SEQUENCE SPECIFIC ASSIGNMENT OF THE PROTON NUCLEAR MAGNETIC RESONANCE SPECTRUM OF BARLEY SERINE PROTEINASE INHIBITOR 2

by

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The sequence specific assignment of the ¹H NMR spectrum of barley serine proteinase inhibitor 2 is described. The sequential assignment procedure has been applied to the residues 22 to 83. The 21 residues in the N-terminal part of the structure were shown to be a random coil for which sequential assignment was not immediately possible. For the C-terminal part of the protein starting at Thr-22 and ending with the C-terminal residue Gly-83, sequence specific assignment of all the H^N, H^α, H^β, H^{β'} resonances was achieved and complete sequence specific assignment of the non labile protons of the amino acid residue types of glycine, alanine, threonine, serine, valine, isoleucine, leucine, asparagine, aspartic acid, glutamine, glutamic acid, phenylalanine, tyrosine and tryptophan is reported.

1. INTRODUCTION

The present paper is the first in a series of papers describing the study by nuclear magnetic resonance spectroscopy of the protein barley

serine proteinase inhibitor 2 (13, 14, 22, 23). The protein was first isolated by MIKOLA and SOULIMNA (1971) (31) and described as an inhibitor of microbial serine proteinases such as

Abbreviations: BSPI-2 = Barley serine proteinase inhibitor 2; (19-83)BSPI-2 = the C-terminal part of BSPI-2 starting at residue 19 and ending at residue 83; CI-I = Barley chymotrypsin inhibitor I; COSY = Two-dimensional correlation spectroscopy; FID = Free induction decay; FT = Fourier transformation. NMR = Nuclear magnetic resonance; NOE = Nuclear Overhauser effect; NOESY = Two-dimensional nuclear Overhauser spectroscopy; ppm = parts per million; 2QS = Double quantum spectroscopy. The nomenclature for the atoms of the amino acid was according to the IUPAC-IUB recommendation. (IUPAC-IUB Commission of Biochemical Nomenclature 1969. Biochem. 9, 3471-3479 (1970)).

subtilisin. However, it has often been referred to as the chymotrypsin inhibitor, CI-2 (42). The inhibitor is present in relatively large amounts in the seeds of the barley variety HIPROLY (21) where it is responsible for the high content of the amino acids proline and lysine found in the seeds of this variety. The protein consists of 83 residues and it is a basic protein (20, 40). The amino acid sequence has been determined using chemical amino acid sequencing (40) and recently from the DNA-sequence of the gene of the inhibitor (53). The amino acid sequence is homologous to the potato serine proteinase inhibitor (41) and has been assigned to the family of inhibitors named after this, which include a number of other inhibitors of plant sources as well as the leech eglin c (42, 44). In this family of inhibitors as well as in the entire group of protein inhibitors the BSPI-2 and eglin c are unique so far in being the only inhibitors which do not contain a disulfide bridge. The three-dimensional structure of the BSPI-2 has been determined by X-ray crystallography firstly in the complex with subtilisin (29) and later in its free form (30). Except for a number of differences in the binding site loop the two structures were in many respects identical. In the binding site loop the peptide bond between Met-59 and Glu-60 was shown to be hydrolyzed by subtilisin (43). A mechanism of inhibition was deduced from this observation, where the inhibitor is bound to the enzyme and subsequently the active site peptide bond is hydrolyzed. However, due to a very tight binding the inhibitor remains in the active site of the enzyme where it prevents access of other substrates. For a number of reasons the physical and chemical properties of BSPI-2 make this protein inhibitor an ideal protein to study by NMR spectroscopy and other methods: the protein is relatively small, it is very soluble, it remains folded in a pH-range of pH 2.8 to above pH 10.5 at 37 °C and it undergoes reversible folding-unfolding at conditions close to the point of denaturation. Therefore, the BSPI-2 can serve as a good model protein for studies of chemical properties such as protein dynamics, protein stability, protein electrostatics, protein folding and protein-protein interactions. In addition, since the gene of the BSPI-2 recently has been expressed in *E. coli*

(FERSHT and LEATHERBOROUGH, personal communication) the protein is subject to chemical modifications employing the techniques of site directed mutagenesis.

The present paper is concerned with the sequential assignment of the ¹H NMR spectrum of BSPI-2 which is a prerequisite for all further studies of this protein by NMR spectroscopy. The proteins studied here are both the 65 residue long C-terminal part of the protein (19-83)BSPI-2 which has been shown to be the core of the protein possessing all the structural features necessary for its function as a proteinase inhibitor (40), and the intact 83 residue long BSPI-2. The sequence specific assignment was achieved by the use of two-dimensional NMR spectroscopy using the information from NOESY, COSY, relayed COSY, 2QS spectra of both the BSPI-2 and the (19-83)BSPI-2. The principles and the structural basis for the method of sequential assignment of the proton two-dimensional NMR spectrum of proteins were first described by WÜTHRICH et al. (55) and BILLETER et al. (6). The method has since been applied to a number of small proteins such as basic pancreas trypsin inhibitor (47) glucagon (51), bull seminal proteinase inhibitor IIA (39), lac repressor headpiece (57), rabbit methallothionein-2 (8, 33), tendamistat (26), α 1-purothionin (12), human epidermal growth factor (11), ribosomal protein E-L30 (45, 46), histidine containing protein from *E. coli* (24, 25), the short scorpion insectotoxin I₂A (3), apamin (50), *E. coli* enterotoxin (34), leech eglin (18) phoratoxin (15), hirudin (38), and histone H5 protein (56) for which essentially complete sequence specific assignments have been achieved using the sequential assignment method.

2. MATERIALS AND METHODS

The barley serine proteinase inhibitor was extracted from seeds of the barley variety HIPROLY obtained from the Department of Plant Breeding of the Carlsberg Research Laboratory. The extraction and the purification of the BSPI-2 and (19-83)BSPI-2 were in principle as described by JONASSEN (20). All chemicals were analytical grade. Deuterium oxide was purchased from Merck.

