Radiation-inactivation analysis of vacuolar H⁺-ATPase and H⁺-pyrophosphatase from *Beta vulgaris* L.

Functional sizes for substrate hydrolysis and for H⁺ transport

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The functional sizes of the vacuolar H⁺-ATPase (V-ATPase; EC 3.6.1.34) and H⁺-pyrophosphatase (PPase; EC 3.6.1.1) from vacuolar membranes of red beet (*Beta vulgaris* L.) were estimated by radiation inactivation, both for substrate hydrolysis and for H⁺ transport. For the V-ATPase, the radiation-inactivation size for H⁺ transport was 446 (403–497) kDa and that for ATP hydrolysis was 394 (359–435) kDa. The low values of both of these estimates suggest that not all subunits which may co-purify with V-ATPases are required for either hydrolysis or transport. For the PPase, the radiation-inactivation size for hydrolysis was 91 (82–103) kDa, suggesting that the minimum functional unit for hydrolysis is the 81 kDa monomer. In contrast to the V-ATPase, the PPase gave a radiation-inactivation size for transport which was 3–4-fold larger than that for hydrolysis (two estimates for transport gave 307 and 350 kDa), indicating that a single catalytic subunit is insufficient for transport activity.

INTRODUCTION

Plant vacuoles are acidified by a typical vacuolar-type H⁺-ATPase (V-ATPase; EC 3.6.1.34). Vacuolar H⁺-ATPases are found in a variety of endomembrane organelles of plants, animals and fungi, and are evolutionarily related to the F₁F₀-ATPases of mitochondria, chloroplasts and eubacteria (Nelson, 1989). The complex subunit composition of these enzymes is now known to a first approximation (Arai *et al.*, 1988; Parry *et al.*, 1989; Matsuura-Endo *et al.*, 1990). In addition, plant vacuoles have a second proton-translocating enzyme, an H⁺-pyrophosphatase (PPase; EC 3.6.1.1), which also appears to serve the purpose of acidifying the vacuole (Rea & Sanders, 1987; Hedrich *et al.*, 1989). The hydrolytic subunit of the latter enzyme has recently been purified and its cDNA has been cloned (see the Discussion section), but much remains to be done to understand its complex kinetics (White *et al.*, 1990) and subunit composition.

An oligomeric structure for the PPase has been suggested by its behaviour in non-denaturing gel electrophoresis (Maeshima, 1990), in cross-linking experiments (Maeshima, 1990) and in gel filtration columns (Sarafian & Poole, 1989). However, these studies did not resolve the question of the minimum functional unit required either for pyrophosphate hydrolysis or for H⁺ transport.

Radiation-inactivation studies have been invaluable in determining the functional size of a variety of enzymes in relation to their polypeptide composition (Kempner & Fleischer, 1989). Several reports have set the radiation-inactivation size of V-ATPases at about 400–500 kDa for ATP hydrolysis, although an estimate of molecular size by summation of associated polypeptides gives values of 650–750 kDa (see the Discussion section). In the case of the PPase, radiation-inactivation sizes of 160 kDa (130–241 kDa) (Chanson & Pilet, 1989) and 88 ± 8 kDa (Fraichard *et al.*, 1991) have been reported for PP_i hydrolysis. The enzyme has been identified on SDS/polyacrylamide gels as a polypeptide of approx. 67 kDa (Britten *et al.*, 1989; Maeshima

& Yoshida, 1989; Sarafian & Poole, 1989); however, the cDNA-deduced size indicates that the correct estimate for the hydrolytic subunit is 81 kDa (Sarafian *et al.*, 1992). It remains therefore to determine whether the minimal functional unit for hydrolysis is a monomer or a dimer.

The radiation-inactivation size for H⁺ transport, as opposed to hydrolysis, has not previously been investigated for either the V-ATPase or the PPase. The present study provides additional information on the radiation-inactivation of these two enzymes, including estimates of functional size for both substrate hydrolysis and H⁺ transport.

