THE COMPLETE AMINO ACID SEQUENCE OF THE P2 PROTEIN IN BOVINE PERIPHERAL NERVE MYELIN

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1. Introduction

The P2 protein is a basic protein localized in peripheral nerve myelin [1]. We have developed purification procedures of the P2 protein and characterized some of its properties [2–7]. The P2 protein is considered to be the most likely candidate of neuritogenic protein to induce experimental allergic neuritis (EAN). However, our attempts to induce EAN in guinea pig or rabbit by injection of purified bovine P2 protein have been unsuccessful [3,7]. We confirmed the finding that bovine P2 protein induces EAN in the Lewis rat [8,9]. In order to clarify the antigenic determinant(s) of the P2 protein to induce EAN, determination of primary structure of the protein is essential. Here we report the complete amino acid sequence of bovine P2 protein.

2. Experimental

2.1. *Isolation of P2 protein*

Myelin was prepared from bovine spinal roots as in [3]. The P2 protein was purified from the acid extracts of the myelin using Sephadex G-75 gel chromatography as in [4].

2.2. *Cyanogen bromide cleavage*

The P2 protein was cleaved by cyanogen bromide (CNBr) in 70% formic acid as in [10] and separated into 4 fragments, CB1–CB4, on Bio-Gel P-6 with 0.1 M acetic acid and on SP-Sephadex C-25 with a linear gradient elution from 0.02–0.5 M pyridine–formic acid (pH 4.2). CB1 was digested with trypsin and separated into 3 fragments, T1–T3, on Bio-Gel P-2 with 0.1 M ammonium bicarbonate buffer (pH 8.0). CB2 was digested with trypsin after citraconylation of the peptide using citraconic anhydride as in [11]. The digests were decitraconylated in 0.07 M formic acid (adjusted to pH 2.5 with pyridine) for 7 h at 37°C and separated on SP-Sephadex C-25.

2.3. *Staphylococcal protease digestion*

The P2 protein was subjected to carboxymethylation with monoiodoacetic acid after reduction with 2-mercaptoethanol as in [12]. The carboxymethylated P2 protein was digested with *Staphylococcus aureus* V8 protease (Miles) (enzyme/substrate, 1/50, mol/mol) in 0.1 M ammonium bicarbonate buffer (pH 8.0) at 37°C for 24 h. The digests were separated on SP-Sephadex as above.

2.4. *Analytical methods*

Peptides were detected by *o*-phthalaldehyde after alkaline hydrolysis or by A_{230} and A_{280}. The purity of the peptides was checked on paper chromatography (*n*-butanol/pyridine/acetic acid/water, 15/10/3/12, by vol.), paper electrophoresis (pyridine/acetic acid/water, 1/1/100, by vol., pH 4.7), amino acid analysis and Edman degradation. Amino acid analyses were performed after hydrolysis in 5.7 N HCl as in [4]. Tryptophan was detected by spraying Ehrlich reagent [13] after paper chromatography or paper electrophoresis. Sequence analysis of each peptide was carried out using manual Edman degradation [11] or using an automated sequence analyzer (JEOL JAS 47K). PTH amino acid derivatives were identified by high-performance liquid chromatography using Partigil 5 (Reeve Angel) [11], by gas–liquid chromatography using 10% silicone SE30 on Gas Chrom Q [14] and by back hydrolysis with 57% hydriodic acid to the free amino acid. Hydrazinolysis and carboxypeptidases

digestion were done as in [11]. The mass spectra were recorded on a field-desorption mass spectrometer (JEOL JMS-D300). Details of the sequencing procedures will be described elsewhere.

3. Results and discussion

CNBr fragmentation of unreduced P2 protein gave rise to 3 distinct peaks termed CB2, CB(3+4) and CB1 on a Bio-Gel P-6 column. By amino acid analysis, cysteine was contained only in CB(3+4), but not in CB2 and CB1. After partial reduction of CB(3+4) with 2-mercaptoethanol for 1 h at room temperature, the material was resolved into 3 peaks, CB3, CB4 and a mixture of both on a SP-Sephadex column. Each of CB3 and CB4 contained 1 mol cysteic acid/mol after performic acid oxidation. This result suggested that CB3 and CB4 were covalently linked to each other by disulfide bond. The amino acid compositions of the isolated CNBr fragments showed that all fragments except CB4 contained homoserine (table 1). As CB4 showed an identical C-terminal residue, Val, to that of intact P2 protein by hydrazinolysis, it became clear that CB4 is the C-terminal fragment of the P2 protein (fig.1).

