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Identification of thiol groups and a disulfide crosslink site in bovine myelin proteolipid protein

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The existence of disulfide crosslinks limits the number of possible folded structures a protein can assume. Thus localization of disulfide and thiol groups is a key to understanding the conformation and orientation of myelin proteolipid protein (PLP) in the myelin membrane. [^{14}C]Carboxamidomethylated PLP was fragmented with chymotrypsin, and the resulting mixture was partially separated by reversed-phase HPLC. Purified ^{14}C-labeled peptides and a disulfide containing peptide were characterized by amino acid analysis. These experiments showed that Cys-32 and Cys-34 are free thiols, and are presumably on the interior of the cell or within the membrane bilayer, and that Cys-200 and Cys-219 are joined by a disulfide bond, and are probably located on the extracellular face of the membrane. Sequence analysis experiments indicate that Cys-5, Cys-6 and Cys-9 are linked by disulfides, probably to other parts of the protein on the extracellular face of the membrane.

Myelin proteolipid protein; Lipophilin; Polypeptide conformation; Disulfide bond; Membrane protein topology

1. INTRODUCTION

Myelin proteolipid protein (PLP), the major protein in central nervous system myelin [1], is thought to play an important role in the formation or maintenance of the multilamellar structure of myelin [2]. On the basis of amino acid sequence, two models for the folding of PLP in the myelin membrane have been proposed, one by Laursen et al. [3] and one by Stoffel et al. [4]. These two models share some common features, such as three transmembrane domains and two *cis*-membrane domains with intervening hydrophilic regions, but differ in regard to the orientation of some of the domains. PLP contains 14 cysteine residues, of which four can be alkylated with a variety of agents [5,6]; the remaining ten are presumed to be involved in disulfide links. Because of the reducing environment of the cytoplasm, it is expected that the thiol groups would be located on the cytoplasmic side and the disulfides on the extracellular side [7]. The location of disulfide bonds puts significant constraints on the possible orientations of the polypeptide chain within the membrane. For these reasons, we have attempted to locate the thiol groups and disulfide bonds in PLP in order to learn in more detail how PLP is organized in the myelin membrane bilayer.

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Abbreviations: CAM-, carboxamidomethyl-; CM-Cys, carboxymethylcysteine; PLP, myelin proteolipid protein; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Tris, tris-(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

The myelin PLP was prepared from a washed chloroform-methanol extract of bovine brain white matter. Lipids were partially removed from the solutes by emulsification and centrifugation as described by Lees and Sakura [8]. This material is referred to as 'crude PLP' because it still contains lipids.

2.1. *Carboxamidomethylation of PLP*

Procedure A: a sample (200 mg) of crude PLP containing 60 mg of protein was added to 20 ml of 50 mM Tris-HCl buffer (pH 8.2, containing 1% SDS, previously deaerated with N_2). The protein was dissolved by sonication in a bath-type sonicator for 20 min. A quantity (20 mg) of [^{14}C]iodoacetamide

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(0.45 μCi/μmol) was then added and allowed to react in the dark at room temperature. After 2 h, the reaction mixture was dialyzed in the dark against 20 mM Tris-HCl (pH 8.0) for 2 days, with renew of buffer twice a day. The slightly turbid solution was used directly for proteolytic cleavage.

Procedure B: this procedure is identical to A except that the 20 mg of [^{14}C]iodoacetamide was added to the reaction buffer *before* addition of 200 mg of the crude PLP sample.

2.2. *Carboxamidomethylation of PLP in myelin membrane*

Intact bovine myelin, prepared by the method of Norton [9], was labeled by a modification of the above procedure B. [^{14}C]Iodoacetamide (20 mg, 0.45 μCi/μmol) was dissolved in 10 ml of Tris-HCl buffer (pH 8.2, previously deaerated with N_2), and to it was added myelin membrane containing 50 mg protein. After 2 h in the dark at room temperature, the myelin was pelleted in an Eppendorf centrifuge. The pellets were suspended in 2 ml of chloroform/methanol (2:1), and undissolved material was removed by centrifugation. Ethyl acetate (1 ml) was added to the supernate to precipitate [^{14}C]CAM-PLP. The precipitate was pelleted and 100 μl of Triton X-100 was added, followed by 5 ml of 50 mM Tris-HCl (pH 8.0, containing 1% SDS). The resulting solution was dialyzed as in procedures A and B.

