Characterization of cuckoo-pint (*Arum maculatum*) mitochondrial adenosine triphosphatases

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The catalytic properties of cuckoo-pint (*Arum maculatum*) mitochondrial adenosine triphosphatase have been analysed. The pH profile, effect of inhibitors, cold-stability and substrate specificity are characteristic of mitochondrial adenosine triphosphatases, although a high guanosine triphosphatase activity does appear to be restricted to plant mitochondrial adenosine triphosphatases. The kinetic properties of nucleoside 5'-triphosphate hydrolysis by membrane-bound and soluble enzymes have been studied by means of double-reciprocal plots. These plots were linear in the absence of an activating anion, which may indicate that the catalytic and/or regulatory mechanism of *Arum maculatum* adenosine triphosphatase is different from that of other enzyme preparations. It is suggested that the differences in subunit composition of plant and mammalian adenosine triphosphatases reported previously [Dunn, Slabas & Moore (1985) *Biochem. J.* 225, 821–824] are structurally, rather than functionally, significant.

INTRODUCTION

In a previous paper (Dunn *et al.*, 1985) we reported the isolation and purification of the catalytic F_1 -ATPase from mitochondria of cuckoo-pint (*Arum maculatum*). The subunit composition of this enzyme was adjudged to be different from that of the rat liver enzyme, purified by an identical procedure, in that the two smallest subunits of the plant enzyme (δ and ϵ) are considerably larger than their mammalian counterparts (Dunn *et al.*, 1985). Similarly, Walker *et al.* (1982) have shown that the δ - and ϵ -subunits of *Escherichia coli* F_1 -ATPase have different structural roles to their mammalian counterparts. The α -subunit of sweet-potato (*Ipomoea batatas*) F_1 -ATPase has recently been shown to be immunologically dissimilar to its mammalian and plastid counterparts (Iwasaki *et al.*, 1984), and Boutry *et al.* (1983) and Hack & Leaver (1983) demonstrated that the α -subunit of plant mitochondrial ATPase is a mitochondrial translation product. The F_1 -ATPase subunits of yeast, *Neurospora* spp. and mammalian mitochondria are all nuclear-coded (Neupert & Schatz, 1981).

Evidently there are several structural and genetic differences between the F_1 -ATPase of animal and higher-plant mitochondria. Whether these differences are also manifested in catalytic properties is not apparent, largely through the lack of information available on the properties of the plant F_1 -ATPase. In the present paper we have analysed the catalytic properties of the membrane-bound and soluble ATPase preparations of *A. maculatum* mitochondria. Apart from a high GTPase activity and the apparent absence of negative co-operativity in the hydrolysis of NTPs, plant mitochondrial ATPase appears to be typical of ATPases prepared from energy-transducing membrane.

EXPERIMENTAL

Submitochondrial particles (SMP) were prepared from mitochondria isolated from locally collected spadices of *A. maculatum* as described previously (Moore & Proudlove, 1983). The oligomycin-insensitive ATPase was chloroform-released from submitochondrial membranes as described by Dunn *et al.* (1984) and was used immediately, except in the cold-lability experiment (see below). ATPase activity was assayed either by the colorimetric determination of P_i (LeBel *et al.*, 1978) or by the ATP-regenerating system of Pullman *et al.* (1960) as indicated below.

The pH optimum of *A. maculatum* ATPase activity was determined by the colorimetric estimation of P_i with the following buffers (all 10 mM): pH 5.0–7.0, Mes; pH 6.5–8.0, Mops; pH 7.5–9.5, Tris; pH 9.0–10.0, Caps. Each assay mixture contained 7.8 μ g of F_1 -ATPase protein and ATP hydrolysis was initiated by the addition of 3 mM-MgATP.

The nucleotide and substrate specificity of *A. maculatum* ATPase was assessed by using the ATP-regenerating system. Assay mixtures contained either SMP (56 μ g) or F_1 -ATPase (7.6 μ g) and the reaction was initiated by the appropriate amount of MgNTP or NTP (when $MgCl_2$ concentration was 0.5 mM). Metal-ion specificity was determined by incubating *A. maculatum* F_1 -ATPase (2.8 μ g) with the appropriate metal ion (3 mM) for 5 min before the addition of ATP, GTP, dATP or dGTP. ATPase activity was assayed colorimetrically.

