FATTY ACID-LINKED PEPTIDES FROM BOVINE P0 PROTEIN IN PERIPHERAL NERVE MYELIN

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ABSTRACT

P0 glycoprotein in bovine peripheral nerve myelin was solubilized in 0.1% triethylamine and digested with trypsin. The tryptic peptides were separated into 12 peaks by high performance liquid chromatography (HPLC) using a nonionic macroreticular polystyrene resin, Hitachi 3013. In addition to the amino terminal and the carbohydrate-linked peptide, four fatty acid-linked peptides were obtained. The fatty acid-linked peptides contained 0.9 mol fatty acids per molecule. The relative molar ratio of palmitate, stearate and oleate linked to the peptide was 8, 3 and 2. The site of esterification of P0 protein appears to be close to the membrane, because the fatty acid-linked peptides included a highly hydrophobic domain. In spite of heterogeneity in the HPLC profiles, all the fatty acid-linked peptides showed similar characteristics in terms of both fatty acid and amino acid compositions. The significance of fatty acids linked to P0 protein is discussed.

Myelin is a compact multilamellar membrane structure surrounding a segment of axon. It has been suggested that integral myelin proteins served as structure elements for formation and stabilization of the highly ordered membrane assembly of myelin (2). P0 protein, which accounts for approximately half the total myelin proteins, is a specific integral glycoprotein of a molecular weight of 28,000 in peripheral nerve myelin (4-6, 11). Considerable extent of post-translational modifications of P0 protein has been reported including glycosylation (3, 19), sulfation (8, 13) and phosphorylation (16). In addition, *in vivo* acylation of P0 protein using radioisotope-labelled fatty acids has been shown (1). Probably, the interaction between P0 protein and fatty acid is important for the myelin structure. In this paper, we characterized the fatty acid-linked peptide fragments of P0 protein and discussed the relationship between this protein and the membrane structure. A preliminary account of this work has been reported (15).

MATERIALS AND METHODS

Materials

Trypsin, treated with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone, was purchased from Worthington. A Hitachi 3013 column and a Chrompack CP Sil 5 column were obtained from Nissei Sangyo and Chrompack, respectively. Reagents for a gas phase sequence analyser were from Applied Biosystems. All the other reagents (analytical grade) were obtained from Wako.

Purification of Bovine P0 Protein

Bovine P0 protein was purified from the peripheral nerve myelin using Sephadex G-200 gel chromatography with 2% sodium dodecyl sulfate (SDS) (11). The isolated P0 protein was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis.

Fig. 1 HPLC pattern of tryptic peptides from bovine P0 protein. Column: Hitachi 3013 (250×4.6 mm ID). Flow rate: 0.5 ml/min. Detection: 280 nm (range 0.64). Primary solvent: 0.1% trifluoroacetate, 10% acetonitrile. Secondary solvent: 0.1% trifluoroacetate, 99.9% acetonitrile. Gradient: 0% to 80% secondary solvent for 60 min, and 80% secondary solvent for 30 min. Column temperature: 60°C

Preparation and Separation of the Tryptic Peptides of P0 Protein

P0 protein (85 mg) was solubilized with 0.1% triethylamine solution and digested with trypsin (0.75 mg) in 0.1 M ammonium bicarbonate (pH 8.4) at 37°C for 1 h. The tryptic digests were lyophilized and solubilized in 0.1% triethylamine containing 1% SDS at 90°C for 30 sec. The peptides were separated by reverse phase high performance liquid chromatography (HPLC) with a Hitachi 3013 column (250×4.6 mm) at 60°C, eluted with a linear gradient of 10–82% acetonitrile in 0.1% trifluoroacetate.

Analysis of Amino Acid Sequence of the Peptides

Automatic Edman degradation of the peptides was carried out with a model 470A gas phase sequence analyser (Applied Biosystems). Manual sequence analysis of the peptides was

also performed by the method previously reported (10).

Fatty Acid Analysis

The peptides containing fatty acids were treated with 1 N NaOH at 80°C for 30 min and neutralized by HCl. The fatty acids were extracted from peptides with hexane and methylesterified in 1 N HCl-methanol at 80°C for 30 min as reported by Stoffel *et al.* (17). The fatty acid esters were again extracted with hexane and identified by gas chromatography mass spectrometry (JEOL DX 300) with Chrompack CP Sil 5 column (10 m×0.32 mm, ID). The analysis was carried out at a temperature gradient of 105–270°C and at the flow rate of 0.5 ml/min. Cleavage of linked fatty acids from the peptides was also carried out with 1 M hydroxylamine (pH 6.6) for 12 h or with methanolic NaOH (0.2 N) for 24 h (1).

