Characterization of Cytochromes from *Methanosarcina* Strain Gö1 and Their Involvement in Electron Transport during Growth on Methanol

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*Methanosarcina* strain Gö1 was tested for the presence of cytochromes. Low-temperature spectroscopy, hemochrome derivative spectroscopy, and redox titration revealed the presence of two b-type (b559 and b556) and two c-type (c552 and c553) cytochromes in membranes from *Methanosarcina* strain Gö1. The midpoint potentials determined were *E*<sub>m</sub>1/2 = +135 ± 5 and +240 ± 11 mV (b-type cytochromes) and *E*<sub>m</sub>2/2 = -140 ± 10 and -230 ± 10 mV (c-type cytochromes). The protoheme IX and the heme c contents were 0.21 to 0.24 and 0.09 to 0.28 μmol/g protein, respectively. No cytochromes were detectable in the cytoplasmic fraction. Of various electron donors and acceptors tested, only the reduced form of coenzyme F<sub>420</sub> (coenzyme F<sub>420</sub>H<sub>2</sub>) and the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) were capable of reducing and oxidizing the cytochromes at a high rate, respectively. Addition of CoM-S-S-HTP to reduced cytochromes and subsequent low-temperature spectroscopy revealed the oxidation of cytochrome b<sub>559</sub>. On the basis of these results, we suggest that one or several cytochromes participate in the coenzyme F<sub>420</sub>H<sub>2</sub>-dependent reduction of the heterodisulfide.

Methanogenic bacteria gain metabolic energy by coupling the conversion of a number of simple substrates such as H<sub>2</sub>-CO<sub>2</sub>, formate, methylamines, methanol, and acetate to the generation of ATP. The methanogenic pathways leading to the conversion of these substrates have been unraveled in the last two decades, and they involve a number of unique cofactors (37). The penultimate step of methanogenesis, the reduction of the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) with either H<sub>2</sub> or reduced coenzyme F<sub>420</sub>(F<sub>420</sub>H<sub>2</sub>) as a reductant, was shown in *Methanosarcina* strain Gö1 to be coupled to electron transport phosphorylation (7, 34). Possible carriers mediating the membrane-linked electron transport from H<sub>2</sub> or F<sub>420</sub>H<sub>2</sub> to CoM-S-S-HTP are iron-sulfur proteins and cytochromes. The presence of cytochromes appears to be restricted to members of the family *Methanomicrobaceae*. This feature coincides with the ability of this methanogenic family to disproportionate methyl groups and to cleave acetate. All other methanogenic families are devoid of cytochromes and incapable of methyl group oxidation and acetate cleavage. Cytochromes have therefore been suggested to play a role in these processes (28). In contrast to this hypothesis, Kenner et al. (25) proposed an involvement of cytochromes in electron transport from H<sub>2</sub> to methyl-CoM in *Methanosarcina barkeri*. Terlesky and Ferry (38) reported on a hydrogenase-linked cytochrome b in *M. thermophila*.

In this paper we present spectroscopic and potentiometric evidence for the presence of two b- and two c-type cytochromes in *Methanosarcina* strain Gö1 and report on electron transport studies which indicate the participation of cytochromes in the energy-conserving electron transport from F<sub>420</sub>H<sub>2</sub> to CoM-S-S-HTP.

**MATERIALS AND METHODS**

**Growth conditions and harvest of cells.** The methanogenic bacterium *Methanosarcina* strain Gö1 (DSM 3647) was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany) and grown in 20-liter carboys under strictly anaerobic conditions in the medium described by Hippe et al. (21) with 150 mM methanol as an energy source and supplemented with 1 g of sodium acetate per liter. Cells in late logarithmic growth phase were harvested either aerobically or anaerobically by continuous centrifugation at 18,000 × g.

