Structure and Function of the Proline-Rich Region of Myelin Basic Protein†

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ABSTRACT: Myelin basic protein (MBP)—the major extrinsic membrane protein of central nervous system myelin—from several species contains a rarely encountered highly conserved triproline segment as residues 99–101 of its 170-residue sequence. Cis peptide bonds are known to arise at X–Pro junctions in proteins and may be of functional significance in protein folding, chain reversal, and/or maintenance of tertiary structure. We have examined the conformation of this proline-rich region using principally ^{13}C nuclear magnetic resonance spectroscopy (125 MHz) both in intact bovine MBP and in several MBP fragment peptides which we synthesized, including octapeptide 97–104 (Arg-Thr-Pro-Pro-Pro-Ser-Gln-Gly). Results suggested an all-trans conformation in aqueous solution for the triproline segment in MBP hexapeptide (99–104), heptapeptide (98–104), and octapeptide. Comparison with the ^{13}C spectrum of intact MBP (125 MHz) suggested that the proline-rich region, as well as all other X–Pro MBP peptide junctures, was also essentially all trans in aqueous solution. Although experiments in which octapeptide 97–104 was bound to a lipid preparation (4:1 dipalmitoylphosphatidylcholine/dimyristoylphosphatidic acid) demonstrated that *cis*-proline bonds do arise (to the extent of ca. 5%) in the membrane environment, a role of linear chain propagation is suggested for the triproline segment of myelin basic protein.

Myelin basic protein (MBP) constitutes 30% of the total protein of central nervous system myelin and is contained on the cytoplasmic side of the membrane sheath [for reviews, see Boggs & Moscarello (1978), Carnegie & Moore (1980), and Boggs et al. (1982)]. Its basic character is produced by the numerous arginine (19) and lysine (13) residues dispersed throughout the protein, which likely participate in electrostatic interactions in vivo between MBP and myelin multilamellae.

Sequence determinations from several sources (Eylar et al., 1971; Carnegie, 1971; Martenson et al., 1971; Martenson, 1981; Small & Carnegie, 1981) have revealed a highly conserved, rarely encountered triproline segment (residues 99–101). Proline residues are known as important structural components of proteins since their conformations are restricted by the carbocyclic pyrrolidine side chain, which is covalently bonded to the protein backbone (Carver & Blout, 1967). Protein backbone rotational angles in proline-rich regions are thus limited to relatively few low-energy values (Madison & Schellman, 1970). In these circumstances, any of several aspects of proline structure could produce a bend in a protein chain. For example, as predicted by Martenson (1981), a proline residue in the cis configuration could redirect a polypeptide chain. Cis peptide bonds, while not normally noted between two amino acids in a protein (which are all trans), are known to arise in imino-type X–Pro and Pro–Pro linkages (Deber et al., 1970; Wüthrich et al., 1972; Dorman & Bovey, 1973a). Cis–trans isomerism about the X–Pro peptide bond has been proposed as a rate-determining step in the renaturation of proteins (Brandts et al., 1975; Lin & Brandts, 1983a,b). During renaturation, the initial, fast-folding step involves the internalization of the hydrophobic side chains into specific domains. The final, slow step then combines these domains by the isomerization of the proline residues, indicating critical involvement of these imino acids.

When in combination with a membrane, MBP may bridge the myelin multilamellae as a monomer (Boggs et al., 1982) and/or via protein/protein associations as dimers (Golds & Braun, 1978a,b; Smith, 1980). Either model could involve a fold around the mid-protein proline-rich region, as originally suggested by Brostoff & Eylar (1971). It has previously been demonstrated that ordered secondary and/or tertiary structure was increased when the protein was bound to membranes/detergents, suggesting that solution and membrane-bound conformations differ considerably (Anthony & Moscarello, 1971; Liebes et al., 1973; Keniry & Smith, 1979, 1981). The function of MBP in its native state in myelin membranes may thus be governed, in part, by specific folding around the proline-rich region—much like conformational transitions in the proline-rich region of immunoglobins might regulate antigen binding by opening and closing from a T to a Y shape (Smith & Utsumi, 1967).

