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Binding of an Intrinsic ATPase Inhibitor to the F_1FoATPase in Phosphorylating Conditions of Yeast Mitochondria[1]

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Yeast mitochondrial ATP synthase has three regulatory proteins; ATPase inhibitor, 9K protein, and 15K protein. A mutant yeast lacking these three regulatory factors was constructed by gene disruption. Rates of ATP synthesis of both wild-type and the mutant yeast mitochondria decreased with decrease of respiration, while their membrane potential was maintained at 170–160 mV under various respiration rates. When mitochondrial respiration was blocked by antimycin A, the membrane potential of both types of mitochondria was maintained at about 160 mV by ATP hydrolysis. ATP hydrolyzing activity of F_1FoATPase solubilized from normal mitochondria decreased in proportion to the rate of ATP synthesis, while the activity of the mutant F_1FoATPase was constant regardless of changes in the rate of phosphorylation. These observations strongly suggest that F_1FoATPase in the phosphorylating mitochondria is a mixture of two types of enzyme, phosphorylating and non-phosphorylating enzymes, whose ratio is determined by the rate of respiration and that the ATPase inhibitor binds preferentially to the non-phosphorylating enzyme.

Key words: ATPase inhibitor, ATP synthesis, F_1FoATPase, localized proton theory, membrane potential.

F_1FoATPase is a ubiquitous enzyme that catalyzes proton transport across bacterial and mitochondrial membranes coupled to hydrolysis of ATP. The enzyme also catalyzes ATP synthesis coupled to proton transport driven by the respiratory system (*1*). The enzyme is composed of two main parts, F_1 and Fo. F_1 is a catalytic sector of the enzyme, and Fo is a proton channel. The bacterial enzyme consists of eight different subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$-$ab_2c_{10}$ (*2–4*). Mitochondrial F_1FoATPase is composed of the same subunits as the bacterial enzyme, together with some extra subunits, such as F_6 protein, subunit d, e, f, g, A6L (*5*), and ATPase inhibitor (*6*). The ATPase inhibitor is thought to be a regulatory protein of the enzyme (*6–8*). Yeast F_1FoATPase has the ATPase inhibitor and two associate proteins, 9K protein and 15K protein, although the latter two proteins were not found in other mitochondrial F_1FoATPases (*9–11*). The 9K protein and ATPase inhibitor each bind to the enzyme in a molar ratio of 1:1 (*12*, *13*). The 15K protein is required for stabilization of a ternary complex of F_1FoATPase·ATPase inhibitor·9K protein (*14*). We have constructed mutant yeast lacking ATPase inhibitor (*15*), 9K protein (*15*), and 15K protein (*16*) to clarify roles of the three regulatory proteins. The deletion of the ATPase inhibitor induced rapid ATP hydrolysis when mitochondria dissipated the membrane potential, and deletion of either 9K protein or 15K protein reduced the action of the ATPase inhibitor (*17*). Thus, it was clear that the ATPase inhibitor and the two associate proteins act to inhibit ATP hydrolysis by the F_1FoATPase under de-energized conditions of mitochondria. The roles of these factors in the process of ATP synthesis, however, were less clear, since their deletion did not bring about unusual reactions in oxidative phosphorylation (*15*, *17*).

In beef heart mitochondria, Sorgato *et al.* reported that the rate of ATP synthesis declines in close relation to the extent of inhibition of the respiratory system, even when the membrane potential is maintained at high level (*18*). This observation is apparently inconsistent with the chemiosmotic theory presented by Mitchell, in which a bulk proton motive force across the mitochondrial membrane is postulated as the driving force of the ATP synthesis (*1*). Several workers suggested a direct coupling of respiratory chain and ATP synthase to resolve the apparent discrepancy (*19*, *20*). The decrease of ATP synthesis under the constant membrane potential, however, can be explained if the phosphorylating fraction of the F_1FoATPase changed corresponding to the rate of respiration. The ATPase inhibitor was the best candidate for a factor to regulate the proportion of phosphorylating F_1FoATPase. Asami *et al.* have re-

