Molecular Size of the Functional Complex and Protein Subunits of the Renal Phosphate Symporter†

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ABSTRACT: The oligomeric structure of the rabbit renal brush-border membrane sodium/phosphate cotransporter was examined with the radiation inactivation and fragmentation technique. The size of its functional complex (its "radiation inactivation size") was estimated from the rate of decay of its sodium-dependent transport activity as a function of the radiation dose. A radiation inactivation size of 223 ± 42 kDa was obtained. The polypeptide constituting the monomeric unit of the Na^{1+}/P_i symporter was detected by immunoblotting with polyclonal anti-peptide antibodies directed against the 14 amino acid C-terminal portion of the symporter molecule. Its apparent molecular size estimated by comparison with standards following SDS–polyacrylamide gel electrophoresis was 64 000. This value is in good agreement with its known molecular mass of 51 797 Da calculated from the amino acid sequence deduced from the nucleotide sequence of its gene since this protein is probably glycosylated. The loss of labeling intensity of the polypeptide of M_r = 64 000 was also measured as a function of radiation dose. The molecular size calculated from these data (its "target size") was 165 ± 20 kDa. The target size estimated for the rat phosphate cotransporter was 184 ± 46 kDa, and its previously reported radiation inactivation size was 234 ± 14 kDa. These results strongly suggest that the renal Na^{1+}/P_i cotransporter exists as an oligomeric protein, probably a homotetramer. The fact that the values obtained for the target size are about 3/4 those obtained for the radiation inactivation size of these cotransport proteins indicates that their subunits are closely associated since most of their subunits appear to be fragmented by a single ionizing radiation hit.

The rate-limiting step in the reabsorption of phosphate from the glomerular filtrate is carried out by a specific sodium-dependent symporter which mediates the concentrative uptake of phosphate coupled to the downhill movement of sodium ions across the apical brush-border membrane of the proximal tubule (Bonjour & Caverzasio, 1984; Mizgala & Quamme, 1985; Gmaj & Murer, 1986; Hammerman, 1986). Although this transport process and its physiological regulation have been studied extensively, limited structural information concerning the cotransporter protein itself has been available until recently (Murer et al., 1991; Berndt & Knox, 1992; Dennis, 1992; Murer & Biber, 1992).

Considerable progress toward the structural identification and characterization of the phosphate symporter has been achieved, however, by the recent cloning into *Xenopus laevis* oocytes of a sodium/phosphate cotransport protein, designated NaPi-1, from rabbit kidney cortex (Werner et al., 1990, 1991). Based on the nucleotide sequence of the cloned cDNA, the phosphate symporter consists of a polypeptide of 465 amino acids with a molecular mass of 51 797 Da, four potential *N*-glycosylation sites, and six to eight membrane-spanning segments (Werner et al., 1991). Western blot analysis of renal brush-border membrane proteins using polyclonal anti-peptide antibodies raised against the 14 amino acid C-terminal portion of this protein revealed proteins of M_r = 64 000 and 65 000 which were specifically labeled in membranes isolated from rabbit and rat kidney cortex, respectively (Biber et al., 1993; Delisle et al., 1994). In addition, a glycoprotein of M_r = 63 000–66 000, purified from methanol/chloroform extracts of the rabbit renal brush-border membrane (Debiec & Lorenc, 1988), has also recently been shown to carry out sodium-dependent phosphate transport when reconstituted in proteoliposomes (Debiec et al., 1992).

Supplementing this information on the monomeric unit of the phosphate symporter, the radiation inactivation technique provides a method for measuring the size of its functional complex in its native membrane environment (Harmon et al., 1985; Beauregard et al., 1987; Jung, 1988; Béliveau & Potier, 1989). This technique has been used to estimate the size of the functional unit of several transport proteins from the renal brush-border membrane of the rat, including the phosphate and glucose symporters (Béliveau et al., 1988a), the Na^{1+}/H^{1+} antiporter (Béliveau et al., 1988b), and six amino acid transporters (Béliveau et al., 1990a). In these experiments, the radiation inactivation size (RIS) was evaluated from the loss of transport activity as a function of irradiation dose.