NMR spectra were recorded on a BRUKER AM 500 NMR-spectrometer equipped with an Aspect 3000 computer. The recorded NMR data were transferred to a VAX 11/750 computer and all further data processing was performed using the Carlsberg Laboratory NMR-software which for certain routines uses the Rowland NMR-Toolkit (17). The following two-dimensional NMR experiments have been used in this work: NOESY (1, 2, 19, 27, 37), COSY (4, 28, 33), 2QF-COSY (35, 36) relayed COSY (16, 49) and 2QS (7, 9, 48). In all cases the spectra were recorded and transformed using hypercomplex 2D FT yielding quadrature detection in both dimensions and pure phase (5, 32, 37). The two data sets were recorded with a 90° relative phase shift of the preparation pulse in the experiments: NOESY, COSY, DQF-COSY and relayed COSY. For the 2QS experiment a 45° relative phase shift of the entire preparation pulse "sandwich" was employed. Typically 2×512 spectra were recorded in the t_1 domain each of 2048 complex points. When processed the NMR data sets were zero-filled up to 2048 complex points in the t_1 dimension and 8192 complex points in the t_2 dimension before the respective Fourier transformations. Chemical shift values are given in ppm relative to the methyl group proton resonances of 4,4-dimethyl-4-silapentane and measured relative to acetone and dioxan as internal standards. The recording of FID's of protein samples in 90% $\text{H}_2\text{O}/10\%$ $^2\text{H}_2\text{O}$ (52) was obtained by continuous selective irradiation at the water resonance frequency in the preparation period and a 3dB attenuation of the irradiation during the evolution and the mixing time. The irradiation was gated during the detection period.

3. RESULTS

3.1. Amino acid spin system identification

The protocol used in the present assignment procedure was as follows. Firstly, the spin systems of the (19-83) BSPI-2 were identified and subsequently, the spin systems of the first eighteen N-terminal residues were identified by comparing the (19-83)BSPI-2 spectrum with the spectrum of the intact BSPI-2 and recognizing

those spin systems in the BSPI-2 spectra absent in the (19-83)BSPI-2 spectra.

The spin system recognition was performed as follows. First, the $\text{H}^{\text{N}}\text{-H}^{\alpha}$ cross peaks were registered as found in the so-called "fingerprint region", of the COSY spectrum of (19-83)BSPI-2, Figure 1. This region should contain 60 cross peaks given that the protein contains 65 residues and 4 of these are prolines. In the spectrum 55 cross peaks are labelled according to the result of the sequential assignment. The peaks of Lys-30 and Arg-67 have been eliminated by the selective irradiation at the water resonance frequency, in this spectrum. They have, however, been observed at other conditions. Two $\text{H}^{\text{N}}\text{-H}^{\alpha}$ cross peaks have never been observed, these are of Leu-20 and Lys-21 in the N-terminal part of the peptide chain. One cross peak, of Val-79 has so low intensity that it is not appearing in the spectrum at the contouring level used.

Subsequently, the search was initiated for the $\text{H}^{\alpha}\text{-H}^{\beta}$ cross peaks corresponding to each of the H^{α} chemical shifts identified in the $\text{H}^{\text{N}}\text{-H}^{\alpha}$ cross peak region of the COSY spectrum. The 19 of the 20 amino acids normally found in proteins have a β -carbon and these can be divided into two groups: one group which contains only one β -proton include threonine, valine, isoleucine and alanine (which has three β -protons whose signals, however, degenerates to one) and one group in which the amino acids have two β -protons consisting of the remaining 15 types of the amino acids normally found in proteins. This latter group can be subdivided into two groups, one which contains the AMX systems: serine, asparagine, aspartic acid, histidine, phenylalanine, tyrosine, tryptophan and cysteine (of which the latter is absent in BSPI-2); and the remaining amino acids which are glutamic acid, glutamine, proline, leucine, methionine, lysine and arginine. Often in COSY spectra of proteins the last group of amino acids, despite of having two β -protons, only shows one strong $\text{H}^{\alpha}\text{-H}^{\beta}$ COSY cross peak, the cross peak of the second $\text{H}^{\alpha}\text{-H}^{\beta}$ coupling having too low intensity to be observed given the signal to noise ratio typically obtained for protein NMR spectra. In order to obtain definitive identification of the amino acid spin systems belonging to the group having two β -protons the $\text{H}^{\beta}\text{-H}^{\beta}$ 2Q coherence observed

Figure 1. ^1H - ^1H COSY spectrum of (19-83)BSPI-2 recorded at 42 °C pH 4.20 in 90%/10% $\text{H}_2\text{O}/^2\text{H}_2\text{O}$. The region shown is the so-called "fingerprint" region where the H^{N} - H^{α} cross peaks have been identified. Each cross peak has been labelled with the residue name using the single letter code for the amino acid residues and the residue number according to the sequence determined by SVENDSEN et al. 1980 (40). Red contours are negative levels and black contours are positive levels.

Figure 2. ^1H - ^1H COSY spectrum of 0.01 M (19-83)BSPI-2 recorded at 42 °C, pH 4.20 in 90%/10% $\text{H}_2\text{O}/^2\text{H}_2\text{O}$. The spectrum shows the H^α - H^β cross peaks for most of the residues in the molecule. The cross peaks have been labelled as described in Figure 1.

in double quantum spectra of these amino acids at the ω_2 frequency corresponding to the H^α was employed. So by combination of the COSY and the double quantum spectrum the division of the amino acid spin systems in the two groups was achieved. Subsequently, the cross peaks of the spin systems of the amino acids glycine, alanine, threonine, valine, isoleucine, asparagine, aspartic acid, serine, phenylalanine, tyrosine, tryptophan, were identified using the information available in COSY, relayed COSY, and double quantum spectra. The identification of cross peaks belonging to the spin systems of the remaining amino acids lysine, arginine, methionine, proline, leucine, glutamine and glutamic acid is less easily achieved due to the chemical shift degeneracy of the methylene proton resonances in these amino acids. The identification of these spin systems was therefore postponed to the state in the analysis, where by sequential assignment the H^N - H^α cross peaks of these amino acids had been identified. The next step in the analysis was the sequential assignment procedure where the neighbouring amino acid spin systems were identified using NOESY spectra. The NOE cross peaks of the type $d_{\alpha\text{N}}(i, i+1)$ and $d_{\text{NN}}(i, i+1)$ were assigned, and by using the information obtained from the identification of the amino acid spin systems in combination with the amino acid sequence, the sequence specific assignment of the individual amino acid spin systems was obtained (54, 57). Finally, the identification of the amino acid spin systems remaining to be completed was performed, where this was possible. The following paragraphs will describe in some detail how the procedure described above was used to obtain the sequence specific assignment of the ^1H NMR spectrum of BSPI-2.

