Biochimica et Biophysica Acta, 376 (1975) 505–518

BBA 46886

RESPIRATION-DEPENDENT UNCOUPLER-STIMULATED ATPase ACTIVITY IN CASTOR BEAN ENDOSPERM MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

YASUAKI TAKEUCHI

Laboratory of Biology, Jichi Medical School, Kawachi-gun, Yakushiji, Tochigi Prefecture (Japan)

(Received August 16th, 1974)

SUMMARY

1. The uncoupler-stimulated ATPase activity of castor bean endosperm mitochondria and submitochondrial particles has been studied. The rate of ATP hydrolysis catalyzed by intact mitochondria was slow and little enhanced by addition of uncouplers at the concentration required for uncoupling the oxidative phosphorylation. ATPase activity was stimulated at higher concentrations of uncouplers.

2. 1-Anilinonaphthalene 8-sulfonate fluorescence was decreased when the mitochondria were oxidizing succinate. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and antimycin reversed the succinate-induced fluorescence diminution. ATP did not induce the fluorescence response.

3. The addition of succinate, NADH or ascorbate/*N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine as electron donor induced high ATPase activity in the presence of low concentrations of uncouplers. Stimulating effect of uncouplers was completely abolished by further addition of antimycin.

4. Submitochondrial particles were prepared by sonication. The particles catalyzed a rapid hydrolysis of ATP and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone at 10^{-8} M did not stimulate the ATPase activity. Addition of succinate induced uncoupler-stimulated ATPase activity. The effect of succinate was completely abolished by further addition of antimycin.

5. The treatment of submitochondrial particles by trypsin or high pH also induced uncoupler-stimulated ATPase activity.

6. The above results were interpreted to indicate that ATPase inhibitor regulated the back-flow reaction of mitochondrial oxidative phosphorylation.

Abbreviations: ANS, 1-anilinonaphthalene 8-sulfonate; Dnp, 2,4-dinitrophenol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; PCP, 2,3,4,5,6-pentachlorophenol; TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine.

INTRODUCTION

The molecular mechanism of electron transport and oxidative phosphorylation in plant mitochondria seems to be essentially the same as in animal mitochondria. The enzymatic characteristics of solubilized ATPase from plant mitochondria [1, 2] parallel those of Factor F_1 from animal sources. However, the behavior of the ATPase in intact plant mitochondria toward uncouplers differs considerably according to the plant species used. Mitochondrial ATPase activity of pea hypocotyls [3], shoots [4] and roots [5] of corn, hypocotyls of mung beans [6] and broad beans [7] are activated by low concentrations of Dnp in accordance with the results obtained with animal mitochondria. On the other hand, the same concentrations of Dnp do not stimulate the ATPase, but do accelerate the State 4 respiration of intact mitochondria in the case of cauliflower [8, 9], cabbage [10], castor bean endosperm [11] and cucumber leaves [12]. These discrepancies necessitate more extensive studies on the mechanism of, as well as on the effect of uncouplers upon, the oxidative phosphorylation in plant mitochondria.

The present author has observed [11] that the ATPase activity of castor bean endosperm mitochondria was stimulated by Dnp provided oxidation of substrates such as succinate or NADH was proceeding. The stimulation was completely suppressed by addition of cyanide or antimycin as well as by anaerobic conditions. Oligomycin also inhibited the respiration-dependent ATPase activity, suggesting this activity was an expression of the backward reaction of oxidative phosphorylation.

More recently similar results were reported from other laboratories with sweet potato [13] and cauliflower mitochondria [14]. In a study on corn shoot mitochondria, Dnp stimulated the ATPase even in the absence of added respiratory substrate. However, further stimulation was observed when oxidation of the exogenous substrate took place. Similar observations have also been reported on animal mitochondria where the ATPase activity of K^+-depleted rat liver mitochondria was enhanced by addition of exogenous respiratory substrate in the presence of Dnp [15, 16].

In connection with these observations, it is interesting that the mitochondrial ATPase inhibitor was reported to suppress the ATP-driven energy-transfer reactions without affecting oxidative phosphorylation [17, 18]. There is also another observation with submitochondrial particles from beef heart [19] that the dissociation of the inhibitor from the mitochondrial ATPase complex was promoted by oxidation of respiratory substrate, thus resulting in a large increase in the uncoupler-stimulated ATPase activity. The proposed interpretation was that the inhibitor blocked the wasteful hydrolysis of ATP and furnished a controlling mechanism for the ATP-driven backflow reactions. Such a regulation appears to operate in spinach chloroplasts [20].

In the present paper, some properties of the respiration-dependent uncoupler-induced ATPase activity of the mitochondria and the submitochondrial particles from *Ricinus communis* endosperms will be reported and possible implications of the result will be discussed.