In the present report, we compare the previously unpublished radiation inactivation size of the rabbit renal phosphate symporter with its target size (TS) estimated from the rate of fragmentation of its constituting polypeptide, detected by Western blot analysis, as a function of irradiation dose. The radiation inactivation size corresponds to approximately 4 times the subunit molecular mass, and the target size

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corresponds to about 3 times this value, indicating efficient energy transfer between the subunits of a homotetrameric complex. Similar conclusions are also reported for the rat renal sodium/phosphate symporter.

EXPERIMENTAL PROCEDURES

Preparation of Brush-Border Membrane Vesicles. Sixteen-week-old male New Zealand rabbits (2 kg) and eight-week-old Sprague-Dawley rats (300–350 g) were purchased from the Charles River Co. (St-Constant, PQ). Kidneys were perfused extensively with 0.85% saline via the renal artery, and brush-border membrane vesicles were prepared from kidney cortex with a $MgCl_2$ precipitation method (Booth & Kenny, 1974). The final pellet containing purified brush-border membranes was resuspended in a cryoprotective medium composed of 150 mM KCl, 14% (w/v) glycerol, 1.4% (w/v) sorbitol, and 5 mM Hepes–Tris, pH 7.5, and stored in liquid nitrogen until use. Protein content was estimated with the bicinchoninic acid assay (Smith et al., 1985) using bovine serum albumin as standard protein. Alkaline phosphatase, a brush-border membrane marker enzyme, was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate (Kelly & Hamilton, 1970). Its specific activity was enriched (10.1 ± 2.4)- and (12.5 ± 2.6)-fold in rabbit and rat membrane preparations, respectively.

Irradiation Procedure. Membrane vesicles were exposed to γ-rays in a Model 220 Gammacell ^{60}Co irradiator (Atomic Energy of Canada) at a dose rate of approximately 1 Mrad/h (Beauregard et al., 1983). During this procedure, the samples were kept at −78 °C with crushed dry ice. The radiation inactivation size (RIS) was calculated from the rate of decay of the sodium-dependent phosphate-transport activity as a function of radiation dose using the following empirical equation (le Maire et al., 1990), which is valid for samples irradiated at −78 °C and was derived from the calibration published by Kepner and Macey (1968):

$$\text{RIS or TS} = 1.29 \times 10^6/D_{37} \quad (1)$$

where D_{37} is the radiation dose (in Mrad) at which the measured activity has been decreased to 37% of its initial value. The D_{37} values were obtained from semilogarithmic plots of the remaining activity versus irradiation dose using a least-squares fit. The target size (TS) was calculated from the rate of disappearance of the immunodetected protein band corresponding to the phosphate cotransporter as a function of irradiation dose using the same equation.

Transport Measurements. Uptake of radiolabeled phosphate was measured at 25 °C with a rapid filtration technique (Hopfer et al., 1973) by addition of 5 μL of brush-border membrane vesicles (20 mg of protein/mL) to 25 μL of 200 μM [^{32}P]KH_2PO_4–K_2HPO_4, 14% glycerol, 1.4% sorbitol, 5 mM Hepes–Tris, pH 7.5, and 150 mM of either NaCl or KCl. The reaction was stopped after 5 s, unless indicated otherwise, by dilution with 1 mL of ice-cold stop solution which had the same composition as the cryoprotective medium described above. The suspension was filtered immediately under vacuum through a nitrocellulose filter (0.45-μm pore diameter), and the filter was rinsed with an additional 7 mL of stop solution. The radioactivity was measured by liquid scintillation counting. For the estimation of intravesicular volume, membrane vesicles were incubated for 4 h at 25 °C in 50 μM [^{3}H]glucose, 14% glycerol, 1.4% sorbitol, 5 mM Hepes–Tris, pH 7.5, and 150 mM KCl. The stop solution had an identical composition except that the radioactive glucose was replaced by 1 mM phlorizin.

