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THE ATPase ACTIVITY OF JERUSALEM-ARTICHOKE MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. Some properties of the ATPase activity of Jerusalem-artichoke mitochondria and submitochondrial particles are described.

2. Intact mitochondria catalyse a slow rate of hydrolysis of ATP, which is only slightly enhanced by the addition of uncouplers, but which is sensitive to oligomycin.

3. Succinate stimulates the rate of hydrolysis of ATP by intact mitochondria but this stimulation is insensitive to oligomycin, and may not therefore involve the classical mitochondrial ATPase (F_1).

4. Broken mitochondria or sonically-prepared submitochondrial particles catalyse a rapid hydrolysis of ATP in the absence of uncoupler which is strongly inhibited by oligomycin.

5. The ATPase of submitochondrial particles is specific for adenine nucleotides.

6. These findings indicate that breakage of the mitochondrial membrane may increase the accessibility of the F_1-ATPase to ATP without losing the substrate specificity.

INTRODUCTION

The phenomenon of uncoupling of oxidative phosphorylation from electron transport in animal mitochondria in the presence of certain weak acids or ionophores has been extensively documented. Thus the addition of a weak acid, such as 2,4-dinitrophenol, to rat-liver mitochondria has been shown (a) to inhibit the synthesis of ATP[1], (b) to stimulate the rate of ADP-limited oxygen uptake[1], and (c) to induce a rapid rate of hydrolysis of exogenously-added ATP[2] that is sensitive to oligomycin[3]. Similar effects of uncouplers have been described for mitochondria prepared from

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; S_{13}, 5-chloro-3-*tert*-butyl-2-chloro-4-nitrosalicylanilide.

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a wide variety of animal tissues and possible mechanisms have been proposed to explain their modes of action (see review in ref. 4).

Uncoupling agents have also been demonstrated to inhibit the synthesis of ATP and to stimulate the rate of ADP-limited oxygen uptake in plant mitochondria[5]. However, the effects of uncouplers on the rates of hydrolysis of added ATP by plant mitochondria (*i.e.* the ATPase activities) appear much more variable than those found in animal mitochondria. Thus, uncouplers have been shown to either stimulate[6–8], inhibit[9] or have no appreciable effect[10–12] on the rate of hydrolysis of added ATP by plant mitochondria. Two recent reports have suggested that active electron transport may play an important role in inducing the ATPase activity of mitochondria isolated from sweet potato[13] or castor-bean endosperm[14]. In addition, the sensitivity of the ATPase to oligomycin appears to vary between mitochondria isolated from different plant tissues[8,13,14].

The present paper describes the ATPase activity of Jerusalem-artichoke mitochondria with particular reference to membrane integrity.

METHODS

Jerusalem-artichoke tubers (*Helianthus tuberosus*) were obtained from local markets and stored in polythene bags at 0–5 °C until required. Mitochondria were isolated initially following the rapid technique of Palmer[15], which effectively prevents any damage caused to the preparation by the acidic cell contents released during homogenization. After centrifugation at $40000 \times g$ for 3 min, the pellet was suspended in a small volume of grinding medium and recentrifuged at $1000 \times g$ for 5 min to remove cell debris. Mitochondria were subsequently precipitated by centrifugation of the supernatant at $8000 \times g$ for 15 min, and suspended at a protein concentration of 25–30 mg/ml. Submitochondrial particles were prepared following sonication[16].

ATPase activity was measured as the release of P_i from ATP by either (a) the colorimetric method described by Lindberg and Ernster[17], or (b) release of $^{32}P_i$ from [γ-^{32}P]ATP. O_2 uptake was determined polarographically using a Clark-type electrode with a cell of 1.5 ml capacity. Protein was estimated using the biuret procedure[18].

[γ-^{32}P]ATP was obtained from Radiochemicals, Amersham; carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were kindly supplied by Dr P. G. Heytler, and other chemicals were purchased from Sigma Chemical Co. and British Drug Houses, Ltd.

