PROTON MAGNETIC RESONANCE INVESTIGATION OF THE CONFORMATIONAL PROPERTIES OF THE BASIC PANCREATIC TRYPSIN INHIBITOR

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1. Introduction

The basic pancreatic trypsin inhibitor (BPTI) from bovine pancreas [1] has a molecular weight of 6500 and consists of 58 amino acid residues. The amino acid sequence [2–5] and the molecular conformation in single crystals [6, 7] are known, and it has been found that the solution conformation of BPTI is unusually stable towards denaturing agents and heat [8]. Some time ago we briefly discussed proton nuclear magnetic resonance (NMR) data which revealed yet another rather unexpected structural feature of BPTI, i.e. very slow exchange of some of the amide protons [9]. This paper presents new data on the denaturation of BPTI and the proton exchange in solutions of BPTI in D_2O.

An obvious goal of the investigations of BPTI is to understand in more detail the mode of action of this inhibitor [6–8]. In addition, since BPTI is by its small size amenable to detailed studies by different techniques, work on this molecule could on a more general basis be particularly valuable for the further development of the investigations of molecular conformations in proteins. In view of the temperature stability of BPTI [8], some of the data on this protein might conceivably even be relevant for the investigation of conformational features in proteins from thermophilic organisms [10].

2. Materials and methods

The basic pancreatic trypsin inhibitor (BPTI; Trasylol®,* was obtained from the Farbenfabriken Bayer AG. For most of the experiments ca. 0.01 M solutions of the protein were used. From additional NMR experiments at variable concentrations between 0.0005 and 0.015 M we found no evidence for intermolecular association [11]. The pD of the aqueous solutions was adjusted by the addition of DCl, or NaOD, respectively, and measured in the NMR tube with a combination electrode. The pD-values are given as read from the pH-meter, without correction for the isotope effect [12].

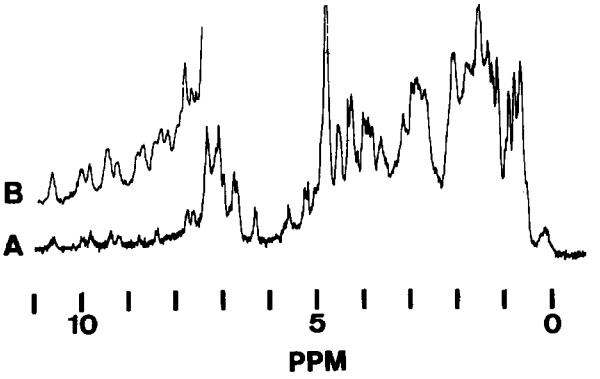

Fig. 1. Proton NMR spectrum at 220 MHz of a 0.01 M solution of the basic pancreatic trypsin inhibitor (BPTI) in D_2O, pD = 7.7, T = 20°. Two spectra were recorded: A) 170 hr and B) 10 min after the preparation of the solution.

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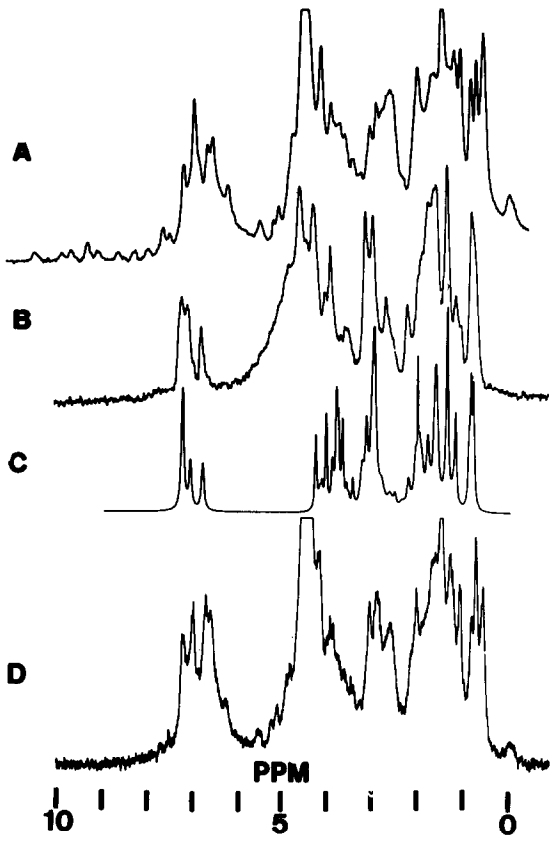

