The Renal Sodium/Phosphate Symporters: Evidence for Different Functional Oligomeric States†

Marc Jetté,‡ Vincent Vachon,‡ Michel Potier,§ and Richard Béliveau*,‡

Laboratoire d'oncologie moléculaire, Département de chimie-biochimie, Université du Québec à Montréal, Montréal, Québec, Canada, H3C 3P8, Groupe de recherche en transport membranaire, Université de Montréal, Montréal, Québec, Canada, H3C 3J7, and Section de génétique médicale, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec, Canada, H1W 2C4

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ABSTRACT: The oligomeric size of the rat renal sodium/phosphate symporters was estimated in brush-border membrane vesicles submitted to radiation inactivation. Altering the electrochemical conditions under which phosphate transport was measured resulted in different molecular size determinations. The radiation inactivation size (RIS) obtained from the radiation-induced loss of transport activity measured in the presence of a sodium gradient was 200 kDa. Under sodium equilibrium conditions, in the presence of a phosphate gradient as the only driving force, transport fell to 13% of the activity measured in the presence of a sodium gradient, and the RIS was 62 kDa. Addition of an outwardly-directed proton gradient increased the transport activity to 29% of that measured in the presence of a sodium gradient. The RIS measured under these conditions was 124 kDa. Under all conditions tested, phosphate uptake by irradiated vesicles was significantly reduced but remained linear during the first 5 s of incubation. The radiation-induced loss of transport activity was thus attributable to a direct inactivation of the transporter rather than to a decrease in the physical integrity of the vesicles. These results are consistent with a tetrameric structure composed of subunits of about 62 kDa and suggest that phosphate transport involves both monomers and tetramers.

The kidney plays a major role in maintaining the plasma level of inorganic phosphate within narrow limits (Berndt & Knox, 1992; Dennis, 1992). Phosphate ions are reabsorbed from the glomerular filtrate predominantly across the cells of the proximal convoluted tubule. Phosphate transport across the luminal brush-border membrane of these cells is mediated by sodium-dependent symporters and constitutes the rate-limiting step of this process (Bonjour & Caverzasio, 1984; Mizgala & Quamme, 1985; Gmaj & Murer, 1986). Several aspects of this transport activity, including its kinetics and regulation, have been studied extensively (Hammerman, 1986; Murer et al., 1991; Murer & Biber, 1992). The main driving force for phosphate transport is the Na^+ electrochemical gradient. Phosphate is also transported, albeit at a slower rate, into renal brush-border membrane vesicles, when sodium concentrations are equilibrated across the membrane. In addition, in the presence of Na^+, an outwardly-directed proton gradient increases the rate of phosphate transport (Sacktor & Cheng, 1981; Amstutz et al., 1985; Strévey et al., 1990) without affecting the affinity of the cotransport system for phosphate (Sacktor & Cheng, 1981).

Recently, a number of renal phosphate symporters have been cloned from rabbit (Werner et al., 1991; Verri et al., 1995), rat (Magagnin et al., 1993), human (Magagnin et al., 1993; Chong et al., 1993), mouse (Collins & Ghishan, 1994; Hartmann et al., 1995; Chong et al., 1995), and flounder (Werner et al., 1994b) kidney cortex, and from the opossum kidney (OK) (Sorribas et al., 1994) and NBL-1 bovine renal (Helps et al., 1995) epithelial cell lines. Based on deduced amino acid sequence comparisons, these transporters were classified into two distinct groups of highly homologous proteins (Biber & Murer, 1994), type I and type II, although the flounder and bovine symporters diverge somewhat from the other members of type II and may belong to different sub-groups (Helps et al., 1995). Both types of phosphate symporters differ in sequence homology and in size: the predicted molecular mass of type I cotransporters is 51−52 kDa, and that of type II cotransporters ranges from 69 to 75 kDa. In contrast with type I cotransporters (Biber et al., 1993; Delisle et al., 1994a), type II cotransporters are expressed at higher levels during dietary phosphate deprivation (Werner et al., 1994a; Verri et al., 1995). Type II cotransporters are also regulated by parathyroid hormone (Kempson et al., 1995) and expressed at lower levels in the genetically deficient *Hyp* mouse (Tenenhouse et al., 1994).

