Immunochemical probing of the structure and cofactor of NADH dehydrogenase from *Paracoccus denitrificans*

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Monospecific antibody to the respiratory NADH dehydrogenase from *Paracoccus denitrificans* was prepared by using as antigen specific immunoprecipitates containing NADH dehydrogenase which were excised from crossed-immunoelectrophoresis plates. The latter were run with selectively solubilized plasma membranes and antibodies against plasma membranes. The antibody immunoprecipitated NADH dehydrogenase from *P. denitrificans* membranes biosynthetically labelled with ^{14}C and solubilized with a wide range of detergents. All immunoprecipitates contained the two subunits of M_r 48000 and 25000, in an approximate 1:1 stoichiometry, that had previously been assigned to NADH dehydrogenase. A polypeptide of M_r 46000 in *P. denitrificans* membranes, previously shown to cross-react with a subunit-specific antibody to mitochondrial NADH dehydrogenase (complex I), was not detected in any immunoprecipitate. Under some conditions a third polypeptide, of M_r 31000, was also detected, but in variable and non-stoichiometric amounts relative to the two other subunits. It was concluded that this polypeptide was incorporated into the immunoprecipitates as an artefact and that the polypeptides of M_r 48000 and 25000 are the sole polypeptides firmly identified in the NADH dehydrogenase. Flavoproteins were specifically radiolabelled by growth of *P. denitrificans* in the presence of [^{14}C]riboflavin. Crossed immunoelectrophoresis of membranes from such cells showed that succinate dehydrogenase contained flavin, but that there was no detectable flavin in NADH dehydrogenase under these conditions. Analysis of excised immunoprecipitates of succinate dehydrogenase showed that flavin was covalently bound to a polypeptide of M_r 56000. Flavin was retained by NADH dehydrogenase under mild conditions of detergent solubilization. Subsequent immunoprecipitation, followed by analysis of the acid-extracted flavin, established that FMN is a cofactor, in common with mitochondrial NADH-ubiquinone oxidoreductase (complex I).

INTRODUCTION

Many of the electron-transport proteins of aerobically grown *Paracoccus denitrificans* have redox centres and sensitivities to inhibitors similar to those of their mitochondrial counterparts [1–4]. There is a difference, however, at the structural level because both the cytochrome aa_3 oxidase [5] and cytochrome bc_1 complex [6] of *P. denitrificans* have considerably simpler polypeptide compositions than the mitochondrial enzymes. The less complex structures of the bacterial proteins can contribute to correlation of structures with function.

The most complex enzyme of the mitochondrial respiratory chain is the NADH dehydrogenase (often known as complex I), which consists of more than 20 polypeptide chains in both bovine heart [7] and *Neurospora crassa* [8]. The characterization from *P. denitrificans* of the corresponding enzyme, which has proved refractory to purification [9], would be of considerable interest, given the expectation that it would have a less complex polypeptide structure, and yet be functionally similar to the mitochondrial enzyme. The original evidence for similarity between the enzymes from *P. denitrificans* and mitochondria was inhibition by rotenone [3] and identification of Fe-S centres by e.p.r. spectroscopy [4]. Subsequently we have identified two subunits (M_r 48000 and 25000) of NADH dehydro-

genase from *P. denitrificans* by a technique involving excision of immunoprecipitate arcs from crossed-immunoelectrophoresis (CIE) plates [9]. A possible interpretation is that these two subunits correspond to the two largest polypeptides of a three-subunit fragment of the mitochondrial enzyme, which is usually termed the flavoprotein fragment [10]. This fragment catalyses transfer of electrons from NADH to a variety of electron acceptors, but does not possess the complete NADH-ubiquinone oxidoreductase activity of complex I [10]. A relationship between the identified subunits of *P. denitrificans* NADH dehydrogenase and the flavoprotein fragment of the mitochondrial enzyme is consistent with the recognition of the largest (M_r 51000) polypeptide of the latter by an antiserum raised against the two identified subunits of the *P. denitrificans* enzyme [11]. The present paper describes the method for preparation of this antiserum and its use in immunoprecipitation experiments designed to identify the flavin cofactor and to characterize further the composition of the enzyme.

