly broader than the other three lines (fig.2). Whereas the latter are essentially independent of temperature between 4°C and 38°C, the lines at 7.58 and 8.22 ppm first broaden, then disappear, and finally merge into a single resonance of intensity corresponding to two protons at 7.90 ppm. This new resonance at 7.90 ppm is quite broad at 38°C, and sharpens as the temperature is further increased. Spectral variations with temperature which would occur simultaneously with those involving the lines at 7.58, 7.90, and 8.22 ppm, could only be detected between 7.1 and 7.4 ppm, where the bulk of the phenylalanine ring proton resonances are usually located. On the basis of the symmetry considerations presented above we conclude that the two lines which are at 7.58 and 8.22 ppm at 4°C correspond to a pair of 2,6- or 3,5-protons of phenylalanine. The life time with respect to 180° 'flips' about the C^β–C^γ axis of one phenylalanine ring in BPTI can thus be estimated to be of the order 1×10^{-2} sec at 4°C, and 8×10^{-4} sec at 38°C. Considering the temperature dependence of the lines at 7.58 and 8.22 ppm, the origin of the above mentioned apparently reduced overall intensity of the aromatic resonances at ambient temperature is now also quite apparent.

There are four resonance lines at 7.79, 7.48, 6.87, and 6.67 ppm which consecutively broaden and disappear when the temperature is raised from 38°C to 72°C (fig.2). Homonuclear INDOR experiments indicated that these four lines come probably from the same aromatic ring. However, considering the additional spectral changes between 7.1 and 7.4 ppm when the temperature is raised from 38°C to 72°C, we cannot at this point definitely rule out that there might be two aromatic residues which would accidentally show a very similar temperature dependence. We conclude that there is one, possibly two aromatic rings in BPTI which have at 38°C a life time of the order 1×10^{-1} sec with respect to 180°-flips about the C^β–C^γ bond.

There is an additional resolved one-proton line at 7.67 ppm which does not vary with temperature between 4°C and 65°C. On the one hand this line could correspond to any proton of an aromatic ring which would be immobilized in the protein over this temperature range. On the other hand it could also be that it corresponds to the C_4 proton of one of the phenylalanines, in which case the dynamic state of the ring would not necessarily be manifested in this NMR line.

With the possible exception of some features at temperatures above 65°C, where denaturation sets

in in acidic solutions of BPTI [3,6], there is no indication in fig.2 of the occurrence of intermediate rotational states of the aromatic rings which would be sufficiently long lived to be manifested in the NMR spectra.

In conclusion the ^{1}H NMR data described in this paper show that for at least 6 of the 8 aromatic rings in BPTI, the frequency of the rotational motions about the $C^{\beta}-C^{\gamma}$ bond axis exceeds 10^2 sec^{-1} at 60°C. For two, perhaps three, of the phenylalanines the transition from a slow to a fast process on the NMR time scale could be followed between 4°C and 72°C. In a subsequent paper [9], these observations will be analysed in the light of the refined single crystal of BPTI [10].

Acknowledgements

We would like to thank Professor Z. Luz for interesting discussions on the subject of this paper, Dr R. Schmidt-Kastner, Farbenfabriken Bayer AG, for a generous gift of BPTI (Trasylol ®), and the Schweizerischer Nationalfonds (Project 3.1510.73) for financial support.

References

[1] Wüthrich, K. (1974) Experientia 30, 577.
[2] Allerhand, A., Doddrell, D., Glushko, V., Cochran, D. W., Wenkert, E., Lawson, P. J. and Gurd, F. R. N. (1971) J. Amer. Chem. Soc. 93, 544.
[3] Masson, A. and Wüthrich, K. (1973) FEBS Lett. 31, 114.
[4] Kassel, B. and Laskowski, M. (1965) Biochem. Biophys. Res. Commun. 20, 463.
[5] Huber, R., Kukla, D., Rühmann, A. and Steigemann, W. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 141.
[6] Vincent, J. P., Chicheportiche, R. and Lazdunski, M. (1971) Eur. J. Biochem. 23, 401.
[7] Karplus, S., Snyder, G. H. and Sykes, B. D. (1973) Biochemistry 12, 1323.
[8] Wagner, G. and Wüthrich, K. (1974). Paper presented at the 6th International Conference on Magnetic Resonance in Biological Systems, Kandersteg, Switzerland, Sept. 16–21, 1974. Submitted to J. Magn. Res.
[9] Wüthrich, K., Wagner, G., Huber, R. and Deisenhofer, W. (1974) to be submitted to J. Mol. Biol.
[10] Deisenhofer, H. and Steigemann, W., private communication.