RESULTS

Table I summarizes the effects of various uncouplers on the respiration and ATPase activity of Jerusalem-artichoke mitochondria. In all cases the addition of uncoupler increases the rate of oxidation of NADH in the absence of ADP or in the presence of ADP and oligomycin. Also, as determined in other experiments the synthesis of ATP, measured from the esterification of $^{32}P_i$ into glucose 6-phosphate in the presence of ADP, glucose and hexokinase is prevented. (For example, the P/O ratio of 1.8 associated with the oxidation of succinate was lowered to 0.04

TABLE I

THE EFFECT OF UNCOUPLERS ON THE RESPIRATION AND ATPase ACTIVITY OF JERUSALEM-ARTICHOKE MITOCHONDRIA

ATPase activity was measured in a medium containing, in 1 ml, 2 mM ATP, 1 mM $MgCl_2$, 0.28 M sucrose and 0.035 M Tris–HCl buffer, at pH 7.2 and 25 °C. Mitochondrial protein was 1 mg. After incubation for 10 min, the reaction was stopped by the addition of 0.2 ml 30% trichloroacetic acid. Following centrifugation at $12000 \times g$ for 2 min, P_i was estimated colorimetrically. For each experiment a control was performed, where trichloroacetic acid was added prior to the mitochondria. The P_i content of the control was subtracted from the experimental values in all cases. O_2 uptake was measured polarographically using a Clark electrode and a cell of 1.5-ml capacity. The reaction medium contained 0.28 M sucrose, 0.035 M Tris–HCl buffer, 1 mM $MgCl_2$ and 3 mM NADH at pH 7.2 and 25 °C. Mitochondrial protein was 2 mg.

Addition	*Expt 1 ATPase activity (nmoles P_i/mg protein per min)*	*Expt 2 O_2 uptake (natoms O/mg protein per min)*
None	3.6	93
Dinitrophenol ($2 \cdot 10^{-5}$M)	4.8	201
S_{13} ($2 \cdot 10^{-7}$M)	4.3	341
CCCP ($2 \cdot 10^{-6}$M)	3.8	
FCCP ($2 \cdot 10^{-6}$M)	4.3	453
Gramicidin (50 ng/mg protein)*	4.0	386
FCCP ($2 \cdot 10^{-6}$M) + oligomycin (10 μg)	0.0	380**

* 1.5 mM KCl present.
** 0.5 mM ADP present.

on addition of uncoupler.) However, all the uncouplers tested fail substantially to enhance the rate of hydrolysis of ATP under conditions similar to those in which O_2 uptake is stimulated. Changing the amount of dinitrophenol in the medium over a range of concentrations from 10^{-7} to 10^{-4} M failed to induce a significant rate of ATP hydrolysis.

Uncouplers also have little effect on the ATPase activity in a medium in which KCl replaces sucrose, which has been shown to inhibit the ATPase activity of certain plant mitochondria[9,19]. The rate of ATP hydrolysis is furthermore not stimulated significantly by changing the tonicity of the assay medium or the ATP concentration. Inclusion in the assay of an ATP-regenerating system (similar to that described by Pullman *et al.*[20]) in order to prevent any possible inhibition by ADP does not enhance the rate of ATP hydrolysis. Hence in artichoke mitochondria, although respiration can be uncoupled from phosphorylation, it is not possible under these conditions to see any stimulation of ATPase activity.

Takeuchi *et al.*[14] reported that the ATPase activity of castor-bean endosperm mitochondria in the presence of uncoupler is stimulated by concomitant oxidation of succinate or NADH, indicating that active electron transport promotes the ATPase activity. The effect of respiratory substrates on the ATPase activity of Jerusalem-artichoke mitochondria is shown in Table II. Addition of succinate to the ATPase assay in the presence of uncoupler causes a stimulation of the rate of

TABLE II

THE INFLUENCE OF OXIDIZABLE SUBSTRATES ON THE ATPase ACTIVITY OF JERUSALEM-ARTICHOKE MITOCHONDRIA

P_i release from ATP was estimated colorimetrically. Mitochondrial protein was 2 mg/ml. 0.1 M succinate, 0.1 M malate, 5 mM NADH, antimycin (5 μg/mg protein), rotenone (2 μg/mg protein), 10^{-6} M CCCP, 10^{-6} M FCCP, and oligomycin (10 μg/mg protein) were added as shown. The anaerobic assay was performed in a "Superseal" bottle after flushing with O_2-free nitrogen for 10 min.

