Membrane-associated NADH dehydrogenase activities in *Rhodobacter capsulatus*: purification of a dihydrolipoyl dehydrogenase

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The presence of several NADH dehydrogenase activities associated with cytoplasmic membrane vesicles of chemoheterotrophically grown *Rhodobacter capsulatus* MT1131 was demonstrated by combining isoelectric focusing with NADH–tetranitrobluetetrazolium activity staining, a procedure that should have general applicability in the analysis of bacterial NADH dehydrogenase activities. Low pI (pI = 5.7), Mid pI (pI = 6.9) and High pI (pI = 8.5) bands were resolved. The Mid pI NADH dehydrogenase activity was purified and identified as a dihydrolipoyl dehydrogenase. Our data indicate that this dihydrolipoyl dehydrogenase is derived from a 2-oxoacid dehydrogenase complex which is associated with the cytoplasmic membrane.

Introduction

The electron transport system of the photosynthetic purple non-sulphur bacterium *Rhodobacter capsulatus* has been extensively studied. The emphasis has been on the components of the light-driven cyclic electron transport system, including the ubiquinol–cytochrome c_2 oxidoreductase (cytochrome bc_1 complex), and upon the cytochrome oxidases. Much less attention has been paid to enzyme activities that are involved in the oxidation of NADH. Indeed, relatively little is known in general about enzyme activities in bacterial cytoplasmic membranes that exhibit NADH dehydrogenase activity (Yagi, 1991; Berks & Ferguson, 1991). On the basis of inhibitor titrations (Klemme, 1969; La Monica & Marrs, 1976; Berks, 1991), EPR studies (Zannoni & Ingledew, 1983; also mentioned in Meinhardt *et al.*, 1989) and molecular genetic analysis (Marrs *et al.*, 1972; Dupuis, 1992), *R. capsulatus* is thought to possess a mitochondrial-type proton-translocating NADH–ubiquinone oxidoreductase. This type of enzyme has not been purified in an intact state from any bacterium. FAD-containing

NADH–quinone oxidoreductases that do not translocate protons are apparently widespread in bacteria (Yagi, 1991) and it has been suggested that an enzyme of this type is present in *R. capsulatus* (Meinhardt *et al.*, 1990). A third type of cytoplasmic membrane NADH dehydrogenase has been purified from *R. capsulatus* by Ohshima & Drews (1981). This enzyme, apparently containing six identical subunits of molecular mass 15 kDa, is not obviously related to any previously described NADH dehydrogenase. A proton-translocating NADH/NADPH transhydrogenase activity is also present in *R. capsulatus* cytoplasmic membranes (Lever *et al.*, 1991).

The present work was undertaken as part of a study aimed at clarifying the complexity and physiological rôles of the multiple membrane-associated NADH dehydrogenases of *R. capsulatus*. To this end, we have been investigating methods to solubilize the membrane-associated NADH dehydrogenase activities in active form, to analyse the enzymes in the crude soluble extract and to purify and characterize each of the enzymes.

Methods

Bacterial growth and cell fractionation. *Rhodobacter capsulatus* strain MT1131 (Zannoni *et al.*, 1980) was grown chemoheterotrophically (aerobically in the dark) at 30 °C with DL-malate as carbon source using the RCV minimal medium described by Weaver *et al.* (1975). The cells were harvested in late exponential phase by centrifugation. Washed bacterial cells were resuspended in a volume of 50 mM-HEPES/NaOH, pH 7.4, 5 mM-MgCl₂, 1 mM-PMSF equal to that of the cell pellet. A few flakes of DNAase were added. The cells were then broken in a pre-cooled French pressure cell (Aminco) at 7.7×10^6 kg m⁻². Unbroken cells and cell debris were pelleted by centrifugation at 40000 g, 4 °C for

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Abbreviations: APAD⁺, acetyl pyridine adenine dinucleotide; BCA, bicinchoninic acid; C₁₂E₈, octaethylene glycol monododecyl ether; CIE, crossed immunoelectrophoresis; DB, 2,3-dimethoxy-5-methyl-1,6-benzoquinone; dNADH, deamino-NADH (reduced nicotinamide hypoxanthine dinucleotide); DTT, dithiothreitol; IEF, isoelectric focusing; PMSF, phenylmethylsulphonylfluoride; TNBT, tetranitrobluetetrazolium.

20 min and discarded. The cell-free extract was then centrifuged at 200 000 g, 4 °C for 2 h. The supernatant was retained as the soluble fraction (cytoplasm plus periplasm). The pelleted membranes were resuspended in 50 mM-HEPES/NaOH, pH 7.4 unless stated otherwise. Detergent solubilizations were carried out by dropwise addition of detergent from a concentrated stock solution followed by incubation for 30 min at 4 °C. Unsolubilized material was removed by centrifugation at 100 000 g at 4 °C for 1 h. Washing of the membranes was carried out in analogous fashion. All purification steps were carried out at 4 °C.

