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PROTON PUMPING BY VESICLES RECONSTITUTED FROM TWO FRACTIONS OF SOLUBILIZED ROSE-CELL PLASMA MEMBRANE ATPase

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Two forms of K^+-stimulated ATPase, which can be solubilized from purified plasma membrane preparations of suspension-cultured rose cells and separated by molecular sieve chromatography, both catalyze the ATP-dependent accumulation of protons into artificial phospholipid/cholesterol vesicles. The higher-molecular weight form of ATPase is highly sensitive to ultraviolet light, and the proton pumping ability of this form is similarly sensitive.

The existence of an electrogenic proton-pumping ATPase in the plasma membranes of plant cells has been postulated on the basis of physiological evidence [1,2]. The most direct evidence for the existence of plant H^+-ATPases comes from studies with microsomal sealed vesicles that demonstrate an ATP-dependent, electrogenic proton transport [3–5]. The purity and the membrane origin of these microsomal vesicles have been questioned, though proton transport in recent experiments [6] has been shown to be sensitive to vanadate, a characteristic of the plasma membrane ATPase. In order to confirm the pumping activity of the plasma membrane ATPase, it will be necessary to solubilize the enzyme, purify it from proteins with related transport functions, and reinsert it in a lipid bilayer in such a way that its pumping can be measured. Several laboratories have successfully solubilized plasma membrane ATPAse from a variety of plant tissues [7,8] and in some cases partially purified it [9]. We now report that two fractions of ATPase activity, solubilized from a preparation of plasma membrane from suspension-cultured rose cells and separated by molecular sieve chromatography, can each catalyze the accumulation of protons into reconstituted vesicles. Furthermore, the pumping activity of one of the fractions, like its ATPase activity, is unusually sensitive to ultraviolet light.

Plasma membrane vesicles were isolated from suspension-cultured cells of *Rosa damascena* by standard methods involving differential centrifugation and discontinuous sucrose gradient centrifugation [10]. Solubilization of the ATPase with 1% sodium cholate removed 99% of the phospholipids and resulted in a loss of 98.5% of the ATPase activity, but the subsequent addition of lipids restored 90% of the original activity. The lipids (Sigma Type IV-S 50% phosphatidylcholine or 99.9% phosphatidylserine) were dispersed by sonication in 2 mM histidine/0.1 mM EDTA/50 μg/ml butylated hydroxytoluene/2 mM Hepes (pH 6.8), and added to the supernatant at the rate of at least 10 mg lipid per mg protein. Detergent-solubilized ATPase fractionated on a Sephadex

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G-150 column was recovered in two distinct, equal peaks [10] with similar enzyme characteristics (Table I, columns 2 and 3). After the addition of lipids, the two fractions had specific activities 13- and 19-fold greater than the initial plasma membrane vesicles.

We tested the transport activity of the ATPase peaks by forming liposomes from the fractionated enzyme preparations and a mixture of 90% phospholipid (Sigma Type IV-S phosphatidylcholine from soybean) and 10% cholesterol. The lipids were dispersed with a 15 s pulse from the sonicator as previously described [10]. The lipids and protein were combined, sonicated for two 5-s intervals, and incubated on ice for 1.5 h. The procedure of Sze [3] was used to isolate sealed membrane vesicles. The protein/lipid suspension (3–4 ml) was layered over a 10% (w/w) dextran T70 cushion prepared in 250 mM sucrose and 2.5 mM Tris-Mes (pH 6.5) (8 ml). After centrifugation for 2 h at $80000 \times g$, a visible band at the sucrose/dextran interface was collected and used as the vesicle preparation.

Decrease in internal vesicle pH was measured by the quenching of fluorescence of the primary amine dye quinacrine [11], which accumulates in acid compartments. Vesicles (100–200 μg protein) were added to the fluorescence assay buffer to give a final concentration of 0.25 M sorbitol and 10 μM quinacrine in 1.5 ml of 25 mM bis-Tris propane adjusted with 25 mM Mes to pH 6.5, plus other salts as indicated. The reaction was initiated by addition of 15 μl of 0.5 M bis-Tris propane/ATP. Fluorescence was measured in a Perkin-Elmer PMF 44-A fluorescence spectrophotometer. Wavelengths for excitation and emission were 425 and 505 nm, respectively.

