J. Biochem. 130, 873–878 (2001)

Deletion of Mitochondrial ATPase Inhibitor in the Yeast *Saccharomyces cerevisiae* Decreased Cellular and Mitochondrial ATP Levels under Non-Nutritional Conditions and Induced a Respiration-Deficient Cell-Type[1]

Yi-Min Lu,[*] Kenichiro Miyazawa,[*] Kou Yamaguchi,[*] Kenichi Nowaki,[*] Hiroyuki Iwatsuki,[*] Yumi Wakamatsu,[*] Naoki Ichikawa,[†] and Tadao Hashimoto[*,2]

[*]*Department of Applied Chemistry, Muroran Institute of Technology, Muroran, Hokkaido 050-8585; and* [†]*Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, 3-138 Sugimoto, Sumiyoshiku Osaka 558-8585*

Received June 25, 2001; accepted October 11, 2001

T_1, a mutant yeast lacking three regulatory proteins of F_1F_oATPase, namely ATPase inhibitor, 9K protein and 15K protein, grew on non-fermentable carbon source at the same rate as normal cells but was less viable when incubated in water. During the incubation, the cellular ATP content decreased rapidly in the T_1 cells but not in normal cells, and respiration-deficient cells appeared among the T_1 cells. The same mutation was also induced in D26 cells lacking only the ATPase inhibitor. Overexpression of the ATPase inhibitor in YC63 cells, which were derived from the D26 strain harboring an expression vector containing the gene of the ATPase inhibitor, prevented the decrease of cellular ATP level and the mutation. Isolated T_1 mitochondria exhibited ATP hydrolysis for maintenance of membrane potential when antimycin A was added to the mitochondrial suspension, while normal and YC63 mitochondria continued to show low hydrolytic activity and low membrane potential. Thus, it is likely that deletion of the ATPase inhibitor induces ATPase activity of F_1F_oATPase to create a dispensable membrane potential under the non-nutritional conditions and that this depletes mitochondrial and cellular ATP. The depletion of mitochondrial ATP in turn leads to occurrence of aberrant DNA in mitochondria.

Key words: ATPase inhibitor, F_1F_oATPase, intramitochondrial ATP, mitochondrial membrane potential, respiration-deficient mutant.

Mitochondrial ATPase inhibitor was first isolated from beef heart mitochondria in 1963 by Pullman and Monroy (*1*), and successively from yeasts (*2, 3*), rat liver (*4*), and skeletal muscle mitochondria (*5*). The role of the inhibitor in mitochondria was first reported by Asami *et al.*: it inhibits ATP-dependent reactions of mitochondria, such as ATP-dependent reduction of NAD^+ and the ATP-driven transhydrogenase reaction, but not oxidative phosphorylation (*6*). Van de Stadt *et al.* proposed the unidirectional action of the inhibitor, assuming an energy-dependent binding and release mechanism of the inhibitor protein on F_1F_oATPase (*7*). We constructed a mutant yeast lacking the ATPase inhibitor and showed that it could grow on non-fermentative carbon sources (*8*). Mitochondria isolated from the mutant yeast synthesized ATP at a normal rate but began to hydrolyze ATP when membrane potential was abolished by uncoupling reagents, while normal F_1F_oATPase was latent under the uncoupled conditions (*9*). From these findings we concluded that the ATPase inhibitor works to inhibit ATP hydrolysis when mitochondria have lost the membrane potential. Recently, however, we demonstrated that F_1F_oATPase in phosphorylating mitochondria is a mixture of two types of enzymes, actively phosphorylating and resting enzymes, and that the ATPase inhibitor binds to the resting F_1F_oATPase (*10*). These observations explained why mitochondrial membrane potential is constant and independent of the respiration rate and raised the question of the physiological role of the inhibitor protein of F_1F_oATPase in resting yeast cells. In the present study, we focused on the action of the inhibitor protein in the resting cells. Comparison of mitochondrial functions in normal and the mutant cells revealed that the ATPase inhibitor works to preserve mitochondrial and cellular ATP under non-nutritional conditions and that the preservation of mitochondrial ATP is particularly important for the protection of the mitochondrial DNA.

MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions—*Saccharo-*

[1] This research was supported by Special Grant-in-Aid for Education and Research of Muroran Institute of Technology.

