Immunological analysis of plant mitochondrial NADH dehydrogenases

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Plant mitochondrial NADH dehydrogenases were analysed by two immunological strategies. The first exploited an antiserum raised to a preparation of SDS-solubilized mitochondrial-inner-membrane particles. By using a combination of activity-immunoprecipitation and crossed immunoelectrophoresis, it was shown that Triton X-100-solubilized membranes contain at least three immunologically distinct NADH dehydrogenases. Two of these were subsequently isolated by line immunoelectrophoresis and analysed for polypeptide composition: one contained three polypeptides with molecular masses of 75, 62 and 41 kDa; the other was a single polypeptide with a molecular mass of 53 kDa. The other approach was to probe plant mitochondrial membranes with antibodies raised to a purified preparation of ox heart rotenone-sensitive NADH dehydrogenase and subunits thereof. Cross-reactions were observed with the subunit-specific antisera against the 30 and 49 kDa ox heart proteins. However, the molecular masses of the equivalent polypeptides in plant mitochondria are slightly lower, at 27 and 46 kDa respectively.

INTRODUCTION

One of the major differences between plant and animal mitochondria is that the former are more flexible and possess multiple pathways for the oxidation of substrates (Moore & Rich, 1985). This is particularly evident when considering the oxidation of NADH. Current evidence indicates the presence of three different enzymes, capable of oxidizing NADH, associated with the mitochondrial inner membrane of most plant species investigated. Two of these dehydrogenases have substrate-binding sites on the matrix side of the membrane, whereas the third faces the intermembrane space.

One of the two enzymes that oxidize endogenous NADH appears to be similar to the mammalian enzyme, the rotenone-sensitive NADH dehydrogenase or complex I (Ragan, 1985). This similarity extends to rotenone-sensitivity, albeit with lower binding affinity (Chauveau & Lance, 1971), three sites of ATP synthesis for NAD⁺-linked substrates (Cunningham, 1964), and the detection of three e.p.r. signals with *g*-values characteristic of the mammalian enzyme (Cammack & Palmer, 1977; Rich & Bonner, 1978).

In addition to the rotenone-sensitive dehydrogenase, there is now increasing evidence for the presence of another internal NADH dehydrogenase that is rotenone-insensitive. The key observations, which have necessitated the postulation of this additional enzyme, are the incomplete rotenone- and piericidin A-sensitivity of the oxidation of NAD⁺-linked substrates and the rotenone-dependent decrease in sites of ATP synthesis from three to two (Ikuma & Bonner, 1967; Bruton & Palmer, 1973; Day & Wiskich, 1974*a,b*; Marx & Brinkman, 1978). Further support for a second internal NADH dehydrogenase has come from temperature-dependency studies of

internal substrate oxidation (Marx & Brinkman, 1979) and an analysis of NADH oxidation by SMP with a mixed polarity of cytochrome oxidase orientation (Møller & Palmer, 1982). The latter study described a rotenone-sensitive dehydrogenase with a *K_m* of 8 μ M and a rotenone-insensitive one with a *K_m* of 80 μ M. It was postulated that this second dehydrogenase functions to allow the turnover of citric-acid-cycle intermediates during periods of slow ATP synthesis.

The operation of the external NADH dehydrogenase on the inner membrane is characterized by the oxidation of exogenous NADH via a pathway which is antimycin-sensitive, independent of the addition of cytochrome *c* (Humphreys & Conn, 1956) and rotenone-insensitive (Wilson & Hanson, 1969). The first two criteria, and a specificity for β - rather than α -NADH, distinguish the external NADH dehydrogenase from another dehydrogenase associated with the outer membrane (Moreau & Lance, 1972; Douce *et al.*, 1973). Other properties of the external dehydrogenase are that it only uses two sites of ATP synthesis (Bruton & Palmer, 1973) and is inhibited by Ca²⁺ chelators such as EGTA (Coleman & Palmer, 1971). Activity can be restored by the addition of Ca²⁺ (Coleman & Palmer, 1971; Moore & Åkerman, 1982). The external NADH dehydrogenase is differentiated from the internal rotenone-insensitive dehydrogenase on the basis of temperature-dependency studies (Marx & Brinkman, 1979) and EGTA-sensitivity in mixed orientation SMP (Møller & Palmer, 1982).