3.1.1. Glycines

The (19-83)BSPI-2 contains three glycines (Gly-29, Gly-54 and Gly-83) and the $\text{H}^{\alpha 1}$ - $\text{H}^{\alpha 2}$

cross peaks from these were immediately identified, Figure 2. For two of these glycines the H^N - $\text{H}^{\alpha 1}$ and H^N - $\text{H}^{\alpha 2}$ cross peaks were both sufficiently intense to be observed, but, for the third of the glycines, Gly-83, only one of the H^N - H^α cross peaks could be identified in the COSY spectrum suggesting that one of the H^N - H^α couplings in this residue is very small. This peak, though, was clearly observed in the relayed COSY experiment. Table I lists the chemical shifts of the protons in the three glycines. The additional three glycines in the N-terminal part of the BSPI-2 are clearly observed in the COSY spectrum of the BSPI-2 as three peaks, however, the overlap is so strong that an attempt to distinguish between the three has been avoided.

3.1.2. Alanines

The three H^α - $\text{H}^{\beta(1,2,3)}$ cross peaks of the three alanines Ala-35, Ala-46 and Ala-77 in (19-83)BSPI-2 are shown in Figure 2. They were distinguished from the three threonine H^β - $\text{H}^{\gamma(1,2,3)}$ cross peaks because by coincidence none of the three threonine H^β resonances had chemical shifts identical to the identified H^α chemical shifts in the spectrum. In the relayed COSY experiments two of the alanine H^N - $\text{H}^{\beta(1,2,3)}$ cross peaks were identified and further evidence for the correctness of these assignments were the NOE cross peaks due to strong H^N - $\text{H}^{\beta(1,2,3)}$ dipolar coupling. The alanine assignments are summarized in Table I. The Ala-14 spin system was unambiguously identified see Figure 3 because Ala-14 is the only new alanine in the additional N-terminal part of BSPI-2 spectrum.

3.1.3. Threonines

Only one of the three H^α - H^β cross peaks of the threonines Thr-22, Thr-55, Thr-58 was observed in the COSY spectrum. The remaining two were identified in the double quantum spectrum, which due to the absence of a diagonal facilitates the identification of J-couplings

Table I. Sequence specific resonance assignment of the proton resonances in BSPI-2^a.

	H ^N	H ^α , H ^{α''}					
Gly 9	8.35	3.97	3.99				
Gly 13	8.35	3.97	3.99				
Gly 15	8.35	3.97	3.99				
Gly 29	9.11	4.42	3.70				
Gly 54	8.85	4.36	3.58				
Gly 83	8.79	4.28	3.12				
	H ^N	H ^α	H ^β	H ^γ			
Thr 12	8.13	4.33	4.31	1.21			
Thr 22	8.29	4.31	4.54	1.12			
Thr 55	7.60	4.11	4.13	1.37			
Thr 58	7.92	4.47	4.59	1.31			
	H ^N	H ^α	H ^β	H ^{γ'} , H ^{γ''}			
Val 3	b)	4.16	2.11	0.94	0.94		
Val 10	b)	4.16	2.11	0.94	0.94		
Val 28	7.18	3.35	2.04	1.02	0.94		
Val 32	8.35	3.64	2.05	0.98	0.97		
Val 38	7.42	3.62	1.99	1.09	1.04		
Val 50	8.42	4.71	1.92	0.86	0.85		
Val 53	8.88	4.05	1.82	0.99	0.98		
Val 57	8.00	4.80	2.36	0.99	0.97		
Val 66	8.22	4.43	1.67	0.74	0.61		
Val 70	8.75	5.38	2.09	0.84	0.65		
Val 79	8.86	3.65	2.13	1.19	0.89		
Val 82	8.48	4.71	2.24	1.31	1.19		
	H ^N	H ^α	H ^β	H ^{γ2}	H ^{γ1'} , H ^{γ1''}		H ^δ
Ile 39	8.08	2.97	1.22	0.12	0.72	-1.10	-0.07
Ile 48	8.25	4.78	1.71	0.67	1.42	0.87	0.68
Ile 49	7.94	4.44	1.60	0.85	1.32	1.13	0.78
Ile 56	8.48	4.12	1.88	0.93	1.65	1.32	0.91
Ile 63	7.96	3.75	1.92	0.97	1.59	1.38	1.02
Ile 76	9.09	4.04	2.58	1.12	1.72	1.21	0.75
	H ^N	H ^α	H ^β				
Ala 14	8.11	4.34	1.41				
Ala 35	8.38	4.07	1.40				
Ala 46	7.95	4.20	1.16				
Ala 77	9.50	4.69	1.45				
	H ^N	H ^α	H ^{β'} , H ^{β''}				
Asp 16	N/A	4.73	2.78	2.81			
Asp 42	7.58	4.75	3.11	3.18			
Asp 64	8.29	4.44	2.68	2.86			
Asp 71	8.79	4.78	2.67	3.48			
Asp 74	8.30	4.21	2.96	3.36			

	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{δ2'} , H ^{δ2''}					
Asn 11	8.53	4.83	2.79	2.92	d)					
Asn 19	8.53	4.83	2.79	2.92	d)					
Asn 19 ^{c)}		4.41	2.97	2.99	7.03	7.68				
Asn 75	8.27	5.40	2.03	3.03	6.59	7.90				
	H ^N	H ^α	H ^{β'} , H ^{β''}							
Ser(1,2) ^{e)}	8.23	4.48	3.89	3.87						
Ser(1,2)	8.37	4.53	3.91	3.89						
Ser 31	8.51	5.00	4.45	4.11						
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^δ	H ^ε	H ^ζ			
Phe 69	9.02	5.71	2.89	2.64	7.28	7.29	7.11			
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^δ	H ^ε				
Tyr 61	8.53	4.89	3.03	2.97	7.17	6.81				
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{δ1}	H ^{ε1}	H ^{ζ2}	H ^η	H ^{ζ3}	H ^{ε3}
Trp 24	8.25	5.18	3.12	2.96	7.16	10.39	7.20	6.68	6.62	7.34
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{δ2}		H ^{ε1}			
His 18	N/A	4.75	3.48	2.64	7.35		8.65			
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{γ'} , H ^{γ''}					
Glu(4,8) ^{h)}	8.43	4.35	2.11	1.98						
Glu(4,8)	8.27	4.32	2.06	1.96						
Glu 23	6.73	5.34	2.18	1.84	2.29	2.17				
Glu 26	10.62	4.42	2.19	2.16	2.59	2.18				
Glu 33	8.25	3.98	2.06	1.98	2.43	2.05				
Glu 34	7.61	4.06	2.21	2.05	2.35					
Glu 45	9.28	4.46	2.12	2.08	2.49	2.13				
Glu 60	8.47	4.34	2.19	2.05	2.45	2.05				
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{γ'} , H ^{γ''}		H ^{ε'} , H ^{ε''}			
Gln 41	7.30	4.15	2.32	2.22	2.52	2.48	g)			
Gln 47	8.87	4.42	1.92	1.84	2.32	2.17	g)			
Gln 78	7.32	4.78	2.15	1.99	2.34	2.30	g)			
		H ^α	H ^{β'} , H ^{β''}		H ^{γ'} , H ^{γ''}		H ^{δ'} , H ^{δ''}			
Pro 7		4.44	2.31	1.93	2.06	2.03	3.65	3.84		
Pro 25		4.68	2.49	1.97	2.29	2.08	3.31	3.92		
Pro 44		4.37	2.38	2.12	2.09		3.77	3.45		
Pro 52		4.83	2.50	1.83	2.12		3.89	3.67		
Pro 80		4.82	2.25	2.10	1.86	1.86	4.33	4.07		