MATERIALS AND METHODS

Preparation of mitochondria

Mitochondria were isolated from endosperms of 4-days dark-grown castor bean (*R. communis*) seedlings according to the method of Yoshida and Sato [21]. Washed

tissues were blotted dry and ground in ice-chilled medium containing 0.5 M sucrose, 0.1 M phosphate buffer (pH 8.3), 1 mM EDTA and 0.1 % bovine serum albumin. Mitochondrial fractions were obtained and washed with 0.25 M sucrose, 20 mM Tris · HCl buffer (pH 7.4) and 0.1 % bovine serum albumin. Precipitate was suspended in 1 M sucrose and placed on 45 % sucrose in a centrifuge tube. After centrifugation at $15000 \times g$ for 20 min, mitochondria were collected in the upper layer and diluted with 10 vol. of washing medium. Mitochondria were washed twice.

Preparation of submitochondrial particles

Mitochondria were suspended in 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$, 1 mM ATP and 1 mM sodium succinate. The protein concentration was adjusted to 20 mg/ml and the suspension was kept at -20 °C overnight. The frozen suspension was thawed and centrifuged for 10 min at $12000 \times g$. The supernatant and the loose fluffy layer were discarded. The packed mitochondria were resuspended in 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1mM ATP and 1 mM sodium succinate to a protein concentration of 30 mg/ml. The suspension was sonicated for 30 s with a Sonifier (Branson Ultrasonic Corp.) at 40 W power output. Sonic disruption was repeated twice with a 2-min interval. pH was adjusted to 7.4 with 1 M KOH, and the suspension was centrifuged for 20 min at $20000 \times g$. The turbid supernatant was centrifuged for 1 h at $100000 \times g$. The reddish-brown pellet was homogenized in 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4) and 2 mM $MgCl_2$ with a Teflon homogenizer and diluted to a protein concentration of 20 mg/ml and washed by centrifugation. The final pellet was suspended in the same medium for use.

Trypsin digestion of submitochondrial particles

The digesting medium contained 0.25 M sucrose, 50 mM Tris · SO_4^{2-} (pH 8.0), 1 mM EDTA and 25 μg/ml of trypsin. The incubation was started by addition of 0.5 ml of submitochondrial particles (100 mg protein/ml) to a final volume of 2.0 ml. After 15 min incubation at 30 °C, the mixture was cooled immediately in an ice bath and centrifuged at $200000 \times g$ for 30 min. The pellet was suspended in 5.0 ml of 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 1 mM EDTA and 3 mM $MgCl_2$ and washed by centrifugation. The precipitated particles were suspended in the same medium to a protein concentration of 20 mg/ml.

Alkali-treated submitochondrial particles

To prepare alkali-treated submitochondrial particles, sonic disruption of the mitochondria was carried out in a medium of which pH was kept at 9.4 with 1 M NH_4OH. The other process of preparation was the same as submitochondrial particles preparation.

Oxidative phosphorylation

O_2 uptake was measured at 30 °C polarographically with a Clark-type oxygen electrode in 3 ml of air-saturated reaction medium. The standard reaction medium was composed of 0.25 M sucrose, 8 mM phosphate (pH 7.4), 10 mM Tris · HCl (pH 7.4), 5 mM $MgCl_2$ and 0.1 % bovine serum albumin. Phosphorylation was measured by determining the inorganic phosphate concentration before and after incubation. The reaction medium contained 20 mM glucose, 2 mM ADP, 0.2 mg/ml of hexokinase

and the components described above. Phosphate concentration was determined by the method of Lindberg and Ernster [22].

Measurement of ATPase activity

ATPase activity was measured by determining the release of inorganic phosphate from added ATP. Basic reaction medium consisted of 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP, 30 μg/ml pyruvate kinase (Boehringer, Mannheim) and 5 mM phosphoenolpyruvate, unless otherwise noted. The reaction was started by addition of 20 μl of the suspension. The final volume was 1.0 ml. Reaction was carried out at 30 °C in test tubes with shaking. After 10 min the reaction was stopped by addition of 1.0 ml of 10 % perchloric acid. The precipitate was removed by centrifugation. Inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow [23]. Protein concentration was determined by the procedure of Lowry et al. [24].

ANS fluorescence

Mitochondria were previously incubated for 3 min in a medium containing 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$ and 20 μM ANS. ANS fluorescence was excited at 375 nm and measured at 485 nm.

RESULTS

Oxidation and phosphorylation

Fig. 1 shows a typical pattern of polarographic record of O_2 uptake by castor bean endosperm mitochondria with succinate as substrate. Transition from State 3 to State 4 was observed by addition of a limited amount of ADP. This transition was repeated by successive addition of ADP. The respiratory control ratio of these mitochondria was 2.2–2.5 and the calculated ADP/O ratio was 1.6–1.8 for succinate. These data indicated that the castor bean endosperm mitochondria used in these experiments were functionally intact. Dnp or FCCP caused uncoupling which appeared to be the stimulation of State 4 respiration. Respiratory control was also observed when NADH was used as substrate.

Effects of uncouplers on the ATPase activity

The ATPase activity of the freshly prepared castor bean endosperm mitochondria was about 0.03 μmol P_i/mg of protein per min. When respiratory substrate and O_2 were present, the ATPase activity was completely abolished [11].

The ATPase activity was not affected by addition of Dnp at the concentration which was sufficient to cause uncoupling of the State 4 respiration. Fig. 2 shows the effect of Dnp and PCP on the ATPase activity at various concentrations. The State 4 respiration was fully activated by 10^{-4} M Dnp or 10^{-5} M PCP, but more than 10-fold concentration was required to stimulate the ATPase activity. Similar result was obtained also in sweet potato mitochondria [13].

