A Fluorescence-Quenching Assay for Measuring Permeability of Reconstituted Lens MIP26

Beth A. Scaglione and David A. Rintoul

A sensitive fluorometric assay for measuring permeability of liposomes containing bovine lens MIP26 has been developed and characterized. Lens membrane proteins were isolated and incorporated into artificial membranes composed of bovine brain phospholipids, sphingomyelin and the fluorescent probe, N-4-nitrobenzo-2-oxa-1,3 diazole phosphatidylethanolamine (NBD-PE). Quenching of the probe with cobalt chloride allowed the measurement of liposome permeability in the presence and absence of lens membrane proteins. Liposomes containing the putative gap junctional polypeptide known as Major Intrinsic Protein 26 (MIP26) were shown to be more permeable than those not containing the MIP26 protein. Permeability was shown to be positively correlated with the amount of MIP26 in the liposomes and with increasing purity of the protein. This method offers a sensitive assay for channel function of the putative junction protein, and provides further evidence that MIP26 from lens membranes is a gap junctional polypeptide. Invest Ophthalmol Vis Sci 30:961–966, 1989

The class of intercellular junctions known as gap junctions is thought to be involved in many aspects of intercellular communication. These include regulation of ion and small molecule flux, modulation of developmental processes, and regulation of growth control. The lens fiber cell gap junction, which is found only in lens tissue and is strongly conserved among many species of vertebrates, has long been known to play a role in mediating ion transport and nutrient waste flux across the cell membranes of this avascular organ. Conversely, development of lens opacification (cataract) has been linked to loss of normal ionic balance. The involvement of the gap junction in maintenance of ionic homeostasis, therefore, has led to its implication as a possible site of early lesions leading to cataractogenesis. Because functions of this channel are difficult to study in situ, various methods have been developed to permit the functional reconstitution of gap junctions into artificial membranes. In 1985 Girsch and Peracchia first reported the development of a method for reconstitution of the junctional protein into liposomes. The method, based upon detection of liposome swelling in the presence of low molecular weight compounds such as sucrose, required a relatively large amount of material and was somewhat insensitive. Subsequently, our laboratory reported the development of an assay based on ascorbate reduction of oxidized cytochrome c trapped in the liposomes. This study also demonstrated that a 26,000 molecular weight membrane protein known as Major Intrinsic Protein 26 (MIP26) was responsible for permeability, as antibodies to synthetic peptides homologous to regions of the MIP26 inhibited ascorbate reduction in the liposomes. Further investigations indicated that changes in pH regulated permeability of the reconstituted gap junction protein. However, reduction of cytochrome c by ascorbate is also sensitive to changes in pH, necessitating the subtraction of appropriate and somewhat variable blanks at each pH studied. Therefore, we saw the need to develop a sensitive assay that would eliminate this complication. In this report we present a reconstitution protocol whereby lens membrane proteins are incorporated into liposomes containing the fluorescent probe NBD-PE, located in both the inner and outer leaflets of the bilayer. Quenching of the fluorescence with cobalt ion allows us to measure the permeability of the liposomes. Some of these data have been previously published in abstract form.

Materials and Methods

Reagents and Materials

Sphingomyelin and NBD-PE were purchased from Avanti Polar Lipids (Pelham, AL). Bovine brain...
Table 1. Phospholipid headgroup composition of bovine brain phospholipids used in preparation of liposomes

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>30</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>28</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>26</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>11</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
</tr>
</tbody>
</table>

Bovine brain tissue was homogenized and extracted as described by Svennerholm. Table 1 shows the composition of bovine brain phospholipid used in these experiments. Reagent grade solvents, buffers, and dialysis tubing were obtained from Allied Fisher Scientific (St. Louis, MO). All other reagents, lipids, and standards were purchased from Sigma Chemical Co. (St. Louis, MO). Octyl glucoside was purchased from Calbiochem (La Jolla, CA), reagents for protein assays were purchased from Pierce Chemical Co. (Rockford, IL), and lenses were purchased from Pel-Freez (Rogers, AK).