The effect of a variety of compounds on soluble and SMP ATPase activity was determined by using the ATP-regenerating system. F_1 -ATPase or SMP (the amount of protein in mg is given in the legend to Table 3) was incubated in the standard assay medium minus

Abbreviations used: F_1 -ATPase, water-soluble catalytic sector of the mitochondrial adenosine triphosphatase complex; SMP, submitochondrial particles; Caps, 3-(cyclohexylamino)propanesulphonic acid; NTP, in this paper, ATP, GTP or ITP; DCCD, dicyclohexylcarbodi-imide; DBCT, dibutylchloromethyltin.

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substrate. The appropriate amount of inhibitor was then added and the incubation was continued for a further 5 min, except when the inhibitor was venturicidin A or DCCD; in these latter cases the enzyme/inhibitor incubation times were 30 and 60 min respectively. At the end of each incubation, MgATP (0.5 mM) was added and the decrease in absorbance was monitored at 340 nm. Ethanol controls and controls in the absence of inhibitor were identical in all cases. The concentration of aurovertin B was determined from the millimolar absorption coefficient of $38.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Linnett *et al.*, 1983). Aurovertin B, DBCT, efrapentin, venturicidin A and the *N*-polymethylenecarboxymaleimides (acid maleimides) were kindly donated by Professor R. B. Beechey (University College, Aberystwyth, Dyfed, Wales, U.K.).

To evaluate cold-lability, *A. maculatum* submitochondrial membranes were shaken with chloroform (Dunn *et al.*, 1984) and the solubilized ATPase was assayed immediately by the ATP-regenerating system. One half of the soluble enzyme preparation was stored at 4 °C and a portion (7.5 µg of protein) was removed after 30, 60, 90 and 120 min and assayed as described above. The remainder of this enzyme preparation was stored overnight at 4 °C as a 60%-(w/v)-(NH₄)₂SO₄ suspension (pH 7.0). The following day the (NH₄)₂SO₄ suspension was briefly centrifuged (1 min, 12000 g) and the pellet was resuspended in 0.25 ml of 0.25 M-sucrose/20 mM-Tris/H₂SO₄ (pH 7.7)/1 mM-EDTA. This ATPase preparation was immediately assayed and then stored at 4 °C. Portions (7.8 µg of protein) were removed after 15, 30, 105 and 180 min and assayed for ATPase activity. An SMP preparation was also incubated at 4 °C and a portion (58 µg) was assayed after 30, 60, 120 and 180 min to assess the cold-stability of the membrane-bound enzyme.

All enzyme assays were performed at least in duplicate and the results presented are representative of these determinations. Protein was determined as described by Peterson (1977).

RESULTS AND DISCUSSION

Fig. 1 shows that the pH optimum of soluble *A. maculatum* ATPase activity is approx. pH 8.0, whereas SMP ATPase activity displayed a pH optimum of 7.5–8.0 (P. P. J. Dunn & A. L. Moore, unpublished work). Both enzyme preparations displayed a gradual increase in ATPase activity with increasing pH and a rapid decline beyond the maximal activities at pH 8.0. ATPase isolated from other energy-transducing membranes, including ox heart (Pullman *et al.*, 1960) and rat liver (Catterall & Pedersen, 1974) mitochondria, as well as thylakoid membranes (Chang & Khan, 1966), have also been shown to exhibit strong pH-dependence, with maximal activities in the range pH 7.5–8.5.

