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Structure and Function of a Menaquinone Involved in Electron Transport in Membranes of *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*

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***Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* contain the same menaquinone. Its structure, determined by thin-layer chromatography, UV absorption spectroscopy, mass spectrometry, and nuclear magnetic resonance spectroscopy, was found to be MK-7 (2-methyl-3-heptaprenyl-1,4-naphthoquinone). The menaquinone is located in the cytoplasmic membranes and is involved in redox reactions of two *b*-type cytochromes present in the clostridia. These reactions were studied with right-side-out membranes prepared from *C. thermoautotrophicum* by using CO as an electron donor. In intact membranes, both cytochromes were reduced, whereas after inactivation of the menaquinone by exposure of the membranes to UV irradiation, reduction of the low-potential cytochrome (E_0', −200 mV) but not of the high-potential cytochrome (E_0', −48 mV) occurred. The reduction of the high-potential cytochrome in UV-irradiated membranes was restored following the addition of oxidized menaquinone and with an excess of CO. The addition of oxidized menaquinone to reduced membranes resulted initially in a preferential oxidation of the low-potential cytochrome. The results obtained indicate that the menaquinone acts between the two *b*-type cytochromes in an electron transport chain.**

Menaquinones are constituents of bacterial cytoplasmic membranes. They play important roles in electron transport, oxidative phosphorylation, active transport, and endospore formation (11, 28, 29). In addition to these functions, the variations in the inherent structures of menaquinones and their uneven distributions among bacteria are considered important in bacterial taxonomy (6).

Clostridium thermoaceticum and *Clostridium thermoautotrophicum* are homoacetogenic bacteria capable of growing heterotrophically on several sugars, formate, and methanol and autotrophically on CO_2-H_2 and CO (4, 23, 30). Under heterotrophic and autotrophic conditions, acetate is totally synthesized from C_1 precursors via the recently established acetyl-coenzyme A pathway (23, 31), and both bacteria operate an electron transport process to provide ATP for growth. This is evidenced by (i) the presence of NADH dehydrogenase, CO dehydrogenase, hydrogenase, and methylenetetrahydrofolate reductase in the membranes (16; D. M. Ivey, Ph.D. dissertation, University of Georgia, 1987); (ii) the presence of cytochromes, menaquinone, and ferredoxin in the membranes (10, 13, 16); (iii) the presence in the membranes of an H^+-ATPase capable of synthesizing ATP driven by a proton motive force (18); and (iv) the occurrence of electron transport and subsequent generation of a proton motive force in membrane vesicles (17).

In this paper we demonstrate that *C. thermoaceticum* and *C. thermoautotrophicum* contain a common menaquinone. The chemical characterization of the isolated menaquinone and its role in redox reactions involving the cytochromes of the membranes are reported.

MATERIALS AND METHODS

Organisms and growth conditions. *C. thermoaceticum* ATCC 39073 and *C. thermoautotrophicum* JW 701/5 were grown on glucose at 58°C under CO_2 as described elsewhere (24, 25). The cells were harvested at the late log phase of growth (A_{660}, about 2).

Membrane preparations. Membranes were prepared from lysates obtained with a French press (18) or by lysozyme treatments and washed with 50 mM potassium phosphate buffer (pH 7.0) as described previously (16). The washed membranes were stored at 4°C in the same buffer together with 20% glycerol. Anaerobic conditions were maintained during the preparation of membranes by using a type B anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.).

Menaquinone isolation. Lipids were extracted from whole cells (100 g of cell paste) or membranes (prepared from 100 g of cell paste with the French press) by stirring with 800 ml of an acetone-methanol (1:1) or chloroform-methanol (2:1) mixture for 14 to 16 h (7, 27). The extract was filtered to remove cell debris and concentrated to 10 to 15 ml in a rotary evaporator at 40°C under reduced pressure. It was then extracted with petroleum ether by using a separatory funnel. The resulting layer of petroleum ether was evaporated to dryness. The yellowish residue was dissolved in a small volume of hexane, and purified menaquinone was obtained from it by employing preparative thin-layer chromatography on silica gel GF-coated plates (500 μm thick, 20 by 20 cm) with benzene-hexane (1:1) as the developing solvent (27). To check for the presence of ubiquinone, the hexane solution was also chromatographed on silica gel-coated plates (250 μm thick, 5 by 20 cm) with petroleum ether-diethyl ether (85:15) as the developing solvent (7). In this system, menaquinones migrate faster ($R_f \approx 0.7$) than do ubiquinones ($R_f \approx 0.4$). Vitamin K_2 (MK-4) and ubiquinone-50 (Sigma Chemical Co., St. Louis, Mo.) were used as standards. Menaquinone was revealed by brief UV irradiation and eluted from the gel with chloroform. The purity of the menaquinone was checked by UV absorption spectroscopy before it was finally

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dissolved and stored in absolute ethanol or chloroform for further studies. Under the conditions of isolation, the menaquinone was obtained in fully oxidized form.

