On the Role of Menaquinone-6 in the Electron Transport of Hydrogen:Fumarate Reductase System in the Strict Anaerobe *Desulfovibrio gigas*

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INTRODUCTION

The genus *Desulfovibrio* includes a great number of micro-organisms exhibiting a strictly anaerobic mode of growth based on reduction of sulphate as terminal electron acceptor (Postgate, 1965). Several species including *Desulfovibrio gigas* grow in a sulphate-free medium in the presence of fumarate (Miller & Wakerley, 1966). The enzymes allowing fumarate dismutation into acetate and succinate were studied in *D. gigas* (Hatchikian & Le Gall, 1970*a, b*) and an increased level of fumarate reductase activity was observed in the particulate fraction prepared from *D. gigas* grown on fumarate or fumarate-sulphate medium compared with that from cells cultivated on lactate-sulphate (Hatchikian & Le Gall, 1972). The particulate H₂:fumarate reductase system contains hydrogenase coupled with fumarate reductase by one or more intermediary electron carriers replaceable by viologen dyes. The presence of a particulate *b*-type cytochrome in *D. gigas* and its role in the reduction of fumarate by molecular hydrogen has already been reported (Hatchikian & Le Gall, 1972), but the role in the electron transport system of membrane-bound menaquinone-6 isolated from this micro-organism (Maroc, Azerad, Kamen & Le Gall, 1970; Weber, Matschiner & Peck, 1970) has not been established. In this paper we report the possible participation of menaquinone-6 in the electron transport from hydrogen to fumarate in *D. gigas* particles.

METHODS

Organism, growth and preparation of particles. *Desulfovibrio gigas* NCIB9332 was grown at 37 °C on various media: lactate-sulphate (Le Gall, Mazza & Dragoni, 1965), fumarate-sulphate and fumarate (Miller & Wakerley, 1966). Methods of preparing extract as well as particulate and soluble fractions have already been described (Hatchikian & Le Gall, 1970*a*).

Preparation of quinone-depleted particles. Quinone-depleted particles were prepared by extraction with a mixture of acetone and water as described for mitochondria by Lester & Fleischer (1961).

Irradiated particles. Ultraviolet irradiation was used to destroy menaquinone-6 (MK-6) present in the particles; this treatment was performed as follows: an original Hanau Q 81 lamp (350 W; maximum emission 360 nm) was plunged into 500 ml of a vigorously stirred suspension of particles (1.5 mg protein/ml) in 0.025 M-tris-HCl buffer (pH 7.6) for 6 min irradiation at 4 °C. The treated suspension was then centrifuged at 65000 *g* in a Spinco centrifuge (rotor 30) for 30 min and the residue was suspended in the same buffer at a concentration of 20 mg/ml. For spectroscopy, lyophilized irradiated (or unirradiated) particles corresponding to 200 mg protein were suspended in 2 ml of pentane and extracted by gentle

stirring at 4 °C for 5 min. After centrifuging at 30000 g for 5 min, the supernatant was diluted with an equal volume of *n*-pentane and the u.v. absorption spectrum determined in cuvettes with 1 cm light path.

Restoration studies. Restoration of the coupled H₂:fumarate reductase activity was attempted in two ways: (i) MK-6 (or menadione) in ethanolic solution was added to the extracted or irradiated particles (0.05 mg/mg protein) and the suspension was incubated for 1 h at room temperature with gentle stirring, (ii) MK-6 suspended in *Desulfovibrio gigas* phospholipids was added to the treated particles; phospholipids extracted from whole cells were prepared by the method of Kanemasa, Akamatsu & Nojima (1967). Phospholipids (160 mg) were homogenized by sonic oscillation in a Sonifier S110 oscillator for 1 min periods in 5 ml of buffer containing 0.25 M-sucrose, 1 mM-EDTA and 0.01 M-tris-HCl (pH 7.6). After centrifuging the suspension at 35000 g for 15 min, MK-6 (2 mg of menaquinone in a minimum amount of ethanol) was added to the supernatant and sonicated for three 10 s intervals at 0 to 5 °C, whereupon a colloidal suspension was formed. This suspension was then incubated with the extracted or irradiated particles for 1 h at room temperature; the amounts used in the mixture were 0.1 ml of the phospholipid suspension/mg of particulate protein.

Preparation of menaquinone-depleted phospholipids. *D. gigas* phospholipid free from natural MK-6 was prepared as follows: a sample of phospholipid (300 mg) was dissolved in chloroform and loaded on a column of silicic acid (10 × 50 mm) packed with chloroform; the column was washed with chloroform to remove MK-6; the phospholipids were then eluted with a mixture of chloroform and methanol (1:1, v/v) and dried under vacuum below 25 °C.

