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Functional molecular masses of vacuolar membrane H^+-ATPase from *Saccharomyces cerevisiae* as studied by radiation inactivation analysis

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The functional molecular masses of the vacuolar membrane H^+-ATPase in *Saccharomyces cerevisiae* under two kinetic conditions for ATP hydrolysis were measured by radiation inactivation. When vacuolar membrane vesicles were exposed to γ-rays from ^{60}Co, the activities catalyzing a single-cycle and multi-cycles of ATP hydrolysis both decreased as single-exponential functions of the radiation dosage. By applying the target theory, the functional molecular masses for single- and multi-cycle hydrolyses of ATP were determined to be approx. $0.9–1.1 \times 10^5$ and $4.1–5.3 \times 10^5$ Da, respectively. *N,N'*-Dicyclohexylcarbodiimide (DCCD) did not inhibit the former reaction but strongly inhibited the latter. It is suggested that the ATPase with a minimal composite of subunits a and b, in which subunit c is not necessarily involved operationally, can catalyze single-cycle hydrolysis of ATP, whereas for multi-cycle hydrolysis of ATP, the ATPase requires a properly organized oligomeric structure with subunits a–c, which may direct a positive cooperative mechanism of ATP hydrolysis and coupled H^+ translocation in a DCCD-sensitive manner.

Vacuolar membrane; ATPase, H^+-; Molecular mass, functional; Radiation inactivation analysis

1. INTRODUCTION

Vacuolar membrane H^+-translocating ATPase of the yeast *Saccharomyces cerevisiae* generates a proton-motive force of 180 mV, inside positive and acidic, in vacuolar membrane vesicles and facilitates acidification of the vacuolar sap [1,2]. The enzyme is composed of three major subunits, subunit a (67 kDa, formerly reported to be 89 kDa [3]), b (57 kDa, formerly reported as 64 kDa [3]), and c (20 kDa) [3]. Subunit a is predicted to be the catalytic site as the ATP analogue 8-azido-ATP binds to this subunit specifically. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) can bind covalently to the subunit in an ATP-protectable manner, and this chemical reaction, which modifies a single tyrosine residue in the molecule, results in enzyme inactivation and loss of ability to form an enzyme-ATP complex, the first intermediate in the hydrolytic reaction [4]. Subunit c has been identified as a DCCD-binding protein [3] and functions as a channel for proton translocation in the enzyme complex [1,2]. The function of subunit b has not yet been identified.

Previous studies in this laboratory indicated that a single cycle of ATP hydrolysis by the purified enzyme proceeds by binding of ATP to a catalytic site of subunit a followed by hydrolysis of the bound ATP to ADP and P_i, which are then slowly released from the enzyme [4]. These findings suggest that the reaction mechanism is similar to those proposed for mitochondrial and bacterial F_0F_1-ATPases [5–8].

Here, we found that the functional molecular masses of the ATPase in single- and multi-cycle

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hydrolysis of ATP were very different. This finding should provide some important clues for further studies on the reaction mechanisms of a family of ATPases definitely located in vacuo-vesicular membrane systems of eukaryotic cells [1].

2. EXPERIMENTAL

Right-side-out vacuolar membrane vesicles that were essentially free of mitochondrial contamination were prepared from the yeast *S. cerevisiae* haploid strain X2180-1A as in [2]. Membrane vesicles with H^+-ATPase activity of 0.4–0.5 U/mg protein [3] were used throughout. [γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham. NBD-Cl was from Sigma. Glucose-6-phosphate dehydrogenase (from yeast, grade II), glucose 6-phosphate and NADP were from Boehringer-Mannheim. Other chemicals used were commercial grade and were as described in [3,4].

Vacuolar membrane H^+-ATPase was assayed under two kinetic conditions: (i) Multi-cycle (steady-state) hydrolysis of ATP. The reaction mixture (100 μl) contained 25 mM Mes/Tris (pH 6.9), 5 mM $MgCl_2$, 25 mM KCl, 5 mM ATP-2Na^+ (pH 7.0, adjusted with Tris) and 10–20 μg protein of vacuolar membranes. The reaction was started by adding ATP at 25°C and terminated as in [4]. (ii) Single-cycle (non-steady-state) hydrolysis of ATP. The assay procedures employed were essentially the same as described for those with purified vacuolar H^+-ATPase [4]. [γ-^{32}P]ATP (1 nM) was incubated at 25°C with 10 μg protein of vacuolar membranes in 100 μl reaction mixture [50 mM Mes/Tris (pH 6.9), 1 mM $MgCl_2$, 1 mM potassium phosphate (pH 6.9), 2 mM NaN_3, 0.1 mM sodium vanadate, 1 mM sodium molybdate] for various times. This amount of enzyme, which was estimated enzymatically and electrophoretically, was calculated to be about 15 nM enzyme consisting of a monomer of equal numbers of subunits a (67 kDa) and b (57 kDa). The reaction was terminated by adding 35 μl of a stop solution (0.2 M triethylamine hydrochloride, 2 M perchloric acid, 5% ammonium molybdate, 1:2:4, v/v) and the $^{32}P_i$ liberated ('acid quench') was measured as in [4]. In 'cold ATP chase' experiments, 50 μl chase solution [50 mM Mes/Tris (pH 6.9), 13 mM $MgCl_2$, 15 mM ATP, and 1 mM potassium phosphate (pH 6.9)] was added to the reaction mixture at various times, and after 5 s incubation, 50 μl stop solution was added to terminate the reaction and the $^{32}P_i$ liberated was measured [4]. Glucose-6-phosphate dehydrogenase was assayed as described [2].