Spectrophotometric characterization of menaquinone was done in ethanolic solutions by the method of Dunphy and Brodie (8). Reversed-phase and Ag^+ thin-layer chromatography were performed by using silica gel GF plates (250 μm thick) impregnated with 5% paraffin oil and 5% $AgNO_3$, respectively (8). After the plates were developed, they were dried and sprayed with 3% rhodamin G for direct visualization of menaquinones, which appeared as red spots on a pink background.

Mass spectrometry and NMR spectroscopy. Electron impact mass spectra of the menaquinone samples were recorded with a model 5985 quadruple mass spectrometer (Hewlett Packard Co., Palo Alto, Calif.), using an electron energy of 70 eV. The sample was introduced to the spectrometer by direct insertion, with the probe temperature programmed from 100 to 250°C, at a rate of 10°C/min. For nuclear magnetic resonance (NMR) analysis, portions (approximately 2 mg) of the menaquinone samples were dissolved in 0.5 ml of $CDCl_3$ (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and transferred into 5-mm NMR tubes (Wilmad 528-PP). The ^1H NMR spectroscopic analyses were performed at 250 MHz with a Bruker AM-250 spectrometer interfaced with an Aspect-3000 computer. The probe temperature was kept at 27°C. ^1H chemical shifts (δ) are expressed in parts per million downfield from internal tetramethylsilane. The shifts were actually measured by reference to the residual $CHCl_3$ solvent signal (δ 7.262 at 27°C), with an accuracy of 0.003 ppm.

Reduction and oxidation reactions of menaquinone. Redox reactions catalyzed by membranes were studied by using anaerobic cuvettes under N_2 and in 50 mM potassium phosphate (pH 7) in a final volume of 0.6 ml (24). Difference spectra of membranes following cytochrome reductions were recorded with an Aminco DW-2 UV-Vis spectrophotometer (American Instrument Co., Silver Spring, Md.) at −196°C. Cytochromes in membranes were reduced either by CO, through the action of membrane-bound CO dehydrogenase, or chemically, with deoxygenated 1 mM dithionite solutions. CO was used either as such (as a gas) or in the form of CO-saturated potassium phosphate buffer (50 mM [pH 7.0]). In experiments in which it was necessary to avoid an excess of CO, the exact amount of CO-saturated buffer needed to completely reduce the cytochromes was established. This amount was determined by titrating the membranes with CO-saturated buffer until the cytochromes were fully reduced. Complete cytochrome reduction occurred within 30 s at 50°C. The minimum amount of CO-saturated buffer giving maximum cytochrome reduction of a membrane preparation containing 3.5 mg of protein per ml was estimated to be 48 μl, and the reduction of the cytochromes was found to be comparable to that obtained with dithionite. The cytochrome content of the membranes was determined from the difference spectra obtained with dithionite-reduced and air-oxidized cytochromes at 560 to 575 nm by using a molar extinction coefficient of 17.5 mM^{-1} cm^{-1} (14). The menaquinone content was determined in ethanolic solutions by recording the absorption difference at 265 to 289 nm between solutions before and after reduction with KBH_4, using a molar extinction coefficient of 14.7 mM^{-1} cm^{-1} (21). Protein was measured by the rose bengal dye-binding assay (9) in the presence of 0.2% Triton X-100 to solubilize the membrane proteins and with bovine serum albumin as the standard.

UV irradiation. Membranes suspended in 0.6 ml of 50 mM potassium phosphate buffer (pH 7.0) were UV irradiated in anaerobic quartz cuvettes at room temperature. The irradiation was done by four 15-W near-UV (254-nm) lamps (Fotodyne Inc., New Berlin, Wis.) placed on each side of the cuvettes at a distance of 22 cm. UV irradiation of isolated menaquinone samples was done in ethanolic solutions in quartz cuvettes as described above.

