NMR of a Peptic Peptide Spanning the Triprolyl Sequence in Myelin Basic Protein†

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ABSTRACT: A 22-residue peptic peptide from rabbit myelin basic protein (r-MBP) that spans the triprolyl sequence has been studied in aqueous solution by high-resolution two-dimensional (2-D) proton NMR. The resonances are assigned with the help of 2-D correlated spectroscopy, pH titration, one-dimensional (1-D) spin-decoupling techniques, and the spectrum of a phosphorylated form of the peptide. The α-CH resonances of Pro-7, Thr-6, and Thr-9 (but not of Thr-20) are markedly shifted downfield with respect to values in small unstructured peptides; similar shifts are observed in the complete r-MBP, suggesting that peptide and protein have similar conformations in the region preceding the Pro_3. Intramolecular interactions between nonadjacent residues are measured by 2-D nuclear Overhauser enhancement spectroscopy (NOESY) and confirmed by 1-D NOE techniques. Such effects are not observed between residues on opposite sides of the triprolyl sequence. ^{13}C NMR shows that the fraction of cis isomer at the X–Pro bonds is in all cases <10%. The results suggest that the triprolyl sequence acts as a rigid spacer that separates a more structured from a less structured region of the polypeptide.

One of the most interesting problems presented by myelin basic protein (MBP) has been elucidation of the structural and functional roles of the sequence of three proline residues (Pro-Pro-Pro) that occurs at positions 99–101 in the primary structures in all species so far examined (Mendz et al., 1983). When the triprolyl sequence in MBP was discovered, it was suggested that it might be the site of a "hairpin bend", which would bring the unique methylated arginine at site 108 opposite the pair of phenylalanine residues at 90–91, resulting in a hydrophobic interaction stabilizing the bend (Brostoff & Eylar, 1971). Possible functions of such a bend region in the protein were discussed (Carnegie, 1971a,b; Boggs & Moscarello, 1978). The first NMR experiment that provided evidence concerning this question was a high-resolution proton study in aqueous solution (Littlemore, 1978), which showed that the resonance of the CH_3 groups of Arg-108 has exactly the same chemical shift in MBP as in the 2-[(4-hydroxyphenyl)azo]-benzenesulfonate monohydrate salt of methylarginine; consequently, there is no observable evidence for a ring-current effect due to nearby phenylalanines.

This paper presents results of an investigation by ^1H and ^{13}C NMR spectroscopy in aqueous solution of a peptide r-(91–112) comprising residues 91–112 of rabbit (r) MBP:[1]

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 1   2   3   4   5   6   7   8   9  10  11
Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro-Pro-
 F   K   N   I   V   T   P   R   T   P   P

                                                12  13  14  15  16  17  18  19  20  21  22
                                Pro-Ser-Gln-Gly-Lys-Gly-Arg-Gly-Thr-Val-Leu
                                 P   S   Q   G   K   G   R   G   T   V   L
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This peptide contains the Pro_3 sequence of interest, with nine residues on its N-terminal side and ten on its C-terminal side. The study reveals no evidence for any bend at the Pro_3 sequence and indicates that the segments of the peptide on opposite sides of the Pro_3 are distinctly separated from each other.

Experimental Procedures

Preparation of the peptide r(91–112) by cleavage of r-MBP with porcine pepsin has been described (Martenson et al., 1981). The peptide was isolated and purified by a combination of gel filtration and ion-exchange chromatography. Its purity was established by amino acid analysis and tryptic peptide mapping. The naturally occurring protein is partially phosphorylated at the threonine residue corresponding to Thr-9 in the peptide. A pure sample of the phosphopeptide was separated by chromatography on CM-cellulose (Martenson et al., 1983).

For NMR runs in which NH groups are exchanged, the peptide was lyophilized in 99.7% D_2O and then in 100% D_2O. For measurements including the NH resonances, the peptide was lyophilized from pure H_2O. Samples were prepared in 5-mm precision tubes. For one-dimensional (1-D) NMR the concentration was 6.6 mM and for two-dimensional (2-D), 25 mM. The pH was adjusted with DCl or NaOD and measured with a Radiometer pHM-64 and an Ingold 6030-02 microelectrode. Values reported are meter readings uncorrected for isotope effect. In the range of concentration from 0.5 to 25 mM, there was no evidence of line broadening or amide proton chemical shift changes. These observations indicate that intermolecular aggregation of the peptide is not appreciable at 25 mM concentration.

Spectra were recorded at 298 K at 400 MHz in the Fourier transform mode with a Bruker WM-400 spectrometer. The 1-D spectra were obtained with a 90° radio-frequency pulse (7–8 μs). The spectral width was 4807 Hz and the acquisition time 1.7 s for typically 1024 scans. Truncated nuclear Overhauser difference spectra (NOED) were obtained with a modified Bruker program with a presaturation time of 1.4 s. Selective decoupling spectra at 200 μW were used for some peak assignments.

The 2-D techniques employed were correlated spectroscopy (COSY) (Nagayama et al., 1980) and nuclear Overhauser enhancement spectroscopy (NOESY) (Kumar et al., 1980). Spectra were acquired by dual quadrature detection (Bax et

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[1] This numbering corresponds to the standard format introduced to maximize sequence homologies (Mendz et al., 1983). The peptide actually consists of residues 88–109 in r-MBP.