Enzyme assays. Dihydrolipoyl dehydrogenase activity was measured as described by Yanagawa (1979). Pyruvate dehydrogenase activity was assayed as in Visser *et al.* (1982). The assay was initiated by the addition of sodium pyruvate to a concentration of 5 mM. For the determination of 2-oxoglutarate dehydrogenase activity, 1 mM-2-oxoglutarate was substituted for pyruvate in the pyruvate dehydrogenase assay system. Malate dehydrogenase activity was assayed as in Markwell & Lascelles (1978) using 270 μ M-NADH, the reaction being started by addition of 200 μ M-oxaloacetate. The production or consumption of NADH in the above assays was measured spectrophotometrically at 340 nm using an extinction coefficient of 6.22 mm⁻¹ cm⁻¹. For NADH, dNADH and NADPH dehydrogenase assays, the concentration of the reduced pyridine dinucleotide was 150 μ M and the electron acceptor was 1 mM-K₃Fe(CN)₆. The reactions were followed spectrophotometrically using an ϵ_{410} of 1.0 mm⁻¹ cm⁻¹. NADH-DB oxidoreductase activity was measured spectrophotometrically (ϵ_{340} 6.8 mm⁻¹ cm⁻¹) using an assay mix of 150 μ M-NADH and 60 μ M-DB. The assay mix for the determination of transhydrogenase activities contained 10 mM-MgSO₄ and 16.5 mM-APAD⁺ (acetyl pyridine adenine dinucleotide). The stock solution of APAD⁺ stock was prepared in phosphate buffer and its concentration was determined from the ϵ_{260} of 16.2 mm⁻¹ cm⁻¹. The reaction was initiated with 150 μ M-electron donor (NADPH or NADH) and the reaction was followed at 450–375 nm in an Aminco DW2000 dual wavelength spectrophotometer. Concentration changes were calculated from an $\Delta\epsilon_{450-375}$ of 5.1 mm⁻¹ cm⁻¹. All enzyme assays were at 30 °C and the assay buffer was 50 mM-HEPES/NaOH, pH 7.4. Detergents above their critical micelle concentrations were included in assays where appropriate. When anaerobiosis was required, stoppered cuvettes containing the samples were alternately evacuated and sparged with argon. When membranes capable of carrying out electron transport to oxygen were present in the assay, advantage was taken of the high affinities of the terminal oxidases to reduce oxygen levels further by inclusion of 10 mM-sodium succinate in the assay samples. The reagent solution employed to initiate the reactions was kept in a sealed anaerobic vial. Withdrawal of the reagent from this vial and transfer to the sample cuvette was by gas-tight Hamilton syringe.

Analytical methods. Protein concentration was estimated using the BCA method (Smith *et al.*, 1985) using BSA (Cohn fraction V) as the standard. SDS-PAGE electrophoresis used the discontinuous buffer system of Laemmli (1970). Samples were heated to 95 °C for 3 min prior to electrophoresis. IEF was carried out in a 1% agarose (BDH IEF agarose) gel using 2% Ampholines pH 3.5–10.0 and, where required, the appropriate detergent above its critical micelle concentration. The anolyte was 0.5 M-H₃PO₄, the catholyte 0.5 M-NaOH. Running conditions were 500 V, 15 mA limiting current for 4 h. The gels were stained for NADH dehydrogenase activity using the method of Owen & Salton (1975) using TNBT as electron acceptor. The pI of focused proteins (at 4 °C) was determined from a linear interpolation between coloured marker proteins (BDH wide range kit, pI values 4.7–10.6, 4 °C). These assignments were confirmed by measuring the pH of solutions prepared by soaking 0.5 cm gel strips cut from blank control lanes in 2 ml 50 mM-KCl overnight at 4 °C. The N-terminal amino acid sequence of *R. capsulatus* dihydrolipoyl dehydrogenase was determined by the method of Pilkington *et al.* (1991). Protein was

separated by SDS-PAGE and semi-dry blotted onto a PVDF filter (Millipore). The filter-bound polypeptide was sequenced directly on an Applied Biosystems 120A gas-phase sequencer employing PTH analysis.

Materials. C₁₂E₈ was obtained from Fluka, Triton X-100 (specially purified for membrane research) was obtained from Boehringer. S-Sepharose, S300HR Sephacryl, Polybuffer Exchanger 94, Polybuffers 96 and 74 and Ampholines were obtained from Pharmacia LKB and Aquacide I was from Calbiochem Novabiochem. DB was synthesized by the method of Wan *et al.* (1975).

Results

Membranes of R. capsulatus have multiple NADH dehydrogenase activities

Extraction of the membrane vesicles from chemoheterotrophically grown *R. capsulatus* MT1131 with a wide variety of detergents released ferricyanide- or dye-linked NADH dehydrogenase activity(ies) whilst diminishing the NADH-ubiquinone oxidoreductase activity of the insoluble residue (Berks, 1991). The soluble extracts did not catalyse appreciable NADH dehydrogenase activity with ubiquinone analogues as electron acceptors and this low activity was insensitive to rotenone, an inhibitor of the proton-translocating NADH-ubiquinone oxidoreductase. The latter result might arise as a consequence of the fragmentation or delipidation of the proton-translocating NADH-ubiquinone oxidoreductase with attendant loss of activity toward ubiquinone analogues.

