On the Role of Menaquinone in the Reduced Nicotinamide Adenine Dinucleotide Oxidative Pathway of *Bacillus brevis*

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SUMMARY

Ultraviolet spectroscopy, partition chromatography and mass spectroscopy showed that the only quinone isolated from *Bacillus brevis* was menaquinone-7. The concentration of quinone present in electron-transport particles was consistent with its participation as an oxido-reduction component. Loss in NADH oxidase activity after ultraviolet irradiation or pentane extraction correlated with the degradation or removal respectively of menaquinone-7 present in the electron transport particles. Attempts to restore NADH oxidase activity either with ultraviolet-treated or pentane-extracted particles using the natural menaquinone were unsuccessful, although lower menaquinone homologues stimulated activity.

INTRODUCTION

Bacillus brevis ATCC10068 contains an active NADH oxidase system (Seddon & Fynn, 1970; Seddon & Fynn, 1971). Electron-transport particles from *B. brevis* possess cytochromes *a*, *b*, *c* and *o* as well as flavoprotein and a menaquinone (Seddon & Fynn, 1971). The particulate-bound NADH oxidase activity of *B. brevis* is mediated by cytochromes *a*, *b* and *c* and flavoprotein but the physiological function of menaquinone or cytochrome *o* in this system is not known. The present investigation was undertaken in an attempt to elucidate the possible participation of quinone in the particulate-bound NADH oxidase pathway of *B. brevis*.

METHODS

Organism, growth and maintenance. *Bacillus brevis* ATCC10068 was maintained and grown as described by Seddon & Fynn (1971). In addition the organism was grown at 30° under rotary shake conditions, and, when required, the glycerol-asparagine (GA) medium was replaced by a peptone-yeast extract (PYE) medium of the following composition (g/l): peptone, 10; yeast extract, 3; NaCl, 5; pH 7.0. Cultures were harvested towards the end of logarithmic growth and washed with 0.1 M-tris-HCl (pH 7.4) (Seddon & Fynn, 1970).

Preparation of electron-transport particles (ETP). A suspension of washed organisms was extruded through a cooled French pressure cell and subcellular fractions obtained as described by Seddon & Fynn (1970). The fraction sedimented at 105,000g was used for studies of ETP NADH oxidase activity. In certain experiments, where the distribution of

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quinone and NADH oxidase activity was assayed, the unfractionated 15,000g supernatant fraction (15S) and the 105,000g supernatant fractions (105S) were used for assay.

Extraction and purification of quinone from whole cells. Six litres of medium were harvested and the organisms (wet pellet) were resuspended in 100 ml of methanol and disrupted with a Polytron macerator at 0 to 5° for 15 s intervals over a period of 2 min. An equal volume of petroleum ether (b.p. 40 to 60°) was added and the mixture was shaken. The two layers were separated and the methanol fraction was twice re-extracted with an equal volume of petroleum ether. The petroleum ether fractions were combined, dried over anhydrous NaSO₄, filtered and evaporated to approximately 5 ml in a rotary evaporator and then evaporated to dryness under nitrogen. The residue was dissolved in cyclohexane and purified by thin-layer chromatography using rhodamine 6G impregnated silica-gel plates (250 μ × 20 cm²) previously washed with ether. The developing solvent was petroleum ether (b.p. 40 to 60°) – diethyl ether (19:1, v/v). Quinones were extracted from the plates with freshly distilled ether.

Extraction of quinone from ETP. Lyophilized ETP were mechanically homogenized with pentane (Szarkowska, 1966). Where the distribution of quinone was investigated the 15S and 105S fractions were assayed. Pentane fractions were evaporated to dryness under nitrogen or with a rotary evaporator.

Identification of quinone. U.v. absorption spectra of all lipid extracts were made as described by Seddon & Fynn (1971) except that cyclohexane replaced ethanol as solvent. Reversed-phase thin-layer chromatography was used to identify the quinone (Randerath, 1966; Seddon & Fynn, 1971) with acetone:H₂O (97:3, v/v) as developing solvent. Standard menaquinones (-5, -7, -9 and -10) were used for comparison. Mass spectroscopy was carried out on a purified quinone sample using an AEI-12 mass spectrometer (source temperature 200°).

Quantitative estimation of quinone. The concentration of quinone was determined from the extinction at 249 nm using a molecular absorbance coefficient of 19,000 (Isler & Wiss, 1959).

Quantitative estimation of cytochromes. All cytochromes were estimated as described by Seddon & Fynn (1971) except that the content of cytochrome *o* was determined using ϵ = 170,000 (415 – 432 nm, i.e. peak minus trough according to Daniel, 1970).