EXPERIMENTAL

Tonoplast preparation

Vacuolar membranes (tonoplast) were prepared from fresh red beet (*Beta vulgaris* L.) roots as described (Rea & Poole, 1985; Sarafian & Poole, 1989). The membranes were suspended in a medium containing 10% (w/v) glycerol, 1 mM-EDTA, 5 mM-dithiothreitol, 2.45 mM-BTP and 2.55 mM-Mes, adjusted to pH 8.0 at 4 °C and stored at –70 °C.

Radiation inactivation

Tonoplast fractions were purged with nitrogen and irradiated in solid CO₂ (–78 °C) with γ-rays in a Gammacell 220 ⁶⁰Co irradiator for various lengths of time according to the method of Beauregard *et al.* (1987). Non-irradiated controls were treated in the same way except for the irradiation. The relationship between radiation dose (Mrad) and molecular size was established using the equation:

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

where D_{37} is the dose required to decrease enzyme activity to 37% of the initial rate and t is the temperature (°C) at which the irradiation was applied (Beauregard *et al.*, 1987).

Abbreviations used: V-ATPase, vacuolar H⁺-translocating ATPase (EC 3.6.1.34); PPase, vacuolar H⁺-translocating pyrophosphatase (EC 3.6.1.1); BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; D_{37} , radiation dose required to decrease enzyme activity to 37% of initial rate.

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Enzyme assays and data analysis

Glucose 6-phosphate dehydrogenase activity was measured as described previously (McIntyre & Churchill, 1985).

The phosphohydrolase activity of the PPase was estimated at 37 °C for 10 min by the method of Ames (1966) in 0.3 ml reaction volumes containing 14.7 mM-BTP and 15.3 mM-Mes (to give pH 8.0), 100 mM-KCl, 5 μ M-Gramicidin D, 0.6 mM-K-PP_i and 0.5 mM-MgCl₂. ATPase assays were performed under similar conditions, except for the substrate which was 1.5 mM-ATP and 3 mM-MgCl₂. Hydrolytic activities of the unirradiated controls (μ mol of substrate hydrolysed/h per mg) were 11–13 for the ATPase and 11–14 for the PPase.

H⁺ transport activity was measured as the initial rate of quenching of fluorescence by the dye Acridine Orange (Bennett & Spanswick, 1983) at room temperature (22 °C) in a Perkin–Elmer Fluorescence Spectrophotometer at excitation and emission wavelengths of 495 and 540 nm respectively. Control and irradiated samples containing 30–50 μ g of protein (constant within each experiment) were transferred to 1 ml of reaction medium containing 2.45 mM-BTP and 2.55 mM-Mes, to give pH 8.0, 50 mM-KCl, 5 μ M-Acridine Orange and either 0.3 mM-PP_i or 1.5 mM-ATP. The reaction was started by the addition of 3 mM-MgSO₄.

In order to minimize post-irradiation damage, assays for H⁺ transport were performed immediately after thawing of the samples (each sample being allowed to thaw separately immediately before assay), and assays for hydrolytic activity were done by adding the assay medium directly to the frozen samples.

For each experimental condition, two to three different membrane preparations were irradiated and assayed on different occasions, with similar results. Each data point for hydrolysis or transport represents the mean result from the irradiation and assay of three independent membrane samples. Regression lines were fitted with the least-squares method. Molecular size estimates given are calculated from $D_{37} \pm \text{s.e.m.}$ values.

SDS/PAGE

One-dimensional SDS/PAGE was performed in a Bio-Rad Protean II Slab Cell according to Laemmli (1970). The irradiated samples were treated with SDS sample buffer, incubated at 4 °C overnight and heated to 60 °C for 15 min immediately prior to loading on 10% acrylamide gels (Sarafian & Poole, 1989). Prestained markers (Bio-Rad) were run alongside the samples.

Western blot and immunodetection

The SDS/polyacrylamide gel was blotted on to nitrocellulose (Millipore HA) in a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) equipped with card electrodes. The transfer was done in Towbin's buffer (Towbin *et al.*, 1979) at 0.1 A overnight, followed by 1 h at 0.7 A. The blot was probed with anti-PPase antibody (1:300 dilution) followed by an incubation in 1 μ Ci of ¹²⁵I-Protein A. The blot was air-dried and exposed to X-ray film. The autoradiogram was used as a guide to locate and cut the immunoreacted bands from the blot. Radioactivity was measured by scintillation counting.

