Analysis of NADH dehydrogenases from plant [mung bean (*Phaseolus aureus*)] mitochondrial membranes on non-denaturing polyacrylamide gels and purification of Complex I by band excision

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The present paper describes the analysis of plant mitochondrial NADH dehydrogenases using a recently developed non-dissociating gradient polyacrylamide-gel-electrophoresis technique [Kuonen, Roberts & Cottingham (1986) *Anal. Biochem.* **153**, 221–226]. Solubilized mung-bean (*Phaseolus aureus*) sub-mitochondrial particles were analysed on 3–22% (w/v) gradient polyacrylamide gels containing 0.1% Triton X-100 and stained for multiple NADH dehydrogenase activities. A rotenone-sensitive NADH dehydrogenase (Complex I) was identified on the basis of co-migration with the purified mammalian enzyme. The polypeptide composition of the plant enzyme was further analysed by band excision and SDS/polyacrylamide-gel electrophoresis.

INTRODUCTION

Plant mitochondria present a considerable challenge in terms of protein characterization because of their low tissue abundance and the complexity of the respiratory chain (Moore & Rich, 1985). In terms of substrate oxidation, plant mitochondria contain at least three immunologically distinct NADH dehydrogenases (Cottingham *et al.*, 1986), a possibility which has long been suggested on the basis of inhibitor sensitivity and substrate membrane polarity (see Palmer & Ward, 1985). In addition, plant mitochondria possess two terminal oxidases, one of which is cyanide-insensitive, but hydroxamic acid-sensitive (see Lance *et al.*, 1985). Despite suggestions to the contrary (Rustin *et al.*, 1983), this oxidase, one of which is cyanide-insensitive, but hydroxamic acid-sensitive (see Lance *et al.*, 1985). Despite enzyme (Walsh *et al.*, 1986; Walsh & Moore, 1987; Elthon & McIntosh, 1987a,b).

In order to characterize further plant mitochondrial NADH dehydrogenases we have used a recently developed Triton X-100-containing non-denaturing gradient polyacrylamide-gel-electrophoresis method, which is particularly suitable for the resolution of membrane components (Kuonen *et al.*, 1986). This technique has permitted the identification of multiple NADH dehydrogenase activities. One of these, which co-migrates with mammalian rotenone-sensitive NADH dehydrogenase, has been further analysed for polypeptide composition by denaturing-gel electrophoresis.

MATERIALS AND METHODS

Plant mitochondria were isolated from etiolated mung-bean (*Phaseolus aureus*) hypocotyls, cuckoo-pint (*Arum maculatum*) spadix and beetroot (*Beta vulgaris*)

roots according to the procedure of Moore & Proudlove (1983), whereas ox heart mitochondria were prepared by the method of Racker (1962). Submitochondrial particles (SMP) were prepared as previously described (Cottingham *et al.*, 1986) by sonication of intact mitochondria. SMP were solubilized for 20 min on ice with 7% (w/v) Triton X-100 at a protein-to-detergent ratio of 1:1; protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard. Insoluble material was removed by centrifugation at 100 000 *g* for 1 h, and the clear supernatant was either used immediately or stored at –70 °C until needed. Purified ox heart Complex I was kindly provided by Dr. C. I. Ragan (Merck, Sharp & Dohme Laboratories, Harlow, Essex, U.K.).

Non-denaturing-gel electrophoresis was performed at 4 °C in 3–22% (w/v) gradient polyacrylamide gels containing 0.1% (w/v) Triton X-100, 100 mM-Tris/glycine buffer, pH 9.0 (Kuonen *et al.*, 1986). Samples were either applied in a single continuous band across the gel, which was subsequently sliced vertically, or in individual wells formed in 3% (w/v) acrylamide.

Staining of gels for NADH dehydrogenase activity was performed by using 100 μ M-NADH in 100 mM-Tris/glycine buffer, pH 7.4, which contained 1 mg of Nitro Blue Tetrazolium/ml.

Excised pieces from the non-dissociating gel were analysed on a denaturing gel as follows: a piece of gel (3 mm $\times$ 6 mm $\times$ 1 mm) was excised from the appropriate NADH dehydrogenase-activity-staining band and soaked in 100 μ l of stacking-gel buffer (Laemmli, 1970), which contained 1% (w/v) SDS and 1% (w/v) mercaptoethanol, for 15 min at 60 °C. The gel slice was then pushed into a well in the stacking gel on a 10–16% (w/v) linear acrylamide gradient denaturing gel. The ion front was run slowly through the stacking gel until within

Abbreviations used: F₁-ATPase, catalytic portion of the proton-translocation ATPase insensitive to oligomycin; SMP, submitochondrial particles.

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Fig. 1. Non-dissociating gradient polyacrylamide-gel analysis of plant and animal solubilized SMP

Solubilized SMP from ox heart (tracks A and E), mung bean (track B), *Arum maculatum* (track C) and beetroot (track D) were applied to non-dissociating gradient polyacrylamide gels and stained for NADH dehydrogenase activity as described in the Materials and methods section.

about 1 mm of the separating gel. The gel slice was then removed and electrophoresis continued in the usual manner. Protein was detected after electrophoresis by staining with silver (Eschenbruch & Burk, 1982).

