Structural relationships between the NADH dehydrogenases of *Paracoccus denitrificans* and bovine heart mitochondria as revealed by immunological cross-reactivities

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An antibody raised against two subunits (M_r 48000 and 25000) of NADH dehydrogenase from *Paracoccus denitrificans* cross-reacts with one of more than 20 polypeptides that form the bovine heart mitochondrial NADH dehydrogenase. The cross-reacting subunit has M_r 51000 and is believed to be the NADH-binding subunit of the enzyme. Antibodies raised against certain subunits of the bovine heart NADH dehydrogenase were tested for cross-reactivity with *P. denitrificans* cytoplasmic membranes. Of those tested, only one, an antibody specific for the 49 kDa subunit of mitochondrial NADH dehydrogenase, cross-reacted with the bacterial membranes. It recognised a polypeptide of approximate M_r 46000. This is an indication for a previously undetected third subunit of NADH dehydrogenase from *P. denitrificans*. The immunological cross-reactions indicate that the NADH dehydrogenases from *P. denitrificans* and bovine heart mitochondria are related structurally.

NADH dehydrogenase (Paracoccus denitrificans) *Immunoblotting* *Respiratory chain* *Mitochondria*

1. INTRODUCTION

There has been considerable discussion as to the possible evolutionary relationship between mitochondria and the bacterium *Paracoccus denitrificans*. This was originally based on the similarities between the respective electron transfer components as judged by spectroscopic properties and inhibitor sensitivities [1,2]. More recently, the purifications of cytochrome *c* oxidase [3] and a cytochrome bc_1 complex [4] from *P. denitrificans* membranes have shown that they possess a very much simpler polypeptide composition than their mitochondrial counterparts whilst remaining functionally analogous to them. Cytochrome *c* oxidase from mitochondria consists of at least 7 polypeptide chains whereas that from *Paracoccus* consists of only two. However, these two subunits have similar M_r values to the largest subunits of the mitochondrial enzyme and are now thought to be equivalent to these two major catalytic subunits of the mitochondrial enzyme. Further evidence to support this view is the fact that an antibody raised to the smaller subunit of *P. denitrificans* cytochrome *c* oxidase cross-reacts immunologically with the polypeptide of similar M_r in yeast mitochondrial cytochrome *c* oxidase and, but to a lesser extent, with subunit II from the bovine heart enzyme. A further example of immunological cross-reactivity has been observed between the cytochromes c_1 of *P. denitrificans* and yeast mitochondria [6] even though the M_r of the *Paracoccus* cytochrome c_1 is approximately twice

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that of the mitochondrial protein. Such cross-reactivities indicate that these proteins must have a considerable amount of structural homology. This is supported by the finding that there is sequence homology between a defined segment of the *Paracoccus* cytochrome c_1 and beef heart mitochondrial cytochrome c_1 [6].

This paper is concerned with the relationships between the NADH dehydrogenases of bovine heart mitochondria and *P. denitrificans*. The NADH dehydrogenase of mammalian mitochondria (often known as complex I) is the most complex and least well-characterised component of the respiratory chain. It consists of more than 20 different subunits [7] with some polypeptides coded for by the nuclear and some by the mitochondrial genome [8]. Recently, an NADH dehydrogenase from *P. denitrificans* has been identified [9]. This enzyme consists of just two subunits, of M_r 48000 and 25000. A procedure has been developed for preparing monospecific antibodies to this enzyme using crossed immunoelectrophoresis methodology even though the enzyme has not been purified to homogeneity [10]. This paper reports the use of such antiserum to test for cross-reactivity with the bovine heart mitochondrial NADH dehydrogenase. Antibodies raised against specific subunits of mitochondrial complex I have also been used to probe for additional cross-reactions with cytoplasmic membranes from *P. denitrificans*.

2. MATERIALS AND METHODS

Bovine heart mitochondrial complex I [11] and cytoplasmic membrane vesicles from *P. denitrificans* [12,13] were prepared as described.

Monospecific antibody to the two-subunit NADH dehydrogenase of *P. denitrificans* was prepared according to George and Ferguson [10]. Antibodies raised against specific subunits of mitochondrial complex I were obtained by the method of Cleeter et al. [14].

SDS-polyacrylamide gel electrophoresis was performed on 10–20% linear gradient gels as in [15], modified by the method of Douglas and Butow [16]. Immunoblotting was carried out according to Towbin et al. [17] with the modification of Batteiger et al. [18]. The substrate for the peroxidase-linked anti-(rabbit immunoglobulin) was 3,3′-diaminobenzidine tetrahydrochloride.