	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^γ	H ^{δ'} , H ^{δ''}		
Leu 20	N/A	4.36	1.63	1.60	1.65	0.98	0.94	
Leu 27	7.88	4.45	1.95	1.76	1.57	0.94	0.87	
Leu 40	7.89	4.11	1.78	1.38	1.50	0.67	0.65	
Leu 51	9.09	5.02	1.37	1.23	1.32	0.82	0.58	
Leu 68	9.06	4.80	1.79	0.97	1.68	0.95	0.85	
Leu 73	8.04	4.44	1.83	1.75	1.81	0.87	0.87	
	H ^N	H ^α	H ^{β'} , H ^{β''}					
Met 59	8.24	4.23	1.57	1.50				
	H ^N	H ^α	H ^{β'} , H ^{β''}		H ^{γ'} , H ^{γ''}		H ^{δ'} , H ^{δ''}	H ^ε
Arg 17	N/A	4.62	1.85	1.74				
Arg 62	8.80	4.54	1.76	1.52	1.47	1.58	3.15	7.89
Arg 65	7.26	5.02	2.23	1.25				
Arg 67	8.98 ^{b)}	4.68	1.84	N/A				
Arg 81	9.25	5.72	2.10	2.00				
	H ^N	H ^α	H ^{β'} , H ^{β''}					
Lys 5	8.24	4.33	1.83	1.75				
Lys 6	7.83	4.31	1.89	1.77				
Lys 21	N/A	4.62	2.30	1.99				
Lys 30	7.86 ^{b)}	4.62	2.09	1.92				
Lys 36	8.48	3.50	1.75	1.73				
Lys 37	6.98	3.88	1.92	1.92				
Lys 43	9.08	4.78	2.12	1.99				
Lys 72	8.15	4.21	1.94	1.94				

^{a)} Chemical shifts are as recorded at pH 4.2 and 42 °C.

^{b)} Sequence specific assignment of the Val-3 H^N and the Val-10 H^N has not been accomplished. The two resonances are observed at 7.93 and 8.01.

^{c)} In (19-83) BSPI-2.

^{d)} Sequence specific assignment for Asn-11 H^{δ2}(1, 2) and Asn-19 H^{δ2}(1, 2) has not been accomplished. The pair of resonances were observed at (6.98, 7.58) ppm and (6.91, 7.63) ppm, respectively.

^{e)} Sequence specific assignment for Ser-1 and Ser-2 has not been accomplished.

^{f)} Sequence specific assignment for Glu-4 and Glu-8 has not been accomplished.

^{g)} The three pairs of Gln H^{ε2}(1, 2) are observed at (6.43, 7.30) ppm (6.67, 7.32) ppm and (6.78, 7.47) ppm.

^{h)} As observed at 22 °C.

between protons having very similar chemical shifts. The H^β-H^{γ2}(1,2,3) of the three threonines in (19-83)BSPI-2 were subsequently identified both in the COSY and in the 2Q spectrum. Note, however, that the H^β-H^{γ2}(1,2,3) cross peak at (4.59, 1.31) ppm in Figure 2 has been eliminated by water resonance suppression. The Thr-12 spin system was likewise identified in the COSY and double quantum spectrum of the intact BSPI-2, being the only additional threonine in the N-terminal part of the protein (Figure 3).

3.1.4. Valines

The (19-83)BSPI-2 contains 10 valines (see Table I) and this residue type therefore is the most abundant in the protein. The typical H^β-H^{γ1}(1,2,3), H^β-H^{γ2}(1,2,3) cross peaks are the "fingerprints" of this residue, they are found, however, in the same region of the spectrum as the H^γ-H^{δ1}(1,2,3), H^γ-H^{δ2}(1,2,3) cross peaks of leucines of which the (19-83)BSPI-2 have six. Obviously, the distinction between the valine and leucine methyl group resonances can be

Figure 3. ^1H - ^1H Double quantum spectrum of BSPI-2 (0.01 M, pH 4.2, 37 °C 99.96% $^2\text{H}_2\text{O}$) mixing time 0.036 s. The spectrum is labelled as in Figure 2. The assignment of the indirect $\text{H}^{\text{B}1}$ - $\text{H}^{\text{B}2}$ coherence effects are indicated.

made only when the complete identification of the valine spin systems is achieved. The H^{a} - H^{b} cross peaks of the valines are shown in Figure 2,

however, the level of contouring is not sufficiently low to show the weak H^{a} - H^{b} cross peaks of Val-52 at (4.80, 2.36) ppm and Val-70

at (5.35, 2.09) ppm. In Figure 4 is shown the region of the COSY spectrum where the $\text{H}^\beta\text{-H}^{\gamma(1,2,3)}$, $\text{H}^\beta\text{-H}^{\gamma(1,2,3)}$ cross peaks of the valines occur. Additional support for the valine assignments was found in the relayed COSY

spectrum, where relayed effects respectively from each of the H^a protons to the γ -methyl group protons were observed. A summary of the chemical shifts of the valine protons is given in Table I. The additional two valines in the

Figure 4. ^1H - ^1H COSY spectrum of (19-83)BSPI-2 (0.01 M, pH 4.2, 90%/10% $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, 42 °C). The region of the spectrum shown displays the methyl group cross peaks of the valines, leucines and isoleucines.

spectrum of the intact protein were observed to have identical chemical shift values for all the side chain proton resonances, however, the two H^{N} signals had slightly different chemical shifts, see Table I.

