Radiation inactivation analysis of H^+-pyrophosphatase from submitochondrial particles of etiolated mung bean seedlings

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Abstract Radiation inactivation analysis was employed to determine the functional masses of enzymatic activity and proton translocation of H^+-pyrophosphatase from submitochondrial particles of etiolated mung bean seedlings. The activities of H^+-pyrophosphatase decayed as a simple exponential function with respect to radiation dosage. D_{37} values of 6.9 ± 0.3 and 7.5 ± 0.5 Mrad were obtained for pyrophosphate hydrolysis and its associated proton translocation, yielding molecular masses of 170 ± 7 and 156 ± 11 kDa, respectively. In the presence of valinomycin and 50 mM KCl, the functional size of H^+-pyrophosphatase of tonoplast was decreased, while that of submitochondrial particles remained the same, indicating that they are two distinct types of proton pump using PP_i as their energy source.

Key words: Functional size; Radiation inactivation; H^+-pyrophosphatase; Proton translocation; Submitochondrial particle

1. Introduction

H^+-translocating pyrophosphatase (H^+-PPase, EC 3.6.1.1) is gradually recognized as essential machinery in the energy metabolism of cells. H^+-PPase from the chromatophore membrane of *Rhodospirillum rubrum* has long been believed to be an alternative coupling factor to the H^+-ATPase system [1,2]. A novel H^+-PPase has been found solely in higher plant vacuoles and was recently characterized [3–5]. Other possible energy-linked membrane-bound PPases from mitochondria of animals [6,7] and yeasts [8,9] and chloroplast of higher plants [10] were also documented. The PPase from plant vacuoles is a proton translocating dimer (approximately 150 kDa) with a single kind of polypeptide [3,4], whereas that from thylakoids is a monomer of 55 kDa with no trace of proton pumping action [10]. The structure and subunit composition of animal mitochondrial PPases are more complicated [11].

Membrane-bound H^+-PPase has also been found in higher plant mitochondria, but it is less well understood. PP_i biosynthesis coupled to the electron transport chain was observed in plant mitochondria [12]. PP_i-dependent proton translocation has also been demonstrated in pea stem submitochondrial particles [13]. This H^+-PPase activity appears to be associated with a protein of 35 kDa bound to the inner mitochondrial membrane [14]. However, other details on the structure and function of plant mitochondrial H^+-PPases are still required.

Radiation inactivation analysis has been used successfully to assess the functional mass and thus elucidate the function–structure relationship of many enzymes [3,15–18]. In this communication, we determined the functional size of H^+-PPase from submitochondrial particles of mung bean seedlings by radiation inactivation. In the presence of valinomycin and KCl, the functional size of H^+-pyrophosphatase of vacuolar membrane is different from that of submitochondrial particles, suggesting that they are probably two distinct types of proton pump using PP_i as their energy source.

2. Materials and methods

2.1. Plant material

Seeds of *Vigna radiata* L. (mung bean) obtained from a local market were soaked overnight in tap water and then germinated at room temperature in the dark. Hypocotyls of 4-day-old etiolated seedlings were excised, chilled on ice, washed with distilled water, and then used as starting material.

2.2. Preparation of submitochondrial particles

Mitochondria were prepared according to the method of Moore and Proudlove [19] with minor modifications. Fresh hypocotyls (0.5–1 kg) were cut into 2 l of chilled extraction medium [30 mM MOPS–KOH buffer (pH 7.5), 300 mM mannitol, 2 mM β-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.6% (w/v) polyvinylpyrrolidone (PVP, $M_r \approx 40\,000$), and 0.1% (w/v) bovine serum albumin (BSA)]. The mixture was ground in a mortar with a pestle and then filtered through eight layers of cheesecloth. The material retained on the cheesecloth was pooled and mixed with 0.5 l of extraction medium. The solution was then homogenized with a Polytron homogenizer at medium speed for 10 s and filtered again as above. The filtered suspension was subjected to differential centrifugation at $1500 \times g$ and $12\,000 \times g$ for 20 min. The pellet was resuspended in washing medium [10 mM MOPS–KOH buffer (pH 7.4), 300 mM mannitol, 1 mM EDTA, and 0.1% (w/v) BSA]. The suspension was subjected to differential centrifugation again at $1500 \times g$ (10 min) and $12\,000 \times g$ (20 min). The pellet containing washed mitochondria was resuspended in washing medium at approximately 50 mg protein/ml and ready for the preparation of submitochondrial particles.