Additions	*nmoles P_i/mg protein per min*
None	2.0
CCCP	1.2
Succinate	0.0
Succinate + CCCP	10.8
Succinate + FCCP (anaerobic)	14.2
Succinate + FCCP + antimycin + rotenone	12.2
Succinate + CCCP + oligomycin	9.0
Malate + CCCP	3.8
NADH + FCCP	3.5

hydrolysis of ATP. Uncoupler is required in the assay to uncouple the oxidation of substrate from phosphorylation; in the absence of uncoupler succinate completely abolishes the hydrolysis of ATP. Malate or NADH when included in the assay do not substantially increase the rate of hydrolysis, while the stimulatory effect of succinate is slightly enhanced in the presence of rotenone and antimycin, or anaerobiosis. These results therefore indicate that, contrary to the results reported for castor-bean endosperm mitochondria[14], the stimulatory effect of succinate on the rate of ATP hydrolysis by artichoke mitochondria is not dependent on active electron transport and can occur in circumstances where succinate oxidation is completely prevented.

Palmer and Wedding[21] have shown Jerusalem-artichoke mitochondria to possess a highly active succinyl-CoA synthetase, demonstrated earlier by Kaufman and Alivisatos[22] to be specific for ATP and ADP, unlike the GTP–GDP specific animal enzyme. The possibility that the stimulatory effect of succinate on the rate of ATP hydrolysis may be related to a reversal of the substrate-level phosphorylation associated with succinyl-CoA synthetase is suggested from the data shown in Table III. Addition of coenzyme A or hydroxylamine, the latter having been suggested by Kaufman *et al.*[23] to react with succinyl-CoA in the presence of succinate to regenerate coenzyme A and form the hydroxamic acid, further increases the rate of ATP hydrolysis. (Although hydroxylamine may also inhibit cytochrome oxidase activity, it does not uncouple oxidative phosphorylation[24], and in artichoke mitochondria hydroxylamine causes no stimulation of ATP hydrolysis in the absence of succinate.) Malonate does not substitute for succinate (Table III), and the succinate stimulation (see Table II) is practically insensitive to oligomycin, indicating that the membrane-located ATPase (F_1) is not involved.

TABLE III

THE POSSIBLE INVOLVEMENT OF SUCCINYL-CoA SYNTHETASE IN THE SUCCINATE STIMULATION OF ATP HYDROLYSIS BY JERUSALEM-ARTICHOKE MITOCHONDRIA

Assay conditions were as described in Table I. 0.1 M succinate, 0.1 M malonate, 0.2 M hydroxylamine (NH_2OH), 0.25 mM coenzyme A (CoA), 10^{-6} M CCCP, $5 \cdot 10^{-6}$ M dinitrophenol and 20 μg oligomycin were added as indicated. Mitochondrial protein was 2 mg per assay.

Additions	*nmoles P_i/mg protein per min*
None	2.0
CCCP	1.2
Succinate + CCCP	10.8
Succinate + CCCP + NH_2OH	17.3
Succinate + CCCP + NH_2OH + oligomycin	13.7
Succinate + dinitrophenol	11.9
Succinate + dinitrophenol + CoA	16.8
Malonate + CCCP	3.7

Triton X-100 breaks the membranes of the mitochondria and, as shown in Fig. 1, when artichoke mitochondria are broken by Triton an extremely rapid rate of ATP hydrolysis is induced. The rate of ATP hdyrolysis can be inhibited up to 90% by oligomycin, indicating the involvement of the membrane-bound ATPase enzyme (F_1). The stimulatory effect of Triton on the ATPase activity may suggest that either (a) the membranes of the intact mitochondria are relatively impermeable to ATP or (b) an endogenous inhibitor may limit the rate of hydrolysis of ATP. Hence the ATPase activity of sonically prepared submitochondrial particles has been investigated. Here the membrane polarity is reversed[16] and therefore (a) there is no necessity for ATP to penetrate the mitochondrial membrane before being hydrolysed[16,25], and (b) any endogenous inhibitor may be removed. Fig. 2 illustrates the

