The Electron Transport System of Hydrogenomonas eutropha

II. REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE-MENADIONE REDUCTASE

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Wosilait and Nason (1) demonstrated that quinones could serve as electron acceptors for the enzymatic oxidation of reduced pyridine nucleotides, and their survey of plant, animal, and microorganism extracts (2) revealed the general occurrence of reduced pyridine nucleotide-quinone reductase activity. This finding and Dam's (3) earlier report, which indicated that vitamin K is a common constituent of plants and microorganisms, suggested that vitamin K may be the physiological acceptor for quinone reductase. The insolubility of vitamin K in aqueous media caused Wosilait and Nason (1) to select the parent compound, menadione, for their pyridine nucleotide-quinone reductase studies in Escherichia coli. Menadione reductase activity subsequently was demonstrated in several purified mammalian flavoprotein enzymes (5) in extracts of Achromobacter fischeri (6), Streptococcus faecalis (7), and Azorhizobacter vinelandii (8), and, recently, in yeast extracts (from which menadione reductase has been crystallized (9)). Weber, Brodie, and Merselis (10) used vitamin K₄ dissolved in Tween 80 and vitamin K requirement for mitochondrial systems (11, 12) has also been shown.

Considerably more direct evidence has established that another quinone (coenzyme Q) serves a major role in oxidation-reduction reactions of mitochondria (13). It is interesting that various homologues of coenzyme Q have been found in microorganisms (14, 15), yet there are no reports of reduced nicotinamide adenine dinucleotide-coenzyme Q reductase (16) or succinate-coenzyme Q reductase activities (17) (with the higher CoQ homologues) in bacterial extracts. Lester and Crane (14), who analyzed Hydrogenomonas eutropha (listed by them as Hydrogenomonas species) for CoQ, found 0.1 μmole of CoQ per g of cells, dry weight. Since the participation of CoQ in electron transport of mammalian tissue has been demonstrated, and as much as appreciable quantities of CoQ₄ occur in extracts of H. eutropha, crude extracts were examined for reduced pyridine nucleotide-CoQ reductase activity.

No pyridine nucleotide-CoQ reductase activity was detected. However, considerable NADH-menadione reductase activity was found, and various benzo- and naphthoquinones could replace menadione as electron acceptor. This report describes the characteristics of NADH-menadione reductase from H. eutropha.

METHODS

Mass cultures of H. eutropha were grown autotrophically in an inorganic salts medium in an atmosphere containing 70% hydrogen, 20% oxygen, and 10% carbon dioxide (18). Cultures were harvested after 90 hours of incubation or approximately at the beginning of the stationary growth phase. Heterotrophically grown cells were cultured in the same salts medium supplemented with 1% glucose and with air in place of the gas mixture. Cells were harvested by centrifugation and were frozen until needed.

Extracts were made with a 10-Kc Raytheon sonic oscillator (10 min) or a 20-Kc Branson Sonifier (3 to 4 min) from 10 to 15 g of frozen cells suspended in 30 ml of 0.01 M phosphate buffer, pH 5. The temperature was maintained between 0° and 5°. After cell breakage, the pH was about 6.5. Large particles were removed by centrifugation at 23,000 X g for 45 min, and the supernatant fluid containing the enzyme was referred to as the crude extract. The crude extract was not routinely centrifuged again to remove small particles; however, when this was done at 144,000 X g for 2 hours, more than 90% of the activity was recovered in the supernatant fluid.

Relatively high concentrations of menadione were required for maximum enzyme activity; to increase the solubility of menadione, BRIJ 35 (19) was added to the reaction mixture. Nevertheless, when each constituent was added separately, some menadione precipitated, after which it slowly redissolved. This could be eliminated by preparing a mixture containing 0.01 M menadione (dissolved in 95% ethanol), 0.3 ml of 10% BRIJ 35, and 0.3 ml 1 M triethanolamine buffer, pH 8.5, made up to 1.80 ml with water (Mixture I). Heating at 35° for several minutes promoted solution of menadione. This mixture, kept at room temperature in a foil-covered amber bottle, gave consistent results throughout each day. The reaction mixture contained 1.80 ml of Mixture I; 0.05 μmole of FMN; enzyme, diluted as required in 0.1 M potassium maleate buffer, pH 6.5; and 0.5 μmole of NADH in a total volume of 3 ml. A blank without NADH was used as reference. The reaction, started by the addition of NADH, was followed at 340 mμ in a Cary model 14 spectrophotometer. Controls without enzyme were periodically included; there was no nonenzymatic oxidation of NADH by menadione. Rates, measured at room temperature, were calculated from initial slopes, which generally remained linear for several minutes. A unit was defined as that amount of enzyme which produced an optical density change at 340 mμ of 0.010 per min. Specific activity was based upon units per mg of protein. Protein was determined by the biuret reaction (20).