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Abbreviations: 905-L_1, mutant yeast lacking 9K protein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CHAPS, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate; D26, mutant yeast lacking ATPase inhibitor; DKD-5D, wild-type yeast; F_1FoATPase, mitochondrial ATP synthase; MOPS, 3-(*N*-morpholino)propanesulfonate; T_1, mutant yeast lacking ATPase inhibitor, 9K protein and 15K protein; TFA, trifluoroacetic acid; TPP$^+$, tetraphenyl phosphonium; W_1, mutant yeast lacking ATPase inhibitor and 9K protein.

ported, however, that the ATPase inhibitor does not inhibit ATP synthesis (*7*), and it was thought not to bind to F_1FoATPase until mitochondria cease phosphorylation by the loss of membrane potential (*8*, *21*, *22*). Therefore it was hard to assume that the ATPase inhibitor regulates the proportion during oxidative phosphorylation (state 3). Recently we noticed that F_1FoATPase solubilized from normal mitochondria of the state 3 respiration reduces the ATP hydrolyzing activity in close relation to the extent of inhibition of the respiration. So we made a systematic study of ATP synthesis and hydrolysis of the enzyme at various respiration rates of mitochondria. To clarify unequivocally whether the ATPase inhibitor and the two associate proteins really participate in the process, we constructed mutant yeast lacking all the three protein factors and compared phosphorylation capacities and ATP hydrolyzing activities of normal and the mutant F_1FoATPases. Results strongly suggest that F_1FoATPase molecule itself is interchangeable between phosphorylating and non-phosphorylating forms corresponding to the respiration rates of mitochondria and that the ATPase inhibitor binds to the non-phosphorylating F_1FoATPase.

MATERIALS AND METHODS

Strains—*Saccharomyces cerevisiae* strain T_1 (***a*** *trp1 leu2 his3 inh1::TRP1 stf1::LEU2 stf2::HIS3*), which contains null mutation in the genes coding for the ATPase inhibitor, 9K protein and 15K protein, was constructed by the reported method (*15*, *16*). Strain W_1 lacking the ATPase inhibitor and 9K protein (*15*), strain D26 lacking ATPase inhibitor (*15*), and strain 905-L_1 lacking 9K protein (*15*) have already been constructed as reported. Strain DKD-5D (***a*** *trp1 leu2 his3*) was used as wild-type control.

Growth Conditions and Preparation of Mitochondria—Yeast cells were cultivated aerobically at 27°C on a medium containing 1% yeast extract, 1% polypepton, and 2% sodium lactate, pH 4.0, collected by centrifugation at 1,700 ×*g* for 3 min at 0°C, and washed four times with ice-cold distilled water. Mitochondria were isolated from the yeast cells by the method of Daum *et al.* (*23*). Protein concentration was determined as described by Lowry *et al.* (*24*) with bovine serum albumin (BSA) as a standard. Protein concentration of mitochondria was adjusted to 10 mg/ml before use.

Assay of ATP Synthesis and Respiration—Respiration rates were measured with a Clark-type oxygen electrode (YSI Inc.) in a 1.7-ml thermostatically controlled chamber as described previously (*15*). Mitochondria (0.6 mg) were incubated at 25°C in the chamber of 1.7 ml of reaction mixture containing 0.6 M mannitol, 50 mM 3-(*N*-morpholino)-propanesulfonate (MOPS), pH 6.5, 5 mM potassium phosphate, 0.1% BSA, and 10 mM succinate. Reactions were started by the addition of 2 mM ADP. Respiration rates were controlled by changing the concentration of malonate. At appropriate intervals, samples of 10 μl were transferred into 490 μl of 0.1 M sodium phosphate, pH 3.0, pre-cooled to 0°C to quench the reaction. ATP synthesis was calculated by measuring changes in the concentrations of adenine nucleotides in reaction mixtures. The nucleotides were analyzed by HPLC on a reversed-phase column, Shim-pack CLC-ODS (6 × 150 mm), which was equilibrated with a mixture of 50 mM sodium phosphate buffer, pH 6.0, and 5% methanol. P/O ratios at various respiration rates were calculated from the polarographic tracings and the amount of ATP formed under the reaction conditions.