Peak 7	Ile-Val-Val-Tyr-Thr-Asp-Lys-Glu-Val-His-Gly-Ala-Val-Gly-Ala-Leu-Val- CHO
Peak 6	Asp-Gly-Ser-Ile-Val-Ile-His-Asn-Leu-Asp-Tyr-Gly-Asp-Asn-Gly-Thr-Phe-Thr-Cys-Asp-Val-
Peak 9	Thr-Ser-Gln-Val-Thr-Leu-Tyr-Val-Phe-Gln-
Peak 10	Thr-Ser-Gln-Val-Thr-Leu-Tyr-Val-Phe-Gln-Lys-Val-
Peak 11	Thr-Ser-Gln-Val-Thr-Leu-Tyr-Val-Phe-Gln-Lys-Val-Pro-Thr-Arg-Tyr-Gly-Val-
Peak 12	Thr-Ser-Gln-Val-Thr-Leu-Tyr-Val-Phe-Gln-Lys-Val-Pro-Thr-Arg-Tyr-Gly-Val-Val-Leu-Gly-Ala- Val-Ile-Gly-Gly-Val-Leu-Gly-Val-Val-Leu-Leu-Ala-Leu-Leu-Leu-Phe-Tyr-Leu-Ile-Arg-Tyr-

Fig. 2 Amino acid sequence of tryptic peptides from bovine P0 protein

Table 1 Amino Acid Composition of Fatty Acid-linked Peptides

	Peak 9	Peak 10 (molar %)	Peak 11	Peak 12
Asp	4.6	4.1	2.8	3.4
Thr	4.9	5.3	5.1	5.1
Ser	4.5	3.4	3.5	4.0
Glu	8.8	5.9	7.5	6.0
Pro	2.6	2.5	3.0	2.6
Gly	13.8	12.7	12.1	13.2
Ala	6.3	5.6	6.2	5.3
Cys*	+	+	+	+
Val	11.3	15.1	13.9	14.4
Met	0	0	0	0
Ile	3.8	4.1	3.8	4.0
Leu	14.8	15.9	17.8	17.4
Tyr	6.1	7.1	6.3	4.2
Phe	3.7	4.1	3.8	4.0
His	0	0	0	0
Lys	3.4	2.8	2.3	3.1
Arg	7.6	7.9	8.1	7.8

*Cysteine was determined as cysteic acid.

RESULTS

Preparation, Separation and Characterization of Tryptic Peptides of Bovine P0 Protein

Biochemical studies on P0 protein have been quite difficult because hydrophobic properties of the protein result in aggregation and precipitation. P0 protein was insoluble in either 8 M urea, 6 M guanidine chloride or 1% Triton X-100 solution. While P0 protein was soluble in high concentration of trifluoroacetic acid or SDS solution, its enzymatic digestion was difficult in such solutions because of denaturation of the enzyme. Recently, we found that the protein could be solubilized and successfully digested by trypsin using 0.1% triethyl-

amine as a solvent. After digestion of P0 protein, the tryptic digests were solubilized in 1% SDS-0.1% triethylamine and separated in 12 peaks by HPLC with a Hitachi 3013 column (Fig. 1). This reverse phase HPLC system was useful for separation of highly hydrophobic peptides, while those peptides were hardly eluted from a conventional C-18 column of HPLC. Amino acid composition and partial amino acid sequence of several peptides were shown in Table 1 and in Fig. 2. The amino terminal sequence of peak 7 was identical with the amino terminal of P0 protein (8), and peak 8 contained the identical amino acid sequence of carbohydrate-linked peptide previously reported (9). On the other hand, each of peaks 9, 10, 11 and 12 was a highly hydrophobic peptide possessing similar amino acid composition and an identical amino terminal sequence.