Electron transport-dependent uncoupler-induced ATPase activity

Uncouplers stimulated the ATPase activity at lower concentrations when succinate and O_2 were provided (Fig. 3). By the addition of succinate, a peak of ATPase

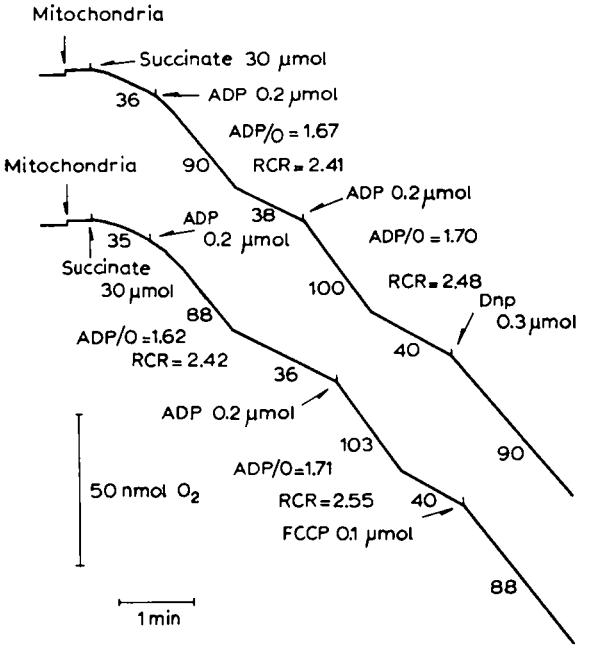

Fig. 1. Polarographic traces showing oxygen utilization by castor bean endosperm mitochondria with succinate as substrate. The reaction medium (3.0 ml) contained 0.25 M sucrose, 10 mM phosphate (pH 7.4), Tris · HCl (pH 7.4), 5 mM $MgCl_2$ and 0.1 % bovine serum albumin. The temperature was 30 °C. The values under each slope indicate the rate of oxygen utilization (nmol O_2/min per mg protein). Mitochondria (0.72 mg of protein), succinate, ADP, Dnp and FCCP were added at the points indicated. RCR indicates respiratory control ratio.

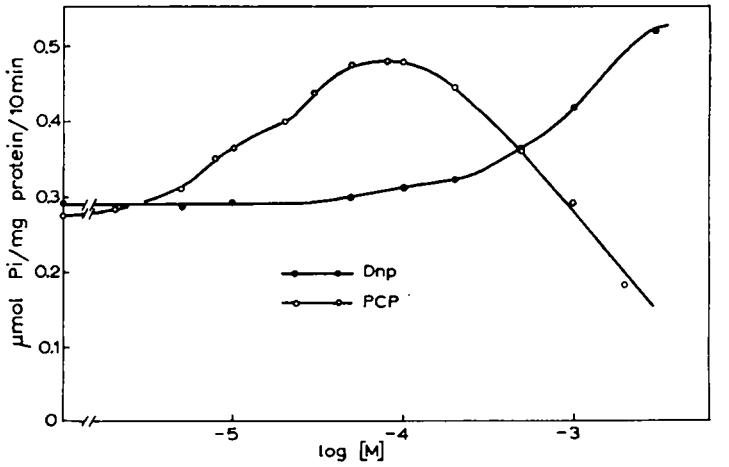

Fig. 2. Effect of Dnp and PCP on castor bean mitochondrial ATPase activity. Reaction medium contained 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$ and 2 mM ATP in final volume of 1.0 ml. The abscissa shows Dnp (●) or PCP (○) in final concentrations. The reaction was started by addition of 0.05 ml of mitochondrial suspension. Mitochondrial protein was 0.91 mg and 0.65 mg for Dnp and PCP experiment, respectively.