The size of the functional unit of renal phosphate symporters has also been studied extensively with the radiation inactivation technique. This approach provides the means of estimating the *in situ* functional molecular size of proteins and the possibility of studying their structure/function relationships without the need to purify or even solubilize them from the membrane (Harman et al., 1985; Beauregard et al., 1987; Jung, 1988; Béliveau & Potier, 1989). The radiation inactivation sizes obtained from the radiation-induced loss of transport activity, measured in the presence of a sodium gradient, were 234, 223, 242, and 172 kDa for

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* Author to whom correspondence should be addressed at the Département de chimie-biochimie, Université du Québec à Montréal, P.O. Box 8888, Centre Ville Station, Montréal, Québec, Canada H3C 3P8. Telephone: (514) 987-3000, ext. 8551. FAX: (514) 987-4054.

‡ Département de chimie-biochimie, Université du Québec à Montréal, and Groupe de recherche en transport membranaire, Université de Montréal.

§ Section de génétique médicale, Hôpital Ste-Justine.

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the phosphate cotransporters of rat (Béliveau et al., 1988a), rabbit (Delisle et al., 1994b), mouse (Tenenhouse et al., 1990), and cow (Delisle et al., 1992) kidney cortex, respectively. Taken together with the above-mentioned molecular masses of their constituent polypeptides, these results indicate that renal sodium/phosphate symporters function as oligomeric proteins, probably homotetramers. Further evidence for such an oligomeric structure was derived from the measurement of the loss of labeling intensity of the polypeptide recognized by antibodies directed against the NaPi-1 transporter (Werner et al., 1991) as a function of the radiation dose (Delisle et al., 1994b). Target sizes of 165 and 184 kDa thus obtained for the rabbit and rat phosphate cotransporters indicate considerable energy transfer between polypeptides following a radiation hit and suggest that the subunits are closely associated (Delisle et al., 1994b).

In the present study, the radiation inactivation size (RIS)[1] was estimated from the radiation-induced loss of transport activity measured under different driving forces. The molecular sizes obtained suggest that different levels of association of monomers are involved in phosphate transport across the renal brush-border membrane.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]Orthophosphate (carrier free) was purchased from ICN Biomedicals, and leucine *p*-nitroanilide and *p*-nitrophenyl phosphate were from Sigma. Other chemicals were of the highest purity available commercially.

Preparation of Brush-Border Membranes. All experiments were performed on renal brush-border membrane vesicles prepared from adult Sprague Dawley male rats (250−300 g) fed on normal Purina lab chow (0.8% (w/w) phosphate). After decapitation, the kidneys were perfused with 0.85% (w/v) NaCl, and brush-border membranes were purified with an $MgCl_2$ precipitation method (Booth & Kenny, 1974). The final pellet was washed and resuspended in 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes/Tris (pH 7.5), and 150 mM NaCl or KCl. For pH gradient experiments, Mes/Tris (pH 5.5) was used instead of Hepes/Tris, and, to generate a sodium electrochemical gradient, KCl and NaCl were replaced by 300 mM mannitol. After incubation at 4 °C for 45 min, the membrane vesicles were stored in liquid nitrogen until use. Enrichment for alkaline phosphatase was 10−12-fold. Alkaline phosphatase (Kelly & Hamilton, 1970) and aminopeptidase N (George & Kenny, 1973) were assayed as described, and protein concentration was measured with the method of Lowry et al. (1951).

Irradiation Procedure. Irradiation was carried out at −78 °C in a Gammacell Model 220 ^{60}Co irradiator at a dose rate of approximately 1.5 Mrad/h as described previously (Beauregard et al., 1983; Béliveau et al., 1988a). The following empirical equation was used to relate the radiation inactivation size (RIS) to $D_{37,t}$, the radiation dose (in Mrad) at which the measured activity has been decreased to 37% of its initial value, and to t, the temperature (in °C) (Beauregard et al., 1987):

$$\log \mathrm{RIS} = 5.89 - \log D_{37,t} - 0.0028t$$

$D_{37,t}$ values were obtained from semi-logarithmic plots of transport versus irradiation dose using a least-squares fit. The experimental errors mentioned in the text and shown in the figures are standard deviations.

