The properties of Mg\textsuperscript{2+}-ATPase in the vacuole of Saccharomyces cerevisiae were studied, using purified intact vacuoles and right-side-out vacuolar membrane vesicles prepared by the method of Y. Ohsumi and Y. Anraku ((1981) J. Biol. Chem. 256, 2079). The enzyme requires Mg\textsuperscript{2+} ion but not Ca\textsuperscript{2+} ion. Cu\textsuperscript{2+} and Zn\textsuperscript{2+} ions inhibit the activity. The optimal pH is at pH 7.0. The enzyme hydrolyzes ATP, GTP, UTP, and CTP in this order and the $K_m$ value for ATP was determined as 0.2 mM. It does not hydrolyze ADP, adenosyl-3'-5'-imidodiphosphate, or p-nitrophenyl phosphate. ADP does not inhibit hydrolysis of ATP by the enzyme. The activities of intact vacuoles and of vacuolar membrane vesicles were stimulated 3- and 1.5-fold, respectively, by the protonophore uncoupler 3,5-di-tetra-butyl-4-hydroxybenzilidenemalononitrile and the K\textsuperscript{+}/H\textsuperscript{+} antiporter ionophore nigericin. Sodium azide at a concentration exerting an uncoupler effect also stimulated the activity. The activity was sensitive to the ATPase inhibitor N,N\textsuperscript{2}-dicyclohexylcarbodiimide, but not to sodium vanadate. The ATP-dependent formation of an electrochemical potential difference of protons, measured by the flow-dialysis method, was determined as 180 mV, with a contribution of 1.7 pH units, interior acid, and of a membrane potential of 75 mV. It is concluded that the Mg\textsuperscript{2+}-ATPase of vacuoles is a new marker enzyme for these organelles and is a N,N\textsuperscript{2}-dicyclohexylcarbodiimide-sensitive, H\textsuperscript{+}-translocating ATPase whose catalytic site is exposed to the cytoplasm.

Recently, we established a procedure for preparing right-side-out vacuolar membrane vesicles of high purity from cells of the yeast Saccharomyces cerevisiae and showed that the vesicles catalyze active arginine transport which is driven by an electrochemical potential difference of protons formed by ATP hydrolysis (1). Subsequent studies on ATP hydrolysis by intact vacuoles and vacuolar membrane vesicles indicated the presence of a new Mg\textsuperscript{2+}-ATPase with a catalytic site oriented externally.

This paper describes the properties of vacuolar membrane-bound Mg\textsuperscript{2+}-ATPase and its characteristic function as a H\textsuperscript{+}-translocating ATPase. The Mg\textsuperscript{2+}-dependent, H\textsuperscript{+}-translocating ATPase of the vacuoles was found to be a new specific marker of these organelles and to differ from mitochondrial ATPase (2) and plasma membrane-bound ATPase (3, 4).

**MATERIALS AND METHODS**

**Chemicals, Strains, and Culture Conditions**—[\textsuperscript{14}C]Methylamine

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1. The abbreviations used were: SF6847, 3,5-di-tetra-butyl-4-hydroxybenzilidenemalononitrile; DCCD, N,N\textsuperscript{2}-dicyclohexylcarbodiimide; MES, 2-(N-morpholinio)ethanesulfonic acid; Tricine, N-tri(hydroxymethyl)methylglycine.
RESULTS

Purity and Yield of Vacuoles—The distributions of marker enzymes (α-mannosidase for vacuoles (18), glucose-6-phosphate dehydrogenase for cytosol (19), succinate dehydrogenase for mitochondria (20), NADPH-cytochrome c reductase for microsomes (11), and chitin synthetase for plasma membrane (21)) were examined by measuring enzyme activities in the vacuole and spheroplast lysate fractions. Table I shows that the recovery of α-mannosidase activity in the vacuole fraction was about 13% and that its specific activity in this fraction was increased 28.5-fold. The recoveries of the four other marker enzyme activities in this fraction were found to be less than 0.1% of their total activities. These results indicate that the vacuole fraction obtained was virtually free from mitochondria and other membranous organelles.

Intactness of Vacuoles—Alkaline phosphatase is known to be localized in the internal space of vacuoles (22). The vacuole

Yeast cells grown in YEPD medium at 30°C harvest at 2 × 10^7 cells/ml by centrifugation (4,500 g, 3 min), wash twice with distilled water at room temperature, suspend in 0.1 M sorbitol at a density of 2 × 10^7 cells/ml, and add Glusulase (0.2 mL) and Zymolyase (10 mg/mL). Incubate at 30°C for 60–90 min with gentle shaking, collect by centrifugation (4,500 g, 30 min), wash twice with 0.1 M sorbitol, and resuspend in 0.1 M sorbitol.