The multiplicity of plant mitochondrial NADH dehydrogenases and the importance of their membrane orientation has impeded the solubilization and characterization of the individual enzymes. For instance, there are two conflicting reports describing the partial purification and characterization of the external NADH dehydro-

Abbreviations used: ubiquinone-1, ubiquinone with a five-carbon isoprenoid side chain; SMP, submitochondrial particles.

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genase (Cottingham & Moore, 1984; Cook & Cammack, 1984, 1985). This confusion has undoubtedly arisen because the best criteria for identifying this enzyme, namely its membrane orientation in intact mitochondria and its sensitivity to inhibition by Ca^{2+} chelators, are lost on solubilization. It is, however, the first firm evidence for the existence of more than one NADH dehydrogenase associated with the inner membrane of plant mitochondria.

We have employed a combination of immunological approaches in order to characterize further and define plant mitochondrial NADH dehydrogenases. The first of these is the analysis of antibodies raised to whole SMP, which are highly purified inner-membrane fragments, by immunoelectrophoresis. This approach has enabled the identification and isolation of two distinct NADH dehydrogenases and implies the existence of a third. The second approach was to probe for plant mitochondrial complex I polypeptides with antibodies raised to purified subunits of the mammalian enzyme. Two cross-reacting polypeptides were detected, which shows that there is a high degree of conservation between the plant and animal enzymes. A preliminary account of this work has already been published (Cottingham *et al.*, 1984).

MATERIALS AND METHODS

Preparation of membranes

Plant mitochondria were prepared from etiolated mung-bean (*Phaseolus aureus*) shoots as previously described (Moore & Proudlove, 1983). SMP were then made as follows: mitochondria, diluted to 5 mg of protein/ml and kept on ice, were sonicated for 4×7 s, with 20 s cooling intervals, on an MSE Soniprep instrument. Intact mitochondria were removed by centrifugation at 18000 *g* for 15 min and then the SMP were collected by centrifugation at 100000 *g* for 1 h. The resulting pellet was resuspended in buffer, which contained 300 mM-mannitol and 10 mM-Mops at pH 7.4, and stored at -70°C until required. SMP were solubilized at a protein concentration of 10 mg/ml by the addition of $\frac{1}{2}$ vol. of 20% (w/v) Triton X-100 and kept on ice for 20 min. Insoluble material was then removed by centrifugation at 100000 *g* for 1 h. The clear supernatant was either used immediately or stored at -70°C until needed.

Ox heart mitochondria were prepared by a standard procedure (Racker, 1962) and made into SMP as described above for mung beans.

Immunological methods

Antisera were raised in New Zealand White rabbits as follows: SMP (5 mg of protein) were solubilized in 0.1% (w/v) SDS for 10 min at room temperature; the resulting solution was then mixed with 0.5 ml of complete Freund's adjuvant and injected at 20 subcutaneous sites on an animal's back; after 4 weeks the same amount of protein was given in two intramuscular injections in incomplete adjuvant. Animals were bled at 2-weekly intervals and boosted by intramuscular injection every 2 months. An IgG fraction was prepared for use in immunoelectrophoresis by the treatment of pooled sera from two animals with 6,9-diamino-2-ethoxyacridine lactate and $(\text{NH}_4)_2\text{SO}_4$ (Hurn & Chandler, 1980).

The purification of ox heart complex I and subunits

with molecular masses of 75, 49, 30 and 13 KDa, and the subsequent production of monospecific antibodies, has already been described in detail elsewhere (Cleeter *et al.*, 1985).