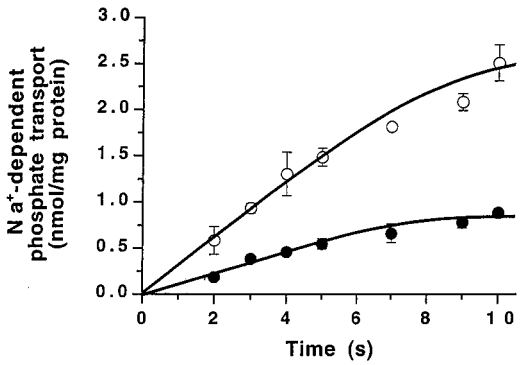

FIGURE 1: Effect of irradiation on the time course of phosphate uptake in the presence of a sodium electrochemical gradient. Vesicles were isolated in 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes/Tris (pH 7.5), and 300 mM mannitol, and irradiated at 0 (○) and 5 (●) Mrad. Phosphate uptake was measured in 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes/Tris (pH 7.5), 150 mM NaCl or KCl, and 200 μM [^{32}P]orthophosphate (3 μCi). The Na^+-dependent phosphate transport activity was calculated as the difference between the uptake values measured in media containing NaCl and KCl. A representative experiment done in quadruplicate is shown.

Phosphate Transport. Phosphate uptake was measured at 25 °C with a rapid filtration technique (Hopfer et al., 1973). The incubation medium contained 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes/Tris (pH 7.5), 150 mM NaCl or KCl, and 200 μM [^{32}P]orthophosphate (3 μCi). The reaction was initiated by the addition of 80−120 μg of membrane protein and stopped, after 5 s unless noted otherwise, by dilution (1/60) with an ice-cold stop solution composed of 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Hepes/Tris (pH 7.5), and 150 mM KCl. The vesicle suspension was filtered under vacuum through a 0.45-μm pore size nitrocellulose filter. The filters were washed with 8 mL of ice-cold stop solution and processed for liquid scintillation counting. Nonspecific binding to the filters was determined with the same procedure, but omitting the vesicles, and subtracted from the raw data.

RESULTS

The effect of irradiation on the time course of phosphate uptake by brush-border membrane vesicles was measured, under zero trans conditions, in the presence of a transmembrane sodium electrochemical gradient (Figure 1). Phosphate uptake by the irradiated vesicles was significantly reduced, but remained linear during the first 5 s of incubation. This indicates that the integrity of the membrane was preserved following irradiation since an alteration of its permeability would have resulted in a faster dissipation of the sodium gradient, a reduced driving force, and a deviation from linearity of the initial uptake.

Phosphate uptake measured in the presence of a transmembrane sodium electrochemical gradient in vesicles exposed to various doses of radiation decreased progressively as a function of the dose (Figure 2A). In contrast, the Na^+-independent phosphate influx, which is mainly due to diffusion, was unaffected at the doses used. The Na^+-dependent influx was calculated as the difference between these two fluxes, and a semi-logarithmic plot of these data revealed a simple exponential decay of activity as a function

[1] Abbreviation: RIS, radiation inactivation size.