Samples for γ-ray irradiation were prepared as follows: vacuolar membrane vesicles (1 mg protein/ml) were suspended in a sample solution consisting of 25 mM Mes/Tris (pH 6.9), 5 mM $MgCl_2$, 25 mM KCl and glucose-6-phosphate dehydrogenase (5 μg protein/ml) as an internal standard with a known molecular mass of 102 kDa [9]. Aliquots (200 μl) of the sample suspension were dispensed in borosilicate glass tubes (12 × 75 mm, Corning), equilibrated with N_2 gas, the tubes sealed with polyethylene caps and Parafilm (American Can) and frozen in dry ice-ethanol at −70°C.

Irradiation of the samples with a ^{60}Co γ-ray apparatus (6000 Ci) was carried out at the Research Center for Nuclear Science and Technology (University of Tokyo). Doses of γ-rays were measured with a Fricke dosimeter. Sample tubes in dry ice-ethanol were placed at a fixed distance (13 cm) from the radiation source and exposed to γ-rays at a dose rate of 0.12 Mrad/h. The samples were removed at appropriate intervals and stored in dry ice-ethanol. After completion of irradiation, all the samples were thawed and remaining enzyme activities were assayed. Samples which had not been irradiated and stored as above were used as controls.

3. RESULTS

3.1. *Single-cycle hydrolysis of ATP by vacuolar H^+-ATPase*

Fig.1 shows the time courses of increase in the total amounts of $^{32}P_i$ formed (acid quench) and [γ-^{32}P]ATP bound to a single site and committed to hydrolysis (cold ATP chase) by the membrane-bound vacuolar H^+-ATPase. Under this non-steady-state condition, or single-cycle hydrolysis of ATP per catalytic site, binding of ATP to the enzyme nearly reached a plateau (60% of the total) in 50–70 s and about 50% of the added ATP underwent single-cycle hydrolysis. The initial rate of $^{32}P_i$ formation at the single site depended on the amount of [γ-^{32}P]ATP bound and at 70 s of incubation, about 80% of the bound substrate was hydrolyzed, as in the case with the purified enzyme [4]. The rest (about 20%) was hydrolyzed rapidly in 5 s of incubation with 5 × 10^6-fold excess ATP, suggesting the operation of a positive cooperative mechanism among the catalytic sites.

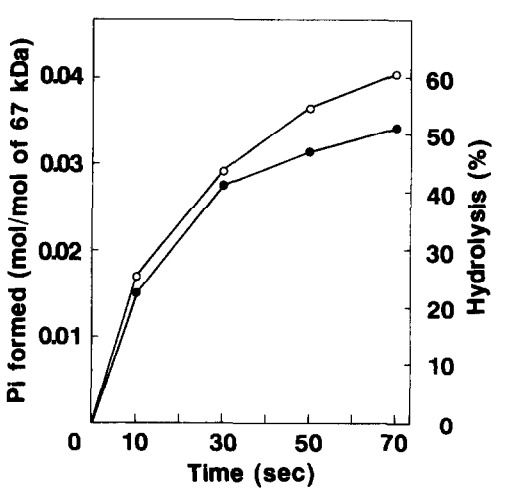

Fig.1. Single-cycle hydrolysis of ATP by vacuolar membrane H^+-ATPase. Assays were carried out as described in section 2. (●) Acid quench, (○) cold chase.

