Sequence analysis of peptide fragments from the intrinsic membrane protein of calf lens fibers MP26 and its natural maturation product MP22

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Calf lens fiber plasma membranes, containing only the intrinsic membrane protein MP26 and its maturation product MP22 were treated with proteolytic enzymes such as trypsin, protease V8 from *S. aureus* or with chemical agents as CNBr in formic acid. The cleavage products, purified by electrophoresis, were analysed for their amino acid composition and N-terminal sequences. Proteolysis gave rise to peptides which were mainly shortened at the C-terminal end of the molecules. While the V8 protease produced a fragment with a similar N-terminal sequence as the maturation product MP22, trypsin yielded another cleavage product. Chemical hydrolysis yielded large fragments (11–15 kDa) with hydrophobic N-terminal sequences. Our results suggest that MP26 is characterised by an N-terminal signal sequence and possesses other hydrophobic domains which could function as untranslocated insertion sequences.

Intrinsic lens fiber membrane protein *Bovine lens* *Amino acid sequence*

1. INTRODUCTION

A prominent feature of the terminal differentiation of the eye lens epithelium into fibers is the biosynthesis of a novel protein of 26-kDa which becomes the major component of the fiber plasma membranes [1,2].

This protein, designated 'MP26', undergoes post-translational cleavage during ageing of the fibers by endogenous protease and accumulates in the membranes of the lenticular nucleus as a polypeptide of 22 kDa, 'MP22' [3,4]. MP26 and MP22 are nearly completely extracted by chloroform–methanol, suggesting that these proteins are closely associated with the lipid environment of the membrane. The intracellular topogenesis [5] of these intrinsic membrane proteins is still questionable. Experiments on translation of different classes of lens polyribosomes in reticulocyte lysate indicate that the messenger RNA encoding MP26 is predominantly localised on a class of polyribosomes associated with the cytoskeleton of the lens fiber [6]. But the nature of the interaction between the membrane compartment in which the MP26 is inserted and the role of this class of polyribosomes in the course of membrane protein synthesis is unclear. Since the N-terminal amino acid sequence comprises a number of hydrophobic residues ([7], here), the insertion into membranes might occur through either a cotranslational mechanism mediated by a signal sequence or the anchorage of a hairpin loop domain of the chain into the lipid bilayer [5,8].

Here we present new data concerning the amino acid sequence of MP26 and MP22 obtained using treatment with trypsin, *S. aureus* protease and

Abbreviations: MP26, intrinsic membrane protein of calf lens fibers; MP22, maturation product of MP26; MP22T, trypsin fragment of MP26; MP22V8, protease V8 fragment of MP26; C15K, CNBr fragment of MP26; C11K, CNBr fragment of MP22; PAGE, polyacrylamide gel electrophoresis; TPCK, l-1-tosylamide-2-phenylethyl chloromethyl ketone

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cyanogen bromide. Our results suggest that MP26 is characterised by an N-terminal hydrophobic signal sequence and comprises hydrophobic domains which may have the function of untranslocated insertion sequences.

2. MATERIALS AND METHODS

2.1. *Preparation of peptides for sequence analysis*

The isolation of bovine lens fiber plasma proteins was done as in [4]. Briefly, the membranes were treated, after urea extraction, with 0.1 N NaOH. The only proteins remaining in the membranes after these treatments were the intrinsic membrane proteins MP26 and MP22 (fig.1).

After preparative PAGE electrophoreses [9] of the membranes (in 3 mm thick 12% or 14% gels containing 2% SDS), the gels were immersed for 2 min in 1 M KCl solution to visualise the polypeptide bands. The cut zones were eluted for 3 h in 20 mM phosphate buffer (pH 7) containing 2% SDS. The extracted polypeptides were precipitated in a mixture of methanol:acetic acid (60:0.5, v/v end concentration). After storing the reaction mixture at −20°C for one night, it was warmed up to room temperature and centrifuged for 20 min at 10000 × *g* at 4°C. The protein pellet was washed in distilled water, centrifuged and stored at −70°C until use.

