Biochimica et Biophysica Acta, 731 (1983) 267–274
Elsevier

BBA 71692

MAJOR INTRINSIC POLYPEPTIDE OF LENS MEMBRANE

BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE MAJOR CYANOGEN BROMIDE FRAGMENT

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(Received December 16th, 1982)

Key words: Lens junction; Major intrinsic polypeptide; Cyanogen bromide cleavage; Immunological characterization; (Bovine and rat eye)

A protein of M_r 26000 has been shown to be the major component of eye-lens junctions, which are similar but not identical to the gap junctions of liver and other tissues. Cyanogen bromide cleavage of the M_r 26000 polypeptide from bovine lenses yields a major fragment of M_r 15000 (fragment 1). However, if the junctions are first treated with trypsin or carboxypeptidase Y, cyanogen bromide treatment yields a fragment of reduced molecular weight. Since protease treatment has been shown to cleave residues almost exclusively from the carboxy-terminal end of the M_r 26000 polypeptide, it follows that fragment 1 represents the carboxy-terminal half of this molecule, part of which is exposed to proteolytic attack outside the membrane. This latter result is corroborated by the fact that antisera which recognize both the M_r 26000 polypeptide and fragment 1 fail to do so after preadsorption with intact membranes. In addition, comparative amino acid and partial sequence analyses of the M_r 26000 polypeptide and fragment 1 indicate that fragment 1 is more hydrophilic in character, suggesting that much of the amino-terminal half of the M_r 26000 polypeptide is buried within the lipid bilayer.

Introduction

Lens cells have been demonstrated, by several means, to be electrically coupled to each other [1,2]. Similar physiological coupling between cells of other tissues has been associated with the presence of gap junctions; these are believed to provide aqueous channels for the passage of ions of low-molecular-weight metabolites ($M_r < 1000$) between cells [3]. Cellular contacts which resemble junctions elsewhere, but display some distinguishing characteristics [4,5], are abundant in the lens. Circumstantial evidence suggests that these lens junctions mediate the extensive exchanges between lens fiber cells. For convenience, they will be referred to as putative gap junctions, but this is not meant to infer that they are necessarily identical to gap junctions in other tissue.

Analysis of purified membrane from lens fiber cells indicates that the major polypeptide species is a component of approximately 26000 daltons [6,7]. This 'major intrinsic polypeptide' is found in the eye lens of all vertebrate species studied, and has been highly conserved throughout evolution [8]. Analysis of purified preparations of lens membrane gap junctions demonstrates that the M_r 26000 polypeptide is the major component [5,9]. Using immunoferritin localization, antisera made against this component bind to lens fiber cell membranes, including gap junctional structures [10].

Of particular interest is structural knowledge on

how the intrinsic M_r 26000 polypeptide molecule is arranged in the lipid bilayer. This would be useful in determining the function of these lens putative junctions and the demonstration, or otherwise, of an aqueous channel spanning the membranes (i.e., the gap junction 'pore', see Ref. 3). As a prelude to these studies, chemical and/or enzymatic methods must be found to cleave the intact polypeptide into fragments suitable for detailed biochemical analyses. This report describes the production and characterization of M_r 15000 cyanogen bromide fragment of the M_r 26000 polypeptide molecule.

Materials and Methods

Purification of the M_r 26000 polypeptide. Bovine eyes were obtained from a local slaughterhouse. Rat eyes were obtained from freshly killed Sprague-Dawley rats. The lenses from cows and rats were immediately removed and decapsulated. Fiber cell membrane from these lenses was prepared essentially according to the method of Alcala et al. [6], as modified by Takemoto et al. [8]. Basically, this procedure involves repeated homogenizations of lens fiber cells in 0.05 M Tris-HCl, pH 7.9, followed by washing with 8 M urea. Purified membrane fractions were dissolved in sample buffer (2% (w/v) SDS/2% (v/v) 2-mercaptoethanol/0.05 M Tris-HCl, pH 6.8) and resolved using 12.5% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [11]. To localize the M_r 26000 polypeptide band, a small amount of membrane was labeled with ^{125}I according to the method of Takemoto et al. [8], and run on an adjacent gel lane. After drying and autoradiography of the gel, the M_r 26000 polypeptide band from the unlabeled sample was excised and incubated with stirring overnight in a large volume of 1 mM NH_4HCO_3. The resulting suspension was filtered through Whatman 3MM paper, then dialyzed exhaustively for 2–3 days against 1 mM NH_4HCO_3 and lyophilized. The protein content of the lyophilized powder was determined [12] using bovine serum albumin as standard. Approximately 50–75% of the M_r 26000 polypeptide was recovered from gels using this elution procedure.