We developed the method of NADH-TNBT activity staining of IEF gels to analyse the NADH dehydrogenase activities of the membrane extracts. The gel shown in Fig. 1 is typical. Three types of activity staining band could be identified, the presence or absence of these bands being dependent on the extraction conditions. A Low pI band (pI = 5.7) was usually a doublet. Staining intensity of this band decreased markedly within a day at 4 °C or after column chromatography. The Low pI band is probably derived from an integral membrane protein as it was not released from the membranes by salt washes (e.g. Fig. 1, lane C). The Mid pI band (pI = 6.9) was also occasionally a doublet. The protein associated with this activity was precipitated by a 40% saturation ammonium sulphate cut and was associated with a protein of size 620 kDa as determined by gel filtration of C₁₂E₈- or NaBr-solubilized membranes. This band was shown to be a peripheral membrane protein as it was partially released from the membrane vesicles by salt washes (e.g. Fig. 1C). The High pI band (pI = 8.5) was of molecular size 60 kDa as determined by gel filtration chromatography of C₁₂E₈- or NaBr-solubilized membranes and was probably associated with a peripheral membrane protein as it was at least partially released from membrane vesicles by salt washes (e.g. Fig. 1C).

It has been observed by Tushurashvili *et al.* (1989) that

Fig. 1. Effect of different washing conditions on membrane association of dihydrolipoyl dehydrogenase: analysis by isoelectric focusing. The gel contained Ampholines 3.5–10, 0.1% (w/v) Triton X-100 and was stained for NADH–TNBT activity. Samples were supplemented with 2% (w/v) Triton X-100 to allow electrophoretic separation. *R. capsulatus* aerobic membranes at 10 mg protein ml⁻¹ were washed in 50 mM-HEPES/NaOH pH 7.4 supplemented as noted. The washed membrane pellet was resuspended in a volume of 50 mM-HEPES/NaOH pH 7.4 equal to the wash volume. Equal quantities (10 µl) of all samples were loaded. Lane A, buffer wash supernatant; B, 20 mM-EDTA wash supernatant; C, 0.5 M-NaCl wash supernatant; D, 2% (w/v) Triton X-100 wash supernatant; E, buffer wash resuspended pellet; F, 20 mM-EDTA wash resuspended pellet; G, 0.5 M-NaCl wash resuspended pellet; H, 2% (w/v) Triton X-100 wash resuspended pellet.

Mg²⁺ induces a time lag in the NADH–ubiquinone oxidoreductase activity of beef heart submitochondrial particles. This suggested that Mg²⁺ might bind to the proton-translocating NADH–ubiquinone oxidoreductase of *R. capsulatus*. When 20 mM-MgSO₄ was added to *R. capsulatus* membranes before solubilization, the High pI NADH–TNBT activity staining band became very prominent. Further, the High pI band appeared under detergent solubilization conditions where it had previously been absent. This enhancement of the High pI band was specific to Mg²⁺; other mono- or divalent cations at the same concentration did not have the same effect. The effect was only apparent if the Mg²⁺ was added prior to solubilization. This suggests that the effect of Mg²⁺ was to release the High pI band enzyme from the membrane rather than to activate the enzyme.

Material that stained for activity but did not focus was also generally observed in our electrophoretic system (e.g. as in Fig. 1). Large enzymes show a propensity toward isoelectric precipitation during isoelectric focusing. Thus one interpretation of this seemingly unfocused material is that it was intact proton-translocating NADH–ubiquinone oxidoreductase that had not focused

properly because of its size. Alternatively, as it was noted that high concentrations of protein stained weakly with the NADH–TNBT activity stain and that the staining of this unfocused material tended to occur after the other bands were fully developed, it is possible that this material stained non-specifically.

From a comparison of rates of rotenone-sensitive reduced nicotinamide nucleotide–DB oxidoreductase activity in membrane vesicles, we established that the *R. capsulatus* proton-translocating NADH–ubiquinone oxidoreductase could utilize deamino-NADH (dNADH) as a substrate as readily as it could NADH (Berks, 1991). Further, the membrane-bound enzyme could oxidize NADPH only at very low rates. It was expected that the electron donor specificity of the membrane-bound proton-translocating NADH–ubiquinone oxidoreductase might be retained by the solubilized enzyme. IEF gels of soluble extracts were therefore stained for dNADH–TNBT, NADPH–TNBT as well as for NADH–TNBT activities. Only the Low pI band was stained when NADPH was supplied as the electron donor and this staining was very weak. With dNADH, both the Low and Mid pI bands were stained, though the staining

Fig. 2. S-Sepharose column chromatography of $\text{C}_{12}\text{E}_8/\text{MgSO}_4$ extracted *R. capsulatus* aerobic membranes. Membrane protein (250 mg) was solubilized at 10 mg protein ml^{-1} , 2.5 mg $\text{C}_{12}\text{E}_8 \text{ ml}^{-1}$ in 10 mM-MES/NaOH pH 6.3 (24 °C), 20 mM- MgSO_4 and loaded on an S-Sepharose column (17 $\times$ 1.6 cm diameter). The column was washed with 40 ml 10 mM-MES/NaOH pH 6.3 (24 °C), 0.25 mg $\text{C}_{12}\text{E}_8 \text{ ml}^{-1}$ then developed with a linear 0–0.5 M-NaCl gradient (—) in the same buffer. The flow rate was 11 ml h^{-1} and 2 ml fractions were collected. (a) ■, NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase. (b) ▲, dihydrolipoyl dehydrogenase; □, 2-oxoglutarate dehydrogenase. Δ , A_{280} in (a) and (b).

intensity of the Mid pI band was relatively somewhat lower than that obtained using NADH as the electron donor (data not shown).