**Membrane preparations.** For the spectroscopic and potentiometric characterization of cytochromes, membranes were prepared under aerobic conditions. The freshly harvested cells were washed twice with 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 M sucrose and 10 mM MgSO<sub>4</sub>. After suspension in 50 mM potassium phosphate buffer, pH 7.0 (KP buffer), and addition of a few crystals of DNase, the cells were lysed by passing them through an Amino French pressure cell at 138 MPa. The resulting crude extract was centrifuged at 10,000 × g for 15 min to remove unbroken cells and cell debris. The supernatant was diluted to 150 ml with KP buffer and centrifuged at 120,000 × g for 2 h. The supernatant (cytoplasmic fraction) was carefully removed and discarded. The membrane pellet was resuspended in the same buffer and centrifuged at 10,000 × g for 15 min to pellet the residual sulfides apparently adhering to the membranes. The supernatant from this low-speed centrifugation was subsequently subjected to ultracentrifugation at 120,000 × g for 30 min. These steps (resuspension of the resulting pellet, low-speed centrifugation, and ultracentrifugation) were repeated two more times. The final membrane pellet was resuspended in the same buffer to a protein concentration of

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approximately 30 mg/ml. For redox titrations, membrane fractions were stored on ice until used; otherwise they were stored at -70°C. Inverted vesicles of Methanosarcina strain Göl (31) were prepared under strictly anaerobic conditions as described previously (7) except that resazurin was omitted from the buffer and the concentration of dithioerythritol (DTE) was reduced to 0.5 mM. For experiments with $p$-chloromercuriphenylsulfonate (pCMPS), DTE was replaced by 0.02 mM Ti(III)-citrate. The washed vesicle preparation was stored under $N_2$ at -70°C until used.

**Room temperature spectroscopy.** Redox difference spectra were recorded with a Perkin-Elmer/Hitachi spectrophotometer (model 556) in the double-beam mode. The sample cuvette was mixed with a few grains of $Na_2S_2O_3$ and measured against an $O_2$- or $H_2O_2$-oxidized reference cuvette. The spectral bandwidth was set to 1 nm, and the scanning speed was 30 nm/min for spectroscopic cytochrome characterizations and 120 nm/min for kinetic investigations, respectively. When anaerobic conditions were required, cuvettes were sealed with rubber stoppers and flushed with $N_2$. Additions were made with microliter syringes (Hamilton, Bonaduz, Switzerland). $H_2$ was added with a gas-tight syringe either as $H_2$-saturated buffer or as hydrogen gas. For the calculation of the concentrations of dissolved $H_2$, an absorption coefficient of 0.01819 was used. Difference spectra [$Na_2S_2O_3$-reduced + CO - $Na_2S_2O_3$-reduced] were obtained by reducing both sample and reference cuvette with a few grains of $Na_2S_2O_3$ and adding 2 ml of CO to the sample cuvette. The spectrum was recorded after 5 min of incubation in the dark.

**Low-temperature spectroscopy.** Spectra taken at 77 K were scanned at 30 nm/min. The samples were frozen in liquid $N_2$ in the presence of 50% glycerol by using the devitrification technique of Estabrook (12). Cuvettes of 1-mm path length and the cryogenic unit of the above-described spectrophotometer were used. Second derivative spectra were recorded with the derivative spectrum attachment. Glycerol was made anaerobic by incubating aliquots of 0.2 ml of 87% glycerol in an anaerobic chamber (M.D.H. microflow anaerobic system; Intermed, Andover, Great Britain) for approximately 3 weeks until a resazurin-containing control turned colorless.

**Determination of heme content.** Protoheme IX was extracted from the membrane fraction with acetone-HCl by the method of Jacobs and Wolin (22) as modified by Kühn and Gottschalk (30). For the identification and quantification of protoheme IX and heme $c$, the acetone-HCl extract and the protein pellet after acetone-HCl extraction were each mixed with 3 ml of 50% pyridine in 0.1 M NaOH in order to prepare the pyridine hemochromes. The difference absorption coefficients used were as follows: $\Delta A_{557-541} = 20.7$ $\text{mM}^{-1}\text{cm}^{-1}$ (protoheme IX (35)) and $\Delta A_{560-540} = 22.1$ $\text{mM}^{-1}\text{cm}^{-1}$ (heme $c$ (26)).