There is no direct evidence as to whether or not the two identified subunits constitute the complete NADH dehydrogenase of *P. denitrificans* [9], although a possible third subunit is suggested by the observation that subunit-specific antiserum against one of the polypeptides (M_r 49000) of the iron-sulphur fragment [12] of mitochondrial NADH dehydrogenase cross-reacts with a

Abbreviations used: Chaps, [3-(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; CIE, crossed immunoelectrophoresis; LDAO, lauryldimethylamine oxide; LDS, lithium dodecyl sulphate; PAGE, polyacrylamide-gel electrophoresis.

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polypeptide (M_r 46000) in the cytoplasmic membranes of *P. denitrificans*. [11]. Thus a particular emphasis of the present paper is detailing the use of the antiserum raised against the two subunits of NADH dehydrogenase from *P. denitrificans* to immunoprecipitate the enzyme under a variety of conditions. The aim of the work was to test for the presence of the M_r -46000 subunit and/or other previously unidentified polypeptides in the immunoprecipitated enzyme.

MATERIALS AND METHODS

Cell growth and membrane isolation

Paracoccus denitrificans (N.C.I.B. 8944) was grown anaerobically in batch culture with succinate as substrate and nitrate as terminal electron acceptor, as described by Burnell *et al.* [13]. Membrane vesicles were prepared from these cells by the method described in [13,14].

For growth of cells on ^{14}C -labelled protein hydrolysate, the sodium succinate concentration of the growth medium was lowered to 1% and the medium was supplemented with 250 μCi of U- ^{14}C -labelled protein hydrolysate. For flavin labelling, the growth medium was supplemented with 250 μCi of D-[2- ^{14}C]riboflavin and was protected from the light at all times to prevent photodecomposition of the riboflavin.

Protein determination

Protein was determined by the method of Lowry *et al.* [15].

Solubilization of *P. denitrificans* membranes

Membrane vesicles were solubilized as described in [9]. Detergent protein ratios used for solubilizing material for running CIE plates were 10 mg/mg of protein for Triton X-100 and 5 mg/mg of protein for LDAO.

For immunoprecipitation experiments, the range of detergents used at various detergent/protein ratios is listed in the Results section (Table 1).

SDS/PAGE and immunoblotting

Linear gradient gels (10–20% acrylamide) were run with the discontinuous buffer system of Laemmli [16] modified as described by Douglas & Butow [17]. ^{14}C -labelled protein standards in the M_r range 12300–200000 were from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). M_r values were estimated by the method of Lambin *et al.* [18]. When denaturing PAGE was carried out at low temperature, lithium dodecyl sulphate (LDS) was used in place of SDS in all buffers. LDS/PAGE was carried out at 4 °C.

Fluorography was performed essentially by the method of Bonner & Laskey [19], but the scintillator used was Amplify from Amersham International.

Stoichiometry of radiolabelled polypeptides in immunoprecipitates was determined by excising bands from gels which had been incubated with Amplify and dried down on to filter paper. Excised pieces of gel were placed face upwards in scintillant and counted for radioactivity: 100–300 d.p.m. was observed after correction for background radioactivity in an analogous slice of the gel taken from a blank lane.

Immunoblotting was performed by the method of Towbin *et al.* [20], as modified by Batteiger *et al.* [21], by using a horseradish peroxidase-conjugated second anti-

body. Peroxidase activity was detected by using Bio-Rad horseradish peroxidase colour-development reagent.

Crossed immunoelectrophoresis

Anti-(*P. denitrificans* membrane vesicle) immunoglobulins were prepared as described in [9].

For preparation of monospecific anti-(NADH dehydrogenase) antiserum, the NADH dehydrogenase immunoprecipitate arcs were excised from 23 wet unstained CIE plates and homogenized with 2 ml of 0.9% NaCl to disperse the agarose. This was then mixed with 2 ml of Freund's complete adjuvant to give a very thick emulsion. This provided enough antigen to inject one New Zealand White rabbit with two initial subcutaneous injections and two subcutaneous booster injections 2 weeks later. The rabbit was bled from the mid-ear artery 4 weeks after the initial injections. Further monospecific antibody was prepared by using the immunoglobulins from the initial bleed to run CIE plates. CIE was performed in water-cooled tanks by the method of Owen & Salton [22] as described in [9]. Immunoprecipitates containing NADH dehydrogenase and succinate dehydrogenase were detected by zymogram staining by the method of Owen & Salton [22]. Non-covalently bound flavin was removed from immunoprecipitates on CIE plates by washing in 10% (w/v) trichloroacetic acid [23].