RESULTS

Preliminary analysis of menaquinone. Initial analysis of the isolated menaquinone from *C. thermoaceticum* and *C. thermoautotrophicum* was done by UV absorption spectroscopy and thin-layer chromatography. Ethanolic solutions of the menaquinone isolated from the bacteria and of MK-4 (used as a reference) gave identical UV absorption spectra with λ_{max} values at 242, 248, 260, 270, and 330 nm, typical of menaquinone (8). No ubiquinone was detected in the two clostridia. In reversed-phase and Ag^+ thin-layer chromatography, the isolated menaquinones behaved identically and gave R_f values of 0.55 and 0.40, respectively. These values are lower than those obtained for MK-4 (0.73 and 0.58, respectively), which demonstrates that the isolated menaquinone has a longer isoprenoid chain than MK-4 does. Actually, a comparison of the R_f values obtained by using Ag^+ thin-layer chromatography with those reported by Dunphy and Brody (8) indicates that the isolated menaquinone is MK-7 (2-methyl-3-heptaprenyl-1,4-naphthoquinone).

Structural characterization of the menaquinone by NMR spectroscopy and mass spectrometry. The ^1H NMR spectra of the menaquinones isolated from *C. thermoaceticum* and *C. thermoautotrophicum* were found to be identical. Figure 1 shows the NMR spectrum of the menaquinone isolated from *C. thermoautotrophicum*. This spectrum closely resembles the ^1H NMR spectra reported for the fully unsaturated isoprenoid menaquinone (5). It contains two complex multiplets of δ 8.082 and δ 7.687 due to the four aromatic ring protons (ϕ—H). The methyl group at the C-2 position (Q-CH_3) can be recognized from the singlet at δ 2.190. The olefinic methyl groups (=C—CH_3) resonate at δ 1.2 to 1.8 ppm, and the olefinic methylene groups (=C—CH_2—) resonate in the broad signal at δ 2.0. Very characteristically, a doublet at δ 3.373 (J = 6.9 Hz) indicates a methylene group attached to the aromatic ring (Q—CH_2), whereas the olefinic methine protons resonate in a broad signal at δ 5.085. From the relative intensity of the latter signal (compared with, e.g., those of the four aromatic protons), it was inferred that the multiprenyl chain length is 7 U. The only major difference between the NMR spectra of the two clostridial menaquinone preparations described here and the spectra of the pure isoprenoid menaquinones (including that of MK-7 [5, 26]) is the presence of a triplet at δ 4.1 and two tripletlike signals at δ 2.8 and δ 2.6 in our spectra. These signals could not be assigned to any proton in the menaquinone structure. Also, the intensity ratio of these signals compared with that of the methylene signal at δ 3.373 was significantly different in both spectra. Therefore, we assumed that the aforementioned signals (asterisks in Fig. 1) might belong to impurities of the samples. To confirm this and also to verify the polyprenyl chain length of the compounds, mass spectrometry was applied to both samples.

When subjected to direct-inlet electron impact mass spectrometry, the two isolated menaquinones produced identical spectra. The mass spectrum obtained for the compound from *C. thermoaceticum* is shown in Fig. 2. Both spectra show a