**Potentiometric titrations.** Redox titrations were performed by the methods of Dutton (9) and Wilson (39) in a custom-designed cuvette (path length, 1 cm; Ochs, Bovenden, Germany) at 25°C. Redox potentials were measured with a micro-redox-electrode type Pt 5700 A (combination electrode; Schott, Hofheim, Germany) in the presence of the following redox mediators: 2,6-diaminodurol ($E_m = +260$ mV), 33 $\mu$M; 1,2-naphthoquinone ($Em = +143$ mV), 34 $\mu$M; phenazine methosulfate ($Em = +80$ mV), 18 $\mu$M; phenazine ethosulfate ($Em = +55$ mV), 16 $\mu$M; duroquinone ($Em = +5$ mV), 33 $\mu$M; pyocyanine ($Em = -34$ mV), 4 $\mu$M; 2,3-dimethyl-1,4-naphthoquinone ($Em = -80$ mV), 83 $\mu$M; 2-hydroxy-1,4-naphthoquinone ($Em = -152$ mV), 31 $\mu$M; antraquinone-2,6-disulfonate ($Em = -184$ mV), 18 $\mu$M; anthraquinone-2-sulfonate ($Em = -224$ mV), 18 $\mu$M; phenosafranine ($Em = -252$ mV), 0.23 $\mu$M; acridinium chloride ($Em = -313$ mV), 19 $\mu$M; diquat ($Em = -349$ mV), 5 $\mu$M; methylviologen ($Em = -449$ mV), 0.4 $\mu$M. To obtain a fast response, the platinum electrode was activated by successive incubations in hydroquinone (in 40 mM sodium oxalate buffer, pH 4.0) and $Na_2S_2O_3$ (in $H_2$O) followed by sandpapering prior to each titration.

Membranes were diluted with oxygen-free argon-gassed 0.1 M 3-morpholinopropanesulfonic acid (MOPS) buffer, pH 7.0, or 0.1 M 2-morpholinooctanesulfonic acid (MES) buffer, pH 6.0 (control), to a final protein concentration of 7 to 15 mg/ml. Titrations were carried out by adding anaerobic solutions of ferricyanide in titration buffer as an oxidant and $Na_2S_2O_3$ in 0.5 M MOPS buffer, pH 7.2, as a reductant with a 5-ml syringe. During the titration the suspension was continuously stirred and flushed with oxygen-free argon. The redox state of the cytochromes was monitored by using a Sigma ZWS 11C spectrophotometer (Biochem, Puchheim, Germany) in the dual-wavelength mode with the $alpha$-peak as measuring wavelength and one of the adjacent minima as reference wavelength; these were 560 - 575 nm for the $b$-type and 549 - 560 nm for the $c$-type cytochromes. The pH (6 or 7) and the preparation method (aerobic or anaerobic) did not affect the midpoint potential of any of the cytochromes. In a control experiment, the mediator system was titrated without membranes to ensure that the spectral changes caused by the redox mediators were negligible. The midpoint potentials were determined by 9 ($b$-type) and 13 ($c$-type) independent titrations, respectively.

**Protein determination.** Protein was determined by the method of Bradford (4) with bovine serum albumin as a standard.

**Preparation of F$_{420}$H$_2$.** The methanogenic cofactor F$_{420}$ was isolated from M.arkeri Fusaro (DSM 804; Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) and chemically reduced to F$_{420}$H$_2$ as described in reference 6. Freshly reduced F$_{420}$H$_2$ was used for each experiment.

**Synthesis of HTP-S-S-HTP and CoM-S-S-HTP.** The homodisulfide of 7-mercaptoheptanoylthreonine (HTP-S-S-HTP) and CoM-S-S-HTP were synthesized by the method of Noll et al. (32) and Bobik and Wolfe (3) except that CoM-S-S-HTP was prepared from HTP-S-S-HTP and CoM-SH in the following way. HTP-S-S-HTP was reduced to 7-mercaptoheptanoylthreonine phosphate (HTP-SH) by adding a few grains of NaBH$_4$ to the anaerobic solution and incubating for 30 min under $N_2$. Excess NaBH$_4$ was eliminated by acidification to pH <1 with 6 M anaerobic HCl and subsequent neutralization with anaerobic 6 M NaOH. A fivefold molar excess of CoM-SH was added, and the solution was oxidized with I$_2$-KI. Excess iodine was reduced with sodium hydrogen sulfite until the solution turned colorless. Purification of CoM-S-S-HTP followed the method of Ellermann et al. (10). Purity was controlled by using thin-layer chromatography. Quantification was done as described below under “Enzyme assays.”