Immunoprecipitation of NADH dehydrogenase

P. denitrificans membranes were solubilized at 4 mg of protein/ml with the detergent concentrations listed in Table 1. Portions (200 μl) of detergent extracts were incubated with an equal volume of pre-immune serum for 1 h at 4 °C, followed by centrifugation at 11 600 g for 10 min. The supernatants were then incubated with 200 μl of anti-(NADH dehydrogenase) serum for a further 1 h at 4 °C, and the pellets obtained after centrifugation were washed with 3 $\times$ 0.5 ml of 20 mM-Hepes/NaOH (pH 7.2)/0.1 M-NaCl and half the concentration of the detergent used in the initial solubilization. The washed immunoprecipitates to be used for determination of polypeptide composition were resuspended in 100 μl of SDS/PAGE sample buffer containing 2% β -mercaptoethanol, boiled for 5 min and then analysed by SDS/PAGE and fluorography. The [^{14}C]flavoprotein-labelled immunoprecipitates were resuspended in 50 μl of immunoprecipitate-washing buffer containing 2% β -mercaptoethanol to disrupt the immunoprecipitate. When large quantities of immunoprecipitated material were required for analysis of unlabelled flavin, membranes were solubilized in taurodeoxycholate (2.5 mg of detergent/mg of protein) and the entire procedure was scaled up 100-fold. The incubation periods were extended to 12 h.

Determination of the flavin cofactor of NADH dehydrogenase

The resuspended immunoprecipitates were treated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid and incubated on ice in the dark for 15 min. Precipitated protein was removed by centrifugation at 11 600 g for 10 min and the supernatant extracted with 4 $\times$ 0.5 ml of diethyl ether. The water layer was applied to a silica-gel plate (DC Kieselgel 60; Merck A.G., Darmstadt, Germany) and run with elution solvent 5% (w/v) Na_2HPO_4 [24]. Standard flavins were detected as

yellow-green fluorescent spots under u.v. light, and radioactive flavins were detected by autoradiography.

Materials

U-¹⁴C-labelled protein hydrolysate, D-[2-¹⁴C]riboflavin and Amplify were purchased from Amersham International (Amersham, Bucks., U.K.). Nonidet P-40, cholic acid, Lubrol PX, sodium taurodeoxycholate, Chaps, riboflavin, FMN, FAD, Chelex-100 and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin were from Sigma. Triton X-100 (scintillation grade) was from BDH, octyl β -D-glucopyranoside and dodecyl β -D-maltoside were from Calbiochem, and LDAO was from Millmaster-Onyx (Oldham, U.K.).

RESULTS

Preparation of monospecific antibody to NADH dehydrogenase from *Paracoccus denitrificans*

In previous work, sufficient immunoprecipitate was obtained by CIE of a partially purified detergent extract of *P. denitrificans* membranes to permit identification of

the NADH dehydrogenase polypeptides by fluorography in conjunction with SDS/PAGE [9]. However, the low intensities of the NADH dehydrogenase immunoprecipitate arcs and overlaps with other immunoprecipitates prevented acquisition of sufficient specific material to use for subsequent production of antibodies (cf. [25]). Consequently, conditions were sought in which NADH dehydrogenase was selectively solubilized from *Paracoccus* membranes, as this was expected to simplify the immunoprecipitation pattern on a CIE plate. It was found that LDAO solubilized NADH dehydrogenase activity with relatively high selectivity, and thus left a much larger insoluble residue than other detergents. When CIE plates were run with a concentrated LDAO extract of *Paracoccus* membranes as antigen, the immunoprecipitation profile was altered sufficiently to permit excision of about half of the NADH dehydrogenase precipitin arc without risk of contamination from other arcs (Fig. 1). The section marked in Fig. 1 was excised from 23 similar CIE plates and injected into a rabbit (see the Materials and methods section).

