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$P_i \rightleftharpoons$ATP exchange in the absence of proton gradient by the H^+-ATPase from yeast plasma membranes

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Purified soluble H^+-ATPase from *Schizosaccharomyces pombe* catalyzes a $P_i \rightleftharpoons$ATP exchange in the absence of a H^+ gradient. When the pH of the assay medium is raised from 5.5 to 8.0 there is a decrease of the ATPase activity and an increase of the rate of $P_i \rightleftharpoons$ATP exchange. At pH 7.0 the addition of the organic solvent dimethyl sulfoxide (20%, v/v) promotes a decrease of ATPase activity and an increase of the $P_i \rightleftharpoons$ATP exchange reaction. The effect of the organic solvent on the $P_i \rightleftharpoons$ATP exchange is related to a decrease of the apparent K_m for P_i.

H^+-ATPase; Proton gradient; P_i-ATP exchange; *(Schizosaccharomyces pombe)*

1. INTRODUCTION

H^+-ATPases from fungi and plant plasma membranes belong to the family of cation-transporting ATPases which also comprises the Ca^{2+}-, the Na^+/K^+- and the H^+/K^+-ATPases from mammalian membranes as well as the K^+-ATPase from bacteria [1]. The mammalian and yeast transport ATPases share the same basic mechanism for ATP hydrolysis. The catalytic cycle comprises a transition between two functional states of the enzyme: one, E_1, being specific for ATP cleavage and the other, E_2, for P_i binding. A covalent aspartyl-phosphate intermediate $E_1 \sim P$ has been identified as well as a noncovalently bound phosphate $E_2 \cdot P_i$ where each of the oxygen atoms from phosphate has an equal probability of exchange with water during the hydration/dehydration step. This sequence of reactions is readily reversible for the mammalian ATPases. In particular, the Ca^{2+}-ATPases from sarcoplasmic reticulum have been studied well in this respect [2]. In contrast, the fungal plasma membrane H^+-ATPase is less reversible; no net synthesis of ATP has been reported so far. Partial reverse reactions were however observed in the absence of a H^+ gradient using the sensitive technique of ^{18}O exchange between [γ-^{18}O]ATP and H_2O or between ^{18}O P_i and H_2O [3]. This approach led to the determination of three rate constants for partial reactions and allowed their comparison with the rate constants of the Ca^{2+}-ATPase similarly obtained [4]. As reported in fig.1, the hydration and dehydration constants k_3 and k_{-3} are much more asymmetric in the H^+-ATPase (k_3/k_{-3} = 162) than in the Ca^{2+}-ATPase (k_3/k_{-3} = 1.6). However the better reversibility of the Ca^{2+}-ATPase is not due to a higher rate constant k_{-3} for the synthesis of aspartyl phosphate $E_1 \sim P$ from bound-phosphate $E_2 \cdot P_i$ but results from slower hydrolysis (k_3) of aspartyl phosphate. The high value of k_3 in H^+-ATPase ex-

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Abbreviation: Me_2SO, dimethyl sulfoxide

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plains, at least partly, the low steady-state level of $E_1 \sim P$ during ATP hydrolysis [5]. In contrast, during ATP hydrolysis, $E_2 \cdot P_i$ must be high in H^+-ATPase as reflected by the $k_3/(k_{-3} + k_4)$ ratio of 76.4 compared to 0.18 in the Ca^{2+}-ATPase.

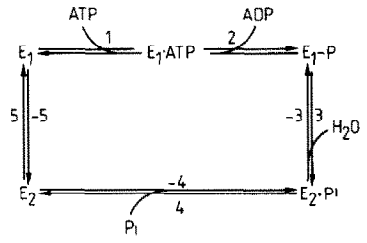
Another partial reaction, the $^{32}P_i \rightleftharpoons$ ATP exchange, has been observed in the yeast H^+-ATPase reconstituted in phospholipid vesicles [6,7]. Most of this exchange activity is inhibited by ionophore and therefore must be driven by the electrochemical ion gradient made during ATP hydrolysis. We now report a $^{32}P_i \rightleftharpoons$ ATP exchange activity by the purified soluble H^+-ATPase from *Schizosaccharomyces pombe* carried out in the absence of H^+ (or any other ion) gradient. This activity is promoted by the addition of dimethyl sulfoxide to the reaction medium and by alkaline pH. As for other energy-transducing ATPases [2] this observation raises the possibility of changes of water activity and of H^+-binding energy during the catalytic cycle for ATP hydrolysis by the yeast H^+-ATPase.

