Biochimica et Biophysica Acta, 723 (1983) 376–382
Elsevier

BBA 41304

MENAQUINONE IS THE SOLE QUINONE IN THE FACULTATIVELY AEROBIC GREEN PHOTOSYNTHETIC BACTERIUM *CHLOROFLEXUS AURANTIACUS*

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(Received December 29th, 1982)
(Revised manuscript received March 8th, 1983)

Key words: Photosynthesis; Menaquinone; Quinone; Bacterial photosynthesis; Electron transport; (Chloroflexus aurantiacus)

The role of quinones was investigated in *Chloroflexus aurantiacus*, a thermophilic green bacterium capable of photosynthetic or respiratory growth. Thin-layer chromatography, ultraviolet difference spectroscopy and high-pressure liquid chromatography showed that menaquinone is the only quinone present in both photosynthetic and respiratory *Chloroflexus* cultures. Menaquinone-10 and menaquinone-8 are the predominant homologues in both cultures. For comparative purposes the quinone compositions in photoheterotrophic cultures of *Chromatium vinosum* and *Chlorobium limicola* were also analyzed. *Chloroflexus* is the only facultatively aerobic photosynthetic bacterium that does not possess ubiquinone. Menaquinone appears to be the only quinone involved in the photosynthetic and oxidative electron transport in this organism.

Introduction

Chloroflexus aurantiacus is a thermophilic green bacterium that uniquely combines certain characteristics of each of the other families of photosynthetic procaryotes. *Chloroflexus* is a member of the family Chloroflexaceae, and is capable of photoheterotrophy, photoautotrophy and chemoheterotrophy [1,2], and in this respect resembles the family of purple nonsulfur bacteria, the Rhodospirillaceae. In contrast, the purple sulfur bacteria (Chromatiaceae) and the green sulfur bacteria (Chlorobiaceae) are obligate anaerobes and are incapable of aerobic growth in the dark. *Chloroflexus* is similar to the Chlorobiaceae in the cytological organization of the photochemical apparatus and in having two different bacteriochlorophyll (BChl) types. Both families of green bacteria contain chlorosomes, which are BChl *c*, *d*, or *e* containing oblong bodies attached to the cytoplasmic membrane [1,3–6]. In the green bacteria the cytoplasmic membrane is the site of primary charge separation, whereas in the purple bacteria the primary photochemical activity takes place on the differentiated intracytoplasmic membrane [3,7–10].

Ubiquinone is found in all purple photosynthetic bacteria (suborder Rhodospirillineae) [11–13]. Some of the purple bacteria also contain menaquinone, but in almost all cases the menaquinone is found only in obligate anaerobes [11–13]. In the green photosynthetic bacteria (suborder Chlorobiineae) all species so far examined have been members of the family Chlorobiaceae and have been found to contain menaquinone and chlorobiumquinone (1′-oxomenaquinone-7) [16–18]. There have been no previous reports on the quinone content of the family Chloroflexaceae.

Quinones are essential constituents in the photochemical electron-transport system of the purple bacteria. In *Rhodopseudomonas sphaeroides* (a member of the Rhodospirillaceae), ubiquinone is

both the primary and secondary electron acceptor in photosynthesis but in *Chromatium vinosum* menaquinone is the primary acceptor and ubiquinone is the secondary acceptor [19,20]. The role of quinones in electron transport in the Chlorobiaceae is unknown.

An analysis of the quinone content of *Chloroflexus* therefore holds multiple interests: besides its relevance to the photochemistry of *Chloroflexus* it may contribute to an understanding of the taxonomic and evolutionary relationships of *Chloroflexus* to the different photosynthetic bacteria, and it may shed additional light on the role of quinones in photosynthesis and respiration. The present study is the analysis of quinones in light-grown anaerobic and dark-grown aerobic cultures of *Chloroflexus*, and for comparative purposes, in *Chr. vinosum* and *Chlorobium limicola*.

Materials and Methods

Organisms and culture conditions

C. aurantiacus strain J-10-fl was grown in the 'Roux' medium of Pierson and Castenholz [1] at pH 8.3 and 53°C. Respiratory cells were grown in the dark with continual stirring and bubbling with moist air. Photosynthetic cells were grown anaerobically in completely filled glass-stoppered Roux flasks at relatively high light intensity (0.5 m from four 60 W 'Lumiline' bulbs).