Spheroplasts

Vacuoles

Vacuoles-

Enzyme Activity

| Enzyme                     | Spheroplast lysate | Vacuole | Ratio B/A | Recovery (b/a × 100) |
|----------------------------|-------------------|---------|-----------|----------------------|
| α-Mannosidase              | 0.02              | 0.68    | <0.02     | <0.002               |
| Glucose-6-phosphate dehydrogenase | 0.11              | 0.1    | <0.005    | <0.003               |
| Succinate dehydrogenase    | 1.9               | 0.01    | <0.01     | <0.005               |
| NADPH-cytochrome c reductase | 0.11              | 0.11    | 0.11      | 0.11                 |
| Chitin synthetase          | 1.23              | 0.11    | 0.11      | 0.11                 |

Protein (mg): 238

a and b, total activity (nmol/min).

Fig. 1. A, effects of cations on ATPase activity of vacuolar membrane vesicles. Assays were carried out under standard conditions except that the divalent cation concentration was as indicated. MgCl2, (○); CaCl2, (●). B, pH profile of Mg2+-ATPase of the vesicles. Assays were carried out under standard conditions but with 10 mM concentrations of MES/Tris, (■); Tris-HCl, (●); or glycine/NaOH, (▲).
TABLE IV
Substrate specificity of Mg\(^{2+}\)-ATPase in vacuolar membrane vesicles

| Substrate | Specific activity unit/mg protein |
|-----------|----------------------------------|
| ATP       | 0.47                             |
| GTP       | 0.44                             |
| UTP       | 0.27                             |
| CTP       | 0.19                             |
| ADP       | 0.04                             |
| pNPP*     | 0.03                             |
| AMP-PNP*  | 0.00                             |

* p-Nitrophenyl phosphate.
* Adenosyl-5'-yl imidodiphosphate.

FIG. 2. Lineweaver-Burk plot of Mg\(^{2+}\)-ATPase. Initial rates were determined under standard conditions with ATP at concentrations of 0.2 to 6.4 mM.

fraction showed a latent phosphatase activity which was activated maximally 4.5-fold on addition of Triton X-100 or cholate (Table II). This indicated that the vacuoles were intact but that the vacuolar membranes were injured by detergents, resulting in breakage of the latency for alkaline phosphatase. The membrane-bound \(\alpha\)-mannosidase activity was activated slightly by the detergents used, indicating that the substrate 4-methylumbelliferone-\(\alpha\)-D-mannopyranoside used was partly permeable through the membrane.

Mg\(^{2+}\)-ATPase activity was detected in the vacuoles with ATP-Mg\(^{2+}\) as an impermeable substrate and this activity was inhibited by a high concentration of detergents (Table II). This suggests strongly that the catalytic site of the enzyme in intact vacuoles is exposed to the outer surface of vacuolar membranes.

**Mg\(^{2+}\)-ATPase as a Marker of Vacuolar Membranes**—Intact vacuoles were treated hypotonically and disrupted me-

TABLE V
Effects of inhibitors on Mg\(^{2+}\)-ATPases of intact vacuoles and vacuolar membrane vesicles

| Addition | Mg\(^{2+}\)-ATPase |
|----------|------------------|
|          | unit/mg protein  |
| None     | 0.13             |
| SF6847   | 0.31             |
| 2        | 0.37             |
| Nigericin| 0.31             |
| 2        | 0.39             |
| Valinomycin| 0.16         |
| 2        | 0.13             |

FIG. 3. Effects of cations on Mg\(^{2+}\)-ATPase. Assays were as described under "Materials and Methods." Membrane vesicles (50 \(\mu\)g of protein/ml) were treated with cations at the concentrations indicated for 5 min at 30 °C before starting the reaction. KCl, (C); NH\(_4\)Cl, (O); CaCl\(_2\), (A); ZnCl\(_2\), (H); CuCl\(_2\), (O).

FIG. 4. Effects of inhibitors on Mg\(^{2+}\)-ATPase. Assays were as described under "Materials and Methods." Membrane vesicles (50 \(\mu\)g of protein/ml) were treated with inhibitors at the concentrations indicated for 5 min at 30 °C before starting the reaction. DCCD, (O); sodium azide, (H); sodium vanadate, (C).
20 min at 25 °C before addition of quinacrine. 

Properties of Mg\(^{2+}\)-ATPase—The enzyme required Mg\(^{2+}\) ion for ATP hydrolysis (Fig. 1A). The optimal ratio of ATP to Mg\(^{2+}\) of 1 indicated that an ATP-Mg\(^{2+}\) complex is substrate for the enzyme. Ca\(^{2+}\) ion had no effect on the activity. The optimal pH of the enzyme was determined to be pH 7.0 (Fig. 1B). The enzyme hydrolyzed ATP and three other ribonucleoside triphosphates, GTP, UTP, and CTP with this order of preference (Table IV). ADP and p-nitrophenyl phosphate, which were not hydrolyzed by the enzyme, did not inhibit the activity. The K\(_m\) value for ATP was determined as 0.2 mM (Fig. 2), which is 8-fold smaller than that of the ATPase of plasma membranes of S. cerevisiae (3).