Antibodies

Polyclonal antibodies raised against mung-bean hypocotyl vacuolar PPase were kindly given to us by Dr. M. Maeshima, Hokkaido University, Sapporo, Japan. The antibodies specifically recognized the 67 kDa PPase polypeptide in red beet root vacuolar membranes.

Protein assay

Membrane protein was measured by a modified Lowry method (Bennett, 1982).

RESULTS

Radiation inactivation of glucose 6-phosphate dehydrogenase

This enzyme has been widely used as an internal marker for radiation-inactivation studies. Its molecular mass, estimated by a variety of methods, is 104 kDa (McIntyre & Churchill, 1985). Glucose 6-phosphate dehydrogenase at 10 μ g/ml was added to vacuolar membranes and, after exposure to various doses of radiation, the size of the enzyme was calculated from triplicate samples using eqn. (1). The deduced molecular mass was 93 ± 2 kDa. Molecular size calculations (below) for the PPase and ATPase have not been corrected for the underestimation of the standard.

Radiation inactivation of the V-ATPase

When tonoplast vesicles were exposed to increasing doses of

Fig. 1. Inactivation of the vacuolar ATPase by γ -radiation

Shown is the ATPase H⁺-pumping activity (a) and hydrolytic activity (b) in red beet tonoplast after exposure to various doses of γ -radiation at -78 °C. Results are expressed as logs of the percentage activity remaining after irradiation. Regression lines were fitted by the least-squares method. Symbols (○, □, △) represent separate membrane preparations, with each point averaged from simultaneous triplicate irradiations. Closed symbols indicate data not used in the computation of the regression. Estimated D_{37} values (means $\pm$ s.e.m.) were 2.88 ± 0.30 ($n = 12$; $r = -0.99$) and 3.26 ± 0.31 ($n = 20$; $r = -0.97$) for H⁺ transport and ATP hydrolysis respectively, giving molecular sizes of 446 (403–497) kDa and 394 (359–435) kDa respectively.

Fig. 2. Inactivation of the vacuolar PPase by γ -radiation

Shown are the pyrophosphatase H⁺-pumping activity (a) and hydrolytic activity (b) in red beet tonoplast (note difference in scales of the abscissae). Activities are expressed as the logs of the percentage activity remaining after irradiation. The two sets of values obtained for H⁺ transport differ mainly as a result of a different activation pattern at low doses in different batches of membranes. Regression lines were fitted by the least-squares method. Closed symbols indicate data not used in the calculation of the regressions. Symbols ($\circ$, $\square$, $\triangle$) represent separate membrane preparations, with each point averaged from simultaneous triplicate irradiations. Estimated D_{37} values (means $\pm$ S.E.M.) were 4.18 ± 0.25 ($n = 5$; $r = -0.99$) for the upper H⁺ transport curve and 3.46 ± 0.20 ($n = 18$; $r = -0.99$) for the lower H⁺ transport curve, with corresponding molecular sizes of 307 (289–326) kDa and 350 (333–371) kDa. The $D_{37} \pm$ S.E.M. for PP_i hydrolysis was 14.05 ± 1.65 ($n = 37$; $r = -0.99$), which yielded a molecular size of 91 (82–103) kDa.

ionizing radiation, both the hydrolytic and H⁺-pumping activities of the vacuolar ATPase decreased in an exponential manner (Fig. 1). However, at relatively low radiation doses, an initial non-linear component was observed in the log-transformed inactivation curve for transport. A more detailed discussion on the possible reasons for this phenomenon is provided later, but for determination of D_{37} values, only those points showing a linear decline in activity were used (open symbols in Fig. 1). The regression line intercept with the ordinate was determined and used as the basis for calculating 37% of initial activity. For the V-ATPase, size estimates from the assay of hydrolytic activity in native membranes and from measurements of H⁺ pumping were 394 kDa (359–435 kDa) and 446 kDa (403–497 kDa) respectively (Fig. 1).

