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PURIFICATION OF THE REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DEHYDROGENASE FROM MEMBRANES OF *ACHOLEPLASMA LAIDLAWII*

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Summary

The ethanol-extracted respiratory chain-linked NADH dehydrogenase of *Acholeplasma laidlawii* has been purified 25—35-fold. This purification involved delipidation of the ethanol-extracted minute non-sedimentable membrane fragments by detergent treatment and gel filtration on Bio-Gel P-200. Sodium deoxycholate-sucrose density gradient centrifugation was followed by dialysis of the active NADH dehydrogenase fractions which caused flocculation of 60% of the membrane proteins while the NADH dehydrogenase remained suspended. Poylacrylamide gel electrophoresis of the purified NADH dehydrogenase gave one major and two minor bands after staining with Coomassie Blue. The purified enzyme gave straight line kinetics in Lineweaver-Burk plots and a K_m = 0.510 mM and V = 0.236 μmol/min. Fatty acid supplementation of *A. laidlawii* membranes had negligible effect on the membrane-bound or ethanol-extracted dehydrogenase, but substantiated the values of the K_m and V. Purification, however, altered the constants by 2—4-fold, suggesting that alteration of the microenvironment or fragmentation of the dehydrogenase was significant. The purified dehydrogenase was very susceptible to a rapid inhibition with heavy metal ions. Mg^{2+} and Ca^{2+} inhibited the enzyme but this inhibition was much slower (90 min) and less complete. Consideration of published purification procedures of NADH dehydrogenase strongly suggested that the purified *A. laidlawii* respiratory chain-linked NADH dehydrogenase was over 90% pure and certainly one of the most purified respiratory chain-linked bacterial NADH dehydrogenases.

Introduction

Membranes of the wall-less bacterium *Acholeplasma laidlawii* contain no cytochromes or quinones [1]. The truncated respiratory chain of these mem-

branes is therefore apparently flavin terminated [2]. The reduced NADH oxidase of these membranes possesses two sites of reduction of ferricyanide, suggesting the presence of at least two enzymes in the oxidase [3]. Extraction of the NADH dehydrogenase from membranes with 9.0% ethanol gives a dehydrogenase which no longer reacts with 0_2 and possesses altered and linear kinetics (i.e. one site of ferricyanide reduction) with ferricyanide as electron acceptor. This NADH dehydrogenase reacts optimally with ferricyanide, efficiently with menadione and C_2 Ind but only very slowly with 0_2, cytochrome *C* and ubiquinone Q_{10}. Dehydrogenase inactivated during purification attempts is not reactivated with FMN, FAD, membrane lipids or Mg^{2+}. It is inhibited by NADH when bound to the membrane but stimulated by NADH when extracted with 9.0% ethanol. The ethanol-extracted gel-filtered dehydrogenase contains about six proteins or peptides, about 30% glycolipid but no iron, which may reflect the uncoupled nature of the oxidase or Site I of the respiratory chain shown by studies of oxygen consumption of membranes.

In this study, we describe the purification of the ethanol-extracted dehydrogenase to one major band and two minor bands on polyacrylamide gels, by sodium deoxycholate-sucrose density gradient centrifugation and dialysis of the active gradient fractions. The specific activity is increased 25—35-fold by our purification scheme.

Materials and Methods

Preparation of membranes

A. laidlawii cells (ATCC No. 14192) were routinely grown on 2% tryptose media at 37°C, and harvested by centrifugation at 10 000 × *g* at 4°C [3]. The cells were then washed three times in buffer β (0.05 M Tris · HCl plus 0.01 M mercaptoethanol and saline). Washed cells were disrupted by sonic oscillation for 3 min (Bronwill ultrasonic disintegrator at maximum power). Membranes were isolated by centrifugation at 100 000 × *g* (Model L2-65B Beckman ultracentrifuge) for 60 min at 4°C of the disrupted cellular milieu. After washing, the membranes were suspended in 2.5 mM Tris · HCl buffer, pH 7.5 (3.6 mg protein/ml) and stored no more than 4 weeks at −20°C. Fatty acid-supplemented membranes were prepared by the method of McElhaney and Tourtellotte [4].