NADH oxidase activity. This was measured either spectrophotometrically or polarographically (Seddon & Fynn, 1970).

Irradiated particles. Slowly stirred volumes (3 to 4 ml) of ETP (40 to 50 mg protein) were irradiated at 366 nm using a model M u.v. lamp (Englhard-Hanovia, Slough, Buckinghamshire) at 0 to 5° similar to the procedure of Brodie & Ballantine (1960). U.v. absorption spectra of pentane extracts in cyclohexane of both irradiated and non-irradiated particles were performed as described by Szarkowska, (1966).

Restoration studies. ETP (40 to 50 mg protein) extracted with pentane (Szarkowska, 1966) as well as ETP extracted by the method of Ernster, Lee, Norling & Persson (1969) were used in these studies. U.v.-irradiated particles, obtained as described above, were also used in attempts to restore NADH oxidase activity. Restoration of activity was attempted in three ways: (i) ethanol solutions (0.05 ml or less) containing quinone (10 to 150 nmoles) were added to the ETP both before and after the commencement of oxidase activity with NADH (controls were conducted with ethanol alone); (ii) pentane-extracted particles were washed with a tenfold excess of quinone (Ernster *et al.* 1969); (iii) suspensions of quinones (formed by dissolving the quinone in a minimum amount of ethanol followed by the addition of distilled water to a final concentration of 3 mM and sonication for three 10 s intervals at

Fig. 1. (a) Spectra of lipid extracts from whole cells of *Bacillus brevis*. All spectra are of petroleum ether (40 to 60°) extracts in cyclohexane. Trace (4) was obtained after purification using rhodamine 6G impregnated silica-gel plates with petroleum ether (40 to 60°) – diethyl ether (19:1, v/v) as developing solvent. (1) Organisms grown on GA medium at 30°; (2) organisms grown on GA medium at 37°; (3) and (4) organisms grown on PYE medium at 30°.

(b) Spectra of pentane extracts of *Bacillus brevis* ETP. Both spectra are for pentane extracts (Szarkowska, 1966) in cyclohexane. (1) ETP from organisms grown on PYE medium at 30°; (2) ETP from organisms grown on GA medium at 37°.

(c) Effect of u.v. irradiation on the spectral characteristics of MK-7 and the endogenous quinone of *Bacillus brevis* ETP. ETP protein (42 mg), obtained from organisms grown on PYE medium at 30°, was divided equally into two 3.0 ml volumes of 0.1 M-tris-HCl buffer (pH 7.4). The sample preparation was covered with a silica disc whereas the control was protected from u.v. irradiation using an aluminium-foil cover. Control and sample preparations of authentic MK-7 (3.0 ml of 60 μ M solutions in ethanol) were treated similarly. U.v. irradiation at 366 nm and 0 to 5° was for a period of 60 min. Irradiated and non-irradiated ETP were extracted with pentane (Szarkowska, 1966) and the spectra made in cyclohexane on a suitably diluted sample. The ethanolic solutions of MK-7 were taken to dryness under nitrogen and spectra made of irradiated and non-irradiated MK-7 samples suitably diluted in cyclohexane. (1), (2) authentic MK-7, non-irradiated and irradiated respectively; (3), (4) quinone extract from irradiated and non-irradiated particles respectively.

0 to 5° whereupon colloidal suspensions formed), 0.05 ml and less, were added to the NADH oxidase assay medium as described in (i).

Protein determination. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin (fraction V) as standard.

Solvents. All solvents were distilled before use; ether was redistilled from reduced iron within 24 h of use. Diethyl ether and petroleum ether (40 to 60°) were dried over Na-Pb alloy before distillation.

Chemicals and nutrients. Neutralized peptone and yeast extract were obtained from Oxoid Ltd., London. NADH (grade III) was purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A. Bovine serum albumin was obtained from the Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex, and *n*-pentane from British Drug Houses Ltd, Poole, Dorset. Standard menaquinones were a gift from Dr O. Isler of Hoffmann-La Roche, Basle, Switzerland.

RESULTS

Quinone of whole cells and subcellular fractions of Bacillus brevis. U.v. absorption spectra (Fig. 1*a, b*) and R_F values (extracted quinone, 0.43; authentic menaquinone-7, 0.45) of the quinone extracted from whole cells and ETP of *B. brevis* grown either on GA or PYE media strongly suggested that the quinone was menquinone-7 (MK-7) (Seddon & Fynn, 1971). Reduction of the oxidized quinone in ethanol using NaBH_4 resulted in changes in the absorption spectra characteristic of menquinone reduction as described by (Kröger & Dadák (1969). Mass spectroscopy data showed a molecular ion at 648 and a base peak at 225 consistent with the structure of MK-7; peaks at 579, 511, 443, 375, 307 and 239 showed sequential loss of isoprene units.

