Radiation-inactivation studies on brush-border-membrane vesicles

General considerations, and application to the glucose and phosphate carriers

Richard BÉLIVEAU,*§ Michel DEMEULE,* Hanane IBNOUL-KHATIB,* Michel BERGERON,†
Guy BEAUREGARD‡ and Michel POTIER‡

*Laboratoire de Membranologie Moléculaire, Département de Chimie, Université du Québec à Montréal, C.P. 8888, succ. A, Montréal, and Groupe de Recherche en Transport Membranaire, Université de Montréal, and

†Département de Physiologie, Université de Montréal, ‡Section de Génétique Médicale, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec H3C 3P8, Canada

Radiation-inactivation studies were performed on brush-border-membrane vesicles purified from rat kidney cortex. No alteration of the structural integrity of the vesicles was apparent in electron micrographs of irradiated and unirradiated vesicles. The size distributions of the vesicles were also similar for both populations. The molecular sizes of two-brush-border-membrane enzymes, alkaline phosphatase and 5'-nucleotidase, estimated by the radiation-inactivation technique, were 104800 ± 3500 and 89400 ± 1800 Da respectively. Polyacrylamide-gel-electrophoresis patterns of membrane proteins remained unaltered by the radiation treatment, except in the region of higher-molecular-mass proteins, where destruction of the proteins was visible. The molecular size of two of these proteins was estimated from their mobilities in polyacrylamide gels and was similar to the target size, estimated from densitometric scanning of the gel. Intravesicular volume, estimated by the uptake of D-glucose at equilibrium, was unaffected by irradiation. Uptake of Na⁺, D-glucose and phosphate were measured in initial-rate conditions to avoid artifacts arising from a decrease in the driving force caused by a modification of membrane permeability. Na⁺-independent D-glucose and phosphate uptakes were totally unaffected in the dose range used (0–9 Mrad). The Na⁺-dependent uptake of D-glucose was studied in irradiated vesicles, and the molecular size of the transporter was found to be 288000 Da. The size of the Na⁺-dependent phosphate carrier was also estimated, and a value of 234000 Da was obtained.

INTRODUCTION

Phosphate transport by brush-border-membrane vesicles from kidney cortex has been studied extensively [1–3]. However, the identity of the carrier molecule and the molecular mechanism of transport still remain unknown in their details. A phosphate-binding proteolipid named phosphorin has been purified [4], and it was suggested that it could be a part of the transport system. Reconstitution has been attempted, but the phosphate-carrier system does not reconstitute very well [5]. The main problem associated with a traditional purification approach, in addition to the reconstitution itself, is that co-transport proteins represent a very small percentage of the total membrane proteins [6].

Radiation inactivation has been used to study the size and structure of both soluble and membrane-bound proteins [7,8]. The main advantage of the radiation-inactivation method is that it permits the measurement of the size of functional units *in situ*, without the need for isolating the protein, which might alter its subunit assembly or functional properties. This method has recently been applied to the brush-border-membrane vesicles to study peptidases [9] and other membrane-bound enzymes [10]. There are, however, very few studies concerning its applicability to transporters from brush-border membranes. The method has been applied to the glucose co-transport system [11–13], for which a tetramer structure was proposed.

The first objective of the experiments reported here is to characterize the effects of irradiation on renal brush-border-membrane vesicles. Experiments showed that this method of study can be applied to brush-border-membrane vesicles to obtain molecular characteristics of transport systems. In the second part, the method is applied to the glucose and phosphate carriers.

EXPERIMENTAL

Membrane vesicles were prepared from rat kidney cortex by the MgCl₂ precipitation method [14]. The final pellet containing brush-border membranes was resuspended in a cryoprotective medium consisting of 0.15 M-KCl, 5 mM-Tris/Hepes, pH 7.5, 14% (v/v) glycerol and 1.4% (v/v) sorbitol. Protein was measured by the method of Bradford [15], by the Bio-Rad protein assay. The frozen samples (–78 °C) were irradiated in a Gammacell model 220 instrument at a dose rate of about 2 Mrad/h. Appropriate controls for non-irradiated preparations were run concurrently under the same conditions, but without irradiation. The empirical eqn. (1) was used to relate molecular size to $D_{37,t}$, the radiation dose (in Mrad) necessary to inactivate an enzyme or a transporter to 37% of its initial value, and to t , the temperature (in °C) [16]:

$$\log M_r = 5.89 - \log D_{37,t} - 0.0028 t \quad (1)$$

§ To whom all correspondence should be addressed.