[2] To whom correspondence should be addressed. E-mail: tadao@mmm.muroran-it.ac.jp

Abbreviations: DKD-5D, yeast strain used as normal control; D26, yeast strain lacking an inhibitor of F_1F_oATPase; D230, yeast strain lacking 9K protein and 15K protein; F_1F_oATPase, mitochondrial ATP synthase; T_1, yeast strain lacking all three regulatory proteins of F_1F_oATPase; YC63, yeast strain derived from D26, harboring an expression plasmid containing the ATPase inhibitor gene; TPP^+, tetraphenyl phosphonium.

myces cerevisiae strain DKD-5D (*a trp1 leu2 his3*) was used as a wild-type yeast control. Strain T_1 (*a trp1 leu2 his3 inh1::TRP1 stf1::LEU2 stf2::HIS3*), which contains null mutations in the genes coding for the ATPase inhibitor, 9K protein and 15K protein (*10*), strain D26 (*a trp1 leu2 his3 inh1::TRP1*), lacking the gene coding for the ATPase inhibitor (*8*), and strain D230 (*a trp1 leu2 his3 stf1::LEU2 stf3::HIS3*), which contains null mutation in the genes coding 9K protein and 15K protein (*11*), were constructed previously as reported. Overexpression of the ATPase inhibitor in mitochondria was achieved as reported previously (*12*) using strain YC63 harboring an expression plasmid originating from YEp51 shuttle vector (*13*). Yeast cells were grown with vigorous shaking at 28°C in a medium consisting of 1% yeast extract, 1% polypeptone, and 2% lactate. For the overexpression of the ATPase inhibitor, yeast cells were treated and grown on 2% galactose medium as reported (*12*).

Determination of Viability of Yeast Cells in Water—Yeast cells harvested at various growth phases were washed three times with sterile water, suspended in the water at a cell density of 10^7 to 10^8 cells per ml, and incubated at 28°C with constant shaking. Viability of cells were determined by colony counting on agar plates containing 1% yeast extract, 1% polypeptone, and 1% glucose (YPD medium). Colonies of respiration-deficient cells on the plate were discriminated from normal cells by overlaying with 2,3,5-triphenyltetrazolium according to the reported method (*14*).

Determination of Adenine Nucleotides of Yeast Cells—During the incubation of cells in water, samples of 1 ml of yeast suspension were taken immediately after vigorous shaking of the cell suspension, frozen with liquid nitrogen and lyophilized. To the lyophilized cells, 1 ml of 0.5 N ice-cold perchloric acid was added to extract adenine nucleotides. The cell debris was spun down by centrifugation and kept for protein assay of the cells. The supernatant, which contains nucleotides, was neutralized with 9.2 N KOH and cooled to 0°C to remove perchlorate. The nucleotides were analyzed by HPLC on a reversed-phase column, Shim-pack CLC-ODS (6 × 15 mm), which was equilibrated with a mixture of 50 mM sodium phosphate, pH 6.6, and 5% methanol.

Other Procedures—Mitochondria were isolated from yeast cells by the method of Daum *et al.* (*15*). ATP synthesis, ATP hydrolysis, respiration and membrane potential of mitochondria were analyzed as reported (*10*). Mitochondrial DNA was prepared from mitochondria and purified by the reported method involving density gradient centrifugation in a CsCl gradient containing 4′,6-diamidine-2-phenylindol (*16*). Protein concentration was determined as described by Lowry *et al.* with bovine serum albumin as a standard (*17*).

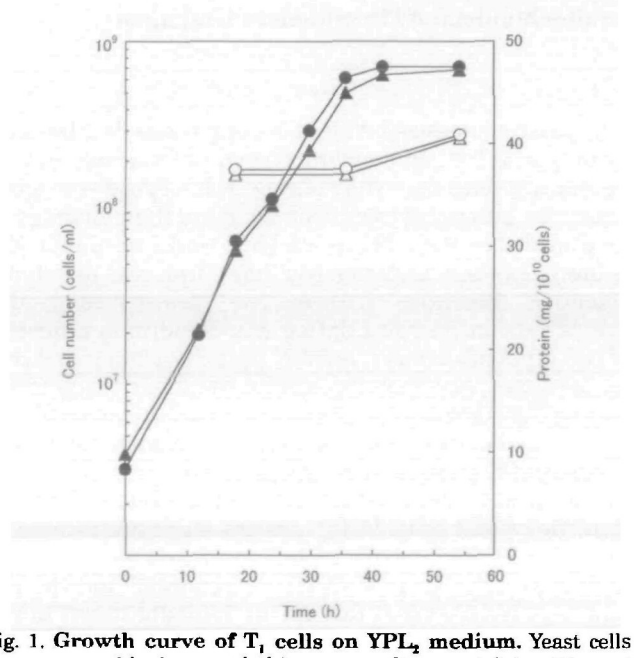

Fig. 1. **Growth curve of T_1 cells on YPL_2 medium.** Yeast cells were grown with vigorous shaking on a medium containing 1% yeast extract, 1% polypeptone, and 2% lactate (YPL_2 medium). At the indicated times, cells were collected from the culture medium and cell growth was checked by measuring colony formation on agar plates containing 1% yeast extract, 1% polypeptone, and 1% glucose. Total proteins were determined after hydrolysis of cells with 2 N NaOH. (●, ○) normal control; (▲, △) T_1 cells. Solid and open symbols are growth rate and total proteins per 10^{10} cells, respectively.