Fig. 2. Proton NMR spectral changes during thermal denaturation of BPTI in D_2O solution containing 0.01 M of the protein and 6 M guanidinium chloride, pD = 7.1. A) T = 20°. B) T = 83°. C) Hypothetical spectrum for BPTI in the random coil form, computed with the amino acid spectra of McDonald and Phillips [14]. D) Spectrum of the solution B after cooling down to 20°.

High resolution proton NMR spectra were recorded on a Varian HR-220 spectrometer. Chemical shifts are in parts per million (ppm) from internal sodium 3-Trimethylsilyl-propionate.

3. Results and discussion

The proton NMR spectrum of BPTI is shown in fig. 1. It contains a series of methyl resonances at around 1 ppm, the remaining resonances of the aliphatic amino acid side chains between 1.5 and 4 ppm, the C_α-proton resonances between 4 and 5 ppm, the solvent resonance at 4.8 ppm, the resonances of the phenylalanyl and tyrosyl residues between 6 and 8 ppm, and 15 readily recognizeable resonances of "exchangeable" protons in the spectral region from 7.5 to 11 ppm. The molecular conformation is manifested in the differences between this spectrum and that of the denatured protein (fig. 2, B and C). Particularly prominent spectral features of native BPTI are the lines at around 0, 5.2, 5.5, and 6.3 ppm, and the appearance of the resonances of a number of "exchangeable" protons (fig. 1B). These qualitative spectral features agree with what we reported previously [9], and seem to coincide essentially with observations made independently by Karplus et al. [13]. In this paper the spectral differences between figs. 1 and 2C are employed for studies of the denaturation of BPTI, and of the exchange of the protons observed between 8 and 11 ppm.

Fig. 2 shows that the thermal denaturation of BPTI in 6 M guanidinium chloride at 83° produces a typical spectrum for a random coil form of the polypeptide chain, as judged from the close similarity in the regions from 0 to 3.5 and 6 to 10 ppm of the spectrum 2B with the computed spectrum 2C [14]. The denaturation is almost completely reversible as judged from the reappearance in the spectral region from 0 to 8 ppm of the prominent spectral features of the native molecule when the solution is cooled down again (fig. 2D). All the exchangeable protons have been replaced by ^2D in the denatured molecule, and hence there are no lines between 8 and 11 ppm in the spectrum D. In a series of measurements of this type the results obtained previously from ORD experiments by Vincent et al. [8] were confirmed, e.g. there were at most very minor changes in the spectral region from 0 to 8 ppm when a neutral solution of BPTI in D_2O was heated to 85°, and in an acidic solution at pD = = 0.7 some spectral changes arose only at temperatures above 70°. In addition we found that a random coil form of BPTI is present at ambient temperature in solutions in trifluoroacetic acid, d_4-methanol, and d_6-DMSO. Upon admixture of D_2O the random coil form of BPTI present in methanol and in DMSO goes over into a molecular conformation with NMR spectral properties which are very similar to those of the native protein. This is illustrated in fig. 3. In DMSO (spectrum A) there are no resonances at 0 ppm and between 5 and 6.5 ppm. The broad resonance at 7 to 9 ppm comes from most of the potentially

