Adenosine Triphosphatase Activity of Cauliflower Mitochondria 1, 2
H. B. Reid, Jr., A. C. Gentile, and R. M. Klein
University of Massachusetts, Amherst, Massachusetts, and

Introduction

Few studies have been conducted on adenosine triphosphatase (ATPase) in plant mitochondria (3, 5, 6). ATPase activity is important in plant metabolism since it results in the loss of the energy of ATP, and is therefore closely associated with reactions involving phosphorylation and utilization of or requirements for ATP. The term ATPase is being used here as defined by Potter (10) to indicate the release of Pi from ATP, not necessarily implying the existence of a single or specific enzyme.

Materials and Methods

Heads of cauliflower (Brassica oleracea L.) were obtained from local markets and refrigerated until used. Mitochondria were prepared from 25 g of buds essentially as described by Laities (8). The mitochondrial pellet was suspended in 6.5 ml of cold 0.45 M sucrose for use in the experiments described. Reactions were carried out in 25 ml Erlenmeyer flasks containing a final reaction volume of 3.0 ml. Unless otherwise stated, reactions were carried out at room temperature with an incubation time of 10 minutes. In most experiments the reaction was started by the addition of mitochondria to an otherwise complete reaction mixture. However, in specified instances, the reaction was initiated by the addition of ATP to a complete mixture containing mitochondria. The reaction media and specific conditions for each experiment are as shown for figure 1; any changes are indicated in the figures or text. In all experiments where the effect of a particular element or compound was investigated, that element or compound was omitted from the controls.

Reactions were stopped by withdrawing a 2 ml sample from each vessel and adding it to 2 ml of 10% trichloroacetic acid. For each experimental sample an appropriate zero time control was prepared by withdrawing a 2 ml sample from a duplicate vessel immediately after the start of the reaction. The trichloroacetic acid mixture was centrifuged to remove precipitated material and the Pi present in a 2 ml aliquot of the clear supernatant solution was determined (13). The difference in Pi released by a sample and its zero time control was used as a measure of the ATPase activity of each treatment. All data presented are from at least 2 experiments; in each experiment all treatments were run in duplicate.

Results and Discussion

Parameters of Hydrolysis. The rate of ATPase activity proceeded linearly with time over a period of 25 minutes (fig 1). At a pH of 5.0 ATPase activity was low; activity increased with increasing pH reaching an optimum of about pH 8.0, after which further increase in pH caused a decline in ATPase activity (fig 2). In the temperature range of 3 to 33° the ATPase activity increased with a temperature coefficient slightly greater than 2.0 (fig 3).

An interesting property of ATPase was its inhibition by excess substrate. In pea mitochondria (3),

---

1 Received April 21, 1964.
2 Supported in part by grants from the National Science Foundation, GB-314 to A. C. Gentile and GB-1664 to R. M. Klein.
ATPase activity was proportional to ATP concentration within a range varying with the concentration of simultaneously present phosphate.

Figure 4 demonstrates that the substrate inhibition observed in cauliflower was similar to that observed in a number of other ATPase systems. ATPase activity increased with increasing ATP concentration up to $6 \times 10^{-3}$ M and fell to zero at $10^{-2}$ M ATP.

**Effect of Phosphate Ions.** Forti (3) found no ATPase activity in freshly prepared pea epicotyl mitochondria incubated in Tris buffer at pH 7.3, nor in samples without buffer. Incubation at the same pH in phosphate buffer as low as 0.004 M activated ATPase activity. A system incubated in Tris alone was not used in the present study since Pi was not necessary to elicit a considerable ATPase activity. Concentrations of Pi of $10^{-4}$ M or lower had little effect on cauliflower ATPase. Pi at $1 \times 10^{-3}$ M which was stimulatory in pea, repressed hydrolysis in cauliflower, while $4 \times 10^{-3}$ M Pi was less stimulatory than in pea mitochondria.

**Effect of Cations.** Mg$^{++}$ have been shown to stimulate mitochondrial ATPase activity. In some systems the ATPase had a specific requirement for Mg$^{++}$ (2,11), while in others, different cations served equally well (5). No enhancement of cauliflower ATPase activity by Mg$^{++}$ supplied as either MgCl$_2$ or MgSO$_4$ was observed in the presence of 0.005 M KCl. Magnesium sulfate or chloride at 0.003 M in the presence of KCl had a stimulative effect; the degree of stimulation, however, was greater in the absence of KCl. The stimulation by Mg$^{++}$ was greater than that by K$^+$ when either ion was added alone. There was a stimulation induced by increasing concentrations of KCl in a medium containing MgSO$_4$ (fig 5). Although the stimulation induced by KCl is small, higher concentrations between $5 \times 10^{-4}$ and $10^{-2}$ M were more effective. Only a slight stimulation was produced by the same concentration of K$^+$ in the absence of added Mg$^{++}$; the degree of stimulation by KCl is independent of Mg$^{++}$.