RESULTS

Cell Growth and Properties of Mitochondria of T_1 Cells—Figure 1 shows the growth curve on YPL_2 medium of T_1 mutant yeast, which lacks all three regulatory proteins of F_1F_oATPase. The growth rate of the mutant was the same as that of normal yeast, in agreement with our previous data that deletion of either the ATPase inhibitor, 9K protein or 15K protein does not influence the cell growth (*12*). Total protein contents of T_1 and normal cells were almost the same, 37 mg to 40 mg per 10^{10} cells. Indices of ATP synthesis of mitochondria prepared from normal and T_1 cells are shown in Table I. ATP synthesis rates and phosphorus/oxygen ratios (P/O ratio) of the two types of mitochondria were almost the same. Respiratory control ratios of these mitochondria were fairly good, though that of T_1 mitochondria was slightly lower than that of normal mitochondria.

Induction of Respiration-Deficient Mutant Cells during Incubation of T_1 Cells in Water—T_1 mutant cells harvested at logarithmic phase were less viable than normal cells and mutated to respiration-deficient cells under non-nutritional conditions. Figure 2A shows the rate of appearance of the respiration-deficient cells among the T_1 cells during the incubation at 28°C in water. Since newly appeared respiration-deficient cells were less viable than the original T_1 cells under these conditions (data not shown), the apparent fre-

TABLE I. **Rate of ATP synthesis, phosphorus/oxygen ratio and respiratory control ratio of mitochondria isolated from normal and T_1 cells.** Mitochondria (0.6 mg) were incubated at 25°C in an oxygen electrode chamber in 1.7 ml of reaction mixture containing 0.6 M mannitol, 50 mM 3-(*N*-morpholino)propanesulfonate, pH 6.5, 5 mM potassium phosphate, 0.1% BSA, 10 mM succinate, and 2 mM ADP. ATP synthesis and phosphorus oxygen ratio (P/O) were measured as described previously (*10*). Respiratory control ratios (RCR) were calculated from the graphic tracing of oxygen electrode aparatus.

	ATP synthesis	P/O	RCR
	(μmol/min/mg prot)		
Normal	0.61 ± 0.19 (n = 9)	1.18 ± 0.13 (n = 9)	3.8 ± 1.0 (n = 9)
T_1	0.60 ± 0.05 (n = 8)	1.16 ± 0.08 (n = 8)	2.5 ± 0.3 (n = 8)

quency of the mutation did not exceed 50% and the frequency often declined when cells were incubated beyond two weeks. Under the same conditions normal cells were stable and virtually all cells were respiration-competent, though prolonged incubation beyond two to three weeks brought about partial cell death and the emergence of respiration-deficient cells. Respiration-deficient cells were also formed from D26 cells, which lack only ATPase inhibitor, though the frequency of mutation was half of that of T_1 cells (Fig. 2B). The mutation rate in D230 cells, which lack 9K and 15K proteins, was even lower and small fraction of the mutation was observed after one week of incubation (Fig. 2B).

The mutation rate depends on the conditions of cell growth. When fermentable carbon sources such as glucose and galactose were used, the respiration-deficient cells appeared even from normal cells after incubation for several days (see below).

Changes in Contents of Cellular Adenine Nucleotides in T_1 Cells—Figure 3 shows changes in adenine nucleotides of T_1 cells during the incubation. ATP content decreased rapidly to about 20% of the original level within four days (Fig.