Assay of ATP Hydrolyzing Activity of F_1FoATPase—Mitochondria (0.35 mg) were incubated at 25°C in 1.0 ml of the reaction mixture. ADP (2 mM) was added to make state 3 respiration. Respiration rates of succinate were controlled by the addition of malonate. NADH (1 mM) and ethanol (80 mM) were also used in various combinations as respiratory substrates to obtain higher respiration rates. F_1FoATPase was then solubilized from mitochondria by the addition of a detergent, 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS), at a final concentration of 0.2%. Samples of 100 μl of the solubilized enzyme were then added to 1.0 ml of the reaction medium containing 50 mM Tris-maleate, pH 7.4, 0.6 M mannitol, 5 mM potassium phosphate, 5 mM $MgSO_4$, 0.1% BSA, and 1 mM ATP to start ATP hydrolysis. At appropriate intervals, samples of 10 μl were transferred into 490 μl of 0.1 M sodium phosphate, pH 3.0, pre-cooled at 0°C, to quench the reaction. ATP hydrolyzing activity was monitored by changes in concentration of adenine nucleotides.

Measurement of Mitochondrial Membrane Potential—Mitochondrial membrane potential was calculated from the distribution of tetraphenyl phosphonium (TPP^+) across the mitochondrial membranes by the reported method (*25*), except that HPLC was used instead of TPP^+-sensitive electrode to estimate TPP^+ concentration. Mitochondria (0.5 mg) were incubated in 0.3 ml of the reaction mixture containing 0.6 M mannitol, 50 mM MOPS, pH 6.5, 5 mM potassium phosphate, 0.1% BSA, 8 μM TPP^+, 10 mM succinate, and 2 mM ADP. After 2 min of incubation at 25°C, mitochondria were spun down by centrifugation (15,000 ×*g* for 2 min at 25°C). Aliquots of 0.1 ml of the supernatant were mixed with the same volume of 80% acetonitrile, 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 0.2% trifluoroacetic acid (TFA). The CCCP added to the final mixture is an internal standard for the measurement of TPP^+, since CCCP migrates just behind TPP^+ on the HPLC column used. Samples of 20 μl of the final mixture were applied onto a reversed-phase column, Wakosil 5C4 column (4.6 × 250 mm, Wako Pure Chemical Industries) equilibrated with 40% acetonitrile containing 0.1% TFA at a flow rate of 0.7 ml/min. TPP^+ and CCCP in the effluent were monitored at 225 nm. The concentration of TPP^+ was calculated by assuming that the intramitochondrial volume is 1.0 μl per mg protein (*26*).

RESULTS

Decrease of ATP Synthesis and Inhibition of F_1FoATPase under a Constant Membrane Potential—Figure 1, A and B shows, respectively, changes in the rates of ATP synthesis of mitochondria of state 3 from normal and T_1 mutant mitochondria. In both cases, the rates of respiration and ATP synthesis decreased in parallel, while the membrane potential did not decrease significantly. The P/O ratios of both mitochondria were almost the same even when respiration was severely inhibited (1.18 ± 0.13, n = 9), though these values were somewhat lower than that of rat liver mitochondria (1.64 ± 0.25, n = 7). Thus, it is likely that the mutant mitochondria, as well as normal mitochondria, regulate the rate of ATP synthesis corresponding to the rate of

respiration, and that the ATPase inhibitor, 9K, and 15K proteins are not responsible for the regulation.

Figure 2 shows changes in the ATP hydrolyzing activities of $F_1FoATPase$ solubilized from normal and T_1 mutant mitochondria of state 3 respiration. The ATP hydrolyzing activity of the enzyme from mutant mitochondria was kept constant, probably fully active, regardless of the decrease of respiration, giving a specific activity of about 3.0 μmol/min/mg protein. On the other hand, the enzyme activity from normal mitochondria decreased with decrease of respiration. The degree of inactivation was largely parallel with the decrease of phosphorylation shown in Fig. 1, though some divergence is observed in the low respiration region, probably due to partial release of the ATPase inhibitor during the solubilization procedures. A reciprocal plot (insert) showed that the lines of ATPase activity of the normal and mutant enzymes converge to the same point on ordinate, giving specific activity of about 3.0 μmol/min/mg protein. The alkaline treatment of the enzyme that abolishes the action of ATPase inhibitor (*27, 28*) raised the specific activities of these partially inactivated enzymes to the same extent given in the reciprocal plot (2.9 ± 0.3 μmol/min/mg protein). Thus, it is clear that $F_1FoATPase$ in mitochondrial membrane of state 3 respiration is a mixture of active and inactive forms of the enzyme, and that the inactive $F_1FoATPase$ remained inactive after solubilization because of the bound inhibitor protein.