The abbreviations used are: CoQ, coenzyme Q; BRIJ 35, polyoxyethylene lauryl alcohol; CMB, p-chloromercuribenzoate.
Reduction of cytochrome $c$ by menadione-dependent NADH-menadione reductase was determined by adding 0.3 ml of 1% mammalian cytochrome $c$ (Sigma, type III) to the menadione reductase assay system and following the increase in absorption at 550 m$\mu$. Reduction of cytochrome $c$ by menadione-independent cytochrome $c$ reductase was assayed without menadione or BRIJ. In both assays the initial linear slopes were used to calculate rates.

RESULTS

Crude extracts of autotrophically grown $H$. eutropha catalyzed a rapid reduction of NAD by molecular hydrogen (21, 22), but on a per mg of protein basis the rate of oxidation of NADH by un-supplemented crude extracts was only 3 to 5% of the rate of NAD reduction. Neither the naphthoquinones, vitamin $K_3$, nor the benzoquinones, CoQ$_4$ or CoQ$_{10}$, were able to serve as electron acceptors for NADH. Addition of vitamin $K_3$ (menadione), on the other hand, resulted in a 15- to 20-fold stimulation of NADH oxidation; vitamin $K_3$ bisulfate was only 15% as effective as menadione. Extracts of heterotrophically grown cells also possessed menadione reductase activity, with essentially the same specific activity.

As shown in Fig. 1B, saturation of the enzyme with respect to menadione occurred at about 0.0012 m. These data were re-plotted in Fig. 1A, and the calculated $K_m$ with respect to menadione was 6.8 $\times$ 10$^{-4}$ m at an NADH concentration of 1.7 $\times$ 10$^{-4}$ m. Neither BRIJ 35 nor the ethanol carried over in the menadione stock solution had any effect on menadione reductase activity. No alcohol dehydrogenase activity was detected in the extracts (see Fig. 5). If BRIJ 35 was replaced by deoxycholate, the rate of menadione reductase was inhibited 35%.

At the beginning of these studies, it was noted that a given enzyme dilution (kept at 0°) showed greater menadione reductase activity after a period of time. Precautions to protect Mixture I from light by using amber bottles covered with aluminum foil did not eliminate this effect. Table I shows the magnitude of these changes with two quantities of the same enzyme dilution assayed at different time intervals after the dilution had been made. The greater change in activity occurred within the first 30 min; the activity became stable after approximately 60 min. If enzyme dilutions were made in different concentrations of maleate buffer or in phosphate buffer (Table II), the activity at zero time and the final activity reflected enzyme stability in these buffers. Again it was observed that the rate of NADH oxidation increased with time, and that this increased rate was proportional to enzyme concentration with all buffers used. The enzyme, not Mixture I, was responsible for the change in rate, for a freshly prepared enzyme dilution assayed with the old Mixture I showed the same increase in rate with time. Table II shows that the enzyme is most active when diluted in 0.1 m potassium maleate buffer; although not shown here, maleate was superior to phosphate buffer at equivalent concentrations. Reliable rate measurements could be obtained if assays were performed 1 hour after the appropriate enzyme dilution was made. Under these conditions activity was proportional to enzyme concentration for well over a 10-fold difference in enzyme concentration.

The optimum pH range for NADH-menadione reductase activity was 8.3 to 0.0 (Fig. 2). Although the plateau shown was determined by only two points, data obtained with other extracts at intermediate pH values indicated that there was no sharp pH optimum. The reaction rate was the same with phosphate, Tris, or triethanolamine buffers in the assay reaction mixture. The stability of the enzyme at various pH values, as de-
FIG. 2. The pH optimum for NADH-menadione reductase activity. Assay conditions were those described in "Methods" except that triethanolamine buffer of the appropriate pH was used in preparing Mixture I. Recorded pH values were determined on the reaction mixture after the experiment. Enzyme was heat-treated crude extract having a specific activity of 464.