Measurement of H₂:fumarate reductase activity. The H₂:fumarate reductase activity of the particulate fraction was determined using the Warburg respirometric method. Initial rates of H₂ uptake during fumarate reduction were measured in the presence of an excess of hydrogenase, and for this purpose a hydrogenase fraction devoid of fumarate reductase activity prepared as already described (Hatchikian & Le Gall, 1972) was added to the particulate fraction. The unit of hydrogenase activity was defined as μmol H₂ oxidized/h with the manometric benzyl viologen assay. The assay conditions for the measurement of H₂:fumarate reductase activity were as follows. Main compartment: tris-HCl buffer (pH 7.6), 100 μmol; hydrogenase, 105 units; methyl viologen (MV) where indicated, 7.5 μmol; enzyme, as indicated. Side-arm: fumarate, 25 μmol. Centre well: 0.05 ml 20 % NaOH. Total volume, 3 ml; temperature, 37 °C; gas phase, H₂.

Analytical methods. Dicarboxylic acids present in the reaction mixture were identified by descending chromatography on Whatman no. 1 paper as described by Miller & Wakerley (1966). The phospholipids were assayed after digestion by estimation of the phosphorus content by the method of Fiske & SubbaRow (1925). Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) and by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Effects of extraction and reincorporation of menaquinone

Acetone-extraction of the particles resulted in the loss of H₂:fumarate reductase activity but with MV the extracted particles regained about 60 % activity. In a typical experiment, a particulate fraction of fumarate-grown *Desulfovibrio gigas* (1.9 mg protein) absorbed 19.5 μmol H₂/h, which increased to 75 μmol H₂/h with 2.5 mM-MV; acetone-extracted particles were inactive but absorbed 45.2 μmol H₂/h with MV. Comparable results were obtained with

particles from lactate-grown *D. gigas*. MV probably couples hydrogenase to fumarate reductase directly, and in the presence of excess hydrogenase the assay simply measures the fumarate reductase activity of the particles. These results suggest that washing the particles with acetone eliminates a lipid-soluble factor involved in the electron transport between hydrogenase and fumarate reductase. A yellow lipid fraction was obtained from the acetone extract by evaporating the solvent, and its u.v. absorption spectrum indicated that a quinone was present. Addition of this fraction or of MK-6 (with or without *D. gigas* phospholipids) failed to restore H_2 :fumarate reductase activity.

Effect of electron transport inhibitors on H_2 :fumarate reductase activity

Dicoumarol and lapachol were tested on the H_2 :fumarate reductase activity of the particles from *D. gigas* grown on fumarate medium. The addition of dicoumarol (5×10^{-4} M) to a system containing 1.4 mg protein produced a 50 % inhibition, whereas lapachol had no inhibitory effect at low concentrations ($< 10^{-4}$ M) and a stimulatory effect at high concentrations ($> 10^{-4}$ M).

Effect of ultraviolet irradiation on particles

Irradiation of the particles resulted in a considerable loss of H_2 :fumarate reductase activity but in the presence of MV the preparations retained 60 % of the activity of unirradiated intact particles with methyl viologen. These results implicate a light-sensitive factor, possibly MK-6, in the electron transport of the particulate H_2 :fumarate reductase activity.

To investigate whether the endogenous MK-6 was altered by the irradiation procedure, the u.v. spectra of pentane extracts from normal and irradiated particles were compared. The spectrum from the extract of unirradiated particles has absorption maxima corresponding to that of the spectrum of MK-6 described by Weber *et al.* (1970). The disappearance of these peaks in the spectrum of the extract of irradiated particles indicated the destruction of the naphthoquinone ring (Brodie & Ballantine, 1960).