MK-7 and NADH oxidase activity. Distribution studies showed that NADH oxidase activity, cytochromes and MK-7 are concentrated into the ETP. With cells grown on PYE media the ETP contained 85 % and 90 % of the total NADH oxidase and MK-7 respectively of the unfractionated 15S fraction; similarly ETP from cells grown on GA medium contained 76 % and 91 % of the total NADH oxidase and MK-7 respectively. The concentration of MK-7 (4.5 and 3.1 $\mu\text{moles/g}$ protein for ETP from organisms grown on PYE and GA medium) is in tenfold excess over the cytochromes.

U.v. irradiation of ETP led to the loss of about 80 % of the NADH oxidase activity on prolonged irradiation (60 min). Such treatment resulted also in the degradation of endogenous MK-7. Authentic MK-7 irradiated in the same manner showed similar changes in spectral characteristics (Fig. 1*c*).

About 90 % of the endogenous menaquinone is removed by three successive extractions according to the method of Szarkowska (1966). Such treatment led also to losses in NADH oxidase of 80 to 90 % of the original activity. Similar results were obtained using the extraction procedure of Ernster *et al.* (1969).

All attempts to restore NADH oxidase activity with MK-7 (the endogenous quinone) failed. The lower menaquinone homologues (MK-2 and MK-3) stimulated NADH oxidase activity of a u.v.-irradiated preparation. NADH oxidase activities of 120, 20, 210 and 120 nmoles NADH oxidized/min/mg protein were obtained for non-irradiated, irradiated (60 min), irradiated + MK-2, and irradiated + MK-3 ETP respectively. Similar results were also obtained with pentane-extracted particles, showing that the lower, water-soluble menaquinones stimulated activity.

DISCUSSION

These results confirm the presence of menaquinone (MK-7) in *Bacillus brevis*. That MK-7 is present in both whole cells and ETP of *B. brevis* and that ubiquinone is absent may be due to menaquinone functioning in electron transport as the quinone participant. Organisms grown on GA medium produce relatively high levels of tyrothricin compared with cells grown on PYE medium (Seddon, 1971). Under conditions of tyrothricin production it has been shown (Seddon & Fynn, 1971) that the antibiotic inhibits the NADH oxidase activity of the isolated ETP. Since MK-7 concentration levels are similar under both conditions of growth it would seem that the quinone function is not related to antibiotic production.

The MK-7 content of ETP is consistent with its participation as an intermediary oxidation-reduction component in electron transport. As is found with *Bacillus megaterium* (Kröger & Dadák, 1969), the concentration of MK-7 in *B. brevis* is in tenfold excess over the cytochromes. U.v. irradiation led to a loss of NADH oxidase activity as in *Mycobacterium phlei* (Brodie & Ballantine, 1960) and the spectrum of the pentane extract from u.v.-irradi-

ated particles indicated that MK-7 was degraded as found by Marquez & Brodie (1970) for MK-8 in the NADH oxidase pathway of a halophilic bacterium. Pentane extraction procedures which removed MK-7 from the ETP also produced corresponding losses in NADH oxidase activity. This evidence therefore implies that MK-7 plays an intermediary role in electron transport between NADH and oxygen.

The failure of MK-7 to restore NADH oxidation of u.v. irradiated or pentane-extracted particles may be due to failure of the techniques used to restore MK-7 to its original site in the ETP or to damage to the structural orientation of the electron-transport system as a whole. Although u.v. irradiation destroys the endogenous menaquinone in *Bacillus brevis* the mere presence of a modified quinone in the irradiated particles could prevent re-incorporation and thus proper functioning of exogenously added quinone (Knook & Planta, 1971). Alternatively u.v. irradiation may destroy some other light-sensitive component, not necessarily a quinone, required for NADH oxidation as shown by Murti & Brodie (1969) in the succinoxidase pathway of *Mycobacterium phlei*.

In conclusion, these results implicate, but do not prove, the participation of MK-7 in the NADH oxidase electron-transport system of *Bacillus brevis*. Work is now in progress to locate the lesion induced by u.v. irradiation and pentane extraction and to effect a more successful method of reincorporating MK-7 to its original physiological site.

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