Cyanogen bromide digestion. Approximately 600 μg of purified protein was dissolved in 0.7 ml of 70% (v/v) formic acid containing 10 mg/ml of cyanogen bromide (Sigma). The tube was sealed in the presence of N_2, then incubated in the dark for 24 h at room temperature. The reaction was terminated by freezing and lyophilization.

Protease digestion. Purified lens membrane containing 28 mg protein was treated with trypsin (Sigma, type X1) in a weight ratio of 1 : 20 (trypsin:lens membrane). Total reaction volume was 2.0 ml of 0.01 M sodium phosphate buffer, pH 7.4. After incubation at 37°C for 2 h, the reaction mixture was pelleted at $48000 \times g$ for 10 min, followed by immediate solubilization of the pellet in sample buffer. For digestion by carboxypeptidase, the same amount of lens membrane in 2.0 ml of sodium phosphate buffer was preincubated at 37°C for 10 min with 44 μg of the endopeptidase inhibitor pepstatin A (Sigma), followed by incubation with 70 μg of carboxypeptidase Y (Pierce Chem. Co.) at the same temperature. Alternatively, 70 μg of carboxypeptidase Y was preincubated with 44 μg of pepstatin A at 37°C for 10 min, followed by addition of 28 mg of lens membrane. After 24 h, membrane was pelleted and the samples prepared in a manner identical to that used with trypsin-treated samples.

Tryptic peptide mapping. Polypeptides were radioiodinated in the presence of SDS as described by Takemoto et al. [8], followed by SDS-polyacrylamide gel electrophoresis. Individual protein bands were excised from the gels, then digested with trypsin and mapped as previously described [8].

Microsequence and amino acid analysis. Polypeptides prepared by elution from polyacrylamide gels were sequenced as previously described [13]. All membrane components sequenced yielded only one sequence, demonstrating the purity of these polypeptides. These same polypeptides were also treated with 6 N HCl under vacuum and analyzed for amino acid content using a Dionex column under accelerated conditions.

Protein blotting. Antisera to purified bovine M_r 26000 polypeptide were prepared and characterized as previously described [10]. To obtain preabsorbed antiserum, 100 μl of antiserum was diluted to a total volume of 800 μl with sodium phosphate buffer, followed by incubation at 22°C

with 27 mg of purified bovine lens membrane. After 16 h, lens membrane was pelleted at 48000 $\times g$ for 20 min, and the supernatant analyzed for protein concentration. Unabsorbed antiserum was diluted with sodium phosphate buffer to the same protein concentration, and equal volumes of either absorbed or unabsorbed antiserum were used in the passive blotting procedure of Tweton and Iandola [14]. Approximately $1 \cdot 10^6$ cpm of ^{125}I-labeled protein A (Pharmacia) was used to label the polypeptides immobilized on nitrocellulose paper. After exposure of the blots with Kodak XRP-1 film for 2 h, the resulting autoradiographs were scanned with a Joyce, Loebl Model MKIIIC microdensitometer.

Results

Proteolytic analysis

Comparison of SDS-polyacrylamide gel electrophoresis of purified M_r 26000 polypeptide (Fig. 1a) with its cyanogen bromide digest (Fig. 1c) indicates that the major fragment obtained is a polypeptide of approximately 15000. This 15000 fragment or fragment 1 fragment is the only polypeptide resolved by 12.5% polyacrylamide gel analysis of the 26 kDa digest. Analysis of the cyanogen bromide digest of the 26 kDa protein using other gel concentrations (from 10 to 20%) failed to detect any additional fragments (data not shown).

