Generation of formate by the formyltransferase/hydrolase complex (Fhc) from *Methylobacterium extorquens* AM1

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Abstract ***Methylobacterium extorquens*** **AM1 possesses a formyltransferase (Ftr) complex that is essential for growth in the presence of methanol and involved in formaldehyde oxidation to CO_2. One of the subunits of the complex carries the catalytic site for transfer of the formyl group from tetrahydromethanopterin to methanofuran (MFR). We now found via nuclear magnetic resonance-based studies that the Ftr complex also catalyzes the hydrolysis of formyl-MFR and generates formate. The enzyme was therefore renamed Ftr/hydrolase complex (Fhc). FhcA shares a sequence pattern with amidohydrolases and is assumed to be the catalytic site where the hydrolysis takes place.**

Key words: Methylotrophic bacterium; Tetrahydromethanopterin; Methanofuran; ^{1}H-nuclear magnetic resonance; Amidohydrolase

1. Introduction

Methylobacterium extorquens AM1 is an aerobic α-proteobacterium capable of growth in the presence of methanol. The central metabolism of this bacterium involves the oxidation of the central intermediate formaldehyde by means of a series of cofactor-dependent enzymes (Figs. 1 and 2) that partly show sequence identity to enzymes involved in methanogenesis. The encoding genes for these enzymes in *M. extorquens* AM1 are essential for growth in the presence of methanol and are located in a single cluster on the chromosome [1]. Among these are the genes encoding the formyltransferase (Ftr) complex that has recently been purified [2]. The 32-kDa subunit of the Ftr complex catalyzes the reversible transfer of the formyl group from N^5-formyl-H_4MPT to methanofuran (MFR). This subunit exhibits sequence identity of about 40% with Ftr enzymes from methanogenic and sulfate-reducing archaea. In contrast to Ftrs from these archaea, the enzyme from *M. extorquens* AM1 forms a complex with three other polypeptides that show sequence identities with the subunits of formyl-MFR dehydrogenase FmdA, FmdB and FmdC [2]. Despite the similarity with FmdABC, the Ftr complex from *M. extorquens* AM1 was not found to catalyze the oxidation of formyl-MFR, and apparently does not possess a molybdopterin-binding site that is present in formyl-MFR dehydrogenases from archaea [2]. The differences found in catalytic and molecular properties prompted us to investigate the hypothesis that the formyl group bound to MFR is not oxidized directly to carbon dioxide but instead hydrolyzed to formate, which then may be oxidized to carbon dioxide by a formate dehydrogenase (Fig. 1). We confirm this hypothesis here by directly demonstrating, via nuclear magnetic resonance (NMR) spectroscopic analysis, that formate is formed from formyl-MFR by the Ftr complex, which therefore is renamed the Ftr/hydrolase complex (Fhc).

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Abbreviations: Ftr, formyltransferase; Fhc, Ftr/hydrolase complex; H_4MPT, tetrahydromethanopterin; H_4F, tetrahydrofolate; MFR, methanofuran; Mch, methenyl-H_4MPT cyclohydrolase

2. Materials and methods

[^{13}C]Formaldehyde was from Aldrich. Tetrahydromethanopterin (H_4MPT) and MFR were purified from *Methanothermobacter marburgensis* [3]. Methenyl-H_4MPT was generated as described by [4]. Formyl-MFR was synthesized from MFR and 4-nitrophenylformate [5]. H_2-forming methylene-H_4MPT dehydrogenase was purified from *M. marburgensis* [6]. Methenyl-H_4MPT cyclohydrolase (Mch) and the Ftr complex from *M. extorquens* AM1 were assayed and purified as described [2,7]. Ftr activity was measured by production of H_4MPT, detected by its spontaneous reduction of cytochrome *c* at 550 nm ($\Delta\varepsilon = 21$ mM^{-1} cm^{-1}) [2] or by measuring the increase in absorbance at 282 nm ($\Delta\varepsilon = 5.1$ mM^{-1} cm^{-1}) [5].