3.1.5. Isoleucines

The two characteristic features of the isoleucine spin systems are the $\text{H}^{\text{a}}\text{-H}^{\text{b}}\text{-H}^{\gamma(1,2,3)}$ and the $\text{H}^{\gamma(1)}\text{-H}^{\delta(1,2,3)}\text{-H}^{\gamma(2)}$ tracks of cross peaks. The inhibitor contains six isoleucines, Ile-39, Ile-48, Ile-49, Ile-56, Ile-63 and Ile-76. Each of the six $\text{H}^{\text{a}}\text{-H}^{\text{b}}$ cross peaks are seen in Figure 2, the Ile-63 ($\text{H}^{\text{a}}\text{-H}^{\text{b}}$) cross peak is, however, of rather low intensity. The six $\text{H}^{\text{b}}\text{-H}^{\gamma(2,3)}$ cross peaks were clearly distinguished from the $\text{H}^{\text{a}}\text{-H}^{\delta(1,2,3)}$ and $\text{H}^{\text{b}}\text{-H}^{\gamma(2,3)}$ cross peaks of alanines and threonines, respectively, due to the large difference in chemical shifts between the H^{b} resonance of isoleucine and the H^{a} and the H^{b} of these two amino acid types. Additional evidence for the $\text{H}^{\text{a}}\text{-H}^{\text{b}}\text{-H}^{\gamma(1,2,3)}$ assignments was seen in the relayed COSY spectrum where the $\text{H}^{\text{a}}\text{-H}^{\gamma(2,3)}$ effects were all clearly observed.

The triplet of the $\text{H}^{\delta(1,2,3)}$ resonances of the isoleucines was clearly observed in the anti-phase COSY cross peaks with 1:2 rectangular pattern of $(\text{H}^{\gamma(1)}, \text{H}^{\gamma(2)})\text{-H}^{\delta(1,2,3)}$ coupling, hence the six pairs of this type of cross peaks were immediately assigned to isoleucines. The remaining identification of the respective $\text{H}^{\text{b}}\text{-H}^{\gamma(1,2)}$ cross peak in the COSY spectrum to complete the assignments of these six spin systems was straightforward given that the primary identification of the two parts of the six spin systems had narrowed the cross peak search. It should be noticed that two isoleucines though are having quite strongly shifted resonances compared to the basis shift of these in model peptides. These are Ile-39 and Ile-76 which as far as the H^{b} chemical shifts are concerned are shifted respectively 0.7 ppm up- and downfield, and more dramatically so is the Ile-39 $\text{H}^{\gamma(1 \text{ or } 2)}$ shifted more than 2.0 ppm to -1.10 ppm.

3.1.6. Aromatic residues

The serine proteinase inhibitor contains only one of each of the aromatic residues tryptophan, tyrosine and phenylalanine. The identification of $\text{H}^{\epsilon(1)}$ and the $\text{H}^{\delta(1)}$ resonances of the tryptophan was accomplished using on the characteristic chemical shift of the $\text{H}^{\epsilon(1)}$ and the coupling seen in the COSY spectra between these. Also the identification of the benzenoid protons of the tryptophan ring was obtained using the multiplet information observed in the antiphase cross peaks in the COSY spectrum. The distinction between the $\text{H}^{\epsilon(2)}$ and the $\text{H}^{\epsilon(3)}$ doublets was established by the observation of the NOE effect between the $\text{H}^{\epsilon(1)}$ and the $\text{H}^{\epsilon(2)}$. Likewise, the assignment of the $\text{H}^{\delta(1,2)}\text{-H}^{\epsilon(1,2)}$ cross peak of the tyrosyl residue was straightforward and the identification of the $\text{H}^{\epsilon(1,2)}$ chemical shifts was obtained from pH-titrations at high pH in which these protons were observed to have relatively

Figure 5. Double quantum spectrum (mixing time 0.036 s) of (19-83)BSPI-2, (0.01 M, pH 4.20 99.97% $^2\text{H}_2\text{O}$ 37°C). The spin system cross peaks of the phenyl group of Phe-69. The $\text{H}^{\delta(1,2)}\text{-H}^{\epsilon(1,2)}$ 2Q coherence detected at the $\text{H}^{\epsilon(1)}$ chemical shift was used to identify the H^{δ} chemical shift, which by coincidence is very close to the chemical shift of the H^{ϵ} .

Figure 6. ^1H NOESY spectrum of (19-83)BSPI-2 recorded with a mixing time of 0.20 s, (0.01 M, 90%/10% $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, pH 4.2, 42°C). The cross peaks shown are the $\text{H}^\delta\text{-H}^\beta$ cross peaks of the aromatic residues, Trp-24, Tyr-61 and Phe-69.

large upfield shifts as expected due to the ionization of the hydroxyl group. The cross peaks of the aromatic protons of the phenylalanine were not all observed in the COSY spectra and only examination of the 2QS spectrum of this residue revealed that the $\text{H}^{\delta(1,2)}$ and the $\text{H}^{\alpha(1,2)}$ resonances were so close in chemical shifts that they were not distinguishable in the COSY spectrum. In Figure 5 is shown the cross peak due to the 2Q coherence of the $\text{H}^{\delta(1,2)}$ and $\text{H}^{\alpha(1,2)}$ at the chemical shift position of the H^ϵ .

The completion of the assignment of these three aromatic residues was accomplished using the NOE effects expected between the $\text{H}^{\delta 1}$ and the two H^β 's of the respective amino acids (6, 45), see Figure 6.

3.1.7. The non-aromatic AMX systems

The (19-83)BSPI-2 contains 2 asparagines one of which is the N-terminal residue, 4 aspartic acids and 1 serine, all AMX spin systems. The AMX spin system with two low field H^β resonances at 4.45 and 4.11, respectively, was assigned to the only serine in the molecule. The Ser-1 and Ser-2 spin systems in the intact BSPI-2 were identified in the COSY spectrum of BSPI-2 using the criteria that the H^β resonances of serine are normally at low field, however, lacking the sequential NOE's in this region, sequence specific assignments were not accomplished for these two spin systems. The four aspartic acid residues were identified using a series of COSY spectra recorded at pH values in the pH region between pH 3 and pH 7 where the aspartic acid normally has its pK. Three of the AMX $\text{H}^\beta\text{-H}^\beta$ cross peaks showed the typical upfield shift with increasing pH, Figure 7, however, one $\text{H}^\beta\text{-H}^\beta$ cross peak which later in the sequential assignment procedure was shown to be the Asp-42 $\text{H}^\beta\text{-H}^\beta$ cross peak showed an abnormal downfield shift for one of the H^β -resonance. This observation could otherwise have indicated that this residue was an asparagine rather than an aspartic acid as determined in the chemical amino acid sequence determination. However, the number of signals of nitrogen bound H^ϵ and H^δ from glutamines and asparagines in the COSY spectrum is in agreement with the expected number of 2 asparagines and 3 glutamines excluding the possibility that Asp-42 should be changed to asparagine in the amino acid sequence of the (19-83)BSPI-2.