2. MATERIALS AND METHODS

The ATPase was solubilized by lysolecithin from *S. pombe* plasma membranes and purified by centrifugation through a sucrose gradient [5]. The enzyme was frozen in aliquots by liquid nitrogen and stored a few days at −80°C before use. When indicated the peak fractions of high ATPase activity after sucrose gradient were lyophilized and conserved at −20°C. The lyophilized powder was suspended in water just before use at the final concentrations of 0.61 mg protein/ml, 1.95 M sucrose, 4 mM ATP, 4 mM EDTA and 50 mM Tris-acetate, pH 7.3. $^{32}P_i$ was purified as described in [8]. ATPase activity was determined by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP [8] or by the Fiske-Subarow method [9], using a regenerating system for ATP (see legend). ATP $\rightleftharpoons P_i$ exchange was assayed by measuring the formation of [γ-^{32}P]ATP from ADP and (^{32}P) P_i [10]. Identification of radioactive ATP and pyrophosphate by autoradiography and ascending thin layer chromatography after extraction of (^{32}P) P_i was as described [11].

3. RESULTS

Fig.2 shows that raising the pH of the assay from 5.5 to 8.0 decreases considerably the ATPase activity. This decrease of activity is reversible as shown by the recovery of 100% ATPase activity measured at pH 6.0 after preincubation at 30°C for 6 min at pH values ranging from 5.5 to 7.0. After preincubation of the enzyme at pH 8.0, 72% of the optimal activity is recovered when the en-



	H⁺ATPase	Ca²⁺ATPase
k_3 (min^{-1} × 10^{-3})	550	31
k_{-3} (min^{-1} × 10^{-3})	31	19
k_4 (min^{-1} × 10^{-3})	4.1	170
k_3/k_{-3}	162	16
$k_3/(k_{-3} + k_4)$	76.4	0.2

Fig.1. Reaction scheme and rate constants for ATP hydrolysis by yeast H^+-ATPase [5] and sarcoplasmic reticulum Ca^{2+}-ATPases [4].

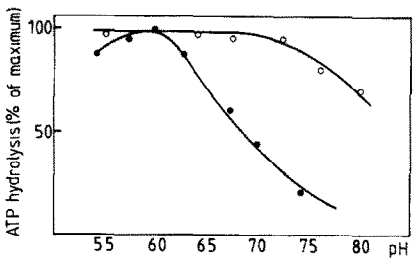


Fig.2. Reversibility of the pH effect on ATP hydrolysis. The assay was carried out at 30°C in 100 μl of 10 mM Mes-KOH, 10 mM Mops-KOH, 50 mM CH_3COOH, 2 mM $MgSO_4$, 15 mM ATP, 25 mM phospho*enol*-pyruvate, 600 U/ml pyruvate kinase, 10 μg/ml lysolecithin. For (●) 0.6 μg of enzyme (buffered at pH 6.0) was added to the medium at the pH indicated and the reaction stopped after 6 min. For (○) 4 μg of enzyme was preincubated for 6 min in the same media (250 μl) at the indicated pH. In a second step, ATP hydrolysis was measured at pH 6.0 during 6 min in the 100 μl reaction mixture described above, using a 10 μl aliquot of the preincubation mixture.

zyme is tested in a subsequent incubation at pH 6.0.

In contrast to the ATPase activity, the $^{32}P_i \rightleftharpoons$ ATP exchange rate increases by a factor of 6 when the pH of the assay is changed from pH 5.5 to 8.0 (fig.3A). Therefore at pH 8.0, the ratio of ATP synthesis/ATP hydrolysis is higher by a factor of about 100 than that observed at pH 6.0 (fig.3B). The ATPase activity measured for 10 min at 35°C and pH 7.0 is inhibited by Me_2SO concentrations higher than 5% in the reaction mixture (fig.4A), and 70% inhibition is observed in presence of 15% Me_2SO. This inhibition is reversible. After preincubation for 20 min at 35°C in the presence of 15% Me_2SO followed by a 20-fold dilution, full ATPase activity is recovered when tested at pH 6.0. However the inhibition of ATPase activity by Me_2SO concentrations above 20% is only partially reversed by a subsequent dilution of Me_2SO. Fig.4A also shows that at pH 7.0, increasing concentrations of Me_2SO from 5 to 20% stimulate $^{32}P_i \rightleftharpoons$ ATP exchange by a factor of 8. At pH 7.0 and 20% Me_2SO, the combined decrease of ATPase activity and increase of $^{32}P_i \rightleftharpoons$ ATP exchange result in an ATP synthesis/ATP hydrolysis ratio which is 110-times higher than the same ratio measured at pH 7.0 in the absence of Me_2SO (fig.4B). Within a certain range the pH and Me_2SO effects are additive so that at pH 7.0 and 20% Me_2SO, rates of