3.2. *Estimation of functional molecular masses of the ATPase under two kinetic conditions*

To determine the minimal molecular entities capable of catalyzing single-cycle and multi-cycle hydrolysis of ATP, we performed a radiation inactivation analysis on the vacuolar membranes. We used glucose-6-phosphate dehydrogenase (G6PDH) as an internal standard. Plots of the residual activities of ATPase and G6PDH as percentages of their original activities vs radiation dosage showed that the activity decays all obeyed a single-exponential function of the dosage. Thus, the target theory could be applied for determination of the functional molecular mass [10]. From three independent experiments, a D_{37} of 6.5 ± 0.6 Mrad for G6PDH was obtained, where D_{37} is the dose of irradiation (in rad) that inactivates the enzyme to 37% of the original activity [10]. This value is close to the D_{37} value of 6.2 Mrad for the enzyme determined previously by Bowman et al. [11]. Using a D_{37} value of 6.5 Mrad, we applied the empirical equation molecular mass = $6.4 \times 10^{11}/D_{37}$ [10] to determine the functional molecular mass of G6PDH under these conditions and obtained a value of 9.8×10^4 Da, which is reasonably close to the value of 10.2×10^4 Da determined using another method [9].

We found that the susceptibilities to irradiation of ATPase for single-cycle and multi-cycle hydrolyses of ATP under the two kinetic conditions were very different. A typical experiment is shown in fig.2. Apparent molecular masses of the vacuolar ATPase were calculated by multiplying the molecular mass of G6PDH (10.2×10^4 Da) by $D_{37(G6PDH)}/D_{37(ATPase)}$, values of 1.1×10^5 and 5.3×10^5 Da being obtained for single- and multi-cycle hydrolyses of ATP, respectively. We repeated the experiment with different vacuolar membranes under the same conditions: molecular masses of 0.9×10^5 and 4.1×10^5 Da, respectively, were obtained with that of G6PDH being within the standard deviation. This may suggest that preparations of vacuolar membranes affect apparent molecular masses and are a major source of experimental errors.

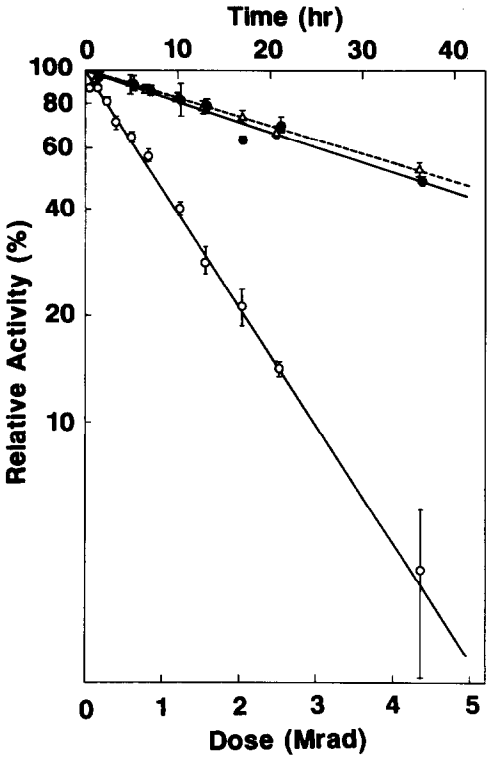

Fig.2. Radiation inactivation of activities of single- and multi-cycle hydrolyses of vacuolar membrane H^+-ATPase. Irradiation with ^{60}Co and assay of enzyme activities were carried out as described in section 2. Points were means for 3 assays. Bars represent the standard error of each point and lines were calculated by least squares. By regression analysis, the D_{37} values were calculated to be 6.7 Mrad for G6PDH (Δ---Δ), and 1.3 and 6.1 Mrad for multi-cycle (○—○) and single-cycle (●—●) hydrolyses, respectively, by vacuolar membrane H^+-ATPase.

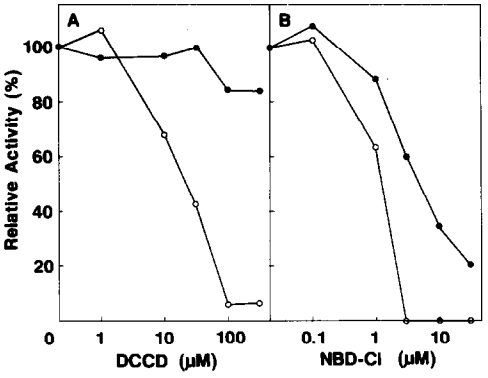

Fig.3. Inactivation of single- and multi-cycle ATP hydrolyses by DCCD and NBD-Cl. Vacuolar membrane vesicles (50 μg protein) were incubated in 500 μl reaction mixture with DCCD (A) or NBD-Cl (B) at the concentrations indicated for 20 min at 25°C before starting the reaction. Single-cycle (●) and multi-cycle (○) ATP hydrolyses were assayed as described in section 2.