The washed mitochondria were diluted with an equal volume of buffer I [10 mM MOPS–KOH (pH 7.0), 250 mM sucrose, 0.5% (w/v) BSA, and 6 mM ATP] and subjected to ultrasonication twice (100 W) for 30 s with a 1 min interval in an ice bath by a Branson 250 sonifier. The unbroken mitochondria were removed by centrifugation at $28\,000 \times g$ for 5 min. The supernatant (submitochondrial particles) was centrifuged at $100\,000 \times g$ for 30 min. The pellet was washed with buffer I without ATP and then centrifuged again. The pellet was resuspended at a final protein concentration of 2 mg/ml in buffer II

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; PPase, pyrophosphatase

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[250 mM sucrose, 20 mM Tris–HCl, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis-(aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and 2 mM $MgCl_2$]. Submitochondrial particles thus obtained were then stored at −70°C until use.

2.3. Tonoplast preparation

Preparation of tonoplast was carried out according to Maeshima and Yoshida with minor modifications as described previously [20,21].

2.4. Radiation inactivation analysis

Samples were irradiated with a ^{60}Co source (29 000 Ci, J.L. Shepherd and Associates, Model 109 Irradiator) at the Division of Isotope Application, National Tsing Hua University. During irradiation, samples were kept at −64°C in a cryothermostat maintained by dry ice in a solution containing ethanol and commercial antifreeze (1:1). The molecular mass of the irradiated sample was calculated from the equation of Beauregard and Potier [22]:

$$\text{Log } M_r = 5.89 - \log D_{37} - 0.0028\, t$$

where M_r is the functional size in Da, t is the temperature (°C) during irradiation, and D_{37} is the dose of radiation in Mrad required to reduce the activity to 37% of that found in unexposed control at temperature t (°C). The dose response of glucose-6-phosphatase dehydrogenase (molecular mass 104 kDa) was as determined previously [16,23] and used as standard marker.

2.5. Measurement of proton translocation

Proton translocation of submitochondrial particles was measured as fluorescence quenching of acridine orange (excitation 495 nm, emission 530 nm) by a Hitachi F-4000 fluorescence spectrophotometer as previously described [4]. The reaction mixture (2 ml) contained 10 mM HEPES–Tris (pH 7.5), 50 mM KCl, 250 mM sucrose, 5 mM $MgSO_4$, 1 mM EGTA, 5 μM acridine orange, and 50 μg submitochondrial particles. Fluorescence quenching was initiated by adding 0.1 mM PP_i to the mixture. An aliquot (20 μl) of nigericin (final concentration 2 μM) was added at the end of each assay.

2.6. Enzyme assay and protein determination

Pyrophosphate hydrolysis was measured as the release of P_i from PP_i [24] in an assay medium [10 mM MOPS–KOH (pH 7.5), 50 mM KCl, 250 mM sucrose, 5 mM $MgSO_4$, 1 mM EGTA, 0.1 mM PP_i, 0.1 mM ammonium molybdate, 0.2 mM sodium vanadate, 0.5 mM sodium azide, 50 mM potassium nitrate and 50 μg submitochondrial particle protein] for 20–30 min at 33°C. The reaction was terminated by adding 2 ml solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) sodium dodecyl sulfate, and 0.02 (w/v) 1-amino-2-naphthol-4-sulfonic acid (ANSA). The amount of released P_i was determined spectrophotometrically at 700 nm.

The protein concentration was measured using a protein assay kit (Bio-Rad) according to Bradford [25].

2.7. SDS–PAGE and immunoblot analysis

A peptide, DVGADLVGKVE, was synthesized to prepare antibody according to the sequence of a conserved fragment from mung bean vacuolar H^+-PPase (cf. [26]). The synthesized peptide was linked to BSA using glutaraldehyde [27]. The conjugate was homogenized with Freund's complete adjuvant for the initial injection and Freund's incomplete adjuvant for the booster injection. After boost for 4 months, anti-peptide serum was collected without further purification. Submitochondrial particles and tonoplast vesicles were subjected to SDS–PAGE [28] and then Western blotting according to standard methods [27].

3. Results and discussion

First, the purity of submitochondrial particles was examined for our studies. Contamination of submitochondrial particles obtained using the current protocol by other organelles, such as endoplasmic reticulum, Golgi bodies, and vacuolar vesicles, was negligible as determined by respective marker enzymes (data not shown; cf. [29]). Moreover, the ATPase activity of our submitochondrial particles could be completely inhibited by 1 mM sodium azide, indicating high purity of this