In order to identify the location of this fragment in the polypeptide chain of 26 kDa, intact lens membrane was subjected to trypsin hydrolysis. Previous studies have demonstrated that treatment of intact bovine lens membranes with proteases of differing specificities results in the cleavage of the M_r 26000 polypeptide to a polypeptide of approximately M_r 22000 [15,8]. All these proteases apparently cleave the M_r 26000 polypeptide at sites external to the lipid bilayer, as judged from the water-soluble nature of the proteases and their ability to cleave sites external to the lipid bilayer in other systems [16]. Using trypsin to generate this fragment, we obtain a polypeptide (M_r 23000 in our hands) which, when treated with cyanogen bromide, produces a major component of M_r 11000, rather than M_r 15000 (Fig. 1b and d). These results suggest that the M_r 11000 fragment

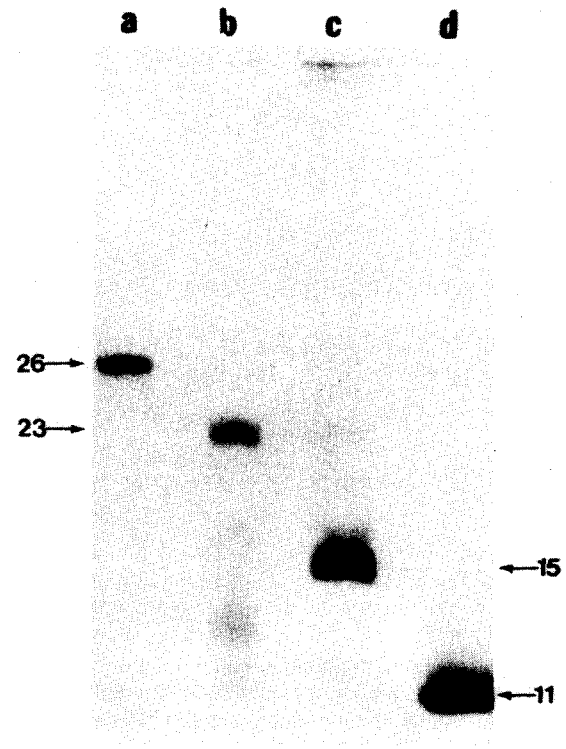

Fig. 1. SDS-polyacrylamide gel electrophoresis of the major cyanogen bromide fragment of the M_r 26000 polypeptide before and after treatment of the intact lens membrane with trypsin. Approximately 10 μg of each polypeptide was resolved on the same 12.5% acrylamide gel, followed by staining with Coomassie brilliant blue R. Trypsin, lysozyme and beta-lactoglobulin were used as markers to determine the approximate molecular weights of the polypeptides denoted by arrows ($\cdot 10^{-3}$). a, Purified M_r 26000 polypeptide; b, purified M_r 23000 fragment obtained after treatment of intact lens membranes with trypsin; c, the major fragment (fragment 1) of M_r 15000 obtained after digestion of purified M_r 26000 polypeptide with cyanogen bromide; d, the major fragment of M_r 11000 obtained after digestion of purified M_r 23000 polypeptide with cyanogen bromide.

results from trypsin cleavage of the M_r 15000 (fragment 1) part of the molecule. To verify this possibility, the purified M_r 15000 and M_r 11000 fragments were radioiodinated and digested completely with trypsin. Comparison of their radioidinated peptide maps indicates very close homology (see peptides 1–6, Fig. 2a and b), indicating similar sequences for these two fragments.

Since at least part of the fragment 1 sequence is exposed and accessible to protease, the C-termi-