For both fractions, the addition of 5 mM ATP : Mg^{2+} caused a substantial decline in fluorescence, leveling off after 15–20 minutes (Fig. 1). Addition of the ionophore FCCP (1 μM) caused an immediate return of fluorescence to the original level. The addition of glucose plus hexokinase to remove ATP or 10 mM EDTA to remove Mg^{2+} caused a gradual return of fluorescence to the original level (data not shown). The transport ac-

TABLE I

INITIAL RATES OF ATPase ACTIVITY AND QUENCH OF QUINACRINE FLUORESCENCE UNDER VARIOUS CONDITIONS

2 ml of cholate-treated, clarified solution were layered on 50 l of Sephadex G-150 and eluted at 4°C with 0.1% sodium cholate/1 mM Tris-Mes (pH 6.5)/1 mM $MgSO_4$. ATPase peaks appeared 5–7 ml and 14–17 ml after the void volume. ATPase was assayed as the release of inorganic phosphate under the same solution conditions used to test fluorescence quench. The control ATPase activity was 316 μmol P_i/mg protein per h for fractions 5–8 and 451 μmol P_i/mg protein per h for fractions 5–8. Fluorescence quench was assayed as described in Fig. 1. Initial rates of quench were calculated from the slope during the first 3 min after addition of substrate and expressed relative to the rate obtained in 'controls' assayed with 3 mM ATP, 3 mM $MgSO_4$ and 50 mM KCl in 0.25 M sorbitol and 25 mM bis-Tris propane/Mes (pH 6.5). The initial rate of quench in the controls was 11.3% min^{-1} for fractions 5–8 and 12.3% min^{-1} for fractions 14–17.

Conditions	ATPase activity (%)		Fluorescence quench (%)	
	Fractions 5–8	Fractions 14–17	Fractions 5–8	Fractions 14–17
Control	100	100	100	100
+15 μM sodium vanadate	12	31	30	38
+5 mM sodium azide	100	100	100	99
+1 mM sodium molybdate	98	98	94	97
−ATP, +3 mM GTP	19	21	16	13
−ATP, +3 mM *p*-nitrophenylphosphate	3	3	1	5
−KCl	38	44	32	29
−KCl, +50 mM NH_4Cl	73	63	81	59
−KCl, +50 mM NaCl	35	39	33	27
−KCl, +50 mM LiCl	21	29	29	31
−KCl, +50 mM KNO_3	99	94	100	93
−KCl, +25 mM K_2SO_4	94	85	87	98

tivity was very similar to plasma membrane ATPase activity in many respects (Table I). Both transport activity and ATPase activity were inhibited 60–70% by 15 μM vanadate and unaffected by 5 mM azide or 1 mM sodium molybdate. Transport was ATP-specific and was stimulated by cations in the sequence $K^+ > NH_4^+ > Na^+ > Li^+$. There was no anion-specific stimulation since KCl, K_2SO_4 and KNO_3 had the same effect. This lack of anion effect on transport is contrary to the results reported by other workers [9] and suggests that our preparations contain different distributions of anion transporters.