2.1. Preparation of [^{13}C]methenyl-H_4MPT$^+$

[^{13}C]Methylene-H_4MPT was prepared by spontaneous reaction of H_4MPT with [^{13}C]formaldehyde [4]. [^{13}C]Methenyl-H_4MPT$^+$ was generated from [^{13}C]methylene-H_4MPT at pH 6.0 by dehydrogenation. The reaction was catalyzed by hydrogen-forming methylene-H_4MPT dehydrogenase [6]. After completion of the reaction, the enzyme was removed by ultracentrifugation using a 30 kDa microconcentrator (Millipore). The compound was purified by reversed-phase high-performance liquid chromatography on LiChrospher 100RP-18 (4 mm × 125 mm, 5 μm, Merck) in 20 mM potassium phosphate buffer, pH 7.0.

2.2. Substrate and product analysis via ^{1}H-NMR spectroscopy

The 0.5 ml direct assay for the determination of formate from formyl-MFR contained 1 mM formyl-MFR in 5 mM Tricine/KOH pH 8.0, 250 mM NaCl, purified Ftr complex (corresponding to 18 mU of formyl-MFR:H_4MPT Ftr[5]), and 10% ^2H_2O. The 0.5 ml coupled assay contained 5 mM Tricine/KOH pH 8.0, 250 mM NaCl, 450 μM [^{13}C]methenyl-H_4MPT$^+$, Mch (3 mU), 0.9 mM MFR, Ftr complex (12 mU Ftr activity, see above), and 10% ^2H_2O. The reaction was started by the addition of protein and incubated at 35°C for 30 min. For a control experiment, Ftr from *Methanosarcina barkeri* [8] (12 mU) was added instead of the Ftr complex of *M. extorquens* AM1.

The reactions were analyzed by ^{1}H-NMR spectroscopy (direct as-

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say). NMR spectra were recorded at 298 K with an Avance DMX500 spectrometer (Bruker). Solvent suppressed spectra were acquired with 64 scans into 16k data points across a spectral width of 6009.5 Hz using a relaxation delay of 1 s. Line broadening apodization function of 0.30 Hz was applied to free induction decay prior to Fourier transformation.

3. Results

Ftr activity can be measured by production of H_4MPT, detected by its spontaneous reduction of cytochrome *c* [2]. The analysis of the stoichiometry of this assay revealed that only sub-stoichiometric amounts of MFR are required in comparison to formyl-H_4MPT. This finding indicated a recycling of the cofactor MFR in the test assay, which could be explained by the hydrolysis of the formyl-MFR formed during the reaction. To prove the hypothesis of the hydrolysis of formyl-MFR to formate by the Ftr complex we have chosen a NMR-based approach that enabled us to directly observe