George & Ferguson (1987) reported that an NADH dehydrogenase that resembles the Flavoprotein fragment of mitochondrial NADH-ubiquinone oxidoreductase was released from the cytoplasmic membrane of *Paracoccus denitrificans* (a close relative of *R. capsulatus*; Woese, 1987) by detergent treatments. In our gel system, Triton-X-100-solubilized cytoplasmic membranes of *P. denitrificans* (grown aerobically on glucose or anaerobically with succinate as electron donor and nitrate as electron acceptor) gave only a single activity staining band (data not shown). The staining of this band (pI = 5.5) was very

much more intense at the same total membrane protein concentration than that of any of the *R. capsulatus* activity staining bands. Thus the NADH-TNBT activity staining pattern of *R. capsulatus* differs from that of *P. denitrificans*, suggesting that the multiple staining pattern seen in *R. capsulatus* is not an artifact of the method.

Isolation of the Mid pI NADH dehydrogenase from the cell membranes of *R. capsulatus*

Extraction of *R. capsulatus* membrane vesicles with the detergent C_{12}E_8 in the presence of 20 mM- MgSO_4 was optimal for the release of all three distinct NADH-TNBT activity staining bands. When such an extract was

Fig. 3. Sephacryl S300HR gel filtration chromatography of Mid pI band NADH dehydrogenase-containing S-Sepharose column fractions. Samples were concentrated to a volume of 2 ml using Aquacide I and loaded onto a Sephacryl S300HR column (1 m $\times$ 1.6 cm diameter). The column buffer was 20 mM-HEPES/NaOH pH 7.5 (24 °C), 130 mM-NaCl, 0.25 mg $C_{12}E_8$ ml $^{-1}$. (a) Pooled NADH- $K_3Fe(CN)_6$ oxidoreductase activity (□) containing fractions from the S-Sepharose column (Fig. 2). Flow rate, 20 ml h $^{-1}$. (b) Pooled NADH- $K_3Fe(CN)_6$ oxidoreductase activity (□) containing fractions from the chromatofocusing column. Flow rate, 13 ml h $^{-1}$. Δ , A_{280} in (a) and (b).

Table 1. Enzymic properties of the purified Mid pI band NADH dehydrogenase

Assay	Specific Activity [μ mol min $^{-1}$ (mg protein) $^{-1}$]	Percentage of NADH $\rightarrow$ $K_3Fe(CN)_6$ oxidoreductase activity
NADH $\rightarrow$ $K_3Fe(CN)_6$	23	100
dNADH $\rightarrow$ $K_3Fe(CN)_6$	3.2	14
NADPH $\rightarrow$ $K_3Fe(CN)_6$	0.048	0.2
NADH $\rightarrow$ DB	0.13	0.6
NADH $\rightarrow$ lipoic acid	1.4	6
NADPH $\rightarrow$ APAD $^{+}$	0	0
NADH $\rightarrow$ APAD $^{+}$	0.89	4

Fig. 4. SDS-PAGE analysis of the purification of the mid pI NADH dehydrogenase. The gel was 12% polyacrylamide/0.3% bisacrylamide and proteins were detected by silver staining. Lane A, chromatofocusing NADH- $K_3Fe(CN)_6$ oxidoreductase activity peak reduced with 50 mM-DTT (11 μ g protein). B, Chromatofocusing NADH- $K_3Fe(CN)_6$ oxidoreductase activity peak reduced with 2% β -mercaptoethanol (11 μ g protein). C, S300HR column pooled NADH- $K_3Fe(CN)_6$ oxidoreductase activity peaks (Fig. 3a) reduced with 2% β -mercaptoethanol (17 μ g protein). D, S-Sepharose column pooled NADH- $K_3Fe(CN)_6$ oxidoreductase activity peaks (Fig. 2) reduced with 2% β -mercaptoethanol (21 μ g protein).

subjected to cation exchange chromatography, not only the majority of the NADH- $K_3Fe(CN)_6$ oxidoreductase activity but also the major part of the extracted protein failed to bind to the column. Elution of the bound protein with a gradient of 0–0.5 M-NaCl released two peaks of NADH- $K_3Fe(CN)_6$ oxidoreductase activity (Fig. 2). When protein from these peaks was subjected to isoelectric focusing the only NADH-TNBT activity staining band found was the Mid pI band (pI = 7.0). If $MgSO_4$ was left out of the extraction buffer, no NADH- $K_3Fe(CN)_6$ oxidoreductase activity peak was observed. $MgSO_4$ was, therefore, always added during the extraction.