FIG. 3. pH stability of NADH-menadione reductase. Dilutions of crude extract (specific activity, 193) were made in 0.1 M potassium phosphate buffers at 45°, and incubation at that temperature was continued for 1 hour. Dilutions were then iced and assayed immediately. The recorded pH was determined on the enzyme dilution after the incubation period.

determined by incubating enzyme dilutions in buffers of different pH for 1 hour at 45°, was found to be optimal at pH 6.5 (Fig. 3).

H. eutropha menadione reductase is NADH-specific; no activity occurs with NADPH (Table III). The saturation curve of NADH indicates that increasing initial rates were obtained up to $1.0 \times 10^{-5}$ M NADH (Fig. 4A), while above this concentration progressive inhibition of NADH oxidation occurred. The $K_m$ with respect to NADH is $2.1 \times 10^{-5}$ M (Fig. 4A). The product of the reaction, NAD, was not destroyed for it was reduced again by the addition of ethanol and alcohol dehydrogenase (Fig. 5). Complete reduction of NAD did not occur in this instance because NADH was simultaneously being oxidized by menadione reductase.

The addition of FMN to the NADH-menadione reductase reaction mixture (Table III) resulted in a 10 to 50% increase in

TABLE III

<table>
<thead>
<tr>
<th>Effect of various substitutions on NADH-menadione reductase</th>
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<tr>
<td>The usual assay system (refer to &quot;Methods&quot;) was used with deletions or additions indicated. Initial rates at 340 μM and 550 μM are given. A partially purified enzyme preparation having a specific activity of 1560 was used.</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Assay system</th>
<th>Change in absorbance per min</th>
</tr>
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<tbody>
<tr>
<td>Complete</td>
<td>0.074</td>
</tr>
<tr>
<td>Minus NADH, plus NADPH*</td>
<td>0.000</td>
</tr>
<tr>
<td>Minus FMN</td>
<td>0.058</td>
</tr>
<tr>
<td>Minus menadione, plus vitamin K,†</td>
<td>0.000</td>
</tr>
<tr>
<td>Minus menadione, plus 3 mg of cytochrome c</td>
<td>0.083 0.410</td>
</tr>
<tr>
<td>Minus menadione, plus 3 mg of cytochrome c</td>
<td>0.002 0.022</td>
</tr>
</tbody>
</table>

* NADPH concentration, $1.7 \times 10^{-4}$ M.
† Vitamin K, concentration, $9 \times 10^{-4}$ M.

FIG. 4. Effect of NADH on the reaction rate at pH 8.5. Refer to "Methods" for assay conditions. The reaction was started by addition of NADH at the final concentration indicated. Rates were average values from four experiments. Crude extract contained 378 units per mg of protein. A, data plotted according to Lineweaver and Burk; B, direct plot of initial reaction rate with respect to substrate concentration.

FIG. 5. Oxidation of NADH by NADH-menadione reductase and further reduction of NAD by alcohol dehydrogenase. After complete NADH oxidation by menadione reductase, 170 μmoles of ethanol were added followed by addition of recrystallized alcohol dehydrogenase (Sigma). NADH-menadione reductase was partially purified (zinc gel eluate) containing 1560 units per mg of protein.
rate, which varied with the particular enzyme preparation used. FAD had no effect either alone or in conjunction with FMN. FMN did not function as an electron acceptor for NADH without added menadione even when the concentration of FMN was increased to $5 \times 10^{-4} \text{ M}$. FMN appears to function as a cofactor between NADH and menadione and not as a terminal acceptor beyond menadione for the following reasons. (a) At an alkaline pH, menadione is rapidly autoxidized (23), obviating the need for an additional terminal electron acceptor. (b) The initial rate of NADH oxidation was the same when the reaction was carried out under aerobic or anaerobic conditions (Fig. 6) except that the extent of oxidation was influenced by the presence of oxygen. (c) Since the concentration of FMN in the reaction mixture was only one-tenth that of NADH, it could not account for the extent of anaerobic NADH oxidation.

The NADH-menadione reductase assay for H. eutropha was compared with that used with E. coli extracts by Woolnaid and Nason (4), the major differences being the absence of flavin and the lower concentration of menadione in their assay. H. eutropha NADH-menadione reductase was about one-third as active in the E. coli assay as under the conditions described here.