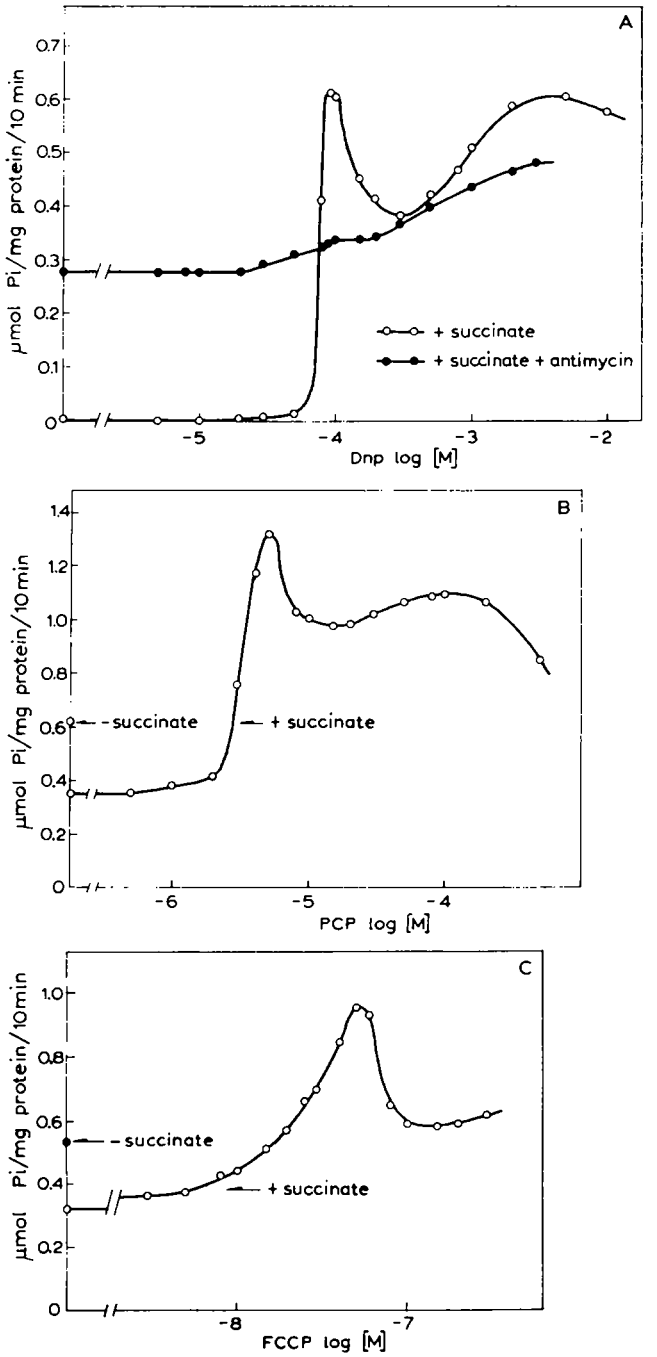

Fig. 3. Effect of uncouplers on ATPase activity in the presence of succinate. Basic reaction medium contained 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$ and 2 mM ATP in 1.0 ml. Each test tube was shaken at 140 Hz. Temperature was 30 °C. Other additions were as follows: (A) Succinate (10 mM), antimycin (0.5 μM), Dnp as indicated, 0.05 ml of mitochondrial suspension (0.50 mg

activity appeared at about 10^{-4} M Dnp in addition to the peak at higher concentrations of Dnp. The stimulation of ATPase activity at higher concentrations of Dnp (Fig. 3A) was probably due to the activating effect of Dnp for the enzyme itself. Our unpublished result indicated that Dnp at about 10^{-3} M stimulated the solubilized ATPase from castor bean mitochondria. The peak of the stimulation curve at lower concentrations of uncouplers was more sharp than that at higher concentrations (Fig. 3A).

This peak of stimulation was completely abolished by further addition of antimycin. By addition of cyanide or by removal of oxygen from the medium, the same result was obtained [11]. This stimulation of ATPase activity was also brought about by addition of other uncouplers. The peaks of ATPase activity at lower concentrations were obtained at about $5 \cdot 10^{-6}$ M and $5 \cdot 10^{-8}$ M, with PCP and FCCP as uncouplers, respectively (Figs 3B and 3C). These concentrations coincided with the concentrations which caused uncoupling of oxidative phosphorylation in animal mitochondria.

Addition of NADH or ascorbate/TMPD as the electron donor gave the same result on the ATPase activity as in the case of succinate addition (Fig. 4).

This uncoupler-induced ATPase activity seemed not to be primed by respiration but required concurrent respiration. In the presence of succinate, Dnp-induced ATPase activity vanished when oxygen was removed from the reaction medium (Fig. 5). Dnp did not stimulate the ATPase activity when it and KCN were added after the mitochondria were preincubated with succinate (data not shown). These results seem to be different from the results with cauliflower mitochondria [14, 25]. Whether the variance is due to the difference in the source material is not clear at present.

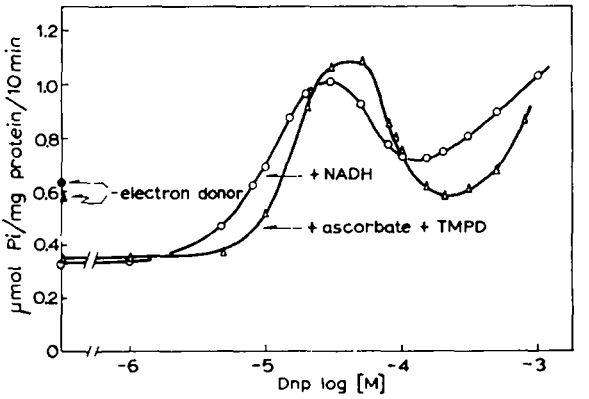

Fig. 4. Effect of Dnp on the mitochondrial ATPase activity in the presence of NADH or ascorbate/TMPD. 2 mM NADH or 20 mM ascorbate + 1 mM TMPD was added as electron donor. Other conditions were as in Figs 3B and 3C. Mitochondria for NADH experiment (●, ○) were 0.40 mg of protein, for ascorbate/TMPD experiment (▲, △) were 0.35 mg.

of protein). (B) Succinate (10 mM), pyruvate kinase (30 μg/ml), phosphoenolpyruvate (5 mM), PCP as indicated, 0.05 ml of mitochondrial suspension (0.65 mg of protein). (C) Succinate (10 mM), pyruvate kinase (30 μg/ml), phosphoenolpyruvate (5 mM), FCCP as indicated, 0.05 ml of mitochondrial suspension (0.70 mg of protein).

