Target Molecular Size of the Red Beet Plasma Membrane ATPase

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ABSTRACT

Radiation inactivation of the red beet (Beta vulgaris L.) plasma membrane ATPase was carried out using γ-ray radiation from a 137Cs source. Inactivation of vanadate-sensitive ATPase activity by γ-ray radiation followed an exponential decline with increasing total dose, indicating a single target size calculated to have a molecular weight of about 228,000. Since the catalytic subunit of the red beet plasma membrane ATPase has been demonstrated to have a molecular weight of about 100,000 by dodecyl-sulfate gel electrophoresis following 32P-phosphorylation, it is suggested that the native enzyme may exist, at least, as a dimer of catalytic subunits.

The plasma membrane ATPase is strongly implicated to represent the pump responsible for driving H+-efflux in higher plant cells (17, 28). During the catalytic cycle of this enzyme, the terminal phosphate group of ATP is transferred to the protein to form a covalent phosphorylated intermediate (4, 5, 30) on the β-carboxyl side chain of an active site aspartyl residue (6). The formation of this covalent intermediate has provided useful information concerning the mechanistic properties of this enzyme and most likely represents a key step in the energy transduction process (3 and references therein). When phosphorylation has been used to label the catalytic portion of the plasma membrane ATPase from corn (4), oat (30), and red beet (5) with 32P, the identification of a 100,000-mol wt subunit has been demonstrated by dodecyl-sulfate gel electrophoresis. This mol wt is similar to that observed for the catalytic subunit of the animal cell Na, K-ATPase (14), the Ca-ATPase of the sarcoplasmic reticulum (9), the gastric mucosal H+, K-ATPase (10), and the fungal plasma membrane ATPase (12). For several of these transport ATPases isolated from animal cells, an oligomeric structure has been suggested for the native enzyme (see “Results and Discussion”). An understanding of the quaternary structure of these transport ATPases is an important consideration in attempting to relate the reaction mechanism of these enzymes to their ion pumping function.

Radiation inactivation analysis has provided a relatively simple method of estimating the native molecular weight of membrane components without the uncertainties associated with techniques involving cross-linking, derivatization, or solubilization with detergents (15 and references therein). This method involves irradiating membrane samples with high energy radiation such as electrons, x-rays or γ-rays, and then analyzing the decrease in enzyme activity with increasing total radiation dose. This relationship between enzyme activity level and radiation dose is then interpreted in terms of target theory (15, 27, 31) where the decrease in enzyme activity due to radiation exposure is an exponential function of the absorbed dose and related to the molecular weight (target size) by the Kepner-Macey equation (16):

\[ \text{Mol wt} = 6.4 \times 10^2 / D_{37} \]

In this equation, \(D_{37}\) is the radiation dose (in Mrad) required to reduce enzyme activity to 37% of the control level. This mathematical relationship has been shown to be valid for both high dose rate exposure by electrons from a linear accelerator (15, 16) and low dose rate exposure by γ-rays from source irradiators (1, 18). A major limitation of the technique is that target theory is based upon the direct effects of radiation on proteins and is confounded by indirect effects due to the presence of radiolysis products (i.e. OH, peroxide) produced in the liquid state (16 and references therein). To minimize these effects, membrane samples are irradiated at low temperature in the frozen or lyophilized state.

In this communication, preparations enriched with plasma membrane ATPase from red beet (Beta vulgaris L.) were irradiated with γ-rays from a 137Cs source irradiator. The results from radiation inactivation analysis suggest that the native enzyme may exist, at least, as a dimer of 100,000 mol wt subunits.

MATERIALS AND METHODS

Plant Material. Red beets (Beta vulgaris L., var Detroit Dark Red) were greenhouse grown in 8-L pots. A complete nutrient solution was applied twice a week and natural light was supplemented with fluorescent lamps. Following harvest at 3 months, the tops of the plants were removed and the storage roots were maintained at 4°C until use.