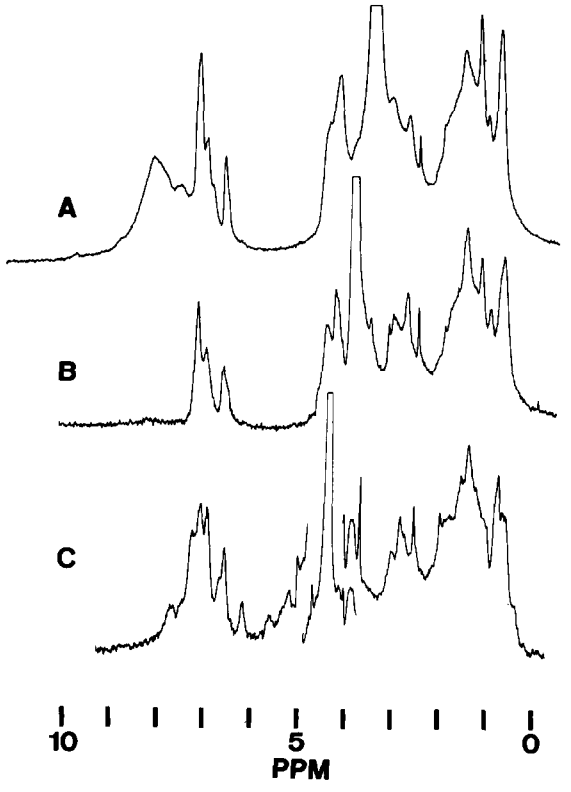

Fig. 3. Proton NMR spectrum at 220 MHz and 20° of 0.01 M solutions of BPTI in mixed solvents of d_6-DMSO and D_2O. A) ≈ 99:1; B) 85:15; C) 65:35, volume percent. The solvent resonances are in the following positions: d_6-DMSO is at 2.5 ppm in all the three spectra, and HDO is observed in A at 3.5 ppm; B, 3.8 ppm; and C, 4.3 ppm.

exchangeable protons. Upon addition of 15% D_2O (fig. 3B) the spectrum is still quite typical for a random coil form of the protein. All the amide protons have been exchanged with ^2D from D_2O. After addition of 35% D_2O (fig. 3C) the NMR spectrum contains the typical features of the native protein except of course for the exchanged protons. A random coil spectrum is again observed if the solution of fig. 3C is heated to ca. 60°.

The appearance of a series of well resolved potentially exchangeable proton resonances between 8 and 11 ppm is a spectral feature which has apparently not been reported for any other protein in D_2O solution. Of the 15 lines of this type which are observed in the freshly prepared solution at neutral pH (fig. 1B and table 1), four decrease visibly in intensity within a few hours at ambient temperature (figs. 1B, 4A, and 4B; ▼), for six additional resonances the intensity is clearly reduced after several hundred hours (fig. 4, C; ■) and the remaining five protons exchange "unmeasurably slowly" under these conditions (fig. 4, ○). At 53° exchange of all these protons can be observed within a few hours, but three lines have not completely disappeared after 1 day (fig. 5). In a freshly prepared acidic solution of BPTI the resonances of ca. 17 to 20 protons are in the spectral region from 8 to 11 ppm (table 1). At pD = 0.9 the exchange of at least 10 of these protons is not complete after one day at

Table 1
Positions and life-times with respect to chemical exchange of the one-proton resonances in the spectral region 7.5 to 11 ppm of BPTI in solutions in D_2O at 22°.

		$\Delta\nu$ (ppm)	$\tau_{1/2}$ (hr)
A. pD = 7.3		7.55	< 2[a]
		7.79	150
		7.87	< 2[a]
		7.98	20
		8.17	20
		8.28	0.4
		8.43	> 1000
		8.67	1.1
		8.80	270
		9.24	> 1000
		9.41	> 1000
		9.41	9
		9.82	> 1000
		9.98	1000
		10.60	> 1000
B. pD = 0.9		7.95	2
	8.1 ...	9.0	> ....[b]
		9.2	> 1000
		9.40	> 1000
		9.45	> 1000
		9.85	> 1000
		10.00	> 1000
		10.60	> 1000
C. pD = 11.0		9.15	15
		9.80	> 100
		10.60	10

[a] This line corresponds possibly to more than one proton.
[b] The resonances between 8.1 and 9 ppm correspond in intensity to 10 to 13 protons. The individual resonances in this spectral region have life-times between 15 hr and > 1000 hr.