2.3. *Chymotryptic cleavage of [^{14}C]CAM-PLP*

The cloudy dialysates of [^{14}C]CAM-PLP were digested with chymotrypsin (enzyme/protein ratio of 1:40) at 37°C. After 6–8 h, chloroform/methanol (2:1) was added, the mixture was shaken and the phases allowed to separate. The distribution of ^{14}C in the upper and lower phases and in insoluble material at the interface was determined by liquid scintillation counting.

2.4. *Purification of peptides by reversed-phase HPLC*

The upper (aqueous) phase was lyophilized and the peptides were dissolved in 0.1% trifluoroacetic acid to give a concentration of about 1 mg/ml, and 100–200 μl was injected onto a C-18 reversed-phase HPLC column, which was then eluted with a linear gradient of 0.05% trifluoroacetic acid in acetonitrile into 0.05% acetonitrile. The eluate was monitored by absorbance at 214 and 254 nm, as well as by liquid scintillation counting and amino acid analysis of individual peaks. Some peptides were further purified by rechromatography using shallower gradients.

2.5. *Sequence analysis of PLP*

Procedure A: crude PLP (1 mg) was dissolved in 100 μl of 98% formic acid, and 10 μl of this solution was added to a polyvinylidine difluoride membrane disk which had been prewetted with methanol. The disk was allowed to air dry (10–20 min) and was then placed in 3 ml of 0.5 ml Tris-HCl (pH 8.2, 10% methanol) and 27 mg of iodoacetamide. After 2 h in the dark, the disk was removed, rinsed with water and allowed to dry. Sequencing of the protein was carried out on an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer.

Procedure B: PLP was applied, as above, to a membrane disk which had been previously soaked for 4 h at 37°C in 3 ml of 0.5 M Tris (pH 8.0, 10% methanol) containing 9 mg of dithiothreitol. The membrane was then placed in an iodoacetamide solution and further treated as in procedure A.

3. RESULTS AND DISCUSSION

PLP contains 14 Cys residues, 4 of which are believed to be in the reduced (thiol) state [5,6] and the remainder disulfides. In order to determine the oxidation state of individual residues, the thiol groups of PLP were radiolabeled and an attempt was made to isolate both labeled and cystine-containing peptides. Because disulfide exchange is a distinct possibility in a protein that contains both thiols and disulfides, PLP was alkylated with [^{14}C]iodoacetamide using several procedures. The general approach was to label free thiol groups with [^{14}C]iodoacetamide, cleave the protein with chymotrypsin, and separate and identify the peptides. In procedure A, no attempt was made to avoid exchange while the protein was being solubilized before alkylation. In procedure B, iodoacetamide was added to the solubilizing buffer before addition of the protein, so that thiols would be blocked as the protein dissolves. Finally, PLP was alkylated in intact myelin, where the protein is presumably in its native state.

The carboxamidomethylated proteins were then digested with chymotrypsin. The resulting peptides were partitioned into chloroform/methanol and the distribution of label in the upper and lower phases and in insoluble interface material was determined. As shown in table 1, in all cases roughly half of the radioactivity appears in the upper (aqueous) phase and half in the insoluble fraction. Very little appears in the lower phase, which was not further analyzed.

The upper phase peptides were then separated by reversed-phase HPLC; alkylated thiol peptides were detected by liquid scintillation counting. Samples of eluted peptides were also subjected to amino acid analysis in order to identify those which contained cystine and CAM-cysteine. Using protein labeled by procedure A, we isolated three radioactive peptides, which accounted for 89% of the total radioactivity in the upper phase. The majority of the radioactivity (75%) was in peaks 1 and 2 (see below), while a minor amount (14%) was found in a fraction corresponding to peak 3a (residues 200–206). On the other hand, when either crude proteolipid or intact myelin was labeled by procedure B, about 90% of the radioactivity could be accounted for by peaks 1 and 2; 3a was not observed. Furthermore, in the latter two

Table 1

Distribution of radioactivity after partition of [^{14}C]CAM-PLP chymotryptic peptides into chloroform/methanol (2:1) and water