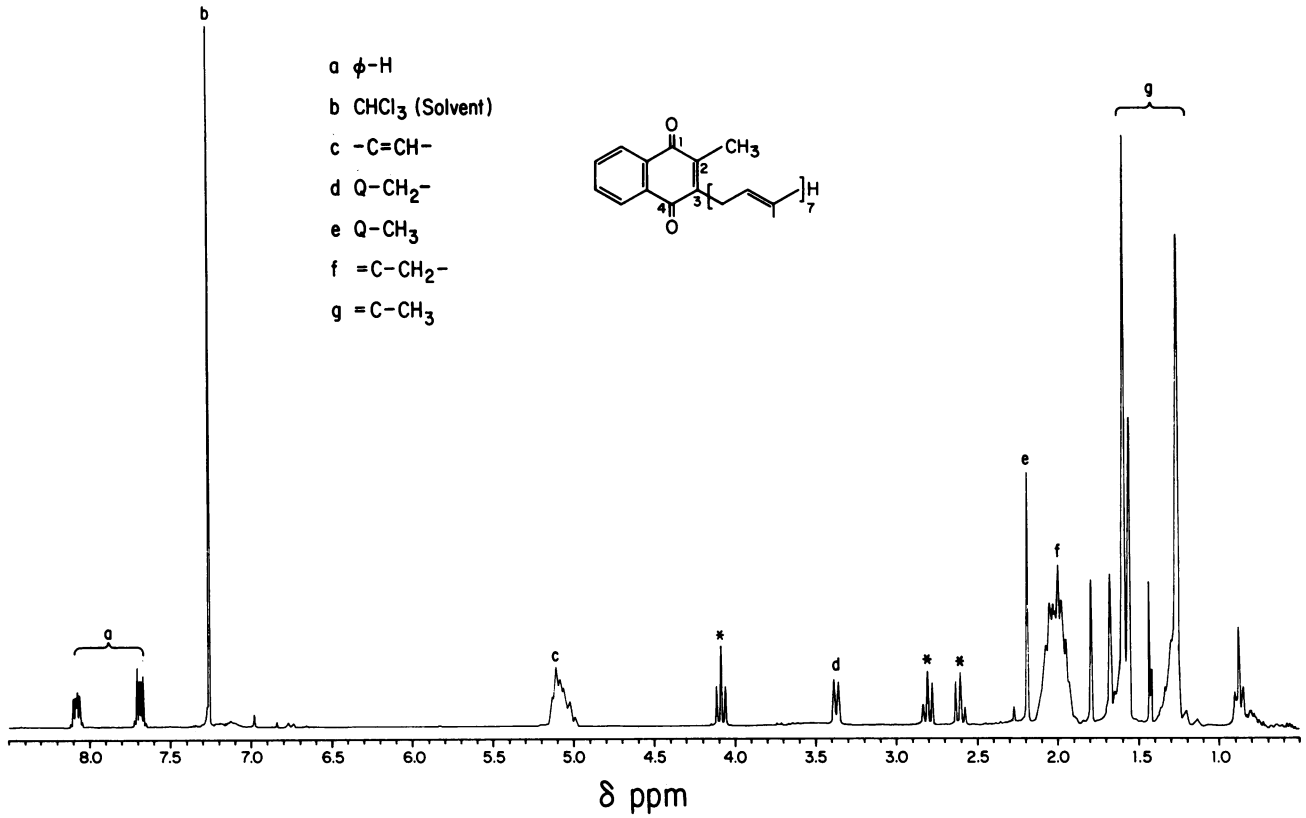

FIG. 1. ^1H NMR spectrum of the menaquinone isolated from *C. thermoautotrophicum*. The spectrum was obtained at 250 MHz, for a solution containing 2.0 mg of sample in 0.5 ml of $CDCl_3$, at 27°C. Signals marked by asterisks are attributed to impurities. Assignments of peaks refer to protons in the structure shown on the graph. ϕ, Phenyl; Q, quinone.

base peak at *m/z* 225 and another rather prominent peak at *m/z* 187. These two peaks are indicative of menaquinones (5, 26). In addition, lower-intensity peaks were observed at *m/z* 307 and 375, pointing to the presence of a multiprenyl side chain. A relatively strong peak at *m/z* 648 was observed. This *m/z* value shows that the multiprenyl side chain is 7 isoprene units long and is fully unsaturated. It should be noted that the mass spectra which we obtained matched that published for MK-7 from *Desulfococcus multivorans* (5). The mass spectra also confirm that the signals observed in