Restoration experiments by reincorporation of MK-6 in the irradiated particles were carried out to determine whether the loss of H_2 :fumarate reductase activity was due to the alteration of endogenous MK-6. The results of these experiments are presented in Fig. 1. After addition of MK-6 or menadione, the coupled activity was partly restored; moreover, the restoration was slightly more efficient in the presence of MK-6 plus *Desulfovibrio gigas* phospholipids. Under these conditions, the H_2 :fumarate reductase activity was restored to a maximum of 17 % of the activity of unirradiated particles, i.e. about three times the residual activity of the irradiated particles. The activity observed with phospholipids alone (Fig. 1 E) was probably due to the presence of menaquinone in the crude preparation. In support of this, the absorption spectrum of the phospholipids indicated that menaquinone was present and the addition of *D. gigas* phospholipids free from MK-6 to the irradiated particles failed partly to restore H_2 :fumarate reductase activity (Fig. 1 G). Furthermore, preincubation of the irradiated particles with pure phospholipids such as phosphatidyl-ethanolamine (10 μ g/3 ml of assay system, dissolved in ethanol) from bacteria (Calbiochem Ltd., Los Angeles, California, U.S.A.) or cardiolipin from bovine heart (Sigma Chemical Co., St Louis, Missouri, U.S.A.) did not increase the enzymic activity. The restored activity appeared to be physiological in that it was inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide (Fig. 1 H) like the H_2 :fumarate reductase activity of the untreated particles (Hatchikian & Le Gall, 1972). The formation of succinate was confirmed in the systems in which the activity of irradiated particles was partly restored by reincorporation of MK-6.

Fig. 1. Restoration by menaquinone-6 of the H_2 :fumarate reductase activity in irradiated particles of *Desulfovibrio gigas*. The reaction mixture contained all the reactants as indicated in Methods. A, unirradiated particles from *D. gigas* grown on fumarate, protein content 3.3 mg. The other systems contained irradiated particles, protein content 3.2 mg. B, irradiated particles alone; C, with MK-6 (0.15 mg); D, with menadione (0.15 mg); E, with *D. gigas* phospholipids; F, with MK-6 + *D. gigas* phospholipids; G, *D. gigas* phospholipids purified on silicic acid column; H, with MK-6 + phospholipids + 2-heptyl-4-hydroxyquinoline-*N*-oxide (10^{-5} M). The phospholipid content in the systems containing these compounds corresponded to 20 μ g of phosphorus.

DISCUSSION

The strict anaerobe *Desulfovibrio gigas* has recently been found to contain MK-6 (Maroc *et al.* 1970; Weber *et al.* 1970). The data presented show that when MK-6 is removed from particles by extraction with acetone or destroyed by u.v. irradiation a concomitant loss of H_2 :fumarate reductase activity is observed. In both cases fumarate reductase activity can still be detected using MV as an artificial coupling factor. Electron transport from H_2 to fumarate is inhibited by dicoumarol, a menaquinone antagonist. However, incubation of treated particles with menaquinone either failed to restore activity (acetone-extracted particles) or only partly restored H_2 :fumarate reductase activity (u.v.-irradiated particles). These experiments also suggested that phospholipids slightly enhanced the restoration of the activity obtained with MK-6, possibly by improving the reincorporation of MK-6 at its site of action in the particle. If we consider that fumarate reductase is inactivated to 40 % by u.v. light as indicated by the results reported here, the level of restoration of the H_2 :fumarate reductase activity by addition of MK-6 to irradiated particles may actually be higher than that mentioned above and can be estimated to be close to 25 %.

The data discussed above strongly suggest that MK-6 is involved in the electron transport between hydrogenase and fumarate reductase in *Desulfovibrio gigas* particles. Similar results, corresponding to a partial restoration of enzymic activities by quinone reincorporation into extracts depleted of these carriers, have already been reported (Knook & Planta, 1971; Kröger & Dadak, 1969). However, in the case of NADH oxidase activity of *Bacillus brevis*, no restoration was observed upon addition of the natural quinone (Fynn, Thomas & Seddon, 1972). The low level of restoration of H₂:fumarate reductase activity of u.v.-irradiated *D. gigas* particles may be due to failure of the techniques used to place MK-6 back to its original site in the particle (Fynn *et al.* 1972). In particular, the presence of altered endogenous menaquinone in irradiated *D. gigas*-particles could prevent reincorporation of exogenously added quinone (Knook & Planta, 1971).

The role of menaquinones in electron transport coupled with fumarate reductase has already been reported in *Bacillus megaterium* (Kröger & Dadak, 1969) and *Proteus rettgeri* (Kröger, Dadak, Klingenberg & Diemer, 1971). Kröger & Dadak (1969) showed that in *B. megaterium*, menaquinone-7 mediates the electron flow between the dehydrogenases (NADH-, malate- or glycerol-1-P-dehydrogenase) and a cytochrome *b*₁ linked to fumarate reductase. The involvement of *Desulfovibrio gigas* membrane-bound cytochrome *b*, now called cytochrome *B*₅₆₀ (Hatchikian, 1972), in the electron transport of the H₂:fumarate reductase system (Hatchikian & Le Gall, 1972) and the possible participation of MK-6 in this electron transport chain, as reported here, suggest that both MK-6 and cytochrome *B*₅₆₀ are needed to couple hydrogenase and fumarate reductase.

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