To determine the peptide bond conformation(s) of this triproline segment, both in solution and in membrane environments, we have synthesized the peptide Arg_1-Thr_2-Pro_3-Pro_4-Pro_5-Ser_6-Gln_7-Gly_8 (comprising bovine MBP residues 97–104) and studied its structure using carbon-13 nuclear magnetic resonance (NMR) spectroscopy. NMR is especially well suited for the study of proline because the cis–trans interconversion is slow enough on the NMR time scale to allow observation of individual isomers and their relative populations (Dorman & Bovey, 1973a; Wüthrich et al., 1972). Spectra of the octapeptide were then compared with spectra of the proline residues within the native protein. The results are discussed in terms of whether or not MBP could contain functional cis X–Pro peptide bonds.

Materials and Methods

Synthesis. Protected L-amino acids were obtained from Bachem (Torrence, CA) and used without further purification.

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Infrared (IR) spectra were obtained on a Beckman IR20A instrument. Thin-layer chromatography (TLC) was performed on silica gel plates (Fisher) using the solvent systems (i) 95% $CHCl_3$/5% MeOH, (ii) 90% $CHCl_3$/10% MeOH, and (iii) $CHCl_3$/MeOH/acetic acid (18:3:1). Products were visualized by using a tolidine spray following exposure to chlorine vapor. Amino acid analyses were performed at the Department of Biochemistry, Research Institute, The Hospital for Sick Children. Final purification by gel filtration employed Sephadex G-25 gel. [^{14}C]Arginine was obtained from New England Nuclear.

Mixed Anhydride: General Procedure. Synthesis of the octapeptide 97–104 region of MBP was accomplished through a combination of mixed anhydride and fragment condensation procedures. In a typical reaction, the *tert*-butyloxycarbonyl (*t*-Boc) amino acid or peptide in tetrahydrofuran (THF) or $CHCl_3$ was cooled to −20 °C in a CCl_4/dry ice bath, and equimolar isobutyl chloroformate was added dropwise, followed by an equivalent of the tertiary base *N*-methylmorpholine (NMM). After 20 min at −20 °C, the C-terminal-protected component was then added, along with an additional equivalent of NMM. The reaction mixture was then allowed to warm to room temperature with stirring overnight.

Products were isolated by successive extractions using equal volumes of H_2O, 5% $NaHCO_3$, and saturated NaCl. Where tetrahydrofuran was the solvent, it was first evaporated and replaced with $CHCl_3$. Following the extractions, the organic layer was dried over anhydrous Na_2SO_4 and the solvent evaporated to yield the crude peptide. Products were generally crystallized or triturated as required for further purification.

Deblocking of Peptides. Removal of the benzyl esters/ethers and benzyloxycarbonyl groups was accomplished by hydrogenation (25 psi) in the presence of 10% palladium on charcoal (Pd/C) catalyst. Samples were dissolved in a suitable solvent, generally *tert*-butyl alcohol/tetrahydrofuran (1:1 v/v), and hydrogenated on a Paar shaker overnight. The catalyst was removed by filtration through Celite and the product isolated by evaporation of the solvents in vacuo.

Removal of *t*-Boc N-protecting groups was accomplished by dissolving peptides in ethyl acetate, cooling to 0 °C in an ice bath, and bubbling HCl gas through the solution for 10 min with efficient stirring. Reactions were monitored by TLC to ensure complete deprotection.

Carbon-13 NMR Spectra. Carbon-13 proton-decoupled NMR spectra were obtained for all protected and unprotected peptide samples—as an aid both for determining peptide purity and for conformational studies. Peptide/protein spectra were collected on Nicolet 360 and Bruker WP500 spectrometers, operating at 90 and 125 MHz, respectively, for carbon-13. Solutions of 25–35 mM were prepared in 10-mm NMR tubes for the Nicolet system and in 5-mm tubes for the Bruker system. D_2O and $CDCl_3$ (Merck Sharp & Dohme) were used as solvents. Spectra were referenced to external tetramethylsilane (Me_4Si) by using the offset frequency obtained from a dioxane (67.85 ppm)/D_2O system (Chapman & Moore, 1978). Peptide spectra (90 MHz) were accumulated in approximately 10 000 transients with a spectral width of 22 000 Hz, a relaxation delay of 1 s, and a pulse width of 12 μs. The Fourier-transformed spectra utilized a line broadening of 3 Hz. Since peak broadening in some samples of the Gly C_α and Gly and Arg carbonyl carbons at intermediate pH values (4.5–7.5) suggested a paramagnetic impurity (London et al., 1978; Irving & Lapidot, 1975), 0.5 mg of ethylenediaminetetraacetic acid (EDTA) was added to all samples; this resulted in sharp peaks at all pH values. The systematic nature of the synthesis allowed for assignments of essentially all resonances by the comparison of intermediate spectra as the synthesis progressed. These assignments were aided by pH titrations and by reference to NMR spectra of peptides of a similar nature (Keim et al., 1973a,b, 1974).