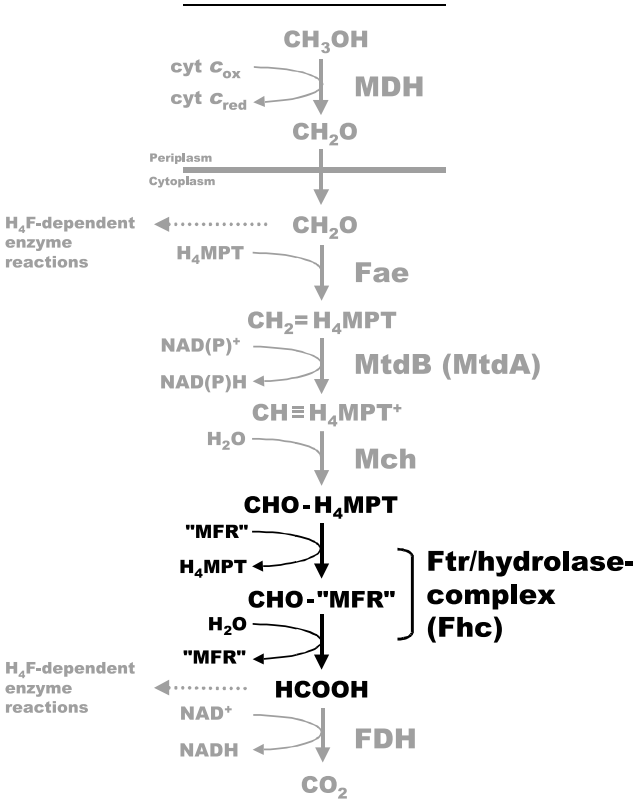

Fig. 1. Proposed enzymatic conversion of formaldehyde to formate by H_4MPT/'MFR'-dependent enzymes in *M. extorquens* AM1. Formaldehyde is produced from methanol by a pyrroloquinoline quinone-dependent methanol dehydrogenase, MDH, and crosses the membrane. Cytoplasmic formaldehyde conversion is initiated by its condensation with dephosphorylated tetrahydromethanopterin (H_4MPT), to methylene-H_4MPT, a reaction catalyzed by Fae [15]. Methylene-H_4MPT is then oxidized to methenyl-H_4MPT$^+$ by NAD(P)-dependent methylene-H_4MPT dehydrogenase (MtdB or MtdA) [18–20]. Hydrolysis of the methenyl-derivative by Mch results in the formation of N^5-formyl-H_4MPT [7]. The formyl group is then transferred to a postulated methanofuran (MFR) analogue, which has not yet been identified and is therefore labeled with quotation marks. The formation of formate by Fhc was shown in this study and is highlighted in black. Links to H_4F-bound C_1 intermediates are indicated by arrows (for a complete overview of the H_4F-dependent reactions see [15,18]). For structure of cofactors see Fig. 2.

the postulated formation of formate. In order to clarify the reading of the spectra, only the region of 6.0 to 9.0 ppm is shown reflecting all the relevant protons for the conversion observed. Furthermore, the protons characteristic of the substrates and products are labeled with arrows (Fig. 2) and indicated with arrows on the spectra (Figs. 3 and 4).

3.1. Demonstration by ^{1}H-NMR spectroscopy of the formation of formate from formyl-MFR catalyzed by the Ftr complex

Fig. 3a shows the spectrum of formyl-MFR. The expected one-proton singlet corresponding to the formyl group bound to the 2-(aminomethyl) group on the furan can be seen at 8.12 ppm (arrow a) and the furan protons in the positions 3 and 5 (6.44 and 7.58 ppm, arrows b) [9,10]. Besides, the spectrum shows a proton signal at 8.45 ppm due to the presence of formate in the sample, which resulted from the chemical synthesis of formyl-MFR from MFR and 4-nitrophenylformate (see Section 2) and an AB quartet from 6.95 to 7.25 ppm, assigned to the *para*-disubstituted benzene moiety. In Fig. 3b the ^{1}H-NMR spectrum is shown after the addition of Ftr complex from *M. extorquens* AM1 and incubation for 15 min at 35°C. The signal of the formyl group of formyl-MFR had completely disappeared and the signals of the furan protons were shifted downfield to 6.67 and 7.67 ppm, respectively (arrows b′), as expected [10]. The formate signal at 8.45 ppm clearly increased (arrow a′). All these differences of the spectra shown in Fig. 3 show the postulated hydrolysis of formyl-MFR to formate and MFR by the Ftr complex.

3.2. Demonstration by ^{1}H-NMR spectroscopy of the formation of formate from [^{13}C]methenyl-H_4MPT catalyzed by the Ftr complex

To follow the entire reaction catalyzed by Fhc, the conversion of N^5-formyl-H_4MPT to formate, we used a ^{13}C-labeling approach. For technical reasons the assays were started with [14a-^{13}C]methenyl-H_4MPT$^+$ (see Section 2) and were thus coupled to the reaction of Mch (Fig. 1). Stoichiometric amounts of [14a-^{13}C]methenyl-H_4MPT$^+$ and MFR were incubated with Mch and Fhc at 35°C. The signals of interest in the resulting spectrum (Fig. 4a) are the doublet at 9.01 ppm (arrow c) with a ^{13}C-^{1}H heteronuclear scalar coupling constant of 212 Hz that corresponds to H14a and the four-protons AB quartet from 7.37 to 7.45 ppm of the para-disubstituted benzene moiety of the [14a-^{13}C]methenyl-H_4MPT$^+$ (arrow d). After 20 min of incubation at 35°C, the two signals of [14a-^{13}C]methenyl-H_4MPT$^+$ decreased (about 40%) with a concomitant appearance of a doublet centered at 8.45 ppm (arrows c′, Fig. 4b) with a ^{13}C-^{1}H heteronuclear scalar coupling constant of 195 Hz of the ^{13}C labeled formate and the AA′XX′ spin system at 6.85 and 7.15 ppm of the para-disubstituted benzene moiety of the H_4MPT (arrows d′, Fig. 4b). A longer incubation time (12 h) resulted in essentially complete conversion to ^{13}C formate and H_4MPT (arrows c′ and d′, Fig. 4c), indicating that the equilibrium is far on the side of product formation due to the hydrolytic step. Furthermore, no traces of carbonate and CO_2 were observed in the ^{13}C-NMR spectrum (data not shown).