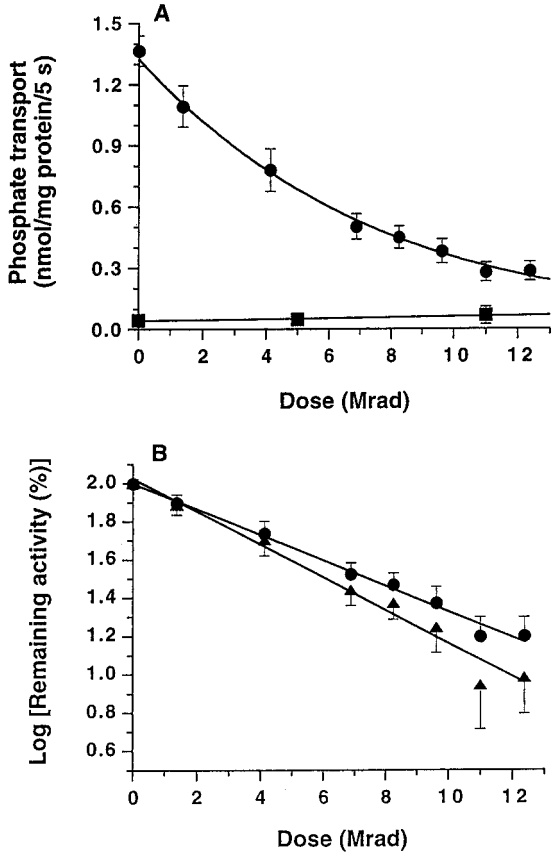

FIGURE 2: Molecular size of the oligomer responsible for sodium electrochemical gradient-driven phosphate transport. (A) Phosphate uptake was measured as described in the legend of Figure 1, in the presence of NaCl (●) or KCl (■). (B) The Na^+-dependent phosphate transport activity (●) was calculated as the difference between the uptake values measured in media containing NaCl and KCl. The uptake values measured in the presence of a phosphate gradient as the only driving force (Figure 3A) were subtracted from these data to estimate the sodium gradient-driven transport activity (▲). The data are derived from four experiments, each done in quadruplicate.

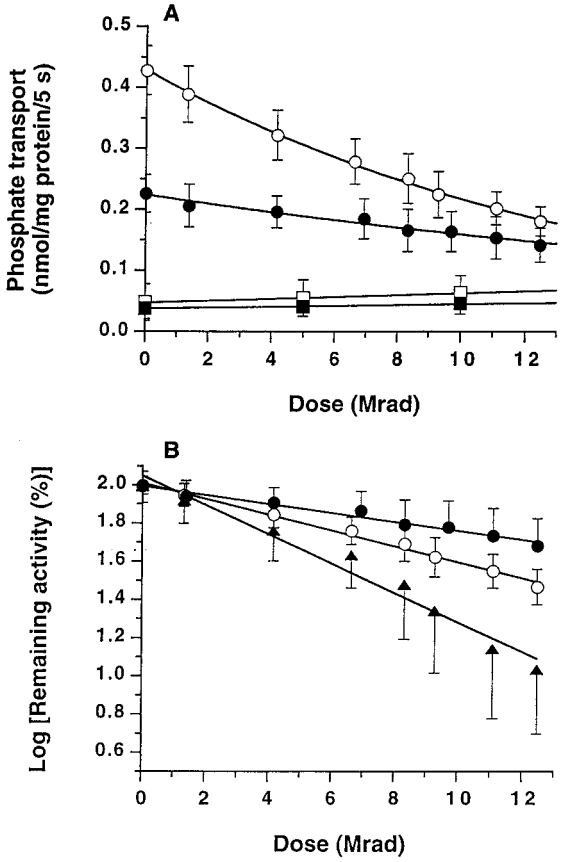

FIGURE 3: Molecular size of the oligomer responsible for pH gradient-driven sodium-dependent phosphate transport. (A) Phosphate uptake was measured in the presence (○ , □) or absence (●, ■) of a pH gradient in vesicles preequilibrated with NaCl (○, ●) or KCl (□, ■) as described in the legend of Figure 4. To avoid formation of NaCl or KCl gradients, phosphate uptake was measured in incubation media similar to the intravesicular media except that each assay was performed with 5 mM Hepes/Tris (pH 7.5) and 200 μM [^{32}P]orthophosphate (3 μCi). (B) The Na^+-dependent phosphate transport activity was calculated as the difference between the uptake values measured in the presence of NaCl and KCl for the data obtained in the presence (○) or absence (●) of a pH gradient. The uptake values measured in the absence of such gradient were subtracted from those measured in its presence to estimate the pH gradient-driven transport activity (▲). The data are derived from five (●) or six (○) experiments, each done in quadruplicate.

of the radiation dose (Figure 2B). The RIS obtained in the presence of a sodium gradient was 200 ± 18 kDa.

The initial rate of Na^+-dependent phosphate uptake measured under sodium equilibrium conditions (Figure 3A) was considerably lower than in the presence of a sodium gradient (Figure 2A): 0.17 ± 0.03 vs 1.33 ± 0.07 nmol (mg of protein)$^{-1}$ (5 s)$^{-1}$. Imposition of an outwardly-directed proton gradient enhanced the uptake of phosphate, under sodium equilibrium conditions, by a factor of 2 [0.38 ± 0.04 nmol (mg of protein)$^{-1}$ (5 s)$^{-1}$] (Figure 3A). Under both conditions, irradiation reduced phosphate uptake without affecting the linearity of the curve during the first 5 s of incubation (Figure 4).