Membrane Isolation. Plasma membrane-enriched fractions
were prepared from the red beet storage tissue as described by Briskin and Poole (5, 7). The membranes recovered from sucrose gradients were diluted with 250 mM sucrose, 1 mM Tris-Mes (pH 7.2), 1 mM dithioerythritol (suspension buffer) to 20 mL, and then centrifuged at 200,000g (42,000 rpm) for 30 min in an IEC A-269 rotor. The resultant pellets were suspended in suspension buffer to a protein concentration of about 5 mg/mL and then distributed into microfuge tubes (200 μL/tube) for irradiation. The ATPase activity of the preparations measured in the presence of 50 mM KCl ranged from 35 to 47 μmol h⁻¹ mg⁻¹ protein. The isolated plasma membranes were rapidly frozen in liquid nitrogen and maintained at liquid N₂ temperature during transit from Logan to Salt Lake City.

Irradiation Procedure. Plasma membrane fractions were irradiated with γ-rays by a modification of the method of Beauregard et al. (1). In preliminary studies, an irradiator containing 1000 Ci of 137Cs was used; however, subsequent studies were carried out using an irradiator containing 5000 Ci of 137Cs. For the 1000 Ci source, the 137Cs formed the walls of a cavity in which the samples were placed. In the 5000 Ci source, the 137Cs source was present as a single rod raised above the samples during irradiation and a uniform dose was maintained by rotating the circular sample rack with a turntable system. The dose rate for the 1000 Ci and 5000 Ci sources was determined to be 438 rad min⁻¹ and 1628 rad min⁻¹, respectively, by dosimetry under conditions appropriate to the radiation exposure of the preparations. Internal standardization of the radiation exposure was carried out with acetylcholinesterase as described by Saccomani et al. (26) for the 5000 Ci cesium source. In both cases, the samples were maintained at −78°C during the course of irradiation. Irradiation of the membrane preparations was carried out for various times, in duplicate, while control samples (also in duplicate) were run concurrently under the same conditions but without irradiation. The control samples were used to account for the slight decrease in activity (~15% max) resulting from storage at −78°C.

Enzyme Assay. Irradiated and nonirradiated samples were assayed for ATP hydrolytic activity as previously described (8) in a 1.0-mL reaction volume containing 0.1 mL of membrane suspension. The assay was carried out at 38°C in the presence of 3 mM ATP (tris salt, pH 6.5), 3 mM MgSO₄, 50 mM KCl, 500 μM sodium molybdate, and 30 mM Tris-Mes (pH 6.5) (titrated from 0.3 M stocks). Sodium vanadate, when added, was present at 50 μM and vanadate-sensitive ATPase (20, 21) represented the difference in activity observed in the absence and presence of the inhibitor.

RESULTS AND DISCUSSION

When red beet plasma membrane fractions were exposed to high energy γ-ray radiation, vanadate-sensitive ATPase activity was reduced in an exponential manner with increasing radiation dose (Fig. 1). Irradiation with a 1000-Ci cesium source only allowed inactivation to 64% of the initial level in 48 h of exposure. However, with the use of a 5000-Ci source, inactivation to 16% of the initial level could be achieved in the same time period. For all the indicated radiation exposures, the vanadate-sensitive ATPase activity in an irradiated sample was compared on a per cent basis to the activity present in a control sample run concurrently under identical conditions but without irradiation. Vanadate-sensitive ATPase activity was examined in this study since it is a more selective indication of plasma membrane ATPase in these preparations (20, 21). A maximum inhibition of about 67% was observed with 50 μM Na₂VO₄. On the other hand, ATPase activity measured in the presence of 50 mM KCl or KCl-stimulated ATPase activity could reflect the activity of other phosphohydrolases such as the putative tonoplast ATPase or mitochondrial ATPase present as contaminants (20 and references therein). Although vanadate has been shown to inhibit

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**Fig. 1.** Inactivation of vanadate-sensitive ATPase activity from red beet by γ-rays. Plasma membrane fractions were exposed to various doses of radiation and subsequently analyzed for vanadate-sensitive ATPase activity as described in "Materials and Methods." Each symbol (■, 1000 Ci source; △, 5000 Ci source) represents a single membrane sample assayed in duplicate. The line is a least-squares fit of the data (r² = 0.957) and the D0 value calculated by linear regression is 2.81 Mrad (se = 0.170 Mrad). The target size by this measurement is 228,000 ± 14,000 D.