**Determination of thiols was performed by the method of Ellman (11) with 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) and with cysteine as a standard. For the determination of disulfides a modification of the method of Zahler and Cleland (41) was used. The sample (up to 30 $\mu$l) was mixed with 100 $\mu$l of 50 mM Tris HCl, pH 9.0, and reduced with 33 $\mu$l of a freshly prepared solution of 3 mM DTE in $H_2$O for at least 20 min. After the addition of 100 $\mu$l of 1 M Tris HCl, pH 8.1, 0.5 ml of 5 mM Na$_2$AsO$_3$, and 0.24 ml of $H_2$O, the samples were mixed well and the reaction was started by the addition of...
100 μl of 1 mM DTNB in 50 mM sodium acetate buffer, pH 5.0. The $A_{412}$ was measured; the reaction time, between 3 and 7 min, was identical for all samples of a test series. Oxidized glutathione (0 to 20 μmol) served as a standard.

Enzyme assays. $F_{420}H_2$ dehydrogenase activity was assayed under anaerobic conditions with a mixture of methylovioligen (MV) and metronidazole as artificial electron acceptors as previously described (14). $F_{420}H_2$:CoM-S-S-HTP oxidoreductase activity was determined in the same way except that the artificial electron acceptors were replaced by CoM-S-S-HTP (final concentration of 90 μM). The benzylviologen-dependent heterodisulfide reductase activity was monitored under anaerobic conditions by the method of Deppenmeier et al. (5) except that N$_2$ gassed 40 mM potassium phosphate buffer, pH 6.6, containing 0.4 M sucrose was used instead of Tris HCl buffer. Whenever p-chloromercuri-riphenylsulfonate (pCMPS) was used as an inhibitor in an enzyme assay, DTE was replaced by 0.1 mM Ti(III)-citrate. $F_{420}$-nonreactive hydrogenase activity was tested as previously described (8), except that the Tris HCl buffer was replaced by the buffer used for preparing inverted vesicles.

Chemicals and gases. Chemicals and gases were of analytical grade. Diquat was a generous gift from the Deutsche ICI GmbH, Frankfurt, Germany.

RESULTS

Cytochromes b and c in Methanosarcina strain Gö1. Methanol-grown cells of Methanosarcina strain Gö1 were fractionated into membranes and cytoplasm and each fraction was tested for the presence of cytochromes. The redox difference spectrum of the membrane fraction showed a typical cytochrome spectrum with α, β, and γ (or Soret) peaks at 556, 514, and 434 nm, respectively, indicating the presence of both b- and c-type cytochromes. Cytoplasmic cytochromes were not detectable in Methanosarcina strain Gö1, and according to the criteria given in references 1, 2, 36, and 42 there were no indications for a periplasmic location. Pyridine hemochrome redox difference spectra taken after acetone-HCl extraction of the membranes revealed the presence of 0.21 to 0.24 μmol of protoheme IX per g of membrane protein and 0.09 to 0.28 μmol of heme c per g of membrane protein. The presence of both b- and c-type cytochromes was furthermore supported by low-temperature redox difference spectra by using the devitrification technique of Estabrook (12). The α peak, in its fine structure, consisted of two peaks at 552 and 559 nm and two shoulders at 547 and 564 nm (Fig. 1A). The second derivative spectrum (Fig. 1B) confirmed the presence of four maxima in the original spectrum by four minima at the appropriate wavelengths indicating the presence of four cytochromes. However, it has to be kept in mind that cytochromes with split α bands have been described (12, 15). One or several cytochromes of Methanosarcina strain Gö1 liganded CO, resulting in a typical CO binding spectrum (not shown). The binding of CO by cytochromes from methanogens was reported previously (27, 29). To verify the presence of four cytochrome species ($c_{547}, c_{552}, b_{559},$ and $b_{564}$), we performed redox titrations in the oxidative and reductive directions. Both ways yielded the same values. The midpoint potentials were $E_{m,7} = -135 ± 5$ mV (β-type cytochromes) and $E_{m,10} = -140 ± 10$ mV (c-type cytochromes). No cytochromes were detectable between −50 and +400 mV.

