DETERMINATION OF THE PURITY OF MEMBRANE FRACTIONS ISOLATED FROM FLOUNDER (*Pleuronectes americanus*) GILLS BY A SELECTIVE BIOTIN EXPOSURE TECHNIQUE

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In the present study a technique was developed for determining the purity of membrane preparations isolated from flounder gills for use in permeability studies (see accompanying paper). Typically the purity of a membrane preparation is determined from the enrichment of marker enzymes that are specific to one membrane region (Kinne-Saffran, E. and Kinne R.K.H., Methods in Enzymology 172: 3-17, 1989, Kinne-Saffran, E. and Kinne R.K.H., Methods in Enzymology 191: 450-468, 1990). However, appropriate enzymes must be identified whenever a new and previously undocumented tissue is investigated. This was the case for the apical membrane of flounder gills. Our goal was to design a reliable and versatile membrane purity assay that could also be used to confirm enrichment values determined using putative marker enzyme activities.

Our approach in determining membrane purity was to label the apical side of the intact epithelium with a chemical probe that could then be used to determine the abundance of the labeled membrane proteins in the final preparation. We chose a biotin derivate with an amino group-specific coupling reagent and a sulfuric acid group primarily because of its relatively substantial molecular weight (556.8 dalton) and high hydrophilicity, which in theory should allow biotin to reach the membrane and binding sites from the outside without penetrating through the membrane. Another favorable property of biotin is its strong affinity for avidin, which in turn can be coupled to an indicator enzyme such as peroxidase.

Flounders (*Pleuronectes americanus*) were obtained from Huntsman, Canada, between July 15 and August 31. After sacrificing the animals the heads were removed from the specimens and immediately put on ice. The branchial arches of the gills were removed and stored on ice in artificial sea water (ASW) until biotinylation. Half of the gill samples were incubated with biotin-amidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester (Sigma product # B 1022) prepared as 0.5mg/ml in 100 ml of ASW. The other half were incubated for an equal period of time in 100 ml ASW only. The latter served as a control. The samples were incubated for either 2, 4, or 30 minutes and then washed three times in 20 ml ASW including 20mM Tris-HEPES to remove excess biotin reagent. Following biotinylation, the gill filaments were removed from the branchial arches and then homogenized in a cooled blender.

A putative luminal membrane fraction was prepared through a combination of differential precipitation using 30 mM $CaCl_2$ and differential centrifugation steps as described by Booth and Kenny (Biochem. J. 142: 575-581, 1974). Also a plasma membrane fraction enriched in basolateral membranes was prepared from the first low speed pellet derived from the homogenized tissue after incubation with $CaCl_2$ (Heidrich, H.G. et al. J. Cell Biol. 54: 232-245, 1972). The protein content of the membrane fractions was determined by the method of Lowry et al. (J. Biol. Chem. 193: 265-275, 1951) using bovine serum albumin as standard. 5 µl of

biotinylated membranes were pipetted into wells of a 96-well plate, mixed with 40 µl of coating buffer, and allowed to stand overnight at 4°C. Coupling of avidin and the subsequent washing steps were performed according to a protocol established in our laboratory (Kipp, H. et al., J. Biol. Chem, submitted).

Biotin content in the membrane fractions was determined with a peroxidase activity assay using the Sigma 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma product # T 8665). Peroxidase assays were measured at room temperature using an ELISA spectrophotometer. Sample dilutions ranged from 1:10 to 1:40 in ASW solution. After pipetting samples into the ELISA sample tray the TMB substrate was added and the level of extinction at 655nm was recorded at one minute intervals up to 30 minutes depending on the reaction speed of the experiment. Samples were mixed before each reading. Biotin content was calculated as the slope of the initial velocity along the peroxidase time course.

Table 1 shows the results of the peroxidase assays. All three incubation times yielded average values of ϵ greater than 2.5, indicating a relative enrichment of biotin in the putative luminal fraction with respect to the basolateral fraction.

Table 1. Biotinylation of putative apical and basolateral membrane fractions isolated from flounder gills

Sample	Incubation Time (min)	Slope BBM (m_a)	Slope PM (m_b)	ϵ	±SD
PER.F05a	2.000	12.660	2.212	5.723	
PER.F05b	2.000	19.905	3.444	5.780	σ
				5.751	**0.040**
PER.F05c	4.000	7.722	3.245	2.380	
PER.F05d	4.000	12.088	4.538	2.664	σ
				2.522	**0.201**
PER.F03	30.000	4.690	0.896	5.234	
PER.F04	30.000	4.450	1.120	3.973	σ
				4.604	**0.892**

The slope of apical and basolateral fractions, ma and mb respectively, are listed in the third and fourth column. The enrichment factor, expressed as expressed as ϵ = ma / mb, is listed in the fifth column with the average value of ϵ for each set of experiments printed in bold face. Standard deviation for ϵ is listed in the sixth column.

An enrichment factor of 5.75 was observed in samples incubated for 2 minutes, which fits well with the six-fold enrichment observed in the same membrane fraction using an ADPase enzyme activity assay (Table 2). No significant peroxidase activity was found in membrane fractions isolated from non-labeled tissue.