Lipid Interactions. Peptide/membrane experiments were performed by ^{13}C NMR on a Bruker WP80SY operating at 20 MHz. Carbon-13 spectra were typically obtained in 30 000 accumulations in 16K data points. A spectral width of 4500 Hz was used with a pulse width of 6.0 μs, an acquisition time of 1.8 s, and a relaxation delay of 0.5 s. Vesicles were prepared by the drying, under nitrogen and under vacuum, of a chloroform solution containing a 4:1 w/w mixture of (neutral) dipalmitoylphosphatidylcholine (DPPC) and (negative) dimyristoylphosphatidic acid (DMPA). (Lipid:peptide ratios were generally 5:1 mol/mol and thus equimolar in peptide and DMPA.) To the dried lipid was added 1.5 mL of D_2O containing peptide at the required concentration, and the mixture was vortexed for 15 min. Peptide samples were treated with 0.5 mg of EDTA prior to lipid addition, and the pH was adjusted to 6.0.

RESULTS

Synthesis. Synthesis of the octapeptide fragment 97–104 of bovine myelin basic protein was carried out by the independent syntheses of two tripeptide fragments, H-Ser(OBz)-Gln-Gly-OBz and *t*-Boc-Pro-Pro-Pro-OH. The former tripeptide was prepared by mixed anhydride coupling *t*-Boc-Ser(OBz)-OH to HCl-Gln-Gly-OBzl. A highly apolar component running with the TLC solvent front (R_f 0.97) after HCl deblocking of *t*-Boc-Gln-Gly-OBzl was determined to be Gln-Gly-diketopiperazine (cyclodipeptide) [see Geiger & Konig (1981)]. This byproduct was removed by its selective solubility in petroleum ether in which the dipeptide hydrochloride did not dissolve.

Synthesis of the required *t*-Boc-Pro_2-OBz and *t*-Boc-Pro_3-OBz peptides was accomplished by the methods described by Deber et al. (1970). ^{13}C NMR spectra of the fully blocked intermediates confirmed that the desired products were obtained (vide infra). After suitable deprotection (see Materials and Methods), the desired hexapeptide was prepared by fragment condensation using the mixed anhydride method [*t*-Boc-Pro_3-OH + HCl·Ser(OBz)-Gln-Gly-OBz → *t*-Boc-Pro_3-Ser(OBz)-Gln-Gly-OBz]. This fragment condensation, with a Pro residue at the C-terminus of the mixed anhydride activated component, minimized the possibility of racemization.

Successive stepwise coupling of *t*-Boc-Thr-OH and then Z_3-Arg-OH (*Z* = benzyloxycarbonyl) completed the octapeptide synthesis. Because of the similar hydrophobic qualities of product and the triprotected Z_3-Arg-OH monomer, trituration proved to be an ineffective method of separation of residual Arg monomer from the octapeptide. The Z_3-Arg-OH impurity was therefore unblocked along with the octapeptide benzyl ester in a hydrogenation step, after which gel chromatography (Sephadex G-25) in aqueous solution was used to remove the Arg monomer from the free octapeptide.

Purity of the final unprotected peptides was confirmed by amino acid analysis (Table I).

Carbon-13 NMR Studies on MBP Peptide Fragments. Determination of the peptide bond conformations at each of the three possible sites of cis–trans isomerism in the triproline segment of octapeptide 97–104 was necessary for subsequent comparison to the situation in native myelin basic protein. In particular, any significant proportion of cis peptide bonds formed at any of the Thr_{98}–Pro_{99}, Pro_{99}–Pro_{100}, and/or

Table I: Amino Acid Analyses of Myelin Basic Protein Peptide Fragments[a]

peptide	mol of amino acids					
	Arg	Thr	Pro	Ser	Gln	Gly
99–104			3.0	0.8	1.0	1.0
98–104		1.1	3.1	1.0	1.0	1.0
97–104	1.0	0.9	2.9	1.0	1.0	1.0

[a] Values given in quantities relative to Gly = 1.0.