Radiation inactivation of H⁺-PPase

As in the case of the V-ATPase, the vacuolar PPase showed an exponential decrease in both hydrolytic and transport activity

Fig. 3. Immunodetection of irradiated PPase by ^{125}I -Protein A

Tonoplast vesicles were irradiated and subjected to SDS/PAGE. Proteins were transferred to nitrocellulose and incubated with antibody to PPase, followed by 1 μCi of ^{125}I -Protein A. The radioactivity associated with the PPase was measured in a scintillation counter. Results are expressed as the log of the radioactivity as a percentage of that in the non-irradiated control. The regression line was fitted by the least-squares method. The D_{37} value was 20.1 ± 1.3 mean $\pm$ S.E.M. ($n = 10$; $r = -0.98$). The corresponding target size for the immunodetected polypeptide was 64 ± 4 kDa.

with increasing doses of radiation over a certain minimum dose (Fig. 2). Molecular size determinations were made using the linear component, excluding the low dose values (Fig. 2, closed symbols).

The radiation-inactivation size of PPase for hydrolysis was 91 kDa (82–103 kDa), which would correspond to a monomer of the 81 kDa subunit predicted from the cDNA sequence.

In the case of transport assays with this enzyme, it became apparent that the different sets of values obtained using different batches of membranes could not be grouped into a single regression line. Even though the slopes of the regression lines through the linear portion of the curves were comparable, large differences in the initial, non-linear, part of the curve for one batch of membranes indicated that the factors causing the rise in activity at low doses were variable. The molecular size estimates from the two inactivation curves for H⁺-transport activity (Fig. 2) are 307 kDa (289–326 kDa) and 350 kDa (333–371 kDa) for the top and bottom curves respectively. Despite the difference between these two estimates, it is clear that the radiation-inactivation size for transport is 3–4-fold larger than that for substrate hydrolysis.

Target size for destruction of the PPase catalytic subunit

Irradiated tonoplast vesicles were solubilized in SDS and subjected to gel electrophoresis. The proteins were blotted on to nitrocellulose and probed with antibodies against purified PPase. Antibody binding was quantified by the use of ^{125}I -Protein A. A single band running at 67 kDa, corresponding to the catalytic subunit, was observed. Fig. 3 shows a regression curve deduced from the radioactivity bound to the PPase after exposure to increasing radiation doses. The target molecular size deduced from quantification of the polypeptide was 64 ± 4 kDa.

These results suggest that radiation destroys only one subunit with each ionizing hit. Although this result is based on a small sample, it serves to confirm that radiation destroys only one 81 kDa subunit with each ionizing hit, i.e. the energy of ionization is not transferred from one polypeptide to another.

The discrepancy between the estimated target size and the size predicted by cDNA cloning is perhaps due to incomplete electrophoretic transfer to nitrocellulose. Several Western blots of irradiated samples showed that lower concentrations of polypeptide are transferred more completely than higher ones, resulting in a lower estimate of the molecular size. Judging from the small amount of protein remaining in the gels, however, this effect would not be large enough to invalidate the above conclusion.

DISCUSSION

Reliability of transport measurements

Measurements of transport across membranes are affected by the size and passive conductance of the vesicles. Previous studies on the glucose and phosphate transporters in brush-border membrane vesicles showed that radiation doses of up to 8.5 Mrad did not significantly alter vesicle size and integrity (Beliveau *et al.*, 1988). Since the effect of radiation beyond this level is not known, samples treated with doses higher than 8 Mrad were not used in the analysis of transport data. In addition, two aspects of the present results suggest that there were no important changes in the size or conductance of the vesicles over the course of the radiation treatments. First, after the initial anomalies at low doses of radiation (see below), the measured transport activities of both enzymes showed the expected simple exponential decay. Secondly, for the V-ATPase, the radiation-inactivation size determined from transport measurements was only slightly different from that determined from hydrolytic activity.