The spectrum shown in Fig. 4b demonstrated diminishing amounts of the product methenyl-H_4MPT$^+$ and at the same time the upcoming products formate and H_4MPT while no intermediates of the catalyzed reaction such as formyl-H_4MPT and formyl-MFR were observed. This indicates that

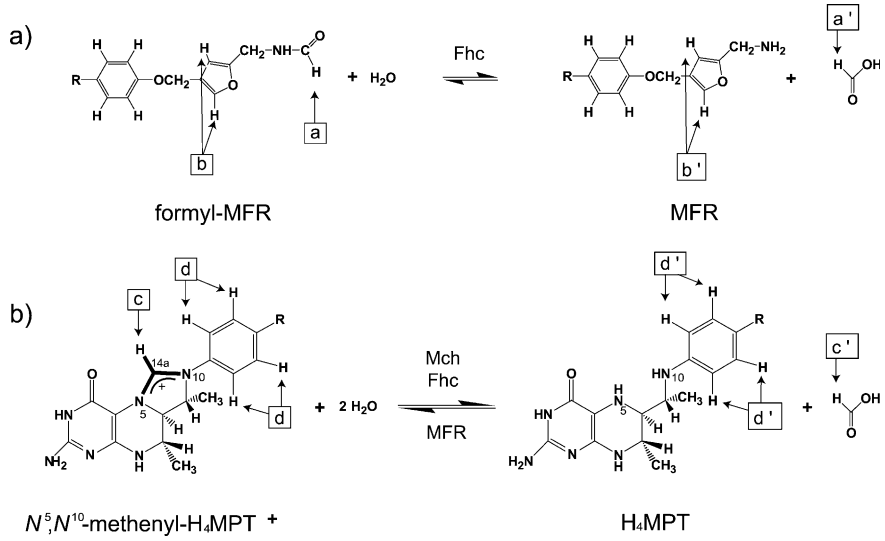

Fig. 2. Reactions studied in vitro by ^{1}H-NMR spectroscopy. a: Hydrolysis of the formyl group of formyl-MFR [9,10] to MFR and formate catalyzed by Fhc. b: Hydrolysis of N^5,N^{10}-methenyl-H_4MPT$^+$ to H_4MPT [21] and formate catalyzed by Mch and Fhc in the presence of MFR [10]. The protons on the substrates and products relevant for the NMR analysis are labeled with arrows.

the Mch reaction was the rate limiting step in the reaction sequence and that the hydrolytic reaction was faster than the formyltransferase reaction under the experimental conditions.

In a control assay we added monofunctional Ftr from the methanogenic archaeon *M. barkeri* (corresponding to 13 mU of activity of formyl-MFR:H_4MPT Ftr) instead of Fhc from *M. extorquens* AM1 to the assay. In this case, the formation of ^{13}C-formyl-MFR could be observed while no formate was formed (data not shown). Addition of Fhc resulted in ^{13}C-formate formation and complete disappearance of ^{13}C-formyl-MFR, as expected. These experiments thus suggest that the hydrolysis of formyl-MFR is catalyzed by the subunits FhcA,B,C (formerly Orf1,2,3 [1]).

3.3. *Sequence-based indication that subunit FhcA of the Fhc may bear a catalytic site for the hydrolysis of formyl-MFR*

Sequence analysis of FhcA (formerly Orf1 [1]), the largest subunit (60 kDa) of the complex, shows about 30% sequence identity with FmdA/FwdA subunits from methanogenic and sulfate-reducing archaea [1]. It also exhibits, within the first 60 N-terminal amino acid residues, sequence identity of about 40% to putative dihydroorotases and hydantoinases (dihydropyrimidinases) deduced from genomic sequences of various organisms. Holm and Sander [11] defined earlier a large superfamily of amidohydrolases that share a common signature pattern. The best-characterized members have a common $(\beta\alpha)_8$ barrel structure and a conserved metal binding site [11]. Members of this superfamily catalyze the hydrolysis of amide or amine bonds in different substrates. Due to the presence of the defined sequence pattern, the FmdA subunits from methanogenic archaea were found to be members of this superfamily [11]. Analysis of the α-subunit of Fhc from *M. extorquens* AM1 revealed that this protein also shares the common sequence signature for amidohydrolases, except for the His residue at position 292, which is replaced by the Asn residue. Asn instead of His is also present in FwdA of *Archaeoglobus fulgidus* and in the FhcA ortholog in the methanotrophic γ-proteobacterium *Methylococcus capsulatus* Bath [12], which was shown to possess the H_4MPT-dependent enzyme activities as well [13]. The presence of this conserved signature pattern in the sequence of FhcA led us to suggest that this polypeptide represents the active site for the hydrolysis of formyl-MFR.