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Table I: Chemical Shifts of Assigned Resonances in the Peptide at 298 K and pH 4.71

residue	no.	α	β_1	β_2	γ_1	γ_2	δ_1	δ_2	ϵ	other
Phe	1	4.280	3.281	3.158			7.294	7.294	7.413	7.413
	GGFA[a]	4.663	3.223	2.991			7.339	7.339	7.339	7.339
Val	5	4.139	2.121		0.906	0.848				
	21	4.166	2.052		0.954	0.948				
	GGVA	4.184	2.130		0.969	0.942				
Ser	13	4.430	3.891	3.896						
	GGSA	4.498	3.885	3.885						
Leu	22	4.330	1.684	1.684	1.638		0.878	0.934		
	GGLA	4.385	1.649	1.649	1.649		0.899	0.943		
Gln	14	4.412	2.092	2.092	2.395	2.395				
	GGQA	4.373	2.131	2.010	2.379	2.379				
Arg	8	4.350	1.769	1.910	1.681	1.681	3.208	3.208		
	18	4.350	1.769	1.910	1.681	1.681	3.208	3.208		2.822
	GGRA	4.396	1.915	1.796	1.719	1.719	3.312	3.312		
Thr	6	4.581	4.111		1.253					
	9	4.593	4.111		1.253					
	20	4.342	4.166		1.184					
	GGTA	4.346	4.220		1.232					
Asn	3	4.693	2.745	2.842						
	GGNA	4.755	2.755	2.831						
Ile	4	4.211	1.864		1.464	1.171	0.855			0.888
	GGIA	4.224	1.894		1.478	1.190	0.885			0.943
Lys	2	4.350	1.730	1.779	1.398	1.430	1.712	1.712	3.016	
	16	4.350	1.730	1.779	1.398	1.430	1.712	1.712	2.995	
	GGKA	4.358	1.747	1.870	1.471	1.471	1.708	1.708	3.023	
Pro	7	4.707	2.387	1.926	2.060	2.060	3.649	3.839		
	10	4.455	2.339	1.978	2.060	2.060	3.737	3.912		
	11	4.386	2.158	2.003	2.060	2.060	3.649	3.737		
	12	4.404	2.312	1.978	2.060	2.060	3.839	3.912		
	GGPA	4.471	2.295	1.981	2.030	2.030	3.653	3.653		
Gly	15	3.987								
	17	3.987								
	19	4.019								
	GGGA	3.972								

[a] Data on tetrapeptides from Bundi & Wüthrich (1979).

al., 1981) with standard Bruker 2-D programs (Bain et al., 1981). Spectra of 1024 × 1024 data points were obtained from 512 individual experiments of 2048 data points by zero filling in one time domain before Fourier transformation. The spectra are absolute-value mode, with sine bell apodization along t_1 and t_2. Accumulation of 64 free induction decays per experiment resulted in total acquisition times of 7 (COSY) or 14 h with a mixing time of 0.35 s (NOESY). The 2-D spectra were symmetrized by the standard Bruker program.

Spectra that include exchangeable hydrogens were measured in a mixture of 80% H_2O–20% D_2O, and the H_2O peak was suppressed by irradiation until the acquisition pulse (Wagner & Wüthrich, 1982). For the COSY spectrum in H_2O, the H_2O resonance was presaturated by irradiation for 1 s at 2.0 mW and gated off before the beginning of the COSY sequence.

The ^{13}C spectra were obtained at 100.62 MHz from 35 mM solution in D_2O in 10-mm precision sample tubes at pH 4.71 and 303 K. Typically 8600 transients were collected with a waiting time of 2.5 s. Spectra are presented after a line broadening of 4 Hz by exponential multiplication.

Proton chemical shifts were assigned with reference to *p*-dioxane as an internal standard, which was calibrated in a separate experiment against internal 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at 3.756 ppm upfield from dioxane. Reported values of chemical shifts were obtained from spin decoupling difference spectra, and their accuracy is estimated as ±0.002 ppm. The ^{13}C spectra are referenced to *p*-dioxane at 67.85 ppm downfield from DSS.

Results

Assignments of Proton Resonances. A 1-D spectrum of the peptide in D_2O at 298 K and pH 4.71 is shown in Figure 1,

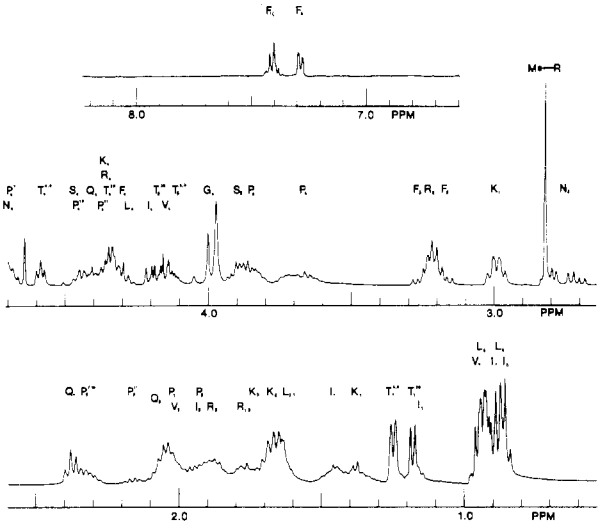

FIGURE 1: Proton NMR spectrum of the peptide at pH 4.71, 298 K, and 6.6 mM. The single-letter codes of the amino acid residues are F (Phe), K (Lys), N (Asn), I (Ile), V (Val), T (Thr), P (Pro), R (Arg), S (Ser), Q (Gln), and L (Leu).

and a COSY spectrum under the same conditions is shown in Figure 2. Assignments of the chemical shifts, δ, are made by examining the correlations in the COSY spectrum, by spin decoupling techniques, pH titrations, and comparisons with the phosphorylated peptide and with values in other peptides. When required, analyses of the spin–spin coupling constants were made by the method of Abraham (1970). The values of the chemical shifts as finally assigned are summarized in Table I.