Crossed immunoelectrophoresis was carried out essentially as described by Owen & Salton (1975), but at 4°C to conserve enzyme activity. Electrophoresis in the first dimension was at 5 V/cm for 4 h. In the second dimension electrophoresis was run at the same voltage but for 18 h. The antibody gel contained 300 μl of IgG fraction, which is equivalent to 1.2 ml of unfractionated serum.

Line immunoelectrophoresis (Kroll, 1981) was performed by using the same buffer and conditions as those used for crossed immunoelectrophoresis. Agarose gels of 2 mm thickness were cast on 820 mm $\times$ 108 mm sheets of Gel Bond (Pharmacia). The antibody-containing gel was then made by excising a 28 mm $\times$ 60 mm window across the middle of the plate and repouring agarose containing 200 μl of antiserum. The sample, solubilized mung-bean SMP, was applied at the cathode end of the plate on a 12 mm $\times$ 60 mm filter-paper wick (Whatman no. 1). Electrophoresis, again at 4°C , was carried out for 18 h at 3V/cm.

After electrophoresis, both crossed- and line-immunoelectrophoresis plates were washed and pressed dry in the same way: soluble antigen and excess antibodies were removed by pressing nearly dry under one sheet of chromatography paper and three sheets of filter paper; the plates were then washed for 15 min in buffer [100 mM-NaCl/20 mM-Tris/HCl (pH 7.4)/5 mM-EDTA/0.1% (w/v) Triton X-100]. Pressing and washing were repeated twice.

NADH dehydrogenase activity was detected by staining with 100 μM -NADH and Nitro Blue Tetrazolium (1 mg/ml) in 100 mM-Tris/HCl buffer, pH 7.4. Colour development required about 30 min. Where appropriate, protein bands were revealed by drying the plate and staining with Coomassie Brilliant Blue R.

Bands which stained for NADH dehydrogenase activity were excised from line-immunoelectrophoresis plates and incubated with Laemmli stacking-gel buffer, made 1% (w/v) with respect to SDS and mercaptoethanol, for 30 min at 60°C . Agarose was then removed by centrifugation at 5000 *g* for 10 min and the supernatant was analysed on gradient 12–16% (w/v)-polyacrylamide Laemmli (1970) gels. Protein bands were then revealed by silver staining (Eschenbruch & Burk, 1981).

Protein blotting was carried out after electrophoresis of SDS-solubilized ox heart and mung-bean SMP on gradient 11–16% (w/v)-polyacrylamide gels. Transfer to nitrocellulose was performed in Laemmli running buffer, to which 20% (v/v) methanol had been added, for 2.5 h at 27 V and 4°C . Antibody identification of transferred polypeptides was performed at room temperature in phosphate-buffered saline, which contained 0.5% (w/v) bovine serum albumin as follows: unoccupied protein-binding sites were blocked by incubating with buffer and shaking for 16 h; antibody binding was performed by incubating with a 200-fold dilution of the appropriate antiserum; unbound antibodies were removed by washing twice for 1 h; bound antibodies were then detected by using a second antibody, donkey anti-rabbit IgG-biotin conjugate, and an enzyme-detecting system (biotin-streptavidin-horseradish peroxidase complex) as recommended by the makers (Amersham International).

Other methods and materials

NADH:ubiquinone-1 oxidoreductase activity was measured as described previously (Cottingham & Moore, 1984). Protein concentration was determined by the method of Lowry *et al.* (1951). All chemicals, unless otherwise stated, were from Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS

The pooled antisera raised to SDS-solubilized mung-bean SMP were initially characterized by the immunoprecipitation of NADH:ubiquinone-1 oxidoreductase activity from Triton X-100-solubilized SMP (Fig. 1). The maximum loss of activity from the supernatant, even with a 2-fold excess of antiserum, was only 65%. The failure to immunoprecipitate all of the supernatant activity implies the presence of an enzyme with NADH dehydrogenase activity which does not cross-react with the mixed antisera to whole SMP. The simplest interpretation of this observation is that there are a minimum of two distinct dehydrogenases present. The immunoprecipitation of rotenone-sensitive activity could not be tested under these conditions because solubilization with Triton X-100 destroys sensitivity to this inhibitor (I. R. Cottingham & A. L. Moore, unpublished work). A similar effect has been observed with mammalian complex I (C. I. Ragan, unpublished work).