Purification of NADH dehydrogenase

Ethanol extraction of membrane. Approx. 40 ml of membrane suspension were extracted with 3.6 ml of ethanol. The membrane suspension was incubated at 43°C for 30 min, then centrifuged at 100 000 × *g* for sedimentation of the large fragments of membrane. The minute non-sedimentable fragments of membrane thus extracted were assayed for enzyme activity and protein content. The 9.0% ethanol extract contained about 51% of the protein and about 96.3% of the activity. The use of ethanol at this step was determined by the optimal ratio of $\Delta A_{340\,nm}$ (oxidase)/$\Delta A_{420\,nm}$ (dehydrogenase) and the polyacrylamide gel patterns from this extraction which gave the least number of protein or peptide bands (Fig. 1).

Agarose gel filtration of the extracted enzyme. Between 35 and 40 ml of

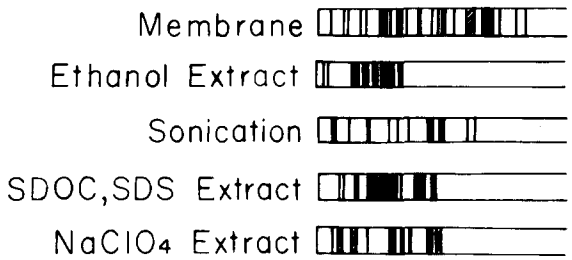

Fig. 1. Polyacrylamide gel electrophoresis of membrane and membrane extracts. 100—300 μg of protein were layered on the gel while suspended in the milieu of extraction. These acetic acid gels (phenol/acetic acid/urea system) were developed for 90 min at 5 mA/tube and then stained with 1% Coomassie Blue. SDS, sodium dodecyl sulfate; SDOC, sodium deoxycholate