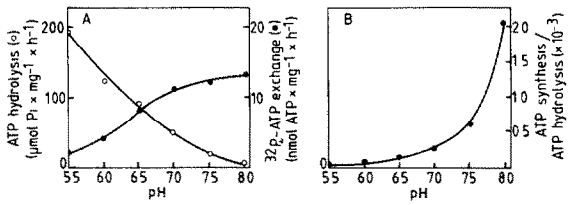


Fig.3. Effect of pH on the activaties of the solubilized H^+-ATPase. (A) ATP hydrolysis (○) and $^{32}P_i \rightleftharpoons$ ATP exchange (●). The assay media (500 μl) contained 25 mM $MgSO_4$, 5 mM ATP, 0.3 mM ADP, 5 mM KH_2PO_4, 10 μCi [γ-$^{32}P_i$]ATP or 500 μCi ^{32}P-labelled P_i, 50 mM Mes-KOH, 50 mM Mops-KOH, 50 mM Tris-HCl. The temperature was 35°C. The pH was as indicated. The reaction was initiated by 10 μg/ml H^+-ATPase and quenched after 20 min by 10% trichloroacetic acid. (B) Ratio ATP synthesis/ATP hydrolysis. The rates of $^{32}P_i \rightleftharpoons$ ATP exchange (ATP synthesis) and ATPase (ATP hydrolysis) were from fig.2.

ATP synthesis up to 1.3 $nmol \cdot mg^{-1} \cdot min^{-1}$ are obtained. This rate is of the order of 1% of the rate for ATP hydrolysis under the same conditions, whereas at pH 6.0 in the absence of Me_2SO, the rate of ATP synthesis is less than 0.01% of that of ATP hydrolysis. Similar data are obtained with lyophilized enzyme which also exhibits dramatic increases of the ratio of ATP synthesis to ATP hydrolysis by the combined effects of alkaline pH and the presence of 20% Me_2SO. Fig.5 obtained with lyophilized enzyme shows that at pH 7.6 the apparent K_m for P_i of the $^{32}P_i \rightleftharpoons$ ATP exchange reaction decreases from 14 to 4 mM P_i by addition of 20% Me_2SO in the reaction medium.

The nature of the compound which incorporates ^{32}P during $^{32}P_i \rightleftharpoons$ ATP exchange was verified. After quenching the reaction and extraction of the inorganic phosphate with ammonium molybdate and isobutanol-benzene, the water phase was adjusted to pH 7.0 and submitted to ascending thin layer chromatography and autoradiography [11]. An aliquot was treated by sarcoplasmic reticulum ATPase before being submitted to chromatography and autoradiography. The radioactivity was found to be associated not only with ATP (~80%) but also with pyrophosphate (~20%).

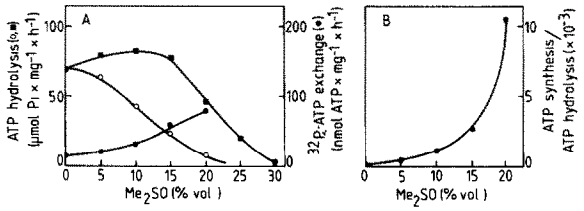


Fig.4. Effect of Me_2SO on the activity of the solubilized H^+-ATPase. (A) ATP hydrolysis (○, ■) and $^{32}P_i \rightleftharpoons$ ATP exchange (●). The assay media (500 μl) contained 25 mM $MgSO_4$, 5 mM ATP, 0.3 mM ADP, 5 mM KH_2PO_4, 10 μCi [γ-^{32}P]ATP or 500 μCi ^{32}P-labelled P_i, 50 mM Mes-KOH, 50 mM Mops-KOH, 50 mM Tris-HCl and the indicated concentration of Me_2SO. The temperature was 35°C and the pH was 7.0. For (○), the reaction was initiated by adding 10 μg/ml H^+-ATPase and quenched by 10% trichloroacetic acid after 20 min. For (■), 50 μg/ml of H^+-ATPase was preincubated during 20 min at 35°C and pH 7.0 in the same medium containing the indicated Me_2SO concentrations. The ATP hydrolysis activity was then measured at pH 6.0 without Me_2SO on a 5 μl aliquot in the 100 μl assay mixture described in fig.2. (B) ATP synthesis/ATP hydrolysis ratio. Data from fig.4A.