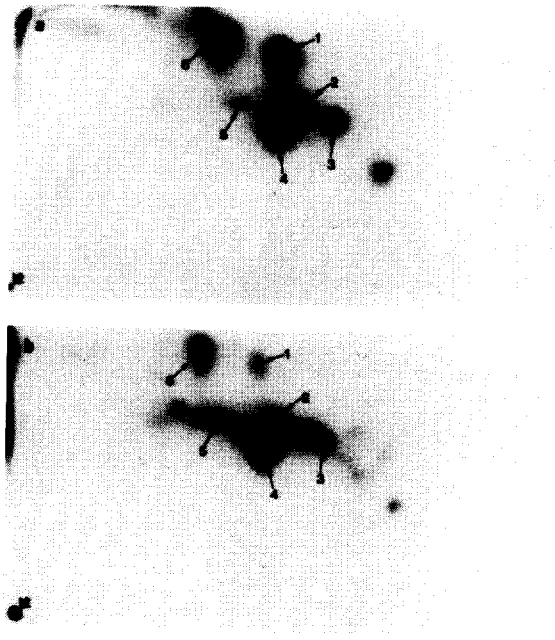

Fig. 2. Analysis of radioiodinated tryptic peptides of the M_r 15000 (fragment 1) and M_r 11000 fragments. See Matrials and Methods for details of radioiodination and trypsin and digestion of proteins. Approximately 50000 cpm were applied to each plate. After two-dimensional resolution of peptides, radioiodinated species were visualized by exposure to Kodak XRP-1 film for 16 h at −70°C. Thick arrows designate origins. First-dimensional electrophoresis was from left to right and second-dimensional chromatography was from bottom to top. a, M_r 15000 fragment (fragment 1); b, M_r 11000 fragment. Peptides are numbered for ease of reference.

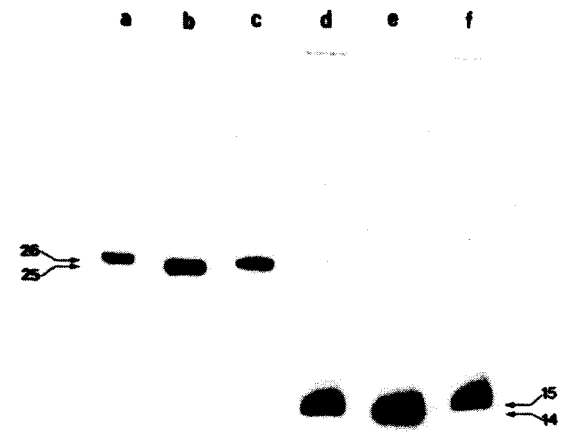

Fig. 3. SDS-polyacrylamide gel electrophoresis of the major cyanogen bromide fragment of M_r 26000 polypeptide before and after treatment of the intact lens membrane with carboxypeptidase Y. Approximately 10 μg of each polypeptide was resolved on the same 12.5% polyacrylamide gel, followed by staining with Coomassie brilliant blue R. Trypsin, lysozyme and beta-lactoglobulin were used as markers to determine the approximate molecular weights of the polypeptides denoted by arrows ($\cdot 10^{-3}$). a, Purified M_r 26000 polypeptide; b, purified M_r 25000 fragment obtained after treatment of intact lens membrane with carboxypeptidase Y; c, purified M_r 26000 polypeptide; d, fragment 1; e, the major M_r 14000 fragment obtained after digestion of the M_r 25000 polypeptide with cyanogen bromide; f, fragment 1.

nal-specific protease carboxypeptidase Y was used to ascertain whether fragment 1 was located at or near the C-terminus of the intact M_r 26000 polypeptide molecule. In agreement with past studies [17], we find that treatment of the M_r 26000 polypeptide in the intact membrane with carboxypeptidase results in a slight reduction in apparent molecular weight, to about M_r 25000 (Fig. 3a–c). This same result was obtained when either carboxypeptidase Y or lens membrane was preincubated with endopeptidase inhibitor pepstatin A prior to the beginning of proteolysis. After treatment with cyanogen bromide, this M_r 25000 component produces a fragment of slightly lower apparent molecular weight (M_r 14000, Fig. 3e) than intact fragment 1 (M_r 15000, Fig. 3d and f).

To verify that the pepstatin-treated carboxypeptidase cleaves only at the C-terminal end of the M_r 26000 polypeptide, the M_r 25000 polypeptide obtained after carboxypeptidase digestion was subjected to microsequence analysis from the N-terminal. Comparison of the first 15 residues with that of intact M_r 26000 polypeptide demonstrated identical sequences (data not shown). These results demonstrate that carboxypeptidase Y cleaves M_r 26000 exclusively at the C-terminus and, therefore, suggest that fragment 1 resides at or close to the C-terminal half of the M_r 26000 polypeptide.