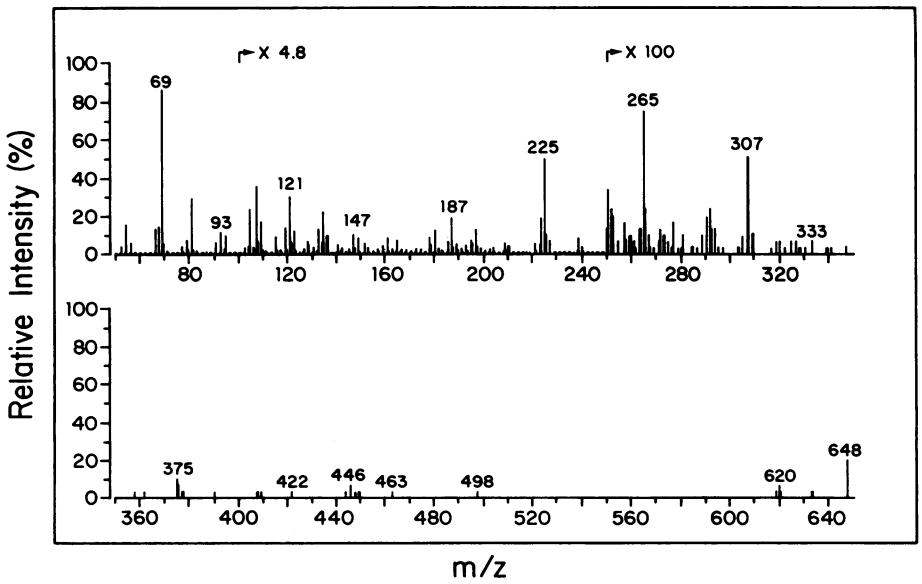

FIG. 2. Electron impact mass spectrum of the menaquinone isolated from *C. thermoaceticum*. Experimental details are given in the text. Arrows indicate that from *m/z* 100 and *m/z* 250 onwards, the intensity of the peaks is multiplied by 4.8 and 100, respectively.

the NMR spectra at δ 4.1, 2.8, and 2.6 belonged to impurities.

Localization of menaquinone. Menaquinone was found almost exclusively (85 to 90%) in the membranes of both bacteria. It was present in a larger amount in *C. thermoaceticum* (0.51 μmol/g of protein in whole cells and 1.8 μmol/g of protein in membranes) than in *C. thermoautotrophicum* (0.37 μmol/g of protein in whole cells and 1.3 μmol/g of protein in membranes).

Roles of menaquinone in reductions of cytochromes. Redox reactions between menaquinone and the two *b*-type cytochromes previously identified in membranes of *C. thermoautotrophicum* (16) were studied by using right-side-out membrane vesicles of *C. thermoautotrophicum* prepared by lysozyme treatment. Cytochrome b_{559} (Cyt b_{559}) (E_0', −200 mV) and Cyt b_{554} (E_0', −48 mV) (16), which were obtained partially reduced in the preparation of the vesicles, were completely reduced by the required amount of CO-saturated buffer (see Materials and Methods). Oxidized menaquinone (in oxygen-free ethanol) at a final concentration of 100 μM was then added, and changes in the difference spectra of the cytochromes over a period of 190 s were recorded (Fig. 3). No spectral change was observed during an initial period of 120 s following the addition of menaquinone. We feel that this may be due to some excess in the CO added to fully reduce the cytochromes; alternatively, it may reflect the time it takes for the exogenously added menaquinone to enter the membrane and react with the cytochromes. During the next 35 s after the initial time period, a preferential oxidation of Cyt b_{559} occurred, as indicated by a gradual decrease in the A_{559}, whereas Cyt b_{554} remained essentially fully reduced. However, after 190 s after the addition of the menaquinone, both cytochromes were oxidized. In the experiment described above, the isolated clostridial menaquinone and MK-4 reacted identically.

Irradiation with UV light destroys menaquinone (2, 19, 28), and menaquinone-mediated electron transfer should not occur in UV-irradiated membranes. The time needed for destruction of the purified menaquinone (MK-7) from the clostridia was determined by exposing a 10 μM ethanolic solution to UV light as described in Materials and Methods. Irradiation for 10, 30, and 50 min destroyed 48, 73, and 100% of the MK-7, respectively. The effect of UV irradiation on cytochrome reduction by CO in membranes of *C. thermoautotrophicum* was then investigated (Fig. 4). In membranes irradiated for 40 min, the reduction of Cyt b_{554} was much inhibited, and after 80 min of UV exposure, reduction did not occur; in contrast, the reduction of Cyt b_{559} was unaffected. To check the intactness of the Cyt b_{554} after the UV treatment, dithionite was added to the membranes. Upon this treatment, Cyt b_{554} was obtained fully reduced, and the cytochrome spectrum obtained was identical to that of fully reduced membranes. The results show that in UV-irradiated membranes, Cyt b_{559} is reduced by electrons coming from CO, whereas Cyt b_{554} is not. The results also suggest that the destruction of menaquinone prevents the reduction of Cyt b_{554}, which in turn indicates that the reduction of Cyt b_{554} is mediated by menaquinone.