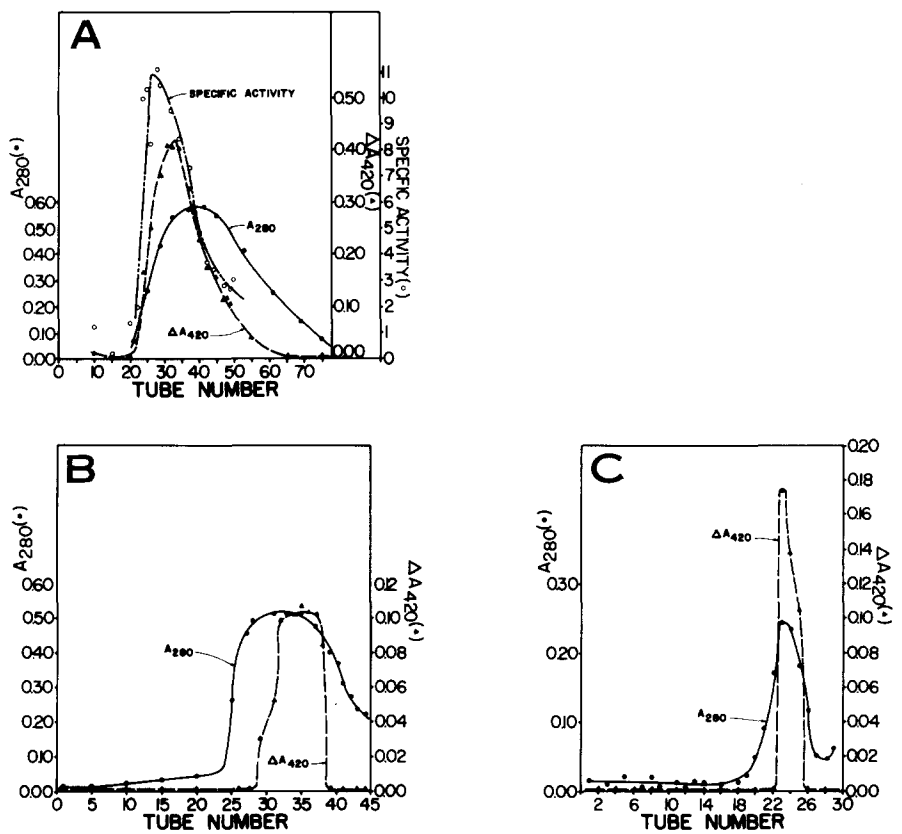

Fig. 2. Gel filtration and density gradient elution patterns of NADH dehydrogenase during purification. (A) Gel filtration on Bio-Gel A-50m of the 9.0% ethanol extract. The A_{280nm}-absorbing material (●———●), the NADH dehydrogenase activity, ΔA_{420nm} (▲———▲) and the NADH dehydrogenase specific activity of the fraction (○———○) determined and plotted for localization of the most purified enzyme fraction. (B) Gel filtration on Bio-Gel P-200 of the highest specific activity fractions for A-50m column. The A_{280nm}-absorbing material (●———●) and the NADH dehydrogenase activity, ΔA_{420nm}, (▲———▲) determined and plotted for localization of the fractions with the most active enzyme for separation from the lipid peak which is eluted in a different zone. (C) sodium deoxycholate-sucrose density gradient of the delipidated most active fractions from the P-200 columns. The A_{280nm}-adsorbing material (●———●) and the NADH dehydrogenase activity, ΔA_{420nm} (▲———▲) were plotted to locate the active enzyme.

ethanol-extracted enzyme at about 2.0 mg protein/ml were layered on a Bio-Gel A-50m column (3.0 × 44 cm with a bed volume of 185 ml of the Agarose gel). Fractions of 60 drops (2.2 ml) were collected with a Buchler Fractomette at a rate of about 0.5 ml/min. The $A_{280\,nm}$ absorption of the fractions was continually monitored (LKB Autoanalyzer) and recorded on a Sargent Welch Model SRG recorder. About 12% of the protein from the original membrane was recovered in this manner while, 57.5% of the activity remained in the active fractions. It is evident from the elution profile of the Agarose A-50m (Fig. 2A) that the enzyme with the highest specific activity was eluted in the void volume, an indication that the enzyme is still in the form of minute non-sedimentable membrane fragments. Properties of this enzyme preparation have been described in detail elsewhere [3].

Bio-Gel P-200 filtration. This column was primarily used to delipidate the minute non-sedimentable membrane fragments eluted from the agarose column [5]. This was done by treatment of the fragments with high concentrations of sodium deoxycholate (16 mg/3.6 mg protein) followed by gel filtration on P-200.

A column 3.0 × 40 cm was packed with a bed volume of 210 ml of Bio-Gel P-200 (reconstituted with 16 mg/ml sodium deoxycholate in 2.5 mM Tris · HCl, pH 7.5). The eluant for this column was 16 mg/ml sodium deoxycholate. The column was prepared for ascending flow to increase flow rate. About 30 ml of the most active fractions from the agarose A-50m column were applied to the column and eluted at about 2.0 ml/h. The fractions collected were 60 drops (2.2 ml). Analysis of the samples showed that 12% of the protein and 20% of the activity was found in the pooled active fractions. These assays were made more difficult by the presence of sodium deoxycholate and the necessity to use only small samples for each assay. The elution profile of this column is shown in Fig. 2B.

Sodium deoxycholate-sucrose density gradient centrifugation. Sodium deoxycholate (16 mg/ml)-sucrose density gradients were prepared in a Buchler gradient mixer with the range of 13—50%. Cellulose nitrate tubes which contain 40.0 ml and fit into the large buckets of the SW-27 rotor (Beckman Instruments, Palo Alto, Calif.) were filled with 28 ml of the gradient and 12.0 ml of the active pooled fractions from the P-200 gel, then layered on the gradient. The gradients were then centrifuged 16 h at 4°C at 95 000 × *g*. After coasting to a stop, the gradients were then fractionated by puncturing the tube and collecting drops. The fractions which contained 60 drops/tube (1.7 ml) were then assayed for $A_{280\,nm}$-adsorbing material and dehydrogenase activity (Fig. 2C). The three or four active fractions were then pooled from two gradients and dialyzed against 2.5 mM Tris · HCl, pH 7.5, overnight at 4°C. A cloudy flocculation containing about 60% of the protein of the dialyzate, was removed by centrifugation at 100 000 × *g* for 60 min at 4°C. The sediment and supernatant were analyzed for Folin-positive protein and dehydrogenase activity.