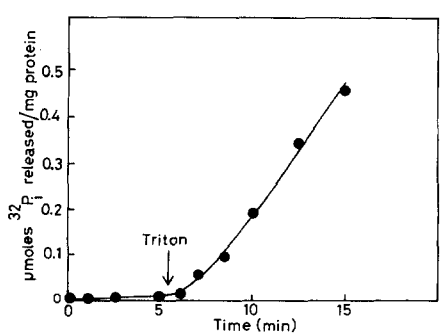
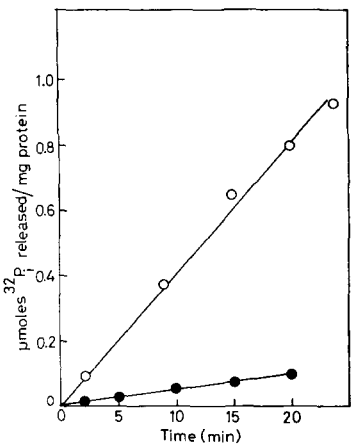


Fig. 1. The effect of Triton X-100 on the ATPase activity of Jerusalem-artichoke mitochondria. The assay contained 40 ml of a medium consisting of 2 mM [γ-^{32}P]ATP, 0.28 M sucrose, 0.035 M Tris–HCl buffer, 80 mg mitochondrial protein at pH 9.0 and 30 °C. Triton (0.5% v/v) was added as indicated. Three 1-ml aliquots were taken at the times shown for $^{32}P_i$ estimation. Each point is the average of the three samples.



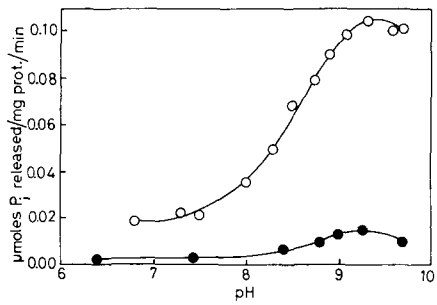


Fig. 2. The ATPase activity of Jerusalem-artichoke submitochondrial particles. The assay medium was as described for Fig. 1, except that 20 mg particles replaced the mitochondria. ATPase activity was estimated from the relase of $^{32}P_i$ from [γ-^{32}P]ATP. Oligomycin (2 μg/mg protein) reduced the particle ATPase to 1.36 nmoles P_i released/mg protein per min. The ATPase activity of the parent mitochondria is included for comparison: ○—○, submitochondrial particles; ●—●, mitochondria.

Fig. 3. The influence of pH on the ATPase activity of Jerusalem-artichoke mitochondria and submitochondrial particles. ATPase activity was assayed colorimetrically as described in Table I; mitochondrial and particle protein was 2 mg/ml and the pH was varied as shown. ○—○, submitochondrial particles; ●—●, mitochondria.

ATPase activity of artichoke submitochondrial particles. The particles catalyse a rapid rate of ATP hydrolysis (40 nmoles/mg protein per min) which is strongly inhibited by oligomycin. This rate of hydrolysis is ten times the rate catalysed by the intact parent mitochondria, also illustrated in Fig. 2. Although soluble proteins may be lost during sonication, the increase in rate of ATP hydrolysis by submitochondrial particles is not caused simply by an increase in the number of F_1 units/mg protein, for in Fig. 1 where the mitochondrial membranes are broken by Triton the rate of ATPase activity increases but the protein content remains constant. The ATPase activity of submitochondrial particles varies somewhat throughout the year (between 40 and 120 nmoles P_i released per mg protein per min). These variations may be of a seasonal or physiological origin relating to the changes in dormancy of the tissue, or in response to prolonged cold storage of the tubers.