The pooled antisera to mung-bean SMP were also analysed by crossed immunoelectrophoresis. At least ten different antibody populations, as judged by staining for protein, were detected (Fig. 2,A). The staining of an identical plate for NADH dehydrogenase activity showed the presence of two enzymes (Fig. 2,B). The intersection of these two peaks means that they represent immunologically distinct enzymes. The small faint peak on the

Fig. 1. Titration of immunoprecipitable NADH:ubiquinone-1 oxidoreductase activity

Triton X-100-solubilized mung-bean SMP (15 µg of protein) were incubated for 16 h at 40 °C with increasing volumes of anti-(mung bean SMP) antisera. NADH:UQ-1 oxidoreductase activity was then measured before and after the removal of insoluble antibody-antigen complexes by centrifugation at 100000 *g* for 30 min. The activity is the supernatant dehydrogenase activity measured after centrifugation expressed as a percentage of the supernatant activity measured before. The total volume of each incubation was 225 µl.

Fig. 2. Crossed-immunoelectrophoresis of Triton X-100-solubilized mung-bean SMP

Crossed immunoelectrophoresis was performed on solubilized SMP (20 µg of protein) as described in the Materials and methods section. Two identical plates were run; plate A was stained for protein and plate B for NADH dehydrogenase activity. During electrophoresis in the first dimension the cathode was on the right-hand side of each plate. It was subsequently moved to the bottom of each plate for electrophoresis in the second dimension.

right-hand side is probably due to aggregation, rather than the presence of another enzyme, because its shape is identical with that of the large right-hand peak.

Crossed immunoelectrophoresis demonstrates the presence of two antibody populations that are capable of immunoprecipitating two different dehydrogenases. Combined with the result of the immunoprecipitation experiment, which showed that not all of the NADH:ubiquinone-1 oxidoreductase activity could be removed from the supernatant (Fig. 1), this observation indicates that the inner membrane of plant mitochondria contains at least three immunologically distinct NADH dehydrogenases. However, it has been reported that plant mitochondria possess both NADH and NADPH dehydrogenase activities (Arron & Edwards, 1980). In view of this, another crossed-immunoelectrophoresis plate was stained with NADPH as substrate. No reaction was observed, indicating that neither of the activities detected by immunoelectrophoresis is specific for this substrate (I. R. Cottingham & A. L. Moore, unpublished work). This negative experiment does, however, function as a control for substrate-independent reactions with Nitro Blue Tetrazolium, the so-called 'nothing dehydrogenase', which have been observed under some conditions (Heeb & Gabriel, 1984). The substrate-specificity of the material which failed to immunoprecipitate after incubation with excess antiserum (Fig. 1) was not tested.

The two NADH dehydrogenases detected after crossed immunoelectrophoresis were further characterized by line immunoelectrophoresis, which has the advantage that immunoprecipitate bands can be separated with a lower risk of cross-contamination. Conditions were chosen to optimize the separation of the two dehydrogenase-staining bands from each other (Fig. 3,B) and from other proteins (Fig. 3,A). These dehydrogenase bands were then cut out from the agarose gel, subjected to SDS/polyacrylamide-gel electrophoresis and silver-stained for protein. The material from the band with the highest mobility, corresponding to the right-hand peak after crossed immunoelectrophoresis (Fig. 2,B), was found to contain five major polypeptides with molecular masses of more than 100, 76, 62, 41, and 25 kDa (Fig. 4, track A). Under the conditions used to prepare the samples for

Fig. 3. Line immunoelectrophoresis of Triton X-100-solubilized mung-bean SMP

Line immunoelectrophoresis was performed on solubilized mung-bean SMP as described in the Materials and methods. A complete plate (only the antibody-containing window is shown in the Figure) was cut in two and the halves stained for protein (A) and NADH dehydrogenase activity (B). The arrow shows the direction in which most proteins, excluding those with pI values over 8.6, migrate. The cathode was applied at the bottom edge of the plate, below the sample wick.