The peaks from the cation exchange column containing NADH- $K_3Fe(CN)_6$ oxidoreductase activity were pooled (as they both contained the Mid pI band), concentrated and subjected to gel filtration chromatography on Sephacryl S300HR (Fig. 3a). Two peaks of NADH- $K_3Fe(CN)_6$ oxidoreductase activity were

<i>R. capsulatus</i> Mid pI NADH dehydrogenase (1–20)	AEFDV I I X G G P X A Y V X A I R
	
<i>P. putida</i> LPD-Val (5–24)	I Q T T L L I G G G P G G Y V A A I R
	:
<i>P. putida</i> LPD-Glc (2–21)	Q K F D V V V I G A G P G G Y V A A I K
	:
<i>P. putida</i> LPD-3 (2–22)	K S Y D V V I I G G G P G G Y N A A I R
	
<i>R. capsulatus</i> carotenoid desaturase I (8–27)	R A V V I G A G L G G L A A A M R L G A
	
<i>R. capsulatus</i> carotenoid desaturase D (6–25)	D V V V I G A R M G G L A A A I G A A A
	:
<i>P. aeruginosa</i> Tn501 mercuric reductase (97–116)	P P V Q V A V I G S G G A A M A A A L K
	: :
<i>H. sapiens</i> glutathione reductase (19–38)	A S Y D Y L V I G G G S G G L A S A R R

Fig. 5. N-Terminal protein sequences of the mid pI NADH dehydrogenase and comparisons with a representative selection of possible sequence similarities. Standard one-letter amino acid codes are used. Numbering is from the N-terminus of the mature proteins or the N-terminus of the predicted gene product. : is a match, . is a conservative substitution or ambiguous sequence position, both relative to the *R. capsulatus* NADH dehydrogenase sequence. A number of other dihydrolipoyl dehydrogenase sequences have been determined. These sequences show approximately the same degree of similarity to the *R. capsulatus* NADH dehydrogenase sequence as the alignments of dihydrolipoyl dehydrogenases displayed. References for the sequences are: *Pseudomonas putida* *lpdV* (LPD-Val) (E_3 from the branched chain 2-oxoacid dehydrogenase complex), Burns *et al.* (1989); *Pseudomonas putida* *lpdG* (LPD-Glc) (E_3 of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes and the L-factor of the glycine oxidation system), Palmer *et al.* (1991a); *Pseudomonas putida* LPD-3, Palmer *et al.* (1991b); *Rhodobacter capsulatus* carotenoid desaturases I and D, Bartley *et al.* (1990); *Pseudomonas aeruginosa* transposon Tn501 mercuric reductase, Brown *et al.* (1983); *Homo sapiens* erythrocyte glutathione reductase, Krauth-Siegel *et al.* (1982).

detected. One eluted at the void volume. The other eluted at a position that corresponded to a size of 620 kDa when the column was calibrated with water-soluble protein standards. Both peaks contained the mid pI NADH–TNBT activity band and both had identical, but complex, SDS-PAGE polypeptide profiles (not shown).

The two activity peaks from the gel filtration column were combined and subjected to chromatofocusing in a pH 8–5 gradient. A single peak of NADH– $K_3Fe(CN)_6$ oxidoreductase activity was detected eluting at a pH of 6.1 (not shown). This peak contained the Mid pI NADH–TNBT activity staining band and this corresponded to the only protein in a Coomassie-Blue-stained IEF gel (not shown). A silver-stained SDS-PAGE gel showed only one polypeptide with an estimated molecular mass of 53 kDa (Fig. 4). No further bands were visible if the gel was stained with Coomassie Blue rather than silver.

If the order of the final two steps of the purification procedure, gel filtration and chromatofocusing, was reversed the same 53 kDa NADH dehydrogenase was purified. However, only a single peak of NADH– $K_3Fe(CN)_6$ oxidoreductase activity was found after the gel filtration step (Fig. 3b). The elution position of this peak was shifted relative to the original purification, the new elution position corresponding to a molecular size of

80 kDa when the column was calibrated with water-soluble standards. This result suggested that the NADH dehydrogenase was part of a larger complex that is fragmented or disaggregated by chromatofocusing, resulting in the release of the NADH dehydrogenase activity as a 53 kDa monomer.

The yield of purified NADH dehydrogenase from 250 mg membrane protein was 75 μ g (0.03%) and the purification factor 125.

Properties of the purified Mid pI band NADH dehydrogenase

Table 1 summarizes the enzymic reactions of the purified NADH dehydrogenase. This NADH dehydrogenase was specific for NADH when assayed with $K_3Fe(CN)_6$ as electron acceptor. dNADH was a relatively poor electron donor, while the reaction with NADPH was only fractionally greater than the non-enzymic oxidation of NADPH by $K_3Fe(CN)_6$, all in agreement with the qualitative results from activity staining (above).

The purified enzyme did not carry out NADPH $\rightarrow$ APAD⁺ transhydrogenation, a reaction characteristic of *R. capsulatus* H⁺-nicotinamide nucleotide transhydrogenase (Lever *et al.*, 1991). In contrast, the enzyme did carry out NADH $\rightarrow$ APAD⁺ transhydrogenation.

