A Novel, Fully Saturated Menaquinone from the Thermophilic, Sulphate-reducing Archaeobacterium *Archaeoglobus fulgidus*

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A recently described extremely thermophilic, sulphate-reducing archaeobacterium, *Archaeoglobus fulgidus*, was investigated for the presence of respiratory lipoquinones. Initial screening using thin-layer chromatography and UV spectroscopy indicated that naphthoquinones were present. A more detailed study, using a combination of HPLC, mass spectroscopy and NMR techniques, indicated that the major respiratory lipoquinone present in this organism is a menaquinone with a fully saturated heptaprenyl side chain (MK-7H₁₄).

INTRODUCTION

Investigations of the respiratory lipoquinone composition of eubacteria have shown that lipoquinones serve as useful chemotaxonomic markers (see Collins & Jones, 1981 for a review). Studies on aerobic archaeobacteria have shown that *Thermoplasma* contains MK-7 and MMK-7, while members of the family *Halobacteriaceae* have MK-8 and MK-8H₂ as the major lipoquinones (Collins, 1985; Collins & Langworthy, 1983; Collins *et al.*, 1981). In contrast, members of the genera *Sulfolobus* and *Acidianus* synthesize an unusual series of benzo-[b]-thiophen-4,7-quinones with fully or partially saturated C6 side chains (Collins & Langworthy, 1983; De Rosa *et al.*, 1977; Lanzotti *et al.*, 1986; Segerer *et al.*, 1986; Thurl *et al.*, 1986). Studies on anaerobic, thermophilic archaeobacteria have shown that *Thermoproteus tenax* contains a menaquinone with a fully saturated hexaprenyl C3 side chain (MK-6H₁₂), but that *Thermococcus celer*, *Desulfurococcus mucosus* and *Desulfurococcus mobilis* are devoid of lipoquinones (Thurl *et al.*, 1985, 1986). Despite the lack of published information on the methanogenic archaeobacteria there is no evidence to suggest that lipoquinones are present in any of the organisms examined to date (M. D. Collins, unpublished; B. J. Tindall, unpublished).

A new type of thermophilic archaeobacterium, *Archaeoglobus fulgidus*, capable of sulphate reduction, has recently been isolated (Achenbach-Richter *et al.*, 1987; Stetter, 1988; Stetter *et al.*, 1987). Studies on the biochemistry and phylogenetic position of this organism suggest that it may represent a novel archaeobacterial branch. In particular, it has been suggested that *Archaeoglobus fulgidus* may occupy an evolutionary position intermediate between the methanogenic and the thermophilic, sulphur-respiring, branches of the archaeobacteria. Considering some of its unusual properties it was of interest to examine this organism for the

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Abbreviations: MK-*n*, menaquinone with *n* isoprene units; MMK-*n*, methylmenaquinone with *n* isoprene units; MK-*n*H_x, menaquinone with saturation in the isoprene side chain.

presence of respiratory lipoquinones. The present paper reports on the structure of the respiratory lipoquinone isolated from the cell membranes of *Archaeoglobus fulgidus*.

METHODS

Organism and growth conditions. *Archaeoglobus fulgidus* (strain VC-16, DSM 4304) was grown anaerobically in the presence of sulphate and lactate in the marine medium described previously (Stetter *et al.*, 1987). Following harvesting of the cells in a continuous-flow centrifuge, the cell pellet was disrupted by sonication, and the cell membranes collected by ultracentrifugation (Speich & Trüper, 1988). The membranes were freeze-dried and examined for their respiratory lipoquinone content.