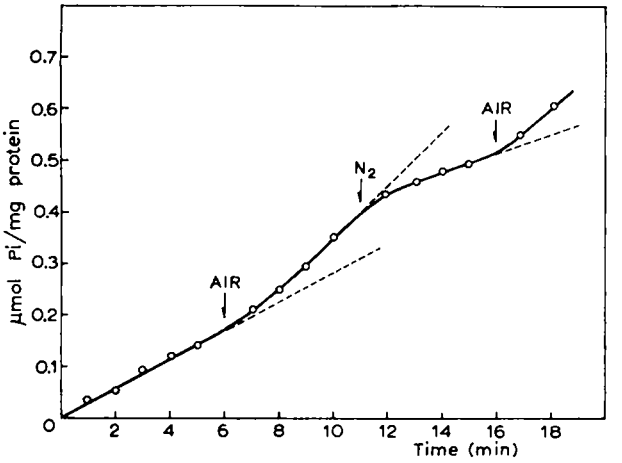

Fig. 5. Effect of aeration and deoxygenization on ATPase activity. The reaction medium contained 0.15 M sucrose, 50 mM KCl, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP, 20 mM succinate, 0.1 mM Dnp and mitochondrial suspension in a final volume of 60 ml in a 100-ml Erlenmeyer flask. The mixture was bubbled with N_2. At intervals, 1-ml aliquots were taken for the determination of P_i released. N_2 was replaced with air at the times indicated.

Response of the mitochondria to ANS fluorescence

Fig. 6 shows response of the mitochondria to ANS fluorescence in the reaction mixture. Addition of mitochondria to the medium containing ANS caused a great increase in the fluorescence of ANS. The addition of succinate induced a significant decrease in the intensity of the fluorescence. This decrease of fluorescence was completely reversed by further addition of antimycin (Fig. 6A). FCCP also reversed the succinate-induced decrease in fluorescence (Fig. 6B). Under the same conditions, addition of ATP did not cause a significant change in the fluorescence (Fig. 6C). The addition of ATP brought about a slight decrease in the fluorescence, but this decrease was not reversed by further addition of FCCP.

It had been shown in animal mitochondria and submitochondrial particles [26–28] or in bacterial membrane particles [26] that the response to ANS fluorescence may correspond to conversion between the energized and de-energized state of the membrane. In the present experiments the ANS fluorescence decreased on addition of succinate. This suggested that the membrane of the mitochondria may be energized by electron transfer as was reported in animal mitochondria. ATP did not induce any energization of the castor bean mitochondrial membranes. A similar result was observed in sonicated particles of *Micrococcus lysodeikticus* and of pea chloroplasts [29].

ATPase activity of submitochondrial particles

The insensitivity of the ATPase to uncouplers in our mitochondria may be due to irreversibility of its oxidative phosphorylation. If this irreversibility was caused by the fact that ATP, being unable to penetrate through the membrane, was not accessible to the enzyme, stimulation of the ATPase activity by uncouplers at low concentrations would be expected if we used submitochondrial particles instead of intact mitochondria. Hence submitochondrial particles were prepared by sonic oscillation (see

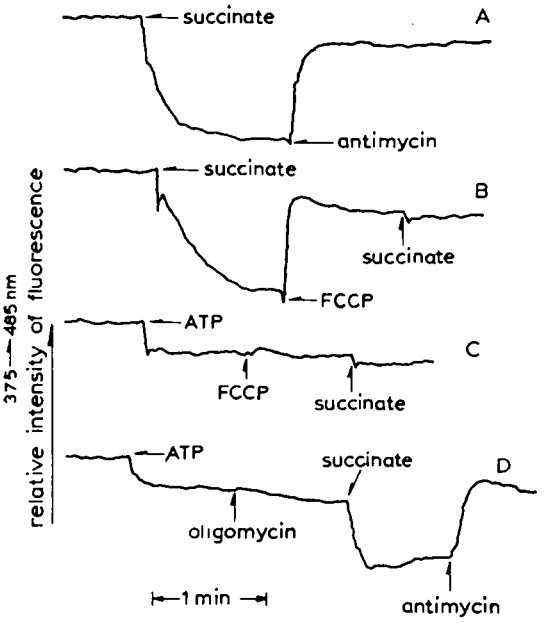

Fig. 6. ANS fluorescence responses in castor bean mitochondria. Mitochondria (2.6 mg of protein) were preincubated for 3 min in a medium (2.5 ml) containing 0.25 M sucrose, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$ and 30 μM ANS. Fluorescence was measured at room temperature. Additions were as follows: (A) Succinate (0.3 mM), antimycin (0.4 μM). (B) Succinate (0.3 mM), FCCP (0.1 μM). (C) ATP (0.5 mM), FCCP (0.1 μM), succinate (0.3 mM). (D) ATP (0.5 mM), oligomycin (2 μg/ml), succinate (0.3 mM), antimycin (0.4 μM).

