Characteristics of MgATP\(^2^-\)-dependent electrogenic proton transport in tonoplast vesicles of the facultative crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L.

I. Struve and U. Lütte
Institut für Botanik, Technische Hochschule Darmstadt, Schnittspahnstrasse 3-5, D-6100 Darmstadt, Federal Republic of Germany

Abstract. Membrane vesicles were isolated from mesophyll cells of *Mesembryanthemum crystallinum* in the C\(_3\) state and in the crassulacean acid metabolism (CAM) state. The distribution of ATP-hydrolysis and H\(^+\)-transport activities, and the activities of hydroxyopyruvate reductase and Antimycin-insensitive cytochrome-c-reductase on continuous sucrose gradients was studied. For isolations carried out routinely a discontinuous sucrose gradient (24%/37%/50%) was used. Nitrate-sensitive ATP-hydrolysis and H\(^+\)-transport activities increased several-fold during the transition from C\(_3\) photosynthesis to CAM. Nitrate-sensitive ATPase showed a substrate preference for ATP with an apparent K\(_m\) (MgATP\(^2^-\)) of 0.19-0.37 mM. In both C\(_3\) and CAM states the ATPase showed a concentration-dependent stimulation by the anions chloride and malate. However, the pH optima of the two states were different: the ATPase of C\(_3\)-M. crystallinum had an optimum of pH 7.4 and that of CAM-M. crystallinum an optimum of pH 8.4. The optical probe oxonol-VI was used to demonstrate the formation of MgATP\(^2^-\)-dependent electric-potential gradients in tonoplast vesicles.

Key words: ATP hydrolysis – Crassulacean acid metabolism (induction) – Membrane potential (tonoplast) – *Mesembryanthemum* (H\(^+\) transport) – Mg\(^{2+}\)-ATPase – Proton transport.

Introduction

Plants performing Crassulacean acid metabolism (CAM) show a large nocturnal accumulation of malic acid in the vacuoles of the photosynthetic cells. Transport of malic acid across the tonoplast into the vacuole is assumed to be driven by a proton-pumping ATPase (Lütte and Ball 1979; Lütte et al. 1982). The uptake of the malate\(^2^-\) anion is secondarily coupled to primary active H\(^+\) transport via the proton-electrochemical gradient established by the ATPase with a stoichiometry of 2 H\(^+\) pumped and 1 malate\(^2^-\) anion transported per 1 ATP hydrolyzed (Lütte et al. 1981; Smith et al. 1982).

Recently, an ATPase associated with vacuoles from *Kalanchoe daigremontiana* was characterized (Smith et al. 1983, 1984a, b; Aoki and Nishida 1984; Lütte et al. 1984) and Mg-ATP-dependent H\(^+\) transport into vacuoles was demonstrated (Jochem et al. 1984; Jochem 1986).

In *Mesembryanthemum crystallinum* (Mesembryanthemaceae), a species where CAM is induced by drought stress and salinity (Winter and von Willert 1972; Winter 1973, 1979; Winter and Lütte 1979) an increase of vacuolar ATPase activity during the transition from C\(_3\) photosynthesis to CAM was shown (Struve et al. 1985). Vacuolar ATPase activity in the C\(_3\) state is not sufficient to drive malic-acid accumulation in the CAM state. This clearly demonstrates the physiological relevance of the vacuolar ATPase for the process of CAM in vivo.

In the present study, tonoplast vesicles were isolated from *M. crystallinum* in the C\(_3\) and CAM states with the aim of comparing the characteristic properties of tonoplast ATPase activity and H\(^+\).
transport and the formation of an electric potential gradient at the tonoplast in the two states.

Material and methods

Plants. Plants of *Mesembryanthemum crystallinum* L. were grown from seeds in glasshouses in soil culture (Einheitserde type ED-73 DIN 11540-80 T, see Struve et al. 1985). Crassulacean acid metabolism was induced by NaCl stress (see Winter and Lüttge 1979; Heun et al. 1981).

Protoplast isolation. Protoplasts were isolated by enzymatic digestion as described by Struve et al. (1985) according to the method of Winter et al. (1982) with the following modifications: Only the upper epidermis was removed; 15 g of tissue were transferred to 40 ml of enzyme medium with Cellulase "Onozuka" RS, 0.5% (w/v), from Yakult Honsha Co., Tokyo, Japan, and Pectolyase Y-23, 0.02% (w/v), from Seishin Pharmaceutical Co, Tokyo, Japan. The suspension of protoplasts and cell fragments was diluted with 500 mM sucrose solution. About 16 ml of the diluted suspension were placed in centrifuge vials and covered with a layer of 0.5 ml 400 mM mannitol (800 mM for salt-treated plants), 100 mM sucrose solution. The protoplasts were mixed by two passages through the steel needle of a syringe (internal diameter 370 gm). Chloroplasts were removed by centrifugation at 1000-g for 5 min at 4 °C. An aliquot of 18 ml of the resulting supernatant was layered on this gradient, centrifugation was the same as described previously (Smith et al. 1984a; Struve et al. 1985) according to the method of Winter et al. (1982).

Membrane preparation. Protoplasts of C3-M. crystallinum were diluted with buffer P, those of salt-treated CAM-M. crystallinum with buffer P without mannitol, both at a ratio 1:3 to get the same final mannitol concentration. Protoplasts were homogenized by two passages through the steel needle of a syringe (internal diameter 370 μm). Chloroplasts were removed by centrifugation at 1000 g for 5 min at 4 °C. An aliquot of 18 ml of the resulting supernatant was layered on a continuous sucrose gradient (15–50% sucrose, w/w, in medium G: 5 mM Hepes/Tris pH 7.6, 2 mM DTT, volume 20 ml) in cellulose nitrate tubes. The gradient was centrifuged in a Beckman SW 27 rotor (Beckman, München, FRG) at 100,000 g for 2 h at 4 °C. Fractions of 1.5 ml were taken from the bottom, frozen in liquid N2 and stored at −70 °C.