Chromatium and *Chlorobium* cultures were obtained from the American Type Culture Collection (Rockville, MD), and were grown at 30–35°C with the same lighting conditions that were described for the anaerobic *Chloroflexus* cultures. Fuller's [21] heterotrophic growth medium, with malate as the carbon source, was used for the growth of *Chromatium*, while Larsen's [22] medium with 0.1% thiosulfate and 0.5% acetate was used for growing *Chlorobium*.

The cells were harvested by centrifugation and rinsed twice in 20 mM Tris-HCl buffer, pH 8.0. Dry weight of the cells was measured by vacuum filtering a measured volume of suspension through a preweighed Millipore filter paper of pore size 0.3 μm, and reweighing the paper after it had been thoroughly dried at 65°C.

Preparation of extracts

The extraction of lipids was an adaptation of the method of Redfearn [23]. The cell suspension was initially extracted at room temperature with 10 vol. of acetone/methanol (1 : 1, v/v) containing 2% of an aqueous 0.1 M $FeCl_3$ solution to assure that the quinones were in the oxidized state. After the mixture was stirred for 5 min, 10 vol. of petroleum ether (b.p. 40–60°C) were added, and vigorous stirring was continued for another 5 min. The two phases were then allowed to separate, the petroleum ether phase was decanted, and extraction of the acetone/methanol phase with petroleum ether was repeated twice more. The three petroleum ether solutions were combined, rinsed five times with methanol.water (90 : 10, v/v) in a separatory funnel, and concentrated to a volume of approx. 1–2 ml by bubbling with nitrogen.

Thin layer chromatography (TLC)

TLC was used to separate the components of each of the four petroleum ether extracts. TLC plates (Analabs, North Haven, CT) precoated with a 250 μm layer of silica gel G, were activated for 30 min at 100°C. After the plates cooled to room temperature, between 0.5 and 1.0 ml of the concentrated extract was applied in aline along the origin. The plates were developed with chloroform in the dark, then removed from the chromatography chamber and allowed to dry.

Bands were located by their color in visible light, by their ultraviolet fluorescence and/or by reaction of a test spot with reduced Nile blue, a reagent that produces a positive reaction with all quinones [24]. Where bands were detected, the adsorbent was scraped off the plate and extracted by repeatedly rinsing with aliquots of absolute ethanol.

Spectrophotometric analysis

Analysis of the ethanolic extracts was carried out as soon as possible; when it was necessary to keep the extracts for more than a few hours they were bubbled with nitrogen, sealed from air, and stored refrigerated in the dark.

A Cary 219 recording spectrophotometer linked to an Apple II computer was used to measure the ultraviolet absorption (225–350 nm) of the ethanolic extracts. Aliquots of a buffered sodium borohydride solution, prepared according to the method of Dadak and Krivankova [25], were suc-

cessively added to the extract until a maximum change in absorption was reached. Difference spectra were obtained by computerized subtraction of the oxidized from the reduced spectra.

Initially, ultraviolet difference spectra were measured for extracts of every band from the TLC plates, in order to determine which extracts contained quinones. Those bands that showed no change in absorption upon reduction were not analyzed further. The type of quinones present in the extracts was determined by their difference spectra.

The modified Craven's test was also employed as an aid in the identification of quinones. The assay was carried out according to the method of Crane and Dilley [26] except that it was necessary to make the absorption measurement immediately because maximum absorption was reached within 2 min and declined thereafter. Ubiquinone and certain tocopherol-quinones are the ony naturally occurring quinones reacting positively to this test, producing a blue color [26].

High-pressure liquid chromatography (HPLC)

Reverse phase HPLC of the extracts was carried out on a Waters Associates liquid chromatograph equipped with a Waters $C_{18}\mu$ Bondapak column (30 cm × 1.9 mm inner diameter, particle size 10 μm) and a 254 nm ultraviolet detector. The mobile phase was degassed HPLC-grade methanol/HPLC-grade isopropanol (80 : 20, v/v). The solvent flow rate was 2.5 ml/min, maintaining a pressure of about 1500 lb/inch^2.