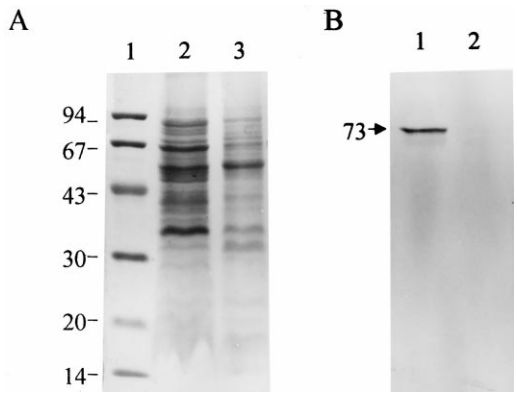

Fig. 1. SDS–PAGE and immunoblotting of tonoplast vesicles and submitochondrial particles. Tonoplast vesicles and submitochondrial particles were isolated as described in Section 2 and then subjected to SDS–PAGE (A) and subsequent immunoblot (B). A: Lane 1, protein markers; lane 2, tonoplast membrane proteins; lane 3, submitochondrial particles. B: Lane 1, tonoplast membrane proteins; lane 2, submitochondrial particles.

preparation. The presence of vacuolar membranes was excluded as determined by the negative result of an immunoassay using antibody against a synthesized peptide of a conserved fragment of vacuolar H^+-PPase (Fig. 1B; cf. [20]). In other words, our submitochondrial particles obviously contain no anti-vacuolar H^+-PPase antibody recognizable proteins, a phenomenon also observed by Vianello et al. [30]. In addition, the H^+-ATPase activity of our preparation was bafilomycin A1 insensitive, confirming the absence of vacuolar vesicles (data not shown) [31]. These results firmly demonstrate that the purity of our submitochondrial particles was high enough for our studies. Furthermore, submitochondrial particles display pyrophosphate hydrolysis and its associated proton pumping reactions (Fig. 2). Both reactions could be completely abolished by 0.2 mM potassium fluoride, a common inhibitor of all known pyrophosphatases. Besides, 2.0 mM P_i alone could not drive the proton translocation, indicating that the observed proton pumping is directly coupled to PP_i hydrolysis rather than the secondary effect of its product (data not shown).

To verify whether this technique is feasible under our conditions, the molecular mass standard glucose-6-phosphate dehydrogenase (104 kDa) was included in the sample solution and subjected to radiation inactivation analysis. A D_{37} value of 10.8 ± 0.2 Mrad and calculated functional size of 108 ± 5 kDa were subsequently obtained for glucose-6-phosphate dehydrogenase (data not shown). This result clearly indicates that radiation inactivation analysis is suitable for our studies.

Upon exposure to high energy irradiation, PP_i hydrolysis activities of submitochondrial particles displayed a simple exponential decay with respect to the increase in radiation dose (Fig. 2A, ●). This semilogarithmic dose–response relationship yields a D_{37} value of 6.9 ± 0.3 Mrad. According to the equation of Beauregard and Potier, a functional mass of 170 ± 7 kDa ($r > 0.98$) was thus obtained for PP_i hydrolysis of submitochondrial particles [22]. In parallel experiments, we also investigated the dose response of PP_i-driven H^+-translocation across the membrane of submitochondrial particles as determined by fluorescence quenching of the ΔpH probe, acridine orange (Fig. 2B). Upon exposure to γ-ray irradiation, PP_i-supported proton translocation of submitochondrial particles