The effect of replacing menadione with vitamins K₃ (Table III) and K₄ (9 $\times\ 10^{-4} \text{ M}$), CoQ₈ (0.5 mg/3 ml), and CoQ₁₀ (0.4 mg/3 ml) was re-evaluated after optimum conditions for NADH-menadione reductase had been determined. No NADH oxidations occurred with these quinones alone; when they were used in conjunction with menadione, the rate of NADH oxidation was the same as with menadione alone or slightly less.

Menadione, which served as an effective electron acceptor from NADH, unlike vitamins K₁, K₂ and CoQ₈ or CoQ₁₀, has an unsubstituted 3-position. Several naphthoquinones and benzoquinones were tested to determine the effect of different substituent groups on the ability of the quinone to act as electron acceptor in this system (Table IV). Solutions of each quinone were prepared in 95% ethanol, but different solubilities of the quinones in ethanol caused some variation in their final concentrations. Mixture I, containing buffer, quinone, and BRIJ 35, could not be used because some of the quinones were unstable at pH 8.5; this was seen as a change in the appearance of the quinone and a loss of effectiveness as an electron acceptor. The constituents were therefore added individually; 0.04 ml of an ethanol-quinone test solution was used in each reaction mixture. Corrections were made for nonenzymatic oxidation of NADH, which in some instances was large. It appears from this limited sampling that 2,3-unsubstituted or 2-methyl naphtho- or benzoquinones function as electron acceptors for the enzymatic oxidation of NADH whereas the 2-amino- or 2,3-substituted naphthoquinones tested are inactive. These results suggest that vitamins K₁ and K₄ and CoQ₈ and CoQ₁₀ may be inactive because the 3-position is substituted.

NADH-menadione reductase was inhibited 32 and 60% by 1.7 $\times\ 10^{-4}$ and 1.7 $\times\ 10^{-4}$ M CMB, respectively. Azide, Amytal, quinacrine, 2,4-dinitrophenol (5 $\times\ 10^{-3}$ M), Dicumarol (2 $\times\ 10^{-3}$ M), arsenite, arsenate, or chelating agents such as α,α-dipyridyl and EDTA had no effect.

Sufficient crude extract was placed in the bottom half of a Petri dish to form a thin layer which just covered the glass surface. This was placed on ice and irradiated for 30 min with ultraviolet light (Mineralight lamp) at a distance of 2 inches. Neither the NADH-menadione reductase activity nor the menadione-independent cytochrome c reductase activity (determined with greater enzyme concentrations) was affected by this treatment.

The crude H. eutropha extract was treated twice with 4 volumes of cyclohexane and assayed for NADH-menadione reductase, CoQ₈ reductase, and menadione-independent cytochrome c reductase activities. Cyclohexane extraction did not alter the original menadione reductase or menadione-independent cytochrome c reductase activities, and CoQ₈ was still not able to function as an electron acceptor for NADH.

H. eutropha crude extracts contain a c-type cytochrome which is reduced under an atmosphere of hydrogen gas and is reoxidized in air (22), but sufficient quantities of this cytochrome have not
yet been purified to permit a study of cytochrome reductase activity with the native cytochrome. Although bacterial systems are often specific for their homologous cytochromes, it was nevertheless of interest to determine whether mammalian cytochrome c (Sigma, type III) was enzymatically reduced by NADH with \textit{H. eutropha} extracts. As shown in Table III, mammalian cytochrome c was readily reduced in the NADH-menadione reductase assay system. In the absence of menadione, the rate of cytochrome c reduction was substantially less.

Nonenzymatic reduction of cytochrome c by reduced menadione has been reported. Menadione reductase was purified 15- to 20-fold by heat treatment, absorption and elution from zinc gel, and chromatography on DEAE-cellulose without evidence of separation of NADH-menadione reductase from the menadione-dependent cytochrome c reductase. The menadione saturation curve was identical for menadione reductase and for menadione-dependent cytochrome c reductase. Flavin stimulation was found, and the reductase activity also was FMN-specific. The pH optimum in the presence of cytochrome c (pH 8.5 to 9.5) was shifted about 0.5 pH unit above the optimum for menadione reductase. Reduction of cytochrome c in this system is thought to be nonenzymatic.