To further ascertain the involvement of menaquinone in the reduction of Cyt b_{554}, UV-irradiated membranes with and without 5 μM oxidized menaquinone were reduced with an excess of CO (Fig. 5). In agreement with the results shown in Fig. 4, Cyt b_{554} was not reduced by CO in the absence of menaquinone. However, after the addition of menaquinone, the reduction of Cyt b_{554} was restored.

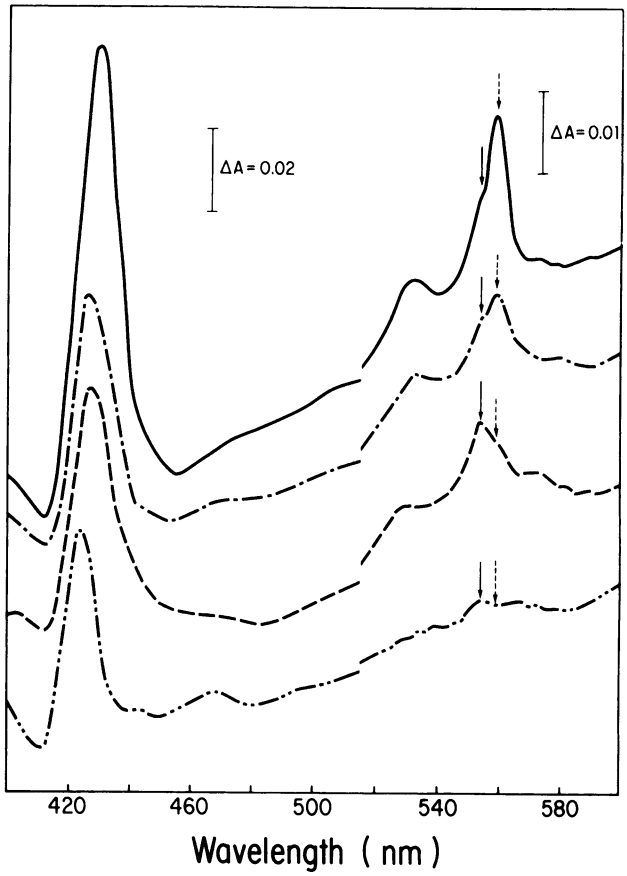

FIG. 3. Oxidation of Cyt b_{559} and Cyt b_{554} in membrane vesicles from *C. thermoautotrophicum* by menaquinone. Right-side-out membranes (3.5 mg of protein per ml) were reduced with CO-saturated buffer at 50°C for 30 s. After equilibration at 25°C, oxidized menaquinone (MK-4) (final concentration, 100 μM) in ethanol was added. Liquid-nitrogen difference spectra (CO-reduced minus air-oxidized cytochromes) were then recorded at 135 s (· – · – ·), 155 s (– – – –), and 190 s (·· – ·· –) after the addition of the menaquinone. The control sample (———) received no menaquinone but received an equivalent amount of ethanol. Wavelengths of absorption by Cyt b_{554} (——→) and Cyt b_{559} (----→) are also indicated.

DISCUSSION

The results of mass spectroscopy, NMR spectroscopy, chromatography, and UV absorption spectroscopy studies demonstrate that *C. thermoaceticum* and *C. thermoautotrophicum* contain the same menaquinone identified as MK-7. MK-7 is the major menaquinone component in many gram-positive spore-forming bacteria (6) and was suggested, on the basis of chromatographic evidence only, to be the major menaquinone in *C. thermoaceticum* (32). The levels of MK-7 (0.4 to 0.5 μmol/g of protein) in the two clostridia were very similar to those found in *Bacillus cereus*, *Bacillus circulans*, *Bacillus megaterium*, and other species of the genus *Bacillus* (15). As it does in other bacteria (1, 21, 22), menaquinone occurred almost exclusively in membrane fractions of the clostridia. It should be noted that in membranes of *C. thermoautotrophicum*, the amount of menaquinone (1.3 μmol/g of membrane protein) is five times larger than that in the cytochromes (0.26 μmol/g of membrane protein). A similar distribution of menaquinone and cytochromes has been reported for other bacteria (21, 22).