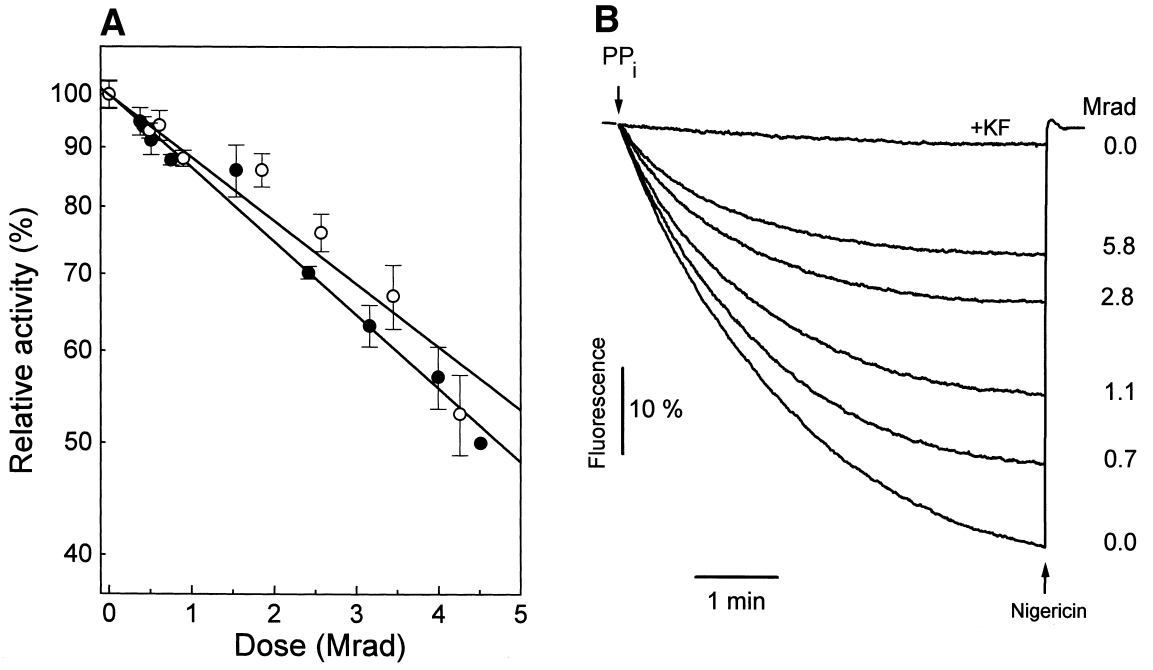

Fig. 2. Radiation inactivation of PP_i hydrolysis and its associated proton translocation of submitochondrial particles. A: Dose response of PP_i hydrolysis and proton translocation. The irradiation and activity assay of H^+-PPase (●) and its associated proton pumping activity (○) were as described in Section 2. All data points are means of at least three independent measurements with line fitted by regression analysis ($r > 0.98$). Control activity of submitochondrial H^+-PPase was approximately 20 μmol PP_i consumed/mg protein/h. B: Reaction trace of fluorescence quenching of acridine orange. Initial rates of fluorescence quenching were determined from the change of fluorescence in the first minute. The concentrations of F^- and nigericin were 0.2 mM and 2 μM, respectively.

was subsequently decreased. The decrease of proton pumping activity also followed a simple exponential decay with respect to the increase of irradiation dose, yielding a D_{37} value of 7.5 ± 0.5 Mrad (Fig. 2A, ○). This value corresponds to a functional size of 156 ± 11 kDa. The similar functional mass of enzymatic activity and proton translocation suggests that these two reactions are well coupled in submitochondrial particles, a phenomenon also observed in the vacuolar proton pumping PPase [4]. However, for vacuolar proton pumping pyrophosphatase, its functional size depends on the electrochemical gradient across the membrane [4]. Uncouplers and ionophores are the membrane perturbation factors that disrupt the pH gradient and the electrochemical potential across the membrane [32]. In the presence of the ionophore valinomycin and KCl, the functional mass of membrane-bound H^+-PPase of tonoplast was decreased from 141 kDa to 63 kDa, which is about the size of the monomer enzyme [4]. In the presence of 2 μM valinomycin and 50 mM KCl, however, the functional size of PPase activity of submitochondrial particles remains the same as in the absence of ionophore (data not shown), indicating that submitochondrial H^+-PPase has a different nature from vacuolar H^+-PPase toward membrane potential.

H^+-PPases were found solely in plant vacuoles and chromatophores of *Rhodospirillum rubrum* and belong to a new category of proton pumps using exclusively PP_i as their energy source [1–3]. Recently, efforts have been made to demonstrate that endomembranes of higher plants other than vacuoles also contain proton pumping pyrophosphatase. For instance, Robinson demonstrated the possibly ubiquitous presence of vacuolar H^+-PPase in many parts of storage parenchyma cells from developing pea cotyledons [33]. Long et al. showed that phloem-specific plasma membrane from *Ricinus communis* seedlings contains a H^+-PPase which resembles that from vacuoles [34]. A preliminary result in this laboratory also suggests the existence of a new endoplasmic reticulum-specific H^+-PPase (Kuo and Pan, unpublished data). Furthermore, Vianello et al. successfully observed a proton pumping activity of pea stem submitochondrial particles [13]. They demonstrated that the molecular mass of mitochondrial H^+-PPase is far smaller than that from vacuoles, indicating a possible new type of H^+-PPase [14]. In this report, we further showed the functional size of submitochondrial particles is similar to that from vacuoles. However, the dose responses of H^+-PPases from submitochondrial particles and vacuoles to ionophore (valinomycin+KCl) are different, suggesting they are distinct types of proton pumping enzyme. The insensitivity of H^+-PPase from submitochondrial particles to anti-vacuolar H^+-PPase antibody indicates, in addition, that they are different types of proton pumping enzyme using identical PP_i as energy source. Taking this evidence together, we believe that distinct types of proton pumping pyrophosphatase are distributed more widely in various endomembranes of higher plants. Subunit identities and detailed characteristics of various proton pumping pyrophosphatases from their respective endomembrane origins require further investigation.

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