Western Immunoblotting. Proteins were separated by SDS–polyacrylamide gel electrophoresis in a Mini-Protean (Bio-Rad) electrophoresis apparatus with the Laemmli (1970) buffer system. The separating gel contained 10% (w/v) acrylamide and 0.3% (w/v) *N,N'*-methylenebis(acrylamide). Prior to electrophoresis, the samples were boiled for 3 min in sample buffer containing 1.0% (w/v) SDS and 2.5% (v/v) β-mercaptoethanol. Electrophoresis was carried out at a constant voltage of 150 V for 1 h. Except when indicated otherwise, each well contained 12 μg of protein. Relative molecular weight determinations were based on a calibration curve prepared with the following proteins (Bio-Rad): myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21 kDa).

Following electrophoresis, the gels were equilibrated in transfer solution (10% (v/v) methanol, 25 mM Tris, and 192 mM glycine, pH 8.3). The proteins were transferred to poly(vinylidene difluoride) (PVDF) microporous (0.45-μm pore diameter) membranes (Millipore) by electroelution in a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad) at a constant voltage of 100 V during 3 h at 4 °C. The remaining binding sites on PVDF membranes were saturated by overnight incubation in 150 mM NaCl, 0.5% (w/v) Tween 20, 0.1% (w/v) bovine serum albumin, and 50 mM Tris, pH 7.5 (buffer A), containing 5% (w/v) powdered milk, at 25 °C. The membranes were washed three times by gentle agitation in buffer A for 10 min and incubated for 1 h with a 1/500 (for rabbit membranes) or a 1/250 (for rat membranes) dilution of anti-peptide antibodies (Delisle et al., 1994) raised in rabbits and directed against the 14 amino acid C-terminal portion of the NaPi-1 sodium/phosphate cotransporter from rabbit kidney cortex (Werner et al., 1991) in buffer A. The membranes were washed three more times in buffer A and incubated for 1 h with a 1/1000 dilution of anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (Amersham) in buffer A. The membranes were finally washed five times for 5 min in buffer A. Labeled antigens were revealed with enhanced chemiluminescence (ECL) Western blotting solutions (Amersham) following the recommendations of the manufacturer. The membranes were exposed for different times to preflashed (1/16 opening) Fuji films which were developed with standard methods. The autoradiograms were analyzed with an Ultroscan XL (Pharmacia) laser densitometer.

RESULTS

Phosphate uptake was assayed in irradiated rabbit brush-border membrane vesicles. Transport activity measured in the presence of a sodium gradient decreased progressively with increasing radiation dose, but the sodium-independent phosphate uptake, which is mainly diffusional, was not significantly affected (Figure 1A). The sodium-dependent phosphate-transport activity decreased exponentially with radiation dose at a rate consistent with a radiation inactivation size of 223 ± 42 kDa (n = 6) for the functional complex of the cotransporter (Figure 1B).

This estimate is based on the assumption that the loss of transport activity is due to a direct radiation-induced inactivation of the cotransporter rather than to a progressive decrease in the physical integrity of the vesicles. An increase in the permeability of the vesicles for sodium ions, for example, would result in a decreased driving force for transport and reduce the time during which phosphate uptake is a linear function of time. Phosphate transport was therefore measured