The 22-residue peptide is composed of 12 different amino acids. Two levels of assignment of the NMR resonances can

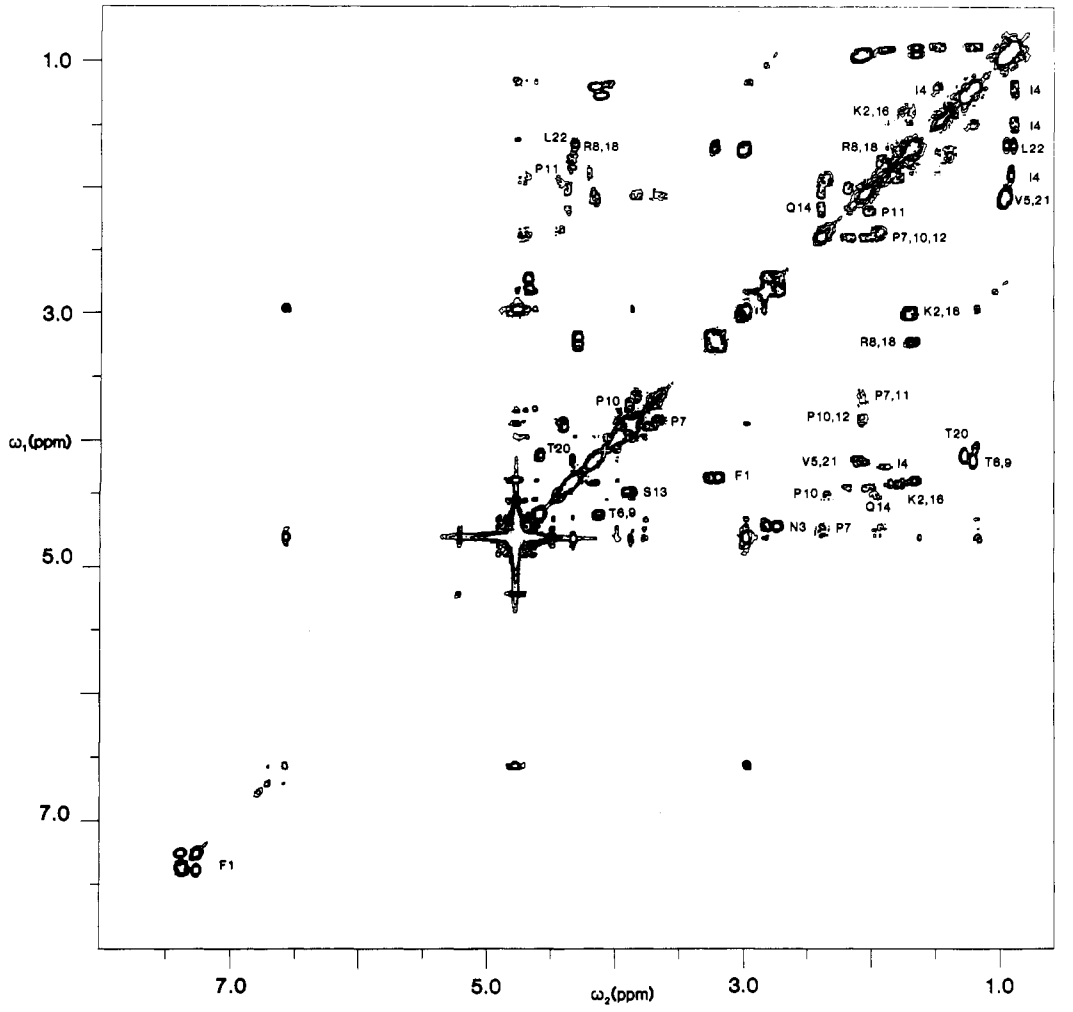

FIGURE 2: Two-dimensional correlated spectrum (COSY) at pH 4.71, 298 K, and 25 mM. The peaks along the diagonal can be identified by reference to Figure 1. The intensity between 6.5 and 7.0 ppm is believed to be due to an impurity of low molecular weight.

be distinguished: to types of residues and to individual residues.

The correlations in the COSY spectrum allow certain spin systems to be assigned unequivocally. Thus, the correlation patterns of Val, Ile, Leu, Pro, Thr, Lys, and Arg are unambiguous. Examples of assignments based on the correlation patterns are shown in the expanded COSY spectra of Figure 3. Since the peptide contains no Glu or Met, the spin system of Gln is also unambiguous. Thus, 16 of the 22 residues can be assigned to definite types on the basis of the COSY spectrum. Spin decoupling difference spectra lead to precise chemical shift values for the resonances concerned.

The remaining types of residues are Phe, Ser, Asn, and Gly. The first three types have ABX/BM spin systems in D_2O solutions and ABMX systems in H_2O solutions. They are readily identified in the 1-D spectrum and 2-D COSY contour maps of the peptide in aqueous solutions. The COSY spectra for Phe and Ser systems are included in Figure 3. The spectral lines of methylene protons appear as characteristic 2 × 4 line resonances in the 1-D spectrum, whose only connectivities are to methine resonances in the 2-D COSY plot in D_2O. Phe-1 is distinguished on the basis of pH titration. Its N-terminal group loses its charge at basic pH, and an upfield shift is observed in its α-CH resonance at pH 7.

Ser and Asn are distinguished on the basis of the characteristic differences in the chemical shifts of the methylene resonances. At pH 4.57, β-CH_2-Ser are found at 3.891 and 3.896 ppm and β-CH_2-Asn at 2.745 and 2.842 ppm. These values are comparable to those observed in small peptides, 3.885, 3.885 and 2.755, 2.831 ppm, respectively (Bundi & Wüthrich, 1979). This assignment also yields much smaller coupling constants for serine β-CH_2 resonances as is usual. A group of lines with an intensity corresponding to four protons about 3.99 ppm and with an intensity of two protons at 4.02 ppm is observed in the 1-D spectrum (Figure 1). No off-diagonal cross peaks are observed in the D_2O COSY plot. The absence of other cross peaks allows the assignment of these spectral lines to glycine methylene protons.

After the resonances in the spectrum have been identified according to the type of residue, the next step is to assign them to individual residues. As unique residue types, Phe-1, Asn-3, Ile-4, Ser-13, Gln-14, and Leu-22 are completely assigned. We thus have still to consider two Val, three Thr, two Arg, four Pro, three Gly, and two Lys.