The antibody obtained from this rabbit was shown to

Fig. 1. Crossed-immunoelectrophoresis pattern of concentrated LDAO-solubilized *P. denitrificans* membrane vesicles against anti-(*P. denitrificans* membrane vesicle) antibodies

The anode is to the left (first dimension) and at the top of the gel (second dimension). Solubilized membranes (80 μ g) were used as antigen. In the second dimension 8 mg of anti-(*P. denitrificans* membrane vesicle) immunoglobulins/ml was incorporated into the agarose. The plate was stained for protein. (b) is marked with broken lines to show the region that could be excised and used for re-injection into a rabbit.

Fig. 2. Crossed immunoelectrophoresis of Nonidet P-40-solubilized *P. denitrificans* membrane vesicles against anti-(NADH dehydrogenase) serum

The anode is to the left (first dimension) and at the top of the gels (second dimension). Solubilized vesicles (50 μ g) were used as antigen. In the second dimension 1 mg of anti-(NADH dehydrogenase) antibody/ml was incorporated into the agarose. (a) Stained for protein; (b) stained for NADH dehydrogenase activity.

Fig. 3. Immunoblot of *P. denitrificans* membranes against anti-(NADH dehydrogenase) serum

P. denitrificans membrane vesicles (80 µg) were run on SDS/PAGE (10–20% gel) and transferred to nitrocellulose paper. This was immunoblotted with a 1:100 dilution of anti-(NADH dehydrogenase) serum, followed by a horseradish peroxidase-linked second antibody and then stained for peroxidase activity. The upper band corresponds to the polypeptide of M_r 48 000, the lower band to that of M_r 25 000.

be monospecific for NADH dehydrogenase. When this antibody was used to run CIE plates with concentrated Nonidet P-40-solubilized membranes as antigen, only one immunoprecipitate arc was seen, and this arc stained

for NADH dehydrogenase activity (Fig. 2). Excision of arcs such as this provided antigen to inject new rabbits for continued production of monospecific antibody.

The monospecific antibody was shown by immunoblotting to contain components that were active against each of the two polypeptides that had been previously identified by fluorography after SDS/PAGE of immunoprecipitates of NADH dehydrogenase taken from CIE plates [9]. Fig. 3 shows an immunoblot, using the anti-(NADH dehydrogenase) antibodies, of an SDS/PAGE run with *P. denitrificans* membranes. The staining pattern clearly shows only the two bands that we had previously characterized.

Immunoprecipitation of NADH dehydrogenase from membranes solubilized in various detergents

Using *P. denitrificans* membranes which had been biosynthetically labelled with ^{35}S , we had shown that excised NADH dehydrogenase immunoprecipitates from CIE plates contained just two ^{35}S -labelled polypeptides [9]. Alternative detergents to the Nonidet P-40 used in the original work were tested with a view to identifying additional polypeptides of NADH dehydrogenase in immunoprecipitates. There is precedent for an antibody raised against less than the total number of polypeptides of a protein complex being capable of immunoprecipitating the entire complement of polypeptides (e.g. [26,27]).

P. denitrificans membranes were biosynthetically labelled with ^{14}C and solubilized with a range of detergents under a variety of different conditions. The NADH dehydrogenase was immunoprecipitated with

Table 1. Polypeptide composition of immunoprecipitated NADH dehydrogenase

Biosynthetically ^{14}C -labelled *P. denitrificans* membrane vesicles were solubilized at a protein concentration of 4 mg/ml under one of the listed sets of conditions. NADH dehydrogenase was immunoprecipitated from 200 µl portions of each detergent extract. Immunoprecipitated material was washed three times in 20 mM-Hepes/NaOH (pH 7.2) and half the concentration of the detergent used in the solubilization. The final immunoprecipitates were resuspended in SDS/PAGE sample buffer containing 2% (v/v) β -mercaptoethanol and boiled for 5 min (unless otherwise stated), then analysed by SDS/PAGE and fluorography. Additions marked in column 3 were present during both solubilization and the washing procedure. Abbreviation: ND, not determined.