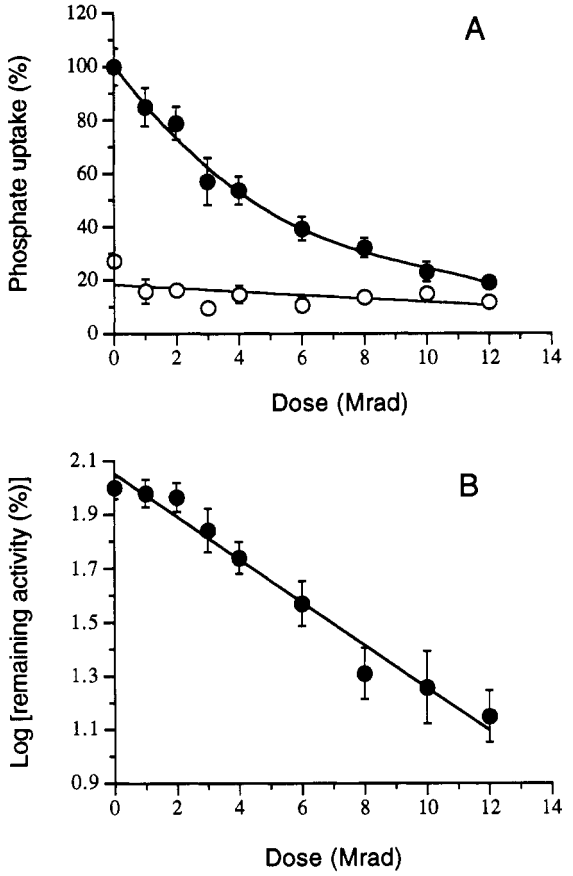


FIGURE 1: Determination of the radiation inactivation size of the rabbit renal sodium/phosphate cotransporter. (A) Brush-border membrane vesicles were irradiated, and phosphate uptake was measured at 5 s in the presence of 150 mM NaCl (●) or KCl (○), as described under Experimental Procedures. (B) Sodium-dependent phosphate transport was calculated as the difference between uptake in the presence of NaCl and KCl and expressed as the log of the percentage of activity remaining relative to that of the unirradiated control. Each value represents the mean ± SD of six experiments in which transport activity was measured in triplicate or quadruplicate.

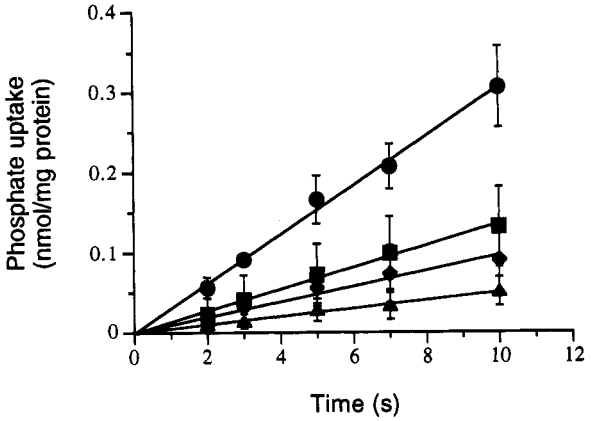


FIGURE 2: Effect of irradiation dose on the initial rate of phosphate uptake. Rabbit brush-border membrane vesicles were exposed to 0 (●), 4 (■), 6 (◆), and 12 Mrad (▲), and phosphate uptake was measured in the presence of 150 mM NaCl as described under Experimental Procedures. Each value represents the mean ± SD of three experiments in which each assay was performed in quadruplicate.

under initial rate conditions (Figure 2). Phosphate uptake remained linear for about 10 s in rabbit brush-border membrane vesicles whether unirradiated or subjected to radiation doses up to 12 Mrad. The transport measurements used for the estimation of the radiation inactivation size, which were done at 5 s, were thus well within the linear portion of the curve.

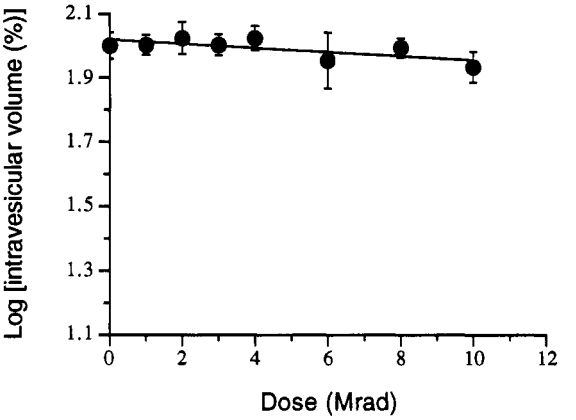


FIGURE 3: Effect of irradiation dose on intravesicular volume. Rabbit brush-border membrane vesicles were irradiated, and the intravesicular volume was determined by measurement of [^{3}H]glucose uptake at equilibrium, as described under Experimental Procedures. Results are expressed as the log of the percentage volume remaining after irradiation. Each value represents the mean ± SD of three experiments in which each assay was done in triplicate.