RESULTS AND DISCUSSION

Mitochondria were solubilized with Triton X-100 and loaded on to non-denaturing gradient polyacrylamide gels, which were stained for NADH dehydrogenase activity and protein. The gel shown in Fig. 1 was overloaded with protein in order to produce denser activity staining for photographic purposes; better resolution was routinely obtained at lower protein loadings (results not shown).

The multiple NADH dehydrogenases of plant mitochondria were investigated (Fig. 1) by comparing the staining pattern on the non-denaturing-gel system of mung-bean mitochondria with that of ox heart (a well-defined pattern), *Arum maculatum* (high in external NADH dehydrogenase activity) and fresh beetroot SMP (low in external NADH dehydrogenase). Ox heart SMP (tracks A and E) show two predominant bands of activity: the major one at position X has been shown by immunoblotting to correspond to the rotenone-sensitive membrane enzyme, Complex I (Kuonen *et al.*, 1986). The identity of the faster-migrating band is unknown, but it has been observed that only 90 % of NADH:ubiquinone 1 oxidoreductase activity in solubilized ox heart SMP is immunoprecipitated by an antiserum raised to purified complex I (Cleeter & Ragan, 1985). Solubilized mung-bean SMP show four bands of NADH dehydrogenase activity (track B), one of which co-migrates with Complex I of ox heart SMP. A comparison of the staining patterns

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified NADH dehydrogenases

Track A, mung-bean NADH dehydrogenase excised from a non-dissociating gel; track B, purified ox heart NADH dehydrogenase (complex I); and track C, molecular-mass markers. The molecular-mass values (in kDa) on the left margin refer to polypeptides visible in track A, whereas those on the right margin refer to those visible in track B. Owing to the faintness of the bands the exact position of each is shown by lines drawn on the left and right margin. Only those discussed in the text are assigned a value.

of *Arum maculatum* (track C) with beetroot SMP (track D) reveals that, as anticipated, both contain Complex I, but differ with respect to the higher-mobility bands. For instance, track C has similar band pattern to that of the mung-bean SMP (track B), apart from an additional band just below Complex I. Note that the band at position Y is much more heavily staining. This band, however, appears to be absent from the beetroot SMP (track D). Since this track is virtually identical with that of ox heart SMP (track E), it is reasonable to assume that the missing band at position Y, which stains for NADH activity, is the external NADH dehydrogenase.

The NADH dehydrogenase-staining band which co-migrates with ox heart Complex I, as identified using an antibody to the purified mammalian enzyme (Kuonen *et al.*, 1986), was excised and run on an SDS-containing gel (Fig. 2). Five major bands are visible (track A), corresponding to polypeptides with molecular masses of 76, 46, 39, 33 and 27 kDa, which approximately correlate with the characteristic ox heart Complex I polypeptides

with masses of 75, 49, 39, 30 and 24 kDa (track B). Other low-molecular-mass bands are visible in the mung-bean enzyme, but these are not as numerous as with the mammalian enzyme. Interestingly, the purified mung-bean enzyme shows no trace of the NAD^+ transhydrogenase, a common impurity of the ox heart enzyme, normally observed in the 100 kDa region. There are, however, traces of material in the 58 kDa region, which are possibly due to the large subunit of the F_1 -ATPase and which are often seen in purified ox heart complex I preparations.

The correspondence between functionally significant polypeptides found in the mammalian and plant enzymes is notable. For instance, three of the four iron-sulphur subunits of the ox heart enzyme, with molecular masses of 75, 49 and 30 kDa (Ohnishi *et al.*, 1985) have equivalents at 76, 46 and 27 kDa respectively in the plant enzyme. The co-identity of the 49 kDa and the 30 kDa subunits with the plant subunits at 46 and 27 kDa has been confirmed by the cross-reactivity with subunit-specific antibodies to the mammalian enzyme (Cottingham *et al.*, 1986). A polypeptide is also present in the 33 kDa region in both proteins. In the mammalian enzyme there is evidence that this could constitute part of the rotenone-binding site (Earley *et al.*, 1987), and cross-reactivity has been further demonstrated between the mammalian polypeptide and an antiserum to intact mung-bean SMP (Cottingham *et al.*, 1986). Despite these similarities, there are some differences between the mammalian and plant enzymes. In particular, there appears to be no polypeptide corresponding to the 51 kDa flavin-containing subunit in the plant enzyme. This requires further clarification by refinement of the purification method. A less significant difference is that less low-molecular-mass polypeptides are present in the plant enzyme, although this could be a simple problem of detection. However, in general terms, the similarity between the ox heart and mung-bean enzymes shows very considerable conservation, which is presumably related to the very highly specialized function of this enzyme as a transmembraneous proton pump.

Conclusion

The present paper demonstrates a one-step purification of Complex I from plant mitochondria by band excision of a non-dissociating gel previously stained for NADH dehydrogenase activity. Complex I purified by this technique is comprised of at least five major polypeptides with molecular masses of 76, 46, 39, 33 and 27 kDa. A combination of increased scale of isolation, electroelution and enhanced antigenicity by conjugation with keyhole-limpet haemocyanin should enable the production of monospecific antibodies by such an approach. This in turn could be used to confirm the identity and composition of the enzyme and allow its fuller charac-

terization and, possibly, eventual molecular cloning. As a method of producing antibodies the use of non-dissociating gels complements immunoelectrophoresis, which has already been applied to the study of plant mitochondria in this laboratory (Cottingham *et al.*, 1986).

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