Phosphate uptake measured under sodium equilibrium conditions in the presence or absence of a proton gradient decreased progressively as a function of the radiation dose, while the Na^+-independent uptake remained unaffected (Figure 3A). The Na^+-dependent uptake values were fitted by single exponentials. The RIS measured under sodium equilibrium conditions with and without a proton gradient were 124 ± 19 and 62 ± 18 kDa, respectively (Figure 3B).

The finding of different RIS values when transport is measured under different driving forces raises the possibility that two transporters, or two oligomeric states of the same transporter, are operating simultaneously and independently of each other. The proton gradient-dependent phosphate transport activity was therefore calculated as the difference between the sodium-dependent uptake values measured in the presence and absence of a proton gradient. The RIS derived from a semi-logarithmic plot of these values (Figure 3B) was 237 ± 54 kDa. Similarly, the sodium-dependent uptake values obtained with a phosphate gradient as the only driving force (Figure 3A) were subtracted from those obtained in the presence of sodium and phosphate electrochemical gradients (Figure 2A). The RIS calculated from these values (Figure 2B) was 259 ± 51 kDa.

Because our experiments on the effects of a pH gradient were carried out on vesicles irradiated at a lower pH, control experiments were done to test whether acidic conditions could favor secondary inactivation by generating free radicals, for example, during irradiation. The radiation inactivation size of two marker enzymes was measured in vesicles irradiated at pH 5.5 and 7.5. For aminopeptidase N, molecular sizes of 55 ± 12 and 58 ± 4 kDa were obtained for vesicles irradiated at pH 5.5 and 7.5, respectively (Figure

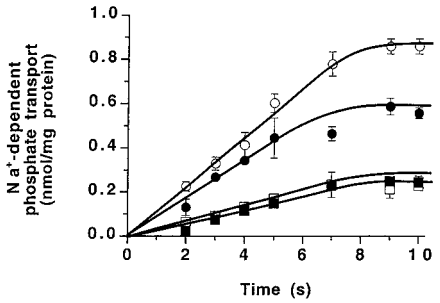

FIGURE 4: Effect of irradiation on the time course of phosphate uptake in the presence of a pH gradient. Vesicles were preequilibrated in 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 5 mM Mes/Tris (pH 5.5) (○, ●) or Hepes/Tris (pH 7.5) (□, ■), and 150 mM NaCl or KCl before irradiation at 0 (○, □) or 5 (●, ■) Mrad. To avoid formation of NaCl or KCl gradients, phosphate uptake was measured in incubation media similar to the intravesicular media except that each assay was performed with 5 mM Hepes/Tris (pH 7.5) and 200 μM [^{32}P]orthophosphate (3 μCi). Na^+-dependent phosphate transport was calculated as the difference between the uptake values measured in media containing NaCl and KCl. A representative experiment done in quadruplicate is shown.