Enzyme assay

Because of its superior sensitivity for this enzyme, the ferricyanide reductase assay was used [3]. Substrate concentrations between 0.144 and 0.586 mM NADH and electron acceptor concentrations between 0.2 and 1.6 mM K_3Fe-

$(CN)_6$ were incubated at 37° C and followed at $A_{420\,nm}$. The incubation mixture was composed of NADH dehydrogenase (0.04—1.15 mg protein), NADH and $K_3Fe(CN)_6$ in 0.05 M phosphate buffer (pH 6.0). The reaction was followed with a Zeiss M4-QIII spectrophotometer with attached recorder (Omnigraphic 3000 Houston Instruments) and held at constant temperature with a Forma circulating constant temperature bath. The reaction was recorded over the first 30 s and if linear was directly related to the μmol of ferricyanide reduced. A unit of NADH dehydrogenase was described as the μmol of NADH oxidized/min per mg protein.

Polyacrylamide gel electrophoresis

Both the phenol-acetic acid-urea [6] and the sodium dodecyl sulfate [7] methods of polyacrylamide gel electrophoresis were used in this study. Tubes 15 × 0.6 cm were used instead of the usual 7.6 × 0.6 cm tubes to allow better resolution of the protein or peptide bands. A stacking gel was incorporated into the sodium dodecyl sulfate gel column for improved resolution of the bands. Among the several dyes tried Coomassie Blue [8] gave the most sensitive results. 70—90 μg of the sample protein were routinely applied to the gels.

Determination of the kinetic constants

Lineweaver-Burk of the dehydrogenase involved at least three determinations of each data point. In these plots, the inverse ferricyanide concentration was plotted against the inverse NADH equivalent of the $\Delta A_{420\,nm}$. The plots were run at 0.144, 0.288 and 0.576 mM NADH and from 0.2 to 1.6 mM $K_3Fe(CN)_6$. The data points were statistically analyzed according to the least squares method [9]. The standard error of the data points was well within those recognized for significant results (5—15%). The K_m and V were calculated from the intercept values of these plots. Kinetic determinations of the K_m and V for membrane-bound and ethanol-extracted enzyme were calculated

TABLE I

PURIFICATION OF THE NADH DEHYDROGENASE FROM MEMBRANES OF *A. LAIDLAWII*

Purification step	Volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg protein)	Recovery (%)	Purification factor
Membrane	44.2	163.5	177.59	1.085	100	0
9.0% ethanolic extract	44.0	83.6	170.96	2.095	96.3	1.93
Agarose gel-filtration (A-50m)	36.5	20.1	102.11	5.065	57.5	4.67
Bio-Gel P-200 filtration	27.4	5.67	35.64	6.085	20.1	5.61
Sodium deoxycholate-sucrose density gradient centrifugation	9.53	2.39	31.92	8.400	18.0	7.75
Dialyzed density gradient fractions	12.4	2.11	29.15	16.100	16.4	14.9
Supernatant of dialyzed density gradient fractions	12.2	0.85	23.97	23.200	13.5	21.4 Range (21.4—24.8)

by computer (Computer Center, State University of New York at Buffalo) using a FORTRAN program which fitted the data to a best-fit hyperbola with the equation $v = V \cdot [S]/K_m + [S]$. Data points were determined using NADH concentrations of 0.282—2.5 × K_m and electron acceptor concentration at 0.2—1.6 mM and five intermediate concentrations. The K_m and V for fatty acid-supplemented membranes and ethanol-extracted and purified dehydrogenase were then calculated using this program.

Metal ion inhibition tests

These tests utilized purified NADH dehydrogenase with the addition of 20 mM metal ions in the described dehydrogenase assay. These included Co^{2+} ($CoCl_2$), Cu^{+} (Cu_2SO_4), Cu^{2+} ($CuSO_4$), Fe^{2+} ($FeCl_2$), Fe^{3+} ($FeCl_3$), Mn^{2+} ($MnCl_2$), Zn^{2+} ($ZnCl_2$), Mg^{2+} ($MgCl_2$) and Ca^{2+} ($CaCl_2$). These salts were either added to the assay directly, or incubated or dialyzed with the purified NADH dehydrogenase, with or without other additives. Reconstitution of the purified dehydrogenase with sodium deoxycholate, lipid and Mg^{2+} or Ca^{2+} was attempted by several reconstitutive methods [10].