2.2. *Chemical treatments of the plasma membranes*

Membrane proteins (25–50 mg) were added to a solution of protease V8 (Sigma) or TPCK-trypsin (Worthington) at 1 mg/ml in NH_4HCO_3 0.5% at pH 8. After 5 h digestion at 37°C, membrane fractions were sedimented during 5 min in a Fisher microcentrifuge. The pellet was washed in bidistilled water and stored at −70°C until use.

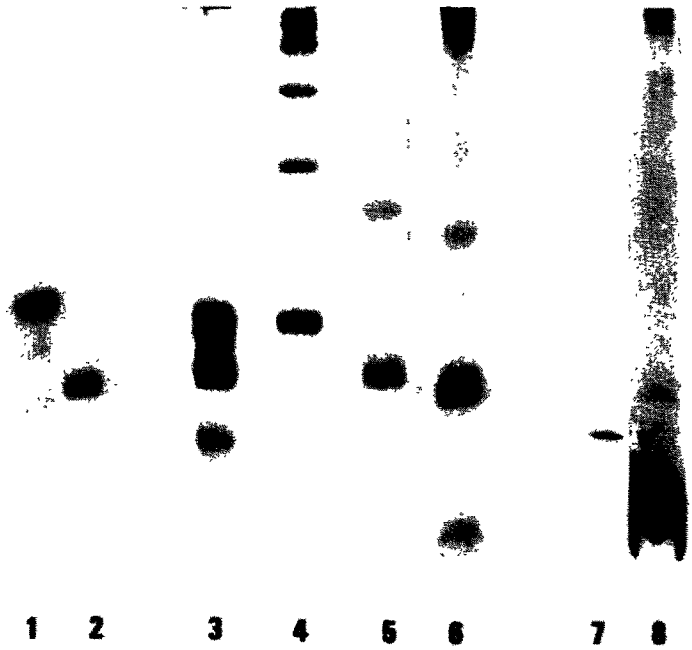

Fig.1. SDS–PAGE of the peptides used for sequence analysis. Electrophoresis was performed in 12% gels. Lane 1, cortex membranes containing only MP26; lane 2, trypsin-treated cortex membranes containing only MP22T; lane 3, nucleus membranes containing MP26 and the natural MP22; lane 4, MP26 purified electrophoretically from nucleus membranes; lane 5, natural MP22 purified electrophoretically from nucleus membranes; lane 6, MP22V8 fragment purified electrophoretically from protease V8-treated nucleus membranes; lane 7, C15K fragment purified electrophoretically from CNBr-treated nucleus membranes; lane 8, C11K fragment purified electrophoretically from CNBr-treated nucleus membranes.

For CNBr cleavage, membranes (6% dry weight) were suspended in 70% formic acid to which 3% CNBr was added. The reaction was carried out under nitrogen and in the dark at room temperature for 24 h. Cleavage was stopped by diluting the reaction mixture in 9 vols water. The membranes were then lyophilised.

2.3. *Sequence analysis*

2.3.1. Automated analyses

Proteins and peptides were analysed by automated Edman degradation in an Applied Biosystems Gas Phase Sequencer. Polybrene was added as a carrier together with glycylglycine to avoid partial blocking by contaminants. Repetitive degradation yields over the first 20 steps were 95%. The phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography on a Waters instrument equipped with a C_{18} μBondapak column, using a sodium acetate/methanol gradient as in [10].

2.3.2. Manual sequence determination

The amino acid sequences of the tryptic peptides were determined by the manual micromethod described by Chang [11] using 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate as the stepwise degradation reagent and thin-layer chromatography on polyamide sheets for the detection of the amino acid thiohydantoin derivatives.

2.4. *Amino acid analysis*

Total acid hydrolysis was performed as in [12]. The hydrolysate was subjected to analysis in a Beckman auto-analyser.