After citraconylation, CB2 was further cleaved with trypsin and separated into 7 fragments, CiT1–CiT7, on SP-Sephadex. The amino acid composition of CB2 was identical to the sum of these 7 fragments. CiT7(107–113) with homoserine and no Arg was determined to be the C-terminal fragment of CB2. Automated Edman degradation on CB2 through 31 cycles(21–51) established the sequence of the N-terminal portion of CB2 and allowed the alignment of CiT1–CiT2–CiT3.

CB1 was N-terminal portion of the P2 protein because the N-terminal of CB1 was blocked as in the native P2 protein. After tryptic digestion, CB1 which contained 2 mol Lys was separated into 3 fragments, T1–T3, on Bio-Gel P-2. T1 with blocked N-terminus was N-terminal portion, and T3 containing homoserine was C-terminal portion of CB1, respectively. Therefore, alignment of these peptides in CB1 was T1–T2–T3. T1, a tripeptide containing Asx, Ser and Lys, was determined to be mol. wt 389 by field desorp-

Table 1
Amino acid compositions and yields of cyanogen bromide fragments from bovine P2 protein

Amino acids	CB1	CB2	CB3	CB4	Total
Asp	3.1 (3)	9.1 (9)		1.0 (1)	(13)
Thr	1.0 (1)	9.8 (11)		1.0 (1)	(13)
Ser	2.3 (3)	4.3 (5)			(8)
Glu	2.0 (2)	9.2 (9)	1.0 (1)	0.9 (1)	(13)
Pro		1.7 (2)			(2)
Gly	1.0 (1)	7.4 (8)			(9)
Ala		4.9 (5)			(5)
Cys[a]			1.0 (1)	1.0 (1)	(2)
Val	1.3 (1)	5.3 (5)	1.4 (2)	2.6 (3)	(11)
Met[b]	0.6 (1)	0.6 (1)	0.5 (1)		(3)
Ile		5.7 (7)		0.9 (1)	(8)
Leu	2.0 (2)	8.0 (8)			(10)
Tyr	1.1 (1)			0.6 (1)	(2)
Phe	2.0 (2)	3.3 (3)			(5)
His					
Lys	2.3 (2)	13.0 (13)	1.0 (1)	2.1 (2)	(18)
Arg		6.2 (6)		0.8 (1)	(7)
Trp[c]	+ (1)	+ (1)			(2)
No. residues	(20)	(93)	(6)	(12)	(131)
N-terminus	Blocked	Lys	Val	Lys	
Yields (%)	80	80	52	62	

[a] Determined as cysteic acid
[b] Estimated from homoserine
[c] Detected with the Ehrlich reagent
The integral values in parentheses are based on the sequence