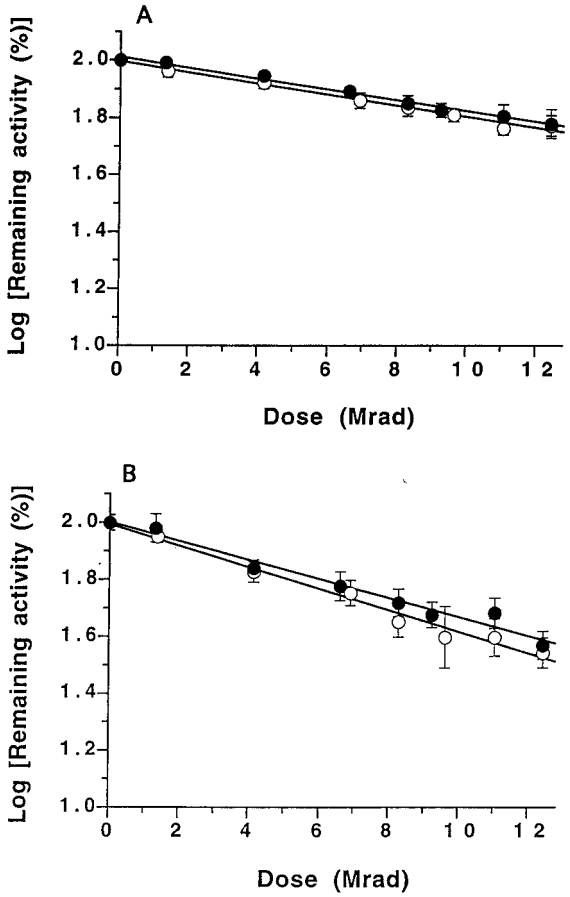

FIGURE 5: Effect of pH on the estimation of the molecular size of aminopeptidase N and alkaline phosphatase. Vesicles were irradiated in 14% (w/v) glycerol, 1.4% (w/v) sorbitol, 150 mM NaCl, and 5 mM Hepes/Tris (pH 7.5) (○) or Mes/Tris (pH 5.5) (●). (A) Aminopeptidase N activity was measured in 300 mM mannitol, 20 mM Hepes/Tris (pH 7.5), and 200 μM leucine *p*-nitroanilide. (B) Alkaline phosphatase activity was measured in 14.4 mM *p*-nitrophenyl phosphate and 1.35 M propanolol buffer (pH 10). The results are derived from three experiments, each done in quadruplicate.

5A). These values are similar to the RIS reported for the aminopeptidase N of the rabbit intestinal brush-border membrane (59 kDa) (Stevens et al., 1986). Under the same conditions, the RIS measurements for alkaline phosphatase were respectively 99 ± 10 kDa at pH 5.5 and 114 ± 16 kDa at pH 7.5 (Figure 5B). These values are not significantly different from each other nor from the RIS previously reported for this enzyme (105 kDa) (Béliveau et al., 1988a).

DISCUSSION

The present study clearly demonstrates that the rate at which phosphate uptake into rat renal brush-border membrane vesicles is inactivated as a function of the radiation dose depends strongly on the conditions under which this transport activity is measured. A 62-kDa protein thus appears to be responsible for phosphate transport when a phosphate gradient is the only driving force. In view of the fact that the carbohydrate moiety of glycoproteins does not contribute to the size measured with the radiation-inactivation technique (Harman et al., 1985; Beauregard et al., 1987; Jung, 1988), this size is remarkably similar to those reported for the cloned type I and type II renal phosphate cotransporters. When transport was measured in the presence of an outwardly-directed proton gradient, the radiation inactivation size was 124 kDa. In the presence of a sodium gradient, the radiation inactivation size (200 kDa) was about 4-fold higher than that obtained under sodium equilibrium conditions in the sole presence of a phosphate gradient. In the present study, the conditions used to impose a sodium gradient also favored the generation of an electrical potential across the membrane due to the diffusion of Cl^- ions into mannitol-loaded vesicles. The measured RIS agrees reasonably well with the previously reported value of 234 kDa which was obtained using similar conditions except that the vesicles contained 150 mM KCl instead of 300 mM mannitol, thus avoiding the presence of an anion gradient (Béliveau et al., 1988a). Similarly large RIS values have also been estimated from the loss of phosphate transport activity measured in the presence of a sodium gradient, but in the absence of an anion gradient, in mouse, cow, and rabbit renal brush-border membrane vesicles (Tenenhouse et al., 1990; Delisle et al., 1992, 1994). These results suggest that the phosphate cotransporters function, in the presence of a sodium gradient, as tetrameric proteins composed of 62-kDa monomers and that a membrane potential has little influence on their apparent size.

These molecular size estimates are based on the assumption that the observed radiation-induced decline in transport activity is due to the inactivation of transporter molecules rather than to increasing damage of the vesicles. Irradiation does not appear to have caused a gross alteration in membrane permeability since, under all conditions tested, the initial period during which transport remained a linear function of time was similar in irradiated and control vesicles. In agreement with this conclusion, the sodium-independent phosphate uptake was not altered by irradiation. Previous studies have also shown that the passive permeability of similarly-prepared renal brush-border membrane vesicles toward $^{22}Na^+$ is only slightly affected at radiation doses comparable to those used in the present study (Béliveau et al., 1988a,b, 1990). Futhermore, during a study of the molecular size of the renal Na^+/H^+ antiporter, it was shown that sodium uptake, driven by an acidic intravesicular pH, remains linear during the first 10 s of incubation in both irradiated and control vesicles, indicating that the driving force of the proton gradient remains unaltered during this

period (Béliveau et al., 1988b). The integrity of irradiated vesicles has also been previously tested by estimating their intravesicular volume from the uptake of glucose at equilibrium (Béliveau et al., 1988a, 1990; Delisle et al., 1994b). Although a small decrease in intravesicular volume was observed, taking its effect into account (Béliveau et al., 1990) leads to modifications of the molecular size estimates which are small in comparison with the dramatic differences reported here for different experimental conditions and the experimental error associated with such measurements.