Phase	Sample/alkylation procedure		
	Crude PLP/ procedure A	Crude PLP/ procedure B	Myelin/ procedure B
Upper (aqueous)	51%	42%	42%
Interface (insoluble)	45%	52%	58%
Lower (organic)	4%	6%	0%

experiments, a cystine-containing peptide (peak 3) was also isolated. When peak 3 was oxidized and rechromatographed, peptides corresponding to peaks 3a and 3b were isolated. Peptide compositions are given in table 2.

peak 1 F C G C G H E A L T G T E K L (31–45)

peak 2 F C G C G H E A L T G T E K L I E T Y (31–49)

peak 3 C A D A R M Y (3a, 200–206)

N A F P G K V C G S N L (3b, 212–223)

These results indicate (i) that Cys-32 and Cys-34 are present both in crude PLP and in myelin primarily as thiols and (ii) that residues Cys-200 and Cys-219 are linked by a disulfide bond. The isolation of a small amount of peak 3a from material labeled by procedure A suggests that some thiol-disulfide exchange had taken place before alkylation. Since we can account for nearly all of the radiolabel in the upper phase, which represents about half of the total label, the remaining two CAM-Cys groups must be in the insoluble fraction (see table 1), which we have not yet been able to fractionate.

PLP contains Cys residues near the NH_2-terminus at positions 5, 6, 9, and 24. In order to ascertain their oxidation state, we first carboxamidomethylated a sample of PLP and subjected it to Edman degradation in a gas-phase sequencer. Although interpretable data could be obtained only through the first 15 or so cycles, no phenylthiohydantoin (PTH) corresponding to CAM-Cys was observed (fig.1). When another sample of PLP was first treated with dithiothreitol to reduce all disulfide bonds, and was then alkylated and subjected to sequence analysis, PTH-CAM-Cys was observed at positions 5, 6, and 9, as expected.

Table 2

Amino acid analyses of derivatized thiol and disulfide peptides[a]

Amino acid	Peak 1	Peak 2	Peak 3a[b]	Peak 3b[b]
Cysteic acid			1.0 (1)	1.0 (1)
CM-Cys	1.5 (2)[c]	1.4 (2)		
Asp			0.9 (1)	1.9 (2)
Thr	1.5 (2)	2.4 (3)		
Ser				0.9 (1)
Glu	1.7 (2)	2.5 (3)		
Pro				0.8 (1)
Gly	3.3 (3)	3.1 (3)		2.1 (2)
Ala	1.0 (1)	1.0 (1)	2.4 (2)	1.2 (1)
Cys/2				
Val				0.9 (1)
Met			0.5 (1)	
Ile		0.9 (1)		
Leu	2.3 (2)	2.2 (2)		1.0 (1)
Tyr		0.8 (1)	0.8 (1)	
Phe	0.8 (1)	1.1 (1)		1.0 (1)
His	1.3 (1)	1.2 (1)		
Lys	0.8 (1)	0.9 (1)		1.0 (1)
Arg			0.6 (1)	
Sequence	31–45	31–49	200–206	212–223

[a] Representative analyses of peptides obtained in several experiments. In a typical experiment, 30 nmol of chymotryptic digest was separated by HPLC; individual peptides were obtained, after rechromatography, in 25–45% yield

[b] Peptides obtained by performic acid oxidation of peak 3

[c] Relative molar ratios; theoretical values are given in parentheses. Analyses are uncorrected for decomposition or incomplete hydrolysis

This experiment shows that Cys-5, Cys-6 and Cys-9 are probably involved in disulfide linkages (assuming the unreduced sample did not become air-oxidized during handling), and confirms earlier suggestions of Jolles et al. [10] that Cys-6 and Cys-9 are in the oxidized form. Furthermore, we had observed earlier [11] that the NH_2-terminal tryptic peptide (Gly-1 to Arg-8) could only be isolated after cleavage of the disulfide bonds, indicating that Cys-5 and/or Cys-6 were crosslinked to other portions of the protein. Intuitively it can also be argued that these three residues must be involved in disulfide linkages. Since they are all in close proximity, they must all be on the same side of the membrane, either outside or inside, and