The first 20 amino acids of the N-terminal sequence of the purified NADH dehydrogenase were determined with three ambiguities (Fig. 5). This sequence was compared with the proteins of the Swissprotein database using the GCG7 implementation of program FAST A (Pearson & Lipman 1988; Devereux *et al.*, 1984). The *R. capsulatus* NADH dehydrogenase sequence showed a high degree of similarity to the flavoprotein disulphide oxidoreductases (Fig. 5), particularly dihydrolipoyl dehydrogenase (dihydrolipoamide:NAD⁺ oxidoreductase; EC 1.6.4.3).

The identity of the purified *R. capsulatus* NADH dehydrogenase with a dihydrolipoyl dehydrogenase was confirmed by assaying for lipoic acid dehydrogenase activity (Table 1). This activity was inhibited by micromolar amounts of arsenite, a reagent specific for vicinal thiols.

Association of dihydrolipoyl dehydrogenase with the cytoplasmic membrane of R. capsulatus

The finding that dihydrolipoyl dehydrogenase could be purified from the membrane fraction of *R. capsulatus* was surprising, since this enzyme is usually a component of the 2-oxoacid dehydrogenase multi-enzyme complexes which are regarded as being water soluble. We therefore investigated the conditions under which dihydrolipoyl dehydrogenase activity was removed from membranes. Membranes were washed with the extraction buffer, 50 mM-HEPES/NaOH, pH 7.4, supplemented variously with 20 mM-EDTA, 0.5 M-NaCl or a representative detergent, Triton X-100. Dihydrolipoyl dehydrogenase activity was removed from the membranes most efficiently by 0.5 M-NaCl (27%). Next most effective was 20 mM-EDTA or Triton X-100 (10% and 11% respectively). Washing with the extraction buffer alone released the least (5%) dihydrolipoyl dehydrogenase activity. In all cases, the majority of dihydrolipoyl dehydrogenase activity remained with the extracted membranes. Electrophoretic analysis of the washes, identifying dihydrolipoyl dehydrogenase activity with intensity of the NADH-TNBT activity of the Mid pI band (Fig. 1) was in broad agreement with the enzyme activity results.

Dihydrolipoyl dehydrogenase was probably derived from a 2-oxoacid dehydrogenase complex. If this was the case, then 2-oxoacid dehydrogenase complexes should have been found in the membrane fraction. Table 2 shows that this was indeed the case. The majority of pyruvate dehydrogenase activity was found to be associated with the membranes. In control experiments malate dehydrogenase, a soluble enzyme of the citric acid cycle, was shown to be virtually absent from the membrane fraction.

Table 2. *Distribution of 2-oxoacid dehydrogenase and malate dehydrogenase activities between soluble and membrane fractions of R. capsulatus*

Washed cells from a 2.5 litre *R. capsulatus* aerobic culture were broken in 100 ml 50 mM-HEPES/NaOH buffer, pH 7.4, by use of a French pressure cell. The membrane fraction contained 53 mg protein, the soluble fraction (cytoplasm + periplasm) 830 mg protein. Activities of membrane-containing fractions were measured anaerobically. The combined soluble fractions possessed an NADH oxidase activity of 4 ± 2 nmol min⁻¹ (mg protein)⁻¹. This activity was not inhibited by anaerobiosis or the NADH-ubiquinone oxidoreductase inhibitor rotenone. The soluble fraction enzyme activities are corrected for this activity. Quoted values are the means ($n = 3$) for experiments on a single day. Repetition of the experiment gave comparable results.

	Malate dehydrogenase	2-Oxoglutarate dehydrogenase	Pyruvate dehydrogenase
Activity in membranes [nmol min ⁻¹] (mg protein) ⁻¹	400 ± 100	25.3 ± 0.7	600 ± 300
Activity in soluble fraction [nmol min ⁻¹] (mg protein) ⁻¹	1100 ± 200	6 ± 3	7.2 ± 0.6
Total activity in membranes (μmol min ⁻¹)	21	1.3	31.5
Total activity in soluble fraction (μmol min ⁻¹)	913	5	6
Activity in membranes (%)	2.2	21	81

Discussion

An NADH dehydrogenase activity has been purified to homogeneity from membranes of aerobically grown *R. capsulatus* and experimental evidence presented is consistent with this purified enzyme being a dihydrolipoyl dehydrogenase. The enzyme reduced lipoic acid in an arsenite-sensitive reaction and exhibited catalytic activities similar to those of authentic dihydrolipoyl dehydrogenase (Table 1; Yanagawa, 1979). Dihydrolipoyl dehydrogenase subunits have molecular masses, determined by SDS-PAGE, in the range 51–59 kDa. The purified *R. capsulatus* NADH dehydrogenase molecular mass determined by this method was 53 kDa. Dihydrolipoyl dehydrogenase is a functional dimer. The purified NADH dehydrogenase was sized at 80 kDa by gel filtration, which does not allow assignment as either a monomer or dimer. It was, however, noted that if samples for SDS-PAGE were not boiled a second band at 125 kDa (presumably a dimer) could be seen. Finally, the high amino acid sequence similarity between the purified NADH dehydrogenase and the N-terminus