(*1*) *Valine.* The peptide contains two valines in positions 5 and 21 (the penultimate C-terminal position). Perusal of the 2-D COSY spectrum and comparisons with the δ of small unstructured peptides result in the identification of the spin systems of these two residues. Although the δ of the valine resonances at pH 4.7 are quite similar, one pair of valine γ-CH_3 doublets displays a marked pH dependence between pH 1.5 and pH 4.7, as shown in Figure 4. These pH-dependent resonances are assigned to Val-21 and the others to Val-5. At pH 4.7, on irradiation of a methyl doublet assigned to Val-21 at 0.954 ppm, an NOED peak appears at 2.052 ppm, which is assigned to β-CH of Val-21. By irradiation at 0.906 ppm, the other β-CH peak at 2.121 ppm is similarly assigned to Val-5. These assignments are supported by pH titration data. Between pH 1.5 and pH 4.7, the β-CH resonance of

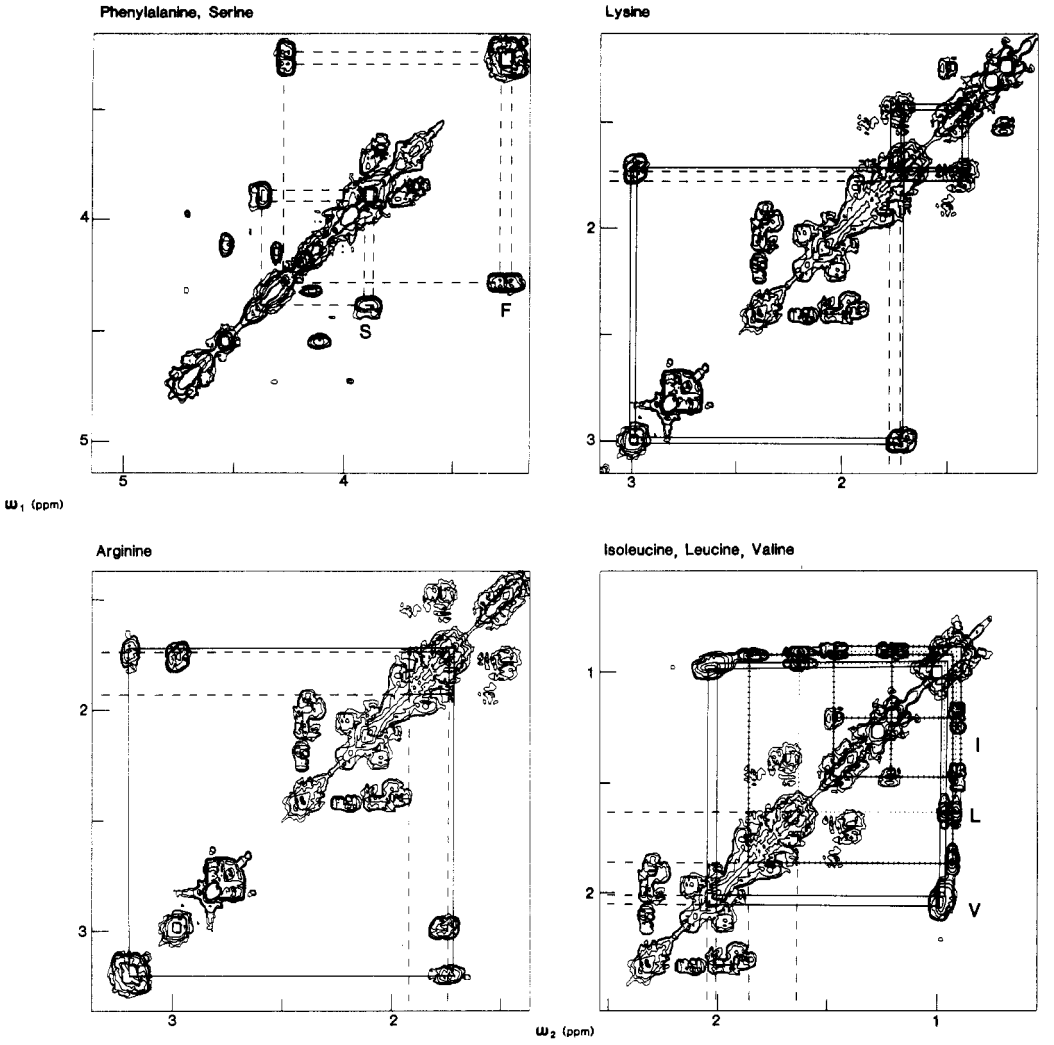

FIGURE 3: Enlarged view of regions of the COSY spectrum showing the characteristic correlation patterns for Ile, Leu, Val, Lys, and Arg residues. The Phe and Ser patterns are also shown. Dashed lines represent β to α connectivities. In the Ile-Leu-Val diagram, solid lines represent Val, dotted lines Leu, and railroad lines Ile.

penultimate Val-21 should display a larger upfield shift than that of the β-CH of Val-5. A group of resonances about 2.10 ppm shifts upfield by about 0.05 ppm in this pH titration. This shift is clearly distinguishable against the background of unshifted resonances.

(*2*) *Threonine.* The three threonine residues are found in positions 6, 9, and 20 in the peptide. They correspond to Thr-96, Thr-99, and Thr-110 in the homology numbering of the rabbit protein. The protons of the threonine residues can be assigned to specific resonances by observing the effect of phosphorylation of Thr-9 in the spectrum of the peptide and with the help of the assignments previously established (Mendz et al., 1983) for the threonine residues in the complete rabbit protein. Residue Thr-99 is partially phosphorylated in vivo, to the extent of about 6% (Martenson et al., 1983). A sample of the phosphorylated peptide was isolated, in which, therefore, Thr-9 is phosphorylated. Comparison of the spectra of the phosphorylated and nonphosphorylated peptides shows that one and only one of the doublets corresponding to Thr-γ-CH_3 protons is shifted downfield in the phosphorylated moiety. On the basis of this modification, we assign it to Thr-9 in the peptide. Furthermore, its chemical shift in the nonphosphorylated peptide (1.263 ppm) is very close to the chemical shift of Thr-99 (1.249 ppm) in the whole protein.