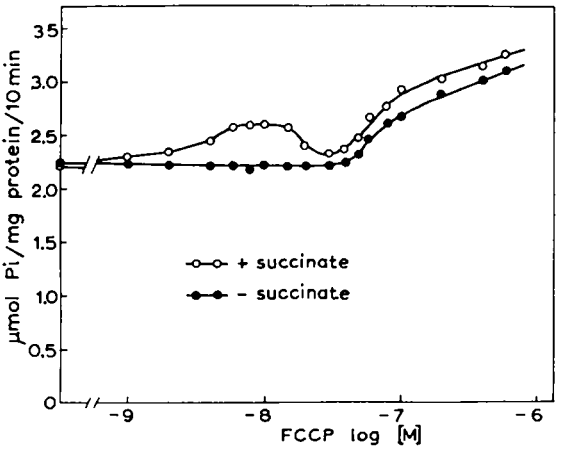

Fig. 7. Effect of FCCP on castor bean submitochondrial ATPase activity in the presence of succinate. Reaction medium contained 0.15 M sucrose, 50 mM KCl, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$, 2 mM ATP, 30 μg/ml pyruvate kinase, 5 mM phosphoenolpyruvate and FCCP at indicated concentrations in a final volume of 1.0 ml. 5 mM succinate was added as indicated. The reaction was started by addition of 0.02 ml of submitochondrial suspension (0.16 mg of protein).

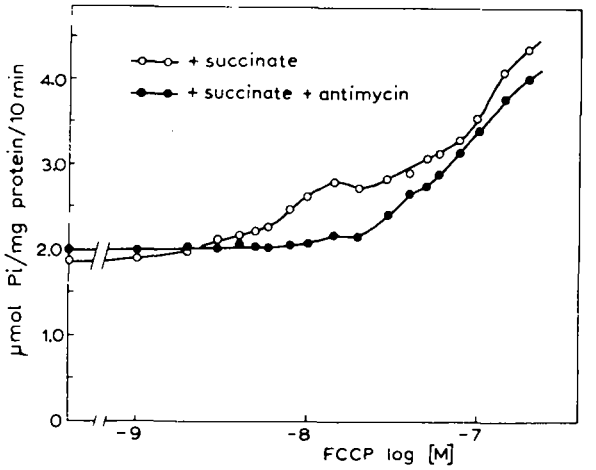

Fig. 8. Effect of antimycin on succinate-induced FCCP-stimulated submitochondrial ATPase activity. Conditions were as in Fig. 6. 0.5 μM antimycin was added as indicated. Submitochondrial protein was 0.22 mg.

Methods). Electron microscopy of negatively stained preparations revealed knob-like particles on the outer surface of the membrane (not shown). P/O ratio of the submitochondrial particles was 0.6 when succinate was the substrate.

Fig. 7 shows the effect of FCCP on the ATPase activity in the submitochondrial particles. In the absence of uncoupler, rate of ATP hydrolysis was about 4 times higher than that in intact mitochondria. When FCCP (10^{-9}–10^{-7} M) was added without succinate the ATPase activity was only slightly activated and no peak was observed on the activation vs FCCP concentration curve. When succinate was added, a peak was observed as in intact mitochondria The optimum concentration of FCCP for the stimulation was about 10^{-8} M. The rate of the stimulation was, however, remarkably lower than that in intact mitochondria. The lower stimulation seemed due to the fact that the coupling of oxidative phosphorylation in the submitochondrial particles was loosened by sonic oscillation. FCCP-induced ATPase activity stimulation in the presence of succinate was completely abolished by further addition of antimycin (Fig. 8). These results showed that the FCCP-induced ATPase activity was electron transport-dependent in the submitochondrial particles as well as in intact mitochondria.

Trypsin digestion and alkali treatment of submitochondrial particles

A specific ATPase inhibitor has been purified from beef heart mitochondria [17, 30]. It is highly sensitive to trypsin. An inhibitor with a mol. wt 13 000 was also purified from chloroplasts [20]. It was also very sensitive to trypsin.

Hence, trypsin-treated submitochondrial particles were prepared from castor bean mitochondria and the effect of FCCP on the ATPase activity was examined (Fig. 9). FCCP at about 10^{-8} M stimulated the ATPase activity in the absence of succinate as in the case of untreated submitochondrial particles in the presence of succinate.

Possible removal of the inhibitor protein during incubation at high pH [17, 30] was investigated. The submitochondrial particles from castor beans were treated at

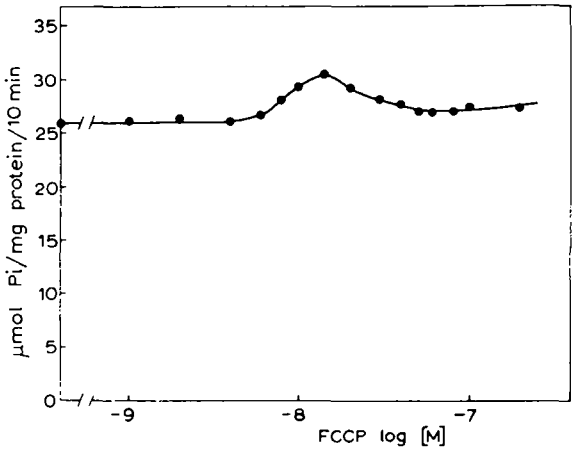

Fig. 9. Effect of FCCP on trypsin-treated submitochondrial ATPase activity. Conditions were as in Fig. 6. Submitochondrial protein was 0.12 mg.

