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TARGET MOLECULAR SIZE SUGGESTING A DIMERIC STRUCTURE FOR THE RED BEET PLASMA MEMBRANE Ca^{2+}-ATPASE

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Key Word Index—*Beta vulgaris*; Chenopodiaceae; beet; membrane transport; transport ATPase; signal transduction; radiation inactivation analysis.

Abstract—The target molecular size of the red beet (*Beta vulgaris* L. var. Detroit Dark Red) plasma membrane Ca^{2+}-ATPase was determined by radiation inactivation analysis involving γ-rays from a ^{60}Co source. The Ca^{2+} transport activity associated with the Ca^{2+}-ATPase in native plasma membrane vesicles declined in an exponential manner with radiation dose and the target molecular size was estimated to be about 245000 ± 5000. As the catalytic subunit (phosphorylated intermediate) of the red beet plasma membrane Ca^{2+}-ATPase has been shown to be about 100000 to 120000, these results suggest that the enzyme exists as a dimer in the native membrane.

INTRODUCTION

The plant plasma membrane Ca^{2+}-ATPase couples ATP hydrolysis to the translocation of Ca^{2+} from the cytoplasm to the cell exterior [1–3]. Together with Ca^{2+} transport systems associated with the endoplasmic reticulum [3] and vacuole [4], this enzyme plays a significant role in plant signal transduction by maintenance of a low cytoplasmic Ca^{2+} concentration near 0.1 μM [2]. During a signal transduction event, transient increases in cytoplasmic Ca^{2+} concentration appear linked to numerous metabolic and developmental processes [5] and Ca^{2+} transport systems then serve to return cytoplasmic Ca^{2+} concentrations to the pre-signal level.

Biochemical studies have shown that the plant plasma membrane Ca^{2+}-ATPase is a P-type transport enzyme which forms a phosphorylated intermediate during the course of ATP hydrolysis [1–3]. Electrophoretic analysis of the radiolabelled phosphorylated intermediate formed using $AT^{32}P$ has shown the catalytic subunit of the red beet plasma membrane Ca^{2+}-ATPase to have a molecular weight of about 100000 to 120000 [6, 7]. As this enzyme has not been purified to homogeneity, the quaternary structure of the native enzyme remains unknown. Nevertheless, an understanding of Ca^{2+}-ATPase quaternary structure will be essential for elucidating its mechanism of energy coupling to Ca^{2+} transport.

Radiation inactivation analysis has proven useful for determining the quaternary structure of enzymes, even when present in crude preparations [8–10]. This method is based upon target theory which assumes that any event of ionizing radiation should inactivate the function of a molecule if it occurs within its effective volume or 'target molecular size' [8, 10]. Given a specific assay for a target molecule, a mathematical relationship relating holoenzyme molecular weight to the applied radiation dose has been shown to be valid for numerous proteins, both soluble and membrane-bound [8, 10]. As ATP-dependent $^{45}Ca^{2+}$ uptake in plasma membrane vesicles can represent a specific assay for activity of the Ca^{2+}-ATPase [1, 2], radiation inactivation analysis of this transport activity was examined in order to elucidate the target molecular size of this enzyme.

RESULTS AND DISCUSSION

Plasma membrane vesicle fractions isolated from red beet storage tissue display ATP-dependent $^{45}Ca^{2+}$ uptake which appears entirely due to activity of the plasma membrane Ca^{2+}-ATPase [2, 6, 11]. Hence, while measurements of ATP hydrolytic activity associated with plasma membrane vesicles may reflect activities of both the plasma membrane H^+-ATPase and Ca^{2+}-ATPase, measurement of $^{45}Ca^{2+}$ uptake under appropriate conditions can represent a specific assay for this latter enzyme [see ref. 1 for discussion]. In order to utilize radiation inactivation analysis, enzyme preparations must be present in a form which minimizes secondary inactivation due to the production of radiological products of water [10]. For this reason, radiation inactivation analysis has typically been conducted on either lyophilized samples or frozen samples maintained at low temperature [8–10]. While red beet plasma membrane vesicles cannot be subjected to lypholization without loss of activity [data not shown], membrane fractions can be frozen under liquid nitrogen, stored at − 50 to − 80° and then thawed

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with retention of ATP-dependent $^{45}Ca^{2+}$ transport [11]. Therefore, radiation inactivation analysis was conducted on red beet plasma membrane vesicle fractions frozen under liquid nitrogen and then maintained at dry ice temperature (− 78°) during irradiation using a ^{60}Co source.