Table II: ^{13}C NMR Chemical Shifts of Pro C_β and Pro C_γ Carbons in Cis and Trans Conformations for Peptide Fragments of Myelin Basic Protein[a]

	chemical shifts (ppm) for peptide fragments[b]			
	tripeptide 3–5	hexa-peptide 3–8	hepta-peptide 2–8	octa-peptide 1–8
β-carbons				
Pro_3 trans	29.70[c]	29.74	29.97	29.41
Pro_3 cis	32.09[c]			
Pro_4 trans	29.36	29.58	29.47	29.41
Pro_4 cis	32.09			
Pro_5 trans	30.73	30.75	30.70	30.64
Pro_5 cis	33.08			
γ-carbons				
Pro_3 trans	25.35[c]	25.34[d]	26.09	26.00
Pro_3 cis	23.20[c]	23.26[d]		
Pro_4 trans	25.99	26.09	26.09	26.00
Pro_4 cis	23.51			
Pro_5 trans	26.06	26.09	26.09	26.00
Pro_5 cis	23.51			

[a] Spectra recorded in D_2O, pH 6.0, and referenced to external Me_4Si. See text for acquisition parameters. [b] Residues in the table are numbered within MBP octapeptide 97–104 according to Arg_1-Thr_2-Pro_3-Pro_4-Pro_5-Ser_6-Gln_7-Gly_8. [c] Trans:cis ratio 50:50. [d] Trans:cis ratio 94:6.

Pro_{100}–Pro_{101} peptide bonds could promote protein folding. While all four proline ring carbons can potentially detect the isomerization state of the adjacent peptide bond, empirical correlations in a wide number of proline peptides have shown that only the Pro C_β and C_γ carbons have sufficient separation of cis/trans resonances (ca. 2–3 ppm) and characteristic chemical shifts to make them useful in diagnosing the relative proportions of the two isomers (Dorman & Bovey, 1973a; Deber et al., 1976; Grathwohl & Wüthrich, 1976; London et al., 1978).

The sequential nature of our synthesis not only allowed for unequivocal peak assignments for each proline residue (see Table II for cumulative chemical shifts) but also afforded the opportunity to study the isomerization state of the triproline segment at various stages of chain elongation. ^{13}C spectra of the H-Pro_3-OH tripeptide itself (not shown) displayed the anticipated pattern of *cis*-Pro C_γ (ca. 23.6 ppm) and *trans*-Pro C_γ (ca. 25.9 ppm) for proline resonances in X–Pro peptide linkages (Table II) in a 50:50 ratio at pH 6. Incorporation of this tri-Pro unit into the hexapeptide H-Pro_3-Pro_4-Pro_5-Ser_6-Gln_7-Gly_8-OH[1] resulted in a marked reduction in the percentage of cis peptide bonds; only a minor Pro C_γ peak was observed (23.26 ppm), corresponding to approximately 6% cis.

While Pro C_γ chemical shifts appear to be relatively insensitive to nearest-neighbor effects, dispersion of the Pro C_β carbons was observed in the hexapeptide (and higher) such that the Pro_5 C_β occurred approximately 1.0 ppm downfield from the (overlapped) Pro_3 and Pro_4 C_β resonances. This

[1] Note that proline residues numbered 3–5 in MBP fragment peptides for simplicity correspond to prolines 99–101 in the protein primary sequence.