Fig.1. Immunoblotting of *P. denitrificans* plasma membranes and complex I with anti-(*P. denitrificans* NADH-dehydrogenase) antibodies. A 10–20% linear gradient SDS-polyacrylamide gel was loaded with 100 μg purified bovine heart mitochondrial complex I (lane 1) and 80 μg *P. denitrificans* plasma membranes (lane 2). The proteins were transferred to nitrocellulose and then incubated with a 1:500 dilution of anti-(*P. denitrificans* NADH dehydrogenase) antibodies. Cross-reaction was detected by incubation with horseradish peroxidase-conjugated anti-(rabbit immunoglobulins), followed by staining for peroxidase activity.

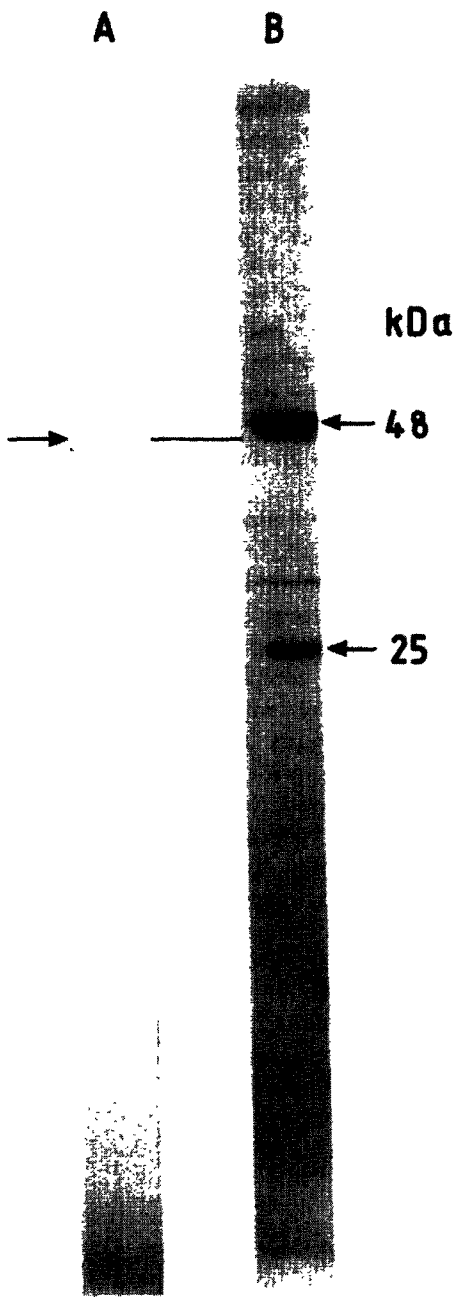

Fig.2. Immunoblotting of *P. denitrificans* plasma membranes with antiserum to the 49 kDa polypeptide of bovine heart mitochondrial complex I. A 10–20% linear gradient SDS polyacrylamide gel was loaded with 80 μg *P. denitrificans* plasma membranes. The proteins were transferred to nitrocellulose and immunoblotted with a 1:100 dilution of antiserum raised against the 49 kDa polypeptide of bovine heart mitochondrial complex I (A). After detection by peroxidase staining the nitrocellulose was washed well and then incubated with a 1:100 dilution of anti-(*P. denitrificans* NADH dehydrogenase) (B). Cross-reaction was detected by incubation with horseradish peroxidase-conjugated anti-(rabbit immunoglobulins), followed by staining for peroxidase activity. The arrow on the left indicates the polypeptide that cross-reacts with the antiserum to the 49 kDa polypeptide of bovine heart NADH dehydrogenase and the arrows and molecular mass on the right denote the two subunits of the *P. denitrificans* NADH dehydrogenase, against which the antibody used in the second cycle of immunoblotting was raised.

Horseradish peroxidase-conjugated anti-(rabbit immunoglobulin) and 3,3′-diaminobenzidine tetrahydrochloride were purchased from Sigma.