For isolations carried out routinely a discontinuous gradient (24%, 37%, 50% sucrose w/w, 5 ml each, in medium G) was used. An aliquot of 23 ml of the 1000 g supernatant was layered on this gradient, centrifugation was the same as with the continuous gradient. Membranes at the interfaces of the discontinuous gradients were collected with a Pasteur Pipette (fraction A = interface supernatant: 24% sucrose; fraction B = 24%:37% sucrose; fraction C = 37%:50% sucrose), diluted fourfold (medium Gc: 300 mM mannitol in medium G) and centrifuged in a Beckman 50.2 Ti rotor at 100,000 g for 90 min at 4 °C. The supernatant was discarded and the pellet was suspended in 300 mM sucrose in medium G to a final protein concentration of 70–100 μg ml−1 (PA = fraction A, pellet). All membrane fractions were frozen in liquid N2 and stored at −70 °C.

Assay of ATPase. The ATPase assays were performed essentially as described previously (Smith et al. 1984a; Struve et al. 1985) with the following modifications: the final assay volume was 225 μl including 50 μl of membrane suspension (diluted 1:10 for C3-M. crystallinum, 1:20 for CAM-M. crystallinum). The reaction mixture contained 0.7 mM magnesium chloride (Na2MoO4·2 H2O) instead of 0.1 mM ammonium molybdate ((NH4)6Mo7O24·4 H2O) because ammonium acts as a permeant weak base, relieving pH gradients across the membranes (Kleiner 1981; DuPont et al. 1982). The standard mixture was incubated at 37 °C for 45 min. In studies of the kinetic parameters of ATP hydrolysis the reaction medium was incubated at 25 °C for 30 min to prevent a substrate deficiency.

For determinations of the pH dependence of ATPase activity the N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine (Tricine)/Tris buffer in the standard test medium was replaced by a mixture of 2-(N-morpholino)ethane sulfonic acid (Mes) and 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bistris-Propane) buffer with pH values between pH 5.5 and pH 9.0. Temperature effects were avoided by determining the pH values at 25 °C and incubating the reaction mixture at 25 °C. The reaction was stopped by the addition of 500 μl 10% (w/v) sodium dodecylsulfate and P1 determined as in Smith et al. (1984a).

Fluorescence assays. Acid interior pH gradients were measured as the degree of fluorescence quenching of the permeant amine dyes, quinacrine and 9-aminoacridine (Deamer et al. 1972). Vesicles were added to an assay buffer of 220 mM sucrose, 1.5 mM DTT, 37 mM Tricine/Tris pH 8.0, 3 μM quinacrine or 30 μM 9-aminoacridine and various salt concentrations to give a final volume of 1.5 ml. Quinacrine was stored as a 5 mM stock solution (H2O) at −20 °C. 9-Aminoacridine was stored as a 3 mM stock solution (10% ethanol) in a refrigerator. Typically, 30–100 μl vesicles (3–10 μg protein) or 100–200 μl vesicles were added for assays with quinacrine and 9-aminoacridine, respectively. Fluorescence was measured at 25 °C with a Sigma ZWS-II photometer (Biochem, München, FRG) equipped with a fluorescence attachment. Excitation was at 427 nm with quinacrine and at 422 nm with 9-aminoacridine and emission was measured through an interference filter (quinacrine: maximal transmission 61% at 530 nm, 9-aminoacridine: 55% at 470 nm). Measurements of ATP-dependent H+ influx were initiated by the addition of 30 μl of 150 mM MgSO4/ATP, adjusted to pH 7.0 with Bistris-Propane.

Absorption spectroscopy of oxonol VI. The formation of transmembrane electric potential gradients was measured with the optical probe bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (OX-VI). Absorption changes of OX-VI and HPR (marker enzyme for peroxisomes) were measured with a Sigma ZWS II dual-wavelength photometer. As shown by Bashford et al. (1979), Bennett and Spanswick (1983) and Scherman and Henry (1980) a decrease in the absorption difference A590nm−610nm indicates an increase in membrane potential (interior positive). Assays were performed in a final volume of 1.2 ml inclusive MgATP, which contains: 220 mM sucrose, 1.5 mM DTT, 2.5 μM OX-VI, 33 mM Tricine/Tris pH 8.0, 20–30 μg membrane protein. The reaction was started by addition of 3 mM MgATP. The optical probe OX-VI was kept at a 0.25 mM stock solution in the refrigerator.

Proteins. Proteins were measured by the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard.

Assay of hydroxypropionate reductase (HPR). The activity of HPR (marker enzyme for peroxisomes) was determined according to Zelitch (1955) using the following reaction mixture: 0.083 mM NADH, 0.1% (w/v) Triton X-100, 50 mM Hepes, pH 7.5 (KOH). The final test volume of 1.2 ml contained 50–150 μl vesicles. Reaction was started by adding 1 mM hydroxypropionate and absorption was measured at 340 nm.

Assay of cytochrome-c reductase (CCR). The activity of CCR (marker enzyme for endoplasmic reticulum (ER)) was measured following Lord et al. (1973). The reaction mixture (final volume 1.2 ml, including 50–150 μl vesicles) was composed of 0.02%