Fig. 1. Electron micrographs of brush-border-membrane vesicles

(a) Control, freeze-thawed, unirradiated vesicles. (b) Irradiated vesicles (8.5 Mrad) from the same vesicle preparation.

Fig. 2. Distribution of vesicles according to their size

Bar histogram derived from electron micrographs of the same brush-border-membrane vesicles as those shown in Fig. 1. The radii of 240 non-irradiated (■) and 385 irradiated (□) vesicles were measured.

$D_{37,i}$ values were obtained from a semi-logarithmic plot of uptake versus dose by using a least-squares fit. Unless otherwise noted, the errors quoted in the text and Tables are standard deviations.

A rapid-filtration technique [17] was used for uptake studies. Incubation media contained 5 mM-Tris/Hepes buffer, pH 7.5, 14% glycerol, 1.4% sorbitol, 150 mM-KCl or 150 mM-NaCl, and 200 μ M- 32 P] P_i (8 μ Ci) or 50 μ M-D- 3 H]glucose (0.3 μ Ci). Na^+ uptake was measured in 5 mM-Tris/Hepes buffer, pH 7.5, containing 14% glycerol, 1.4% sorbitol and 150 mM- 22 NaCl. Alkaline phosphatase and 5'-nucleotidase were assayed by standard procedures [18]. The mean enrichment of alkaline phosphatase, compared with the cortex homogenate, as measured by hydrolysis of *p*-nitrophenyl phosphate was 11.8-fold. Uptake studies performed in triplicate at 25 °C were initiated by the addition of 80–120 μ g of brush-border-membrane protein. After

Fig. 3. Radiation-inactivation curves for alkaline phosphatase and 5'-nucleotidase in membrane vesicles

Membrane vesicles were irradiated at the indicated doses, and phosphatase (a) and 5'-nucleotidase (b) activities were measured with *p*-nitrophenyl phosphate or AMP as substrate ($n = 4$).

incubation, the reactions were stopped by the addition of 1 ml of ice-cold stop solution. The stop solutions used for the phosphate- and Na^+ -transport experiments contained 5 mM-Tris/Hepes buffer, pH 7.5, 150 mM-KCl, 14%

Fig. 4. Polyacrylamide-gel electrophoresis of irradiated membrane vesicles

Vesicles were irradiated and electrophoresis was performed in the presence of SDS, under reducing conditions (a); 80 μg of protein was used per lane. Staining was done with Coomassie Blue. Lane 1 shows molecular-mass standards. Other lanes show samples irradiated at doses of 0 (lane 2), 2.2 (lane 3), 4.4 (lane 4), 6.6 (lane 5), 8.8 (lane 6) and 11 (lane 7) Mrad. The gels were scanned with a Gelman densitometer at 550 nm (b). The positions of protein α and protein β are indicated. The absorbances of the peaks for proteins α (■) and β (□) were plotted as a function of the dose of irradiation (c), to estimate the target size, by using eqn. (1). The size of protein α was 355000 and protein β 167000 Da ($n = 2$).

glycerol and 1.4% sorbitol. This stop solution was found to be effective on the basis of the lack of phosphate uptake or release by the vesicles when the membranes which were incubated in the uptake medium for various times were left for 1 min in the stop solution, before being filtered, as compared with membrane vesicles that were filtered immediately after addition of the stop solution. Addition of arsenate, a competitive inhibitor of phosphate, to the stop solution caused no change. High ionic strength and low temperature were, however, found to be critical for reproducible results (not shown). For glucose-transport experiments, the stop solution contained 5 mM-Tris/Hepes buffer, pH 7.5, 150 mM-NaCl, 14% glycerol, 1.4% sorbitol and 1 mM-phlorizin. The vesicle suspension was applied to 0.45 μ m-pore-size filters under vacuum. Filters were washed with 8 ml of ice-cold stop solution and processed for liquid-scintillation counting.