Chemicals

FAD, FMN and NADH were obtained from Sigma Chemical Co. Bio-Gel A-50m and Bio-Gel P-200 were obtained from Bio-Rad Laboratories. The sodium deoxycholate used in the early part of these studies was obtained from Mann Research Laboratories and was an enzyme grade specified as heavy metals less than 2 ppm and bile pigments practically nil. Later, because this product could not be obtained, we used sodium deoxycholate recrystallized from 50% ethanol in the presence of ethylenediamine tetraacetic acid (EDTA).

Results

Purification of NADH dehydrogenase

The effects of purification on the dehydrogenase are quantitated and summarized in Table I. Since specific activity curves of the various isolation procedures were single peaked, (Fig. 2) the results of these purification steps indicated that only one NADH dehydrogenase is found in the membranes of *A. laidlawii*. Because this NADH dehydrogenase is firmly bound to the membrane (as evidence by the requirement for release of high concentrations of sodium deoxycholate), it is usually characterized as an integral membrane protein.

The most noteworthy step in this purification scheme was the sodium deoxycholate-sucrose density gradient step, characterized by flocculation of proteins upon dialysis of the density gradient fractions. Although the NADH dehydrogenase did not flocculate more than 50% of the contaminating membrane proteins were removed by sedimentation of the flocculate. The dehydrogenase was purified 6-fold by density gradient centrifugation and dialysis without significant loss of yield. Preliminary experiments suggested that the precipitate inhibits the suspended dehydrogenase. The NADH dehydrogenase which remained suspended during dialysis and clarification was probably no longer a minute non-sedimentable membrane fragment. This purification scheme required six steps to obtain a purification factor of about 25 and a yield of about 13%. Upon occasion, a second sodium deoxycholate-sucrose density gradient of the

TABLE II

PURIFICATION OF THE NADH DEHYDROGENASE WITH A SECOND DENSITY GRADIENT

Purification step	Protein (mg/ml)	Activity (units/ml)	Specific activity (μmol/min per mg protein)	Recovery (%)	Purification factor
First sodium deoxycholate-sucrose density gradient centrifugation	0.315	3.34	8.40	100	7.75
Second sodium deoxycholate-sucrose density gradient centrifugation	0.073	0.84	11.05	25.2	10.15
Dialyzed density gradient centrifugation fractions	0.033	1.25	38.50	37.4	35.5
Centrifuged dialyzate *	0.073	0.315	4.30	9.43	4.0

* Addition of FMN, FAD, or Mg^{2+} did not restore activity.

active fractions from the first gradient yielded an enzyme which, after dialysis was purified thirtyfive-fold (Table II). A drawback of this second gradient was that only a 40% recovery of the first density gradient or only about 5.0% of the membrane-bound activity was recovered. Reactivation of the sediment after dialysis and clarification of the fractions from this gradient was not successful by addition of Mg^{2+}, FMN, FAD or membrane lipids.

Polyacrylamide gel electrophoresis

A comparison of the polyacrylamide electrophoresis gel patterns after various steps of the purification scheme (Fig. 3) shows a dramatic loss of protein or peptide bands from the membrane-bound enzyme to the purified NADH dehydrogenase. The membrane contained 20—30 bands, the A-50m gel filtered 10—15 bands, six major bands, the P-200 gel-filtered ethanol extract six bands, and the density gradient centrifuged one major and two minor bands. Similar results were obtained with both the sodium dodecyl sulfate procedure and the phenol-acetic acid-urea method of polyacrylamide gel electrophoresis. Another indication of increased purity by these gels is the gradual loss of a residual band of non-penetrating proteins on top of the gel.

Enzyme kinetics

Lineweaver-Burk plots of this NADH dehydrogenase (1/ferricyande concentration vs. 1/NAD formed per min) gave straight line functions between ferricyanide concentrations 0.2—1.6 mM (Fig. 4). These plots represent the average value of the data point after 3—6 determinations. The line was determined from 42 determinations and the slope of the line calculated by the least squares method. A similar function has been shown for the 9.0% ethanol-extracted enzyme. The slope of this curve, is more gradual than slopes of curves determined similarly for the ethanol-extracted and membrane-bound enzymes (the intercepts also vary). These changes in the slope and intercepts during purification are also shown by the gradual increase of the V and decrease of the K_m. These