Extraction and purification of the lipoquinones. Freeze-dried cell membranes were extracted by a modification of the method of Redfearn (1967), using methanol/hexane (2:1, v/v). The cell membranes were extracted for 1 h, and the lipoquinone material partitioned into hexane by the addition of 1 vol. hexane. After decanting the hexane layer, extraction was continued for a further 30 min, followed by the addition of 1 vol. hexane and 0.1 vol. 0.3% (w/v) aqueous NaCl. The hexane phases were combined, concentrated in a rotary evaporator, and the lipoquinones purified on silica gel thin-layer plates developed in hexane/*tert*-butyl methyl ether (9:1, v/v). Quantitative analysis of the lipoquinone mixture was carried out using an LDC/Milton Roy HPLC equipped with a CI-10 integrator. Lipoquinones were separated on an ODS-2 column (250 × 4.6 mm), using methanol as mobile phase, and with detection at 269 nm.

Physicochemical analyses. UV spectra were recorded in *iso*-octane using a Beckman ACTA MVI spectrophotometer. Mass spectra were recorded on an AEI MS50 instrument, employing a direct insertion probe, an ionizing voltage of 70 eV, and a temperature of 180 °C. ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker WH500 spectrometer using CDCl₃ as solvent. A 2-D COSY-45 spectrum was run using 2K × 2K data points, with sine-bell windows and symmetrification. Multiplicities were determined by the DEPT method.

RESULTS AND DISCUSSION

Extraction of the cell membranes using the method of Collins (1986) or a modified method of Redfearn (1967), and subsequent examination for the presence of a respiratory lipoquinone by silica gel thin-layer chromatography, revealed the presence of a single UV-absorbing band co-chromatographing with an authentic menaquinone standard. Examination of the UV spectrum of this material in *iso*-octane showed absorption maxima at 242, 248, 260, 269 and 326 nm, typical of menaquinones (Collins, 1986). Analysis of the menaquinone mixture by HPLC indicated that about 98% of the material was a menaquinone with a retention time (59.5 min) considerably greater than that of MK-7 (15.2 min) and MK-8 (21.5 min) standards.

Mass spectral analysis of the major lipoquinone revealed a comparatively simple fragmentation pattern, with intense peaks at m/z 662 (M^+) and at m/z 187. A less intense peak at m/z 648 could be attributed to the loss of a CH₃ group from the naphthoquinone nucleus. The lack of fragmentation in the region m/z 187 to 648 was indicative of the absence of unsaturation in the C3 isoprenoid side chain (Thurl *et al.*, 1985, 1986). In fully or partially saturated lipoquinones isolated to date, the first isoprene unit is unsaturated, which results in cleavage of the side chain at C'1–C'2 and C'3–C'4 and produces the characteristic peaks at m/z 187 and 225, attributable to the naphthoquinone nucleus. In the absence of a double bond at the first isoprenoid unit, cleavage at C'3–C'4 does not occur, and the absence of a peak at m/z 225 provides a rapid method for detecting menaquinones with saturation in the first isoprene unit (Thurl *et al.*, 1985). Similarly, fully unsaturated lipoquinones show cleavage at diallylic bonds, giving a characteristic cleavage pattern based on the rule $(M^+ - 69) - 68 \times (n - 1)$. In the absence of such a fragmentation pattern and the lack of a peak at m/z 225 this suggests that the major lipoquinone is a menaquinone with a fully saturated side chain, consisting of seven isopranyl units (Fig. 1). Examination of the mass spectrum of the menaquinone mixture also revealed the presence of a second lipoquinone in small amounts, with a molecular ion (M^+) at m/z 592, indicating the presence of a fully saturated menaquinone with six isopranyl units.

¹H-NMR studies confirmed the presence of a 2-methyl-1,4-naphthoquinone ring nucleus, with resonances at δ 2.177 (singlet; methyl at C2), δ 7.65–7.7 (multiplet; C6 and C7), and at δ 8.04–8.1 (multiplet; C5 and C8). Resonances due to the isoprenoid C3 side chain showed the absence of unsaturation. The presence of a multiplet centred at δ 2.61 (C'1; aliphatic methylene)

Fig. 1. Structure and mass spectral fragmentation pattern of the novel menaquinone from *Archaeoglobus fulgidus*. The major peaks in the mass spectrum may be attributed to the molecular ion (M^+ ; m/z 662), the loss of the C2 methyl (1; m/z 648), and cleavage at C1–C2 (2; m/z 187).