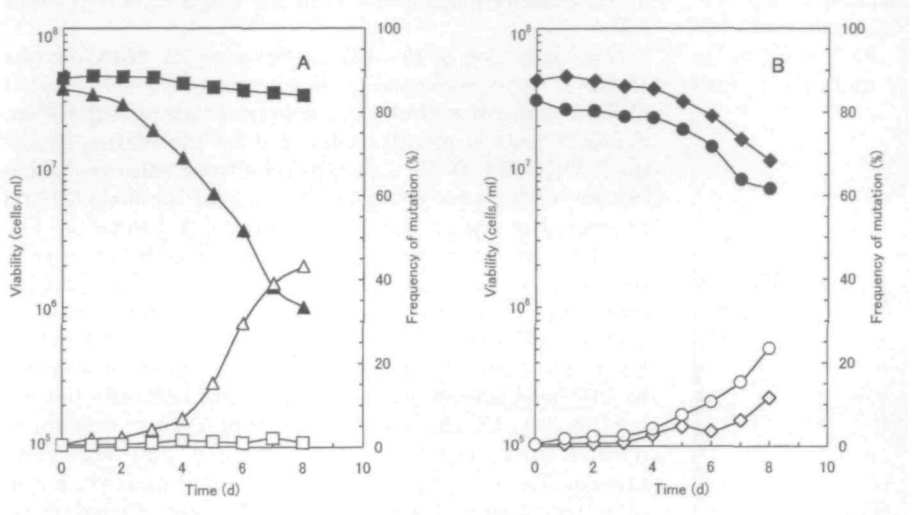

Fig. 2. **Appearance of respiration-deficient mutants during the incubation in water.** Yeast cells were grown on YPL_2 medium and harvested at mid-logarithmic phase. Cells were washed with sterile water three times and suspended in the water. At the indicated times, cell viability was checked by colony formation on YPD agar plates. The mutation rate to respiration-deficient cells was determined as described in "MATERIALS AND METHODS." A (■, □), normal control; (▲, △), T_1 cells. B (●, ○), D26 cells; (◆, ◇), D230 cells. Solid and open symbols indicate viability and mutation rate, respectively.

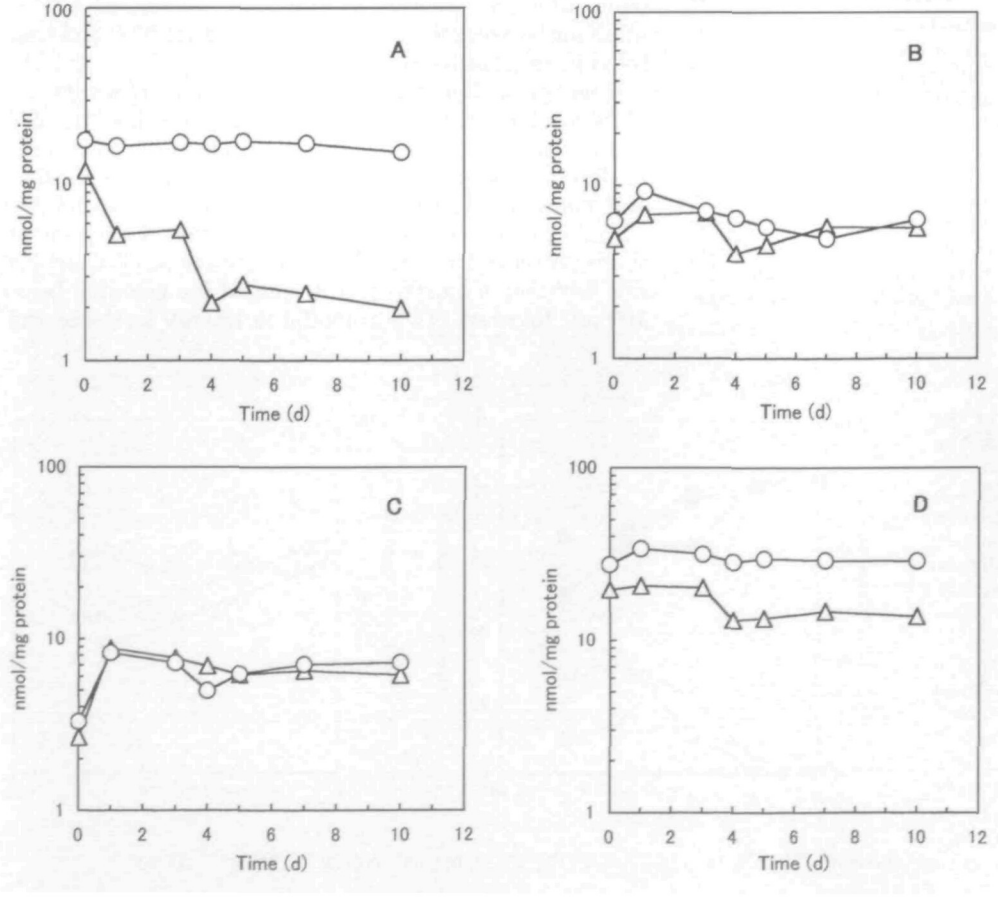

Fig. 3. **Changes in adenine nucleotides in T_1 cells during incubation in water.** Yeast cells were obtained by the same procedure as given in Fig. 2 and were suspended in the sterile water. At the indicated times, cells were withdrawn from the cell suspension. Adenine nucleotides were extracted and determined as described in "MATERIALS AND METHODS." Changes in ATP, ADP, AMP, and total adenine nucleotides are shown in A, B, C, and D, respectively. ○, normal control; △, T_1 cells.