3. RESULTS AND DISCUSSION

Three types of peptides from MP26 were examined (table 1): those resulting from natural cleavages mainly MP22, those obtained through proteolytic cleavages by TPCK-treated trypsin (cleavage at the K–X or R–X peptide bonds), by *S. aureus* protease (cleavage at the E–X bonds) [4] and finally fragments resulting from chemical hydrolysis by CNBr in 70% HCOOH (cleavage at the M–X bonds) [13]. In view of the very low solubility of MP26 and MP22, enzymatic and chemical cleavages were performed on membrane preparations. The purity of the peptide fragments obtained were checked by SDS–PAGE (fig.1).

3.1. *Amino-terminal region*

The automated Edman degradation of MP26 yielded a single sequence for the first 33 positions of the chain: 24 out of the 33 residues have a hydrophobic or at the very least a non-polar side chain (fig.2). Starting with methionine, this part of the protein could well correspond to a signal sequence, similar to that usually removed from secreted proteins before release in the extracellular space. A more direct evidence that the amino-terminus of the MP26 corresponds to an uncleaved leader sequence is given by the in vitro translation of this lens membrane polypeptide [8]. Cell-free translation of messenger RNA indicates that MP26 requires the co-translational presence of microsomal membranes and the 'signal recognition particles' confers specificity on membrane integration.

The naturally occurring MP22 has an N-terminal sequence which is identical to that of MP26 from position 17 onward. Since the apparent difference in molecular mass (4 kDa) (table 1) is too large to be explained by the natural removal of 16 residues, one would have to conclude that MP22 also has a shorter C-terminal sequence. This then in turn would suggest that MP26 has both the N-terminal and the C-terminal parts exposed to external proteases.

3.2. *Analysis of tryptic and* S. aureus *protease peptides*

The overall hydrophobic character of MP26 has made the isolation and purification of peptides a very difficult task. Recoveries were in general poor and quite variable. In addition, the big size of the fragments illustrates the high resistance of MP26 to proteolytic cleavages. Represented here are the N-terminal sequences of some of the tryptic (T) or *S. aureus* protease peptides which could be obtained in sufficient yields to perform amino acid analysis (table 1) and automatic Edman degradation (fig.2). Fragment MP22T had an N-terminal sequence which corresponds to residues 6 and onwards of native MP26. Again, because of the difference in molecular mass, we conclude that its C-terminal portion is also shorter than that of MP26.

Table 1

Amino acid composition of MP26 and its degradation product by proteases and cyanogen bromide (CNBr)

Amino acids	Number of residues					
	MP26	MP22nat	MP22T	MP22 V8	C15K	C11K
D	9.44	9.14	9.35	6.80	7.98	4.22
T	9.23	8.67	8.21	6.82	6.57	3.23
S	19.00	14.90	15.00	14.46	12.88	7.70
E	14.60	16.70	9.20	10.30	13.60	5.50
P	27.70	16.50	21.00	24.40	15.50	10.00
G	24.80	24.70	21.50	20.16	13.85	9.40
A	28.60	20.50	27.30	24.60	12.79	12.70
V	19.13	15.10	17.98	16.80	8.50	7.30
M	5.30	4.16	4.35	4.40	0.00	0.00
I	5.80	7.20	5.70	4.53	4.20	3.89
L	32.60	24.20	28.10	27.60	14.88	15.00
Y	8.70	7.50	7.57	6.80	4.85	3.30
F	16.70	14.90	16.30	14.50	8.70	7.80
H	6.65	4.90	6.40	7.30	2.70	2.90
K	3.80	4.20	1.50	1.90	4.80	3.30
R	14.50	11.60	9.50	14.40	8.10	7.00
W	3.27[a]	N.D.	2.00[a]	2.00[a]	3.00[b]	N.D.
Total number of residues	247.70	214.30	211.20	208.00	140.00	103.20
Nb hydrophobic residues / Total Nb of residues	53.00	51.00	56.20	53.00	47.00	54.30
M_r	26822.00	21860.60	21894.50	21909.00	15534.50	11036.40