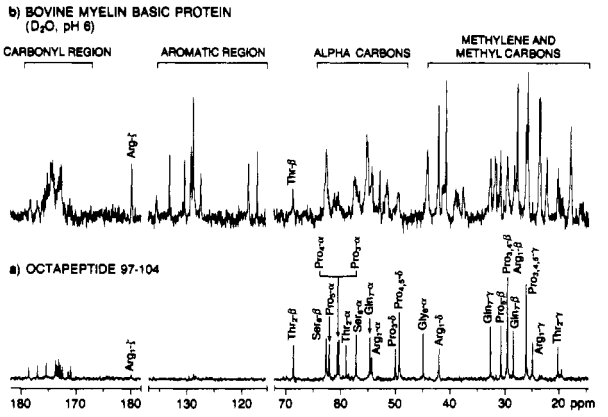

FIGURE 1: Natural-abundance ^{13}C NMR spectra (125 MHz) of (a) the synthetic octapeptide fragment H-Arg_1-Thr_2-Pro_3-Pro_4-Pro_5-Ser_6-Gln_7-Gly_8-OH, corresponding to residues 97–104 of bovine myelin basic protein (30 mg/mL, D_2O, pH 6), and (b) intact bovine myelin basic protein (45 mg/mL). General categories of resonances in various regions of the protein spectrum are indicated in (b). Octapeptide peak assignments were aided by spectra of intermediate peptides during synthesis (see text); proline chemical shifts are collected in Table II.

chemical shift separation was insufficient to suggest a *cis*-Pro C_β resonance. Further analysis of all the spectra indicated that the Pro_5 C_β signal was at the "normal" position for a proline residue in a Pro–X link but that the Pro_3 C_β and Pro_4 C_β were shifted "upfield", apparently because of the presence of the proline residues situated on their C-terminal side.

Further elongation of the triproline segment through the addition of the Thr and Arg components produced hepta- and octapeptide MBP fragments for which ^{13}C NMR spectra in the *cis*-Pro C_γ region remained free of signals from other amino acid components. In this region (ca. 23.5–24.0 ppm) (Deber et al., 1976), no resonances could be detected which would be attributable to *cis*-Pro C_γ (see Figure 1a). The hepta- and octapeptides were therefore completely in the trans conformation at all proline junctions.

Since the residues of greatest interest—the three prolines—retain their essentially all-trans conformational integrity through three chain-elongation steps, the triproline region was considered to have become structurally stable. The octapeptide 97–104 was thus judged to be of sufficient length to allow valid conformational comparisons with the corresponding region in intact MBP.

Comparison of Myelin Basic Protein and Its Peptide Fragment 97–104. Natural-abundance ^{13}C NMR spectra of bovine myelin basic protein and octapeptide fragment 97–104 (125 MHz) are compared in Figure 1a,b. Spectral resolution at this field strength did allow for observation of several individual protein peaks. Nevertheless, most protein resonances represent composites; i.e., MBP contains a total of 12 prolines which produce the resonances labeled Pro C_β and C_γ, as shown in detail in Figure 2. From their chemical shifts and relative intensities (and close correspondence to positions of Pro resonances in the octapeptide), it appears on inspection that the vast majority of all MBP proline residues are in the trans orientation. Direct evaluation of the *cis*-proline content using the most reliable empirical indicator, the *cis*-Pro C_γ resonance, is impeded by the partial overlap on the upfield edge of its expected region by other protein resonances (Figure 2b). However, it is possible to make an indirect assessment by using, for example, the integrated intensity of the (26) glycine α-carbons (single resonance near 45 ppm, Figure 1b) as a reference to determine the relative number of *trans*-Pro C_γ carbons (sufficiently resolved at 25.8 ppm from Arg C_γ carbons; Figure 2b). By cutting and weighting techniques, it was