MgATP is the substrate for mitochondrial ATPases (Ulrich, 1964; Selwyn, 1967). Fig. 2 shows that when the NTP concentration is varied in the presence of 0.5 mM-MgCl₂, the NTPase activity of F₁-ATPase and SMP gradually increases up to 0.5–0.6 mM-NTP, after which there is a gradual decline in enzyme activity. Ulrich (1964) reported that maximal rat liver ATPase activity was achieved at an Mg²⁺/ATP ratio of 1:1.5 (pH 8.5), whereas Selwyn (1967) showed that free ATP and free Mg²⁺ are competitive antagonists of ox heart soluble ATPase activity. Free GTP and free ITP are also

Fig. 1. pH optimum of *A. maculatum* F₁-ATPase activity

ATPase was assayed by the colorimetric determination of P_i. Each assay contained 7.8 µg of F₁-ATPase and one of the following buffers (10 mM): Mes (●), Mops (■), Tris (▲) or Caps (○). The reaction was started by the addition of 3 mM-MgATP.

inhibitors of NTP hydrolysis by mitochondrial ATPases (Fig. 2). When MgCl₂ and ATP concentrations are varied, but at a fixed ratio of 1:1, the ATPase activity of *A. maculatum* SMP and F₁-ATPase reaches substrate saturation at 0.7 mM-MgATP. However, analogous to the results shown in Fig. 2, the increase in NTPase activity observed up to a MgCl₂/NTP ratio of 1:1 was reversed with subsequent inhibition in activity when the added MgCl₂ concentration exceeded the added ATP concentration (P. P. J. Dunn & A. L. Moore, unpublished work). Fig. 2 shows that free NTP can result in as much as 50% inhibition of maximal NTPase activity and that, similar to the mammalian enzymes, MgNTP is the substrate for *A. maculatum* mitochondrial ATPase. The maximal GTPase activity of F₁-NTPase was 50% greater than the ATPase activity and 150% greater than the ITPase activity (Fig. 2a). SMP, GTPase and ATPase activities were both approximately double the ITPase activity (Fig. 2b). Grubmeyer & Spencer (1977) had previously reported that the soluble ATPase of pea (*Pisum sativum*) cotyledon mitochondria was capable of hydrolysing GTP 3-fold faster than ITP and 5-fold faster than ATP, whereas pea cotyledon SMP hydrolysed GTP twice as fast as ATP (Grubmeyer & Spencer, 1977). Partridge *et al.* (1985) have also demonstrated that corn (*Zea mays*) mitochondrial ATPase has a high GTPase activity. The rate of hydrolysis of NTPs by the soluble corn ATPase followed the order GTP > ITP > ATP (Partridge *et al.*, 1985). Pullman *et al.* (1960) reported that ox heart F₁-ATPase hydrolysed ATP, GTP and ITP equally. Whether high GTPase activity is of functional significance to the plant mitochondrial ATPase remains to be seen.

Although the soluble ATPase of *A. maculatum* mitochondria hydrolyses GTP more rapidly than ATP, the enzyme exhibits a lower *K_m* when MgATP is substrate. Table 1 shows that the *K_m* for MgATP is 0.63 mM, whereas that for MgGTP as substrate is 1.11 mM. These values were derived from the Lineweaver-Burk plots of *A. maculatum* ATPase activity at different

Fig. 2. Substrate specificity of *A. maculatum* mitochondrial ATPase

ATPase activity was assayed by the ATP-regenerating system and contained 7.6 μg of F₁-ATPase (a) or 56 μg of SMP (b). The MgCl₂ concentration was 0.5 mM and the assays were initiated by the appropriate concentration of ATP (○, ●), GTP (△, ▲) or ITP (□, ■).

MgNTP concentrations (not shown). To date there has been no systematic survey of K_m and V_{max} values for a particular ATPase preparation hydrolysing either ATP, GTP or ITP. The comparison of the kinetic parameters of NTP hydrolysis by *A. maculatum* membrane-bound ATPase with those of the soluble enzyme shown in Table 1 reveals that the membrane-bound enzyme has a lower K_m for NTP than the soluble enzyme. The NTP specificity for SMP ATPase is in the order ATP > ITP > GTP, whereas for soluble ATPase it is ATP > GTP > ITP. Substrate specificity of ox heart and rat liver membrane-bound ATPases is in the order ATP > ITP > GTP, but, in both cases, substrate specificity is lost once the ATPase is detached from the membrane (Pedersen, 1975). The Michaelis-Menten constants for the hydrolysis of MgATP by *A. maculatum* SMP

Table 1. NTP specificity of *A. maculatum* mitochondrial ATPase

NTPase activity of SMP and soluble (F₁-ATPase) ATPases was assayed by the ATP-regenerating system. Assays contained the appropriate concentration of MgATP, MgGTP or MgITP and either 56 μg of SMP or 7.6 μg of F₁-ATPase. K_m and V_{max} values were calculated from intercepts on the x- and y-axes respectively of Lineweaver-Burk plots (not shown).