Table 2. Enrichment and specific activity of marker enzymes in the membrane fractions isolated from flounder gills

	ADPase	Na,K-ATPase
Homogenate	2.52 ± 0.19	3.02 ± 0.26
Putative Apical Membrane Fraction	15.62 ± 0.93 (6.20)	2.94 ± 0.18 (0.97)
Basolateral Membrane Fraction	2.08 ± 0.20 (0.82)	10.29 ± 0.96 (3.40)

The enzyme activities are given in µmoles/hr/mg protein. Mean values of 3 experiments ± SD are shown. Numbers in brackets give the enrichment factor, which represents the ratio between the activity in the membrane fractions and the activity in the homogenate.

Our studies using different incubation times indicate that there is apparently effective labelling of the luminal membrane surface in about two minutes. As indicated by the decrease in the labelling ratios, longer incubation leads to an increased labelling of the basolateral membrane fraction suggesting penetration of the biotin conjugate through leaky tight junctions or into leaky cells.

The Na,K-ATPase marker enzyme used in this study for the basolateral membrane is well established in its basolateral localization from both functional and comparative studies. The ADPase was used in several tissues as luminal marker enzyme based on histochemical studies by Novikoff et al. (*In*: The Interpretation of Ultrastructure, Plenum Press, pp. 149-192, 1962). In our laboratory, for example, the enzyme was useful to characterize the luminal membranes of the renal thick ascending limb of Henle's loop (Kinne-Saffran, E. and Kinne, R.K.H., J. Membrane Biol. 159: 231-238, 1997). With regard to the flounder gill membrane fractions the specific activity of the ADPase also appears to estimate the amount of luminal membranes in the apical membrane fraction correctly since the biotin labelling ratio is only slightly lower than the ratio obtained comparing the marker enzymes. This slightly lower labelling with biotin of the apical membrane might be due to a limited access to the apical membrane surface which is easy to comprehend when the complex morphology of the gill is considered (Figure 1).

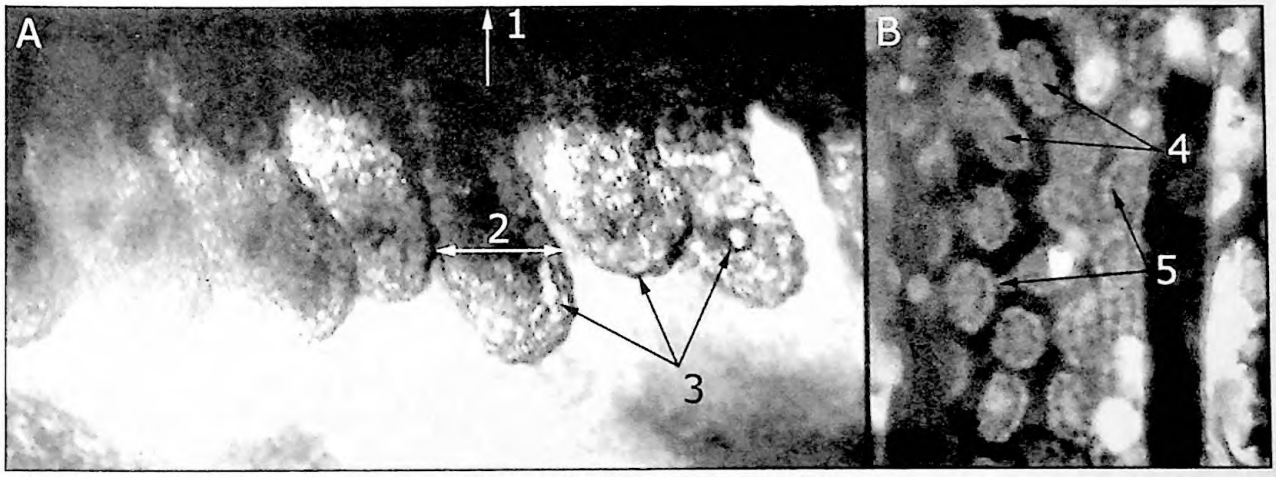

Figure 1. Confocal optical images of the flounder gill, taken at 200 times magnification (A) and 100 times magnification (B) respectively, using flounder gill tissue mounted on a glass dissecting slide. A: lateral view of secondary lamella (2) stemming from the primary lamella (1). Individual pavement cells are discernable (3). B: Cross section of secondary lamella showing a monolayer of epithelial cells (5) surrounding what is presumably the capillary blood space (4). Overlap between adjacent secondary lamella may prevent or delay binding of biotin to those overlapped regions of the apical membrane.

The biotin-labelling technique was initially introduced in cell culture to label apically located proteins (Lisanti M.P. et al., J. Cell Biol. 109: 2117-2127, 1989) and applied at the MDIBL to the operculum of the killifish (Lankowski, A. et al., MDIBL Bulletin 41:77-78, 2002). However, in these studies no direct comparison with marker enzymes was performed. Our experiments indicate that even when the cell surface morphology is very complex biotinylation can be used to label apical membranes and to determine their enrichment in membrane fractions obtained from such tissue. There is also growing evidence that, at a given time, plasma membrane components

can reside to a significant extent in other—mostly intracellular—vesicles en route to the apical or the basolateral membrane (Kipp, H., see above, Peters, K.W. et al., MDIBL Bulletin 41: 81-83, 2002). Thus, compared to the use of marker enzymes extracellular labelling with defined compounds that do not penetrate plasma membranes or the tight junctions might also be of advantage—as long as care is taken that the cells to be labelled remain intact.

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