Fig. 4. Analysis of excised line immunoelectrophoresis NADH dehydrogenase-staining bands by SDS/polyacrylamide-gel electrophoresis

Dehydrogenase-staining bands were excised and applied to SDS/polyacrylamide gels as described in the Materials and methods section. Protein bands were revealed by silver staining. Track A corresponds to the NADH dehydrogenase shown in the uppermost band, nearest the anode, in Fig. 3. Track B is from the lower band in the same Figure. The origin is at the top. Molecular masses (in kDa) are marked and were determined from the relative mobility of standards run in adjacent tracks.

electrophoresis [SDS and mercaptoethanol were added to a final concentration of 0.1% (w/v) and the sample incubated at 60 °C for 2 min], IgG molecules do not dissociate fully and are thus visible as broad bands with a molecular mass in excess of 100 kDa. However, some breakdown does occur, and the broad band at the 25 kDa position is probably attributable to the IgG light chain. The three remaining protein bands are likely components of a plant mitochondrial NADH dehydrogenase. The line-immunoelectrophoresis band of lower mobility was found to contain only one polypeptide, in addition to the IgG bands, with a molecular mass of 53 kDa (Fig. 4, track B).

The second immunological approach examined the cross-reaction between antibodies, raised to the entire mammalian complex I and to four purified subunits, with the equivalent enzyme in plant mitochondria. Antibodies were produced to SDS-denatured ox heart complex I and subunits with molecular masses of 75, 49, 30 and 13 kDa as described elsewhere (Cleeter *et al.*, 1985). The antiserum to whole complex I cross-reacts strongly with numerous polypeptides in ox heart SMP (Fig. 5, track A). These include proteins with molecular masses of 75, 51, 49, 42, 39 and 24 kDa, and other smaller ones, including the 13 kDa polypeptide. Two bands representing impurities in complex I are also visible: the transhydrogenase with a molecular mass in excess of 85 kDa and one of the large ATPase subunits at 58 kDa. The band just below the 75 kDa subunit is likely to be due to breakdown. The antisera raised to the 75, 49, 30 and 13 kDa subunits all gave highly specific cross-reactions

Fig. 5. Protein blotting of ox heart and mung-bean SMP

Two identical SDS/Laemmli gradient polyacrylamide gels were run: one with SDS-solubilized ox heart SMP applied in a continuous band across the gel (tracks A–E) and the other with mung-bean SMP (tracks F–J). After electrophoretic transfer to nitrocellulose sheets, both blots were cut into strips and treated separately with various antisera. Tracks A and F were blotted with anti-(ox heart complex I) antibodies; tracks B and G, with anti-(75 kDa polypeptide) antiserum; tracks C and H, with anti-(49 kDa polypeptide) antiserum; tracks D and I, with anti-(30 kDa polypeptide) antiserum; and tracks E and J with anti-(13 kDa polypeptide) antiserum. Molecular masses are shown in kDa.

with single polypeptides in ox heart SMP (Fig. 5; tracks B, C, D, and E respectively).