Maintenance of Membrane Potential by ATP Hydrolysis under Non-Respiring Conditions—Figure 3A shows the decrease of the membrane potential of mitochondria by the inhibition of respiration. Antimycin A was added to the mitochondrial suspension in the presence of 10 mM succinate, and 5 mM ATP, to block the state 4 respiration. Before addition of the antibiotic, both normal and mutant mitochondria had membrane potentials of about 200 mV. Addition of antimycin A decreased the potential to about 160 mV, the same level of potential as that of state 3 respiration (see Fig. 1). Addition of antimycin A to the mitochondrial suspension without ATP decreased the membrane potential to 100 mV in the case of both normal and mutant mitochondria (Fig. 3B). Recovery of the potential up to 150 mV was attained by the addition of ATP. However, in normal mitochondria, the level of the recovery decreased with increase in the incubation time without ATP.

Reactivation of Mitochondrial $F_1FoATPase$—Table I summasizes the relationships among ATPase activities of solu-

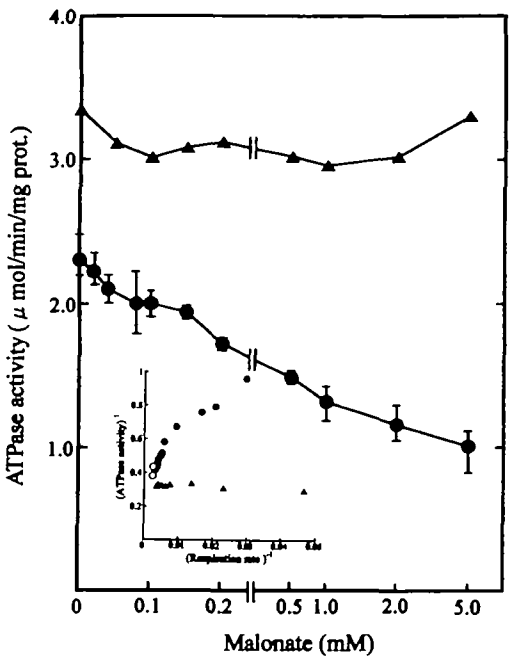

Fig. 2. **ATP hydrolyzing activity of $F_1FoATPase$ solubilized from mitochondria of state 3 respiring at various rates.** Mitochondria (0.35 mg) were incubated at 25°C in 1.0 ml of the reaction mixture given in Fig. 1. $F_1FoATPase$ was solubilized by a detergent, CHAPS, and the ATP hydrolyzing activity was measured as described in "MATERIALS AND METHODS." (●) DKD-5D, wild-type control (n = 3); (▲) T_1 mutant yeast. Open circles in reciprocal plot (inset) are ATP hydrolyzing activities of $F_1FoATPase$ solubilized from wild-type mitochondria oxidizing NADH (1 mM), succinate (10 mM), and ethanol (80 mM) with various combinations.