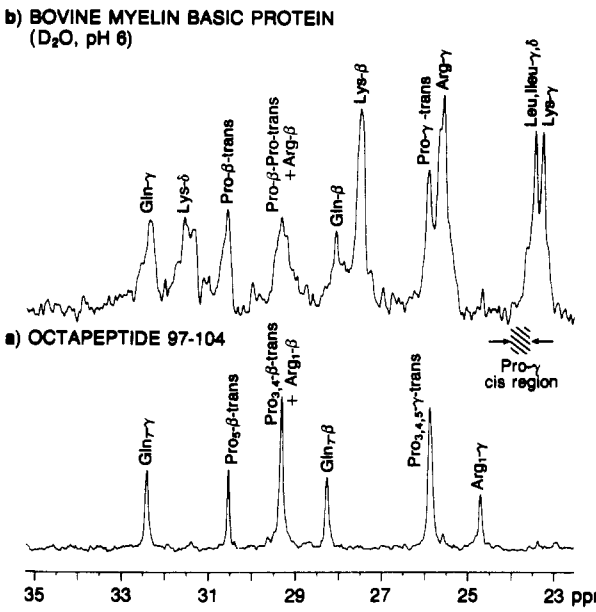

FIGURE 2: Natural-abundance ^{13}C NMR spectra (125 MHz) of (a) the synthetic MBP octapeptide fragment 97–104 and (b) intact bovine MBP, expanded in the region containing proline C_β and C_γ resonances. Protein resonances, which in each case are composites of all such carbons of the complete protein, were assigned from empirical correlations of amino acid chemical shifts (Wüthrich, 1976). The hatched area denotes the expected region for *cis*-Pro C_γ resonances (Deber et al., 1976).

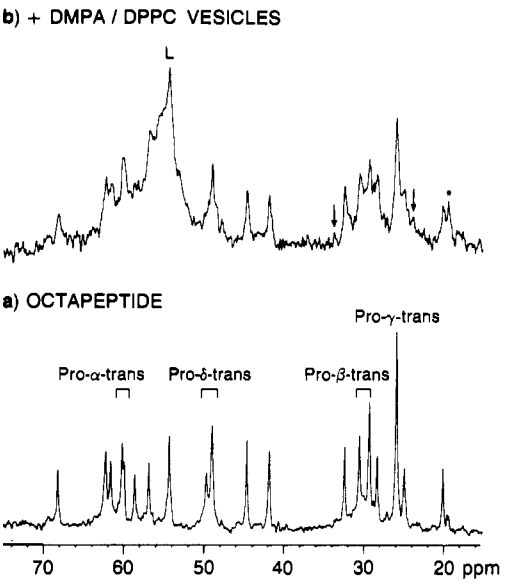

FIGURE 3: Natural-abundance ^{13}C NMR spectra (20 MHz) of MBP octapeptide fragment 97–104 (a) in aqueous solution and (b) in the presence of 5-fold molar excess of gel-phase multilamellar vesicles prepared from DPPC/DMPA (4:1) w/w). Proline resonances indicated by arrows in (b) are in the *cis*-Pro C_β and C_γ regions. The starred resonance (*) is residual ethanol.

found that all of the 12 total Pro C_γ carbons (±5%) are accounted for by the integrated intensity of the MBP resonance at 25.8 ppm.

It may be further noted that the region between ca. 32.5 and 33 ppm in the protein spectrum (Figure 2b) is essentially devoid of resonances, other than the downfield shoulder of the protein Gln C_γ resonance envelope. This region would be expected to contain *cis*-Pro C_β resonances arising from cis X–Pro peptide bonds where Pro is not followed by a second proline. This observation provides additional evidence that the 10 such Pro residues in MBP (including the Pro_4–Pro_5 peptide bond in the fragment octapeptide) are involved in essentially all-trans peptide bonds.

Although the combined results of this phase of the investigation argue against a predominantly cis X–Pro junction as a feature of MBP conformation in aqueous solution, they do not exclude a few percent of cis peptide bonds occurring at various X–Pro sites throughout the MBP sequence.

Conformation of the Pro-Rich Region in Membrane-Bound Octapeptide 97–104. While various physicochemical studies differ in detail concerning the extent of bilayer penetration of uncharged MBP residues [see Fraser & Deber (1984) and references cited therein], a chain reversal induced by membrane interaction of the triproline sequence in the protein could play a biologically functional role. Indeed, amphiphilic molecules such as phospholipids may promote a functional conformation in peptides and proteins that generally lack a specific or rigid conformation in solution (Kaiser & Kezdy, 1983; Taylor et al., 1983). Further, proline cis:trans ratios can vary with solvent polarity [e.g., see Deber et al. (1970) and Grathwohl & Wüthrich (1976a,b)]. It is thus conceivable that a membrane environment may create conditions suitable for a "hairpin" turn through the formation of cis peptide bonds involving proline. Since the octapeptide 97–104 does not exhibit an appreciable population of cis isomers in aqueous solution, and has been shown (vide infra) to mimic native MBP in this respect, its relatively simple ^{13}C spectra provided an excellent opportunity to evaluate the conformation of the triproline segment upon interaction with phospholipid.

