Purification of F_1 -ATPase from cuckoo-pint (*Arum maculatum*) mitochondria

A comparison of subunit composition with that of rat liver F_1 -ATPase

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Plant mitochondrial ATPase has been chloroform-solubilized and purified by gel filtration from spadices of cuckoo-pint (*Arum maculatum*). The subunit composition of purified plant and rat liver ATPase were compared by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The δ - and ε -subunits of the plant enzyme are larger than their supposed rat liver counterparts and, as such, *A. maculatum* mitochondrial ATPase shows structural homologies with the enzyme from *Escherichia coli* [Futai, Sternweis & Heppel (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2725–2729] rather than with the rat liver enzyme.

The catalytic portion, or F_1 -ATPase, of the ATPase complex of mitochondria and bacteria has been purified from a range of animal tissues and yeast (Criddle *et al.*, 1979) and from *Escherichia coli* (Futai *et al.*, 1974). In most cases, purified F_1 -ATPases have been shown to consist of five detergent-separable subunits which have generally been termed α , β , γ , δ and ε in decreasing order of M_r . However, it has previously been shown, by subunit-size and amino-acid-sequence analysis, that the subunits of *E. coli* and ox heart F_1 -ATPases are not necessarily equivalent (Walker *et al.*, 1982). Most strikingly, *E. coli* δ -subunit was found to be equivalent to *oscp* of ox heart F_0 -ATPase rather than the subunit of ox heart F_1 -ATPase (Walker *et al.*, 1982).

Thus far there has been little research on the F_1 -ATPase of higher-plant mitochondria. Recently, however, sweet-potato (*Ipomoea batatas*) F_1 -ATPase has been purified by an elaborate four-step procedure from submitochondrial particles (Iwasaki & Asahi, 1983). This enzyme consisted of six subunits. In contrast, we report here a rapid two-step purification of five-subunit F_1 -ATPase from *Arum maculatum* (cuckoo-pint) submitochondrial particles. The same procedure was used for

purification of rat liver F_1 -ATPase to enable a direct comparison of subunit composition. The results presented show that, on the basis of M_r , the subunits of *A. maculatum* F_1 -ATPase are more analogous to those of *E. coli* F_1 -ATPase than to those of rat liver F_1 -ATPase.

Experimental

Mitochondria were isolated from locally collected spadices of *Arum maculatum* (cuckoo-pint) as described previously (Moore & Proudlove, 1983) and were stored at -80°C . Mitochondria were also prepared from rat liver by the method of Johnson & Lardy (1969) and were stored likewise. Submitochondrial particles were prepared by using an Aminco French pressure cell as described by Moore & Proudlove (1983) and stored at -80°C until use. F_1 -ATPase was released from submitochondrial membranes with chloroform as described by Dunn *et al.* (1984). *A. maculatum* and rat liver F_1 -ATPases were subsequently purified as follows. Ultrogel AcA34 (LKB, South Croydon, Surrey, U.K.) was degassed and then used to make a $2.6\text{ cm} \times 90\text{ cm}$ column, which was equilibrated with 20 mM-Tris/HCl (pH 7.7)/50 mM-NaCl/1 mM-EDTA/1 mM-ATP/0.5 mM-PMSF. The column was calibrated with Blue Dextran (void volume 134 ml) and the following molecular-mass standards (obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.): thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa). ATPase released by chloroform treat-

Abbreviations used: F_1 -ATPase, catalytic portion of the proton-translocating ATPase insensitive to oligomycin; F_0 -ATPase, membrane sector or ionophore portion of the ATPase; SDS, sodium dodecyl sulphate; PMSF, phenylmethanesulphonyl fluoride.