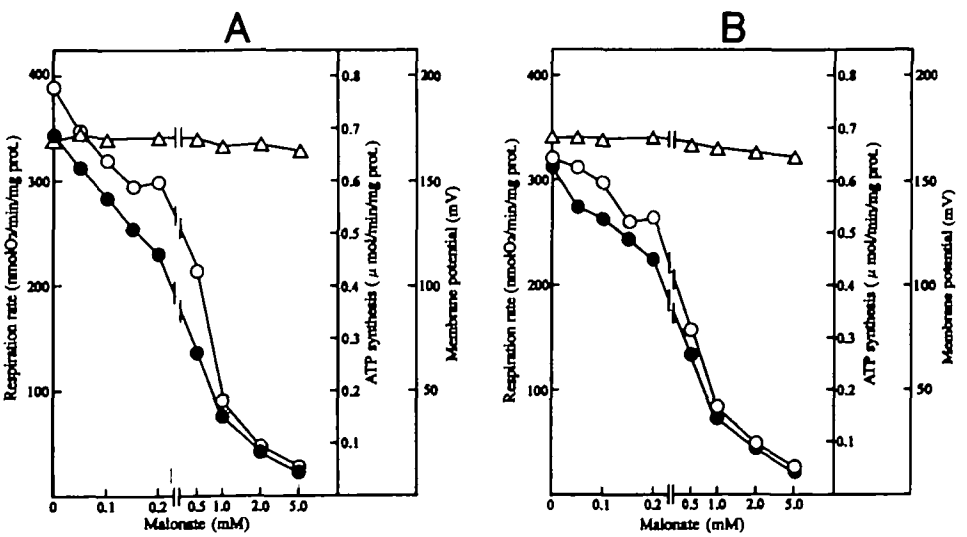

Fig. 1. **ATP synthesis of mitochondria of state 3 respiring at various rates.** Mitochondria (0.6 mg) were incubated at 25°C in an oxygen electron chamber of 1.7 ml containing 0.6 M mannitol, 50 mM MOPS, pH 6.5, 5 mM potassium phosphate, 0.1% BSA, 10 mM succinate, and 2 mM ADP. Respiration rate was controlled by changing the concentration of malonate as indicated. At appropriate intervals, samples of 10 μl of the reaction mixture were mixed with 490 μl of 0.1 M sodium phosphate, pH 3.0, pre-cooled at 0°C to quench the reaction. Rate of ATP synthesis (○) was calculated from changes in concentration of adenine nucleotides in the reaction mixture. The rate of respiration (●) and membrane potential (△) were determined as described in "MATERIALS AND METHODS." A, DKD-5D, wild-type control; B, T_1 mutant.

bilized F_1FoATPase, membrane potential, and respiration rates of normal mitochondria. Consistent with the data presented above, the inhibition of respiration by malonate under state 3 respiration reduced the ATP hydrolyzing activity of the F_1FoATPase but did not decrease the membrane potential. Inhibition of the respiration by antimycin A in the absence of ATP reduced the membrane potential to about 100 mV. Once the potential was reduced to this level, re-establishment by ATP hydrolysis was partial, due to gradual binding of the ATPase inhibitor to F_1FoATPase. However, respiration *via* cytochrome *c* in the presence of ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) brought about increment of the potential concomitant with the increase of ATP hydrolyzing activity. We have already reported that addition of CCCP to mitochondria of state 3 respiration induced maxim activation of respiratory activity with rapid binding of ATPase inhibitor to F_1FoATPase (*17*). Thus, the activation of the electron transport system itself is not a factor in the displacement of the ATPase inhibitor from the binding site; rather, the electrochemical gradient of proton generated by the respiratory system provides the driving force for the displacement. Thus, the electrochemical gradient of protons generated by electron transport provides a strong driving force for displacement of the ATPase inhibitor from the binding site of the enzyme.

Inhibition of F_1FoATPase of Various Mutant Mitochondria—We have reported that the 9K protein binds to F_1FoATPase *in vitro* forming an equimolar complex with the enzyme, and that the formation of the complex decreases the ATP hydrolyzing activity of the enzyme 40% (*13*). The 15K protein also forms an equimolar complex with the F_1FoATPase and stabilizes the binding of the ATPase inhibitor and 9K protein to the enzyme without changing their inhibitory activity (*14*). Figure 4 shows changes in the ATP hydrolyzing activities of the F_1FoATPases solubilized from mitochondria lacking those protein factors. Mutant F_1FoATPase lacking both the ATPase inhibitor and 9K protein exhibited high level of ATP hydrolyzing activity, like the T_1 mutant, regardless of the respira-