of dihydrolipoyl dehydrogenase is evident (Fig. 5). The *R. capsulatus* NADH dehydrogenase sequence shows greater similarity to dihydrolipoyl dehydrogenase sequences than it does to those of glutathione reductase or mercuric reductase. Further, both glutathione reductase and mercuric reductase have N-terminal extensions relative to the dihydrolipoyl dehydrogenases. The sequence of the purified enzyme is not that of one of the carotenoid desaturases of *R. capsulatus* strain B10 (the parent strain of MT1131) (Bartley *et al.*, 1990). It can thus be asserted with confidence that the purified NADH dehydrogenase of *R. capsulatus* is a dihydrolipoyl dehydrogenase. On the basis of the catalytic properties, it is certain that the dihydrolipoyl dehydrogenase purified in this work is not one of the NADH/NADPH dehydrogenases purified from a soluble fraction of *R. capsulatus* strain B10 by Laurinavichene *et al.* (1987). It is distinct from the membrane NADH dehydrogenase activity purified by Ohshima & Drews (1981). It may, however, account for the H⁺-nicotinamide nucleotide transhydrogenase-independent NADH → APAD⁺ activity of *R. capsulatus* membrane vesicles (Lever *et al.*, 1991).

Dihydrolipoyl dehydrogenase (Carothers *et al.*, 1989; Pai, 1991; Williams, 1992) is normally a component of soluble 2-oxoacid dehydrogenase complexes or the soluble glycine cleavage system (Roche & Patel, 1989; Patel & Roche, 1990; Perham, 1991; Mattevi *et al.*, 1992) and so purification of this enzyme from a membrane fraction was unexpected. The result was of particular interest because there are a number of observations in the literature of membrane-associated dihydrolipoyl dehydrogenases in prokaryotes (for a review of reports of dihydrolipoyl dehydrogenases with novel functions and of the enzyme bound to the plasma membrane of eukaryotes see Danson, 1988). Dihydrolipoyl dehydrogenase has been found by Owen *et al.* (1980) in crossed immunoelectrophoresis (CIE) experiments to be associated with the membranes of *E. coli* grown aerobically on succinate/citrate. It was not detected by George & Ferguson (1987) in analogous CIE experiments in *Paracoccus denitrificans*, a close relative of *R. capsulatus* (Woese, 1987). This might be because no antibodies to the *P. denitrificans* dihydrolipoyl dehydrogenase were produced. However, no *R. capsulatus* dihydrolipoyl-dehydrogenase-like band is visible in IEF gels of *P. denitrificans* membranes from cells grown aerobically on glucose or anaerobically on succinate/nitrate (this work). In the thermophile *Thermoplasma acidophilum*, 30–50% of the total cellular dihydrolipoyl dehydrogenase activity was found to be associated with the membrane-containing fractions (Smith *et al.*, 1987). In the mycoplasma *Acholeplasma laidlawii*, a portion of the total pyruvate dehydrogenase activity is recovered in mem-

brane fractions (Constantopoulos & McGarrity, 1987; Wallbrandt *et al.*, 1992). The purinolytic anaerobe *Clostridium cylindrosporum* possesses a glycine cleavage system, the dihydrolipoyl dehydrogenase component of which shows strong N-terminal homology to other dihydrolipoyl dehydrogenases (Dietrichs *et al.*, 1990). As determined by immunogold electron microscopy, 65% of the dihydrolipoyl dehydrogenase involved in the glycine cleavage system was associated with the cytoplasmic membrane. In contrast, after the cells had been broken in a French pressure cell, 95% of the enzyme was found in the soluble fraction (Dietrichs *et al.*, 1991). Finally, in *Staphylococcus aureus* dihydrolipoyl dehydrogenase was suggested to be bound to membrane-associated ribosomes (Adler & Arvidson, 1984, 1987; Hemilä, 1991). Similar claims have been made for pyruvate dehydrogenase component E₂ (dihydrolipoamide acyltransferase) in *Bacillus subtilis* (Hemilä *et al.*, 1990) and in *Neurospora crassa* mitochondria (Kreader *et al.*, 1989; Russell & Guest, 1991).

It seems probable that the purified dihydrolipoyl dehydrogenase is part of a 2-oxoacid dehydrogenase complex associated with the membrane. There is reasonable evidence, for example, that mitochondrial proton-translocating NADH-ubiquinone oxidoreductase can bind pyruvate and 2-oxoglutarate dehydrogenases (Sumegi & Srere, 1984; Porpaczy *et al.*, 1987; Fukushima *et al.*, 1989). We have shown that under the cell breakage conditions described in Methods, a substantial proportion of the pyruvate and 2-oxoglutarate dehydrogenase molecules in *R. capsulatus* were associated with the cytoplasmic membranes (Table 2). Some of these membrane-bound 2-oxoglutarate dehydrogenase complexes were present in the C₁₂E₈/MgSO₄ extract. Therefore, it is most likely that the purified dihydrolipoyl dehydrogenase was derived from 2-oxoacid dehydrogenase complexes and was not associated with any novel function. Evidence that supports this view came from the chromatofocusing experiments, which caused a change in the apparent size of dihydrolipoyl dehydrogenase as measured by gel filtration from ≥ 600 kDa to 80 kDa. This suggests that dihydrolipoyl dehydrogenase is dissociating from a larger complex under these chromatographic conditions. Dihydrolipoyl Dehydrogenase is non-covalently bound to the core E₂ proteins in 2-oxoacid dehydrogenase complexes. If chromatofocusing can release dihydrolipoyl dehydrogenase molecules from these complexes, then it is perhaps not surprising that IEF gives a single band from gel filtration NADH-K₃Fe(CN)₆ oxidoreductase activity peaks of differing molecular sizes. The strongest NADH-TNBT activity staining band in the soluble extract of aerobic *R. capsulatus* has the same pI as the purified membrane-associated dihydrolipoyl dehydrogenase (not shown),