The ATPase activity of artichoke submitochondrial particles is markedly dependent on the pH of the assay medium. This is illustrated in Fig. 3. The maximum rate of hydrolysis of ATP is observed at pH 9.0–9.3. At this pH there is also a slight stimulation of the rate of hydrolysis of ATP by intact mitochondria.

Surprisingly, the ATPase of Jerusalem-artichoke submitochondrial particles is highly specific towards adenine nucleotides. As shown in Table IV, only ATP and to a lesser extent ADP is hydrolysed at a significant rate. GTP, UTP and glucose 6-phosphate are hydrolysed to only an extremely small extent. The hydrolysis of ADP may relate to some adenylate kinase activity during incubation.

TABLE IV

SUBSTRATE SPECIFICITY OF THE ATPase OF JERUSALEM-ARTICHOKE SUB-MITOCHONDRIAL PARTICLES

All substrates were added at concentrations of 2.5 mM. Other conditions were as described in Table I.

Substrate	*nmoles P_i/mg protein per min*
AMP	0.11
ADP	11.90
ATP	49.10
GTP	3.22
UTP	4.91
Glucose 6-phosphate	0.11

DISCUSSION

A state where oxidative phosphorylation may be uncoupled from electron transport without any apparent stimulation of the ATPase activity, or where electron transport is actually influencing the functioning of the ATPase enzyme, suggests that either (a) ATP may not reach the site of hydrolysis in the absence of active electron transport, (b) there may be an inhibitor of ATP hydrolysis within the mitochondria, or (c) possibly the terminal steps of ATP synthesis in plant mitochondria are different from those thought to occur in animal mitochondria.

It is clear from the data presented in this paper that in artichoke mitochondria uncoupled electron transport does not induce the activity of the oligomycin-sensitive ATPase, and it is therefore unlikely that the process of ATP synthesis here is in any way different from that proposed for animal mitochondria[25,26]. The stimulatory effect of succinate on the oligomycin-insensitive ATPase activity of artichoke mitochondria probably relates to a reversal of the substrate-level phosphorylation associated with succinyl-CoA synthetase. This may therefore indicate a basic difference between mitochondria isolated from artichoke tubers and castor-bean endosperm[14].

Investigations of the translocation of adenine nucleotides in artichoke mitochondria[27] exclude the possibility that an adenine nucleotide translocator, similar to that described for animal mitochondria[28,29], is controlling the entry of ATP. It is suggested, therefore, that perhaps an endogenous inhibitor prevents the hydrolysis but not the synthesis of ATP in artichoke mitochondria. Such an inhibitor might correspond to that described by Pullman and Monroy[30] and could be closely bound to F_1. Sonication or treatment with detergent may remove or denature the inhibitor, while freezing and thawing of artichoke mitochondria can enhance the ATPase activity, possibly as a result of membrane disruption (Passam, H. C., unpublished). Partial purification of the supernatant from the sonication procedure has indicated the presence of an inhibitor of phosphorylation (Passam, H. C., unpublished), but more experiments are necessary to further define its nature.

An interesting feature of Jerusalem-artichoke submitochondrial particles is the specificity of the ATPase for ATP. The nucleotide specificity of the phosphoryla-

tion reactions of animal mitochondria has been shown to be imposed by a translocator situated within the cristae membrane[28,29]. Where the membrane polarity is reversed, such as in sonically-prepared submitochondrial particles, the nucleotide specificity is decreased, and Pullman *et al.*[20] have shown that the purified ATPase of beef heart mitochondria (where the membrane, and hence the adenine nucleotide translocator, is absent) actively hydrolyses several nucleotide triphosphates. In artichoke mitochondria the specificity for adenine nucleotides is apparently imposed by the ATPase enzyme. The adenine nucleotide specificity of the succinyl-CoA synthetase of plant mitochondria[22] is also presumably controlled by the specific nature of this enzyme, and it may be that plant mitochondria utilize only adenine, and not other, nucleotides.

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