Analytical Methods

Bovine brain phospholipids were analyzed by twodimensional thin layer chromatography. Phospholipid concentrations were determined by phosphate assay. Protein concentrations were determined in the presence of 1% SDS, using the bicinchoninic acid method and bovine serum albumin as a protein standard.

Membrane Preparation

Frozen bovine lenses were thawed, decapsulated, homogenized and used to prepare plasma membranes according to the method of Russell et al, with the following exceptions. Crude lens membrane preparations were resuspended in 50% sucrose and sequentially overlaid with 7 ml each of the following sucrose solutions (w/w in 1 mM NaHCO₃, pH = 8.0): 45%, 41%, 37% and 31%. Sucrose gradients were centrifuged at 85,000 g for 4 hr. Membranes at the top three interfaces were removed, dialyzed with NaHCO₃ and pellets at 190,000 g for 1 hr. These membranes were then processed according to the method of Russell et al. Membranes designated as "untreated" were purified merely by the above sucrose gradient method. Those designated as "urea-treated" were purified further by incubation in 7 M urea; those designated as "urea/NaOH-treated" were then incubated in NaOH as described. These membrane preparations were solubilized in octylglucoside (2% w/v in 50 mM Tris/HCl, pH = 7.4) for 12–18 hr at 3°C. Octylglucoside extracts were centrifuged at 8000 g for 15 min using a Beckman microfuge; pellets containing nonsolubilized membranes were discarded. SDS gel electrophoresis was performed and gels were stained as described by Andrews and Chrambach.

Preparation of Gel-Eluted MIP26

A crude lens membrane preparation (corresponding to 1 mg of protein and processed through the sucrose gradient step) was electrophoresed on a 1.5 mm SDS-polyacrylamide gel according to the methods described by Andrews and Chrambach. A small section of the gel was stained with Coomassie Blue and the location of the 26 kD band was determined. The area of the gel containing the 26 kD band was removed, cut into small pieces and placed in Buffer A (0.01 M Tris-acetate, 0.5% SDS w/v, pH = 8.6). A clear portion of the gel was also isolated and treated similarly. These fractions were electrophoresed using an ISCO gel eluting apparatus. Electrode compartments were filled with 75 ml Buffer B (0.04 Tris-acetate, 0.5% SDS, pH = 8.6). Inner compartments were filled with 90 ml Buffer A. Gel fragments were placed in the well and on the bridge of the sample cup. Samples were eluted for 5 hr. Excess SDS was precipitated from the solution using the method of Bok et al.

Reconstitution and Liposome Preparation

Solvent was evaporated from 1 μmol bovine brain phospholipid (Table 1), 1 μmol sphingomyelin and 33 nmol of the fluorescent probe NBD-PE under nitrogen. These ratios and amounts of lipid were used in all experiments described in this report. Lipids were solubilized by addition of 2% octylglucoside (w/v) in 50 mM Tris/HCl (pH = 7.4) to give a final volume of 0.5 ml. Lens membrane protein (in octylglucoside) was added to give the desired protein concentrations. After repeated dispersal through a 27 gauge needle, these suspensions were injected rapidly into 10 ml of 50 mM Tris/HCl (pH = 7.4), placed in dialysis tubing (12,000–14,000 MW cutoff) and dialyzed at 3°C against 100 ml of the same buffer. The dialysis buffer was changed after 4 hr and the volume increased to 1000 ml; dialysis was continued overnight. All fluorescence quenching experiments were performed within 24 hr of the conclusion of this second dialysis.

Preparation of Gel-Eluted MIP26

A crude lens membrane preparation (corresponding to 1 mg of protein and processed through the sucrose gradient step) was electrophoresed on a 1.5 mm SDS-polyacrylamide gel according to the methods described by Andrews and Chrambach. A small section of the gel was stained with Coomassie Blue and the location of the 26 kD band was determined. The area of the gel containing the 26 kD band was removed, cut into small pieces and placed in Buffer A (0.01 M Tris-acetate, 0.5% SDS w/v, pH = 8.6). A clear portion of the gel was also isolated and treated similarly. These fractions were electrophoresed using an ISCO gel eluting apparatus. Electrode compartments were filled with 75 ml Buffer B (0.04 Tris-acetate, 0.5% SDS, pH = 8.6). Inner compartments were filled with 90 ml Buffer A. Gel fragments were placed in the well and on the bridge of the sample cup. Samples were eluted for 5 hr. Excess SDS was precipitated from the solution using the method of Bok et al.