When plasma membrane vesicles were exposed to high energy γ-rays ranging up to 5 Mrad, ATP-dependent $^{45}Ca^{2+}$ uptake decreased in an exponential manner with increasing radiation dose (Figure 1). This decrease in $^{45}Ca^{2+}$ uptake was not due to an increase in the passive permeability of the vesicles since radiolabel movement into the vesicles in the absence of ATP was unaffected by γ-ray irradiation. Hence, this decrease in ATP-dependent $^{45}Ca^{2+}$ uptake reflected an inactivation of the Ca^{2+}-pump. This decrease of $^{45}Ca^{2+}$ transport activity in a simple exponential manner ($R^2 = 0.992$) with radiation dose indicates that this activity is associated with a single target size [8–10]. The functional molecular weight for the Ca^{2+}-ATPase can be estimated from the following relationship describing such a plot [see ref. 10]:

$$\ln [A/A_0] = -[M/k] \times D$$

where A represents the activity recovered following irradiation, A_0 represents the activity of the control sample, M represents the target molecular size in Da, k represents an empirical constant (6.4×10^5) [10, 12] and D represents the radiation dose in Mrad. Using this relationship and the estimated slope of the plot (− 0.382), the target molecular size of the red beet plasma membrane Ca^{2+}-ATPase was determined to be about 245 000 ± 5000.

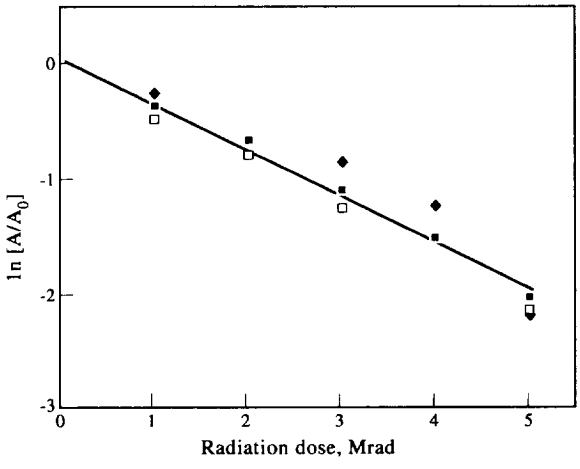

Fig. 1. Inactivation of ATP-dependent $^{45}Ca^{2+}$ transport in plasma membrane vesicles from red beet storage tissue by γ-ray irradiation. Plasma membrane vesicle fractions were exposed to various doses of radiation at − 78° with residual ATP-dependent $^{45}Ca^{2+}$ uptake assayed after vesicle fractions were thawed. Each symbol represents a single enzyme sample assayed in duplicate with a best fit line determined by linear regression ($R^2 = 0.992$). From the estimated slope of the line (slope = − 0.382), the target molecular size was calculated according to: $\ln [A/A_0] = -[M/k] \times D$ where variables are defined in 'RESULTS AND DISCUSSION'. From this relationship, the target molecular size was estimated to be about 245 000 ± 5000.