Samples of vesicles were fixed for 20 min with 2.5% glutaraldehyde solution, post-fixed for 2 h at 4 °C in buffered 1% OsO₄ solution, and impregnated at 37 °C in aqueous OsO₄ solution for 1 day. The tissues were washed, dehydrated and embedded in an epoxy resin (Epon). Thick sections (0.3 μ m) were made on a Reichert microtome and examined with a standard transmission electron microscope (Philips 300) at 80–100 kV.

Proteins were analysed by SDS/polyacrylamide-gel electrophoresis [19]. Samples (80 μ g of protein) were dissolved in 50 mM-Tris/HCl, pH 6.8, containing 1% SDS, 10% glycerol, 10 mM-mercaptoethanol and 0.00125% Bromophenol Blue. Gels contained 30% acrylamide and 0.8% bisacrylamide. Protein bands were stained with Coomassie Blue in 30% methanol/7% acetic acid. The gels were scanned at 550 nm with a Gelman densitometer.

RESULTS

Structural integrity of irradiated vesicles

Membrane vesicles from irradiated and non-irradiated populations were examined by electron microscopy. As shown in Fig. 1, there was no alteration of vesicle structure for an irradiated population of vesicles, compared to the control. Fig. 2 shows the distribution of vesicles according to their size. The dominant population had a radius of 50 nm, and most of the vesicles had a radius in the range 25–100 nm. This distribution was similar in control and irradiated samples. These results indicate that irradiation itself does not cause a physical alteration leading to major changes in the size of irradiated vesicles.

Molecular-size determination of brush-border-membrane enzymes

In order to establish the validity of the irradiation method in our system, the inactivation of two intrinsic enzyme markers was investigated. For alkaline phosphatase, the inactivation curve gave a molecular size of 104800 ± 3500 Da (Fig. 3a). This value is similar to that reported for calf kidney alkaline phosphatase (115000) estimated by the same method [11] and for isolated alkaline phosphatase [20] purified by SDS/polyacrylamide-gel electrophoresis (120000). The estimated molecular size of 5'-nucleotidase was 89400 ± 1800 Da (Fig. 3b). This is similar to the value estimated in

fibroblasts (80000) [21], but somewhat higher than that found in rabbit kidney (58000) [12].

Gel electrophoresis of membrane proteins

Membrane proteins were separated by SDS/polyacrylamide-gel electrophoresis. As shown in Fig. 4(a), there was no alteration of the general protein profile of the gel, as the radiation was increased from 0 to 11 Mrad (at -78 °C). Proteins with molecular masses higher than 60000 Da are progressively destroyed by the irradiation. Proteins with molecular masses lower than 60000 were much less affected by the irradiation procedure. We have chosen two proteins as examples for validating the irradiation method of estimating molecular masses. The electrophoresis gel was scanned and the peaks corresponding to proteins α and β were located (Fig. 4b). The heights of these peaks were measured and plotted as a function of the irradiation dose (Fig. 4c). This allowed the estimation of the target size of protein α (355000 Da) and protein β (167000 Da). A calibration plot obtained with standard proteins was used to estimate the molecular mass of the same two proteins: 345000 Da for protein α and 147000 Da for protein β . The results obtained by the radiation-inactivation method are thus in good agreement with those obtained from a SDS/polyacrylamide-gel-electrophoresis calibration plot. This confirms the validity of this method of measuring the molecular size of proteins in minimally altered renal brush-border-membrane vesicles.

Radiation inactivation of glucose- and phosphate-transport systems

In view of the experimental procedure related to the inactivation, we investigated the effects of the freeze-thaw cycle and cryoprotective medium to detect any alteration of the transport properties that could arise in the non-irradiated control vesicles. Fig. 5 shows that the Na⁺-gradient-dependent uptake of phosphate obtained with these vesicles was similar to that obtained with native vesicles [22], with similar overshoots, time courses of influx and intravesicular volumes. Intravesicular volumes

Fig. 5. Time course of phosphate uptake by brush-border-membrane vesicles

The vesicles were prepared as described in the text. Phosphate uptake was measured from a cryoprotective solution containing 0.2 mM-[³²P]P_i (8 μ Ci), 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 0.15 M-NaCl (□), or from a solution containing 0.2 mM-[³²P]P_i, 100 mM-mannitol, 5 mM-Tris/Hepes, pH 7.5, and 0.15 M-NaCl (■) ($n = 3$).