Reconstitution and Liposome Preparation

Solvent was evaporated from 1 μmol bovine brain phospholipid (Table 1), 1 μmol sphingomyelin and 33 nmol of the fluorescent probe NBD-PE under nitrogen. These ratios and amounts of lipid were used in all experiments described in this report. Lipids were solubilized by addition of 2% octylglucoside (w/v) in 50 mM Tris/HCl (pH = 7.4) to give a final volume of 0.5 ml. Lens membrane protein (in octylglucoside) was added to give the desired protein concentrations. After repeated dispersal through a 27 gauge needle, these suspensions were injected rapidly into 10 ml of 50 mM Tris/HCl (pH = 7.4), placed in dialysis tubing (12,000–14,000 MW cutoff) and dialyzed at 3°C against 100 ml of the same buffer. The dialysis buffer was changed after 4 hr and the volume increased to 1000 ml; dialysis was continued overnight. All fluorescence quenching experiments were performed within 24 hr of the conclusion of this second dialysis.

Preparation of Gel-Eluted MIP26

A crude lens membrane preparation (corresponding to 1 mg of protein and processed through the sucrose gradient step) was electrophoresed on a 1.5 mm SDS-polyacrylamide gel according to the methods described by Andrews and Chrambach. A small section of the gel was stained with Coomassie Blue and the location of the 26 kD band was determined. The area of the gel containing the 26 kD band was removed, cut into small pieces and placed in Buffer A (0.01 M Tris-acetate, 0.5% SDS w/v, pH = 8.6). A clear portion of the gel was also isolated and treated similarly. These fractions were electrophoresed using an ISCO gel eluting apparatus. Electrode compartments were filled with 75 ml Buffer B (0.04 Tris-acetate, 0.5% SDS, pH = 8.6). Inner compartments were filled with 90 ml Buffer A. Gel fragments were placed in the well and on the bridge of the sample cup. Samples were eluted for 5 hr. Excess SDS was precipitated from the solution using the method of Bok et al.
Permeability Assay

An aliquot of the liposome preparation was preincubated in either 7.4 μM lasalocid (a divalent cation ionophore, dissolved in ethanol) or ethanol alone at 25°C in the cuvette chamber of a SPEX 1902 Fluorolog equipped with a magnetic stirrer. The fluorescence intensity was measured at 533 nm (band pass = 20 nm) with excitation at 467.5 nm (band pass = 10 nm). Cobalt chloride was added to a final concentration of 1.75 mM and fluorescence intensity was again determined after 2 min. Final concentrations (in 2.3 ml of 50 mM Tris/HCl, pH = 7.4) in the cuvette were as follows: 12.4 nM bovine brain phospholipid, 12.4 nM sphingomyelin, 0.41 μM NBD-PE and 7.4 μM lasalocid. Lens membrane protein concentrations ranged from 1.4 to 17.3 μg/ml. The final concentration of ethanol in all cuvettes was 1.1%.

Relative fluorescence quenching (RFQ) was calculated as follows, where I = intensity:

\[
RFQ = \frac{I(\text{total}) - I(+\text{coLab})}{I(\text{total}) - I(+\text{lasalocid})} \quad (1)
\]

Thus, for unilamellar vesicles with fluorescent NBD-PE distributed equally between the inner and outer lipid monolayers, “relative fluorescence quenching” theoretically equals approximately 0.5 for vesicles that are impermeable in the absence of lasalocid. This value corresponds to quenching by cobalt of roughly 50% of the NBD-PE fluorescence (that originating from the outer lipid monolayer) in the absence of ionophore and complete quenching, again without any spectral shift, is seen upon addition of the ionophore. In preliminary experiments, A23187, another divalent cation ionophore, was tested; the level of quenching achieved was not as significant as was achieved with lasalocid (data not shown). These results indicate that lipid vesicles prepared as described are impermeable with respect to cobalt ion, unilamellar, and that the NBD-PE fluorophore is evenly distributed on both sides of the membrane bilayer.