Although only one phosphate symporter (NaPi-2) has, so far, been cloned from rat renal cortex (Magagnin et al., 1993), both type I and type II cotransporters are probably present. Transporters of both types have been cloned from rabbit (Werner et al., 1991; Verri et al., 1995), humans (Magagnin et al., 1993; Chong et al., 1993), and mouse (Collins & Ghishan, 1994; Hartmann et al., 1995; Chong et al., 1995), and the presence of NaPi-2-related mRNA species in the renal cortex of several animal species has been demonstrated by Northern blot analysis (Magagnin et al., 1993). In addition, a 65-kDa protein was labeled specifically in Western blots of rat kidney brush-border membrane proteins with antibodies raised against the C-terminal portion of the rabbit type I cotransporter (NaPi-1) (Delisle et al., 1994a,b), a region in which NaPi-1 and NaPi-2 differ markedly (Werner et al., 1991; Magagnin et al., 1993). The monoexponential decay of the sodium gradient-dependent phosphate transport activity as a function of the radiation dose suggests that both transporters have a similar size and probably both function as tetrameric proteins.

This interpretation is supported by the results of a previous study in which the size of the phosphate cotransporter was estimated from the radiation-induced disappearance of a 65-kDa polypeptide specifically labeled with antibodies raised against NaPi-1 (Delisle et al., 1994b). The target size thus measured was about 3-fold larger than the monomeric size of the phosphate cotransporter, indicating that the subunits are closely associated and that the energy absorbed by a monomer can spread to adjacent subunits and cause their fragmentation. The results of the present study, on the other hand, suggest that phosphate transport is mediated by different transporters or by different oligomeric associations of the same transporter. The target size estimated from the rate of radiation-induced fragmentation of transporter polypeptides should therefore reflect the proportion of each of these molecular structures. The fact that irradiation was carried out in the absence of any driving force implies that these different oligomeric complexes existed in the membrane before the vesicles were irradiated.

These considerations are consistent with the involvement of proteins of about 62 kDa consisting of type I and type II cotransporter monomers when phosphate transport is measured in the presence of a phosphate gradient as the only driving force. This interpretation obviates the need to invoke the involvement of still another protein which would have to share not only a similar size but also a similar K_m for phosphate (Sacktor & Cheng, 1981) with type I and type II cotransporters. Although the RIS measured in the presence of a proton gradient corresponds to that expected for a dimer, it is not clear whether dimers exist in the membrane before irradiation or whether the proton gradient can prevent the monomers from functioning independently by forcing them, for example, to associate into dimers. The results derived from the measurement of phosphate uptake driven by proton or sodium gradients are therefore probably best explained by assuming that these monomeric proteins continue to function as sodium-dependent phosphate permeases independently of the presence of such gradients. Subtraction of the uptake values attributable to the free monomeric transporters yields RIS estimates of 237 and 259 kDa for the proton gradient-dependent and sodium gradient-dependent phosphate transport activities, respectively. Both values are close to that expected for a tetramer composed of subunits of about 62 kDa. This interpretation is also consistent with the fact that an outwardly-directed proton gradient is known to stimulate phosphate uptake both in the presence and in the absence of a sodium gradient (Strévey et al., 1990).

The experimental data are thus consistent with the existence, in the rat renal brush-border membrane, of a 62-kDa phosphate transporter which functions independently of the transmembrane sodium and proton gradients. Although it cannot be excluded that this protein may represent a new, hitherto unidentified phosphate transporter, it likely consists of type I and type II renal phosphate cotransporters. This study also contributes further evidence that the sodium and proton gradient-dependent phosphate transport activities of these cotransporters are mediated by tetramers.

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