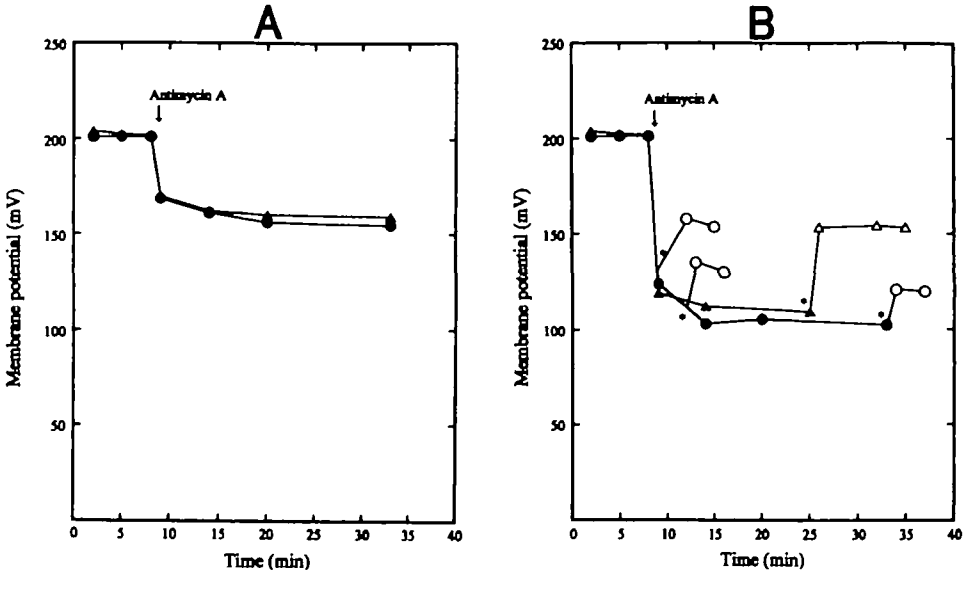

Fig. 3. **Maintenance of membrane potential by ATP hydrolysis under non-respiring conditions.** A: Mitochondria (5 mg) were incubated at 25°C in 3.0 ml of reaction mixture containing 0.6 M mannitol, 50 mM MOPS buffer, pH 6.5, 10 mM potassium phosphate, 0.1% BSA, 8 μM TPP$^+$, 10 mM succinate, and 5 mM ATP. Antimycin A (0.6 μg/mg protein) was added at the times indicated. Samples of 200 μl of the mitochondrial suspension were subjected to centrifugation (15,000 ×*g* for 2 min) and aliquots of 100 μl of the supernatant were mixed with the same volume of 80% acetonitrile containing 50 μM CCCP and 0.2% TFA. Membrane potential was calculated as described in "MATERIALS AND METHODS." ●, normal mitochondria; ▲, T_1 mutant mitochondria. B: Mitochondria were incubated under the same conditions except that ATP was omitted from the incubation system. Membrane potential was calculated as in A. ●, normal mitochondria; ▲, T_1 mutant mitochondria. At the times indicated (asterisks), ATP (5 mM) was added and the changes in membrane potential was checked. In case of normal mitochondria, the incubation system was triplicated. ○, normal mitochondria; △, T_1 mutant mitochondria.

TABLE I. **Reactivation of ATP hydrolyzing activity of F_1FoATPase by increment of membrane potential.** Mitochondria (1.67 mg) were incubated at 25°C in 1.0 ml of reaction mixture containing 0.6 M mannitol, 50 mM MOPS, pH 6.5, 10 mM potassium phosphate, 0.1% BSA, 8 μM TPP$^+$, and 10 mM succinate. Experiment 1: ADP (2 mM) was added to make state 3 respiration. Experiment 2: Measurements were made 10 min after the addition of malonate (5 mM). Experiment 3: Measurement were made 10 min after the addition of antimycin A (4 μM). In experiment 4 and 5, ATP (2 mM) was added 10 min and 20 min, respectively, after addition of antimycin A. In experiments 6 and 7, ADP (2 mM), ascorbate (10 mM), and TMPD (0.1 mM) were added 5 min and 20 min, respectively, after addition of antimycin A.

