Isolation and Kinetic Analyses of the Soluble F_1 ATPases from Mitochondria of Wheat and Pearlmillet

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The mitochondrial F_1 ATPases from two cereal crops, wheat and pearlmillet, were purified and studied. The wheat F_1 ATPase could be purified to homogeneity and is apparently composed of six subunits with apparent molecular weights of 55 kDa (α and β), 35 kDa (γ), 26 kDa (δ') and 22 kDa (δ). The ϵ subunit was barely detectable. Both enzymes reveal typical non-linear kinetics but show variability in their response to bicarbonate and chloride. While the wheat F_1 ATPase is stimulated by bicarbonate and chloride, the pearlmillet F_1 ATPase is inhibited by both anions. The two enzymes are Mg^{2+} dependent ATPases and are competitively inhibited by Ca^{2+}, unlike maize, pea and turnip ATPases. Both the enzymes also possess a GTPase activity which is two fold higher than the ATPase, unlike rice, sorghum and oat root F_1 ATPases.

Introduction

The mitochondrial F_o-F_1 ATPase is a membrane-bound multi-subunit complex that is highly conserved not only in the lower and higher eukaryotes but even amongst counterparts in chloroplasts and bacterial membranes. It consists of mainly two components – a membrane-bound lipophilic F_o component, attached to a hydrophilic F_1 component (that can be studied *in vitro* as ATPase).

In vivo, the complex acts as a channel for the dissipation of the proton gradient, which is generated by movement of protons and electrons across the membrane through the electron transport chain, and it stores the energy released during the dissipation of the proton gradient as ATP. The ATP synthase has been studied in great detail in a number of organisms (Amzel and Pedersen, 1983) and even the X-ray crystallographic studies of the bovine ATPase were recently published (Abrahams *et al.*, 1994).

In plants, the study of the F_1 ATPase has been hampered due to problems involved in its purification and it is only in the last decade that F_1 ATPases from plants such as maize (Hack and Leaver, 1983; Spitsberg *et al.*, 1985), sweet potato (Iwasaki and Asahi, 1983), arum (Dunn *et al.*, 1985), pea (Horak and Packer, 1985), beet (Ouazzini and Berville, 1991), turnip (O'Rourke and Wilson, 1992), etc. have been purified and studied. The studies reveal that plant mitochondrial F_1 ATPases are essentially similar to their mammalian counterparts but are characterized by a lower specific activity. One of the features of some plant mitochondrial ATPases has been the presence of an additional subunit (24–26 kDa) that has been reported to be homologous to the oligomycin sensitivity conferring protein of animal ATPases (Horak *et al.*, 1989). This subunit is not a characteristic feature of all purified plant F_1 ATPases. It is occasionally observed in some preparations such as pea (Horak and Packer, 1985) and turnip (Horak *et al.*, 1990) but is reportedly absent from purified monocot plant F_1 ATPases such as maize (Hack and Leaver, 1983; Spitsberg *et al.*, 1985), *Arum maculatum* (Dunn *et al.*, 1985) and *Avena sativa* (Randall *et al.*, 1985). Our group has been interested in the study of F_1 ATPases from monocots to investigate the differences in their properties and more importantly to ascertain if monocot F_1 ATPases as a group are 5 subunit enzyme complexes without δ' subunit. Our studies indicate that like dicot F_1 ATPases, plant monocot F_1 ATPases such as those from wheat, pearlmillet as well

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as from sorghum and rice (Sane *et al.*, 1996a,b), are also six subunit enzymes. The additional δ' subunit thus appears to be a characteristic of most plant F_1 ATPases.