Results

Cobalt Quenching of NBD-PE

Figure 1 shows the excitation and emission spectra of NBD-PE fluorescence in the absence of CoCl₂ and upon addition of CoCl₂ and lasalocid. Addition of 0.5 mM CoCl₂ quenches approximately 35% of the fluorescence; increasing the cobalt concentration to 1.75 mM quenches approximately 50% of the fluorescence. Cobalt addition does not shift the peak of either the excitation or emission spectra. The excitation maximum was 467.5 nm and emission maximum was 533 nm. Increasing the cobalt concentration to 3.5 mM results in little additional quenching (data not shown). These results indicate that approximately half of the NBD-PE is accessible to externally added CoCl₂ in the absence of ionophore. This represents the fraction of the probe in the outer monolayer of the liposome bilayer. Nearly complete quenching, again without any spectral shift, is seen upon addition of the ionophore. In preliminary experiments, A23187, another divalent cation ionophore, was tested; the level of quenching achieved was not as significant as was achieved with lasalocid (data not shown). These data indicate that lipid vesicles prepared as described are impermeable with respect to cobalt ion, unilamellar, and that the NBD-PE fluorophore is evenly distributed on both sides of the membrane bilayer.

Purification of MIP26

The SDS-PAGE of untreated bovine lens membrane proteins, those treated with 7 M urea, and those treated with urea and 0.1 mM NaOH, as described by Russell et al., is shown in Figure 2. As can be seen in Lane 1, MIP26 is the major protein even in crude membrane preparations. Lane 3 shows that MIP26 is highly enriched in the urea/NaOH treated membranes.
Fig. 2. SDS-PAGE of bovine lens membrane proteins purified by the method of Russell et al.1) Fifty micrograms of protein was applied to each lane, electrophoresis and staining was performed as described in Materials and Methods. Migration positions of marker proteins of designated molecular weights are shown on the left. Lane 1—untreated membranes, purified through sucrose gradient step only; Lane 2—urea-treated membranes; Lane 3—urea/NaOH-treated membranes.

Fig. 3. Relative fluorescence quenching of liposome preparations containing untreated, urea-treated, and urea/NaOH-treated bovine lens membrane protein. Membranes were purified and extracted as described in Materials and Methods. After solubilization and re-constitution of 200 µg of membrane protein from each of these preparations, fluorescence quenching of NBD-PE by 1.75 mM CoCl₂ was measured and calculated as described. After dilution into buffer, each assay cuvette contained 5.8 µg of protein. Data shown are means of triplicate experiments; error bars indicate the standard errors.

Fig. 4. Relative fluorescence quenching of liposome preparations containing 200, 400 and 600 µg of urea/NaOH-purified membranes. Membranes were purified, extracted, and solubilized as described in Materials and Methods. Liposomes containing purified lens membrane proteins were prepared as described in Materials and Methods. After dilution into 50 mM Tris/HCl (pH = 7.4) at 25°C, quenching of NBD-PE fluorescence by 1.75 mM CoCl₂ was measured as described. For preparations labeled 200, 400 and 600 µg, the final protein amounts per permeability assay were 3.8, 11.5 and 17.3 µg of membrane protein respectively. Relative fluorescence quenching was calculated as described in Materials and Methods. Data shown are means of triplicate determinations; error bars indicate standard errors.

Discussion

The experiments presented in this report indicate that MIP26 is a channel-forming protein, that functional channel proteins can be purified electrophoretically in the presence of SDS, and that cobalt quenching of NBD-PE fluorescence can be used to assess channel functions. Previous workers have concluded that MIP26 is a channel-forming protein, and
a component of the fiber cell gap junction, based on both immunocytochemical evidence\textsuperscript{24,27} and reconstitution assays.\textsuperscript{11-13,28} It should also be noted that functional reconstitution of electroeluted material (Figure 5) provides further evidence that MIP26 monomers can reassociate to yield a functional channel, which is thought to be a tetramer of the MIP26 protein.\textsuperscript{3,29} Preliminary electron microscopic evidence (data not shown) indicate that the liposomes formed by our technique are 240–260 nm in diameter. This is in good agreement with previous observations on liposomes obtained with this technique.\textsuperscript{25} Based on this information, we can calculate that preparations containing 50 \( \mu \)g of electroeluted MIP26 would have a lipid/protein mole ratio of approximately 1000/1, and therefore would have approximately 120–130 channels (tetramers of the MIP26 monomer) per liposome. It is not possible to discern from the data presented whether all of these channels are functional. However, even if we assume that only a fraction of the channels were actually functioning to allow cobalt into the liposome, it is probable that each liposome would contain several serviceable channels. Other assumptions are required when similar calculations are made for protein preparations of lower purity. However, it seems clear that most of the liposomes should contain many channels, even if the MIP26 preparations were only 70–80\% pure.