In the rabbit protein, the γ-CH_3 resonances of Thr-96 and Thr-110 are found 0.007 and 0.067 ppm upfield from the γ-CH_3 resonance of Thr-99, respectively. In the non-

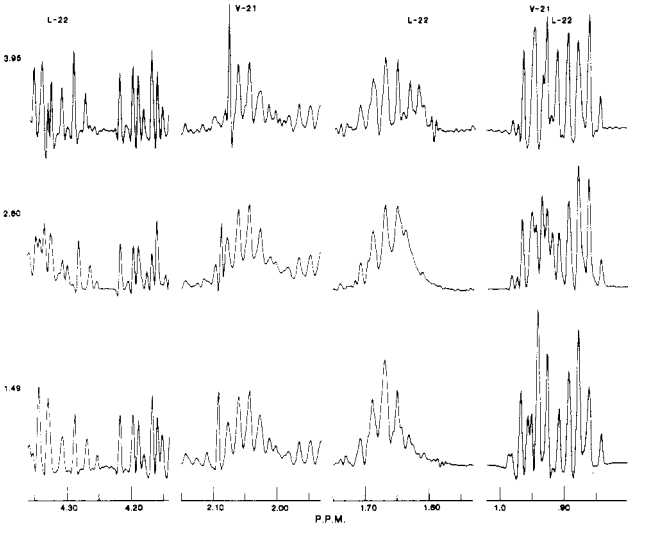

FIGURE 4: Variation of chemical shifts with pH in titration of the peptide at 298 K and 6.6 mM. Resolution enhanced with Gaussian multiplication with line broadening parameter −3 Hz and Gaussian broadening parameter 0.19.

phosphorylated peptide one of the methyl resonances overlaps that of Thr-9 and the other is found 0.079 ppm upfield from it. Consequently, we tentatively assign the former to Thr-6 and the latter to Thr-20.

Table II: Chemical Shifts of Threonine Resonances at 298 K and pH 4.71 in Rabbit MBP and in Peptide Fragments

	α	β	γ
peptide			
6	4.58	4.11	1.253
9	4.59	4.11	1.253
20	4.342	4.166	1.184
9[a]	4.69	4.50	1.35
protein			
96	4.585	4.112	1.242
99	4.595	4.131	1.249
110	4.280	4.170	1.181
Gly-Gly-Thr-Ala	4.346	4.220	1.232

[a] Phosphorylated.

The chemical shifts of the α-CH and β-CH protons of Thr-6 and Thr-20 resulting from these assignments are very close to those of Thr-96 and Thr-110 in the complete protein (Table II). Moreover, relative to the standard values found for small peptides (Bundi & Wüthrich, 1979), the α-CH resonance of Thr-96 is downfield shifted by 0.239 ppm, and its β-CH resonance is shifted 0.108 ppm upfield; the corresponding shifts for Thr-6 are 0.234 and 0.110 ppm. On the other hand, the chemical shifts of α-CH and β-CH of both Thr-110 in the protein and Thr-20 in the peptide are close to the standard values in Gly-Gly-Thr-Ala.

(*3*) *Arginine.* The peptide contains two arginine residues at positions 8 and 18. The latter is partially methylated to *N*-monomethyl- and *sym*-*N*-dimethylarginine. On the basis of data for rabbit MBP itself, 35% of Arg-18 is dimethyl and 19% monomethyl (Deibler & Martenson, 1973). The resonances of these methyl groups are two barely resolved singlets, with an intensity ratio of ~2. The spin systems of the β,γ-CH_2 protons of Arg are superimposed on resonances due to Lys, Leu, and Pro residues. From the COSY spectrum, however, the assignments in Table I are obtained, following the connectivities between protons of the side chain. The residues Arg-8 and Arg-18 yield overlapping resonances, and it is not possible to distinguish them. All the Arg chemical shifts are slightly upfield from those in a small peptide Gly-Gly-Arg-Ala (Bundi & Wüthrich, 1979).

(*4*) *Glycine.* The three glycine residues, 15, 17, and 19, are all on the C-terminal side of the triprolyl sequence. Glycine resonances can be detected by comparing the 1-D spectrum and a 2-D COSY spectrum and noting that these resonances have no connectivities. Two of the resonances are superimposed and the third one is shifted 0.03 ppm downfield. Irradiation of the downfield resonance at 4.019 ppm leads to an NOED peak at 4.34 ppm, the value for the α-CH of Thr-20. This α_i-CH_2 to α_{i+1}-CH NOE suggests assignment of this Gly resonance to Gly-19.

(*5*) *Proline.* The four prolines are at position 7 and the triplet at 10, 11, and 12. A proline spin system consists of 1α, 2β, 2γ, and 2δ protons. In the peptide Gly-Gly-Pro-Ala, one observes two distinct β-CH_2 chemical shifts at 1.98 and 2.30 ppm, one γ-CH_2 at 2.03 ppm, and one δ-CH_2 at 3.65 ppm (Bundi & Wüthrich, 1979). Systematic selective decoupling of the resonances between 2.10 and 1.95 ppm showed that irradiation at 2.06 gave a group of peaks in the difference spectrum between 3.65 and 3.91 ppm. These are assigned to the δ-CH_2 of the four prolines. Cross peaks between these resonances and one at 2.06 are observed in the COSY. Four pairs of δ-CH_2 protons can then be assigned in the COSY (shown expanded in Figure 5): (a) 3.649, 3.737; (b) 3.649, 3.839; (c) 3.737, 3.912; (d) 3.839, 3.912 ppm. The four pairs of γ-CH_2 protons, however, have almost identical chemical shifts at 2.06 ppm, so that it is not possible to follow the spin system of each individual proline residue in the COSY spectrum from the various δ-CH_2 through the correlated γ, β, and α protons.

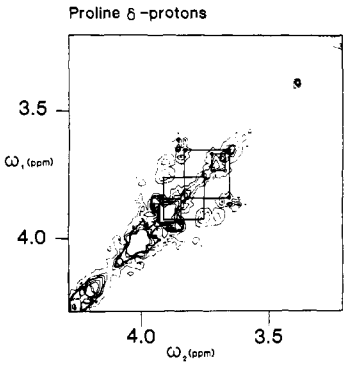

FIGURE 5: An expanded COSY spectrum showing the regions of the resonances from proline residues in the peptide.