Redox reactions of $F_{420}H_2$ and CoM-S-S-HTP with cytochromes. It was now of interest to gain information on the physiological role of these electron carriers in the methanogenic metabolism. For this purpose, typical methanogenic electron donors and acceptors were tested for their ability to interact with the cytochromes in vesicles of Methanosarcina strain Gö1. The addition of 17 μM $F_{420}H_2$ (final concentration) to anaerobic washed vesicles of Methanosarcina strain Gö1 resulted in an increase of the ratio of the reduced cytochromes from 6 to 60%, as is apparent from Fig. 2. The kinetics of the cytochrome reduction was too rapid to be resolved with the method employed. As a control, buffer was treated in the same way as $F_{420}$ for its reduction to $F_{420}H_2$. The addition of this buffer did not affect the redox state of the cytochromes. The heterodisulfide (13 μM final concentration), which serves as the physiological electron acceptor for the $F_{420}H_2$:CoM-S-S-HTP oxidoreductase system (7), led to a rapid oxidation of the reduced cytochromes as evident from a decrease in the reduction level from 77 to 36% (Fig. 2). A control which received aerobic water did not induce this effect. $F_{420}$, NAD$,^+$, NADP$,^+$, and HTP-S-S-HTP could not oxidize the cytochromes, and CoM-SH, CoM-SH + HTP-SH, NADH, and NADPH were unsuitable as reductants for cytochromes. Under the conditions employed, H$_2$ supported no or only a slow reduction of the cytochromes. This was due to a limitation in hydrogenase activity, for the buffer used (N$_2$-gassed, 0.5 mM DTE) was not sufficiently

![Figure 1](http://jb.asm.org/)
various was Methanosarcina strain cytochrome oxidation. To be assumed can disulfide, spectra sary for the other hand on the solid arrow, 13 nmol of CoM-S-S-HP was added. Volume was 1 ml; protein content was 2.9 mg/ml. (C) Kinetics of the cytochrome reduction by $F_{430}H_2$. At the time indicated by the open arrow, 17 nmol of $F_{430}H_2$ was added. Volume was 1 ml; protein content was 1.45 mg/ml.

reduced to restore full hydrogenase activity. Applying the reactivation conditions described (8) led on one hand to a complete restoration of the hydrogenase activity, but caused on the other hand a complete reduction of the cytochromes, preventing the investigation of the H2-dependent cytochrome reduction. Therefore, the DTE concentration of the test buffer was increased from 0.5 to 2 mM. This led to a partial reactivation of the hydrogenase (42.7 nmol of MV reduced per min per mg of protein) without reducing the cytochromes beyond a level of 44%. Nevertheless, at 6 to 600 μM dissolved H2, the rate of H2-dependent cytochrome reduction was less than 0.1% of the rate of H2-dependent MV reduction, suggesting that the flow of electrons from hydrogenase to the cytochromes was indirect and therefore unphysiological.

A number of electron transport inhibitors were tested for their effect on the $F_{430}H_2$ or CoM-S-S-HP-mediated reduction or oxidation of cytochromes. Antimycin A (90 μM), 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) (5 μM), rotenone (0.6 mM), myxothiazol (16 μM), or KCN (0.5 to 20 mM) did not affect these reactions. The effect of CO could not be judged because of a shift in the absorbance spectrum caused by the binding of CO. The only effective inhibitor was pCMPS (0.5 mM), which prevented the reduction or oxidation of the cytochromes by $F_{430}H_2$ or the heterodisulfide. Since pCMPS reacts with reduced sulfur compounds, it can be assumed that thiol groups or FeS centers are necessary for the observed reactions.

Low-temperature spectra of CoM-S-S-HP-induced cytochrome oxidation. To clarify the oxidation sequence of the various cytochrome species upon the addition of the heterodisulfide, spectra were taken at 77 K. The redox difference spectrum in Fig. 3A shows two peaks and two shoulders after the addition of 1 to 2 μl of 20 mM Na2S2O4 and accordingly four minima in the second derivative spectrum (Fig. 3B). Then, both the sample and the reference cuvette were thawed under N2, and 65 nmol of heterodisulfide was added in a volume of 1 μl to the sample cuvette. The cuvettes were devitrified again, and a spectrum (Fig. 3C) and its second derivative (Fig. 3D) were recorded. A change in the fine structure of the α band became evident. The peak at 559 nm in Fig. 3A appeared now as a shoulder, whereas the shoulder at 564 nm and accordingly the minimum at 564 nm in the second derivative spectrum had disappeared.
These observations suggest the oxidation of a b-type cytochrome by CoM-S-S-HTP. The further sequence of cytochrome oxidation could not be resolved. Neither O2 nor the substrate analog HTP-S-S-HTP was able to induce this effect, arguing in favor of the specificity of the reaction. pCMPS was an effective inhibitor of the reaction, as was expected from the experiments described above.