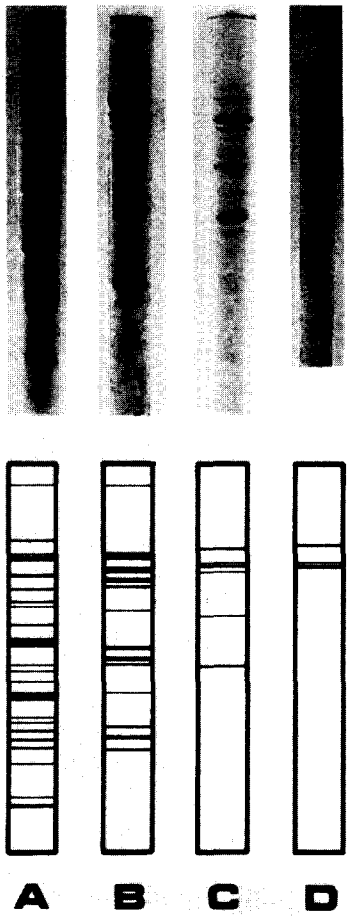

Fig. 4.

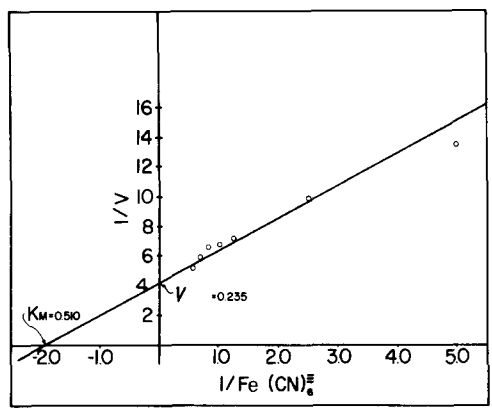

Fig. 3. Polyacrylamide gel electrophoresis of the NADH dehydrogenase after various stages of purification. The photographs of the gels are shown in the top row and the diagrammatic representation in the bottom row. These sodium dodecyl sulfate gels were developed for 6 h at 5 mA/tube and stained with 1% Coomassie Blue. Line A, membrane; B, agarose A-50m gel filtered; C, P-200 gel filtered NADH dehydrogenase and D, the density gradient-centrifuged NADH dehydrogenase.

Fig. 4. The Lineweaver-Burk plot of the purified NADH dehydrogenase. The function was determined at 0.288 mM NADH and at varying mM $Fe(CN)_6^{3-}$ concentrations. The $1/V$ legend represents the inverse of the μmol NADH oxidized per min. The straight line was determined by least squares method.

kinetic constants indicate that the enzyme becomes less controlled as the lipids and other membrane proteins are removed from the enzyme preparation. This conclusion is reinforced by preliminary data showing that the flocculated proteins of the dialysis step inhibit the supernatant. Purification of the dehydrogenase also increased the rate of reaction of the enzyme.

Additional evidence of the less controlled state of the purified enzyme was obtained by NADH inhibition studies (Fig. 5). These studies on the membrane-bound and ethanol-extracted dehydrogenase have shown correspondingly inhibition and stimulation of these enzyme forms with NADH. Our data suggests that between 0.144 and 0.288 mM NADH, about 25% inhibition occurs with

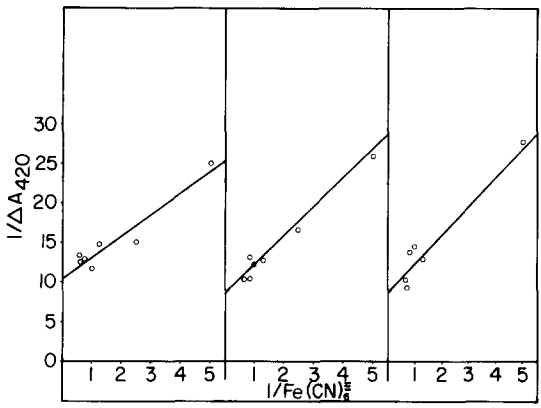

Fig. 5. The inverse plots of the purified NADH dehydrogenase at 0.144 mM(A), 0.288 mM(B) and 0.576 mM(C) NADH. The abscissa represents varying mM $Fe(CN)_6^{3-}$ concentrations. The straight lines were determined by the least squares method.

membrane-bound and 12.1% stimulation occurs with ethanol-extracted dehydrogenase. NADH inhibited the purified enzyme between 0.144 and 0.288 mM NADH by only 10% and no increase in inhibition was observed at 0.576 mM NADH. The purified enzyme is therefore refractile to NADH at concentrations above 0.288 mM, and much less inhibited than the membrane-bound enzyme. (These data points showed a much greater standard deviation and the curves were determined by least squares). This loss of control when compared to the membrane-bound or ethanol-extracted enzyme, is evidence of a more nearly purified dehydrogenase.