Proton transport activity was measured as the initial rate of quenching of the dye Acridine Orange. A theoretical justification of this widely used method is given by Bennett & Spanswick (1983). When the experimental conditions are properly chosen, the initial rate of change of either fluorescence or absorbance of the dye can give a good measure of the relative rates of proton transport. For example, in a study of vacuolar H⁺-ATPase, Palmgren & Sommarin (1989) found that the initial response of the dye closely paralleled the rate of ATP hydrolysis over a wide range of enzyme activity. In the present work, it is not possible to say whether the difference in radiation-inactivation size for transport (446 kDa) versus hydrolysis (394 kDa) by the ATPase reflects a real property of the enzyme, or non-ideal behaviour of either the Acridine Orange fluorescence or the vesicle permeabilities. In any case, the relatively close agreement between the radiation-inactivation sizes for transport and hydrolysis by the V-ATPase serves as an internal control for the more interesting case of the PPase, in which the two estimates are markedly (3–4-fold) different.

Anomalies of the inactivation curves

Deviations from the simple exponential decay of activity were seen at low radiation doses for the transport activities of the V-ATPase and the PPase (Figs. 1 and 2). Such deviations, which represent an increase in activity of those enzyme molecules not 'hit' by irradiation, are more commonly found with membrane enzymes than with soluble ones (Kincaid *et al.*, 1981). The deviations have been interpreted either in terms of the inactivation of a large-molecular-mass inhibitor (Harmon *et al.*, 1980), or in terms of the dissociation of oligomers of the enzyme into monomers with increased activity (Kincaid *et al.*, 1981). In the present case, this would mean dissociation of aggregates of the holoenzyme into single functional units of about 400 kDa each (for the V-ATPase) or about 320 kDa each (for the PPase).

It is important to consider whether the anomalies seen at low radiation doses in any way invalidate estimates of molecular size

from the subsequent exponential decay. When such anomalous effects cannot be clearly distinguished, they can lead to erroneous values of radiation-inactivation size (Simon *et al.*, 1982; Potier & Giroux, 1985). In the present case the anomalies are limited to low doses, and are followed by simple exponential decay curves. In this case, regardless of the interpretation of the initial activation, the final slopes and corresponding radiation-inactivation sizes should not be affected (Harmon *et al.*, 1980; Verkman *et al.*, 1984). Nevertheless, it seems possible that these initial activation effects are responsible for the slight difference in final slope between the two curves for H⁺ transport of the PPase in Fig. 2.

Radiation-inactivation size of the V-ATPase

The molecular sizes calculated from Figs. 1, 2 and 3 for the V-ATPase and the PPase under various conditions are collated in Table 1. For the V-ATPase, the estimates for hydrolysis and for transport are in the same range, around 400–450 kDa. These estimates are in agreement with previously reported radiation-inactivation sizes for this enzyme. In corn coleoptiles, values of 387 ± 19 kDa (Wang *et al.*, 1989) and 386 kDa (309–520 kDa) (Mandala & Taiz, 1985) have been reported, as well as an estimate of 405 ± 25 kDa from mung-bean hypocotyls (Wang *et al.*, 1989) and a slightly higher value of 542 kDa (374–642 kDa) from maize roots (Chanson & Pilet, 1989). In fungi, a size of 410–530 kDa has been reported for yeast (Hirata *et al.*, 1989) and 520 kDa for *Neurospora* (Bowman *et al.*, 1986).

The similarity in inactivation sizes for ATPase hydrolytic and pumping activities suggests that the same minimal functional size is required for both functions. This conclusion was also reached by Anraku and his colleagues (Uchida *et al.*, 1988; Hirata *et al.*, 1989), based on the observation that hydrolysis under normal conditions, either in native membranes or in the Zwittergent-solubilized enzyme, is sensitive to dicyclohexylcarbodi-imide, which blocks the proton channel of V-ATPases (Sun *et al.*, 1987) in the same manner as for F₁F₀-ATPases. This group also showed (Hirata *et al.*, 1989) that the radiation-inactivation size of yeast V-ATPase for normal hydrolysis was 410–530 kDa, corresponding to the above estimates, but that the functional size decreased to 90–110 kDa for single-cycle hydrolysis, under which conditions it was insensitive to the carbodi-imide. The latter value would correspond to a single heterodimer of the A and B subunits, which are normally present in three copies in the catalytic head of the enzyme (Arai *et al.*, 1988).