Fig. 2. Allocation of the ^{13}C -NMR signals obtained from the menaquinone from *Archaeoglobus fulgidus*. Values are expressed as p.p.m. The two signals marked * are interchangeable.

and a doublet at δ 0-975 (aliphatic methyl; first isoprene unit) confirm that the first isoprene unit is saturated. Menaquinones containing an unsaturated first isoprene unit are characterized by the presence of a doublet at δ 3-36 (C'1; allylic methylene) and a singlet at δ 1-77 (*trans*-methyl; first isoprene unit). Saturation in the terminal (end of chain) isoprene unit was confirmed by the presence of a doublet at δ 0-850 (two equivalent terminal methyls) and the absence of a signal due to *cis*-end of chain methyls (singlet at δ 1-667). Resonances centred at δ 0-83 (internal aliphatic methyls) had an integral of 5, and taken together with the absence of resonances in the region δ 4-85-5-1, attributable to unsaturation, confirm the presence of a fully saturated heptaprenyl C3 side chain. COSY-45 ^1H -NMR spectra of the isoprenyl side chain region confirm these structural conclusions.

^{13}C -NMR of the lipoquinone (Fig. 2) provided further, confirmatory evidence for the nature of the major lipoquinone in this organism. Resonances attributable to carbon atoms in the naphthoquinone ring nucleus are consistent with the presence of a 2-methyl-3-polyisoprenyl-1,4-naphthoquinone. All remaining signals were in the region 19.4–39.3 p.p.m., indicating that only aliphatic methines, methylenes and methyls were present. Resonances for carbon–carbon double bonds are found at higher values, and these data provide conclusive proof for the presence of a fully saturated C3 isoprenyl side chain.

The data presented provide unequivocal evidence for the structure of the major lipoquinone from the cell membranes of *Archaeoglobus fulgidus*. The UV spectrum, mass spectra, ^1H - and ^{13}C -NMR data are consistent with the presence of a 2-methyl-3-polyisoprenyl-1,4-naphthoquinone. The characteristic fragmentation pattern of the major lipoquinone in the mass spectrometer indicates the absence of unsaturation in the isoprene side chain. It has already been shown that in partially or fully unsaturated lipoquinones the absence of predicted peaks in the isoprenoid fragmentation pattern is indicative of the points of saturation (Collins, 1986). In the absence of such a fragmentation pattern it may be concluded from the mass spectrum that the C3 side chain is fully saturated. Data provided from the ^1H -NMR and ^{13}C -NMR are in agreement with the proposed structure.

The data indicate that the major respiratory lipoquinone present in the archaeobacterium *Archaeoglobus fulgidus* is a fully saturated menaquinone homologue, and corresponds to

2-methyl-3-I,II,III,IV,V,VI,VII-tetradecahydro-heptaprenyl-1,4-naphthoquinone (MK-7H₁₄) (Fig. 1).

This study shows that the archaeobacterial sulphate-reducer, *Archaeoglobus fulgidus*, like its eubacterial sulphate-reducing counterparts, contains a menaquinone as the major respiratory lipoquinone (Collins & Widdel, 1986). However, examination of the menaquinone composition of eubacterial sulphate-reducers has shown that the C3 isoprenoid side chains of the lipoquinones in these organisms are either fully unsaturated, or dihydrogenated in the terminal isoprenoid unit. In contrast, the presence of a fully saturated side chain in the menaquinone from *Archaeoglobus fulgidus* indicates similarities to *Thermoproteus tenax* and the fully saturated side chains found in 'Caldariella-' and 'Sulfolobus-quinone'. This feature of a fully saturated C3 side chain in the respiratory lipoquinones of certain archaeobacteria may prove to be a characteristic marker for one branch of the archaeobacterial lineage. Further studies are in progress to test this hypothesis.

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