Menaquinone standards were the kind gift of Dr. K. von Berlepsch and Dr. P. Loeliger of F. Hoffman-LaRoche & Co., Basel, Switzerland. Standard ethanolic solutions of 0.1 mg menaquinone/ml were prepared with menaquinone-6, -7, -8, -9 and -10 and were chromatographed under the above conditions. When HPLC of one of the bacterial extracts produced a peak close to that of one of the standards, the extract was co-chromatographed with a small amount of standard to verify whether the two peaks did in fact coincide.

HPLC was also used as a second method for the quantitative estimation of menaquinone in the extracts. Known volumes of the standard menaquinone solutions were chromatographed, and a calibration curve for each homologue was prepared based on the peak heights that resulted. Comparison of the appropriate calibration curves to the peak heights in chromatograms of the extracts allowed quantitative determinations to be made.

Results

TLC of the four lipid extracts (*Chloroflexus* (light- and dark-grown), *Chromatium* and *Chlorobium*) separated several fractions that reacted positively to Nile blue. All these fractions had ultraviolet difference spectra resembling those of various known quinones. The reduced minus oxidized difference spectrum of menaquinone is characterized by a maximum increase in absorption at 245 nm, and a maximum decrease at 263 and 272 nm [25,27]. Each of the four cultures examined produced a band after TLC that displayed the typical menaquinone spectrum (Fig. 1). Both *Chloroflexus* cultures contained no additional quinones. One fraction isolated from *Chromatium* by TLC showed a difference spectrum identical with that of ubiquinone [28] with a decrease at 275 nm and a slight increase at 290 nm. A second quinone fraction separated by TLC of *Chlorobium* appeared to be chlorobiumquinone (Fig. 1). It showed a difference spectrum with a maximum increase in absorption at 251 nm and a maximum decrease at 265 nm. In its oxidized state, the absorption maximum of this compound was at 254 nm with a shoulder at 265 nm. Powls and Redfearn [15] described the absorption spectrum for oxidized chlorobiumquinone as having a maximum at 254 nm and a shoulder at 265 nm, while the absorption maximum of reduced chlorobiumquinone was at 247 nm. Our findings agree with earlier reports that menaquinone and ubiquinone are present in *Chromatium* and that *Chlorobium* contains menaquinone and chlorobiumquinone [14,16,17,29].

The quinone difference spectra were also used to calculate the amount of quinone present in each organism (Table I). Light-grown *Chloroflexus* contained about the same amount of menaquinone on the basis of cell dry weight as *Chlorobium* and *Chromatium*, but dark-grown *Chloroflexus* contained five to ten times that amount. However, even this amount of menaquinone did not match

TABLE I

CHROMATOGRAPHY AND QUANTITATIVE DETERMINATION OF QUINONES ON THREE PHOTOSYNTHETIC BACTERIA

Organism	Identification of quinone [a]	R_F on TLC [b]	Amount of quinone present (μmol quinone/g cell dry wt.)
Light-grown *Chloroflexus*	menaquinone	0.72	0.16 [c] 0.18 [d]
Dark-grown *Chloroflexus*	menaquinone	0.72	1.61 [c] 1.84 [d]
Chromatium	menaquinone	0.76	0.32 [c] 0.28 [d]
Chromatium	ubiquinone	0.54	2.60 [c]
Chlorobium	menaquinone	0.73	0.15 [c] 0.23 [d]
Chlorobium	chlorobiumquinone	0.42	n.d.

[a] Identified by ultraviolet difference spectra, Craven's test and HPLC.
[b] Extracts of each organism were chromatographed on silica gel G plates, 250 μm thick, using $CHCl_3$ as solvent.
[c] Calculated from ultraviolet difference spectra using the following millimolar extinction coefficients: menaquinone, $\Delta\epsilon_{245\,nm} = 28.7$ $mM^{-1}\cdot cm^{-1}$; ubiquinone, $\Delta\epsilon_{275nm} = 12.9$ $mM^{-1}\cdot cm^{-1}$ [27].
[d] Calculated from HPLC peak heights.

the level of ubiquinone in *Chromatium*.

