Menaquinone Function in the Electron Transport System of an Antibiotic-producing Strain of *Bacillus brevis*

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Bacillus brevis ATCC 10068 grown under appropriate growth conditions produces a mixture of tyrocidine and gramicidin, which are potent inhibitors of electron transport and oxidative phosphorylation processes. We have recently characterized the electron-transport system of this strain and have identified a menaquinone (MK-7) by thin-layer chromatography and by u.v. and mass spectroscopy to be the sole quinone component (Fynn, Thomas & Seddon, 1972). Pentane extraction (Szarkowska, 1966) and acetone extraction (Fynn & Redfearn, 1964) were used to remove the endogenous quinone in an attempt to confirm a functional role for MK-7 in the electron transport system. Although these methods were successful in removing 90 to 95 % of the quinone and largely abolishing the NADH oxidase activity of the preparation, the addition of MK-7 in various carrier systems failed to restore the latter activity. However, from the literature it is clear that while restoration of succinoxidase activity of acetone-extracted preparations has been successfully demonstrated, the reactivation of the NADH oxidase pathway has been more difficult to achieve. Also the pentane extraction technique suffers from the disadvantage that it can only be successfully employed to extract lyophilized material. The lyophilization process itself involves some structural damage to the electron transport particle since the NADH oxidase activity falls to 40 to 60 % of its original value. We now report on the u.v. irradiation of fresh material as a means of inactivating the endogenous menaquinone and providing the basis for a further investigation of quinone function in the organism.

METHODS

U.v. irradiation. Irradiation with 364 nm light was most effective in abolishing the NADH oxidase activity of the freshly prepared 105 P material (Seddon & Fynn, 1971): shorter wavelength (254 nm) was less effective and produced a general inactivation of the enzyme activities of the preparation. Irradiation was carried out with a 125 W Phillips 57236 F/70 mercury arc source placed at a distance of 10 cm above a Petri dish containing a 3 mm depth of 105 P preparation in tris-HCl buffer (0.1 M, pH 7.4). The preparation was stirred on ice by means of a magnetic stirrer and kept covered with a borosilicate glass plate to avoid evaporation losses.

Quinone/phospholipid micelles. The appropriate phospholipid (see Results) to give a final concentration of 15 mg/ml was dissolved in a minimum volume of chloroform and the calculated volume of a 10^{-2} M-ethanolic solution of quinone added. The mixture was evaporated to dryness on a rotary evaporator at 20 °C and resuspended in 0.01 M, pH 7.8, tris-EDTA buffer. The suspension was sonicated for 15 min at 0 °C using a MSE-Mullard type 4200 60 W ultrasonic disintegrator fitted with a stainless-steel vibrator probe of 6 mm end diameter and 9:1 end ratio. The sonicated material was centrifuged at 100000g for 45 min and any insoluble residue discarded.

Enzyme assays

NADH-quinone reductase. The assay system contained: tris-HCl buffer, pH 7.4, 250 μ mol; NADH, 0.33 μ mol; quinone, 0.30 μ mol; total volume 3 ml in a 1 cm quartz cuvette. The system was rendered anaerobic by flushing with N₂ and the reaction started by the addition of 0.1 to 1.0 mg enzyme protein. The decrease in extinction at 340 nm was measured on a Unicam SP 800 recording spectrophotometer. The initial slope of the curve was used to calculate the rate of quinone reduction.

The measurements of NADH-oxidase, NADH-ferricyanide reductase, NADH-cytochrome *c* reductase, cytochrome oxidase and difference spectra were performed as described previously (Fynn & Seddon, 1971; Seddon & Fynn, 1972).

Chemicals. NADH (grade III) and L- α -lecithin (commercial, type II-S from soy beans) were obtained from the Sigma Chemical Co. Ltd, Kingston-upon-Thames, Surrey. Menaquinones were a gift from Dr O. Isler of Hoffman-La-Roche, Basle, Switzerland.

RESULTS

U.v. irradiation for 15 min inhibited by approximately 95 % the NADH oxidase activity of the 105 P preparation. A time course study showed that this irradiation-resistant residual activity was due to a KCN-insensitive component which by-passed the cytochrome system of the respiratory chain. In one experiment the KCN-insensitive NADH oxidase activity constituted 13 % of the total oxidase activity. After 20 min of irradiation the total NADH oxidase activity had fallen to 5.5 % of the control value and this residual activity was completely insensitive to cyanide (and also to 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and antimycin A).

The u.v. spectrum of the endogenous MK-7 extracted from the irradiated preparation revealed that the characteristic peaks at 244, 249, 262 and 269 nm were no longer discernible, indicating that extensive degradation of the menaquinone had occurred concomitant with the loss of NADH oxidase activity.

Evidence was obtained that the site of inhibition of electron flux through the electron transport system of the irradiated preparation lay between the NADH flavoprotein and cytochrome *c*. Firstly, in an irradiated preparation where the NADH oxidase activity had been reduced to 8 % of the control, the NADH-cytochrome *c* reductase activity declined to 4.5 % whereas the NADH-ferricyanide reductase, the NADH-K₃ reductase and cytochrome oxidase activities fell to values of 64 %, 98 % and 88 % respectively. Secondly, difference spectra obtained from the irradiated preparation indicated that the cytochromes were no longer reducible on the addition of NADH.