[a] Tested by fluorescence technique in the presence of guanidinium chloride
[b] Minimal number of residues measured by automatic sequence

N.D., undetermined

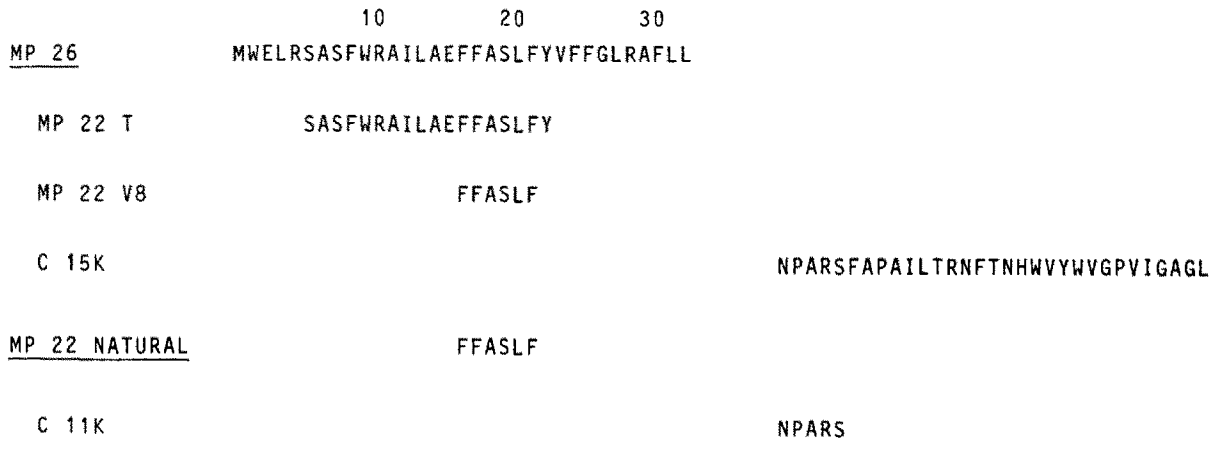

Fig.2. N-terminal sequence analysis of MP26 and its peptide fragments. The peptides are tagged as in fig.1.

It is remarkable that fragment MP22V8 has the same N-terminal sequence as the naturally occurring MP22 suggesting that the same E–F peptide bond was cleaved. Since natural or artificial proteolysis occurs at the N-terminal as well as at the C-terminal region, it can be assumed that both terminals of MP26 are located at the same membrane side.

3.3. *Chemical cleavages*

The treatment of MP26 containing membranes with CNBr in 70% HCOOH resulted in the breakdown of the protein into fragments considerably smaller than those obtained by proteolytic hydrolysis. Two of these 'C' peptides were examined. C15K yielded a sequence of 31 unambiguously identified residues (fig.2). This sequence contained about 15 hydrophobic residues including 2 tryptophans, 2 phenylalanines and one tyrosine. In addition, in contrast to the N-terminal sequence of MP26, there were three prolines. A smaller 11-kDa fragment had the same N-terminal sequence as C15K and is presumably shorter at the C-terminal side (table 1,fig.2). Most probably the 11-kDa fragment is generated by chemical cleavage of the natural MP22. In fact, the C11K amino acid analysis indicates that the hydrophobicity of this fragment is higher than that of C15K. This is explained by the loss of the hydrophilic C-terminal by the natural MP22 and thus by its cleavage fragment C11K.

In conclusion, the amino acid analysis and the primary structure of the MP26 and its natural or artificial degradation products are characterised by a high degree of hydrophobicity (table 1) typical of intrinsic membrane proteins. Probably de novo synthesis of MP26 and co-translational membrane integration is mediated by a signal sequence specific translocator and other hydrophobic domains such as those identified in the C15K and C11K fragment, which have the function of stop transfer sequences.

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