The lengths of the menaquinone isoprenoid chains in each organism were determined by reversed phase HPLC (Fig. 2). In this system, polar substances emerge from the column earlier than nonpolar ones. Retention times of the menaquinone standards were as follows (in min): menaquinone-6, 3.0–3.2 (slight variation between different chromatograms); menaquinone-7, 4.0–4.2; menaquinone-8, 5.1–5.4; menaquinone-9, 6.7–7.2; MK-10, 9/1–9.8. A small amount of menaquinone standard of known side-chain length was added to any sample whose chromatogram contained a peak with a retention time close to that of the standard. If the retention time of the unidentified peak matched the menaquinone standard, a single higher peak appeared in the chromatogram. Calibration curves that related the quantity of menaquinone homologue to its HPLC peak height were used to calculate the amount of menaquinone present in each organism. Table I shows that the amount of quinone calculated by this method was in fairly good agreement with the amount determined from the difference spectra.

The HPLC data show that menaquinone-10 and menaquinone-8 comprise the major menaquinones in both *Chloroflexus* cultures (Fig. 2). In *Chloroflexus*, 1.56 and 0.16 μmol menaquinone-10/g cell dry wt. were present in dark-grown and light-grown cultures, respectively. The amounts of menaquinone-8 present in the two respective cultures were 0.28 and 0.02 μmol menaquinone-8/g cell dry wt. In dark-grown *Chloroflexus*, a small amount of menaquinone-9 may also be present, but the peaks produced by co-chromatographing the extract with menaquinone-7 and menaquinone-6 do not appear to match any peaks in this chromatogram. The menaquinone band of light-grown *Chloroflexus* produced a chromatogram with a minor peak whose retention time did match that of the menaquinone-6 standard, but no detectable menaquinone-7 or menaquinone-9 were present in the light-grown culture.

The HPLC chromatograms of the menaquinone-containing fractions from *Chlorobium* and *Chromatium* each showed a single peak matching menaquinone-7 in the former organism and menaquinone-8 in the latter. In addition, the sub-