Enzyme preparation	Substrate	K_m (mM)	V_{max} (μmol/min per μg)
SMP	MgATP	0.10	0.56
	MgGTP	0.27	0.64
	MgITP	0.12	0.30
F ₁ -ATPase	MgATP	0.63	5.30
	MgGTP	1.11	10.00
	MgITP	1.25	5.00

Table 2. Metal-ion requirement and substrate specificity of *A. maculatum* F₁-ATPase

ATPase activity was estimated by the colorimetric determination of P_i. Assays contained 2.8 μg of soluble plant ATPase and the appropriate metal ion (3 mM) as the chloride salt. In one assay, 1 mM-CaCl₂ was included with 3 mM-MgCl₂ as shown. Enzyme activity was initiated by the addition of 3 mM-NTP or 3 mM-NTP 5 min after the addition of the metal ion.

Substrate	ATPase activity (μmol/min per μg)	ATPase (as % of MgATPase)
None	0.01	0.3
MgATP	3.57	100
MgGTP	3.89	109
MgdATP	3.41	96
MgdGTP	3.68	103
CaATP	2.57	72
MnATP	1.06	30
Ca ²⁺ + MgATP	2.74	77

(Table 1; $K_m = 0.1$ mM) and soluble ATPase (Table 1; $K_m = 0.63$ mM) are in agreement with published values (reviewed by Pedersen, 1975). Interestingly, the membrane-bound ATPase also has a lower K_m for MgGTP ($K_m = 0.27$ mM) and MgITP ($K_m = 0.12$ mM) than the soluble ATPase ($K_m = 1.11$ mM for MgGTP and 1.25 mM for MgITP), as revealed in Table 1. It is conceivable that release of the enzyme from the submitochondrial membranes results in a conformational change, thus giving rise to the altered kinetic properties. It is noteworthy that the Lineweaver-Burk plots (not shown) are linear in the absence of an activating anion, which is normally required to prevent negative co-operativity (Ebel & Lardy, 1975). It is impossible to say at this stage whether the linear Lineweaver-Burk plots reflect an atypical mitochondrial ATPase mechanism.

The ability of *A. maculatum* mitochondrial ATPase to hydrolyse GTP and ITP shows that the amino group on the 6-position of the purine moiety is not essential.

Table 3. Effect of inhibitors on soluble and membrane-bound ATPases of *A. maculatum* mitochondria

ATPase was assayed by the regeneration of ATP. F₁-ATPase or SMP was incubated in assay medium with inhibitor for the appropriate period of time. The amounts of protein present were as follows: ^a90 µg; ^b70 µg; ^c50 µg; ^d10 µg; ^e5 µg. ATPase activity was initiated by the addition of 0.5 mM-MgATP. Percentage inhibition for each inhibitor is taken from a control assay minus inhibitor. Abbreviations used: AM 11, acid maleimide with 11 methylene groups (etc.); ND, not determined.

Inhibitor	SMP		F ₁ -ATPase	
	Concn. [nmol/mg (µM)]	Percentage inhibition	Concn. [nmol/mg (µM)]	Percentage inhibition
Oligomycin	17.2 (1.24) ^b	97	248 (1.24) ^d	0
DCCD	5.6 (0.4) ^b	75	50 (0.25) ^d	15
Venturicidin A	18.5 (1.33) ^b	82	26 (1.33) ^e	0
DBCT	1.74 (0.15) ^a	40	70 (0.35) ^e	9
Efraeptin	0.25 (0.018) ^b	38	18.3 (0.09) ^e	42
Aurovertin B	22.5 (1.62) ^b	35	100 (0.54) ^e	36
Trifluoperazine	560 (28) ^c	50	2600 (29) ^d	50
AM 11	556 (40) ^b	42	8000 (40) ^e	46
AM 7	556 (40) ^b	31	8000 (40) ^e	0
AM 5	556 (40) ^b	20	8000 (40) ^e	0
AM 2	556 (40) ^b	15	ND	ND