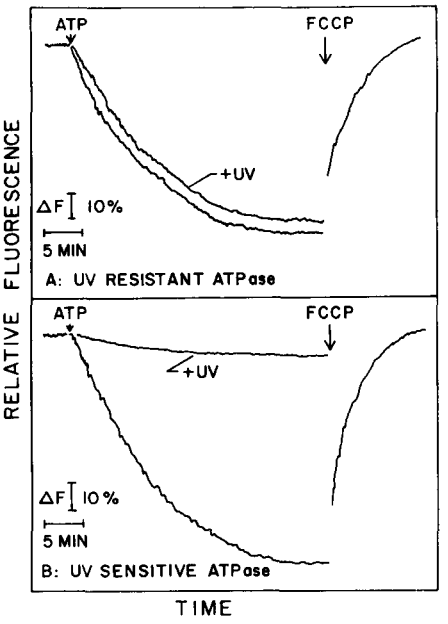

Fig. 1. Time-course of quenching of quinacrine fluorescence. The fractionated enzyme preparations were reconstituted in liposomes composed of 90% phospholipid (approx. 50% phosphatidylcholine from soybean) and 10% cholesterol. Sealed vesicles were collected after centrifugation over a 10% dextran T70 cushion. Vesicles (100–200 μg protein) were incubated with 10 μM quinacrine in 1.5 ml of a mixture of 0.25 M sorbitol, 50 mM KCl, and 25 mM bis-Tris-propane/Mes (pH 6.5). Quench was initiated by addition of 15 μl of 0.5 M bis-Tris propane ATP (first arrow). Quench was released by addition of 1 μM FCCP, a protein ionophore (second arrow). Quench was also measured using sealed vesicles irradiated with 600 $J \cdot m^{-2}$ of 290 nm light from an Oriel 250 W Hg lamp fitted with a Jobin-Yvon holographic grating monochrometer ('UV'). This fluence inactivated 10% of the ultraviolet-resistant ATPase and 90% of the ultraviolet-sensitive ATPase. (A) Fractions 14–17 from Sephadex chromatography of solubilized plasma membranes; (B) fractions 5–8.

In a previous report [10], we showed that one of the fractions of ATPase, the leading fraction from the G150 column, was at least 15-times more sensitive than the other fraction to irradiation with near, intermediate, and far-ultraviolet light. Ultraviolet light inhibited the proton pump prepared from the ultraviolet-sensitive ATPase but not the ultraviolet-insensitive ATPase (compare Fig. 1B to Fig. 1A).

The effect of ultraviolet light on the sensitive enzyme is strong enough to suggest that it may be of physiological significance. Though the peak sensitivity of the ATPase is at 290 nm, slightly below the shortest solar wavelengths that reach the ground, the ATPase can be inactivated by wavelengths as high as 365 nm [10], well within the range of solar radiation. In one test in which plasma membrane vesicles were exposed to direct summer sunlight, the sensitive ATPase lost activity with a half-life of 16 min. This suggests a mechanism by which solar ultraviolet light might directly affect ion transport, membrane potential, and other cell activities. Ultraviolet light sensitivity has been demonstrated in plasma membrane-enriched vesicles from wheat roots [12]; the ultraviolet light sensitivity of ATPase from shoot tissues has not yet been tested.

The ATPase preparation described here confirms the existence of an electrogenic proton-pumping ATPase in the plasma membrane of plant cells. The enzyme behaves as an intrinsic membrane protein, requiring strong detergents for solubilization and phospholipids for activity [13]. This preparation is similar in pH optimum, K^+ stimulation, and inhibitor sensitivity to preparations from corn [7] and oat [9]. Apparently all these preparations contain the same enzymes at different stages of purification. In common with the transport ATPases of animal [14] and fungal membranes [15], plant ATPases form a phosphorylated intermediate [16,17].

The significance of the two peaks of ATPase activity is still unclear. Both peaks have the characteristics ascribed by other workers to plasma membrane ATPase (Table I; Ref. 6). We have carefully tested the possibility that one enzyme comes solely from a contaminating organelle in

our plasma membrane preparations. Though a ultraviolet-sensitive ATPase is present in Golgi membranes, we calculated that less than 2% of our 'plasma-membrane' ATPase was associated with Golgi or mitochondrial markers [10]. For a number of reasons we believe it unlikely that one form of ATPase is formed artifactually by our extraction procedures. That both ATPase activities are functional in proton translocation supports the contention that both are native, undegraded forms.

While most references address 'the' plasma membrane ATPase there is no reason to assume that only one ATPase is present, or that any one ATPase has only one function. Other workers have suggested that there might be separate ATP-dependent transport systems operating in opposite directions [18], and that the H^+-extruding ATPases might function in different modes at various potassium concentrations [19]. The question has been raised whether *Chara corallina* has a single ATPase that can switch its H^+-translocating stoichiometry under different physiological conditions, or whether there are at least two different ATPases having 2 H^+/1 ATP and 1 H^+/1 ATP transport stoichiometries [20]. Our two peaks may represent two such different ATPases. An alternative hypothesis is that the low molecular weight form is produced from the high molecular weight form through a proteolytic processing step occurring within the plasma membrane. A reason for the processing could be a change in function, or it could relate to ultraviolet-regulation of the activity.

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