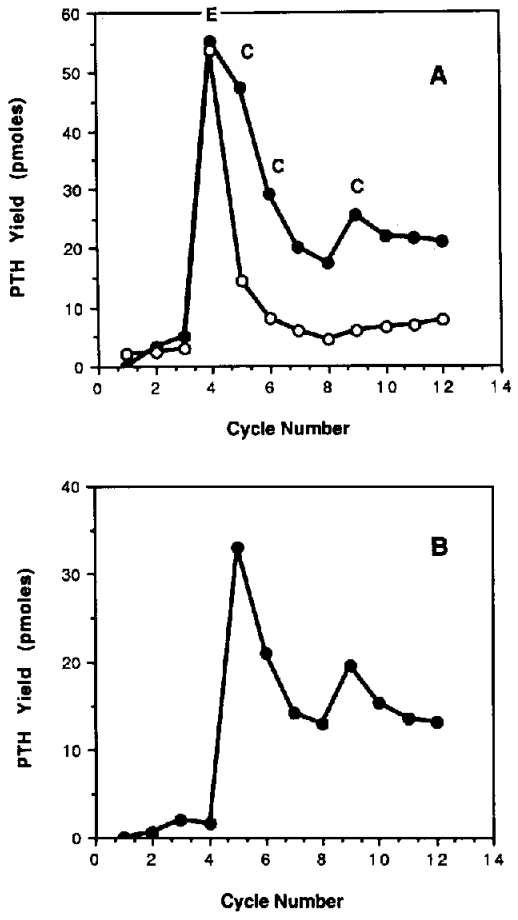

Fig.1. (A) Plot of yields of PTH-CAM-Cys + PTH-Glu (which coelute on HPLC analysis) versus sequencing step. The lower curve, obtained by sequencing unreduced CAM-PLP, shows only PTH-Glu at position 4; the upper curve, obtained for reduced and carboxamidomethylated PLP, shows in addition to Glu-4, CAM-Cys at positions 5, 6 and 9. (B) Subtraction of the lower curve in A from the upper. This shows more clearly, despite the rising baseline, the yields of PTH-CAM-Cys in positions 5, 6 and 9. Although the yield in step 6 is significantly lower than in step 5, it is much more than can be accounted for by carryover from step 5.

therefore all oxidized or all reduced, respectively [7]. Since there are 14 Cys residues in PLP, even numbers of Cys residues must be in the disulfide and thiol forms. If Cys-5, -6 and -9, as well as Cys-32 and -34, were thiols, then there would have to be a total of at least six thiols (an even number), rather than the observed four.

In earlier studies [12], we isolated a fragment of PLP comprising residues 181–276 by cleavage at Trp-180 and Trp-211. The fact that this fragment consisted of two connected chains, showed that Cys residues 183, 200, 219 and 227 are interconnected by disulfide bonds, and that these Cys residues are not joined to other parts of the protein. Since we have now shown that Cys-200 is linked to Cys-219, it follows that Cys-183 and Cys-227 are also linked. This argument assumes that Cys-183 and Cys-227 are not thiols, which is unlikely because topological considerations require them to be on the same face (presumably outer) of the membrane bilayer.

We suspect that the remaining two cysteine residues are at positions 24 and 168, which are both in hydrophobic domains of the protein and are presumably embedded in the membrane bilayer where they could not easily form crosslinks. This leaves Cys residues 108, 138 and 140 to crosslink with Cys residues 5, 6 and 9, assuming that there are only four thiols and that the other ten are actually involved in disulfide crosslinks. While this model agrees with our earlier one [2] in regard to the Cys-200 to Cys-219 and Cys-183 to Cys-227 crosslinks, it is in better agreement with the model of Stoffel et al. [3], with respect to orientation of the extramembrane segments comprising residues 36–58 and 91–150. Furthermore, the hydrophobic segment comprising residues 10–35, which we had proposed earlier [2] to be a *cis*-membrane domain, would have to be a *trans*-membrane in the new model. Yet even this revised model must be regarded as tentative, since recent observations [13] suggest that at least one thiol group may be acylated by a fatty acid and would not have been alkylated by iodoacetamide. Thus the total number of Cys residues in the reduced form may be greater than four and the number of disulfide crosslinks less than five.

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