Similar microsequence analysis of the M_r 23000 polypeptide fragment obtained after trypsin treatment of intact membrane demonstrated a small amount of cleavage from the N-terminus of the M_r 26000 polypeptide (Nicholson, B.J., unpublished

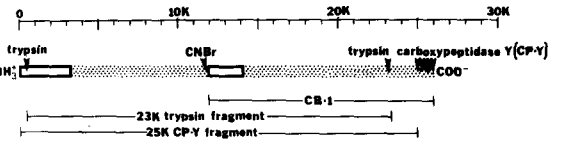

Fig. 4. Diagramatic representation of bovine lens M_r 26000 polypeptide. Boxed regions represent portions of known sequence. Arrows represent enzymatic cleavage sites accessible while the protein is still part of the lipid bilayer, or chemical sites accessible when the protein is solubilized. The trypsin cleavage site nearest the N-terminus was determined from sequence studies of the M_r 23000 trypsin fragment (Nicholson, B., Takemoto, L. and Hunkapiller, M., unpublished data).

data). Based upon all these findings using the various proteolytic and chemical methods of cleavage, the probable cleavage sites for cyanogen bromide, carboxypeptidase Y, and trypsin are summarized in Fig. 4.

Amino acid and N-terminal sequence analysis

The amino acid composition of fragment 1 was determined (Table I). Calculation of the relative polarity [18] from the amino acid compositon demonstrates the increased hydrophilic nature of fragment 1 when compared with the intact M_r 26000 polypeptide. The polarity index of fragment 1 (42.7%) is indicative of soluble proteins, while the polarity index of the M_r 26000 polypeptide (33.0%) previously published [7] is indicative of extremely hydrophobic membrane proteins that can only be solubilized by detergents or organic solvents [18]. These results would suggest that, of the portion of the bovine M_r 26000 polypeptide located outside the lipid bilayer, the majority is in the C-terminal half of the molecule (fragment 1).

While proteolytic analysis suggests that 3000–4000 daltons of the C-terminus of fragment 1 lie external to the lipid bilayer, partial sequence

TABLE I

AMINO ACID COMPOSITION OF FRAGMENT 1

Amino acid	mol% (fragment 1)
Aspartic acid/asparagine	9.7
Threonine	3.1
Serine	6.2
Glutamic acid/glutamine	13.0
Proline	6.7
Glycine [a]	25.6
Alanine	7.6
Cysteine	0.8
Valine	3.3
Methionine	0
Isoleucine	1.3
Leucine	6.8
Tyrosine	1.5
Phenylalanine	3.6
Histidine	6.3
Lysine	4.3
Arginine	0.1
Polarity [b]	42.7

[a] Unusually high values probably due to the presence of free glycine in the electrophoresis buffers.

[b] Total mol% of aspartic acid/asparagine, threonine, serine, glutamic acid/glutamine, histine, lysine and arginine [18].

analysis of fragment 1 suggests that part of fragment 1 may also be externally exposed (Fig. 5). Although fragment 1 contains a short stretch of hydrophobic amino acids from residues 6 to 11, most of the remaining residues are of hydrophilic nature. It is of interest that within the hydrophilic region are two potential tryptic sites (between residues 4 and 5 and 13 and 14) which are not cleaved when intact junctions are digested with trypsin. These hydrophilic domains of fragment 1 may be exposed outside the lipid bilayer, suggesting possible multiple insertion points for the M_r 26000 polypeptide in the lens membrane.

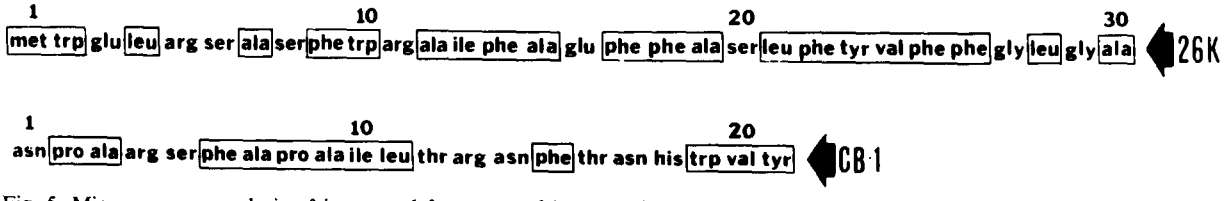

Fig. 5. Microsequence analysis of intact and fragments of lens membrane M_r 26000 polypeptide. The boxes enclose hydrophobic residues. CB-1, fragment 1.

In contrast to fragment 1, partial sequence analysis of the intact bovine M_r 26000 polypeptide demonstrates that this portion of the protein has a much more hydrophobic character. A possible lipid-associated sequence (residues 12–30) containing only one charged residue (glutamic acid, No. 16) has been identified near the N-terminus of both bovine and rat 26 kDa proteins (Nicholson, B.J., unpublished data). This region is analogous to the transmembrane sequences of other intrinsic membrane proteins previously studied [19–22].