Ca$^{++}$ can also replace Mg$^{++}$ as activators of ATPase. Ca$^{++}$ were effective in stimulating the ATPase of pea mitochondria (3) but inhibited yeast ATPase (7). CaCl$_2$ at $1 \times 10^{-3}$ M, the concentration used to stimulate pea mitochondrial ATPase activity, inhibited cauliflower mitochondrial ATPase activity about 30%.

**Substrate Specificity.** The term ATPase is used in a general sense, not implying a specific enzyme of a definite activity. This definition is necessary because of the varying substrate specificity, as well as other properties of the enzymes from different sources. Mg-activated ATPase of muscle splits 2 Pi groups from ATP. Forti (3) has shown that pea epicotyl mitochondrial ATPase split only the terminal Pi group from ATP; the release of the second Pi group was the result of combined ATPase and adenylyl kinase activity. Inorganic pyrophosphatase activity was lacking in pea preparations, ADP was not hydrolysed, and ADP completely inhibited the hydrolysis of ATP when the 2 were present in equal concentrations. Although the ability of cauliflower mitochondria to hydrolyse nucleoside triphosphates other than ATP was not investigated, an attempt was made to determine the degree of specificity by comparing the rates of hydrolysis of ATP and ADP at substrate concentrations of $5 \times 10^{-4}$ M. The cauliflower mitochondria hydrolysed ATP more rapidly than ADP. This ATPase was, however, not specific for the triphosphate grouping of ATP since it is able to release Pi from ADP at about half the rate at which it hydrolysed ATP. The combined values of ATP alone and ADP alone were greater than the value of the combined system, indicating a slight but not complete inhibitory effect of ADP on ATPase activity as has been reported (2).

**Sucrose Tonicity.** Lowering the sucrose concentration from 0.45 M to 0.3 M or less caused an increase in ATPase activity (fig 6). The degree of stimulation induced by a 0.3 M solution was not fur-
ther increased by lowering the sucrose concentration to 0.2 M or 0.1 M. Increased activity in the presence of sucrose solutions of low tonicity, in contrast to that in 0.45 M sucrose, was inhibited by dinitrophenol (DNP) at the concentrations tried. The DNP inhibition did not lower the activity beyond that of the control in 0.45 M sucrose, although it reached that level in several instances.

**Inhibitors of ATPase Activity.** Ethylenediaminetetraacetate (3,12) is a strong chelator and would be expected to bind Mg$^{2+}$ in solution. EDTA (0.01 M) inhibited ATPase activity about 55% in a system containing both Mg$^{2+}$ and K$^+$. It can be supposed that part, if not all, of this inhibition was due to the binding of Mg$^{2+}$ and other cations of the system. Although this possibility was not thoroughly investigated, it appeared that the degree of inhibition by EDTA was greater than the stimulation induced by Mg$^{2+}$.

NaF at 0.001 M was found to inhibit cauliflower ATPase activity about 80%. NaCl at the same concentration was not effective, indicating that it was, indeed, the fluoride ion which was responsible for the observed inhibition.

Deoxycholate, an agent reported to cause mitochondrial rupture, has been shown to stimulate mitochondrial ATPase (1). At a concentration of 0.1%, deoxycholate produced a 30 to 50% increase in ATPase activity of cauliflower mitochondria. This deoxycholate stimulation occurred in the absence of but was greater in the presence of Mg$^{2+}$.

DNP in a concentration of 3 \times 10^{-3} M produced almost 100% increase in pea mitochondrial ATPase activity in the absence of added Pi (3). This ATPase activity was proportional to DNP concentration up to 6 \times 10^{-3} M. The response to DNP was greater in media containing NaCl or KCl. In some systems, however, DNP had no effect or was inhibitory to ATPase (9).

Concentrations of DNP from 10^{-3} M to 5 \times 10^{-1} M had no effect on cauliflower mitochondrial ATPase in a system containing both MgSO$_4$ and KCl, as well as in the absence of either or both of these ions.

**Conclusions**

A number of similarities were noted between the properties of cauliflower mitochondrial ATPase activity and those of pea mitochondria (3). ATP hydrolysis as a function of time was quite similar in both, and both exhibited substrate inhibition at high ATP concentrations. NaF and EDTA inhibited pea mitochondrial ATPase activity and also that of cauliflower. Both systems were stimulated by treatments known to induce mitochondrial swelling.