The cross-reaction of these antisera with mung-bean SMP was much weaker than with ox heart SMP (Fig. 5; tracks F–J). Before discussing the cross-reactions of individual antisera, it should be noted that an 'artefactual band', the appearance of which depends only on the presence of the substrates of the enzyme-detection system, is visible in the position corresponding to a molecular mass of 78 kDa. The anti-(complex I) antisera cross-reacts with five polypeptides in mung-bean SMP with molecular masses of 58, 55, 50, 46 and 36 kDa (Fig. 4, Track F). The two highest-molecular-mass bands probably correspond to the large subunits of the plant mitochondrial F_1 -ATPase, since the ox heart preparation contains these as contaminants. Evidently the conservation of antigenic sites between the two tissues is greater for the ATPase than for complex I. The subunit with a molecular mass of 50 kDa might correspond to the large subunit, found at 51 kDa in ox heart, of the flavoprotein fragment of complex I. The band, with a molecular mass of 46 kDa, could be related to the 49 kDa polypeptide, because an equivalent band was observed with the antisera specific for that polypeptide (Fig. 4, track H). The band with the lowest molecular mass could be equivalent to either the 42 or the 39 kDa polypeptide in ox heart.

The cross-reaction of the subunit-specific antisera with mung-bean SMP is very weak. No band is visible with the anti-(75 kDa polypeptide) antiserum, although the possibility exists that any cross-reaction is obscured by the artefact. In order to check that this was not the case, immunodetection of a similar blot was carried out by using ^{125}I -protein A followed by autoradiography; no cross-reaction was detected (I. R. Cottingham & A. L. Moore, unpublished work). In view of the susceptibility of the 75 kDa subunit in ox heart SMP to extensive proteolysis (M. W. J. Cleeter & C. I. Ragan, unpublished work), a 'cocktail' of proteinase inhibitors was added before solubilization with SDS, but again no cross-reaction was detected. This shows that, if the equivalent polypeptide exists in mung-bean complex I, it is either extremely labile or so antigenically different that it fails to cross-react with polyclonal antibodies to the ox heart enzyme. This lack of conservation of antigenic sites on ox heart and mung-bean polypeptides may also explain the absence of cross-reactivity between the ox heart transhydrogenase (the high-molecular-mass band on track A, Fig. 5) and the equivalent polypeptide in mung-bean SMP (Fig. 5, track F). Two of the three remaining subunit-specific antisera cross-react with polypeptides in mung-bean SMP. The antiserum raised to the 49 kDa polypeptide cross-reacts with a protein of 46 kDa molecular mass (Fig. 5, track H). It is unlikely that this difference in size is due to proteolysis, since it is unaffected by the inclusion of proteinase inhibitors. The strongest cross-reaction was observed with the antiserum to the 30 kDa polypeptide, but again at a decreased molecular mass of 27 kDa (Fig. 5, track I). Finally, the antiserum to the smallest subunit failed to cross-react with mung-bean SMP (Fig. 5, track J).

It is reasonable to assume that, if antibodies raised to ox heart complex I cross-react, albeit weakly, with mung-bean SMP, then the converse should be observed. This was tested by blotting purified ox heart complex I with the pooled antisera raised to mung-bean SMP

Fig. 6. Protein blotting of ox heart complex I

Purified ox heart complex I (20 μg of protein) was resolved by electrophoresis on an SDS/Laemmli gradient polyacrylamide gel and then blotted on to nitrocellulose. The blot was then treated with 100 μl of the mixed antisera raised to SDS-solubilized mung-bean SMP. Molecular masses were determined by a comparison of relative mobility with known polypeptides on the original gel after staining for protein. Molecular masses are shown in kDa.

(Fig. 6). Cross-reaction with five polypeptides with molecular masses of more than 80, 60, 56, 33 and 30 kDa was observed, in addition to some small breakdown products. Taken in order of decreasing molecular mass, these probably represent the transhydrogenase, the two largest subunits of the ATPase and two complex-I subunits. The strongest of the cross-reactions with complex I, as might be predicted from the results with the subunit antibodies (Fig. 5), was with the 30 kDa polypeptide.

It is interesting that the antibodies to mung-bean SMP identify the 33 kDa polypeptide in complex I. This is consistent with two recent observations. Firstly, that the mitochondrially coded translation product of an unassigned reading frame, URF1 (Chomyn *et al.*, 1985), has been identified as the 33 kDa polypeptide of bovine complex I (S. Patel, C. I. Ragan, A. Chomyn & G. Attardi, unpublished work). The second observation is that a close sequence homology with the mammalian gene, URF1, has been detected in the mitochondrial genome of maize (*Zea mays*) (Scazzocchio *et al.*, 1983). Such sequence homology might be expected to produce the antigenic similarity indicated by our results.