Immunoanalysis

To support further the external disposition of at least part of the fragment 1 segment, antisera against purified M_r 26000 polypeptide were first absorbed against intact lens membrane to remove components directed against externally displayed antigenic sites of in situ M_r 26000 polypeptide. Previous immunological localization of this same antisera has demonstrated that it readily binds to purified lens membrane [10]. In addition, reaction of this antisera with a blot of total lens membrane proteins indicated that the M_r 26000 polypeptide is the only component to which the antisera binds (Takemoto, L.J., unpublished data). Fig. 6 demonstrates that the unabsorbed antisera binds well to both purified M_r 26000 polypeptide and to fragment 1. Apparently, fragment 1 must contain major antigenic site(s) that are recognized when the purified M_r 26000 polypeptide was originally injected into rabbits for the production of antisera. This is verified by the protein blot of M_r 26000 and fragment 1 reacted against antisera preabsorbed with intact lens membrane. All of the antigenic reactivities to both fragment 1 and M_r 26000 were removed, strongly suggesting that in the intact membrane at least part of fragment 1 is exposed outside the lipid bilayer.

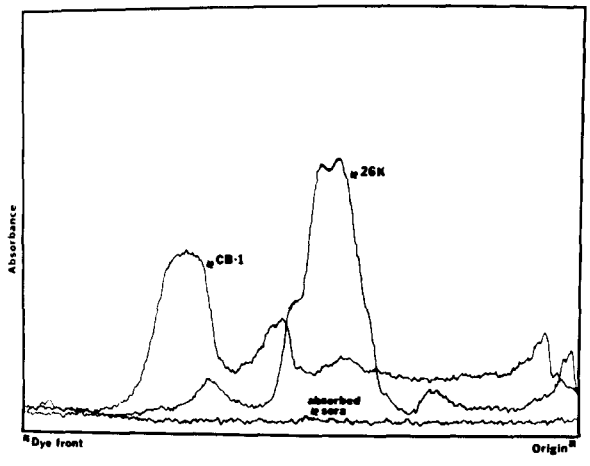

Fig. 6. Treatment of the M_r 26000 polypeptide and (CB-1) fragment 1 blots with M_r 26000 polypeptide antisera and M_r 26000 polypeptide antisera preabsorbed against intact lens membranes. All scans were done at the same instrument settings. The blot scan of M_r 26000 polypeptide plus absorbed antisera is identical to that of fragment 1 plus absorbed antisera and is, therefore, not shown.

Discussion

Based upon a wealth of circumstancial evidence, it is probable that gap junctional structures play a key role in the mediation of cell-cell communication via intracellular pores [3]. Purification of gap junction-like structures from both the lens and liver indicate that the major polypeptide species have similar molecular weights (26000 and 28000, respectively) [9,23]. However, peptide mapping and partial sequences of the proteins from both these tissues indicate no apparent homologies [23], although additional sequencing will be necessary to complete the comparison of the two proteins. While initial results indicate no homology between putative gap junction components from the lens and the liver, there is definitely homology of the putative gap junction component from the same tissue of different species. Peptide mapping studies of the M_r 26000 polypeptide of the lens or M_r 28000 polypeptide of the liver indicate extensive tissue-specific conservation throughout evolution (Ref. 8, and Nicholson, B.J., unpublished data). In addition, comparison of the first 30 residues of bovine and rat lens M_r 26000 polypeptide indicate almost identical sequences (Nicholson, B.J., unpublished data).

These results could indicate a tissue-specificity for the putative gap junction protein. However, morphological differences between the lens junctions and the gap junctions of other tissues has led to the suggestion that the structures in lens may not be subserving the same role as gap junctions. Further analysis of the structure and primary sequence of the major component of these lens junc-

tions (M_r 26000 polypeptide) will be crucial in resolving this issue. Such analysis could determine whether or not the M_r 26000 polypeptide can form the walls of a hydrophilic channel (1.5–2 nm in diameter) connecting the cytoplasms of adjacent cells – a channel which is considered characteristic of gap junctions [3,24,25].