Fig. 3 shows the effects of various cations on Mg\(^{2+}\)-ATPase. The activity was not affected by K\(^+\) or Na\(^+\) (data not shown), but was inhibited strongly by Cu\(^{2+}\) and Zn\(^{2+}\) ions. These cations inhibited the activity noncompetitively; the K\(_{1/2}\) values of inhibition by Cu\(^{2+}\) and Zn\(^{2+}\) ions were determined as less than 10 \(\mu\)M and about 20 \(\mu\)M, respectively. Ca\(^{2+}\) and NH\(_4\)\(^+\) ions stimulated the activity at concentrations of more than 0.1 mM. We are now investigating these stimulating effects with respect to energy coupling of active cation transport by the vesicles.

The activity was inhibited by DCCD and the K\(_{1/2}\) value of inhibition was determined as 6 \(\mu\)M (0.15 \(\mu\)mol of DCCD/mg of membrane protein) (Fig. 4). However, unlike the H\(^+-\)translocating ATPase of submitochondrial particles from S. cerevisiae (2), the Mg\(^{2+}\)-ATPase of the vacuoles was not inhibited by sodium azide and was in fact stimulated at concentrations of more than 1 mM sodium azide, probably due to its uncoupler effect (23). Sodium vanadate, which is reported to be an inhibitor of Mg\(^{2+}\)-ATPase in the plasma membranes of S. cerevisiae (3), did not inhibit the activity noticeably (Fig. 4).

Evidence that Mg\(^{2+}\)-ATPase of Vacuoles is H\(^+-\)Translocating ATPase—The Mg\(^{2+}\)-ATPase activities of intact vacuoles and vacuolar membrane vesicles were stimulated 3- and 1.5-fold, respectively, by the protonophore uncoupler SF6847 (24, 25) and the K\(^+/H^+\) antiporter ionophore nigericin (Table V), indicating that these reagents decreased the proton gradient formed by ATP hydrolysis. Valinomycin had no effect on the activities.

ATP hydrolysis-dependent formation of a proton gradient was directly demonstrated by recording the change in quenching of quinacrine fluorescence. The fluorescence signal of quinacrine was quenched by incubating the vacuolar membrane vesicles from strain X2180-1A (p') with ATP, reflecting uptake of protons and formation of \(\Delta\psi\) (Fig. 5). These results confirm those on the quenching of 9-aminacridine fluorescence reported previously (1), and indicate that the uptake of protons is coupled with ATP hydrolysis by Mg\(^{2+}\)-ATPase. ATP-dependent alkalinization of reaction mixture containing vesicles and ATP in 2 mM glycylglycine buffer, pHi 6.3, was also observed with a pH electrode (data not shown).

The electrochemical potential difference of protons across the vacuolar membrane generated upon ATP hydrolysis was determined quantitatively by the flow-dialysis method with \(^{14}\)C]methylamine or \(^{14}\)C]KSCN to measure the formation of \(\Delta\psi\) and \(\Delta\psi\)KSCN for measuring the membrane potential (Fig. 6). The \(\Delta\psi\) thus calculated (16) was 180 mV, with contribution of 1.7 pH units, interior acid, and of a membrane potential of 75 mV, interior positive.

**DISCUSSION**

The first half of this paper describes biochemical criteria for the preparations of intact vacuoles and vacuolar membrane
vesicles according to the marker concept of cellular organelles of de Duve (26). Our results indicate that intact vacuoles, which had high α-mannosidase activity and were virtually free from other cellular organelles, were obtained reproducibly, judging by the recovery of marker enzymes (Tables I and II), and that they were converted to right-side-out vesicles (1) with high recovery of α-mannosidase and Mg\textsuperscript{2+}-ATPase by brief hypotonical treatment and centrifugation (Table III and Scheme 1).

We found a new Mg\textsuperscript{2+}-ATPase in the vacuolar membranes and concluded that it is an important marker enzyme of these organelles. The characteristic properties of the enzyme are summarized in Table VI in comparison with those of mitochondrial and plasma membrane Mg\textsuperscript{2+}-ATPase of S. cerevisiae. It is clear that vacuolar membrane ATPase differs from the mitochondrial enzyme in its pH optimum and sensitivities to oligomycin, sodium azide, and Cu\textsuperscript{2+} ion. In addition, the vacuolar membrane enzyme is not inhibited at all by ADP. It also differs from the plasma membrane enzyme (3) with respect to its pH optimum, \( K_{m} \), value for ATP, and sensitivity to sodium vanadate.

We obtained evidence that the vacuolar membrane Mg\textsuperscript{2+}-ATPase is characterized as a H\textsuperscript{+}-translocating ATPase and generates an electrochemical potential difference of protons of 180 mV, interior acid, across the membrane (Table V and Figs. 5 and 6). A good correlation was found between the extents of inhibition of Mg\textsuperscript{2+}-ATPase activity by DCCD and Cu\textsuperscript{2+} ion and of arginine uptake by the vesicles (1). These facts indicate that the enzyme is a fundamental energy-donating system for various specific antiporters for amino acids (1) and Ca\textsuperscript{2+} ion\textsuperscript{2} in the vacular membrane. The protein-chemical properties of the enzyme are under investigation in our laboratory.

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