Detergent used	Detergent concn. in solubilization (w/v)	Other varied conditions	$10^{-3} \times M_r$ values of ^{14}C -labelled polypeptides in immunoprecipitate	Approximate stoichiometry of polypeptides in immunoprecipitates
Cholate	4%	—	48, 25	1:1
LDAO	2%	—	48, 25	1:1
Triton X-100	4%	—	48, 31, 25	1:3:1
Triton X-100	2%	—	48, 31, 25	1:3:1
Triton X-100	4%	Sample for SDS/PAGE unboiled	90, 48, 25	0.5:0.5:1
Triton X-100	2%	+2% (v/v) β -mercaptoethanol	48, 31, 25	1:2:1
Cholate	2%	—	48, 31, 25	1:1.5:1
Lubrol PX	2%	—	48, 31, 25	1:1:1
Octyl β -D-glucopyranoside	2%	—	48, 31, 25	1:0.6:1
Dodecyl β -D-maltoside	1%	—	48, 31, 25	1:0.4:1
Taurodeoxycholate	0.5%	—	48, 31, 25*	1:1.4:1
Chaps	2%	—	†	ND
LDAO	2%	+5 mM-NADH	48, 25	ND
Cholate	4%	+5 mM-NADH	48, 25	ND

* Plus minor contaminating polypeptides (see the text).

† Polypeptide composition very similar to that of membrane vesicles.

Fig. 4. Fluorogram showing SDS/PAGE of immunoprecipitated NADH dehydrogenase

^{14}C -labelled *P. denitrificans* membranes were solubilized at a protein concentration of 4 mg/ml in either 4% Triton X-100 (lanes 1 and 2) or 4% cholate (lanes 3 and 4). NADH dehydrogenase was immunoprecipitated from 200 μl of these detergent extracts. After washing, the immunoprecipitates were resuspended in 200 μl of SDS/PAGE sample buffer containing 2% β -mercaptoethanol. Each sample was then divided in two; one half was boiled for 5 min (lanes 2 and 4), and the other half was left at room temperature (lanes 1 and 3). Then 50 μl of each sample was run on SDS/PAGE (10–20% gradient gel), which was subsequently fluorographed. The M_r scale on the left is based on the mobility of standard proteins with the following M_r values: myosin (H-chain), 200000; phosphorylase b, 92500; bovine serum albumin, 68000; ovalbumin, 43000; α -chymotrypsinogen, 25700; β -lactoglobulin, 18400; cytochrome c, 12300.

the monospecific antibody and characterized by using SDS/PAGE followed by fluorography. In all cases the immunoprecipitates clearly contained the same two polypeptides, of M_r 48000 and 25000, that had been previously identified (Table 1). When certain concentrations of detergent were used for solubilization, e.g. 4% cholate or 2% LDAO, these two components were the only polypeptides seen (Fig. 4). Excision of bands from SDS/PAGE gels followed by counting for radioactivity established that these two polypeptides were present in an approx. 1:1 molar ratio (Table 1). When the immunoprecipitation was carried out with Triton X-100 or Nonidet P-40, a third polypeptide band was generally

seen (Fig. 4). This band was of M_r 31000 if the SDS/PAGE sample was boiled in the presence of 2% β -mercaptoethanol, but of M_r 90000 if the sample contained 2% β -mercaptoethanol but was not boiled before SDS/PAGE. In the unboiled sample the intensity of the M_r -48000 band was substantially decreased relative to the boiled sample, whereas the M_r -25000 band remained constant. This suggests that the band of apparent M_r 90000 could reflect an association of the M_r -31000 and -48000 polypeptides, with the aggregate electrophoresing slightly anomalously. The M_r -31000 band was also seen when low concentrations of detergents such as cholate or LDAO, which clearly gave only two bands when used at higher concentrations, were used for the solubilization, as well as with several of the other detergents tested (Table 1). However, this band was present in non-stoichiometric and variable amounts relative to the two polypeptides definitely assigned to NADH dehydrogenase. With some detergents it appeared as the dominant polypeptide in the immunoprecipitate, whereas in other cases it was only a minor component (Table 1). Furthermore, there was a substantial decrease in the intensity of this band when the solubilization and immunoprecipitation procedures were carried out in the presence of 2% β -mercaptoethanol. Under these conditions the M_r -48000 and -25000 bands were clearly visible. This suggests that the extra polypeptide represents an artefactual association of another polypeptide from the *Paracoccus* membranes with the NADH dehydrogenase immunoprecipitate. This is consistent with the absence of such a band after SDS/PAGE of NADH dehydrogenase immunoprecipitates excised from CIE plates run with Nonidet P-40-solubilized membranes in our original work [9].