DISCUSSION

The present paper is the first to describe the use of immunological methods for the analysis of plant mitochondrial NADH dehydrogenases. In addition to confirming the existence of three different dehydrogen-

ases, a possibility which has long been suspected, this approach has enabled the preliminary characterization of two of these enzymes. One contains some or all of three polypeptides with molecular masses of 76, 62 and 41 kDa. The other comprises a single polypeptide with a molecular mass of 53 kDa. The employment of an immunological approach has also confirmed the presence, on the basis of antibody cross-reactivity, of an enzyme equivalent to mammalian complex I.

So far it has not been possible to show which of the two dehydrogenases, identified by immunoelectrophoresis, is responsible for either the rotenone-sensitive or rotenone-insensitive activities. However, two of the polypeptides of the three protein dehydrogenase, with molecular masses of 76 and 62 kDa, are similar in size to two of the polypeptides, at 76 and 65 kDa, reported in a purified preparation of the external NADH dehydrogenase (Cottingham & Moore, 1984). Despite this, the possibility that this preparation contains components of complex I, rather than the external NADH dehydrogenase, cannot be eliminated, because of the failure of the antibody to the 75 kDa subunit to cross-react with the mung-bean enzyme. The other preparation of the external NADH dehydrogenase (Cook & Cammack, 1984, 1985) contains three polypeptides with molecular masses of 55, 39.2 and 33.9 kDa. This bears no resemblance to the three polypeptide dehydrogenases identified from line immunoelectrophoresis, but the 55 kDa polypeptide may correspond to the 53 kDa protein of the other dehydrogenase.

Another possibility for the 53 kDa dehydrogenase is that it represents a subfraction of complex I. This is suggested by the observation that mammalian complex I can be chaotropically resolved into fragments which include an active, but rotenone-insensitive, ubiquinone-1 reductase. Polypeptide analysis of this flavoprotein fraction shows the presence of three proteins with molecular masses of 51, 24 and 10 kDa (Galante & Hatefi, 1979). The possibility of complex I breakdown has already been invoked to explain the isolation of a two polypeptide NADH dehydrogenase, with molecular masses of 48 and 25 kDa, from *Paracoccus denitrificans* (George & Ferguson, 1984). It could be that, in the case of the 53 kDa dehydrogenase, the presence of the 25 kDa polypeptide is obscured by the IgG band.

If it is subsequently found that the 53 kDa dehydrogenase from plant mitochondria is not a fragment of complex I, then it could set an interesting precedent. To date the simplest mitochondrial dehydrogenase, which donates reducing equivalents directly at the level of ubiquinone, is glycerol-3-phosphate dehydrogenase. This consists of a single polypeptide with a molecular mass of 76 kDa, and contains an FAD prosthetic group (Cole *et al.*, 1978; Cottingham & Ragan, 1980). It would be very interesting if the same function could still be performed by an enzyme 50% smaller.

The detection of two polypeptides in plant SMP membranes, at 46 and 27 kDa, which cross-react with antibodies to the mammalian subunits gives a good demonstration of the common ancestry of the mitochondrial enzymes in two different kingdoms. However, the lack of cross-reactivity with antibodies to the 75 kDa polypeptide is surprising. In the mammalian enzyme this protein contains one of the redox-active iron-sulphur centres which are functionally important in energy conservation at the first site of ATP synthesis (Ohnishi

et al., 1985). As such it might be expected to be highly conserved, but this does not appear to be the case. It is also puzzling that the transhydrogenase was only detected by the antibodies to plant SMP and not by the antibodies to mammalian complex I. The one-way cross-reaction does, however, indicate that this protein is also present in plant mitochondria.

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