Soluble ATPase will tolerate at least a minor alteration in the pentose ring, since it hydrolyses dATP and dGTP to approximately the same extent as it does ATP and GTP (Table 2). In contrast, soluble ATPases isolated from corn (Partridge *et al.*, 1985) and ox heart (Dorgan *et al.*, 1984) hydrolyse dATP more rapidly than ATP. Table 2 also discloses that Ca²⁺ and Mn²⁺ can support the hydrolysis of ATP by *A. maculatum* F₁-ATPase in the absence of added Mg²⁺. Ca²⁺ is almost as effective as Mg²⁺, but MnATPase activity is only a fraction (30%) of MgATPase activity. It seems likely that the metal ions bind to the same site(s) on the enzyme, since Ca²⁺-plus-MgATPase activity is lower than that of MgATPase alone (Table 2). The metal-ion requirement of mitochondrial and bacterial ATPase appears to vary from one enzyme preparation to another, and the literature is suitably confusing (reviewed by Pedersen, 1975). However, Senior *et al.* (1980) have recently demonstrated that there are two bivalent-metal-ion-binding sites associated with mitochondrial and bacterial ATPases. One site was shown to be important structurally (i.e. removal of tightly bound Mg²⁺ from this site resulted in enzyme depolymerization) and the other catalytically at which Mn²⁺, Ca²⁺ or Zn²⁺ could substitute for Mg²⁺; Senior *et al.*, 1980).

SMP and soluble ATPase activities of *A. maculatum* mitochondria are inhibited by a variety of compounds (Table 3), most of which have been documented as inhibitors of mitochondrial, plastid and bacterial ATPases (Linnett & Beechey, 1979). Oligomycin, DCCD, DBCT and venturicidin A all inhibit ATP hydrolysis by *A. maculatum* SMP, but not F₁-ATPase, activity (Table 3). These inhibitors are also specific for mammalian and bacterial membrane-bound ATPases. Aurovertin and efraeptin inhibit both enzyme preparations (Table 3), and the results of Cross & Kohlbrenner (1977) indicate that efraeptin binds to the catalytic site. We have recently shown that the antibiotic aurovertin binds to plant mitochondrial ATPase and this binding is associated with antibiotic fluorescence (Dunn *et al.*, 1985). Trifluoperazine also inhibits both the membrane-

bound and the soluble ATPase activities of plant mitochondria (Dunn *et al.*, 1984), although the amount of phenothiazine required to inhibit ATPase activity by 50% is several orders of magnitude greater than the amounts of the aforementioned antibiotics (Table 3). Interestingly, kinetic studies suggest that trifluoperazine is a competitive inhibitor of plant mitochondrial ATPase (P. P. J. Dunn & A. L. Moore, unpublished work). The *N*-polymethylenecarboxymaleimides (acid maleimides) inhibit plant SMP ATPase activity and this inhibition is related to the length of the methylene side chain (Table 3). Thus the greatest inhibition was attained by the acid maleimide (AM) with eleven methylene groups in the side chain (AM 11) and the least inhibition by AM 2, which has only two methylene groups in the side chain (Table 3). Only AM 2 inhibited the soluble ATPase, but 8 µmol of AM 11/mg of protein (40 µM) was required to cause 46% inhibition of ATP hydrolysis. The acid maleimides have been used to probe the position of thiolate ions in membrane proteins: the carboxy group at the end of the methylene side chain controls the extent of membrane penetration. For example, Griffiths *et al.* (1984) have recently probed the catalytic activities of ox heart mitochondria ATPase by using the acid maleimides. ATP-P_i exchange activity and the ATP-driven membrane potential of SMP were substantially inhibited by 500 µM-AM 10 and AM 11 but, in contrast with the results shown in Table 3, the ATPase activity of ox heart SMP was not affected by any of the acid maleimides up to 500 µM (Griffiths *et al.*, 1984). Griffiths *et al.* (1984) concluded that there are two essential thiol groups near the membrane surface that are functional in proton translocation. The mode of inhibition of plant mitochondrial ATPase shown in Table 3 is not clear, but probably does not involve a specific interaction with F₁-ATPase, since very high concentrations of AM 11 were required for partial inhibition. The stepwise increase in inhibition of SMP ATPase with increasing chain length may reflect non-specific hydrophobic interactions with the membrane sector of the ATPase complex.