The decline of the vanadate-sensitive component of ATPase activity in a simple exponential manner indicates that this parameter of ATPase activity is representative of a single target size (15, 27, 31) and most likely a single enzyme. From an analysis of the data using linear regression, the radiation dose required to decrease the activity to 37% of the control value (D37) was found to be 2.81 Mrad. This D37 value implies a target size (mol wt) of about 228,000 D (se = ±14,000 D). Since the mol wt of the catalytic subunit of the red beet plasma membrane ATPase has been shown to be about 100,000 (5), this would suggest a dimeric structure consisting of two catalytic subunits for the native enzyme. This proposal, however, may represent a minimal estimate for the native mol wt since the radiation inactivation technique depends upon efficient energy transfer between associated subunits (27, 31). In addition, there is some uncertainty as to whether or not there are other noncatalytic subunits associated with the enzyme complex. Studies are currently underway to substantiate this proposed dimeric structure by limited cross-linking techniques using the ³P-labeled phosphoenzyme to tag the catalytic subunit.

Using radiation inactivation analysis in conjunction with information obtained from dodecyl sulfate gel electrophoresis, oligomeric structures have been proposed for several ion transporting ATPases isolated from animal cells which are representative of the class of ATPase which form covalent phosphoryl intermediates (see 3 for discussion). The Na⁺ K-ATPase is believed to be a complex consisting of two 100,000 mol wt catalytic subunits and two 37,000 mol wt noncatalytic glycoproteins (14, 16, 19, 22) while a trimeric (26) or tetrameric (24) arrangement of 100,000 mol wt subunit has been suggested for the gastric mucosal H⁺-K-ATPase. Recent studies have also suggested a dimeric structure for both the Ca-ATPase of the SR (13 and references therein) and the Ca-ATPase of the erythrocyte plasma

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Abbreviation: SR, sarcoplasmic reticulum.
membrane (18). For the Na, K-ATPase (23, 25), SR Ca-ATPase (13 and references therein) and erythrocyte Ca-ATPase (18 and references therein), cross-linking studies and other techniques have supported the structures suggested by radiation inactivation studies. The proposal for an oligomeric structure for the higher plant ATPase suggests a further similarity to these other well characterized transport enzymes isolated from animal cells.

An oligomeric structure for these various transport ATPases is also significant in terms of attempting to correlate the reaction mechanism of these enzymes to their ion-pumping function. In this respect, an oligomeric structure could imply that the interface between adjacent subunits could represent a hydrophilic channel for ion translocation (13 and references therein) or individual subunits could contain hydrophilic channels within a single polypeptide chain that repeatedly crosses the membrane (22 and references therein). For both the Na, K-ATPase (14), and the Ca-ATPase of the SR (9), evidence has been presented which indicates that the minimal unit of ATP hydrolytic activity is a 100,000 mol wt catalytic subunit (and a 37,000-mol wt glycprotein in the case of Na, K-ATPase); however, it is unclear whether or not this is also the minimal unit which can couple ATP hydrolysis to ion transport (9, 14). An oligomeric structure for linking ATP hydrolysis to transport, however, would be consistent with the proposal by Boyer et al. (2) for the requirement of dual catalytic sites acting out of phase with one another. Such models have been proposed for the Na, K-ATPase (14), SR Ca-ATPase (29), and the gastric H, K-ATPase (10). Therefore, these types of proposals for a structure-function relationship in these well characterized transport ATPases isolated from animal cells may be relevant to the higher plant plasma membrane ATPase and may represent a perspective for further study.

LITERATURE CITED

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