Effects of fatty acid supplementation

The fatty acid content of *A. laidlawii* was altered by supplementation of various fatty acid-poor media (Table III). These experiments show agreement

TABLE III

KINETIC CONSTANTS OF THE MEMBRANE-BOUND, ETHANOL-EXTRACTED AND PURIFIED NADH DEHYDROGENASE OF *A. LAIDLAWII*

Enzyme preparation	Fatty acid supplement	K_m (mM)	Error (%)	V (μmol/min)	Error (%)
Membrane-bound NADH dehydrogenase		2.05	29.0	0.059	19.9
	C_{12} lauric	1.39	22.1	0.059	13.2
	C_{14} myristic	1.20	23.1	0.069	12.4
	C_{16} palmitic	0.800	26.8	0.048	12.7
	C_{18} stearic	1.233	14.2	0.073	7.6
	C_{18} oleic	1.065	20.2	0.048	10.1
Ethanol-extracted NADH dehydrogenase		0.91	10.4	0.058	4.8
	C_{12} lauric	1.06	22.6	0.043	11.3
	C_{14} myristic	0.98	17.9	0.072	8.8
	C_{16} palmitic	1.36	12.0	0.066	6.7
	C_{18} stearic	2.45	20.7	0.081	14.6
	C_{18} oleic	1.28	10.2	0.058	5.5
Purified NADH dehydrogenase		0.510	11.2	0.236	3.9

TABLE IV

INHIBITION OF THE PURIFIED NADH DEHYDROGENASE WITH METAL IONS

Additions	Stimulation	No reaction	Inhibition
None (dialysis)	—	+	—
Co^{2+}, Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, or Zn^{2+}	—	—	+
Mg^{2+}, or Ca^{2+}	—	+	—
Mg^{2+} or Ca^{2+} (incubation at 37°C)	+0 min	—	+90 min
Mg^{2+} or Ca^{2+} (dialysis)	—	—	+
Sodium deoxycholate + lipid + Mg^{2+}	—	+	—
Sodium deoxycholate + lipid + Mg^{2+} (incubation at 60, 20, 4°C) *	—	—	+

* The slow annealing method has been very fruitful with these membranes [10].

of K_m and V values for membranes and ethanol-extracted dehydrogenase independent of fatty acid supplements. (The significance of the 5—10% variations between bound and ethanol-extracted dehydrogenase is debatable). No order of relationship seemed to exist between the fatty acid chain length and the values of the constants. By contrast, the dramatic changes in these constants after various stages of purification, indicate that the enzyme properties are more closely related to the stage of purification than to fatty acid content of the preparation. The percent error of these computer programs is considered very good if the K_m falls within 15% error and the V within 10% error; many of our values are within these limits. Also, the general decrease of the percent error with increasing purity indicates a more homogeneous system and therefore lower percentage error of the K_m and V in the more purified preparation. These experiments show that the fatty acid content of the enzyme preparation has little effect on the function of the NADH dehydrogenase.

Inhibition with metals

Metals are very inhibitory to this NADH dehydrogenase (Table IV). Inclusion of Co^{2+}, Cu^{+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} or Zn^{2+} in the incubation mixture inhibited the reaction. Mg^{2+} and Ca^{2+} also caused inhibition of this enzyme, but required incubation for up to 90 min for 56% inhibition. This delayed type of inhibition also could be shown by dialysis against these metals. Dialysis without metals, however, did not cause inactivation of the enzyme. This slow inactivation was not inhibited by the presence of sodium deoxycholate and lipids. Reconstitution experiments were not fruitful because no liposomes containing active NADH dehydrogenase could be prepared in the presence of metals. These data suggested two types of metal inhibition: a rapid inactivation by the first group of metals and a slow inactivation by Mg^{2+} and Ca^{2+}.