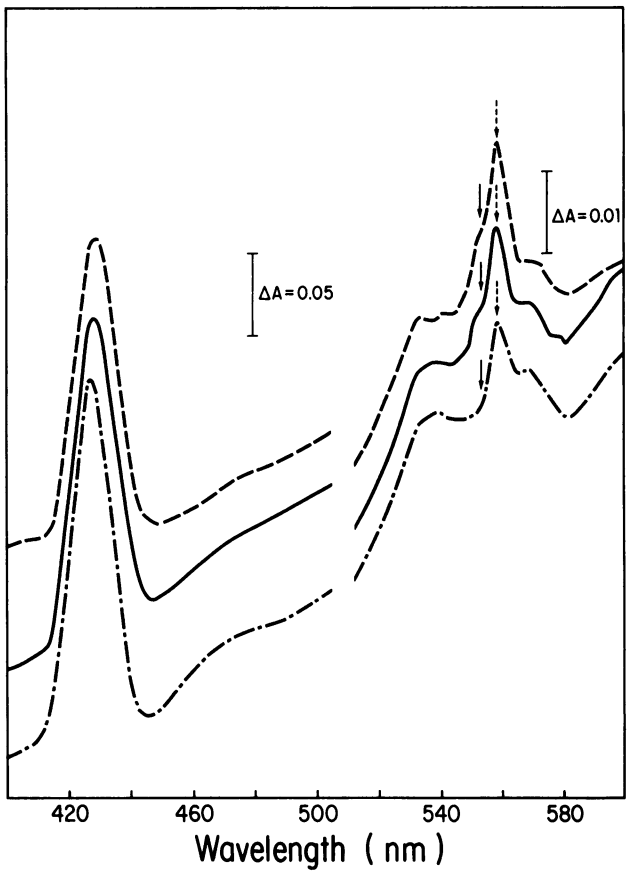

FIG. 4. Effect of UV irradiation on the reduction of Cyt b_{559} and Cyt b_{554} with CO in membrane vesicles of *C. thermoautotrophicum*. Membranes (3.5 mg of protein per ml) in quartz cuvettes were irradiated with UV light (254 nm) before being reduced with 48 μl of CO-saturated buffer at 50°C. Liquid-nitrogen difference spectra were recorded for membranes irradiated for 40 min (———), for 80 min (· – · – · –), and without irradiation (– – – –) (control). Cyt b_{554} (⟶) and Cyt b_{559} (----›) are also indicated.

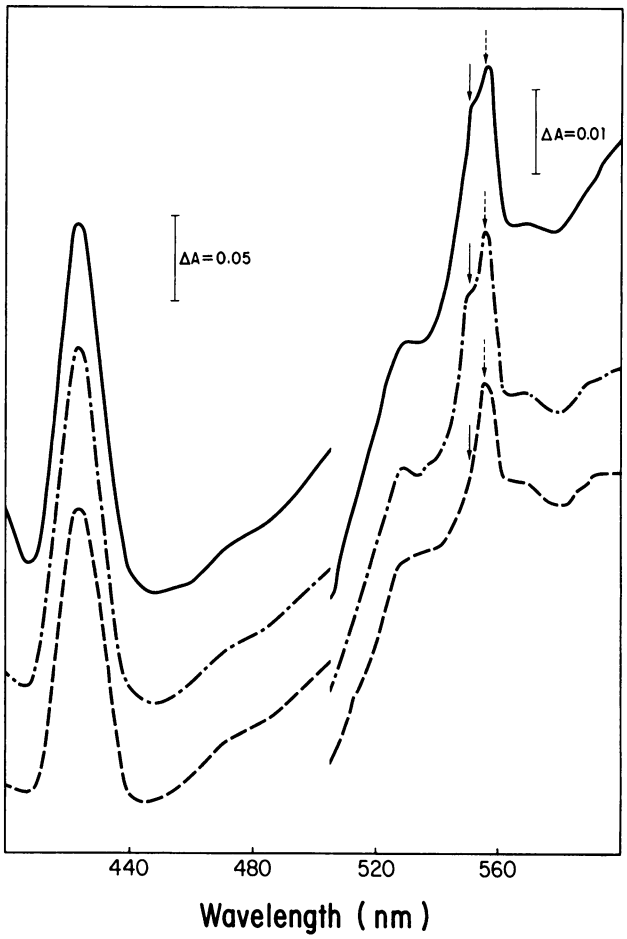

FIG. 5. Effect of exogenously added menaquinone on the reduction of Cyt b_{559} and Cyt b_{554} with CO in UV-irradiated membranes of *C. thermoautotrophicum*. Membranes (6.5 mg of protein per ml) were UV irradiated as described in the legend to Fig. 4. Irradiated membranes were treated with 5 μM menaquinone (MK-4) for 300 s before being reduced with excess CO gas at 50°C for 30 s. Membranes were reduced after irradiation for 80 min (– – – –), after irradiation for 80 min and treatment with menaquinone (· – · – · –), and without irradiation or treatment with menaquinone (———) (control). Cyt b_{554} (⟶) and Cyt b_{559} (----›) are also indicated.