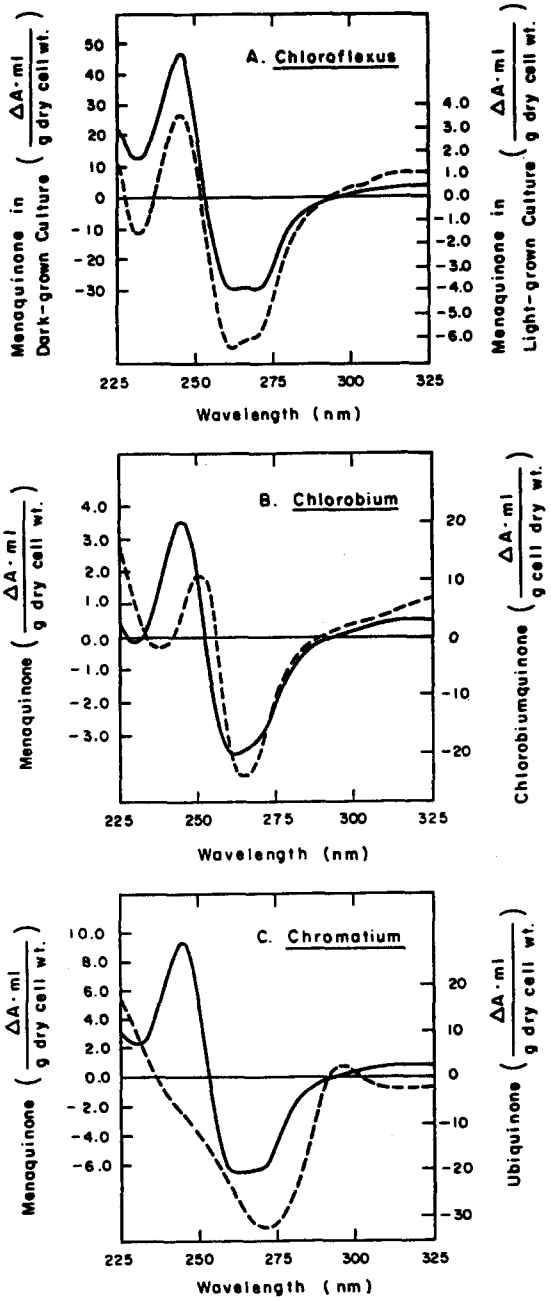

Fig. 1. Reduced minus oxidized difference spectra of the quinones in three photosynthetic bacteria. (A) Menaquinone in dark-grown (———) and light-grown (— — —) *Chloroflexus*. (B) Menaquinone (———) and chlorobiumquinone (— — —) in light-grown *Chlorobium*. (C) Menaquinone (———) and ubiquinone (— — —) in light-grown *Chromatium*.

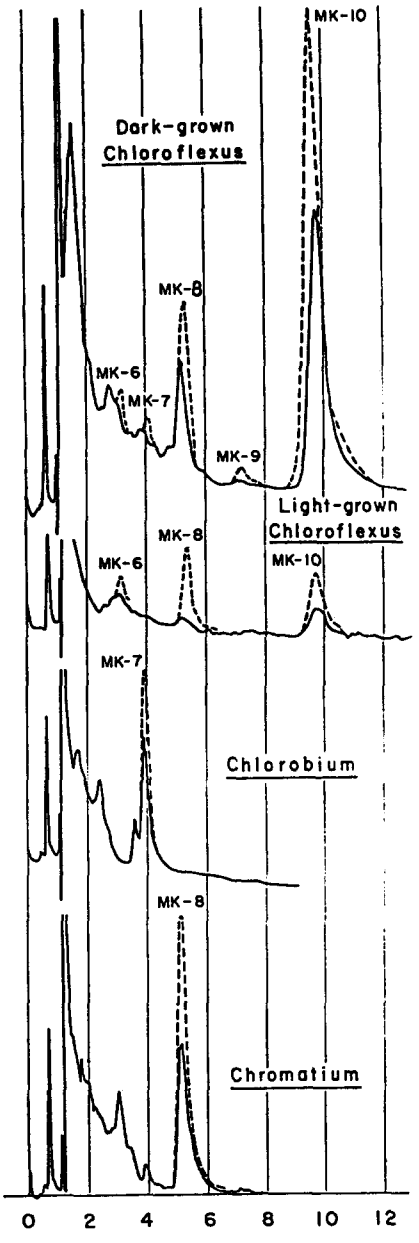

Fig. 2. HPLC chromatograms showing the side-chain length of menaquinones in various bacterial extracts. Menaquinone-containing fractions were separated from the bacterial extracts by TLC. Purified menaquinone fractions whose chromatograms (———) showed a peak having a retention time close to the previously measured retention time of one of the menaquinone standards were combined with a small amount of that standard (menaquinone (MK) -6, -7, -8, -9 or -10) and re-chromatographed. Each dashed peak (— — —) is produced by a separate co-chromatography experiment using the indicated menaquinone standard. When a purified menaquinone sample is co-chromatographed with a given menaquinone standard,

only the peak with the same retention time as that standard shows any change in absorbance. The enhanced peaks from several such 'spiking' experiments are superimposed on the original chromatogram of the 'unspiked' sample.

stance tentatively identified as chlorobiumquinone in *Chlorobium* had an HPLC retention time of 2.7 min, indicating that it is more polar than menaquinone-6. HPLC of the ubiquinone-containing *Chromatium* fraction gave a peak with retention time of 2.0 min, but the side-chain length of this ubiquinone was not determined.

Discussion

Our findings indicate that chlorobiumquinone is not involved in either photosynthesis or respiration in the facultative green bacterium *Chloroflexus*. Powls and Redfearn [15] suggested that chlorobiumquinone may be associated with the sulfide metabolism of the Chlorobiaceae; since the amount of chlorobiumquinone found in *Chloropseudomonas ethylicum* was much higher when the bacteria were grown on a sulfide-containing medium.