The radiation-inactivation size of the V-ATPase under normal conditions appears, however, to underestimate the proposed size (759 kDa) arrived at by addition of the associated subunits in their stoichiometric ratios in clathrin-coated vesicles (Arai *et al.*,

Table 1. Summary of results obtained for radiation-inactivation size

Results are molecular sizes estimated from D_{37} (means ± S.E.M.) values using eqn. (1).

	Molecular mass (kDa)	
	ATPase	PPase
Polypeptide subunit		64 ± 4 81*
Hydrolysis	394 (359–435)	91 (82–103)
H ⁺ transport	446 (403–497)	307 (289–326) 350 (333–371)

* Estimated from cDNA sequence.

1988). Additionally the molecular sizes of V-ATPase-associated subunits in plants, using the same plausible stoichiometry, gives total sizes of 709 kDa (based on Parry *et al.*, 1989) or 647 kDa (based on Matsuura-Endo *et al.*, 1990). In the case of mitochondrial ATPase, the radiation-inactivation size (460 kDa) more closely approximates to the value (530 kDa) obtained by addition of subunit sizes (Bowman *et al.*, 1986). The discrepancy between estimates of the size of the V-ATPase obtained using the two methods remains to be elucidated. It may be that not all of the subunits found to be associated with the purified enzyme are required for either hydrolysis or transport. In particular, the radiation-inactivation data suggest that a large subunit of 100–115 kDa, sometimes associated with purified preparations of V-ATPase in both plants (Parry *et al.*, 1989) and animals (Nelson, 1989), is unlikely to be required for either ATP hydrolysis or H⁺ transport. There is, however, evidence that a homologous polypeptide in yeast is required for V-ATPase assembly (M. F. Manolson, personal communication).

Radiation-inactivation size of the H⁺-PPase

The radiation-inactivation size for PPase activity (Table 1) was found to be 91 kDa (82–103 kDa), in agreement with a recently reported value of 88 ± 8 kDa (Fraichard *et al.*, 1991) for PPase in *Acer* vacuolar membranes, but different from a previously reported estimate of 130–241 kDa (Chanson & Pilet, 1989). The size of the polypeptide has been determined from the cDNA sequence to be 81 kDa (Sarafian *et al.*, 1992), somewhat larger than the SDS/PAGE estimates of about 67 kDa for the purified hydrolytic polypeptide (Britten *et al.*, 1989; Maeshima & Yoshida, 1989; Sarafian & Poole, 1989).

Purified preparations of vacuolar PPase contain a single polypeptide of about 67 kDa (Britten *et al.*, 1989; Maeshima & Yoshida, 1989). Antibodies to this polypeptide inhibit PPase activity (Maeshima & Yoshida, 1989). Moreover, labelling of the polypeptide and inhibition of enzyme activity by *N*-ethylmaleimide are both protectable by MgPP_i (Britten *et al.*, 1989). Since purified preparations are active in hydrolysis, and no other polypeptides co-purify with enzyme activity, it is concluded that no other polypeptides are required for PP_i hydrolysis. The functional unit is therefore a monomer of the 81 kDa polypeptide.

In contrast to the above results for PP_i hydrolysis, the radiation-inactivation size for H⁺ transport by the PPase was found in two different preparations to be 307 kDa (289–326 kDa) and 350 kDa (333–371 kDa). The minimum functional size for transport is therefore 3–4-fold larger than that for hydrolysis. Recent evidence suggests that purified preparations of the 81 kDa polypeptide can be reconstituted in a form competent in H⁺ transport (C. Britten & P. A. Rea, personal communication), which would suggest that the functional unit for transport activity probably consists of several identical subunits of the 81 kDa polypeptide.

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