NOED spectra reveal each β-CH_2 resonance that arises from the same ring as the given δ-CH_2 resonance. The COSY spectrum is then used to correlate β-CH_2 resonances with the α-CH of the same residue. With this procedure, the group of resonances belonging to a given residue is obtained. These data, however, do not permit the assignment of a given set of correlated resonances to a particular proline residue. The four spin systems that result are as follows:

	α	β_1	β_2	$\gamma_{1,2}$	δ_1	δ_2
I	4.707	2.387	1.926	2.060	3.649	3.839
II	4.386	2.158	2.003	2.060	3.649	3.737
III	4.404	2.312	1.978	2.060	3.839	3.912
IV	4.455	2.339	1.978	2.060	3.737	3.912

The use of the phosphorylated peptide allows the resonances of Thr-6 and Thr-9 to be distinguished since the electronegativity of the phosphate group deshields the methyl of Thr-9. The through-space charge effect of the phosphate group causes an observed upfield shift in a Pro-δ-CH_2 resonance at 3.72 ppm, which is thus assigned to Pro-10. This assignment is confirmed by an NOED experiment on the phosphorylated peptide. On irradiation at 1.35 ppm, which is the position of the γ-CH_3 proton of phosphorylated Thr-9, an NOED peak is observed at 3.72 ppm, which is very close to the position of a δ-CH_2 proton of either system II or system IV at 3.74 ppm in the nonphosphorylated peptide. In the nonphosphorylated peptide there is a 1-D NOE from the α-CH of Thr-9 to the α-CH and both δ-CH of system IV. Thus, proline spin system IV can be assigned to Pro-10.

On irradiation of the peak at 3.839 ppm (Pro-δ-CH_2 protons in systems I and III), NOED peaks are observed at 4.43 and 4.11 ppm. The former is due to α-CH proton of Ser-13 and the latter to the β-CH_2 proton of Thr-6. Thus, spin systems I and III must belong to Pro-7 and Pro-12. On irradiation at 3.649 ppm, an NOE occurs at 4.581 (Thr-6-α-CH). Thus, either system I or system II must be Pro-7, so that I must belong to Pro-7 and III to Pro-12. It then follows that Pro-11 belongs with system II, an assignment concordant with the anomalous chemical shifts of this system and the unusual situation of Pro-11 between two other prolines. The α-CH of Pro-7 at 4.707 ppm is markedly shifted downfield as is the adjacent α-CH of Thr-6.

(*6*) *Lysine.* The two lysine residues, 2 and 16, display slightly different ϵ-CH_2 resonances at pH 4.6, 2.995 and 3.016 ppm. Only these ϵ-CH_2 resonances show any chemical shift differences between the two lysines so that we cannot use the NOESY (H_2O) spectrum to make an assignment based on α-CH and α-NH resonances. When the Lys-ϵ-CH_2 resonance

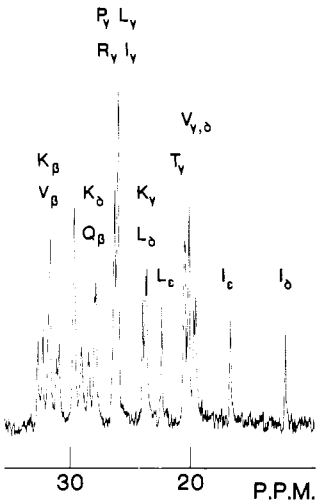

FIGURE 6: A proton-decoupled NOE-enhanced ^{13}C spectrum at 100.62 MHz of the peptide at pH 4.71 and 303 K, showing the region of Pro-γ-CH_2 peaks. A pulse width of 13 μs (70°) was used to excite the ^{13}C nuclei with a recycling time of 2.5 s, which is $>5T_1$ for the $-CH_2$ carbons.

at 3.016 ppm is irradiated, an NOE peak is observed at the position of the Phe-β-CH_2. This NOE is either a nearest-neighbor effect between Phe-1 and Lys-2 or the result of a structure that brings the N-terminal Phe-1 close to the opposite end of the molecule. In the context of all the NOE data to be discussed in the next section, the former possibility is much more probable, and the ϵ-CH_2 resonance at 3.016 ppm has therefore been entered in Table I as assigned to Lys-2.

^{13}C Spectra: Cis and Trans Prolines. The ^{13}C chemical shifts of all protonated carbons of proline depend upon the cis or trans configuration of the X–Pro peptide bond (Smith et al., 1972). The ^{13}C chemical shift of Pro-γ-CH_2 is the most specific indicator of cis–trans isomerism (Dorman & Bovey, 1973). Its value for cis prolyl residues ranges from 1.70 to 2.55 ppm upfield from that for trans (Deslauriers & Smith, 1980).

The upfield (aliphatic) region of a fully decoupled NOE-enhanced 100.62-MHz ^{13}C spectrum is shown in Figure 6. Tentative assignments depicted on the figure were made on the basis of the intensity of the spectral lines and by comparison with the assignments of resonances in the whole MBP protein (Chapman & Moore, 1978). Selective irradiation of the Pro-γ-CH_2 proton resonances at 2.060 ppm confirmed the ^{13}C assignment. Figure 7a shows the region from 20 to 35 ppm in a selective NOE-enhanced, proton-coupled ^{13}C spectrum obtained with continuous irradiation of the Pro-γ-CH_2 protons. Three groups of peaks, at 25.66, 25.91, and 31.36 ppm, appear enhanced. The resonances at 31.36 ppm had been tentatively assigned to Val-β-CH, and since the proton of one of the valine residues resonates at only 0.008 ppm upfield from those of Pro-γ-CH_2, selective irradiation of the latter at 0.02 W also saturates the former. Thus, Val-β-CH is observed at 31.36 ppm and the other two peaks correspond to Pro-γ-CH_2. The greater enhancement in the peak at 25.91 ppm suggests that three of the Pro-γ carbon resonances occur in that position, whereas at 25.66 ppm the resonance of one Pro-γ is overlapped by Arg-γ, Ile-γ, and Leu-γ lines.