3A), while that of normal cell was fairly constant throughout the incubation time. Since decrease of ATP in the mutant cells precedes the decrease of their viability, the decrease of ATP content is due not to cell death but to decrease of ATP level in individual T_1 cells. Contents of ADP (Fig. 3B) and AMP (Fig. 3C) changed in parallel in normal and mutant cells. Of the three nucleotides, the decrease of ATP content in T_1 cells is striking, but the decrease of total adenine nucleotides is less remarkable compared to that of normal cells (Fig. 3D).

Mitochondrial DNA of Newly Appeared Respiration-Deficient Cells—To check what kind of changes were induced in mitochondrial DNA of the newly appeared respiration-deficient yeasts, we selected several respiration-deficient colonies that appeared on the 7th day of the incubation in water. As a control of cytoplasmic petite mutants, T_1 cells were grown on YPD medium and spontaneously appeared respiration-deficient cells were selected. Respiration-competent T_1 cells were used as a source of normal mitochondrial DNA. Mitochondrial DNA was prepared and purified by the reported method (*16*). Figure 4, A and B, shows patterns of agarose-gel electrophoreses of the fragmented DNAs from three types of yeast cells; respiration-competent T_1 cells, spontaneously appeared cytoplasmic petite mutant cells, and newly appeared respiration-deficient cells. In the electrophoretic profile of the DNA fragments, the newly appeared respiration-deficient cells were very similar to the cytoplasmic petite mutant recovered from YPD medium and differed from the respiration-competent cells.

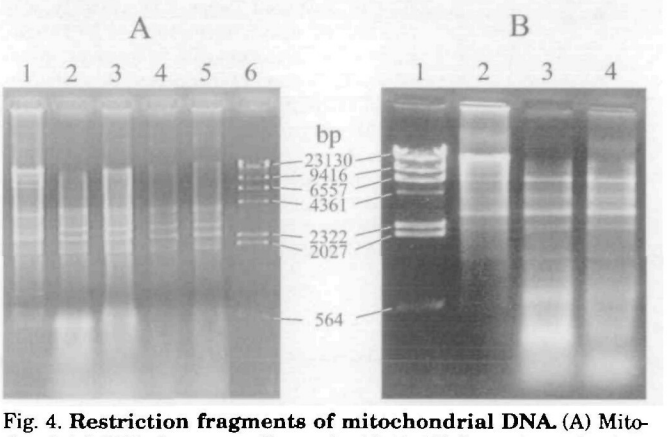

Fig. 4. **Restriction fragments of mitochondrial DNA.** (A) Mitochondrial DNA fragments digested with *Eco*RI. Lane 1, respiration-competent mitochondrial DNA of T_1 cells; lane 2, DNA of a cytoplasmic petite mutant of T_1 cells grown on YPD medium; lanes 3–5, mitochondrial DNAs of respiration-deficient cells of three randomly selected strains (present study). (B) Mitochondrial DNA fragments digested with *Hin*dIII. Lane 1, size marker, λ DND digested with *Hin*dIII; lane 2, DNA of respiration-competent cells; lane 3, DNA of a cytoplasmic petite mutant from YPD medium; lane 4, a respiration-deficient mutant of the present study. Mitochondrial DNAs were prepared and purified by the reported method (*16*). Digested fragments were developed in agarose-gel and visualized by UV radiation with ethidium bromide.

Overexpression of the ATPase Inhibitor in Mitochondria Reduced the Induction of Respiration-Deficient Mutant Cells—As mentioned above, the appearance of respiration-deficient cells is greatly influenced by the carbon source used. DKD-5D, YC63, and D26 cells were cultured on galactose medium according to the reported method (*12*) and harvested at logarithmic growth phase. As shown in Fig. 5A, the viability of these cells decreased less than that of T_1 cells (Fig. 2). It is clear that the frequency of mutation reduced by overexpression of the ATPase inhibitor in mitochondria of YC63 cells. Changes in the cellular ATP level of these cells were also checked (Fig. 5B). A gradual decrease in ATP level was observed in normal and D26 cells, but not inYC63 cells. We checked the content of ATPase inhibitor in normal cells harvested at logarithmic and stationary phases. The amount of the inhibitor protein was very low in cells harvested at logarithmic growth phase, though it increased at stationary phase (data not shown). Thus, it is likely that the presence of sufficient amount of ATPase inhibitor is required preserve mitochondrial ATP and keep the cells respiration-competent.