Materials and Methods

Isolation of F_1 ATPase

Seeds of wheat (*Triticum aestivum*) and pearlmillet (*Pennisetum vulgare*), obtained from the local market, were placed in trays over a sheet of wet cotton pad and sown in dark at 30–32 °C. Mitochondria were isolated from 6–8 day old etiolated seedlings as described previously (Nath *et al.*, 1993). The crude mitochondrial suspension was passed through a cushion of 0.6 M sucrose containing 10 mM HEPES.KOH (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.2, 1 mM EGTA [ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid] and 0.1% bovine serum albumin and pelleted after centrifugation at 12,500 x *g* for 20 min at 4 °C. The pellet was resuspended in the same buffer but containing 0.4 M mannitol instead of sucrose and bovine serum albumin and re-pelleted at 12,500 x *g* for 15 min. The mitochondrial pellet was then frozen at -70 °C for 20 min, thawed and suspended in 1 ml of 250 mM sucrose, 10 mM Tris.HCl [tris(hydroxymethyl)aminomethane] pH 7.6 and 1 mM PMSF (phenylmethylsulfonylfloride). It was immediately diluted with 20 ml of hypotonic buffer containing 10 mM Tris.HCl pH 7.6 and 1 mM PMSF and kept in ice for 30 min at 4 °C and then suspended in 800 µl of a buffer containing 250 mM sucrose, 10 mM Tris.HCl pH 7.4, 1 mM EDTA and 1 mM PMSF. The suspension was then mixed vigourously with 0.5 vol of chloroform for 20 sec to release the membrane-bound mitochondrial F_1 ATPase. The suspension was centrifuged at 5,000 x *g* and the aqueous layer was collected and diluted with 1/9 volume of chilled methanol. This suspension was then centrifuged at 100,000 x *g* for 30 min at 4 °C and the F_1 ATPase from the supernatent was precipitated by 50% ammonium sulfate saturation, incubated for 30 min in ice. This was collected by centrifugation at 19,000 x *g* for 10 min and the pellet was suspended in 200 µl of 20 mM Tris.HCl pH 7.6, 12.5% methanol and 1 mM each of ATP, EDTA and PMSF. This suspension was then loaded on a 20–40% glycerol gradient equilibrated with 20 mM Tris.HCl pH 7.5, 1 mM EDTA, 2 mM ATP, 1 mM PMSF and 12.5% methanol. The gradient was centrifuged at 100,000 x *g* for 16 h at 4 °C in a SW50.1 swing-out rotor (Beckman). Fractions of 300 µl were collected from the bottom of the tube, checked for ATPase activity and used further or stored at -70 °C.

Assay

The mitochondrial F_1 ATPase was assayed by the coupled- enzyme ATP-regenerating assay described by Pullman *et al.* (1960). Reactions were carried out in a final volume of 0.5 ml in an assay buffer containing 50 mM Tris.HCl pH 8.0, 5 mM $MgSO_4$, 10 units each of pyruvate kinase and lactate dehydrogenase, 2.5 mM PEP (phospho*enol*-pyruvate) and 170 µM NADH. Reactions were started by addition of ATP (5 mM) and the decrease in optical density of NADH at 340 nm was monitored for 2 min.

The effect of various anions and inhibitors was studied by their addition to the reaction mixture just prior to addition of ATP.

All assays were performed with at least three independent preparations of ATPases.

Analysis on SDS-PAGE

Active fractions of the F_1 ATPases were denatured and reduced with 50 mM dithiothreitol and analysed on a 16.5% denaturing SDS polyacrylamide gel in a continuous buffer system (Laemmli, 1970). Bands were visualised by silver-staining as described by Sammons *et al.* (1981). Estimation of protein was carried out by the method of Peterson (1977).

Results and Discussion

Purification of the mitochondrial F_1 ATPases

Aliquots of the glycerol gradient fractions showing peak activity (usually the 8th fraction from the bottom) were loaded on a 16.5% denaturing SDS-polyacrylamide gel for resolution of the F_1 ATPase subunits. A silver-stained profile of the F_1 ATPase from wheat revealed a six subunit enzyme (Fig. 1). Bands indicating an apparent molecular weight of 55 kDa (α+β), 35 kDa (γ), 26 kDa (δ') and 22 kDa (δ) could be clearly identified while the ε subunit was barely detectable. The difficulty

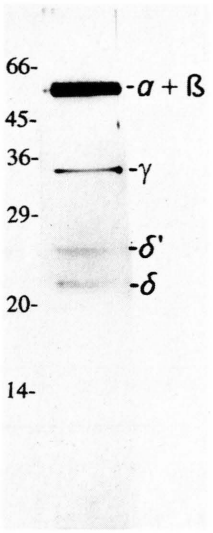

Fig. 1. Detection of the glycerol gradient purified wheat mitochondrial F_1 ATPase on a denaturating 16.5% SDS-polyacrylamide gel by silver-staining. Numbers on the left indicate sizes of the molecular weight markers in kDa.