In the region 1.7–2.6 ppm upfield from the trans Pro-γ-CH_2 resonances, where cis Pro-γ lines are expected to appear, there is a group of peaks, between 23.17 and 23.50 ppm, with an intensity corresponding to three nuclei, which are tentatively assigned to Lys-γ and Leu-δ carbons. To ascertain the possible contribution of the cis Pro-γ carbon to this group, we obtained

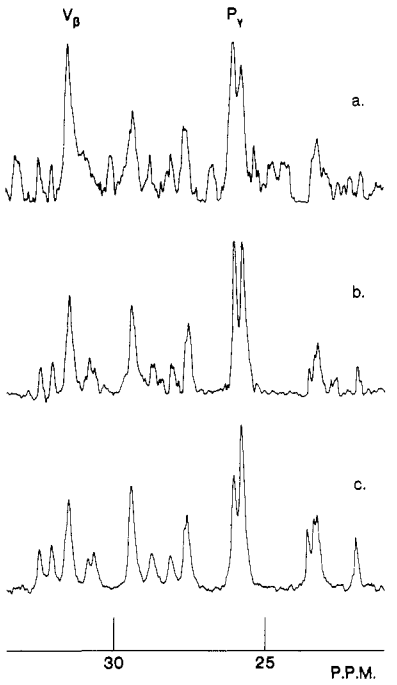

FIGURE 7: (a) Region of the ^1H-coupled ^{13}C spectrum shown in Figure 6, with continuous irradiation of the Pro-γ-CH_2 protons. (b) Selective NOE enhanced (Pro-γ-CH_2) fully decoupled ^{13}C spectrum. The pulse width was 13 μs (70°) with a recycling time of 4.0 s, which is $>8T_1$ for the $-CH_2$ carbons. (c) NOE-enhanced fully decoupled ^{13}C spectrum.

the selective NOE-enhanced fully decoupled spectrum shown in Figure 7b. This spectrum results from selective irradiation of the Pro-γ-CH_2 protons and gated broad-band decoupling.

A maximum content of 10 ± 4% cis Pro-γ is calculated by comparing the intensity ratios of resonances at 25.66 and 25.91 ppm to those at 23.17, 23.28, and 23.50 ppm in the selective NOE-enhanced decoupled spectrum (Figure 7b) and in the NOE-enhanced spectrum (Figure 7c) with the assumption of a maximal NOE for the Lys-γ and Leu-γ carbons.

In the case of the tripeptide, Pro-Pro-Pro, the observed range of the Pro-γ-CH_2 resonances in D_2O at neutral pH was 2.2–2.6 ppm. The contents of all cis Pro isomers derived from the relative intensities of these lines were less than 10%.

Nuclear Overhauser Effects. Nuclear Overhauser effects between nonbonded protons within range of mutual dipolar relaxations have been a major source of information on the conformations of proteins and peptides. Even when a quantitative calculation of internuclear distance is not feasible, the NOE data under appropriate conditions can indicate which protons are within a range of about 500 pm from one another. Two-dimensional NOE spectra (NOESY) provide a graphic display of NOE effects between different parts of a molecule. Such a spectrum is shown in Figure 8.

In addition to the expected intraresidue NOE effects, a number of interresidue effects are clearly observed between nearest neighbors in the peptide on the N-terminal side of the triprolyl sequence: Phe-1/Lys-2, Asn-3/Ile-4, Val-5/Thr-6, and Thr-6/Pro-7. More interesting from the point of view of revealing structure in this region are the NOE's between nonadjacent residues: Phe-1/Asn-3, Phe-1/Ile-4, Phe-1/Val-5, Phe-1/Thr-6, Phe-1/Pro-7, Asn-3/Thr-6, Ile-4/Pro-7, and Val-5/Arg-8. All the NOE's cited have been confirmed by 1-D NOE experiments. No NOE effects are found between any residues on opposite sides of the triprolyl sequence.

In the region between the triprolyl sequence and the C terminus, fewer NOEs are observed. Interactions between

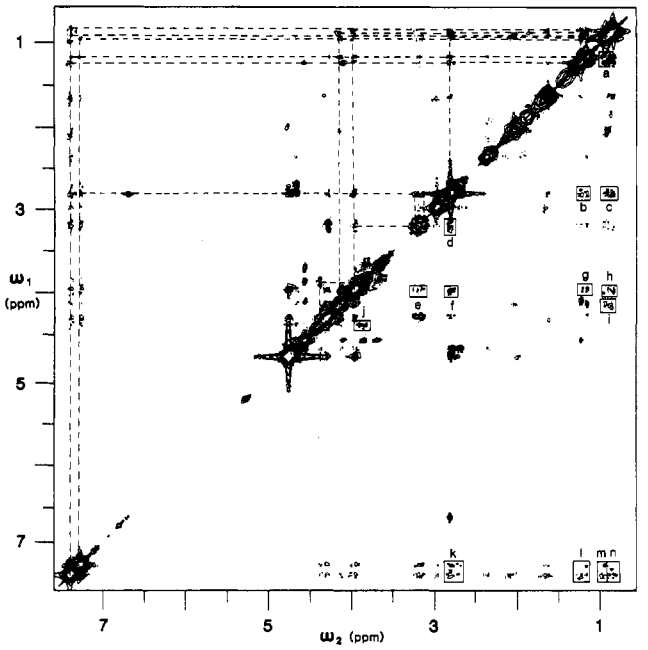

FIGURE 8: Two-dimensional nuclear Overhauser difference spectrum (NOESY) at pH 4.71 and 25 mM. The interresidue NOE's are indicated as follows: (a) Ile-4-γ/Val-5-γ; Thr-6-γ/Val-5-γ; Thr-20-γ/Val-21-γ; (b) Asn-3-β/Thr-6-γ; (C) Asn-3-β/Val-5-γ; Val-5-γ/Arg-8-δ; (d) Phe-1-β/Asn-3-β; (e) Phe-1-β/Pro-7-δ; (f) Ser-13-β/Arg-18-δ; (g) Ile-4-γ/Pro-7-δ; Thr-6-γ/Pro-7-δ; (h) Gly-15,17-α/Leu-22-δ; (i) Ile-4-δ,γ/Thr-6,9-β; (j) Thr-9-α/Pro-10-δ; (k) Phe-1-δ,ϵ,ζ/Asn-3-β; (l) Phe-1-δ,ϵ,ζ/Thr-6,9-γ; (m) Phe-1-δ,ϵ,ζ/Val-5-γ; (n) Phe-1-δ,ϵ,ζ/Ile-4-δ. [Unassigned cross peaks of HDO, Me-R, upfield methyls, and impurity (cf. Figure 2) are artifacts due to T_1 noise.]