suggesting that the dihydrolipoyl dehydrogenase activity in both fractions is due to the same enzyme. This again argues that the membrane-bound dihydrolipoyl dehydrogenase is derived from a 2-oxoacid dehydrogenase complex and that it is not a component of a novel enzyme.

If the purified dihydrolipoyl dehydrogenase was derived from a 2-oxoacid dehydrogenase complex, it was possible that dihydrolipoyl dehydrogenase and the dehydrogenase activity would co-migrate on the S-Sepharose column used as the first step in the dihydrolipoyl dehydrogenase purification procedure. Fig. 2(a) shows that this is not the case. No pyruvate dehydrogenase activity was bound to the column but two peaks of 2-oxoglutarate dehydrogenase activity were detected. The major 2-oxoglutarate dehydrogenase activity peak co-migrated with a minor dihydrolipoyl dehydrogenase and NADH- $K_3Fe(CN)_6$ oxidoreductase activity peak. A second, small, 2-oxoglutarate dehydrogenase activity peak co-migrated with the main dihydrolipoyl dehydrogenase and NADH- $K_3Fe(CN)_6$ oxidoreductase activity peaks. A possible explanation is that some of the 2-oxoglutarate dehydrogenase complexes lose dihydrolipoyl dehydrogenase subunits during chromatography. This has been observed (Williams, 1976). It is suggested that dihydrolipoyl dehydrogenase and NADH- $K_3Fe(CN)_6$ oxidoreductase activities are inhibited in the intact complex by steric factors and/or regulatory mechanisms forcing the enzyme to operate in the physiological reaction direction. It follows that if dihydrolipoyl dehydrogenase were released from the 2-oxoglutarate dehydrogenase complex during chromatography, then these activities would be expected to increase.

The membrane-association of dihydrolipoyl dehydrogenase in *R. capsulatus* is not an artifact caused by part of the cell soluble fraction being trapped inside the membrane vesicles. Dihydrolipoyl dehydrogenase activity is detectable in unsolubilized membrane preparations. Further, addition of 1% (w/v) Triton X-100 to 1 mg membrane protein ml^{-1} in the assay mix (sufficient to cause a loss of sample turbidity and of NADH oxidase activity, indicating total membrane solubilization) did not change the dihydrolipoyl dehydrogenase activity.

A simple explanation of the membrane association of the 2-oxoacid dehydrogenase complexes in *R. capsulatus* could have been that it is an artifact resulting from the use of high speed centrifugation to pellet the cell membrane vesicles. The 2-oxoacid dehydrogenase complexes are large enzymes and might sediment during the high speed centrifugation needed to recover the *R. capsulatus* membrane vesicles (200 000 g for 2 h) or used to pellet unsolubilized membrane components after detergent treatment (100 000 g for 1 h). This explanation

is not consistent with the observation that a greater proportion of *R. capsulatus* pyruvate dehydrogenase complexes than 2-oxoglutarate dehydrogenase complexes are membrane associated (Table 2) when both complexes are likely to be of comparable size. It also does not explain why no obvious dihydrolipoyl dehydrogenase activity band is seen in membranes from *P. denitrificans* centrifuged and solubilized under the same conditions as the *R. capsulatus* membrane samples (B. C. Berks, unpublished observations). Further, a membrane-associated dihydrolipoyl dehydrogenase was detectable in *E. coli* even though spins of 30 000 g or less for 30 min were used in the vesicle preparations (Owen *et al.*, 1980).

The procedure for determining NADH dehydrogenase activities using isoelectric focusing should be of general value in identifying proteins with these activities in bacterial membranes. In addition to the Mid pI band NADH dehydrogenase that we characterized, the work described in this paper enabled us to identify two additional NADH dehydrogenase activities in *R. capsulatus*. The Low pI band was probably derived from the mitochondrial-type NADH-ubiquinone oxidoreductase as it was derived from an integral membrane protein and exhibited the correct substrate specificities. Further, it had a similar pI, to, and was unstable like, the Flavoprotein fragment of *P. denitrificans* NADH-ubiquinone oxidoreductase (George & Ferguson, 1987). The High pI band is possibly associated with the NADH dehydrogenase purified from *R. capsulatus* membranes by Ohshima & Drews (1981). Further research will be necessary to determine the validity of these suggestions.

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