The remaining two AMX systems of asparagine were subsequently assigned, one of these were of the N-terminal Asn-19 for which the H^α was found at 4.42 ppm. In the intact BSPI-2 the H^α of Asn-19 shifts to 4.83 ppm as expected, and is found in precisely the same position as that of the Asn-11, Figure 3. The $\text{H}^\beta\text{-H}^\beta$ cross peak in the 2Q spectrum at the chemical shift position of this H^α has a cross peak intensity corresponding to two $\text{H}^\beta\text{-H}^\beta$ cross peaks, and for this reason the resonances of the two Asn-residues in the N-terminal peptide are believed to have identical chemical shifts, as far as the H^N , H^α , $\text{H}^{\beta 1}$, and $\text{H}^{\beta 2}$ are concerned. The cross peaks of the two pairs of $\text{H}^{\beta 2}$ for these two

Figure 7. ^1H - ^1H phase sensitive COSY spectra of (19-83)BSPI-2, (0.01 M, 90%/10% $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ 35°C). The three spectra shown were recorded at pH 3.28 (black), 4.20 (red) and 6.45 (blue). The cross peaks whose chemical shifts are strongly influenced by the pH are the H^γ - H^γ cross peaks of glutamic acid, and the H^β - H^β cross peaks of aspartic acid.

residues are seen at four separate chemical shifts at 7.58, 6.89 and 7.63, 6.91 but these have not been assigned with respect to sequence. The resonances of Asp-16 in the N-terminal part of BSPI-2 were identified, by their pH-dependence. The remaining His-18 AMX system was identified on the basis of its chemical shifts in model peptides (10, 54).

3.1.8. The sequential assignment

The sequential assignment procedure which has been extensively described by WÜTHRICH and co-workers (54, 57) employs the nuclear Overhauser effect between hydrogens of neighbouring residues in the amino acid sequence and the amino acid sequence determined by chemical methods to obtain sequence specific assignment of the NMR spectrum of a protein. The

Table II. Sequential assignment of the (19-83)BSPI-2 NOESY spectrum

	d _{αN} (i,i+1)	d _{NN} (i,i+1)	d _{ωδ} (i,i+1)	d _{βN} (i,i+1)
T22 – E23		8.28 6.73		
E23 – W24	5.34 8.26			
W24 – P25			5.17 3.92 5.17 3.31	
P25 – E26				3.92 10.63
E26 – L27		10.63 7.88		
L27 – V28		7.88 7.17		
V28 – G29	3.34 9.12			
G29 – K30		9.12 7.78		
K30 – S31	N/A	N/A	N/A	
S31 – V32	4.99 8.35			
V32 – E33		8.35 8.24		
E33 – E34		8.24 7.61		
E34 – A35		7.61 8.38		
A35 – K36		8.38 8.48		
K36 – K37		8.48 6.97		
K37 – V38		6.97 7.43		
V38 – I39		7.43 8.07		
I39 – L40		8.07 7.87		
L40 – Q41		7.87 7.29		
Q41 – D42		7.29 7.57		
D42 – K43		7.57 9.07		
K43 – P44			4.78 3.45 4.78 3.78	
P44 – E45	N/A	N/A	N/A	N/A
E45 – A46		9.28 7.95		
A46 – Q47	4.20 8.86			
Q47 – I48	4.42 8.25			
I48 – I49	4.78 7.94			
I49 – V50	4.43 8.42			
V50 – L51	4.71 9.09			
L51 – P52			5.02 3.89 5.02 3.67	
P52 – V53	4.83 8.88			
V53 – G54	4.05 8.85			
G54 – T55	3.58 7.60	8.85 7.60		
T55 – I56	4.11 8.48			
I56 – V57	4.12 8.00			
V57 – T58	4.80 7.92			
T58 – M59	4.47 8.24			
M59 – E60	N/A	N/A		
E60 – Y61	4.34 8.53			
Y61 – R62	4.89 8.80			
R62 – I63	4.53 7.95			
I63 – D64	3.74 8.29	7.96 8.28		
D64 – R65		8.29 7.26		
R65 – V66	5.02 8.22			
V66 – R67	4.43 8.96			
R67 – L68	N/A	N/A		

	$d_{\alpha\text{N}}(i,i+1)$	$d_{\text{NN}}(i,i+1)$	$d_{\alpha\alpha}(i,i+1)$
L68 - F69	4.80 9.02		
F69 - V70	5.71 8.75		
V70 - D71	5.38 8.79		
D71 - K72	4.78 8.15		
K72 - L73		8.15 8.04	
L73 - D74		8.04 8.29	
D74 - N75	4.21 8.27		
N75 - I76	5.40 9.09		
I76 - A77	4.04 9.50		
A77 - Q78		9.50 7.32	
Q78 - V79	4.78 8.86		
V79 - P80			3.65 4.34
			3.65 4.08
P80 - R81	4.82 9.25		
R81 - V82	5.72 8.48		
V82 - G83	4.71 8.80		

typical sequential NOE's are of the type $d_{\alpha\text{N}}(i,i+1)$ and $d_{\text{NN}}(i,i+1)$ (55), however, other such as the $d_{\beta\text{N}}(i,i+1)$, the $d_{\alpha\text{N}}(i,i+3)$, the $d_{\alpha\text{N}}(i,i+4)$ and the $d_{\text{NN}}(i,i+2)$ are useful types of sequential NOE's in the sequential assignment method. For sequences involving prolines that have no amide hydrogen the $d_{\alpha\alpha}(i,i+1)$ can be used. The sequential NOE's observed in the NOESY spectrum of the (19-83)BSPI-2 are listed in Table II and in Figure 8, Figure 9, and Figure 10 are shown the three regions of the NOESY spectrum of (19-83)BSPI-2, respectively, where the $d_{\text{NN}}(i,i+1)$, the $d_{\alpha\text{N}}(i,i+1)$, and the $d_{\alpha\alpha}(i,i+1)$ are found.