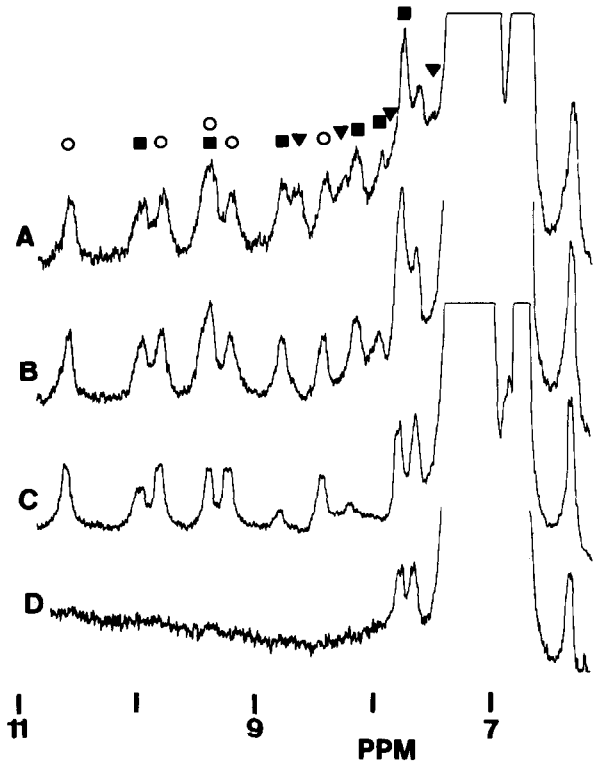

Fig. 4. Spectral region from 7 to 11 ppm of the proton NMR spectrum at 220 MHz of BPTI at different times after preparation of a 0.01 M solution in D_2O, pD = 7.3, T = 22°. A) 25 min; B) 150 min; C) 660 hr; D) after standing at 85° for a few minutes.

53° (fig. 6). In a freshly prepared solution of BPTI at pD = 11, there are only three resonances of slowly exchanging protons (table 1). If a solution of BPTI in neutral H_2O is compared with the spectrum of fig. 1B, no additional resonances can be detected in the spectral region at low field from 9 ppm.

On the basis of what is generally observed in peptides and proteins [15, 16] the above data seem to imply that the slow exchange of some protons in native BPTI comes about because some of the potentially exchangeable protons are shielded from interaction with the solvent. This interpretation would be consistent with the observation that all the labile protons in BPTI are rapidly exchanged when a random coil type NMR spectrum is observed (figs. 2B, 3B). From inspection of the molecular model the resonances between 8 and 11 ppm (fig. 1) have previously been

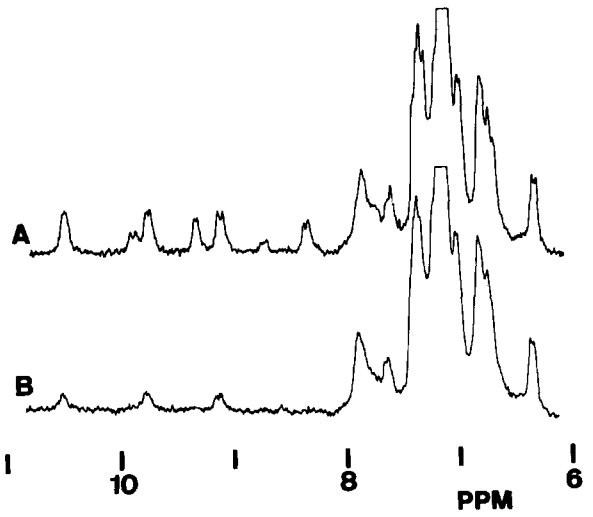

Fig. 5. Spectral region from 7 to 11 ppm of the proton NMR spectrum at 220 MHz of a 0.01 M solution in D_2O of BPTI, pD = 7.3, after being kept at 53° for: A) 2.5 hr; and B) 23 hr.