Under the conditions of detergent solubilization used for the immunoprecipitations (Table 1), NADH dehydrogenase was insensitive to rotenone. Sensitivity to rotenone was retained only under conditions of solubilization, including up to 2% (w/v) of Chaps or lower concentrations of bile salts than shown in Table 1, from which immunoprecipitates contained a profile of polypeptides very similar to that for whole membranes. (Table 1).

When *Paracoccus* membranes solubilized in 0.5% taurodeoxycholate were used in the immunoprecipitation (as was the case for flavin analysis), contaminating bands of considerably fainter intensity were seen in the fluorogram. These could principally be assigned to the α and β bands of ATPase, a major component of the membrane (C. L. George & S. J. Ferguson, unpublished work). Immunoprecipitations performed in the presence of the enzyme substrate, NADH, which might be expected to keep the enzyme in its native conformation and so help to retain any putative additional subunits, gave results identical with those obtained in the absence of NADH (Table 1).

The use of ^{14}C -labelled material in this work also eliminates the possibility that a low- M_r polypeptide with zero or close-to-zero cysteine and methionine content went undetected in our previous work with ^{35}S -labelled membranes.

Identification of the flavin cofactor of NADH dehydrogenase

Membranes of *P. denitrificans* prepared from cells grown in the presence of [^{14}C]riboflavin were analysed by

Fig. 5. Autoradiograms of crossed-immunoelectrophoresis plates run with [^{14}C]riboflavin-labelled *P. denitrificans* membranes

The anode is to the left (first dimension) and at the top of the gels (second dimension). Nonidet-P-40-solubilized [^{14}C]riboflavin-labelled membranes (20 μg) were used as antigen. In the second dimension 2 mg of anti-*(P. denitrificans* membrane vesicles) immunoglobulins/ml were incorporated into the agarose. The plates were incubated for 30 min at 4 $^{\circ}\text{C}$ as follows then dried on to filter paper and autoradiographed: (a) treated with distilled water; (b) treated with 10% trichloroacetic acid to remove non-covalently bound flavin.

CIE followed by autoradiography (Fig. 5) (cf. [23,27]). It was found that one major and several minor immunoprecipitates were radiolabelled, but surprisingly none of these immunoprecipitates corresponded to the single immunoprecipitate that stained for NADH dehydrogenase activity. The major radiolabelled arc stained for succinate dehydrogenase activity. Only the succinate dehydrogenase immunoprecipitate possessed flavin in a covalently bound form, as judged from autoradiograms of CIE plates which had been treated with trichloroacetic acid to release non-covalently bound flavin (Fig. 5b). Under these conditions all the minor immunoprecipitates were lost, but the major radiolabelled arc remained (Fig. 5b). Examination by LDS/PAGE and fluorography of [^{14}C]flavin-labelled membranes showed that a single polypeptide of M_r 56000 contained ^{14}C . This is consistent with it being the major polypeptide of succinate dehydrogenase, which is found to contain covalently bound flavin and have an M_r in this range in the enzymes examined from other sources [27–32].

The data above show that [^{14}C]riboflavin had been specifically incorporated into flavoproteins in *P. denitrificans*, but no radiolabelled flavin was found in the NADH dehydrogenase immunoprecipitate under conditions used in CIE. NADH dehydrogenase was expected to be a flavoprotein, containing either FAD, by analogy with some other bacterial NADH dehydrogenases, e.g. *Escherichia coli* [33] and *Bacillus subtilis* [34], or FMN, by analogy with the mitochondrial enzyme [35]. Thus it was suspected that flavin was lost either on solubilization with Nonidet P-40 or during the CIE procedure. To test whether this was the case, the following procedure was used. *P. denitrificans* membranes were prepared from cells grown in the presence of [^{14}C]riboflavin. The NADH dehydrogenase was immunoprecipitated, as described previously, from such membranes, which had been solubilized in a range of different detergents. Flavin was then extracted from the immunoprecipitate with trichloroacetic acid and the extracted flavin analysed by t.l.c. in conjunction with autoradiography. When the [^{14}C]flavin-labelled membranes were solubilized in 4% cholate, 2% Triton X-100

Table 2. Values of standard flavins and flavin extracted from immunoprecipitated NADH dehydrogenase

Immunoprecipitated NADH dehydrogenase was acid-extracted to remove non-covalently bound flavin. This material was concentrated and treated with Chelex to remove metal ions. Portions of this sample were spiked with standard FMN (0.5 μg), FAD (0.5 μg) or water and run on a t.l.c. plate with 5% Na_2HPO_4 as solvent. Spots were detected by fluorescence under u.v light.