Table 1. Effect of irradiation on intravesicular volume and Na⁺ uptake

Membrane vesicles were irradiated as described in the Experimental section. Uptake of glucose at equilibrium (60 min) was measured by incubating the vesicles in a solution containing 14% glycerol, 1.4% sorbitol, 5 mM-Hepes/Tris, pH 7.5, 50 μ M-D-[³H]glucose and 0.15 M-NaCl. Na⁺ uptake was measured in 14% glycerol, 1.4% sorbitol, 5 mM-Hepes/Tris, pH 7.5, and 0.15 M-²²NaCl ($n = 2$).

Irradiation dose (Mrad)	Intravesicular volume (μ l/mg of protein)	Na ⁺ uptake (pmol/5 s per μ g of protein)
0	2.526 $\pm$ 0.474	36.81 $\pm$ 2.99
3	2.614 $\pm$ 0.351	35.43 $\pm$ 3.54
6	2.345 $\pm$ 0.418

Fig. 6. Initial rates of phosphate and D-glucose uptake by brush-border-membrane vesicles

Vesicles were isolated and irradiated as described in the Experimental section. The membranes were exposed to different doses: $\square$, 0 Mrad; $\blacksquare$, 3 Mrad; $\bullet$, 6 Mrad. Phosphate uptake (a) was measured as described in Fig. 5. D-Glucose uptake (b) was measured from a solution containing 42 μ M-[³H]glucose, 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl ($n = 3$).

were estimated from the values of D-glucose uptake at equilibrium, in the presence of NaCl. D-Glucose was chosen because this substrate does not bind to renal brush-border membranes [23]. Its equilibrium value thus represents the actual intravesicular space that it occupies. As shown in Table 1, there was no effect of irradiation on

Fig. 7. Determination of the molecular size of the D-glucose carrier

(a) D-Glucose uptake was measured under the same conditions as in Fig. 6. (b) Na⁺-dependent D-glucose transport was calculated as the difference between the uptakes from incubation media containing Na⁺ ($\square$) and K⁺ ($\blacksquare$). The results are expressed as log of the percentage of activity remaining ($n = 3$).

the intravesicular volume between irradiation doses of 0 and 6 Mrad.

To eliminate the possibility of a dose-dependent decrease in the driving force caused by an increase in the Na⁺ permeability of the membrane, initial rates of phosphate and glucose uptake were compared between irradiated and non-irradiated vesicles (Figs. 6a and 6b). The uptake was linear for at least the first 5 s of incubation independently of the irradiation dose, indicating that there was no alteration of the driving force for the short time of incubation used in our experiments. Any decrease in the driving force should have caused a pronounced effect on the linearity of the uptake. In addition, there was no difference in the initial rate of Na⁺ influx between control and irradiated vesicles (Table 1).

Glucose uptake was measured in vesicles subjected to irradiation at doses up to 9 Mrad (Fig. 7a). There was a progressive loss of transport activity measured in the presence of Na⁺ as a function of the dose administered to the vesicles. In contrast, Na⁺-independent D-glucose influx, which is mainly diffusional, was totally unaffected at these doses. The difference between these two fluxes was calculated as the Na⁺-dependent influx, and a semi-logarithmic plot of these data indicates a simple exponential dependence on radiation (Fig. 7b). The

Fig. 8. Determination of the molecular size of the phosphate carrier

(a) Uptake was measured as described in Fig. 5 at 5 s. (b) Na⁺-dependent phosphate transport was calculated as the difference between the uptake from incubation media containing Na⁺ (□) and K⁺ (■) and are expressed as the log of the percentage of activity remaining ($n = 7$).

$D_{37,-78}$ value was 4.47 ± 0.27 Mrad, which corresponds to a molecular size of 288 000 Da for the glucose transporter.

Phosphate uptake was measured under the conditions described in Fig. 8. Na⁺-independent phosphate uptake, which is also diffusional, was totally unaffected by the irradiation, but uptake measured in the presence of Na⁺ showed a dose-dependent loss of activity (Fig. 8a). The Na⁺-dependent influx was calculated and plotted in Fig. 8(b). The $D_{37,-78}$ value was 5.47 ± 0.31 Mrad, which corresponds to a molecular size of 234 000 Da for the phosphate transporter.