**Study of F420H2:CoM-S-S-HTP oxidoreductase with inhibitors.** Various inhibitors were tested for their ability to inhibit the F420H2:CoM-S-S-HTP oxidoreductase and the partial activities of this enzyme system (F420H2 dehydrogenase and heterodisulfide reductase). Cyanide (6 mM), propylidione (30 mM), and several specific electron transport inhibitors such as antimycin A (90 μM), myxothiazol (7 μM), HQNO (5 μM), and rotenone (0.6 mM) did not affect the F420H2:CoM-S-S-HTP oxidoreductase reaction at all. In contrast, sodium azide (10 mM), carbon monoxide (saturated) and pCMPS (0.5 mM) inhibited this reaction 50, 66, and 100%, respectively, but their site of action could be attributed to the F420H2 dehydrogenase. Similarly, the inhibition of the F420H2:CoM-S-S-HTP oxidoreductase by pyridine (120 mM; 82%) and hydroxylamine hydrochloride (16 mM; 20%) was attributable to the inhibition of the heterodisulfide reductase. Phenanthroline disulfonate (4.2 mM) inhibited both F420H2 dehydrogenase and heterodisulfide reductase completely.

N3-, CO3-, pyridine, and NO (resulting from NH3OH·Cl-) are known to specifically inhibit metal-containing proteins. Pyridine inhibits cytochrome-containing proteins, whereas phenanthroline disulfonate chelates iron and inhibits a wide range of iron-containing proteins. The inhibitory effect of pCMPS indicates the importance of reduced sulfur compounds for the reactions tested. Especially interesting was the effect of CO because of its ability to bind to the cytochromes. The inhibition of the F420H2 dehydrogenase by CO was additionally demonstrated with the purified enzyme (not shown). It is obvious that none of the inhibitors tested affected only the entire reaction without inhibiting the partial activities. Therefore, no conclusions as to whether cytochromes participate in the electron transfer between F420H2 and CoM-S-S-HTP can be drawn from these experiments. However, the results presented are in accordance with the fact that both F420H2 dehydrogenase and heterodisulfide reductase are FeS proteins (14, 19).

**DISCUSSION**

The spectrophotometric data presented in this paper are in accordance with the presence of two b- and two c-type cytochromes in *Methanosarcina* strain G01. Since this organism belongs to the family *Methanosarcinaceae* and is capable of utilizing methanol and acetate as substrates (unpublished data), this result is not unexpected. There have been several other reports on cytochromes in methanogens since the first description of cytochromes in this group of organisms by Kühn et al. (29). A common feature of the cytochromes from methanogens is their ability to bind CO. Analysis of a CO difference spectrum of the membranes from *Methanosarcina* strain G01 (not shown) according to the criteria of Wood (40) suggested the presence of a low-spin-state cytochrome(s). The midpoint potentials of the cytochromes from various methanogens vary somewhat: −325 mV and −138 mV are the Em7 values published for the b-type cytochromes of *M. vaucluata* Zhilina grown on methanol, and similar values were also reported for methanol-grown *M.arkeri*. The latter organism contained an additional cytochrome b with an Em7 of −250 mV after growth on acetate (30). In *Methanobalbus tindarius*, two b-type cytochromes with midpoint potentials of −90 mV and −290 mV and one c-type cytochrome with an Em7 of −190 mV could be distinguished (27). The E′m7 values determined here for the cytochromes of *Methanosarcina* strain G01 (b type: E′m7 = −135 mV and −240 mV; c type: E′m7 = −140 and −230 mV) are well within this range. The observation of one b- and one c-type cytochrome with almost identical midpoint potentials is remarkable.