Characterization of the Fatty Acid-linked Peptides

Fatty acid contents of the tryptic peptides were examined. After the peptides were treated in 1 N NaOH at 80°C, the fatty acids were extracted, methylated and analysed by gas chromatography mass spectrometry. A reconstructed ion chromatogram and mass chromatograms of fatty acid methylesters from peak 12 are shown in Fig. 3 and Fig. 4, respectively. Peaks 3, 4 and 5 in Fig. 3 were identified as palmitate, stearate and oleate, respectively, based on the mass spectrograms shown in Fig. 4A, 4B and 4C. The peak 12 contained 0.9 mol of fatty acids per molecule. The relative proportion of palmitic acid: stearic acid: oleic acid was 8:3:2 based on molar basis in the peak 12. Similar results were obtained when peaks 9, 10 and 11 were analysed. However, no fatty acid was detected in other peaks.

Fig. 3 Reconstructed ion chromatogram of fatty acid methylester from peak 12. Column: Chrom-pack CP Sil 5 (10 m $\times$ 0.32 mm ID). Flow rate: 0.5 ml/min (He). Column temperature: 105–270°C. Gradient: 8°C/min

Fatty acids linked to the peptides was retained following extraction with acidified chloroform/methanol or chloroform/methanol. However, almost all fatty acids were released from the peptides by the cleavage with 1 M hydroxylamine or with methanolic NaOH (0.2 N) treatment, indicating the covalent linkage of fatty acids to the peptides.

DISCUSSION

For biochemical characterization P0 protein, we developed effective methods of solubilization, tryptic digestion of the protein in 0.1 M triethylamine and separation of the peptides by HPLC with a Hitachi 3013 resin. This nonionic macroreticular polystyrene resin has advantages of high peak resolution, excellent chemical and mechanical stability and wide sample capacity range (7). These procedures may be useful for analysis of other hydrophobic membrane proteins.

In addition to the amino terminal peptide and the glycopeptide, four highly hydrophobic peptides were obtained by the HPLC. All these four peptides contained fatty acids such as palmitic acid, stearic acid and oleic acid. The fatty acid can not be accounted for by contamination from known membrane lipids nor removed by isolation methods of the peptides including extensive chloroform-methanol extraction. The results indicate that the fatty acids are covalently linked to the peptides.

The types of linkage of the fatty acid moiety to the acyl proteins have been classified into three, *i.e.* *O*-ester, thiolester and amide bond

(14). P0 protein was shown to link the fatty acid covalently by ester bond, based on its lability to alkaline methanolysis and susceptibility to the hydroxylamine treatment. Although the precise site of esterification is not known, it is postulated that the alcoholic groups of hydroxylamino acids, such as serine and threonine residues in the amino terminal region of the peptides, are involved. It is also possible, however, that the fatty acids are linked to the peptides by a thiolester bond, since the peptide contains at least one cysteine residue in the peptides.

Although four fatty acid-linked peptides showed clearly different retention time during the HPLC elution, they appear to be an identical or similar peptide derived from the same region of P0 protein, because they showed not only similar amino acid composition and an identical amino terminal sequence, but also similar fatty acid composition. There is a possibility that these peptides have different chain lengths, which causes the difference in retention time in the HPLC.

Agrawal *et al.* (1) reported that fatty acids were actively incorporated into rat P0 protein *in vivo*, using radioactive fatty acids. In this paper, we made it clear that native P0 protein contains fatty acids. Since the composition of fatty acids linked to the peptides resembles the ratio of incorporation reported (1), it is likely that fatty acids linked to P0 protein are evenly exchanged. As fatty acid-linked peptides include a highly hydrophobic region which probably penetrate the membrane (12), the site of esterification appears to locate close

Fig. 4 Mass spectrograms of fatty acid methylesters. Spectrograms of peaks 3, 4 and 5 in Fig. 3 are shown in Fig. 4A, 4B and 4C, respectively. The patterns of Fig. 4A, 4B and 4C were quite similar with those of standard palmitic acid, stearic acid and oleic acid, respectively.

to the membrane, suggesting a possible interaction between fatty acids and adjacent membrane lipids.

In the central nervous system, acylation of the proteolipid protein in myelin has been shown (18). Acylated forms of P0 protein and the proteolipid protein may play a similar function in both the peripheral and central

nervous system myelin. The biological role of esterified fatty acids in P0 protein remains unknown. However, covalently linked fatty acids would increase the hydrophobicity of the protein and may perform specific functions concerning the characteristic membrane assembly of myelin.

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