The differences between the 2 plant mitochondrial ATPases are, however, more numerous than the similarities. CaCl$_2$ inhibited, DNP did not affect, and Pi did not greatly stimulate cauliflower ATPase activity. Aging was stimulatory to pea ATPase activity but aging decreased cauliflower ATPase activity. The ATPase of pea was shown to be highly specific, being completely inhibited by ADP, that of cauliflower hydrolysed ADP about half as rapidly as ATP, and was only slightly inhibited by ADP when both substrates were present in equal concentrations.

Studies with various cations indicate that the ion effect on ATPase activity is the result of more than simply ionic strength, since the nature of ion involved is at least as important as its concentration. Mg$^{2+}$ and K$^+$ stimulated mitochondrial ATPase, Ca$^{2+}$ inhibited, and Na$^+$ were without effect.

There may be more than 1 type of ATPase activity present in cauliflower mitochondria, as seems to be the case for rat liver mitochondria (1). The activity of untreated mitochondria was not influenced by any of the concentrations of DNP tested, while the ATPase activity stimulated by low concentrations of sucrose was inhibited by DNP. This hypotonic-induced activity was stimulated to a greater extent by Mg$^{2+}$ than the activity with higher sucrose solutions. Deoxycholate-induced ATPase activity differs from the activity induced by low sucrose tonicity by being so insensitive to DNP.

Our evidence does not show whether a single enzyme with the ability of hydrolysing phosphate from both ATP and ADP, or 2 separate enzymes, 1 specific for each substrate, are in operation. Since pyrophosphatase activity was not investigated, the possibility remains that the breakdown of ATP may be either the result of a hydrolysis of the terminal group, or of the 2 terminal phosphate groups in a single step followed by pyrophosphatase activity. The evidence presented indicates that activity is greater with ATP than with ADP.

In many studies of mitochondrial ATPase activity, the existence of a latent ATPase has been proposed. Latent ATPase is absent in freshly prepared mitochondria, but appears following treatment with either DNP or other such stimulating agents, or following mitochondrial rupture brought about by physical or chemical means. In this study such an activation of latent ATPase was noted following both incubation with deoxycholate and with hypotonic sucrose solutions.

Stimulation of ATPase activity by DNP has been reported in nearly every system on which it has been tested. One explanation for the absence of a stimulatory effect of DNP on the cauliflower mitochondrial ATPase might be that the membranes of freshly prepared mitochondria are impermeable to DNP. That this may be the case is indicated by the fact that hypotonic sucrose solutions induce a sensitivity to DNP. The ability of DNP to stimulate ATPase activity has been proposed as an index of the structural integrity of mitochondria. The fact that digitonin extracts of mitochondria have demonstrated large degrees of stimulation by DNP seems rather inconsistent with this proposal, unless it is assumed that the mitochondrial subunits resulting from digitonin treat-
ment are still highly structured, are active, and have intact membranes.

In the majority of studies, DNP has shown a stimulatory effect on systems of low activity or has induced activity in systems in which it was otherwise absent. In some investigations DNP failed to stimulate further systems previously activated by other treatments (4). The ATPase activity of freshly prepared, untreated mitochondria from cauliflower buds was found to be high, which may indicate that ATPase in these mitochondria is already being stimulated by other factors, thus preventing further increased activity following DNP treatment.

Summary

The mitochondrial adenosine triphosphatase of cauliflower buds has optimum activity at pH 8.0 and a temperature coefficient of approximately 2.0. Optimum activity occurs at a concentration of adenosine triphosphate of $3 \times 10^{-3} \text{M}$; higher concentrations of substrate ($10^{-2} \text{M}$) completely inhibit activity. Potassium and magnesium ions stimulate, calcium ions inhibit, and sodium ions do not affect adenosine triphosphatase activity. Phosphate hydrolysis is greatly increased in a medium of low sucrose tonicity; this increased activity is inhibited by dinitrophenol while that of suspensions incubated in 0.45 M sucrose is not affected. Aging of mitochondria for 30 minutes in ice cold 0.45 M sucrose has an inhibitory effect on phosphatase activity.

The enzyme system shows a preference for adenosine triphosphate over adenosine diphosphate as substrate; the latter is hydrolysed at about half the rate of the former. Adenosine diphosphate slightly inhibits phosphate hydrolysis when the 2 substrates are present in equal concentrations.

Concentrations of dinitrophenol between $10^{-5}$ and $5 \times 10^{-4} \text{M}$ do not affect the adenosine triphosphatase activity. Fluoride ion and ethylenediaminotetraacetate inhibit this activity; the degree of inhibition by ethylenediaminetetraacetate is greater than the stimulation by magnesium ions. Adenosine triphosphatase activity is stimulated by treating the mitochondria with deoxycholate; this increased activity is not sensitive to dinitrophenol.

Literature Cited