In previous studies, the catalytic subunit of the red beet plasma membrane Ca^{2+}-ATPase has been shown to have a molecular weight of 100 000 by Williams *et al.* [6] and 124 000 by Thomson *et al.* [7]. This was shown by formation of a radiolabelled phosphorylated intermediate associated with the enzyme reaction mechanism and then analysis by dodecyl-sulphate gel electrophoresis [ref. 13 for discussion]. Using a similar approach, Hsieh *et al.* [14] also found a catalytic subunit molecular weight of 120 000 for the carrot plasma membrane Ca^{2+}-ATPase. Given this data for the molecular size of the catalytic subunit, radiation inactivation analysis would suggest that the plasma membrane Ca^{2+}-ATPase exists as a dimeric arrangement of catalytic subunits in the native membrane. While our estimate of the target molecular size for the red beet plasma membrane Ca^{2+}-ATPase appears lower than that determined for the radish plasma membrane Ca^{2+}-ATPase (i.e. 270 000) [15], the catalytic subunit size for that enzyme has not been ascertained. A dimeric arrangement for the red beet plasma membrane Ca^{2+}-ATPase would be similar to what has been observed for the Ca^{2+}-ATPase of the erythrocyte plasma membrane [16] and sarcoplasmic reticulum [17] where results from radiation inactivation analysis have been corroborated by limited cross-linking studies. A dimeric arrangement for the plant plasma membrane Ca^{2+}-ATPase could indicate that such a structure is important for energy coupling to transport and/or the regulation of enzyme activity.

EXPERIMENTAL

Plant material. Red beets (*Beta vulgaris* L., cv Detroit Dark Red) were purchased commercially. The beets were detopped and stored at 4° in the dark for at least 10 days to insure uniformity in membrane isolation [18]

Prepn of plasma membrane vesicles for irradiation. Plasma membrane vesicle frs were isolated from red beet storage tissue as described by Williams *et al.* [6]. The final vesicle fr. was suspended in 250 mM sucrose, 1 mM BTP/Mes pH 7.2, 1 mM DTE to a protein concn of 5 mg ml^{-1} and then 200 μl aliquots were distributed into Eppendorf tubes. The membrane vesicles were frozen under liquid N_2 and then maintained at − 80° until transit to the irradiation facility.

Radiation inactivation analysis. Radiation inactivation analysis was performed as described by Briskin and Reynolds-Niesman [19] using a ^{60}Co γ-ray source at the Neely Nuclear Research Center (Georgia Institute of Technology) but without an int. standard. During irradiation the dose was maintained at 2.5 Mrad hr^{-1} as determined using dosimetry under conditions appropriate to the radiation exposure of the prepns. With dosimetry conducted in this manner, there was no observed temp. effect on the profile of radiation inactivation [see ref. 20 for discussion]. For each irradiated sample, a parallel control sample was maintained at identical conditions except without radiation. The plasma mem-

brane frs were maintained at dry ice temp. (− 78°) both during irradiation and transit between Urbana and Atlanta. Following exposure to γ-ray radiation, the membrane fractions were thawed and $^{45}Ca^{2+}$ uptake into the vesicles was determined. Irradiation of the plasma membrane frs was conducted in triplicate for each dose tested.

Measurement of ATP-dependent $^{45}Ca^{2+}$ uptake. Radiolabelled $^{45}Ca^{2+}$ uptake was measured by the method of Giannini *et al.* [11]. The assay was conducted in a 500 μl reaction vol. containing 250 mM sorbitol, 25 mM BTP/Mes pH 7.5, 3.75 mM $MgSO_4$, 3.75 mM ATP, 100 mM KNO_3, 0.4 mM NaN_3 and 15 μM $CaCl_2$ (containing 2.5 μCi $^{45}Ca^{2+}$). Uptake was initiated by addition of plasma membrane vesicles (40 μg ml^{-1} final concn). At timed intervals, 150 μl aliquots were removed and the vesicles were collected by rapid filtration on 0.45 μm metricel membrane filters (Gelman). The filters were rapidly washed with two 4 ml aliquots of wash buffer (250 mM sorbitol, 25 mM BTP/Mes pH 7.5, 3.75 mM $MgSO_4$, 100 mM KNO_3, 0.4 mM NaN_3, 15 μM $CaCl_2$) with radioactivity determined by liquid scintillation spectroscopy in scintillation cocktail (5 ml, Bio-safe II, RPI Co.). For each dose, ATP-dependent transport was considered as the difference in radiolabel uptake observed in the presence and absence of ATP.

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