The most interesting question regarding the cytochromes aims at their function. Since metabolically versatile organisms such as *M.arkeri* produce cytochromes in similar amounts under methlyloxytrphic, acetotrophic, and hydrogenotrophic growth conditions (30), it cannot be ruled out that these electron carriers participate in electron transport under all growth conditions, i.e., also during growth on H2-CO. Accordingly, Kenmer et al. (25) observed an H2-dependent reduction and a methyl-CoM-dependent oxidation of the cytochromes of acetate-grown *M.arkeri*. This observation was taken to indicate the involvement of cytochromes in methanogenesis from H2 and methyl-CoM. However, our observation that the H2-dependent cytochrome reduction proceeded at a much lower rate than the F420H2-dependent one suggested that the reduction of the cytochromes was indirect and unphysiological. In contrast, the F420H2-dependent reaction was so rapid that it could not be kinetically resolved with the method employed (Fig. 2). Similarly rapid was the reoxidation of the cytochromes by the heterodisulfide. On the basis of these kinetics we suggest one or several cytochromes to participate in the electron transport of the F420H2:CoM-S-S-HTP oxidoreductase system of *Methanosarcina* strain G01. Spectroscopic experiments performed at low temperature indicate the participation of a cytochrome b564 in this process. This membrane-bound energy-conserving system was proposed to play an essential role in methylotrophic organisms during methyl group oxidation. The electrons gained by methyl group oxidation are mainly transferred to F420H2. The F420H2 thereby produced is subsequently oxidized by the F420H2:CoM-S-S-HTP oxidoreductase.

The participation of cytochromes in the F420H2-dependent heterodisulfide reduction is furthermore supported by their midpoint potentials. The midpoint potential of the F420H2: F420H2 redox couple is sufficiently negative (−340 mV to −350 mV [23]) to act as an effective reductant for all cytochromes. The midpoint potential of the heterodisulfide has not yet been experimentally determined. However, since most other disulfides exhibit redox potentials of about −200 mV (24), the E′m7 value of the CoM-S-S-HTP/CoM-SH + HTP-SH couple can be expected to be in the same range (17, 18). Hence, the low potential cytochromes (E′m7 = −240 and −230 mV) could easily transfer electrons to the heterodisulfide. The two cytochromes with the more positive redox potentials (E′m7 = −135 and −140 mV), on the other hand, will only be in the oxidized state at a high [CoM-S-S-HTP]/[CoM-SH] [HTP-SH] ratio.

In inverted vesicles of *Methanosarcina* strain G01 the F420H2:CoM-S-S-HTP oxidoreductase was shown to catalyze proton translocation in response to electron transport (7). On the basis of the involvement of cytochromes in this redox reaction, their direct participation in proton translocation deserves further consideration. A Q-cycle type of proton translation appears unlikely because of the lack of quinones in methanogens. A hydrogen-cycling mechanism, as proposed for certain sulfate-reducing bacteria (33), is even less likely because several methylotrophic methanogens.
such as Methanobacterium tindarius and Methanococcoides methylutens are devoid of hydrogenase.

A number of chemical agents (myxothiazol, HQNO, antimycin A, and rotenone) which are specific inhibitors of cytochrome-linked electron transport chains did not affect the F$_{420}$H$_2$-dependent heterodisulfide reduction. This is not surprising since these inhibitors are mainly active in aerobic systems. On the other hand, there are also anaerobic electron transport systems such as those of Desulfovibrio gigas or Wolinella succinogenes that are sensitive to antimycin A and HQNO or 2-nonyl-4-hydroxyquinoline-N-oxide (16, 26). The insensitivity of the F$_{420}$H$_2$-dependent electron transport to CoM-S-S-HP could be an indication for an entirely different organization of the electron transport system in methanogens.

So far we have only considered a role of cytochromes in F$_{420}$H$_2$ oxidation. Since obligately acetotrophic methanogens also possess cytochromes, they must fulfill a different function in this group of organisms. This view is supported by the fact that the in vitro methanogenesis from acetate in M. barkeri is not dependent on F$_{420}$ (13) and that the F$_{420}$ level is lower in acetate-grown cells than in cells grown on other substrates (20). So it has to be assumed that the F$_{420}$H$_2$:CoM-S-S-HTP oxidoreductase is not of importance during methanogenesis from acetate. Experiments by Terlesky and Ferry (38) suggest a linkage of a cytochrome b with hydrogenase in acetate-grown M. thermophila. Future experiments will be needed to clarify the exact role of cytochromes during methanogenesis from acetate.

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