The results described here demonstrate that menaquinone participates in the redox reactions of the *b*-type cytochromes of *C. thermoautotrophicum*, and they suggest that menaquinone has a position between Cyt b_{559} and Cyt b_{554} in an electron transport chain in this bacterium. The basis for this conclusion is, first, that the addition of oxidized menaquinone to membranes containing reduced cytochromes results in the oxidation of the low-potential Cyt b_{559} in preference to the higher-potential Cyt b_{554} (Fig. 3). Second, Cyt b_{559} is reduced, whereas Cyt b_{554} is not reduced, in membranes treated with UV light that destroys the menaquinone. Third, the addition of menaquinone to UV-treated membranes restores the reduction of Cyt b_{554}.

It has been suggested that the final electron acceptor in the acetogenic clostridia is methylenetetrahydrofolate (12, 16, 29), which is an intermediate in the acetate biosynthesis pathway (23, 31). On the basis of the values of redox potentials, it is likely that methylenetetrahydrofolate (E_0', −120 mV) (3) can accept electrons from Cyt b_{559} (E_0', −200 mV) rather than from Cyt b_{554} (E_0', −48 mV) or menaquinone (E_0', −74 mV) (29), since the two latter electron carriers have redox potentials higher than −120 mV. However, this has not yet been verified experimentally.

Given the redox potential values of the electron carriers

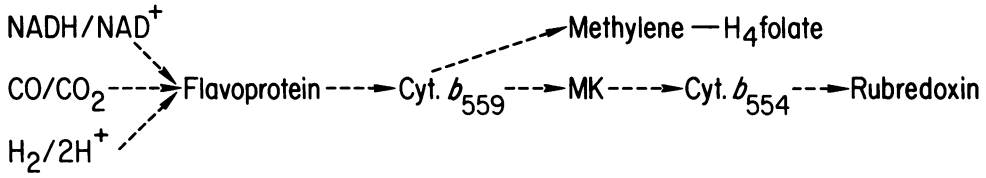

FIG. 6. Proposed electron transport chain operating in acetogenic clostridia.

and redox couples (13, 16, 29) found in *C. thermoautotrophicum*, a hypothetical scheme for an electron transport chain can be postulated (Fig. 6). In this scheme, we have placed the menaquinone between the two cytochromes on the basis of evidence presented in this paper. However, a possible alternative is that menaquinone is more directly reduced, for instance by the flavoprotein, and that Cyt b_{559} is bypassed. Our results demonstrate, however, that Cyt b_{559} reduces menaquinone, which in turn is needed for the reduction of Cyt b_{554}. The postulation of electron transfer from Cyt b_{554} to rubredoxin is based on the presence of two *C. thermoaceticum* rubredoxins having redox potential values of −27 mV and +20 mV (33) and the fact that electron paramagnetic resonance studies have given evidence for the presence of rubredoxin in *C. thermoautotrophicum* membranes (J. Hugenholtz, T. V. Morgan, and L. G. Ljungdahl, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K-147, p. 269).

The electron transport chain shown in Fig. 6 is also believed to operate in *C. thermoaceticum*, since the composition of electron carriers as well as other physiological properties of this bacterium are essentially the same as those in *C. thermoautotrophicum* (Ivey, Ph.D. dissertation). The sequence of cytochrome-menaquinone-cytochrome in electron transport as suggested here has also been postulated in the formate-fumarate system of the anaerobe *Wolinella succinogenes* (formerly designated *Vibrio succinogenes*), in which fumarate is the final electron acceptor (20).

The function and coupling of the electron transport chain with respect to energy generation and oxidative phosphorylation in the acetogenic clostridia is not yet understood. However, the generation of a proton motive force is observed in the membrane vesicles, with CO as the electron donor and ferricyanide as an artificial electron acceptor (17).

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