The sequential assignment was based on the initial identification of the spin systems of the 11 amino acid types described previously. In Figure 11 the sequence is listed showing the positions of these amino acids in the sequence. Four of the amino acids occur only once in the sequence, these are Trp-24, Ser-31, Tyr-61 and Phe-69, thus when identified these spin systems were unambiguously assigned. The presence of pairs of neighbouring amino acid residues, which consist of one or two of the 11 amino acids initially identified, and which are unique in occurring only once in the sequence are shown in Figure 11. The four unambiguously assigned

spin systems as well as the unique pairs of neighbouring amino acids have been the fixed points for the sequential assignment process. Throughout the sequence the positions of amino acids of the types, which have not been identified initially, form gaps no longer than three contiguous positions.

In six cases the sequential NOE from one residue to the subsequent was not observed in the NOESY spectrum of (19-83)BSPI-2, Table II. Two cases involve the Lys-30 and the Arg-67 for which the H^{α} resonance is eliminated by the irradiation at the frequency of the water resonance and one case involve the Pro-44 in which neither the $d_{\alpha\text{N}}(\text{Pro-44, Glu-45})$ nor the two $d_{\beta\text{N}}(\text{Pro-44, Glu-45})$ are observed which could be expected in case the Pro-44 was in the trans conformation (54), neither is the $d_{\alpha\alpha}(\text{Pro-44, Glu-45})$ observed which could have been expected in case Pro-44 had been in the cis-conformation. Finally, a sequential connectivity between the Met-59 and Glu-60 was not observed in the NOESY spectrum. The peptide bond between these two residues in the active site of the inhibitor, and the lack of a sequential NOE in this case might be due to a large degree of flexibility around this bond which reduces the size of the possible sequential NOE's over the

Figure 8. ^1H -NOESY spectrum of (19-83)BSPI-2 recorded with a mixing time of 0.20 s. The region shown is the d_{NN} region. The labelling of the $d_{\text{NN}}(i, i+1)$ cross peaks is so that the first sequence position is that of the chemical shift of the H^{N} in the ω_1 -direction and the second of the ω_2 -direction.

peptide bond connecting these two residues so much that it is not observed in NOESY spectra recorded with the mixing time of 0.20 s applied. Alternatively, an explanation for this could be that the amide exchange of the Glu-60 H^{N} is so fast that saturation transfer from the H_2O saturation pulse is reducing the intensity of this resonance so much that NOE's involving this proton become very small. Finally, in case of the three N-terminal residues of the (19-83)BSPI-2 no

sequential NOE's were observed. For the Leu-20 H^{N} this is most likely because this peptide hydrogen is in fast exchange with solvent. In the COSY spectrum recorded at 22°C the H^{N} - H^{α} cross peak of Leu-20 is clearly observed, however, at higher temperatures this cross peak disappears. This observation is in agreement with the explanation that solvent exchange in combination with water resonance suppression eliminates NOE's to this hydrogen. For Lys-21 the

absence of NOE's is due to the H^a resonance of this residue being close to the water resonance. In case of the intact BSPI-2 no sequential NOE's were observed between any of the additional 18 amino acid residues in the NOESY spectra recorded and sequential assignment was therefore not possible in this end of the molecule. The use of longer mixing times than 0.30 s and lower temperatures might solve this problem, but such experiments have so far not been done.

3.1.9. Glutamic acid and glutamine

The spin systems of the six glutamic acids in the (19-83)BSPI-2 were identified as follows. The H^N , H^a , H^b , $\text{H}^{b'}$ resonances were identified from the COSY and the 2Q spectra, as described previously. The identification of the H^b - H^{γ} cross peaks of the glutamines, the glutamic acids and the prolines is difficult in protein COSY spectra because these are typically found in the region around the diagonal between 1.8 to 2.5 ppm, and only in a few favourable cases the H^b - H^{γ} cross peak could be observed in these. However, often in NOESY spectra the cross peak of the H^a - H^{γ} dipolar coupling can be seen, and from this at least one of the H^{γ} chemical shifts was measured. In glutamines and glutamic acid the H^{γ} - H^{γ} cross peaks are relatively intense, hence from the information about one of the H^{γ} the other was found from the $\text{H}^{\gamma 1}$ - $\text{H}^{\gamma 2}$ cross peaks. The $\text{H}^{\gamma 1}$ - $\text{H}^{\gamma 2}$ cross peaks of the glutamic acid residues shift towards higher field with increasing pH. In the COSY spectrum, Figure 7, are shown the six $\text{H}^{\gamma 1}$ - $\text{H}^{\gamma 2}$ cross peaks of the six glutamic acids at pH 3.28 pH 4.20 and pH 6.45. The remaining three $\text{H}^{\gamma 1}$ - $\text{H}^{\gamma 2}$ cross peaks which did not show the typical shift were assigned to glutamines, and identified in agreement with the sequential assignment.

The intact BSPI-2 contains in addition two glutamic acid residues, Glu-4 and Glu-8. The H^N , H^a , H^b , $\text{H}^{b'}$ resonances of these two residues are slightly different, however, for both residues these chemical shifts are close to those of glutamic acid in model peptides, Table I, (10, 54).

3.1.10 Arginine, proline, lysine, leucine and methionine

The assignment of the spin systems of the remaining five types of amino acids was based

primarily on the information achieved in the sequential assignment that the H^N - H^a cross peak according to sequential NOE's belonged to a given amino acid spin system in the sequence. Accordingly, the additional information available on the spin system considered, was examined in order to check that this corresponded to that expected for this type of amino acid.

For the prolines the $\text{H}^{\gamma 1}$ - $\text{H}^{\gamma 2}$ resonances were assigned partly on $d_{\alpha\beta}(i,i+1)$ couplings in the NOESY spectra partly on their typical chemical shift values between 3 and 4.5 ppm. The assignment of the H^a resonances of the four prolines Pro-25, Pro-44, Pro-52, and Pro-80 was based, partly on the finding that they were the only H^a resonances which did not couple to an H^N in the amide region and partly on the observation that three of the four in the NOESY spectrum were showing dipolar coupling to the subsequent H^N in the sequence. The assignments of resonances of the H^b and the H^{γ} 's were obtained thus from the COSY and the 2Q spectra.