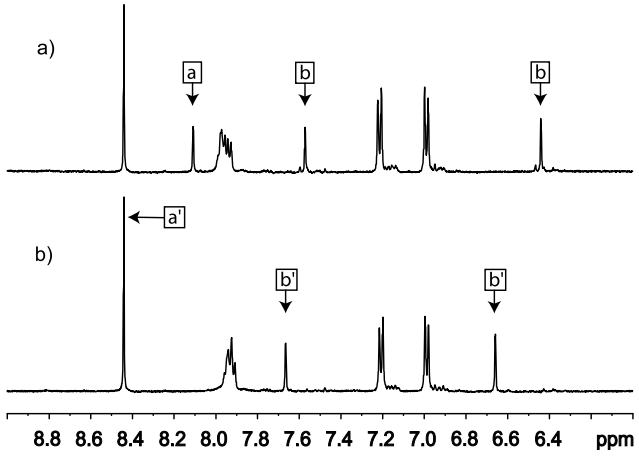

Fig. 3. ^{1}H-NMR spectra showing the conversion of the formyl group of formyl-MFR to formate by Fhc. a: The 0.5 ml test assay contained 11 mM formyl-MFR and 30 mM formate in 4 mM Tricine/KOH pH 8.0, 200 mM NaCl, and 10% ^2H_2O at 298 K. The spectrum shows the one-proton singlet at 8.12 ppm (arrow a) corresponding to the formyl group bound to the 2-(aminomethyl) group on the furan and the furan protons in the positions 3 and 5 (6.44 and 7.58 ppm, arrows b) [10]. The presence of formate is shown by the proton signal at 8.45 ppm. b: Spectrum after addition of Fhc (corresponding to 18 mU of activity of formyl-MFR:H_4MPT Ftr) and 15 min of incubation at 35°C. The signal of the formyl group of formyl-MFR had completely disappeared and the signals of the furan protons were shifted downfield to 6.67 and 7.67 ppm (arrows b′). The formate signal at 8.45 ppm had clearly increased (arrows a′). The spectra prove the conversion of the formyl group to formate. Only the region of 6.0 to 9.0 ppm is shown reflecting all the relevant protons for the conversion observed.