The identification of menaquinone-10 as the predominant homologue in *Chloroflexus* is somewhat surprising because menaquinone homologues with side chains longer than nine isoprenoid units are rare in the gram-negative bacteria, except in the genus *Bacteroides* in which the homologues range from menaquinone-7 to menaquinone-14 [12]. Except for *Chloroflexus* the only menaquinone homologues occurring in the phototrophic bacteria are seven, eight or nine isoprenoid units in length. However, The presence of long-chain quinones in *Chloroflexus* is reasonable, given the nature of the fatty acids comprising the membrane of this organism. The fatty acids of *Chloroflexus* are longer and more saturated than those of the other green bacteria [30,31] as might be expected from the thermophilic nature of this organism and the role of fatty acid composition in membrane fluidity.

In addition to menaquinone-10, smaller amounts of menaquinone-8 and possibly menaquinone-9 and menaquinone-6 are also present. These homologues may be degradation products or precursors of menaquinone-10 or they may represent additional pools of electron acceptors. Bruce et al. [7] have presented kinetic data suggesting that two separate quinone pools may be involved in the primary photochemistry of *Chloroflexus*. They found that in the presence of *o*-phenanthroline, the postillumination kinetics of the return of electrons from the primary and scondary electron acceptors to the donor complex bore a close resemblance to those of other photochemical systems in the purple bacteria under the same conditions. In *Rps. sphaeroides*, *o*-phenanthroline is known to block electron transfer between the two ubiquinone pools comprising the primary and secondary electron acceptors [11,32,33]. It also blocks electron flow between menaquinone and ubiquinone in *Chromatium* [34] and between the two plastoquinone pools in plants [35, 36]. It is thus likely that the observations of Bruce et al. reflect the presence of two photochemically active pools of menaquinone in *Chloroflexus*.

Menaquinones are widespread in photosynthetic bacteria, and Takamiya [37] showed that menaquinone is photochemically active in the green bacterium *Chlorobium*, while Feher and Okamura [20] found that menaquinone is probably the primary electron acceptor in *Chromatium* with ubiquinone serving as subsequent acceptors. Both of these organisms are obligate anaerobes, so it is not surprising to find menaquinone in anaerobic *Chloroflexus*, but the occurrence of menaquinone in aerobic *Chloroflexus* is more unusual. Of those photosynthetic bacteria previously found to contain menaquinone, only *Rps. gelatinosa* is capable of growth under fully aerobic conditions, while *Rhodospirillum fulvum* and *R. molischianum* can only tolerate oxygen under microaerophilic conditions. The rest are obligate anaerobes. Although menaquinone is found in a few nonphotosynthetic microorganisms capable of respiration, *Rps. gelatinosa* and *Chloroflexus* are the only phototrophs to possess menaquinone. The identification of menaquinone in *Chloroflexus* does not conclusively show that menaquinone is active in the electron-transport system, although no other quinone appears to be present that could function in electron transport in either respiration or photosynthesis.

A significant difference between *Chloroflexus* and *Rps. gelatinosa* is that ubiquinone is present only in the latter. *Chloroflexus* is the only facultatively aerobic photosynthetic bacterium that does not contain ubiquinone, and there are only four other groups of gram-negative bacteria that do not contain ubiquinone but do grow aerobically [12]. One of these groups is the thermophilic genus *Thermus* and another is the gliding bacteria. Ther-

mophilic and gliding properties are also found in *Chloroflexus*.

Previous work has shown that many characteristics of the photochemistry and growth physiology of *Chloroflexus* resemble the purple bacteria while *Chloroflexus'* morphology and antenna pigments are typical of the green bacteria [1,3,7,38]. These data, together with the present finding that menaquinone is the sole quinone in *Chloroflexus*, reinforce the argument that *Chloroflexus* may be an evolutionary intermediate between the green and the purple bacteria or a progenitor of both.

Acknowledgements

We thank Dr. Reiner Feick and Professor Richard Dilley for helpful advice and discussions. We thank Dr. K. von Berlepsch and Dr. P. Loeliger for a generous gift of menaquinone standards. This work was supported by NSF grant No. PCM-7915326 (to R.C.F.), NSF equipment grant No. PRM-8110744 (to R.E.B.) and USDA/CSRS Office of Competitive Research Grants, grant No. 59-2259-0-1-473-0 (to R.E.B.). The early portion of this project was performed while M.B.H. was supported by NSF Undergraduate Research Participation grant No. SPI 8025861.

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