DISCUSSION

The structural integrity of vesicles was found to be unaffected by irradiation: they remained sealed, with similar dimensions and identical intravesicular volumes. Cryoprotective medium was found to play an essential role for the preservation of structure, as previously reported [23]. Functional integrity of the membranes was also demonstrated on the basis of the absence of an effect of irradiation on Na⁺-independent influx of either glucose or phosphate, which are diffusional. These results are in agreement with studies on water and urea transport, which showed no variation in water transport caused by

irradiation [24]. Water transport was found to be a passive process, which does not require a protein carrier [24]. Possible artifacts arising from alteration of the driving forces caused by irradiation were also eliminated on the basis of measurements performed in initial-rate conditions. The irradiation effect was thus not caused by a decrease in the driving force, but resulted from the inactivation of the carrier. In addition, Na⁺ uptake itself was unaffected by the inactivation protocol, confirming the fact that the Na⁺ gradient was identical for treated and untreated vesicles. Here again, the cryoprotective medium was essential for the preservation of Na⁺ permeability, as was also indicated by others [11,23].

As brush-border membranes are composed of a great variety of membrane proteins, it was necessary to verify the validity of the radiation-inactivation method in this system. The sizes of two arbitrarily chosen proteins estimated by a standard SDS/polyacrylamide-gel-electrophoresis procedure and compared with those obtained by the radiation-inactivation method combined with densitometric measurement of stained proteins [25] were found to be similar.

The molecular size of 288 000 Da estimated in our study for the glucose transporter is in good agreement with the results of others, obtained with brush-border membranes from bovine kidney, 345 000 Da [23]. Higher values were, however, obtained for the glucose transporter in rabbit kidney brush border (1 000 000 Da [11]) and for purified transporter from rabbit kidney which was irradiated before or after reconstitution (328 000 or 358 000 Da [11]). Phlorizin is a good competitor of glucose at the binding site of this carrier [22]. The molecular size of a phlorizin-binding protein was estimated as 110 000 Da [12] and 230 000 Da [26]. On the other hand, estimation of SDS/polyacrylamide-gel electrophoresis of the molecular mass of the purified transporter gave a value of 165 000 Da for a dimer composed of two identical monomers of 85 000 Da [11]. Estimation of the size of an azidophlorizin-binding protein by the SDS/polyacrylamide-gel-electrophoresis method gave a value of 72 000 Da [27]. These results led to the proposal of a model in which phlorizin-binding protein represents only a part of an oligomeric carrier which is responsible for the co-transporter function [26]. It was suggested that the glucose transporter is a tetramer composed of subunits of molecular mass close to 85 000 Da [11]. The correspondence between the radiation-inactivation size measured by the inactivation method and the molecular characteristics of a given enzyme is delicate to evaluate, when the molecular composition (subunits) of the enzyme is unknown. The radiation-inactivation size is the mass of 1 mol of protein structure whose associated biological activity is abolished after a single hit by ionizing radiation [16]. In oligomeric proteins, the radiation-inactivation size could correspond either to the subunits or the whole oligomer [8,16]. However, the relatively large value of radiation-inactivation size for the Na⁺ symporter suggests that it is an oligomer.

Compared with the glucose carrier, very little is known about the phosphate carrier. The recent use of phosphonoformic acid as an inhibitor of Na⁺-dependent phosphate transport in renal membranes [28] should help in the future characterization of this transport system. The only reported evidence pertaining to the identification of a molecular structure possibly related to

phosphate transport is the existence of a proteolipid with phosphate-binding characteristics [4]. The molecular size of this compound (14000 Da) is much smaller than that of the phosphate carrier reported here (234000 Da). The proteolipid would thus constitute only part of the transport protein. The high molecular mass reported here for the phosphate carrier suggests the possibility of a multimeric enzyme. In view of the fact that the Na⁺ gradient is responsible for the induction of important conformational changes in this carrier [29,30], it would be of future interest to see if these conformational changes involve any association and dissociation of monomers.

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