The effect of radiation dose on the physical integrity of the vesicles was also tested by estimating the intravesicular volume from the uptake of glucose at equilibrium (Figure 3). The intravesicular volume decreased by about 10% at 10 Mrad, the highest dose tested, and by only about 4% at 5.8 Mrad, the D_{37} value obtained for the effect of radiation on the sodium-dependent phosphate-transport activity of these vesicles (Figure 1). The loss of transport activity that can be accounted for by the decrease in intravesicular volume is thus small in comparison with the experimental error associated with these measurements.

Polyclonal antibodies raised against the 14 amino acid C-terminal portion of the NaPi-1 sodium/phosphate cotransporter from rabbit kidney cortex were used to detect the cotransport protein in Western blots. Such antibodies have recently been shown to label specifically a polypeptide of M_r = 64 000 from rabbit renal brush-border membranes (Biber et al., 1993; Delisle et al., 1994). These antibodies also detect specifically a protein of M_r = 65 000 from rat brush-border membranes (Delisle et al., 1994). The amount of immunolabeled polypeptide detected was a linear function of the amount of protein loaded onto the gel for both rabbit and rat brush-border membranes (Figure 4).

Exposure of the membrane vesicles to ionizing radiation resulted in a dose-dependent decrease in labeling intensity of the rabbit (Figure 5A) and rat (Figure 6A) antigens. In both cases, the radiation-induced degradation of the cotransporter molecule was a simple exponential function of radiation dose (Figures 5B and 6B). The target sizes calculated from these results were 165 ± 20 kDa (n = 3) for the rabbit phosphate cotransporter (Figure 5B) and 184 ± 46 kDa (n = 3) for that of the rat (Figure 6B).

DISCUSSION

The radiation inactivation size reported here for the rabbit sodium/phosphate cotransporter (223 kDa) corresponds to about 4 times the molecular size of its monomeric subunit estimated from its electrophoretic mobility on SDS–polyacrylamide gels followed by Western blot analysis (M_r = 64 000) or calculated from the amino acid sequence deduced from the nucleotide sequence of the NaPi-1 gene (52 kDa) (Werner et al., 1991). The renal phosphate cotransporter of the rat also has a radiation inactivation size (234 kDa) (Béliveau et al., 1988a) which is about 4-fold larger than the size of its monomeric subunit estimated by Western blot

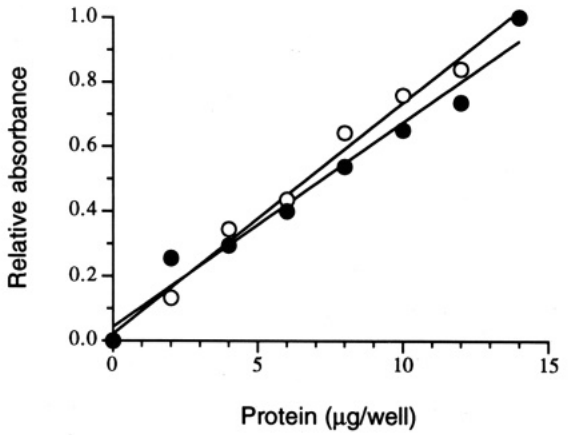
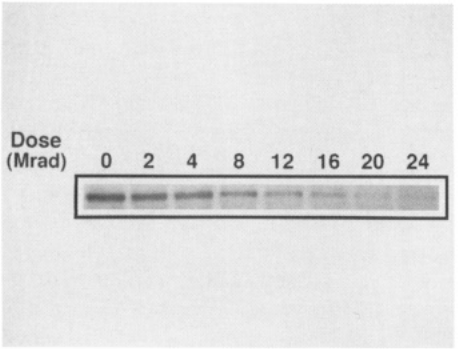


FIGURE 4: Calibration of immunoblots. Unirradiated brush-border membrane proteins were resolved by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The Western blots were incubated with affinity-purified antibodies directed against the 14 amino acid C-terminal portion of the rabbit Na^{1+}/P_i cotransporter, followed by donkey anti-rabbit IgG horseradish peroxidase-linked antibodies. The labeled antigens were revealed by autoradiography with ECL Western blotting solutions. The intensity of the specifically labeled protein bands corresponding to proteins of M_r = 64 000 from the rabbit (●) and M_r = 65 000 from the rat (○) was measured by densitometric scanning of the autoradiograms. Each value represents the average of two independent experiments.