nearest neighbors are found for Pro-12/Ser-13, Gly-19/Thr-20, and Thr-20/Val-21. An interresidue effect is detected between Lys-16-ϵ-CH_2 and Leu-22-γ-CH_3. The ϵ-CH_2 resonances of the two lysine residues are found at 2.995 and 3.016 ppm, the latter being tentatively assigned to Lys-2. In a sequence of NOED experiments, the irradiation frequency is moved upfield from 3.00 at intervals of 0.025 ppm (10 Hz). The first NOE to be observed is that previously assigned to be between Phe-1 and probably Lys-2. When the irradiation frequency is moved further upfield, another NOE peak appears that, on the basis of this assignment, can be ascribed to interaction between Lys-16-ϵ-CH_2 and Leu-22-γ-CH. This result suggests that the negatively charged carboxyl end group of the terminal leucine may approach the positively charged side chain of Lys-16. Other interresidue dipolar effects observed between nonadjacent residues in the C-terminal part of the peptide are Pro-12/Gln-14, Ser-13/Leu-22, Gln-14/Arg-18, Gln-14/Leu-22, Gly-15(or Gly-17)/Leu-22, and Thr-20/Leu-22.

Discussion

Ever since the primary structure of a myelin basic protein was published (Carnegie, 1971; Eylar et al., 1971), the role of the unusual triprolyl sequence has been the subject of intense discussion. The potential of high-resolution NMR spectroscopy to help solve this problem has been apparent, but cogent data could not be obtained from the complete protein because of the difficulties in assigning resonances to the many relevant residues. The peptic peptide 91–112 from rabbit MBP has a sufficient number of residues on both sides of the Pro-Pro-Pro to suggest that it is a good model for the corresponding region of the protein structure. The complete assignment of the threonines in the proton NMR of MBP from five species, including rabbit, has permitted the correlation of the resonances from specified threonine residues in the protein with those in the peptide. It is then seen that the anomalous chemical shifts of the α-CH and β-CH resonances of Thr-96 and Thr-99 in the whole protein are closely paralleled by similar shifts in the peptide. The anomalous shifts in both cases are believed to be due to ring-current effects from the phenylalanine. In the peptide, NOE interactions are observed between Phe-1 and Thr-6,9, which support this interpretation. The methylarginine resonance at 2.822 ppm in the peptide is close to the 2.801 ppm in the protein. These correspondences provide evidence that the local structures of peptide and protein in aqueous solution are similar in the region of the triprolyl sequence.

The assignment of the resonances in this 22-residue peptide was accomplished by current 2-D NMR methods. The 2-D NOE spectra then revealed the general pattern of interresidue distances in the peptide structure. The conformation on the N-terminal side of Pro_3 is compact and ordered, whereas the conformation on the C-terminal side is less compact and more disordered. Circular dichroism spectra of this peptide, however, give no evidence of any α-helicity in its structure in aqueous solution. There is no evidence for any interaction between the two parts of the peptide on opposite sides of the Pro_3. Thus, the triprolyl sequence appears to act not to bend the peptide chain back on itself but, just the opposite, to propagate the chain forward, forming a rigid spacer that keeps the two parts of the molecule rigorously separated.

The peptide on the N-terminal side of the Pro-Pro-Pro is richer in hydrophobic residues as compared to the C-terminal side. On one scale (Levitt, 1976), the average hydrophobicity of residues 1–9 in the peptide is 1.5 kJ mol^{-1} greater than that for residues 13–22.

The C-terminal side of the peptide contains three glycine residues, which, lacking side chains and being interspersed among other residues, promote freedom and mobility of the peptide chain. The NOE's observed between residues on the N-terminal side and between those on the C-terminal side of the Pro_3 sequence cannot be explained by any unique model of the peptide conformation. It is therefore believed that a dynamic equilibrium must exist between several different conformations, each of which has sufficient stability to yield observable interresidue NOEs.

The NOE observed between the terminal leucine and Lys-16 may be caused by a Coulombic interaction. Such an interaction would not occur in the complete protein, since it is an adventitious effect of the cleavage at Leu-112. Thus, this particular evidence for preferred conformation in the C-terminal half of the peptide is believed to be without functional relevance to the protein itself. In general, the NOE data suggest that both the N-terminal Phe-1 and the C-terminal Leu-22 traverse a range of positions that yield dipolar interactions with other residues on the same side of the Pro_3. In no case, however, are such interactions observed across the Pro_3 barrier.

A "hairpin" bend involving the Pro_3 sequence is energetically favored only if one of the proline residues has the cis configuration (Martenson, 1981). Calculations show that in glycyltriprolylglycine cis configurations are thermodynamically markedly less favorable than trans (J. Bremer, unpublished calculations). The calculations were based on the empirical conformational energy potentials of Momany et al. (1975) and a grid search of the energies with respect to torsional angles ψ_2 and ψ_3 in steps of 15°. The lowest minimum for the all-trans peptide was 5 kJ mol^{-1} below that for any configuration including a cis Pro bond.

The NMR data support the conclusion that the Pro-Pro-Pro sequence is not a bend region. A bend conformation of low energy would require at least one of the proline–proline bonds to be in the cis configuration. The ^{13}C spectra show that cis prolines occur to a negligible extent in this peptide. An all-trans configuration of the Pro_3 sequence is consistent with a function as a spacer. A somewhat similar function has been suggested for prolines in the hinge region of the γ-chains of certain immunoglobulins (Welscher, 1970).

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