Recovery of Membrane Potential by ATP Hydrolysis of Mitochondria from Various Yeast Cells—Mitochondria of T_1 and normal cells were prepared from cells cultured on YPL_2 medium. YC63 cells were cultured on galactose medium and mitochondria were prepared by the reported method (*12*). As shown in Fig. 6, under the state 4 respiration, these mitochondria had membrane potential of about 200 mV. Addition of antimycin A decreased the potential below 100 mV. Recovery of the potential to 150 mV by the hydrol-

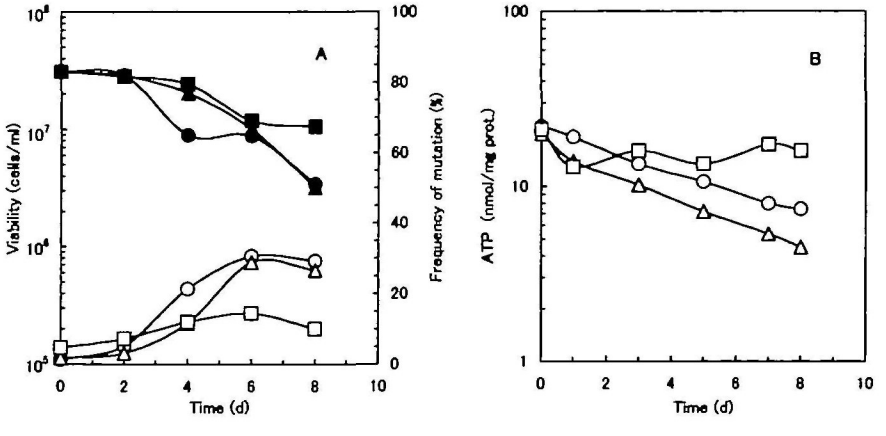

Fig. 5. **Effect of overexpression of the ATPase inhibitor in YC63 cells.** (A) Viability of cells and frequency to respiration-deficient phenotype. Normal control yeast, YC63 cells harboring an expression vector of the ATPase inhibitor and D26 cells were grown on galactose medium according to the reported method (*12*). Cells were harvested at mid-logarithmic growth phase and incubated in sterile water under the conditions given in Fig. 2. Viability and mutation rates to respiration-deficient mutants were determined by the procedure given in Fig. 2. (▲, △), normal control; (●, ○), D26; (■, □), YC63. Solid and open symbols indicate viability and mutation rates to respiration-deficient mutant, respectively. (B) Changes in cellular ATP level in the three strains. ATP contents were determined by the method given in Fig. 3. (△), normal control; (○), D26; (□), YC63.

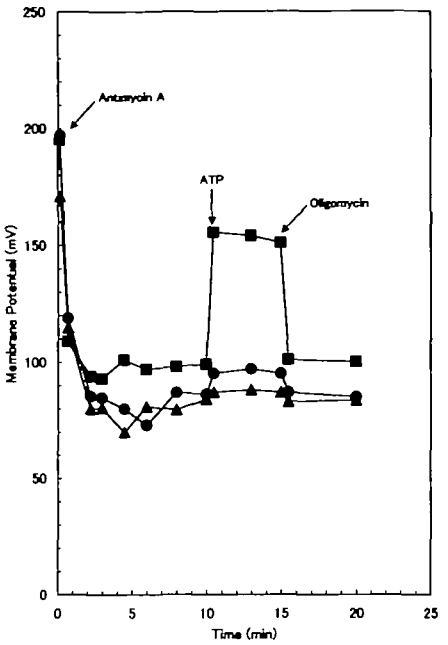

Fig. 6. **ATP hydrolysis maintained membrane potential of T_1 mitochondria.** Normal and T_1 cells were cultured on YPL_2 medium and YC63 cells were cultured on the galactose medium. Mitochondria (5.0 mg) prepared were incubated at 25°C in 3.0 ml of reaction mixture containing 0.6 M mannitol, 50 mM 3-(*N*-morphorino)propanesulfonate buffer, pH 6.5, 10 mM potassium phosphate, 0.1% bovine serum albumin, 8 μM tetraphenyl phosphonium (TPP^+) and 10 mM succinate. Antimycin (2.5 μg per ml), ATP (5 mM), and oligomycin (10 μg/mg protein) were added at the times indicated by arrows. Samples of 0.2 ml of the mitochondrial suspension were centrifuged at 15,000 ×*g* for 2 min, and aliquots of 0.1 ml of the supernatant were mixed with the same volume of 80% acetonitrile containing 50 μM carbonyl cyanide *m*-chlorophenylhydrazone. Membrane potential was calculated from the distribution of TPP^+ across the mitochondrial inner membrane according to the equation of Nernst as described before (*10*). ■, T_1 mitochondria; ●, normal mitochondria; ▲, YC63 mitochondria.

