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Menadione reductase from *Desulfovibrio gigas*

Menadione reductase (NAD(P)H:2-methyl-1,4-naphthoquinone oxidoreductase, EC 1.6.5.2) has been studied in a number of bacteria[1-3]. However, there appear to be no reports concerning the existence of this enzyme in the genus Desulfovibrio, which includes a large number of microorganisms exhibiting a strictly anaerobic mode of growth based on the reduction of sulfate as terminal H-acceptor. We report here that in extracts of *Desulfovibrio gigas* there exists considerable NAD(P)H–menadione reductase activity; the enzyme has been purified 25-fold and some of its properties have been determined.

Cultures of *D. gigas* were grown in an "Amsco" fermentor, as described previously[4]. The bacteria (80 g wet weight) were suspended in 70 ml 0.01 M Tris–HCl (pH 7.6) and disrupted with a French pressure cell. The crude extract and the soluble protein fraction were prepared as already described[5].

Solid $(NH_4)_2SO_4$ was then added to the soluble protein suspension to give 45% saturation. After stirring for 20 min, the precipitate was removed by centrifugation, redissolved in 35 ml of 0.5 M Tris–HCl (pH 7.6) and dialyzed against 0.01 M Tris–HCl (pH 7.6). The dialyzed suspension was applied to a TEAE-cellulose column previously equilibrated with 0.01 M Tris–HCl (pH 7.6) and the fraction which was not adsorbed contained the activity. The suspension containing the enzyme was placed on a column of Sephadex G-200 (3.5 cm × 85 cm) equilibrated with 0.01 M Tris–HCl (pH 7.6) and filtered with the same buffer. The fraction containing the activity was applied to a hydroxylapatite column equilibrated with 0.001 M phosphate buffer (pH 7.6), and menadione reductase was eluted using 0.024 M phosphate (pH 7.6). The enzyme was purified approx. 25-fold; pertinent data are given in Table I.

The menadione reductase of *D. gigas* exhibited a great instability and the different steps of purification resulted in considerable loss of activity.

The effect of pH upon the activity of the purified enzyme was studied with

TABLE I

ENZYME PURIFICATION

The reaction mixtures contained the following (μmoles): acetate buffer (pH 5.2), 100; FMN, 0.05; NADPH, 0.5; menadione, 0.5; and enzyme in a total volume of 3 ml. NAD(P)H menadione reductase activity is measured at 22° by following the oxidation of reduced pyridine nucleotides at 340 nm. A unit of enzyme was defined as that amount of enzyme which produced an absorbance change at 340 nm of 0.010 per min.

Enzyme fraction	*Enzyme units*	*Specific activity (units/mg protein)*
Crude extract	1 605 600	74,5
Soluble protein	1 080 000	107
45% $(NH_4)_2SO_4$	820 000	120
TEAE-cellulose	592 850	138
Sephadex G-200	403 200	640
Hydroxylapatite gel	288 880	1862

Abbreviation: PCMB, *p*-chloromercuribenzoate.

TABLE II

COMPARATIVE MENADIONE REDUCTASE ACTIVITY WITH NADH AND NADPH; EFFECT OF FLAVIN NUCLEOTIDES

The components of the reaction mixture are given in Table I with the deletions or additions indicated. The partially purified enzyme preparation having a specific activity of 1862 was used (approx. 10 μg protein per experiment).

Assay system	*Flavin concentration (μM)*	*Change in absorbance at 340 nm per min**
NADH		
− Flavin	—	0.030
+ FMN	16.6	0.096
	33.2	0.098
+ FAD	16.6	0.037
NADPH		
− Flavin	—	0.048
+ FMN	16.6	0.160
	33.2	0.165
+ FAD	16.6	0.056

* Corrected for nonenzymatic oxidation of pyridine nucleotides.

phosphate buffer (pH 5.8–7.8) and acetate buffer (pH 4.2–5.6). The optimum pH range for menadione reductase activity was 4.8–5.4.

The menadione reductase of *D. gigas* is not NADH-specific; it oxidizes both NADH and NADPH. However, NADPH is more effective (Table II). The purified enzyme preparation reduced menadione in the absence of added flavin nucleotide. Addition of FMN increased the activity of the preparation three times but FAD had no effect (Table II). A difference spectrum of the purified preparation (oxidized *minus* reduced form) demonstrated that flavin (absorption maximum 446 nm) was present.

The rates of oxidation of reduced pyridine nucleotides were measured with

TABLE III

QUINONE REQUIREMENT OF MENADIONE REDUCTASE

The standard assay procedures were used except that the menadione was replaced by the indicated quinone added in 0.05 ml methanol (except for *p*-benzoquinone which was in aqueous solution). The partially purified enzyme preparation having a specific activity of 1862 was used.

Quinone	*Concentration (mM)*	*Change in absorbance at 340 nm per min**
1,4-Naphthoquinone	0.20	0.130
2-Methyl-1,4-naphthoquinone (menadione)	0.16	0.162
Vitamin K_1	0.38	0.000
p-Benzoquinone	0.15	0.188
p-Toluoquinone	0.17	0.192
Ubiquinone (Co-Q_6)	0.20	0.000

* Corrected for nonenzymatic oxidation of pyridine nucleotides.

various concentrations of substrates. The apparent K_m determined by Lineweaver–Burk curves were found to be approx. 2.10^{-5} M, 5.10^{-5} M and $1.45.10^{-4}$ M at pH 5.2 with menadione, NADPH and NADH, respectively. Substrate inhibition of the reaction occurred with NADH at higher substrate concentrations (0.5 mM).

Several quinones were tested to determine their ability to act as electron acceptor in this system (Table III). The need for menadione could be replaced by 1.4-naphthoquinone, *p*-benzoquinone and *p*-toluoquinone but not by vitamin K_1 and ubiquinone. Ferricyanide can be an electron acceptor for *D. gigas* menadione reductase, but the enzyme showed no cytochrome c reductase activity. Recently, a menaquinone (MK-6) was isolated from *D. gigas*[6,7]. This quinone (kindly furnished by Dr. O. Isler, Hofmann-La Roche Co.) tested for its ability to replace menadione with *D. gigas* purified enzyme was inactive.

The response to inhibitors showed that the enzyme is sensitive to *p*-chloromercuribenzoate (PCMB) and dicumarol. The presence of 2.10^{-5} M PCMB and 5.10^{-5} M dicumarol inhibited enzyme activity 64 and 57%, respectively.

Menadione reductase activity was described in extracts of many bacteria but its function remained in doubt. However, WEBER *et al.*[8] demonstrated its participation in the electron transport systems of *Mycobacterium phlei* and *Corynebacterium creatinovorans*. Menadione reductase is present in species of the genus Desulfovibrio other than *D. gigas* (*Desulfovibrio vulgaris* hildenborough, *Desulfovibrio desulfuricans* El Agheila Z), but its functional role remains to be investigated.

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