in detecting the ϵ subunit by silver staining has been documented even in earlier studies by Dunn *et al.* (1985) and by Sane *et al.* (1996a,b). Unlike the wheat F_1 ATPase, the pearlmillet enzyme could not be purified by the existing method. In addition to the major subunits, the silver-stained profile of the pearlmillet F_1 ATPase also revealed a few other polypeptides besides a general smear in all the preparations (data not shown). The subunit composition of the wheat and pearlmillet ATPases investigated in this study demonstrates that both wheat and pearlmillet F_1 ATPases as well as those from sorghum (Sane *et al.*, 1996a) and rice (Sane *et al.*, 1996b) are six subunit F_1 ATPases unlike animal F_1 ATPases. The 26 kDa polypeptide is present as an additional subunit in all these cereals like in the dicot preparations of sweet potato (Iwasaki and Asahi, 1983), pea (Horak and Packer, 1985), beet root (Ouazzini and Berville, 1991) and turnip (O'Rourke and Wilson, 1992). While it is occasionally observed in some of the dicot preparations (pea, turnip), it was consistently present in all preparations of wheat, pearlmillet, rice and sorghum. However, there do exist differences between the intensities of the δ' and the δ subunits among the plant species. In wheat as well as sorghum, the δ' is almost equivalent in intensity to the δ subunit, while in rice it was at a much lower intensity. On the other hand, maize F_1 ATPase (Hack and Leaver, 1983; Spitsberg *et al.*, 1985) was found to lack the δ' subunit while oat root ATPase (Randall *et al.*, 1985) has been reported to occasionally contain an additional subunit *larger* than the δ in their F_1 ATPase preparations (which is likely to be δ' subunit observed in our preparations). Horak *et al.* (1989) have previously reported that the 26.5 kDa polypeptide (designated as δ in pea) is homologous to the oligomycin sensitivity conferring protein (OSCP) while the 22 kDa polypeptide (designated as δ') is actually the δ equivalent of animal and fungal ATPases (Horak *et al.*, 1990; Morikami *et al.*, 1992). In all probability, the 26 kDa subunit observed in wheat, pearlmillet, rice, sorghum (and oat root) may be similar to the 26 kDa subunit observed in sweet potato, pea, turnip and beet root and is likely to be the OSCP. If so, its presence as the sixth subunit in most plant F_1 ATPases may indicate its higher affinity for the F_1 ATPase in plants, unlike in animals, as suggested by Morikami *et al.* (1992). The differences in the relative intensity of the 26 kDa subunit and its presence/occasional association with the F_1 ATPases may merely reflect the ability of the method, being used for isolation of the ATPases, to effectively extract the OSCP.

Kinetic analyses

Lineweaver-Burk plots of the active fractions of wheat and pearlmillet F_1 ATPases revealed typical non-linear kinetics with Hill coefficients of 0.65 ± 0.04 and 0.7 ± 0.06 respectively (Fig. 2). Specific activities of 12 ± 2 and 3 ± 0.5 μmol ATP/min x mg protein were obtained for the unstimulated enzymes of wheat and pearlmillet respectively, while the sulfite-stimulated enzymes showed specific activities of 20 and 6.3 μmol ATP/min x mg protein respectively.

Effect of anions and cations

Since mitochondrial F_1 ATPases, in general, are known to exhibit variable sensitivity to several anions especially oxyanions and divalent cations, a study of the effects of some ions on the F_1 ATPase activities from wheat and pearlmillet was undertaken.

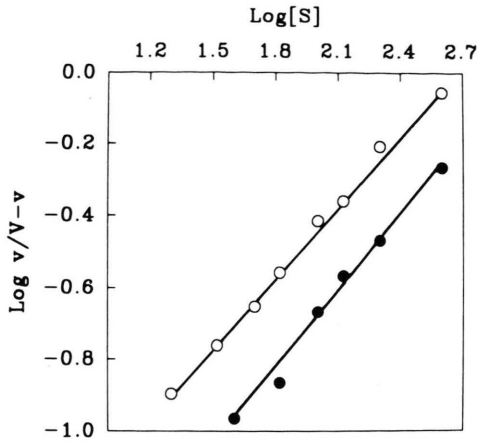

Fig. 2. Hill plot of the wheat and pearlmillet F_1 ATPase for determination of Hill coefficients.
v, velocity at a given ATP concentration,
V, maximum velocity.
Assays were carried out by the coupled enzyme ATP regenerating assay as described in Materials and methods on a SLM Aminco DW2C spectrophotometer in a final volume of 0.5 ml.
○–○ = Wheat, ●–● = pearlmillet.