Chloroform solubilization of F₁-ATPase from ox heart

Fig. 3. Cold-lability of *A. maculatum* ATPase

SMP (▲, 58 μg), chloroform-released ATPase (■, 7.5 μg) or (NH₄)₂SO₄-precipitated enzyme (●, 7.8 μg) was assayed for ATPase activity by the ATP-regenerating system (0.5 mM-MgATP) after various periods of incubation at 2 °C.

SMP yields a near-homogeneous preparation that is cold-stable and has a specific activity of 10–20 μmol of ATP hydrolysed/min per mg of protein (Beechey *et al.*, 1975). Other methods of preparing ox heart F₁-ATPase yield a cold-labile enzyme with a specific activity of 100 μmol of ATP hydrolysed/min per mg of protein (for one example, see Knowles & Penefsky, 1972). Fig. 3 shows the effects of low-temperature incubation on the activities of chloroform-released *A. maculatum* F₁-ATPase and the (NH₄)₂SO₄-precipitated enzyme. Chloroform treatment of *A. maculatum* SMP as described previously (Dunn *et al.*, 1984) produces a cold-stable soluble ATPase, but (NH₄)₂SO₄ precipitation (and overnight storage at 4 °C) renders the enzyme distinctly cold-labile with 75% decrease in hydrolytic activity after 30 min incubation at 4 °C (Fig. 3). The chloroform-released enzyme will endure 2 h incubation at 4 °C without a significant depreciation in hydrolytic activity and thus resembles the membrane-bound enzyme (Fig. 3). Cold-stability and low hydrolytic activity displayed by chloroform-released ATPases [e.g. Beechey *et al.* (1975) and Fig. 3] have been attributed to the association of the inhibitor protein (Pullman & Monroy, 1960) with the soluble enzyme and, as such, these enzyme preparations may more closely resemble the ATPase *in vivo* than preparations with high hydrolytic activity. (NH₄)₂SO₄ precipitation of the chloroform-released enzyme does induce cold-lability, but does not activate hydrolytic activity (Fig. 3). We have, however, previously shown that *A. maculatum* F₁ATPase can be activated by passage through a gel-filtration column and the resulting purified enzyme does not appear to have an inhibitor protein associated with it (Dunn *et al.*, 1985).

The MgATP complex is the substrate for *A. maculatum* mitochondrial ATPase: free ATP and free Mg²⁺ are both inhibitory. In terms of nucleotide specificity, the soluble

and membrane-bound ATPase of plant mitochondria appear to be different from their mammalian counterparts in the high GTPase activity displayed by the plant enzyme and the maintenance of substrate specificity after the enzyme has been detached from the membrane. *A. maculatum* mitochondrial ATPase does not display negative co-operativity with respect to its substrate, which may suggest that NTP hydrolysis by this enzyme may proceed by a mechanism different to that of well-characterized mammalian ATPases. Negative co-operativity can generally be overcome by the presence of an activating anion such as bicarbonate (Ebel & Lardy, 1985). In the case of *A. maculatum* ATPase we have been unable to detect any significant anionic or cationic activation (P. P. J. Dunn & A. L. Moore, unpublished work). It is difficult to ascertain at this stage whether the unusual nucleotide and substrate specificity is of functional significance to *A. maculatum* mitochondria or whether it merely reflects the mode of isolation or the composition of the assay medium. Functionally, therefore, *A. maculatum* ATPase is similar to well-characterized energy-transducing ATPases but, as previously shown, there are differences in the subunit composition of the plant and mammalian ATPases. It must be concluded that differences between the subunit compositions of plant, bacterial and mammalian ATPases reflect the different structural roles of the smaller subunits (δ and ε), which will be verified by antigenic and amino-acid-sequence analysis.

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