Given that the leucine H^N , H^a , H^b , $\text{H}^{b'}$ resonances were assigned based on the sequential assignment and the H^{γ} - $\text{H}^{\delta 1(1,2,3)}$, H^{γ} - $\text{H}^{\delta 2(1,2,3)}$ of the leucines were distinguished from the valine H^b - $\text{H}^{\gamma 1(1,2,3)}$, H^b - $\text{H}^{\gamma 2(1,2,3)}$ cross peaks by the assignment of these latter, the search for H^b - H^{γ} COSY cross peaks was considerably narrowed but resulted nevertheless only in the complete identification of one leucine spin system, that of Leu-27. The assignments of the remaining leucine spin systems were therefore based on intra-spin system NOE's from either the H^a 's or from the H^b 's to the δ -methyl groups. The assignment of five of the leucines was accomplished by these procedures and the assignment of the remaining Leu-20 spin system was based then on elimination and supported by the finding that the chemical shifts of this leucine spin system were identical to those found for leucine in model peptides (10, 54).

The assignment of the methionine, arginine and lysine spin systems was not fully accomplished in this work. For the arginines the five H^e -($\text{H}^{\delta 1}$, $\text{H}^{\delta 2}$) were observed and so were six of the eight H^e -($\text{H}^{\epsilon 1}$, $\text{H}^{\epsilon 2}$) couplings of the lysines, however, only in case of Arg-62 a complete assignment was achieved partly based on NOE's from the H^a to the H^{γ} 's.

Figure 9. ^1H -NOESY spectrum of (19-83)BSPI-2 conditions as described in Figure 8. The region shown is the $d_{\alpha\text{N}}$ -region. The labelling scheme is as follows. The first sequence position is that of the H^α whose chemical shift in the ω_1 -axis corresponds to the cross peak position and the second sequence position is then equally corresponding to the H^N chemical shift position on the ω_2 -axis. NOE-cross peaks in which other proton types than H^α and H^N are involved have been labelled with Greek letters referring to the IUPAC-IUB recommendation.

Figure 10. ¹H-NOESY spectrum of (19-83)BSPI-2 as above. The spectrum shows the $d_{\alpha\delta}(i,i+1)$ cross peaks involving the δ protons of the four prolines in the sequence.

4. DISCUSSION

The determination of the spatial structure of the BSPI-2 is one of the main aims for studying this protein by proton NMR spectroscopy. The sequence specific assignment of the proton NMR spectrum is a prerequisite for the assignment of the NOESY spectrum which again is the main basis for the determination of the three-dimensional structure. Such studies will be the subject of the subsequent papers, however, the results obtained of just the sequence specific assignments reveal some obvious features of the BSPI-2 structure which will be commented here. It is obvious from the studies that the intact BSPI-2 is composed of two structurally different compartments. The N-terminal part of the protein is clearly not engaged in forming a globular structure with the rest of the molecule for which both NMR spectroscopy and X-ray crystallography provide evidence for a well defined globular structure. The evidence for the lack of an ordered globular structure in the first 21 residues is based on the observation of the assigned NMR signals of the hydrogens of the amino acid residues 1 through 21. They have in common the following two features 1) the resonances have much smaller

linewidths than those of the remaining part of the molecule and 2) the resonances of the protons concerned have chemical shifts very close to those measured for the equivalent resonances in random coil polypeptides. Furthermore, the sequential NOE's $d_{\alpha N}(i,i+1)$ and $d_{NN}(i,i+1)$ which are common in globular proteins were not observed in NOESY spectra of the protein recorded with mixing times up to 0.300 s at 22 °C. Each of these observations suggests that the first 21 residues of the BSPI-2 do not engage in a well defined structure like the rest of the molecule. This is in agreement with the observations by X-ray crystallography that no reflections of the atoms of this part were observed (29, 30). It is also in agreement with the presence of the two forms of the protein BSPI-2 and (19-83)BPSI-2 in the extract of barley seeds where proteinases could easily hydrolyze off the amino acid residues of the unstructured part of BSPI-2.

It has previously (20) been shown that BSP1-2 exists in solution as a dimer. However, the NMR spectrum so far assigned, reveals no signs of dual sets of resonances for particular protons, which might have been expected for protons at the interphase of the two monomer units in the

1-10:	Ser	Ser	Val	Glu	Lys	Lys	Pro	Glu	Gly	Val
11-20:	Asn	Thr	Gly	Ala	Gly	Asp	Arg	His	Asn	Leu
21-30:	Lys	Thr	Glu	Trp	Pro	Glu	Leu	Val	Gly	Lys
31-40:	Ser	Val	Glu	Glu	Ala	Lys	Lys	Val	Ile	Leu
41-50:	Gln	Asp	Lys	Pro	Glu	Ala	Gln	Ile	Ile	Val
51-60:	Leu	Pro	Val	Gly	Thr	Ile	Val	Thr	Met	Glu
61-70:	Tyr	Arg	Ile	Asp	Arg	Val	Arg	Leu	Phe	Val
71-80:	Asp	Lys	Leu	Asp	Asn	Ile	Ala	Gln	Val	Pro
81-83:	Arg	Val	Gly							

Figure 11. The amino acid sequence of BSPI-2. Unique pairs of amino acids involving one of the 11 types of amino acids first assigned are connected with a bar. The positions in which one of the 11 types occurs are indicated by red lettering.

complex. A qualitative analysis, however, comparing the strongly shifted H¹² and H¹³ of Trp-24 with the structure determined by X-ray crystallography shows that neither Phe-69 or Tyr-61 which are the only two other aromatic residues in BPSI-2 could be responsible for these shifts. It is a possibility, therefore, that the tryptophans of the two monomers in the complex are in close contact and oriented in such a way that they give rise to the observed shifts. A quantitative examination of all the ring current shifts and other chemical shift anisotropy effects calculated on the basis of the X-ray structure and those observed is presently in progress in order to clarify this problem.

The sequential assignment procedure has relied on the chemically determined amino acid sequence, still however, the NMR study is at the same time an alternative method of examining the results of the chemical amino acid sequence determination. In particular, the positions in the amino acid sequence held by amino acids which are unambiguously identified by the NMR examination can be checked. The present work has in all respects confirmed the amino acid sequence as determined by automated amino acid sequencing and from the DNA-sequence of the gene (53).

The present work has resulted in the assignment of resonances of 447 hydrogen atoms in the structure of (19-83)BSPI-2. This corresponds to the assignment of 80% of the total number of protons whose resonances are normally expected in ¹H NMR-spectra of proteins. The complete assignment of the resonances from the arginines, lysines, and methionines is presently in progress.

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