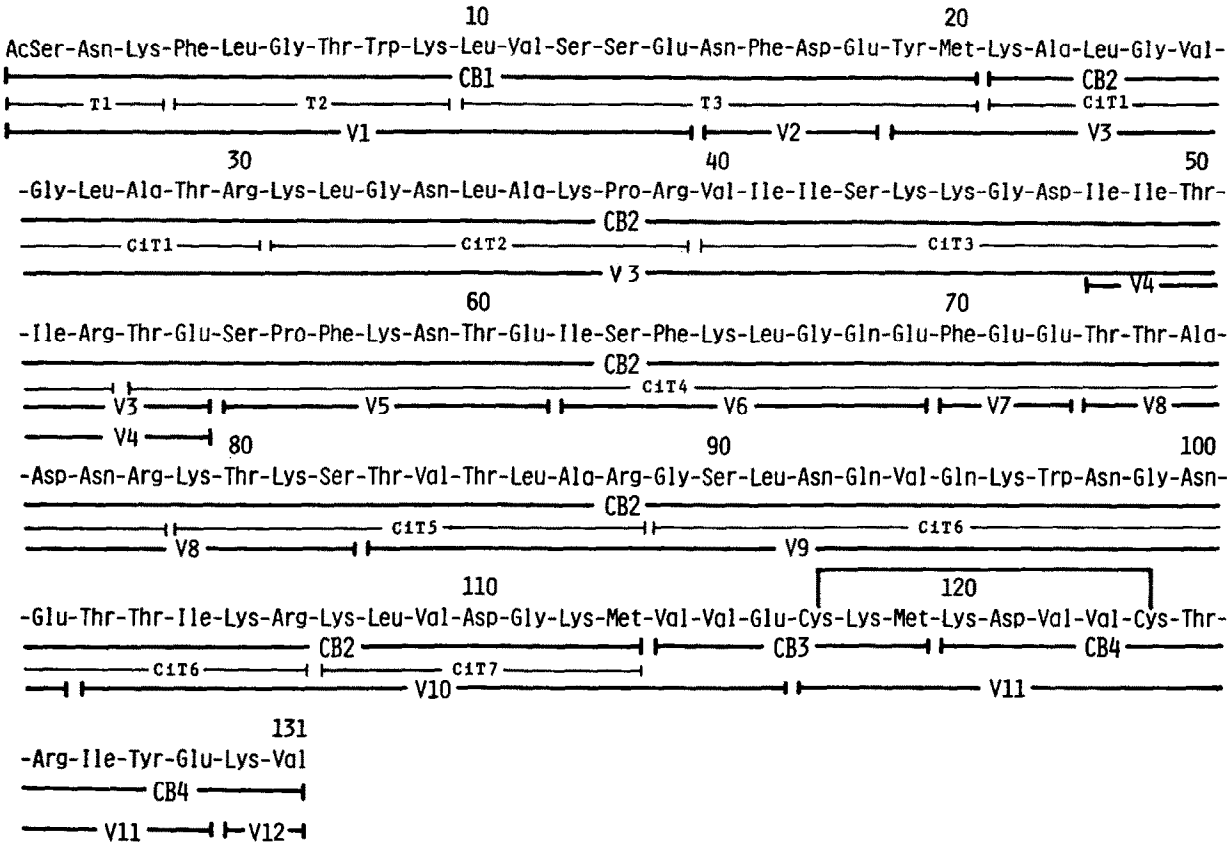

Fig. 1. The complete amino acid sequence of bovine P2 protein. The peptides isolated by cyanogen bromide cleavage (CB) and by Staphylococcal protease digestion (V) of the P2 protein are shown below the sequence. T and CiT refer to tryptic peptide of CB1 and citraconylated CB2, respectively.

tion-mass spectrometry. Sequence of the C-terminal part of T1 was determined by carboxypeptidase A and B digestion as –Asn–Lys. From these results, the sequence of T1 was decided to be acetyl-Ser–Asn–Lys.

Twelve fragments were isolated from Staphylococcal protease digests of reduced and carboxymethylated P2 protein using SP-Sephadex. All of these fragments covered the whole sequence of the P2 protein (fig.1). It is interesting to note that Staphylococcal protease cleaved at Ser residue (pos. 82) in addition to Glu and Asp residues at positions 14, 18, 47, 54, 61, 69, 72, 101, 116 and 129. This is in agreement with an observation that this protease will cleave at certain Ser residues [15]. The alignments of CB1–CB2, CB2–CB3 and CB3–CB4 were obtained from the peptides V3, V10 and V11, respectively. The alignments of CiT1–CiT2–CiT3–CiT4–CiT5–CiT6–CiT7 were also obtained from the peptides V3, V4, V8, V9 and V10.

Combination of the above results led to whole sequence of the P2 protein as shown in fig.1. According to this primary structure, the P2 protein consists of 131 amino acid residues with following composition:

Asp 5, Asn 8, Thr 13, Ser 8, Glu 10, Gln 3, Pro 2, Gly 9, Ala 5, Cys 2, Val 11, Met 3, Ile 8, Leu 10, Tyr 2, Phe 5, His 0, Lys 18, Arg 7, Trp 2

The molecular weight calculated on the basis of the sequence is 14 859. The charged residues are irregularly distributed throughout the molecule except at positions 14–18, 69–72, 78–81, 105–107. Comparison between the known sequence of an encephalitogenic protein [16] and that of the P2 protein does not reveal considerable sequence homology.

The knowledge of the primary structure of the P2 protein is a prerequisite for determination of its antigenic determinant(s) to induce EAN. Such an analysis is now feasible and is in progress.

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