tentatively assigned to amide protons involved in the hydrogen bonds of the secondary structure elements located in the interior of the BPTI molecule [9]. The doublet structure which can be recognized in several of these resonances (figs. 4–6) provides additional

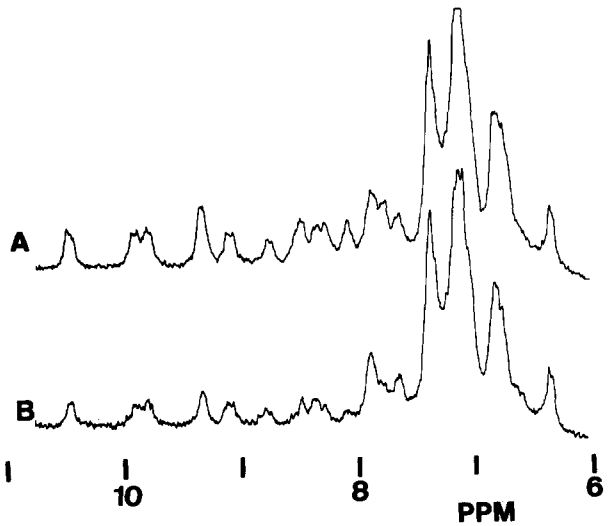

Fig. 6. Spectral region from 7 to 11 ppm of the proton NMR spectrum at 220 MHz of a 0.01 M solution in D_2O of BPTI, pD = 0.9, after being kept at 53° for: A) 2.5 hr; B) 22 hr.

evidence for the assignment to amide protons of the peptide backbone. Overall it is rather surprising that groups of atoms located in the interior of this small protein molecule appear to have an extremely small probability to get in contact with solvent molecules. This seems to indicate that the solution conformation of BPTI is rather rigid, and that there is a small probability for the occurrence of molecular species which differ appreciably from the average form manifested in the NMR spectrum.

It seemed of interest to compare the conditions where the solution conformation of BPTI becomes sufficiently dynamic for all the amide protons to be exchanged rapidly, as judged from the disappearance of the resonances between 8 and 11 ppm, and those where the average conformation is modified, as judged from the spectral changes in the region 0 to 8 ppm. In all these experiments we found that the resonances of all the exchangeable protons disappeared well before there were any modifications in the spectrum of the non-exchangeable protons. The following are a few examples to illustrate this point. In a solution of BPTI in D_2O at neutral pH all the resonances of exchangeable protons disappear within minutes at 65°, and there is no evidence for denaturation of the protein at 85°. In a neutral BPTI solution in 6 M guanidinium chloride, the proton exchange is rapid at 50°, and denaturation begins at ca. 75° until a random coil spectrum is seen at 83° (fig. 2). In a mixed solvent of DMSO and D_2O (1:1) all the resonances of exchangeable protons disappear at 43°, and denaturation to a random coil form is observed at ca. 70°.

In conclusion it was the purpose of this paper to present some unusual features of the solution conformation of BPTI. The present NMR studies have confirmed the previously described stability of this molecule towards certain denaturing agents [8]. On the other hand it was found that BPTI is in a random coil form at ambient temperature in DMSO, TFA, and CH_3OH. NMR has revealed another unusual feature in this molecule, i.e. the extremely slow rate at which certain exchangeable protons are replaced by deuterium in solutions of BPTI in D_2O. Observation of these protons provides a means to investigate the dynamics of the solution conformation of BPTI under variable non-denaturing conditions. Work is in progress to relate these data to particular features in the amino acid sequence [2–5] and the single crystal conformation [6, 7] of the molecule.

Acknowledgements

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