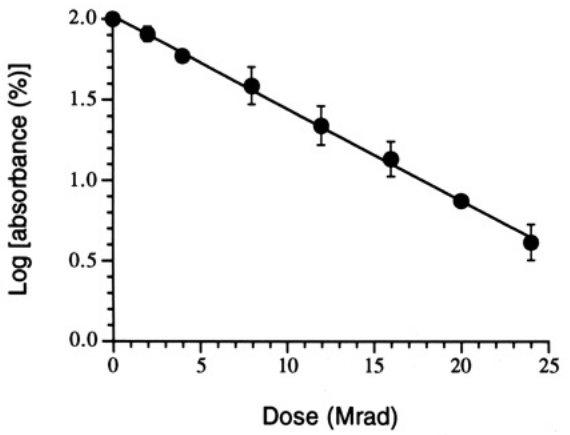
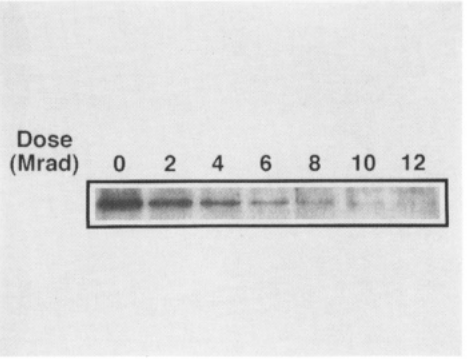


FIGURE 5: Determination of the target size of the rabbit renal sodium/phosphate cotransporter. Brush-border membrane vesicles were irradiated and analyzed as described in the caption of Figure 4. (A) Autoradiogram showing the effect of radiation dose on the structural integrity of the protein of M_r = 64 000 which corresponds to the rabbit renal Na^{1+}/P_i cotransporter. (B) The disappearance of the immunolabeled polypeptide measured by laser densitometry. The results are expressed as the log of the percent area remaining under each peak relative to the unirradiated control. Each value represents the mean ± SD of three independent experiments.

analysis (M_r = 65 000). These large radiation inactivation sizes could in principle be due to the immunodetected polypeptide plus other, as yet unidentified, proteins. This possiblity appears unlikely, however, since the injection of a



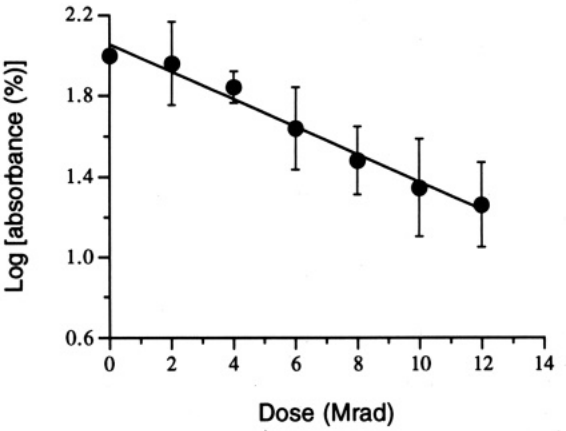


FIGURE 6: Determination of the target size of the rat renal sodium/phosphate cotransporter. Brush-border membrane vesicles were irradiated and analyzed as described in the caption of Figure 4. (A) Autoradiogram showing the effect of radiation dose on the structural integrity of the protein of M_r = 65 000 which corresponds to the rat renal Na^{1+}/P_i cotransporter. (B) Disappearance of the immunolabeled polypeptide was measured by laser densitometry. The results are expressed as the log of the percent area remaining under each peak relative to the unirradiated control. Each value represents the mean ± SD of three independent experiments.

single cRNA species coding for a single protein was sufficient for the expression of sodium-dependent phosphate-transport activity in *Xenopus laevis* oocytes (Werner et al., 1991). These results thus strongly suggest that the sodium/phosphate cotransporters from both species exist as tetrameric protein complexes in the renal brush-border membrane.