It should also be noted that quenching of NBD-PE by cobalt is nonlinear with respect to protein concentration in the reconstituted liposomes (Figs. 4, 5). Several explanations for this deviation from linearity are possible. Since the rate of permeation of cobalt into the liposomes is an exponential function, it is possible that the time period chosen (2 min in all cases) is too long, and that measurements of the rate of quenching might be a better way to assess linearity of the response. Earlier evidence from our laboratory, using the rate of cytochrome C reduction as the assay parameter, indicated that initial rates of permeation were proportional to the protein concentration used in the reconstitution.\textsuperscript{12} Since the isolation and reconstitution methods described in this earlier report are essentially identical to those used in the experiments described in the current report, it is likely that measurements of rates of quenching would also be linear with respect to protein concentration. In fact, such measurements have been made and this prediction is borne out (Scaglione and Rintoul, manuscript in preparation).

Finally, it appears that MIP26 alone is sufficient to form a functional channel, based on the evidence presented in Figure 5. This final conclusion is important in light of recent evidence that a protein of 70 kD, which is immunocytochemically related to MIP26, is also found in lens junctional regions.\textsuperscript{30} Since this 70 kD protein is not found in nonjunctional regions of fiber cell membranes, these authors proposed that it might play a role in junction activity or structure. Our data would indicate that this protein is not required for the permeability function of the MIP26; it may be important in regulation of permeability, intercellular recognition, or perhaps other, unrecognized functions of the lens fiber cell junction. Likewise, our data do not rule out a channel function for the MP70 protein, they merely indicate that channel function of the MIP26 does not require the presence of MP70.

In conclusion, we report the development of a novel and sensitive assay to detect permeability of vesicles. We have used this assay to study the channel-forming protein MIP26 from bovine lens, using as little as 1.4 \( \mu \)g of electrophoretically purified MIP26 per assay. Increasingly purified preparations of the solubilized membrane protein, or gel-eluted protein (pure MIP26) contain increasingly effective channel-forming activities, consistent with the hypothesis that this channel-forming ability is associated with MIP26 protein.

**Abbreviations**

MIP26 — Major Intrinsic Protein, 26 kD, from lens fiber cell membrane

MP70 — Membrane protein, 70 kD, from lens fiber cell membrane

**Fig. 5.** Relative fluorescence quenching of liposomes containing gel-eluted MIP26. Membranes were purified and extracted as described in Materials and Methods. Preparative SDS-PAGE and gel elution were performed as described. Liposomes were prepared which contained either MIP26 (50 and 100 \( \mu \)g protein) or an equivalent extract from a portion of the gel which contained no protein. Relative fluorescence quenching in the presence of 1.75 mM CoCl\textsubscript{2} was measured and calculated as described in Materials and Methods; protein content per assay was 1.4 and 2.9 \( \mu \)g of protein, respectively, for the MIP26-containing preparations. Data shown are means of triplicate determinations; error bars indicate standard errors.
NBD-PE—N-4-nitrobenzo-2-oxa-1,3 diazole phosphatidylethanolamine
SDS-PAGE—Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis

Key words: MIP26, gap junction, reconstitution, liposomes, fluorescence quenching

Acknowledgments

We wish to thank Dr. R. Welti of this Division for helpful discussions, Dr. James Guikema of this Division for helpful discussions and assistance regarding ionophores and electrophoresis, and Linda Edelman and Kara Cundy for assistance with electrophoresis and gel elution.

References