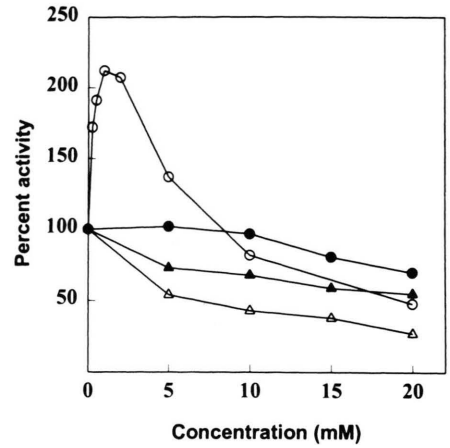

Fig. 4. The effect of various anions on the pearlmillet F_1 ATPase activity.
○–○, sulfite; ●–●, bicarbonate; △–△, nitrate; ▲–▲, chloride.
Anions were added just prior to the assay and the reactions were carried out as described in Materials and Methods. The activity of the F_1 ATPase in absence of any anions (3 μmol ATP/min x mg protein) was taken as 100%.

Effects of sodium salts of different anions and chloride salts of different cations were tested on the F_1 ATPases from wheat and pearlmillet. The results are shown in Fig. 3 (wheat) and Fig. 4

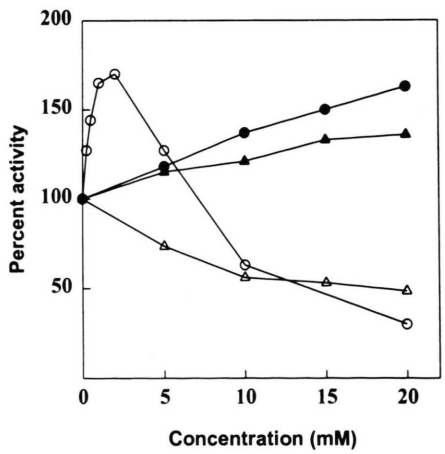

Fig. 3. The effect of various anions on the wheat F_1 ATPase activity.
○–○ = sulfite, ●–● = bicarbonate, △–△ = nitrate, ▲–▲ = chloride.
Salts were added just prior to the assay and the reactions were carried out as described in Materials and methods. The activity of the F_1 ATPase in absence of any anions (12 μmol ATP/min x mg protein) was taken as 100%.

(pearlmillet) respectively. Bicarbonate was found to stimulate the wheat enzyme from 1.6–1.8 fold at concentrations of 20 mM bicarbonate and 5 mM ATP. However, at low ATP concentrations, (20–100 μM ATP), this stimulation was negligible (about 1.2 folds, data not shown). Surprisingly, bicarbonate did not stimulate the pearlmillet enzyme. In fact, there was a decrease in its activity at concentrations above 10 mM bicarbonate.

Sulfite had similar effects on the wheat and pearlmillet enzymes. At low concentrations of sulfite (upto 2 mM), the two enzymes were stimulated upto two folds. With increasing sulfite concentrations, an inhibition of the ATPase was observed. While concentrations of about 7 mM sulfite brought the activity to the original (unstimulated enzyme activity), concentrations above this inhibited the enzymes strongly with upto 50–80% inhibition being observed at concentrations of 20 mM sulfite. This marked stimulation and inhibition by sulfite appears to be a common feature for the cereal F_1 ATPases and was also exhibited by sorghum and rice (Sane *et al.*, 1996a,b). It differs from the dicot F_1 ATPase preparations of turnip where even 100 mM sulfite was able to stimulate the ATPase upto five folds.

Stimulation of the wheat enzyme was also obtained with chloride. However, this stimulation never went beyond 1.3 folds even at concentrations of 20 mM chloride. In contrast, the pearlmillet ATPase was inhibited by chloride at a concentration of 20 mM. Inhibition of upto 40% of the activity was obtained. Nitrate inhibited the wheat as well as pearlmillet ATPases. The inhibition increased gradually with increasing concentration of nitrate. A concentration of 20 mM nitrate reduced the activity of the F_1 ATPases to 50% of the original in wheat and 30% in pearlmillet.

The above studies thus show that while there is a similarity in the kinetic properties of the cereal ATPases of wheat, pearlmillet, rice, sorghum and oat, the pearlmillet enzyme is quite different from the other four in terms of its sensitivity to bicarbonate and chloride. It was the only ATPase that was inhibited in response to bicarbonate and chloride.