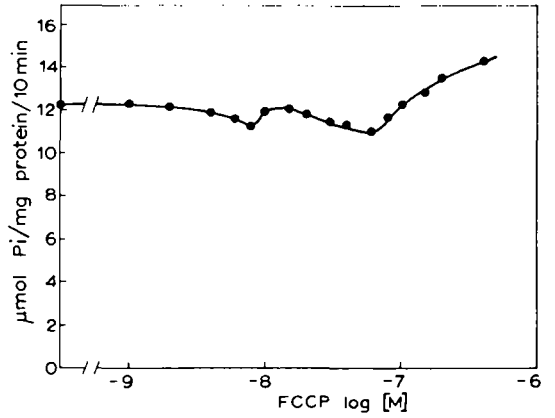

Fig. 10. Effect of FCCP on alkali-treated submitochondrial ATPase activity. Conditions were as in Fig. 6. Submitochondrial protein was 0.20 mg.

pH 9.4. Fig. 10 shows the effect of FCCP on the ATPase activity of the treated particles in the absence of succinate. The ATPase activity somewhat decreased toward higher concentrations of FCCP, but the peak of the activity was observed at about 10^{-8} M FCCP, coinciding with its optimum concentration for trypsin-treated particles.

DISCUSSION

The effect of electron transport on the uncoupler-induced ATPase activity has been reported from several laboratories. In rat liver mitochondria, Hemker [31] reported the inhibition of Dnp-induced ATPase activity by amytal or antimycin at concentrations required for inhibition of respiration. More recently, Skulachev and coworkers [32] observed in sonicated submitochondrial particles that rotenone, antimycin and cyanide inhibited the Dnp-induced ATPase activity. They thought that the

result was difficult to be directly related either to the chemical theory or the chemiosmotic theory of oxidative phosphorylation. Some other molecular mechanisms may be involved.

Takeuchi et al. [11] reported that Dnp stimulated the ATPase activity of castor bean endosperm mitochondria only when substrate oxidation was proceeding. Carmeli and Biale [13] also showed the respiration-stimulated Dnp-induced ATPase activity in sweet potato mitochondria. More recently, Gomez-Puyou et al. [15, 16] observed respiration-dependent Dnp-stimulated ATP hydrolysis and [^{32}P]ATP exchange reaction by K^+-depleted rat liver mitochondria in a medium containing valinomycin. They suggested that respiration-dependent K^+ influx was coupled to ATP influx via an adenine nucleotide carrier. In cauliflower mitochondria, Jung and Hanson [14, 25] reported a similar respiration-dependent Dnp-induced ATPase activity and discussed the mechanism, comparing it with the ATPase activity of corn mitochondria which was stimulated by Dnp even in the absence of any respiratory substrate. They suggested that the nucleotide translocator of cauliflower mitochondria or other plant mitochondria of the same type was latent in the resting state and was activated by mobilization of the respiratory chain. In this mechanism, ATP added from outside was translocated inside and hydrolyzed when the translocator was in the active state.

In castor bean mitochondria, contrary to cauliflower mitochondria, ATPase activity was sensitive to oligomycin even in the absence of respiratory substrate [11]. Consequently, it was unlikely that exogenous ATP did not enter into the mitochondria. Nevertheless, ATPase activity was insensitive to Dnp at a concentration which was sufficient to cause uncoupling. In cabbage mitochondria, Lotlikar and Remmert [10] reported that uncouplers did not stimulate the ATPase activity and Dnp completely inhibited [^{32}P]ATP exchange reaction. In our laboratory, respiration-dependent Dnp-induced ATPase activity was also found in these mitochondria (unpublished). Uncoupler-induced ATPase and [^{32}P] ATP exchange reactions are probably carried out on the internal surface of mitochondria. These results mentioned above do not support the view that respiration-induced nucleotide translocation is the main cause of the induced ATPase activity.

In submitochondrial particles obtained by sonication, ATP has free contact with ATPase without being impeded by intervening membranes. The ATPase activity of castor bean endosperm submitochondrial particles was about four times higher than that of intact mitochondria. ATPase activity of the particles was not stimulated by FCCP at a concentration required for uncoupling. However, respiration-dependent uncoupler-induced ATPase activity was observed when succinate was added as a respiratory substrate. The profile of the ATPase activation curve was the same as in intact mitochondria. This respiration-dependent uncoupler-induced ATPase activity is not the result of mobilization of ATP translocator, since transmembrane transport of ATP does not explain the results for submitochondrial particles.