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ment (6mg) was applied to the column and proteins were eluted with the equilibration buffer at a flow rate of 28ml/h. Fractions (2.8ml) were collected and assayed for ATPase activity by using the ATP-regenerating system of Pullman *et al.* (1960). Active fractions were pooled, dialysed extensively against equilibration buffer minus NaCl, and freeze-dried for subsequent analysis by polyacrylamide-gel electrophoresis. Protein was assayed by A_{280} or by the method of Peterson (1977). 'Native' (i.e. non-SDS) polyacrylamide-gel electrophoresis was performed on slab gels with a linear polyacrylamide concentration gradient from 5 to 10% (w/v) as described by Knowles & Penefsky (1972), except that the ratio (w/w) of acrylamide to bisacrylamide was 75:4 and that sucrose, Tris and EDTA were omitted from the acrylamide/bisacrylamide stock. Subunit structure was analysed by electrophoresis on 12.5%-(w/v)-polyacrylamide gels containing 0.1% SDS (Laemmli, 1970). Gels were silver-stained for protein as described by Morrissey (1981). SDS/polyacrylamide gels were calibrated with the following molecular-mass standards (purchased from Sigma): bovine serum albumin (68kDa), ovalbumin (43kDa), α -chymotrypsinogen (25.7kDa), cytochrome *c* (11.7kDa). Densitometer scans of polyacrylamide gels were obtained with a Joyce-Loebl microdensitometer. Aurovertin fluorescence of protein released by chloroform treatment was observed by the method of Beechey *et al.* (1975). Aurovertin B was kindly donated by Professor R. B. Beechey (University of Aberystwyth, Aberystwyth, Dyfed, Wales, U.K.).

Results and discussion

The densitometer scan of a native polyacrylamide gel (Fig. 1a) shows that one major protein and six to seven minor proteins were released with chloroform from *A. maculatum* submitochondrial membranes, as judged by silver staining. Beechey *et al.* (1975) utilized chloroform to solubilize ox heart F_1 -ATPase to near homogeneity. However, in this case, the less sensitive Coomassie Blue stain was used to reveal proteins (Beechey *et al.*, 1975). When proteins released by chloroform were treated with aurovertin B before electrophoresis, the antibiotic bound to the major protein, as revealed by the fluorescent band shown in Fig. 1(b). Aurovertin forms a fluorescing complex with solubilized ox heart (Beechey *et al.*, 1975) and rat liver (Lardy & Lin, 1969) mitochondrial ATPases. Fig. 1(b) shows that solubilized plant mitochondrial ATPase also produces a fluorescent complex with aurovertin. The specific activity of *A. maculatum* F_1 -ATPase released by chloroform was 2.3 units/mg of protein (1 unit = 1 μ mol of ATP

Fig. 1. Electrophoresis of *A. maculatum* chloroform-released proteins on 'native' polyacrylamide gels

The gels were 5–10%-(w/v)-polyacrylamide slab gels, silver-stained and scanned (to show protein bands) with a Joyce-Loebl microdensitometer (a); '(–)' is the origin and '(+)' the dye front. In (b), chloroform-released protein (2 μ g) was incubated with 10nmol of aurovertin B at 39°C for 15min before electrophoresis and ATPase-associated fluorescence was observed by the method of Beechey *et al.* (1975).

hydrolysed/min per mg of protein), whereas that for rat liver F_1 -ATPase prepared in the same manner was 11.6 units/mg of protein. For both enzymes this represents a recovery of 75% of total enzyme units from submitochondrial-particle ATPase activity. Initial ATPase activities were 0.21 and 1.34 units/mg for *A. maculatum* and rat liver submitochondrial particles respectively.

Fig. 2 shows the elution profile for gel-filtration purification of *A. maculatum* F_1 -ATPase. Plant mitochondrial F_1 -ATPase was purified to homogeneity, as revealed by 'native' polyacrylamide-gel electrophoresis. The purified enzyme had a specific activity of 30 units/mg, which is equivalent to a 140-fold purification from submitochondrial ATPase activity, and a recovery of 50% of the total enzyme units. The elution volume from the calibrated gel-filtration column was 202ml, indicating that *A. maculatum* F_1 -ATPase has an M_r of 386000. Similarly, Iwasaki & Asahi (1983) reported an M_r of 370000 and specific

Fig. 2. Chromatography of chloroform-released proteins on Ultrogel AcA34

A. maculatum chloroform-released proteins (6 mg) were applied to the gel-filtration column, which was equilibrated in, and eluted with, 20 mM-Tris/HCl (pH 7.7)/50 mM-NaCl/1 mM-EDTA, 0.5 mM-PMSF. ●, Protein (detected by absorbance at 280 nm); ○, Enzyme activity [units (μmol of ATP hydrolysed/min)/ml].