Extracted flavin spiked with	$100 \times R_F$ values
FAD	29.0, 50.8*
Water	29.7
FMN	30.0

* The major spot corresponding to FAD, which is known to migrate further than FMN in this system [26].

or 2% LDAO the autoradiogram was blank, indicating that under these conditions no flavin was present in the immunoprecipitated NADH dehydrogenase. However, when the membranes were solubilized in 2% cholate a spot was seen on the autoradiogram, indicating that at least some flavin was retained by the NADH dehydrogenase under these conditions. When the autoradiogram was aligned with the t.l.c. plate and the standard flavins (FMN, FAD and riboflavin) on the t.l.c. plate were observed under u.v. light, the R_F value for the radioactive spot was considerably lower than that for FAD, but was also slightly lower than that for FMN. The acid extract was known to have chromatographed anomalously, because the solvent front tended to flow around rather than through the applied sample.

In order to determine whether the large volume of the applied sample and its chemical contents were affecting the behaviour of the extracted flavin on t.l.c., a large-scale immunoprecipitation was carried out from unlabelled *Paracoccus* membranes solubilized with 0.5% tauro-

deoxycholate. Taurodeoxycholate was chosen because it was effective at solubilizing NADH dehydrogenase under conditions (low concentration of detergent) that favoured retention of flavin in the NADH dehydrogenase immunoprecipitate. The immunoprecipitate from taurodeoxycholate was extracted with trichloroacetic acid and the concentrated acid extract incubated with Chelex-100 for 5 min to remove any bi- or ter-valent metal ions. The Chelex-100 was then removed by centrifugation, leaving an extract that had lost its brown colour (probably owing to removal of iron, as the trichloroacetic acid treatment would also have extracted any non-haem iron from the immunoprecipitate). Samples of the Chelex-100-treated extract were then spiked with standard FMN, FAD or water, and run on a t.l.c. plate. This clearly indicated that the acid extract contained FMN and not FAD (Table 2). It was also shown that FMN and FAD that had been added to the acid extract had retarded R_F values on t.l.c. relative to standard samples of FMN and FAD that had been applied in water to the t.l.c. plate. An equivalent retardation can therefore be taken into account for the radiolabelled flavin extracted from the immunoprecipitate obtained from vesicles solubilized with 2% cholate. This correction gave the extracted radiolabelled flavin a very similar mobility to the FMN standard that was chromatographed on the same plate. Thus the radioactive flavin observed in the cholate extract was also identified as FMN.

DISCUSSION

The objectives of the present investigation were to prepare a monospecific antibody to the NADH dehydrogenase of *P. denitrificans*, in the absence of a purification procedure for this enzyme, and to probe for putative subunits of the enzyme additional to those previously identified [9]. Realization of the first of these allowed immunoprecipitates obtained under a variety of conditions to be analysed. If the polypeptide of M_r 31000 is not considered to be a true component of the NADH dehydrogenase, for the reasons given in the Results section, the conclusion from Table 1 is that only the M_r -48000 and -25000 subunits can be identified as components of *P. denitrificans* NADH dehydrogenase. No definite explanation can be given for the putative association of the M_r -31000 subunit with that of M_r 48000. If additional subunits of the enzyme do exist, including that of M_r 46000 which was recognized by subunit-specific antiserum against the M_r -49000 polypeptide of mitochondrial NADH dehydrogenase [11], then they must dissociate during the solubilization and/or immunoprecipitation procedures. This would imply that the NADH dehydrogenase is relatively labile, because a functional ubiquinol-nitrate oxidoreductase [36] and an ATP synthase complex (C. L. George, A. L. Craske & S. J. Ferguson, unpublished work) have been prepared from *P. denitrificans* under similar conditions to those used both in the present work and previously [9] for solubilizing NADH dehydrogenase. The extensive range of solubilization procedures examined in the present work might nevertheless have been expected to reveal conditions in which putative extra subunit(s) is(are) retained. Such an approach is illustrated by recent work with succinate dehydrogenase from *E. coli*, which was immunoprecipitated as a single polypeptide from membranes solubilized with Triton X-100,

but contained four polypeptides when the membranes were solubilized with Lubrol PX before immunoprecipitation [37].