For both species, the target size obtained from the rate of disappearance of the immunolabeled protein band was about 3/4 of the radiation inactivation size obtained from the loss of sodium-dependent phosphate-transport activity. This observation provides further evidence in support of the multimeric structure of the transporter molecule. It also indicates that the subunits are associated closely enough to allow the energy absorbed by a single subunit to spread to other subunits and, on the average, cause the fragmentation of three of the four subunits. Within a tetramer, each monomer could be in close proximity to two other subunits. The absorbed energy could then transfer efficiently to adjacent subunits, but not to the fourth monomer. As a consequence of this finding, however, the oligomeric structure required for activity cannot be ascertained from these results because the whole quaternary structure appears to be destroyed by a single hit (Beauregard et al., 1987; Béliveau & Potier, 1989). The cotransporter protein could be composed, for example, of four identical subunits, all contributing to a single transport unit, or each of the subunits within the tetramer could constitute an independent transport unit although the unfragmented subunit clearly cannot carry out phosphate transport after destruction of the other monomers.

Intersubunit energy transfer has been reported for a number of integral membrane proteins (Jhun et al., 1991), but appears to be much less common in water-soluble oligomeric proteins (le Maire et al., 1990). The fact that significant noncovalent transfer of energy can occur between the subunits of oligomeric membrane proteins was nevertheless clearly demonstrated by Jhun et al. (1991) who showed that the erythrocyte glucose transporter retains a large target size even after its 12 transmembrane α helices are cut individually into small membrane-bound fragments by pepsin digestion. Interestingly, the present results differ somewhat from those reported for the intestinal glucose cotransporter, another well-studied sodium-dependent cotransport protein, for which the target size corresponds to that of the monomer, while the radiation inactivation size corresponds to that of a homotetramer (Stevens et al., 1990).

Phosphonoformic acid, a competitive inhibitor of sodium-dependent phosphate transport across the renal brush-border membrane (Szczepanska-Konkel et al., 1986), has been used as a probe to study the sodium/phosphate cotransporter (Szczepanska-Konkel et al., 1987; Yusufi et al., 1989). In a previous study (Béliveau et al., 1990b), the radiation inactivation size of a protein mediating phosphate-displaceable sodium-dependent phosphonoformic acid binding in the rat renal brush-border membrane was found to correspond to a molecular mass of 130 kDa. This result suggests, among other possibilities, that the renal phosphate transporter could be composed of two such subunits of 130 kDa. This hypothesis is contradicted, however, by the results of the present study because, following a radiation hit, the biological activity of the protein complex cannot be preserved in half of its subunits, while three out of four subunits become fragmented. Phosphonoformic acid binding may therefore detect primarily a protein other than the sodium/phosphate symporter in the renal brush-border membrane.

The radiation inactivation size of the rabbit renal sodium/phosphate cotransporter (223 kDa) is similar to those reported earlier for the renal phosphate cotransporters of rat (234 kDa) (Béliveau et al., 1988a), mouse (242 kDa), (Tenenhouse et al., 1990) and cow (172 kDa) (Delisle et al., 1992). Despite their similar sizes, the phosphate cotransporters from different species can differ greatly in their structural details as was recently demonstrated by the small degree of homology between the amino acid sequence of the NaPi-1 protein from the rabbit and the NaPi-2 protein from the rat (Magagnin et al., 1993). On the other hand, NaPi-2 is highly homologous to the NaPi-3 protein from man (Magagnin et al., 1993). In addition, there is growing evidence for the existence of more than one high-affinity sodium/phosphate cotransporter in the brush-border membrane of the proximal tubule (Murer et al., 1991). The fact that our antibodies directed against a segment of the rabbit NaPi-1 cotransport protein can detect specifically a protein from the rat kidney despite this poor homology with the rat NaPi-2 protein provides further support for such multiplicity.

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