Cation effects

The effects of three divalent cations viz. Mg^{2+}, Mn^{2+} and Ca^{2+} (as their chloride salts) were also tested. Both enzymes were found to use Mg ATP as the most preferred substrate. While Mn^{2+} could substitute Mg^{2+} at low concentrations, concentrations above 1 mM Mn^{2+} inhibited the F_1 ATPase activity in both pearlmillet and wheat. Neither of the enzymes was found to possess any Ca^{2+} ATPase activity. In fact, Ca^{2+} acted as a competitive inhibitor for the Mg ATPase. Concentrations of 5 mM Ca^{2+} drastically inhibited the Mg^{2+} ATPase activity to 15% of the activity at 5 mM Mg^{2+}. The activity could be recovered by increasing the Mg^{2+} concentration. This is similar to ATPases from sorghum and rice but very much in contrast to ATPases from maize (Partridge *et al.*, 1985), pea (Grubmeyer *et al.*, 1977) and turnip (O'Rourke and Wilson, 1992) where the Ca^{2+} ATPase activity is equivalent to the Mg^{2+} ATPase. Sequence alignments of the α and β subunits of wheat, rice and maize, however, did not provide any conclusive information on amino acid changes that may be related to the Ca^{2+} ATPase activity (data not shown).

Inhibitors

Sulfhydryl group inhibitors viz. NEM (N-ethylmaleimide) and thimerosal (sodium ethylmercurithiosalicylate) both inhibited the F_1 ATPases of wheat and pearlmillet. While greater than 50% inhibition was obtained with 6 mM NEM, less than 10 μM thimersol could almost completely inhibit the two ATPases (Table I). The results are similar to those reported for the other plant ATPases of oat root (Randall *et al.*, 1985), turnip (O'Rourke and Wilson, 1992), sorghum (Sane *et al.*, 1996a) and rice (Sane *et al.*, 1996b).

Table I. Effect of some inhibitors on the activities of the glycerol gradient purified F_1 ATPases from wheat and pearlmillet. Reactions were carried out as described in Materials and methods.

Inhibitor	Concentration for 50% inhibition		Concentration for >90% inhibition	
	Wheat	Pearlmillet	Wheat	Pearlmillet
NEM	6 mM	3 mM	ND	ND
Thimerosal	3 μM	3 μM	5 μM	6 μM
Azide	10 μM	10 μM	30 μM	40 μM

ND = not done.

Azide was also a potent inhibitor of the F_1 ATPase. Complete inhibition of the ATPases could be obtained at a concentration of less than 40 μM.

Nucleotide specificity

The ability of the F_1 ATPases to hydrolyse nucleoside triphosphates other than ATP was also tested. The results are shown in Table II. Both ATPases could use GTP and ITP as substrates. GTP was preferentially used over ATP, with the rate of GTP hydrolysis being almost two folds higher than that of the ATP hydrolysis. Although this is lower than that reported for maize (Partridge *et al.*, 1985), it is much higher than those of oat (Randall *et al.*, 1985), sorghum (Sane *et al.*, 1996a) and rice (Sane *et al.*, 1996b). Unlike ATP hydrolysis, the hydrolysis of GTP increased over a

Table II. Activities of the wheat and pearlmillet F_1 ATPases with different nucleoside triphosphates. Assays were carried out as described in Materials and methods. Activity with 5 mM ATP was taken as 100%.

Nucleoside triphosphate (5 mM each)	Percent activity	
	Wheat	Pearlmillet
ATP	100	100
GTP	200	180
ITP	127	105
CTP	< 5	< 5

period of time, taking about 4–6 min for maximum activity. The rate of hydrolysis of ITP was marginally higher than that of ATP hydrolysis. CTP was not utilised as a substrate by either of the ATPases.

The present studies thus bring out the differences that exist in kinetic properties of even a highly conserved enzyme such as F_1 ATPase not only amongst different plant species but even within the monocot species. It is interesting to note that the F_1 ATPases of wheat, rice and sorghum, inspite of belonging to different subfamilies, show a great deal of similarity in structure and kinetic properties whereas F_1 ATPases from maize, sorghum and pearlmillet differ in structure and kinetic properties inspite of belonging to the same subfamily. The differences in the anion activation especially bicarbonate (pearlmillet vs the rest), Ca^{2+} ATPase activity (maize vs the rest) and the varying GTPase activities of the cereal ATPases are worthy of further investigations particularly in relation to amino acid sequences of α and β subunits. This aspect is presently under study in our laboratory.

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