In the absence of evidence to the contrary, consideration must still be given to the possibility that the two-subunit NADH dehydrogenase is the complete enzyme of the *P. denitrificans* respiratory chain. The strongest argument against this notion is intuitive; two polypeptides do not seem sufficient to accommodate all the iron-sulphur centres known to be associated with NADH dehydrogenase activity in cytoplasmic membranes of *P. denitrificans*. In the mitochondrial enzyme these centres, including up to 24 iron atoms, are distributed amongst at least five polypeptides [38], including those of not only the flavoprotein and iron-sulphur fragments but also the hydrophobic residue that remains after dissociation of these two fragments from the enzyme [39]. The finding that the NADH dehydrogenase activity of *P. denitrificans* membranes loses rotenone-sensitivity upon solubilization with detergent is not a very compelling argument for the existence of additional subunits. Solubilization of the bovine heart mitochondrial inner membrane with Triton X-100 results in loss of rotenone-sensitive NADH-ubiquinone oxidoreductase activity, and yet essentially the full complement of more than 20 polypeptides can be immunoprecipitated under these conditions [40]. The NADH dehydrogenase from *N. crassa* mitochondria can be prepared with more than 20 polypeptides in Triton X-100 solution and yet has very low NADH-ubiquinone oxidoreductase activity that is only partially sensitive to rotenone [8]. Thus it would be premature to equate loss of rotenone-sensitivity in the *P. denitrificans* system with loss of polypeptides, rather than with, for example, disruption of a rotenone-binding site. The question of whether the two identified polypeptides constitute the complete NADH-ubiquinone oxidoreductase will only be answered when sufficient quantities of purified enzyme are available to permit determination of whether it contains all the known iron-sulphur centres and has the capacity to translocate protons.

FMN was identified in immunoprecipitates of NADH dehydrogenase. These immunoprecipitates, from membranes solubilized with either 2% cholate or 0.5% taurodeoxycholate, contained the polypeptide of M_r 31000 as well as the two identified polypeptides of NADH dehydrogenase (Table 1). However, there was no correlation between the presence of the M_r -31000 polypeptide and FMN content, because the flavin was not detectable in immunoprecipitates from either 2% Triton X-100 or 2% LDAO, yet this polypeptide was a major component of the immunoprecipitate from the former detergent, but was absent when the latter was used for solubilization. Thus we conclude that the data in the present paper provide evidence for a role for FMN in the *P. denitrificans* enzyme. This is a further similarity with the mitochondrial enzyme, in which the M_r -51000 polypeptide, which is recognized by the antibodies to the M_r -48000 and -25000 polypeptides from *P. denitrificans* [11], is thought to bind FMN [10].

Purified NADH dehydrogenase from *E. coli* [33] and *B. subtilis* [34] contain FAD and thus differ from the corresponding enzymes from *P. denitrificans* and mitochondria. What is known of the subunit composition of these enzymes [33,34] also suggests that they are not related to the proton-translocating NADH dehydro-

genases of mitochondria and *P. denitrificans*. The only bacterial NADH dehydrogenases for which FMN has been suggested to be a cofactor are those from *Bacillus stearothermophilus* [41] and *Acinetobacter calcoaceticus* [42]. However, in these cases the evidence was that the enzymes were activated by added FMN; flavin was not actually identified as a component of the purified enzymes. Thus the extraction of FMN from immunoprecipitated *P. denitrificans* is the first direct finding of a bacterial NADH dehydrogenase that contains FMN.

C.L.G. thanks the Rowbotham Fund of Birmingham University for research grants. S.J.F. was a Nuffield Foundation Science Research Fellow in 1984–85.

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Received 13 August 1986/15 January 1987; accepted 5 March 1987