activity of 39 units/mg for the purified sweet-potato F₁-ATPase, although this represents only a 30% recovery of enzyme units and 7-fold purification. The F₁-ATPase released by chloroform from rat liver was also purified to homogeneity by gel filtration on Ultrogel AcA34 (results not shown). Purified rat liver F₁-ATPase had a specific activity of 85 units/mg (60-fold purification and 65% recovery of total enzyme units) and, with an elution volume of 200 ml from the gel-filtration column, an *M_r* of approx. 396 000. The *M_r* values reported here for purified plant and rat liver F₁-ATPases are similar to those previously reported for mitochondrial and bacterial F₁-ATPases (Penefsky, 1979).

The subunit compositions of purified *A. maculatum* and rat liver F₁-ATPases were analysed by SDS/polyacrylamide-gel electrophoresis (Figs. 3a and 3b). Silver staining for protein by the method of Morrissey (1981) revealed four subunits for each enzyme, which we have designated as α, β, γ, and δ according to established nomenclature. For *Arum maculatum* F₁-ATPase the subunit molecular masses are 5 kDa (α), 52 kDa (β), 35 kDa (γ), 22 kDa (δ) (Fig. 3a, whereas for rat liver the calculated values are 553 kDa (α), 51 kDa (β), 33 kDa (γ), and 12 kDa (δ) (Fig. 3b). A fifth subunit (ε) was not detected by silver staining, but was visible when Coomassie Blue protein staining was used. It would therefore appear that the silver-staining technique used in the present study does not stain the ε-subunit of F₁-ATPases. Rat liver ε-subunit has a molecular mass of 7.5 kDa, whereas the subunit from *A. maculatum* is slightly larger at 11 kDa (P. P. J. Dunn, unpublished work). Iwasaki & Asahi (1983) reported similar subunit molecular masses for sweet-potato F₁-ATPase, although two

Fig. 3. Subunit analysis of plant and animal F₁-ATPase. Subunit analysis of *A. maculatum* (2 μg) (a) and rat liver (2 μg) (b) F₁-ATPases was performed on 12.5% (w/v) polyacrylamide slab gels, containing 0.1% SDS, silver-stained for protein and scanned with a Joyce-Loebl microdensitometer; '(-)' is the origin and '(+)' the dye front.

δ-subunits (δ and δ') were apparently present. Those authors suggest that one of the δ-subunits (δ') may have arisen from proteolytic digestion. The results shown in Fig. 3(b) for the subunit composition of rat liver F₁-ATPase concur favourably with those published previously for animal F₁-ATPases (Criddle *et al.*, 1979). The three largest subunits of *A. maculatum* F₁-ATPase (α, β and γ) are nearly identical in size with those of rat liver F₁-ATPase. There is, however, a considerable discrepancy between the molecular masses of the δ-subunits, the plant δ-subunit being 10 kDa larger than the rat liver δ-subunit (22 kDa as compared with 12 kDa respectively). On the whole, the purified plant F₁-ATPase described here, displays a subunit composition very similar to that reported for *E. coli* F₁-ATPase by Futai *et al.* (1974). The molecular masses of *E. coli* δ- (20.5 kDa) and ε- (12 kDa) subunits (determined by SDS/polyacrylamide-gel electrophoresis; Futai *et al.*, 1974) show close similarity with δ- (22 kDa) and ε- (11 kDa) subunits from *A. maculatum* F₁-ATPase (see Fig. 3). Walker *et al.* (1982) have shown, by amino-acid-sequence analysis, that *E. coli* and ox heart δ-subunits are not equivalent. Ox heart δ-subunit is homologous in sequence with the ε-subunit of *E. coli*, whereas the *E. coli* δ-subunit shows sequence and function homologies with ox heart oSCP

(Walker *et al.*, 1982). If δ - and ϵ -subunits of plant and bacterial F_1 -ATPases also show sequence and function, as well as size, homologies, then important answers may be found for the evolutionary development of mitochondria in higher plants and animals.

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