3.3. *Single-cycle hydrolysis of ATP is not sensitive to DCCD*

DCCD inhibited H^+-ATPase activity in vacuolar membrane vesicles (fig.3A) as well as H^+ translocation across the membrane under steady-state conditions [2–4], but did not inhibit the ATPase activity at a single catalytic site (fig.3A). NBD-Cl inhibited both activities (fig.3B) like those of the purified enzyme [4]. These results suggest that subunit c is not involved in single-cycle hydrolysis of ATP but that an oligomeric organization of subunits a and b, in which subunits c are operationally assembled, is necessary for steady-state hydrolysis of ATP coupled with H^+-translocating activity.

4. DISCUSSION

We proposed previously that the mechanism of ATP hydrolysis by yeast vacuolar H^+-ATPase may be similar to those by mitochondrial and bacterial F_0F_1-H^+-ATPases [4]. Vacuolar ATPase also has a multi-subunit structure, and in the mechanism under non-steady-state conditions, we proposed that ATP initially binds to the NBD-Cl-sensitive catalytic site to form an enzyme-ATP complex. Then, the bound ATP, most of which is committed to hydrolysis, is split into ADP and P_i. The hydrolytic step is stimulated by the presence of excess ATP [4].

The present work addressed the question of the apparent molecular masses of the H^+-ATPase bound to vacuolar membranes for single- and multi-cycle hydrolyses of ATP. As demonstrated by Housley et al. [12], target size analysis based on γ-ray irradiation is a convenient method for estimating the functional molecular structure of a membrane protein complex in situ. However, it has been documented that this method can be subject to systematic errors when one is less cautious of temperature and secondary radiation effects or applies it to low molecular mass species [13]. We chose G6PDH as an internal standard to optimize the experimental system because its use as a standard is widespread. We determined the molecular mass of the enzyme to be 9.8×10^4 Da, which was within the range of reported values (92–114 kDa) [14].

The apparent molecular mass for multi-cycle hydrolysis of ATP was determined to be $4.1–5.3 \times 10^5$ Da. We would like to suggest that this deviation may not be systematic error due to our experimental system but, rather, may depend on complex molecular organization of the H^+-ATPase and/or change in susceptibility of the enzyme to γ-ray irradiation in different vacuolar membrane preparations. Since the estimation of the functional molecular masses of H^+-ATPase in vacuolar membranes were semi-quantitative, we wish to emphasize here the 4-fold difference in functional molecular masses under the two kinetic conditions. The functional molecular mass for steady-state hydrolysis of ATP ($4.1–5.3 \times 10^5$ Da) indicates that the enzyme is oligomeric in terms of a catalytic site and requires the whole structure for activity, suggesting strong interaction among the catalytic sites. There are reports that the vacuolar H^+-ATPases in maize and *Neurospora* have similar large molecular sizes of 400–520 kDa [15,16]. If it is assumed, based on these observations and other available data, that the enzymes are mainly composed of three subunits, subunit a (67–72 kDa), b (57–62 kDa), and c (16–20 kDa) [3,4,15,16], the oligomeric ATPase complex capable of multi-cycle hydrolysis of ATP coupled to H^+ translocation may have the molecular composition $(a,b)_m c_n$, where $m \geqq 3$ and $n > m$, by analogy with the molecular organization of F_0F_1–H^+-ATPase $(\alpha\beta)_3\gamma\delta\epsilon a_1 b_2 c_{6-12}$ [17]. Possibly several minor components are also present in the enzyme complex.

In contrast, the functional molecular mass of ATPase for a single cycle of ATP hydrolysis was found to be $0.9–1.1 \times 10^5$ Da. This value clearly shows that single-cycle hydrolysis of ATP is catalyzed by enzyme with a much smaller molecular mass, probably with only one catalytic site, and thus that the reaction does not require any interaction among catalytic sites. Single-cycle hydrolysis of ATP was not inhibited by DCCD. This indicates that subunit c is not involved in single-cycle hydrolysis. One molecule each of subunits a and b is the minimal component capable of catalyzing the reaction in a DCCD-insensitive manner as the two subunits exist in the membrane in equimolar amounts [1], subunit a being the catalytic component and subunit b probably playing a regulatory role.

Recently, Zimniak et al. and Bowman et al. determined the nucleotide sequences of a '70 kDa'

subunit of vacuolar H^+-ATPase from carrot [18] and *Neurospora* [19]. There is extensive homology between these subunits and subunit α of H^+-translocating ATPase from the plasma membrane of *Sulfolobus acidocaldarius* [20,21]. This striking observation suggests a common ancestral origin in the evolution of archaebacterial plasma membrane H^+-ATPase and vacuo-vesicular H^+-ATPases in fungal, plant and animal cells [1,22]. Further biochemical and molecular biological studies will give clues to the stoichiometry of the subunit composition of holo-H^+-ATPases and the subunit interaction capable of evoking positive cooperativity in ATP hydrolysis.

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