ysis of ATP was only observed in T_1 mitochondria. Recovery was partial in normal mitochondria and negligible in YC63 mitochondria. ATP hydrolyzing activity of the T_1 mitochondria was 0.07 ± 0.01 μmol ATP per min per mg protein, while those of normal and YC63 mitochondria were each about 0.01 ± 0.005 μmol. Thus, it is highly likely that T_1 cells lose cellular ATP so quickly because of extra consumption of mitochondrial ATP by inhibitor-deleted F_1F_oATPase to maintain dispensable membrane potential.

DISCUSSION

Total deletion of three regulatory proteins of F_1F_oATPase does not bring about reduction of growth rate or the rate of protein synthesis on YPL_2 medium. The observations were consistent with data that both normal and T_1 mitochondria synthesize ATP at the same rates and with the same P/O ratios (Table I). T_1 cells harvested at logarithmic phase, however, were less viable (Fig. 2A) and lost cellular ATP rapidly (Fig. 3A) when they were incubated in water. During incubation respiration-deficient mutants of T_1 cells were induced (Fig. 2A). In D26 cells, the frequency of mutation was lower than that of T_1 cells, probably due to the action of 9K protein, which partially inhibits F_1F_oATPase (*18*). Since respiration-deficient mutants of D230 cells were also observed, 15K protein seemed to share some functions to prevent the mutation. This observation is consistent with data that the ATPase inhibitor functions effectively in cooperation with 9K protein and 15K protein (*9, 11*). Of the three regulatory factors, however, the ATPase inhibitor appears to have a special role to prevent the mutation. As shown in Fig. 2B, the deletion of the ATPase inhibitor had a more marked effect than deletion of 9K protein and 15K protein.

The induction of respiration-deficient cells in relation to the content of mitochondrial ATP was first reported by Šubík *et al.* (*19*). Almost 100% of induction was attained when yeast cells were allowed to grow on glucose medium in the presence of antimycin A and bongkrekic acid. We confirmed their results but found that the growth of cells is not obligatory. The mutation could be induced in normal cells after growth on YPL_2 medium by re-suspension in a medium containing YPD, antimycin A, bongkrekic acid, and tunicamycin, in which no bulk growth of cells was observed (data not shown). Omission of either bonkrekic acid or antimycin A failed to produce the induction. Thus, it is likely that depletion of ATP in mitochondria is necessary for the mutation. Isolated T_1 mitochondria consume more ATP and maintain a higher membrane potential than normal mitochondria when antimycin A, an antibiotic that inhibits mitochondrial respiration, was added to the mitochondrial suspension (Fig. 6). Once membrane potential has decreased, the ATPase inhibitor binds strongly to F_1F_oATPase, and normal and YC63 mitochondria can not re-establish their potential by the reverse reaction of F_1F_oATPase (Fig. 6). The, mitochondria1 ATP level of T_1 cells incubated for 7 days in water decreased greatly to 3% of the original level, while that of normal mitochondria was 25%. Thus, it is highly likely that deletion of the ATPase inhibitor increases the consumption of mitochondrial ATP under non-nutritional conditions and eventually leads to mutation.

Recent studies by Graud *et al.* indicate that loss of membrane potential and/or loss of cellular ATP are important for elimination of mitochondrial DNA. A high frequency of mutation to the respiration-deficient cells was observed when the δ-subunit of F_1F_oATPase was deleted, which brings about cessation of ATP production and induces permeability of mitochondria to protons though the mutated F_1F_oATPase (*20*). Data in the present study, however, suggested that preservation of ATP level in mitochondria is more important than maintaining the membrane potential of mitochondria. Actually, yeast strains are viable for a long time under low temperature and anoxia, unfavorable conditions to keep mitochondrial membrane potential. Our conclusion in this study is that the ATPase inhibitor binds to resting F_1F_oATPase, preventing its action in the reverse direction and hence preserving ATP in cells under such severe non-nutritional conditions. In relation to these observations, it is noteworthy that m-RNA of the ATPase inhibitor protein increased 11-fold in yeast cells cultured in minimal nutritional conditions compared with the cells cultured in rich medium (*21*).

The authors thank Drs. Y. Shinohara and H. Terada of Tokushima University for providing bonkrekic acid, and Dr. K. Tagawa, pro-

fessor emeritus of Osaka University, for his generous help and advice throughout this research.