**DISCUSSION**

It is peculiar that although \textit{H. eutropha} contains coenzyme Q\textsubscript{4} whole sonic preparations or crude extracts do not catalyze a reaction analogous to the NADH CoQ reductase (16) found in mitochondrial preparations. Crude \textit{H. eutropha} preparations were extracted twice with cyclohexane in an attempt to demonstrate the requirement for lipid-soluble factors. After extraction with cyclohexane, menadione reductase and menadione-independent cytochrome c reductase activities were unchanged. Neither CoQ\textsubscript{4} nor CoQ\textsubscript{10} served as an electron acceptor before or after extraction.

Extracts exposed to ultraviolet light to inactivate light-sensitive naphthoquinones (24) were unchanged with regard to menadione reductase and menadione-independent cytochrome c reductase activities. Neither vitamin K\textsubscript{1} nor K\textsubscript{2} was stimulatory. Wosilait and Nason (4) and Brodie and Brodie (25) found no flavin requirement for menadione reductases from \textit{E. coli} and \textit{M. phlei}; similarly no exogenous flavin was required for yeast menadione reductase, but the crystallized enzyme from yeast was shown to have FAD as the prosthetic group (9). Other menadione reductases from \textit{H. eutropha}, \textit{A. fischeri} (6), and \textit{S. faecalis} (7) were activated by added FMN. This suggests that menadione reductase is a flavin enzyme, and, depending on the source of the enzyme, the flavin may be more or less firmly bound.

Brodie (26) found that menadione reductase activity in \textit{M. phlei} was associated with the soluble fraction required for oxidative phosphorylation. Earlier it was shown (25) that menadione reductase from this organism was insensitive to 10\textsuperscript{-4} M CMB and by this means could be distinguished from a menadione-independent cytochrome c reductase which was sensitive to CMB. Results presented in this paper show that \textit{H. eutropha} menadione reductase, unlike the \textit{M. phlei} enzyme, is inhibited by CMB; 1.7 \times 10\textsuperscript{-4} M and 1.1 \times 10\textsuperscript{-4} M CMB inhibited 32 and 67%, respectively.

Dicumarol has been shown to interfere with the K vitamins, presumably because of the structural similarity of the aromatic nuclei. Dicumarol competitively inhibits menadione reductase from \textit{E. coli} (4) and the diphosphopyridine nucleotide-triphosphopyridine nucleotide ("DT") diaphorase (27), and it was reported to cause 50% inhibition (at 3 \times 10\textsuperscript{-4} M) with crystalline yeast menadione reductase (9). The \textit{H. eutropha} menadione reductase was insensitive to 2 \times 10\textsuperscript{-5} M Dicumarol.

The effect of 2,4-dinitrophenol on menadione reductase is not uniform. Brodie and Gray (28) reported that NADH oxidation by menadione with \textit{M. phlei} extracts was inhibited by the presence of dinitrophenol, but in a subsequent paper (26) a dinitrophenol-treated supernatant fraction containing menadione reductase was shown to inhibit oxidative phosphorylation without inhibiting oxygen uptake. Dinitrophenol (10\textsuperscript{-4} M) caused only 7% inhibition with \textit{E. coli} extracts (4) and no inhibition with the \textit{H. eutropha} enzyme.

**SUMMARY**

Extracts of autotrophically grown \textit{Hydrogenomonas eutropha} contain a menadione reductase which is specific for reduced nicotinamide adenine dinucleotide. The reaction has a pH optimum of 8.3 to 9.0. Activity was stimulated by flavin mononucleotide but not by flavin adenine dinucleotide. The \textit{Km} values with respect to menadione and NADH were 5.8 \times 10\textsuperscript{-4} M and 2.1 \times 10\textsuperscript{-5} M, respectively. Added cytochrome c was rapidly reduced, although this probably represented nonenzymatic reduction of cytochrome c by reduced menadione. Various naphtho- and benzoquinones could replace menadione as electron acceptor if the 3-position was unsubstituted. No vitamin K\textsubscript{1} or K\textsubscript{2} or coenzyme Q\textsubscript{4} or Q\textsubscript{10} reductase activity was detected.

**REFERENCES**

3. Dam, H., \textit{Advances in Enzymol.}, 2, 280 (1942).
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