Asami et al. [18] reported that ATPase inhibitor efficiently blocked ATP-driven energy-transfer reactions in EDTA-particles from beef heart mitochondria and that the respiratory flow abolished this blocking effect. Van de Stadt et al. [19] observed in beef heart submitochondrial particles that the inhibitor dissociated from the ATPase when oxidation of a substrate was going on, and that the dissociation resulted in a marked activation of uncoupler-induced ATPase activity.

This regulating effect of ATPase inhibitor seems to operate also in plant mitochon-

dria. Trypsin-treated castor bean submitochondrial particles, whose inhibitor was deprived or damaged, showed FCCP-induced ATPase activity without any addition of respiratory substrate. The induced ATPase had a peak at about the same uncoupler concentration as required for uncoupling of oxidative phosphorylation. Moreover, the profile of the activation curve was the same as that of trypsin-untreated submitochondrial particles with their respiratory-chains working. When the inhibitor was removed by alkali treatment, the submitochondrial particles gave a similar result. In membrane vesicles from *Mycobacterium phlei*, it was reported [33] that ATP-enhanced ANS fluorescence was observed only when vesicles were trypsinized.

These results seem to support the view that the ATP-driven energy-linked process is controlled by the ATPase inhibitor. The regulation is likely to take the form of association and dissociation of the ATPase inhibitor to and from the enzyme. In castor bean mitochondria, the control of "wasteful" hydrolysis of ATP may be more marked than in other, especially animal, mitochondria.

ACKNOWLEDGEMENTS

The author would like to thank Dr K. Nagano, Dr S. Sato and Dr K.Yoshida for their helpful discussions and encouragement throughout the course of this work.

REFERENCES

1 Peterson, T. G. and Heisler, C. R. (1963) Biochem. Biophys. Res. Commun. 12, 492–497
2 Yoshida, K. and Takeuchi, Y. (1970) Plant Cell Physiol. 11, 403–409
3 Forti, G. (1957) Physiol. Plant. 10, 898–909
4 Stoner, C. D., Hodges, T. K. and Hanson, J. B. (1964) Nature 203, 258–261
5 Bottrill, D. E. and Hanson, J. B. (1969) Aust. J. Biol. Sci. 22, 847–855
6 Blackmon, W. J. and Moreland, D. E. (1971) Plant Physiol. 47, 532–536
7 Matlib, M. A., Kirkwood, R. C. and Smith, J. E. (1972) J. Exp. Bot. 23, 886–898
8 Laties, G. G. (1953) Physiol. Plant. 6, 199–214
9 Reid, H. B., Gentile, A. C. and Klein, R. M. (1964) Plant Physiol. 39, 1020–1023
10 Lotlikar, P. D. and Remmert, L. F. (1968) Plant Physiol. 43, 327–332
11 Takeuchi, Y., Yoshida, K. and Sato, S. (1969) Plant Cell Physiol. 10, 733–741
12 Matsumoto, H., Wakiuchi, N. and Takahashi, E. (1971) Physiol. Plant. 25, 353–357
13 Carmeli, C. and Biale, J. B. (1970) Plant Cell Physiol. 11, 65–82
14 Jung, D. W. and Hanson, J. B. (1973) Arch. Biochem. Biophys. 158, 139–148
15 Gómez-Puyou, A., Sandoval, F., Tuena, M., Pena, A. and Chávez, E. (1971) Biochem. Biophys. Res. Commun. 45, 104–111
16 Gómez-Puyou, A., Sandoval, F., Chávez, E., Frietes, D. and Tuena, M. (1972) Arch. Biochem. Biophys. 153, 215–225
17 Pullman, M. E. and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762–3769
18 Asami, K., Juntti, K. and Ernster, L. (1970) Biochim. Biophys. Acta 205, 307–311
19 Van de Stadt, R. J., De Boer, B. L. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 338–349
20 Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 7657–7662
21 Yoshida, K. and Sato, S. (1968) J. Fac. Sci. Univ. Tokyo, Sect. III 10, 49–62
22 Lindberg, O. and Ernster, L. (1956) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 3, pp. 1–22, Interscience, New York
23 Leloir, L. F. and Cardini, C. E. (1957) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. III, pp. 843–844, Academic Press, New York
24 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

25 Jung, D. W. and Hanson, J. B. (1973) Biochim. Biophys. Acta 325, 189–192
26 Chance, B. (1970) Proc. Natl. Acad. Sci. U.S. 67, 560–571
27 Jasaitis, A. A., Kuliene, V. V. and Skulachev, V. P. (1971) Biochim. Biophys. Acta 234, 177–181
28 Azzi, A. A., Gherardini, P. and Santato, M. (1971) J. Biol. Chem. 246, 2035–2042
29 Grinius, L. L., Ilina, M. D., Mileykovskaya, E. I., Skulachev, V. P. and Tikhonova, G. V. (1972) Biochim. Biophys. Acta 283, 442–445
30 Horstman, L. L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344
31 Hemker, H. C. (1963) Biochim. Biophys. Acta 73, 311–323
32 Skulachev, V. P. (1971) in Current Topics in Bioenergetics (Sanadi, D. R., ed.), Vol. 4, pp. 127–190, Academic Press, New York
33 Aithal, H. N., Kalra, V. K. and Brodie, A. F. (1974) Biochemistry 13, 171-178