If this were the case, at least part of the M_r 26000 polypeptide would have to be buried in the lipid bilayer. The total amino acid composition of bovine lens M_r 26000 polypeptide shows that this protein has a very hydrophobic character and probably contains large segments embedded in the lipid bilayer. This is also apparent from the partial sequence of intact lens M_r 26000 polypeptide, which contains a stretch of 19 amino acids (residues 12–30) that contains only one charged residue. This segment is a possible candidate for a transmembrane sequence, as has been shown for other membrane proteins [22]. The presence of the hydrophilic amino acids glutamic acid and serine in this sequence would be thermodynamically unfavorable in a hydrophobic environment, but in the case of the M_r 26000 polypeptide they may comprise part of the hypothesized hydrophilic channels of gap junctions. Alternatively, the negative charge of glucose could be neutralized by ionic pairing with a basic residue in an adjacent transmembrane span of the protein.

In contrast, fragment 1 possesses characteristics of an externally located segment of the molecule. Its total amino acid composition is less hydrophobic in character than that of the intact M_r 26000 polypeptide, and is more indicative of soluble proteins. Sequence analysis demonstrates the presence of more hydrophilic amino acids, with an absence of long sequences of hydrophobic residues in the portion sequenced so far. Based upon cleavage with carboxypeptidase Y, the C-terminus of fragment 1 is either at or near the C-terminus of the M_r 26000 polypeptide. With an apparent molecular weight of 15000, fragment 1 must, therefore, occupy approximately half of the C-terminal side of the M_r 26000 polypeptide. Whether or not fragment 1 is either at or near the C-terminus must await further verification by C-terminal determination of both fragment 1 and intact M_r 26000 polypeptide. Repeated attempts by our laboratories to determine the C-terminal residues of either of these two polypeptides has so far proved unsuccessful.

In addition, we have so far been unsuccessful in resolving the remaining CNBr fragment(s) of the M_r 26000 polypeptide. Based upon previously published amino acid analysis [7], there are approximately 3–5 residues of methionine per mol of the M_r 26000 polypeptide. The N-terminal of the intact molecule and the adjacent residue to the N-terminal of fragment 1 account for two of these residues. The remaining residues probably belong to the very hydrophobic N-terminal half of the M_r 26000 polypeptide. It is possible that after CNBr cleavage these segment(s) are insoluble in the SDS-containing buffer used for dissolution. Alternatively, small fragment(s) could be produced that are not resolved by the polyacrylamide gels used in this study.

Reactivity with antisera against M_r 26000 polypeptide indicates that at least part of the fragment 1 segment must be exposed outside of the lipid bilayer. This is supported by the observation that trypsin, which cleaves approximately 3000–4000 daltons from the M_r 26000 polypeptide in situ, removes a similar portion from fragment 1. Based upon this observation, most of the trypsin cleavage is occurring at the C-terminal side of the M_r 26000 polypeptide, although N-terminal sequence analysis of the M_r 23000 fragment produced by trypsin cleavage indicates a small amount of proteolysis also occurs near the N-terminus of the M_r 26000 polypeptide (Nicholson, B.J., unpublished data). Electron microscopic examination of both lens and liver gap junctions before and after trypsin treatment has indicated no changes in junctional morphology in the extracellular region that joins the two opposing lipid bilayer (Nicholson, B.J., unpublished data). This is not a surprising observation, since the size of the trypsin molecule (over 50 angstroms in diameter) is such that it may well be excluded from the 20–30-angstrom wide extracellular gap between membranes and/or the 20-angstrom-wide aqueous channels of the junction. This possibility could explain why the potential cleavage sites identified in the hydrophilic portion near the N-terminus of fragment 1 are not cleaved when intact junctions are treated with trypsin. Together, these observations make it tempting to speculate that proteolysis may be re-

stricted to the cytoplasmic faces of the gap junction, where the C-terminus of the M_r 26000 polypeptide and, therefore, a portion of fragment 1 are exposed. Further studies, including more sequence data and in situ localization with monoclonal antisera will be needed, however, to establish this supposition definitively, and establish further the disposition of the M_r 26000 polypeptide in the membrane.

Acknowledgments

This research was supported by grants from the National Institutes of Health to L.J.T. (EY 02932-04), J.-P.R. (GM 06965), and J.H. B.J.N. was supported by a fellowship from the Ross Foundation.

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