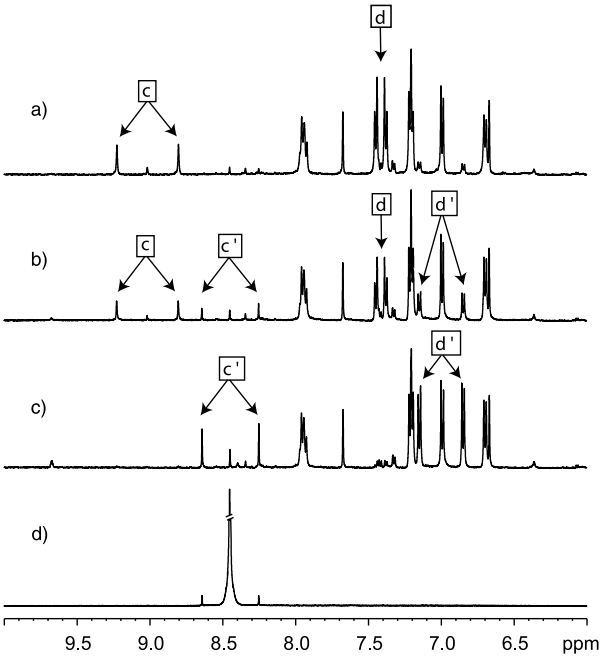

Fig. 4. ^{1}H-NMR spectra showing the conversion of [14a-^{13}C]methenyl-H_4MPT$^+$ to ^{13}C-formate by Mch and Fhc. The spectra show only the region of 6.0 to 10 ppm, reflecting all the relevant protons for the conversions observed. a: The 0.5 ml test assay contained 450 μM [14a-^{13}C]methenyl-H_4MPT$^+$ and 400 μM MFR in 4 mM Tricine/KOH pH 8.0, 200 mM NaCl, and 10% $^{2}H_2O$ at 298 K. The 14a protons of [14a-^{13}C]methenyl-H_4MPT$^+$ gave a chemical shift at 9.01 ppm (contamination by unlabeled precursor) and appeared mainly as a doublet at 9.22 and 8.80 ppm due to ^{1}J coupling to ^{13}C (arrows c). b: Spectrum after the addition of Mch (3 mU) and Fhc (corresponding to 12 mU of activity of formyl-MFR: H_4MPT Ftr) and 20 min of incubation at 35°C. About 40% of the [14a-^{13}C]methenyl-H_4MPT$^+$ was converted to ^{13}C formate (at 8.45 ppm (arrows c′), doublet at 8.64 and 8.25 ppm). The protons H_{2b}–H_{6b} and H_{3b}-H_{5b} of the disubstituted aromatic ring of methenyl-H_4MPT from 7.37 to 7.45 ppm (quartet, arrow d) declined while the H_{2b}–H_{6b} and H_{3b}–H_{5b} protons of H_4MPT appeared as a AA′XX′ spin system at 6.85 (doublet) and 7.15 ppm (doublet, arrows d′). c: Spectrum after 12 h of incubation. [14a-^{13}C]Methenyl-H_4MPT was quantitatively converted to ^{13}C formate and H_4MPT. d: Spectrum of 100 mM formate.

4. Discussion

It was shown that Fhc (formerly the Ftr complex) from *M. extorquens* AM1 catalyzes not only the transfer of the formyl group from N^5-formyl-H_4MPT to MFR but also the hydrolysis of formyl-MFR to formate. H_4MPT-dependent formaldehyde oxidation is presumed to be the main catabolic route for formaldehyde oxidation in *M. extorquens* AM1. Thus, generation of free formate as an intermediate of formaldehyde oxidation via Fhc is an essential component in the metabolism of this methylotrophic proteobacterium. Formate released by the complex may then be oxidized to CO_2. A tungsten-dependent formate dehydrogenase was recently purified and characterized from *M. extorquens* AM1 [14].

In this study we analyzed the reactions catalyzed by Fhc qualitatively rather than quantitatively. A comparison of in vitro rates of the reactions catalyzed by Fhc with other reactions involved in the H_4MPT/'MFR' pathway is difficult at this time since the authentic MFR analogue from *M. extorquens* AM1 is not yet available and is expected to have different kinetic properties than MFR from the archaeon *M. marburgensis* used in this study. Investigations are under way using the cytochrome *c*-dependent Ftr assay [2] to isolate the MFR analogue from *M. extorquens* AM1 to allow structural analysis. So far, we can only state with confidence that the hydrolysis of the formyl group of formyl MFR was not the rate limiting step in the reaction described in Section 3.2.

The release of formate by Fhc directly connects the H_4MPT-linked and the tetrahydrofolate (H_4F)-linked C_1 transfer pathway in this bacterium (Fig. 1). It is therefore conceivable that the pool of H_4F-bound intermediates which are essential to feed the assimilatory serine cycle and other biosynthetic reactions is replenished from formate. It is therefore an open question as to which extent the condensation of formaldehyde with H_4F (initiated by the spontaneous condensation of formaldehyde and H_4F), and the conversion via H_4MPT-dependent enzymes (initiated by the formaldehyde activating enzyme, Fae, [15]) through formate, respectively, contribute to the formation of C_1-bound H_4F derivatives.

Insights into the H_4MPT/MFR-dependent pathway of *M. extorquens* AM1 will also help to understand the metabolism of other methylotrophic bacteria. It appears very likely that other methylotrophic bacteria such as methanotrophic bacteria which exhibit H_4MPT-dependent enzyme activities [13] also possess the enzymatic hydrolytic step described in this study as an integral part of their metabolism. This is in agreement with in vivo labeling experiments which have shown that formate is a central intermediate upon oxidation of formaldehyde to CO_2 in *Methylosinus trichosporium* OB3b [16] and another type II methanotroph closely related to *Methylocystis parvus* [17].

During revision of the present manuscript, mutants defective in formate oxidation were generated in *M. extorquens* AM1, and these accumulated formate in growth medium during growth on methanol (L. Chistoserdova, M. Laukel, J.C. Portais, J.A. Vorholt and M.E. Lidstrom, unpublished results). This finding supports the conclusion presented in this report that formate is the main product of the reaction catalyzed by Fhc.

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