Binding of MBP octapeptide 97–104 to a 5-fold molar excess of multilamellar lipid vesicles (4:1 DPPC/DMPA) produced the ^{13}C spectrum shown in Figure 3. The ratio of neutral to negative lipids simulates the situation in whole myelin; sufficient DMPA is present on a molar basis to promote the overall membrane affinity of the net positively charged octapeptide. Spectral interpretation of Figure 3b was aided by the fact that signals arising from this gel-phase DMPA/DPPC mixture are broadened beyond detection at 30 °C (except the relatively mobile choline head-group resonance). Thus, it was possible to observe the proline C_β and C_γ regions of the membrane-bound peptide without any interference from lipid resonances. Broadening of all peptide signals occurred upon membrane binding; for example, the *trans*-Pro C_γ resonance increased in line width from 4.8 to 14.4 Hz upon addition of lipid. However, all the peptide signals could be accounted for (with the exception of those masked under the choline head group at 55 ppm), and there was no selective broadening or selective loss of integrated intensity nor any specific chemical shift changes of any peptide resonances. This latter result suggests that despite the hydrophobic character of the pyrrolidine side chains of proline residues, the peptide is bound at the vesicle surface with little or no penetration into the (gel-phase) bilayer.

With respect to peptide bond conformation in the membrane environment, minor resonances were observed reproducibly which could be attributable to octapeptide *cis*-Pro C_β and *cis*-Pro C_γ peaks (indicated by arrows in Figure 3b), which constituted ca. 5% of the total resonance intensity. Thus, while >95% of all proline resonances in membrane-bound octapeptide remain in trans regions of the spectrum, the membrane environment does induce a measurable percentage of cis peptide bonds.

Discussion

Structural evaluation of the highly conserved triproline sequence (residues 99–101) of myelin basic protein in aqueous solution by ^{13}C NMR at 125 MHz, supported by the specific

analysis of the structure at each X–Pro peptide bond of the synthetic MBP fragment octapeptide 97–104, revealed an all-trans conformation for all peptide bonds involving proline, both in the octapeptide and in the intact protein. Using polyproline oligomers as a model, the triproline segment corresponds, in effect, to one turn of a left-handed (all-trans) polyproline II helix (Carver & Blout, 1967). Not only does this structure not allow for the possibility of a reversal in chain direction but also, as suggested by Martenson (1981), it suggests the linear propagation of the chain. Moreover, the pyrrolidine ring of each proline residue constitutes a rigid structure due to the rotational restrictions imposed by locking the protein backbone into the cyclic Pro side chain. A 180° reversal of the protein chain direction—which would arise if the cis isomer predominated at one of these three X–Pro linkages (or, indeed, at any X–Pro junction throughout the protein)—thus seems unlikely.

In accord with the known susceptibility of the proline peptide bond conformation to the polarity of the surrounding environment, a study of the MBP octapeptide 97–104 in association with a phospholipid membrane demonstrated that while the majority of X–Pro bonds remain in an all-trans conformation, similar to the situation prevailing in aqueous solution, a measurable population (ca. 5%) of cis X–Pro bonds may arise. In this context, the question as to how the percentage of such a species relates to its possible biological function must remain an open one. It should also be noted that the high-frequency occurrence of Pro residues in β-turns (Chou & Fasman, 1977; Smith & Pease, 1980)—four-residue segments which can also reverse a protein chain direction by 180°—represents an additional mode through which MBP chain reversal can arise. The intramolecular 1–4 hydrogen bonds which stabilize these β-turns may be more favored in the less polar environment of a membrane and may eventually prove to be a feature of MBP functional tertiary structure. No ready NMR approach exists to diagnose the presence of such turns, if any, in a membrane-bound protein.

Our findings are consistent with the results of an NMR study of a 22-residue peptic peptide (91–112) of rabbit MBP (Nygaard et al., 1984) in which the triproline sequence was similarly found to be essentially all trans in aqueous solution and was thus proposed to act as a rigid spacer segment between other regions of the peptide. The triproline segment apparently creates this highly stable, all-trans conformation (vide infra) in an MBP fragment peptide as small as six residues (99–104). Thus, the combined results of the present study of MBP and its fragment peptides suggest a role of chain propagation for the proline-rich region in the middle of the myelin basic protein primary sequence.