REFERENCES

1. Pullman M.E. and Monroy G.C. (1963) A naturally occurring inhibitor of mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **238**, 3762–3769
2. Hashimoto, T., Negawa, Y., and Tagawa, K. (1981) Binding of an intrinsic ATPase inhibitor to mitochondrial ATPase—Stoichiomitry of binding of nucleotides, inhibitor, and enzyme. *J. Biochem.* **90**, 1151–1157
3. Dianoux, A.C. and Hoppe, J. (1987) Complete amino-acid sequence of the natural ATPase inhibitor from the mitochondria of the yeast *Candida utlis*. *Eur. J. Biochem.* **163**, 155–160
4. Cintron, N.M. and Pedersen, P.L. (1979) A protein inhibitor of the mitochondrial adenosine triphosphatase complex of rat liver. Purification and characterization. *J. Biol. Chem.* **254**, 3439–3443
5. Yamada E.W., Shiffman F.H., and Huzel N.J. (1980) Ca^{2+}-regulated release of an ATPase inhibitor protein from submitochondrial particles derived from skeletal muscles of the rat. *J. Biol. Chem.* **255**, 267–273
6. Asami, K., Junti, and Ernster, L. (1970) Possible regulatory function of a mitochondrial ATPase inhibitor in respiratory chain-linked energy transfers. *Biochim. Biophys. Acta* **250**, 307–311
7. Van de Stadt, J.R., De Boer, B.L., Van Dam, K. (1973) The interaction between the mitochondrial ATPase (F1) and the ATPase inhibitor. *Biochim. Biophys. Acta* **292**, 338–349
8. Ichikawa, N., Yoshida, Y., Hashimoto, T., Ogasawara, N., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) Activation of ATP hydrolysis by an uncoupler in mutant mitochondria lacking an intrinsic ATPase inhibitor in yeast. *J. Biol. Chem.* **265**, 6274–6278
9. Mimura H., Hashimoto, T., Yoshida, Y., Ichikawa, N., and Tagawa, K. (1993) Binding of an intrinsic ATPase inhibitor to the interface between α- and β-subunits of F_1F_oATPase upon de-energization of mitochondria. *J. Biochem.* **113**, 350–354
10. Iwatsuki, H., Lu, Y-M., Yamaguchi, K., Ichikawa, N., and Hashimoto, T. (2000) Binding of an intrinsic ATPase inhibitor in phosphorylating conditions of yeast mitochondria *J. Biochem.* **128**, 553–559
11. Yoshida, Y., Sato, T., Hashimoto, T., Ichikawa, N., Nakai, S., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) Isolation of a gene for a regulatory 15-Kda subunit of mitochondrial F_1F_oATPase and construction of mutant yeast lacking the protein. *Eur. J. Biochem.* **192**, 49–53
12. Ichikawa, N., Fukuda, M., Hashimoto, T., and Tagawa, K. (1998) The carboxyl-terminal region of yeast ATPase inhibitor is indispensable for the stability of the protein in mitochondria. *J. Biochem.* **124**, 886–891
13. Broach, J.R., Li, Y-Y., Wu, L.-C. C., and Jayaram, M. (1983) Vectors for high-level, inducible expression of cloned genes in yeast in *Experimental Manipulation of Gene Expression* (Inoue, M., ed.) pp. 83–117, Academic Press, Orlando, FL
14. Ogur, M., St. John, R., and Nagai, S. (1957) Tetrazolium overlay technique for population studies of respiratory deficiency in yeast. *Science* **125**, 928–929
15. Daum, G., Böhni, P.C., and Schatz, G. (1982) Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257**, 13028–13033
16. Dujon, B. (1981) *Molecular Biology of the Yeast Saccharomyces cerevisiae* (Strathern, J.N., Jones, E.W., and Broach, J.R., eds.), Cold Spring Harbor Laboratory Press, New York
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
18. Hashimoto, T., Yoshida, Y., and Tagawa, K. (1987) Binding properties of 9K protein to F_1ATPase: A counter ligand to the ATPase inhibitor. *J. Biochem.* **102**, 685–692
19. Šubík, J., Takácscová, G., and Kováč, L. (1978) Intramitochondrial ATP and cell functions: Growing yeast cells depleted of intramitochondrial ATP are losing mitochondrial gene. *Mol. Gene. Genet.* **166**, 1003–1016
20. Giraud, M-F. and Velours, J. (1994) ATP synthase of yeast mitochondria. Isolation of the F_1 δ-subunit, sequence and disruption of the structural gene. *Eur. J. Biochem.* **222**, 851–859
21. Wodicka, L., Dong, H., Mittman, M., Ho, M-H., and Lockhart, D.J. (1997) Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nature Biotechnol.* **15**, 1359–1366