Experiment No.	Addition			Respiration rate	ATPase activity of solubilized F_1FoATPase	Membrane potential
	1st	2nd	3rd	(nmolO_2/min/mg prot.)	(μmol/min/mg prot.)	(mV)
1	ADP	—	—	355	2.30	161
2	ADP	Malonate	—	23	1.04	162
3	Antimycin A	—	—	1	0.24	100
4	Antimycin A	ATP	—	2	0.40	120
5	Antimycin A	ATP	—	2	0.28	114
6	Antimycin A	ADP	Ascorbate+TMPD	353	1.00	154
7	Antimycin A	ADP	Ascorbate+TMPD	353	0.90	164

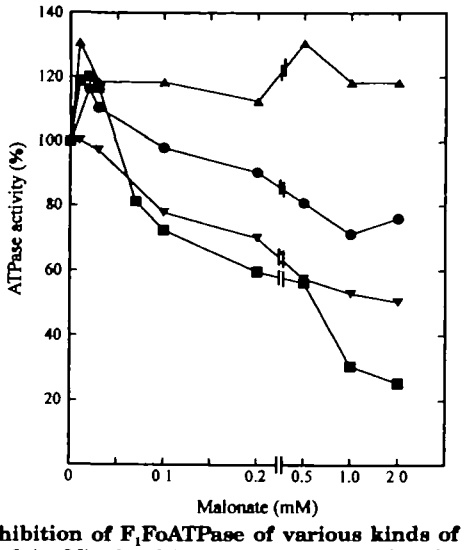

Fig. 4. **Inhibition of F_1FoATPase of various kinds of mutant mitochondria.** Mitochondria (0.35 mg) were incubated under the conditions given in Fig. 2. ATP hydrolyzing activity of each F_1FoATPase was determined as described in "MATERIALS AND METHODS." Symbols ■, ▲, ●, and ▼ represent ATP hydrolyzing activities of F_1FoATPase solubilized from wild-type control, W_1 yeast, D26 yeast, and 905-L_1 yeast mitochondria, respectively.

tion rate of the mitochondria. Decrease of the activity of F_1FoATPase harboring 9K protein and 15K protein but lacking ATPase inhibitor was partial, since the 9K protein inhibits the ATPase activity 40%. The enzyme from the mutant mitochondria harboring the ATPase inhibitor and 15K protein but lacking 9K protein showed considerable inhibition. These observations indicate that the inhibition of the ATP hydrolyzing activity is mainly due to the action of the ATPase inhibitor.

DISCUSSION

In the present study we showed that normal and T_1 mutant mitochondria synthesize ATP at the same rate and same P/O ratio under a constant membrane potential, regardless of changes in the respiration rate (Fig. 1). We also found that the ATP hydrolyzing activity of F_1FoATPase solubilized from normal mitochondria decreases with decrease of respiration, indicating that the ATPase inhibitor binds to the F_1FoATPase even under state 3 respiration. Concerning the binding and dissociation of inhibitor protein on mitochondrial membrane, Van de Stadt *et al.* have reported that the dissociation of ATPase inhibitor is dependent on the energy pressure exerted by the electron transport chain (*8*). The inhibitor protein has been assumed not to bind to the F_1FoATPase until mitochondria lose the membrane potential. The present observations raise serious problem concerning the regulatory mechanism of F_1FoATPase in ATP synthesis. So we have reevaluated the role of the ATPase inhibitor and regulatory mechanism of the F_1FoATPase in ATP synthesis.