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Registry No. DPPC, 2644-64-6; DMPA, 30170-00-4; octapeptide 97–104, 97102-07-3; triproline 3–5, 19285-44-0; hexapeptide 99–104, 97102-08-4; heptapeptide 98–104, 97102-09-5; H-Ser(OBz)-Gln-Gly-OBz, 97170-29-1; *t*-Boc-Pro-Pro-Pro-OH, 29804-51-1.

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Analysis of the Specific Association of the Eighth and Ninth Components of Human Complement: Identification of a Direct Role for the α Subunit of C8†

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ABSTRACT: The basis for the physical association between C8 and C9 in solution was examined by isolating the noncovalently associated α–γ and β subunits of C8 and determining their respective affinities for C9. Results indicate that only α–γ associates with C9 and this association, though reversible, is complete at near equimolar ratios of each component. Further experiments using purified α or γ revealed that only α was capable of forming a stable complex with C9. Although the strength of this interaction was dependent on salt concentration, association was observed in buffer of physiological ionic strength and in human serum. These results establish that the domain on C8 responsible for interaction with C9 is located entirely within α. In related experiments, addition of β to preformed dimers of either (α–γ + C9) or (α + C9) resulted in complete association of this subunit. These particular results indicate that there are two physically distinct sites on α that separately mediate association of α with β and with C9. Furthermore, occupation of one site does not impair interaction at the other.

Complement-mediated lysis of cell membranes occurs as a result of specific interaction between C5b, C6, C7, C8, and C9 (Bhakdi & Tranum-Jensen, 1983; Podack & Tschopp, 1984). Assembly of the cytolytic complex on target membranes is initiated by formation of C5b and proceeds in the sequential manner

$$\text{C5b} \xrightarrow{\text{C6}} \text{C5b-6} \xrightarrow{\text{C7}} \text{C5b-7} \xrightarrow{\text{C8}} \text{C5b-8} \xrightarrow{\text{C9}} \text{C5b-9}$$

The high-affinity membrane binding site that mediates association of the nascent complex with cell membranes develops with formation of C5b-7. Subsequently, C8 and several C9 molecules bind to yield C5b-9, the macromolecular complex that produces membrane lysis.

Human C8 consists of three nonidentical subunits: α (M_r 64 000), β (M_r 64 000), and γ (M_r 22 000) (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). These subunits occur as a disulfide-linked α–γ dimer that is noncovalently associated with β. We previously established that β alone mediates the binding of C8 to C5b-7, as evidenced by the ability of purified β to associate with this complex (Monahan & Sodetz, 1981). Importantly, the resulting C5b-7β complex does not support subsequent incorporation of C9, thus emphasizing that α–γ and β are required for C9 binding (Brickner & Sodetz, 1984). We also established that γ has no direct role in C8 function by demonstrating that C8′, a derivative composed of only α and β, is functionally similar to normal C8 (Brickner & Sodetz, 1984). This derivative is able to interact with C5b-7 to form C5b-8′, a complex that can bind C9 and cause cell lysis. These observations indicate α and β but not γ are essential for incorporation of C9 into C5b-9.

Although C8 is required for C9 binding, there is no conclusive evidence that these proteins physically interact during C5b-9 formation or within the fully assembled cytolytic complex. Indeed, cross-linking studies of intact C5b-9 suggest that few, if any, of the multiple C9 molecules are in close physical proximity to C8 (Monahan et al., 1983). This observation is consistent with two possible explanations for the known requirement for C8. One is that C8 participates only indirectly by inducing a conformational change within the complex or membrane, leading to formation of a C9 binding site or sites that are distant from C8. Another possible explanation is that C8 interacts directly but transiently with C9, facilitating its polymerization and insertion into the membrane.

Current evidence favors at least some direct interaction between C8 and C9. The ability of these proteins to specifically associate in solution has been demonstrated and cited as indirect evidence that interaction occurs during C5b-9 assembly (Kolb et al., 1973; Podack et al., 1982). If so, the inability of β and the requirement for α to mediate incorporation of C9 into the nascent complex suggest that the site of interaction with C9 either is located on α or is shared between α and β. In this study, we investigated these possibilities and

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