3. RESULTS AND DISCUSSION

Monospecific antiserum raised against the two-subunit NADH dehydrogenase of *P. denitrificans* has been shown previously to contain components that cross-react immunologically with both of the two identified subunits of the enzyme [10]. This antibody was tested for cross-reactivity with complex I from beef heart mitochondria. Fig.1 shows that a single polypeptide component of complex I, of M_r ~51000, was recognised by the anti-(*P. denitrificans* NADH dehydrogenase) serum. The specificity of this reaction was further confirmed by the finding that immunoblotting of sub-mitochondrial particles with the antiserum against *P. denitrificans* NADH dehydrogenase also showed only the same single cross-reacting band as seen with complex I. Thus, either the 48 or 25 kDa subunits of the bacterial NADH dehydrogenase must have structural features in common with the 51 kDa subunit of the mitochondrial enzyme. The 51 kDa subunit of complex I probably plays a central role in the catalytic activity of the enzyme as it is thought to be the binding site for NADH [19].

Complex I can be split using chaotropic agents to give 3 distinct fractions [20]. These are the flavoprotein fragment which contains FMN [21] and two iron-sulphur centres [22], an iron-sulphur fragment which contains 4 iron-sulphur centres [23], and an insoluble residue which contains a further 2 or 3 iron-sulphur centres. The flavoprotein

fragment consists of only 3 subunits of M_r 51000, 24000 and 9000–10000 yet retains NADH dehydrogenase activity with ubiquinone analogues [21]. The finding of cross-reactivity between the two-subunit NADH dehydrogenase from *Paracoccus* and the 51 kDa subunit of complex I is in accord with the previously noted, probable relationship between the flavoprotein fragment of complex I and the bacterial enzyme [9]. It has been questioned whether the two subunits of the *Paracoccus* NADH dehydrogenase represent the complete NADH-ubiquinone oxidoreductase or whether they are a flavoprotein fragment analogous to that of complex I which may be produced by splitting of the enzyme during detergent solubilisation [10]. It is known from EPR studies [24] that *Paracoccus* membranes contain a similar array of iron-sulphur centres associated with NADH dehydrogenase activity to those found in complex I. In complex I these are distributed over many polypeptide chains [25]. It is hard to imagine how these could all be accommodated on just the two subunits of the bacterial enzyme. This prompted us to consider whether there might be one or more additional subunits to the *Paracoccus* enzyme which were lost under the conditions used for detergent solubilisation or crossed immunoelectrophoresis. Such putative additional subunits might be expected to have the same role in the bacterial enzyme as the subunits of the iron-sulphur fragment do in complex I. To probe for such additional polypeptides, antibodies specific for subunits of the iron-sulphur fragment of M_r 75000, 49000, 30000 and 13000 were tested by immunoblotting for cross-reaction with cytoplasmic membranes from *P. denitrificans*. Of these only the antiserum raised against the 49 kDa subunit showed cross-reactivity (fig.2A). The cross-reaction was with a polypeptide of very similar molecular mass to the 48 kDa polypeptide of the *Paracoccus* NADH dehydrogenase. To distinguish whether this cross-reactivity represents the finding of a distinct subunit of the bacterial NADH dehydrogenase or whether the antibody recognises the previously identified 48 kDa subunit, a second cycle of immunoblotting was performed using the anti-(*P. denitrificans* NADH dehydrogenase) serum (fig.2B). This clearly showed that the polypeptide that cross-reacted with the antiserum against the mitochondrial 49 kDa subunit was distinct from the 48 kDa subunit of the bacterial enzyme. An M_r of 46000 was estimated for the cross-reacting subunit.

The present work provides further evidence for the structural relationship between the components of the respiratory chain in *P. denitrificans* and those in mitochondria. Cross-reaction between antibodies to *Paracoccus* proteins and proteins from yeast mitochondria have been observed before [5,6]. However, it is surprising that although the respiratory chain complexes have been best characterised from bovine heart mitochondria there has been only one report of a rather weak cross-reaction between *Paracoccus* proteins and their counterparts in bovine heart mitochondria [5].

The present observations indicate that the NADH dehydrogenase of *P. denitrificans* is related to the mitochondrial enzyme. These results, together with the complexity of complex I, and the generally simpler polypeptide composition of the electron transfer components of *Paracoccus* in comparison to their mitochondrial counterparts, give further impetus to the study of NADH dehydrogenase from *P. denitrificans*. To date this enzyme has proved refractory to purification although two polypeptides and its FMN cofactor have been identified by immunological methods [9,10]. The cross-reaction of *P. denitrificans* cytoplasmic membranes with antiserum to the 49 kDa subunit of mitochondrial complex I suggests that there could be a third subunit of the *Paracoccus* enzyme that ought to copurify, under appropriate conditions, with the previously described two subunit enzyme.

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