The observation that the rate of ATP synthesis in mitochondria is independent of the magnitude of the proton motive force was first reported by Sorgato *et al.* (*18*). This important observation should have given a clue for understanding of the action of the enzyme in ATP synthesis. Indeed, they properly stated that the data could be explained by postulating that a limited proportion of F_1FoATPase is active in ATP synthesis (*18*), but they argued against their own idea and reached the conclusion known as the localized proton theory (*18–20*). Evidence from other studies, however, such as ATP synthesis by acid-base transition on thylakoid membranes (*29*), and ATP synthesis using purified bacterial F_1FoATPase embedded in liposome membrane (*30*), and the ATP synthesis by purified mitochondrial F_1FoATPase with combinations of bacteriorhodopsin on liposome membrane (*31*), failed to support the proposed theory. At the beginning of this study, we considered that one possible settlement would be that a limited proportion of F_1FoATPase is active in ATP synthesis, in which the ATPase inhibitor acts to determine the proportion of inactive F_1FoATPase. As shown in Fig. 1B, however, mitochondria of T_1 mutant synthesized ATP in the same manner as normal mitochondria. This observation excluded the possibility of the participation of the inhibitor protein even under a severely inhibited state of respiration. In contrast to the observation of ATP synthesis, as shown in Fig. 2, a striking difference in ATP hydrolyzing activity was observed in solubilized F_1FoATPase from mutant mitochondria. The hydrolyzing activity was fully active and independent of the changes in rates of respiration, indicating that the decrease of ATP hydrolyzing activity observed in normal mitochondria is due to the action of inhibitor protein (see also Fig. 4). Since the ATPase inhibitor cannot be responsible for regulation of ATP synthesis, it must bind to non-phosphorylating F_1FoATPase. Thus, two types of F_1FoATPases exist in mitochondria of state 3 respiration: actively phosphorylating and non-phosphorylating forms. The ATPase inhibitor selectively forms a complex with the non-phosphorylating enzyme and keeps the ATPase activity latent even after the solubilization of the enzyme-inhibitor complex. The non-phosphorylating enzyme in the mutant mitochondria unavoidably exhibits ATP hydrolyzing activity when solubilized due to lack of the inhibitor protein. In this regard, Sanchez-Bustamante *et al.* have reported that only a limited number of F_1FoATPases are in an active state during steady-state phosphorylation, though they did not mention the role of the inactive enzyme (*32*). Based on these observations, we considered a mechanism by which respiration rate determines rate of ATP synthesis without changing the membrane potential, as follows. Phosphorylating mitochondria at a definite rate of respiration transiently lose the potential due to ATP production, but the mitochondria can regain the original potential by resting phosphorylation of some fractions of their ATP synthases. Accordingly, a definite number of enzymes are always involved in ATP production under the given respiration rate. Observable membrane potential is not the value of an individual mitochondrion but the average for a mixture of phosphorylating and non-phosphorylating mitochondria. Upon addition of malonate, populations of active ATP synthase decrease in proportion to the decrease of the respiration, so that little change in the membrane potential could be observed and *vice versa*. In this connection, it is noteworthy that there is a threshold proton motive force of about 150 mV, below which no phosphorylation is induced (*30, 33*).

Matsuno-Yagi *et al.* have proposed a model of the regulatory mechanism of ATP synthesis by F_1FoATPase that in-

volves two kinetic modes of the enzyme, low K_m–low V_{max} and high K_m–high V_{max} modes, whose interconversion is dependent on the energy supply from the respiratory chain (*34*). Under the low energy conditions, mitochondria constrain the kinetics to the former mode, low turnover capacity while at high levels of energy, the kinetics is converted to the latter mode, high turnover capacity. Their proposal may be true for their experimental conditions, under which ATP synthase is limited by *N,N*-dicyclohexyl carbodiimide in order to increase proton flow through active enzyme under a constant respiration rate. In normal mitochondria, however, the amount of ATP synthase is sufficient to correspond to increase of respiration and there is no need to change the kinetic pattern to the high turnover mode. Indeed, upon addition of 1 mM NADH in the presence of 10 mM succinate, the respiration rate of normal mitochondria increased to 420 nmol O_2/min/mg protein with increase of the ATP hydrolyzing activity to 2.6 μmol/min/mg protein and without change in the P/O ratio (Fig. 2, insert). From these observations, we conclude that ATP synthesis under a constant membrane potential is controlled by changing the molecular species of active F_1FoATPase, and that the ATPase inhibitor binds to non-phosphorylating F_1Fo-ATPase, though the rate of binding is slow and does not correspond to the changes in phosphorylation rates.

In the present study we have not mentioned the physiological role of the ATPase inhibitor. Recently, however, we found that T_1 mutant cells were less viable in non-nutritional conditions, and that the content of adenine nucleotides, especially the cellular ATP level, decreased. We also observed the appearance of respiration-deficient cells from the T_1 mutant during prolonged incubation in water. These observations will be described elsewhere.

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