Discussion

The NADH oxidase of *A. laidlawii* has been isolated as a minute non-sedimentable membrane fragment [3]. Here we report the isolation of the primary respiratory chain-linked NADH dehydrogenase from that oxidase. In general,

bacterial respiratory chain-linked NADH dehydrogenases have been difficult to isolate and have only been purified 2—10-fold [11—13]. This dehydrogenase demonstrated a purification factor of 25—35-fold. Although purifications of bacterial NADH dehydrogenases have usually relied upon gel filtration patterns as indications of homogeneity, this purification and at least one other from *Peptostreptococcus elsdenii* [11] have been closely monitored by polyacrylamide gel electrophoresis. Both of these NADH dehydrogenases represent about a 20—30-fold purification [11] from membrane and demonstrate only one major band in polyacrylamide gel patterns. These results suggest that purification factors of 20—30-fold of bacterial NADH dehydrogenases represent 90% purification of these enzymes. This generalization is also applicable to mitochondrial respiratory chain-linked NADH dehydrogenases, which are called homogeneous protein preparations at purifications of 30—60-fold [14—17]. The NADH dehydrogenase from *A. laidlawii* definitely possesses a purification factor similar to the most purified respiratory chain-linked NADH dehydrogenases which have been isolated. The yield of purified dehydrogenase was also within the range obtained with similar schemes, approx. 10—30%. The specific activities of this dehydrogenase and of other bacterial dehydrogenases were usually 40—50-fold less than those of mitochondrial enzymes. Purified preparations gave specific activities of 20—40 while mitochondrial dehydrogenase specific activities were 1000—1600. Variations in apparent K_m and V verified these differences [11—17].

Release of the dehydrogenase after treatment with sodium deoxycholate and gel filtration in the presence of sodium deoxycholate eluant resulted in a heavy loss in yield, probably due to the removal of lipids from the microenvironment of the dehydrogenase. The increase in specific activity which accompanies the loss of activity here may be due to the non-specific activation usually observed with release of an enzyme from the membrane matrix [18].

The linearity of the Lineweaver-Burk plots of the purified dehydrogenase substantiated the homogeneity of the dehydrogenase and the conditions of assay. The K_m and V from a typical plot prepared by least squares agreed closely with the constants computed with our FORTRAN program. The agreement of the several data points and the relation of these points to the line were also encouraging. Significant alterations of K_m and V values were, however, observed as the enzyme was purified. On the other hand, the fatty acid content of the membrane-bound ethanol-extracted dehydrogenase had little effect on the K_m and V values of that stage of purity. We interpret this as a lack of fatty acid composition effect on the NADH dehydrogenase of this membrane. Other researchers obtained similar results with the NADH oxidase from this membrane using Arrhenius plots [19]. It is possible that the alterations of the kinetic constants as the enzyme is purified represent not only molecular fragmentation of the dehydrogenase [20], but also alterations in the microenvironment of the enzyme. The lack of effect of fatty acids on this oxidase are unusual; discontinuities in the Arrhenius plots of NADH oxidase from several yeast mitochondria [21,22] and *Escherichia coli* [23] have been reported. This may be a result of the simplicity of the NADH oxidase from *A. laidlawii* membranes. It may also reflect the independence of the NADH dehydrogenase from membrane lipids.

The inhibitory effect of various metals on this NADH dehydrogenase could be divided into two categories: a rapid, nearly complete and slower, incomplete (56%) inhibition. The rapid inhibition was observed immediately after introduction of the heavy metal ions to the assay mixture. Inhibition studies of the NADH oxidase of *E. coli* [24] show that Zn^{2+} inhibits NADH oxidase activity between the NADH dehydrogenase and cytochrome *b* in this chain. The primary NADH dehydrogenase activity of this chain is refractile to Zn^{2+}. Our NADH dehydrogenase is inhibited by Zn^{2+}. The slow inhibition caused by Mg^{2+} and Ca^{2+} may be explained by activation of a protease in the enzyme preparation. The loss of activity only if Mg^{2+} or Ca^{2+} is present during dialysis, and the length of the time required for only partial inhibition, lend support to this hypothesis.

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