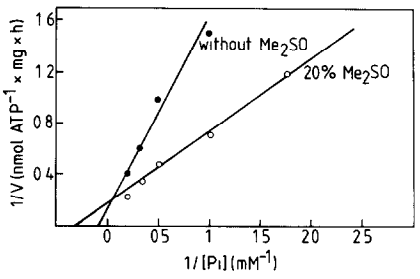


Fig.5. Effect of Me_2SO on the K_m for P_i in $^{32}P_i \rightleftharpoons$ ATP exchange activity. The media contained (400 μl) 50 mM Tris-HCl, 0.2 mM ADP, 25 mM $MgCl_2$, 0.6 mM ATP, and the indicated concentration of P_i. 20% Me_2SO was added in (○). The reaction was initiated by adding 53 μg/ml of lyophilized purified ATPase and quenched after 10 or 60 min by 10% trichloroacetic acid. The pH was 7.0 and the temperature was 35°C.

4. DISCUSSION

In reconstituted proteolipid vesicles, ATP hydrolysis rates of 15 μmol·min^{-1}·mg^{-1} and $^{32}P \rightleftharpoons$ ATP exchange rates of 25 nmol·min^{-1}·mg^{-1} were reported for *Saccharomyces cerevisiae* plasma membrane H^+-ATPase [6,7]. In the presence of ionophores, the ATP hydrolysis rate was stimulated by 10% and the $^{32}P_i \rightleftharpoons$ ATP exchange rate was inhibited by 98% indicating that the H^+ concentration gradient was required as driving force for the synthesis of [γ-^{32}P]ATP. Under these conditions, the ratio of ATP synthesis/ATP hydrolysis was about 10^{-3} in the presence of a H^+ gradient and 10^{-5} in the absence. We now report conditions which promote significant $^{32}P_i \rightleftharpoons$ ATP exchange rates with the soluble purified ATPase from *S. pombe* in the absence of phospholipid vesicles and thus in the absence of a H^+ gradient. Under the optimal conditions tested, $^{32}P_i \rightleftharpoons$ ATP rates of 1.3 nmol·min^{-1}·mg^{-1} and ATP synthesis/ATP hydrolysis ratios up to 10^{-2} were observed. Two parameters, alkaline pH and organic solvents were found to promote reversal of ATP synthesis in the absence of a H^+ gradient. The reversible effects of pH between 5.5 and 7.5 on ATP hydrolysis and $^{32}P_i \rightleftharpoons$ ATP exchange can be analyzed in terms of modifications of the concentration of the ionic species transported by the ATPase similarly to the effects of Ca^{2+} on the Ca^{2+}-ATPase activities [2,12,13]. Reversal of the Ca^{2+}-ATPase is stimulated by the presence of 1 mM Ca^{2+} sufficient to saturate the low-affinity site for Ca^{2+} transport [2,12]. In contrast, we have observed that reversal of the yeast H^+-ATPase is stimulated at low H^+ concentrations (above pH 7.0). ATPase synthesis is thus favored by desaturation of a H^+-binding site of high affinity. This would indicate that the E_1 form in fig.1 contains a H^+-binding site of high affinity which must be saturated in order to form ATP ~ P during ATP hydrolysis. When the pH of the medium rises above pH 7.0 this site becomes significantly deprotonated and ATP hydrolysis decreases while ATP synthesis increases. There is presently no experimental evidence that this protonation site is involved in H^+ transport even though this might seem likely by analogy with the Ca^{2+}-ATPase. The reversible effects of Me_2SO up to 20% can be analyzed in terms of modification of the concentration of water in the assay medium as for the Ca^{2+}-ATPase [2]. Addition of organic solvent has been shown to promote the reversal of several ATPases and pyrophosphatases [2]. The simplest explanation given to these observations is that the catalytic sites of these enzymes are hydrophobic and therefore the entry of phosphate from the external aqueous medium is promoted by addition of solvents which decrease the difference of hydrophobicity between the environment and the catalytic site [2,14,15]. This interpretation is in agreement with our observation that the K_m for P_i during the $^{32}P_i \rightleftharpoons$ ATP exchange of yeast H^+-ATPase is decreased by the addition of 20% Me_2SO. If so, the inhibition by Me_2SO of ATP hydrolysis and the stimulation of ATP synthesis rate indicate that the partition of P_i between the external medium and the active site of E_2 (step 4 in fig.1) is a limiting step in ATP synthesis from external P_i.

The detection of appreciable amounts of PP_i during $^{32}P_i \rightleftharpoons$ ATP exchange is surprising: it might indicate phosphorolysis of aspartyl ~ P by inorganic phosphate under our experimental conditions which requires high concentrations of phosphate. This synthesis of $^{32}PP_i$ does not occur in the absence of enzyme. The mechanistic meaning of this activity carried out by the yeast H^+-ATPase has to be assessed by further experimental work.

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