Attempts to reactivate the NADH oxidase activity of the irradiated preparation were made with a variety of quinone compounds (Table 1). The apparent activation obtained with a number of quinones was non-physiological since it was not inhibited by the addition of KCN; such stimulation is apparently due to the activity of NADH-quinone reductase in this organism which readily catalyse the reduction of the more hydrophilic of the added quinones. These reduced quinones are autoxidizable at pH 7.4 and give rise to the observed rates of KCN-insensitive NADH oxidation. This is confirmed by the observation that the rate of reduction of a given quinone under anaerobic conditions (determined as the rate of NADH oxidation as described under Methods) is the same as the rate of NADH oxidation under aerobic conditions in the presence of KCN and the same quinone.

Reconstitution of the electron transport system of the irradiation particle was achieved

Table 1. *The effect of menaquinones on the NADH oxidase and NADH-quinone reductase activities of u.v.-irradiated 105 P particles from Bacillus brevis*

Final concentration of KCN was 10 mM and quinone 0.1 mM in all experiments. MK-/PL refers to quinone solubilized with a commercial preparation of L- α -lecithin.

Additions	*Enzyme activity (μ mol NADH oxidized/ min/mg protein at 25 °C)		
	†NADH oxidase		NADH-quinone reductase
	—	KCN	
—	0.04	0.03	—
1,4-Napthoquinone	1.57	1.53	1.54
K ₃	1.52	1.50	1.51
MK-1	1.55	1.55	1.55
MK-2	1.03	0.95	0.97
MK-5	0.06	0.05	0.01
MK-5/PL	0.15	0.05	0.01
K ₁	0.05	0.04	0.00
K ₁ /PL	0.21	0.05	0.00
MK-7	0.05	0.04	0.00
MK-7/PL	0.30	0.04	0.00
MK-9	0.03	0.03	0.00
MK-9/PL	0.08	0.04	0.00

* Enzyme assays were carried out as indicated in the text.

† NADH oxidase activity of the non-irradiated control was 0.49 μ mol NADH/min/mg protein.

only with the higher menaquinones, MK-5, MK-7 and K-1, and to a limited extent by MK-9, after these had been solubilized with phospholipid from soy-bean source. Synthetic mixtures of cephalin and lecithin, commercial and purified grades of lecithin and lipid extracts from *Bacillus brevis* were also used to prepare quinone-phospholipid micelles, but none of these was successful in restoring the NADH oxidase activity. The restoration by the higher MK homologues was specific since other menaquinones or ubiquinone homologues solubilized in the same way were unable to restore KCN-sensitive NADH oxidase activity.

The reconstituted preparation, i.e. irradiated 105 P+MK-7/PL, behaved essentially as the control. Difference spectra on the addition of NADH showed the appearance of the characteristic peaks of the reduced cytochrome.

DISCUSSION

Among the criteria that remain to be satisfied before MK-7 can be accepted as a functional member of the respiratory chain of *Bacillus brevis* is that removal of this component should inhibit electron transport and replacement should restore this activity. U.v. irradiation does not remove the endogenous menaquinone but from the u.v. spectrum it is clear that the quinone is largely destroyed *in situ* by this treatment. The concomitant loss of NADH oxidase is almost complete except for a residual KCN-insensitive oxidation the nature of which is the subject of further investigation. The site of inhibition of the electron transport system is consistent with the assigned locus for quinone function in both mitochondrial and bacterial systems. Although there is no loss of endogenous phospholipid during u.v. irradiation it has been established that the reinsertion of a hydrophilic quinone into the membrane matrix of the electron transport system is greatly facilitated when the quinone is bound in a suitable phospholipid micelle.

In common with the experience of other groups (e.g. Asano & Brodie, 1964) a plant phospholipid mixture was the most effective vehicle for the solubilization of the quinone in a form which would reactivate. It is not clear why quinone micelles formed from phospholipid from other sources, including a phospholipid fraction from *Bacillus brevis*, were not successful in producing reactivation. Successful KCN-sensitive reactivation also appeared to be specific for naphthoquinones with more than 20 carbons in the isoprenoid side chain. Unfortunately the corresponding benzoquinone compound to MK-7 was not available for testing but Q-6 and Q-9 isoprenalogues of ubiquinone were inactive.

The reconstituted preparation had an NADH oxidase activity which was approximately 60 % of the control. This activity was KCN-sensitive and the difference spectra obtained confirmed that the electron flux was via the cytochrome system.

Thus the evidence presented in this paper suggests that MK-7 is the functional quinone component of the electron transport system of *Bacillus brevis*. In this respect the respiratory chain of this antibiotic-producing bacterium shows no abnormal differences in composition from the respiratory chains of other aerobic Gram-positive bacteria.

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