Function of a Glutathione-Dependent Formaldehyde Dehydrogenase in *Rhodobacter sphaeroides* Formaldehyde Oxidation and Assimilation[†]

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ABSTRACT: Despite its reactivity with many biological molecules, formaldehyde can be commonly encountered by virtually all cells. The widespread existence of glutathione-dependent formaldehyde dehydrogenases (GSH-FDH) in procaryotes and eucaryotes suggests this enzyme plays a central and universal role in biological formaldehyde oxidation. This work sought to determine the role of GSH-FDH in the facultative phototrophic bacterium *Rhodobacter sphaeroides*. Growth phenotypes of wild-type and mutant cells, changes in enzyme specific activities, and the pattern of ^{13}C-labeled compounds detected by NMR spectroscopy cumulatively suggest that *R. sphaeroides* GSH-FDH can play a critical role in formaldehyde metabolism under both photosynthetic and aerobic respiratory conditions. In photosynthetic cells, the data indicate that GSH-FDH generates reducing power, in the form of NADH, and one-carbon skeletons that are oxidized to carbon dioxide for subsequent assimilation by the Calvin−Benson−Bassham cycle. For example, use of methanol as a sole photosynthetic carbon source increases the specific activities of GSH-FDH, an NAD-dependent formate dehydrogenase, and the key Calvin−Benson−Bassham cycle enzyme, ribulose-1,5-bisphosphate carboxylase. This role of GSH-FDH is also supported by the pattern of [^{13}C]formaldehyde oxidation products that accumulate in photosynthetic cells and the inability of defined GSH-FDH or Calvin cycle mutants to use methanol as a sole carbon source. Our data also suggest that GSH-FDH acts in formaldehyde dissimilation when aerobic respiratory cultures cometabolize methanol and succinate.

Formaldehyde is a ubiquitous compound that a variety of organisms encounter from biological and environmental sources that include the photooxidation of atmospheric hydrocarbons (*1*, *2*), emissions from industrial products or processes (*3*), and the enzymatic oxidation of methylated compounds (*4*−*8*). The reactivity of formaldehyde with proteins, membranes, and DNA means that cells must efficiently remove this potentially lethal compound (*9*−*11*). Although a variety of routes for formaldehyde metabolism have been characterized (*12*−*14*), glutathione-dependent formaldehyde dehydrogenase (GSH-FDH;[1] *14*) is found in a large and diverse group of prokaryotes (*15*−*17*) and eukaryotes (*18*−*21*).

Members of the GSH-FDH family oxidize *S*-(hydroxymethyl)glutathione (HMGSH), an adduct formed spontaneously between formaldehyde and glutathione (*14*, *22*; eq 1), to *S*-formylglutathione (FGSH) with concomitant reduction of NAD (eq 2):

$$\mathrm{HCHO} + \mathrm{GSH} \xrightarrow{\text{spontaneous}} \mathrm{HMGSH} \qquad (1)$$

$$\mathrm{HMGSH} + \mathrm{NAD^+} \xrightarrow{\text{GSH-FDH}} \mathrm{FGSH} + \mathrm{NADH} \qquad (2)$$

In turn, FGSH is often hydrolyzed to formate ($HCOO^-$) and glutathione (GSH) by *S*-formylglutathione hydrolase (*23*, *24*; eq 3):

$$\mathrm{FGSH} + \mathrm{H_2O} \xrightarrow{\text{hydrolase}} \mathrm{HCOO^-} + \mathrm{GSH} \qquad (3)$$

Identification of both GSH-FDH and *S*-formylglutathione hydrolase in a wide variety of organisms has been taken as evidence that they play an important and conserved role in

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[1] Abbreviations: cfu, colony-forming units; CO_2, carbon dioxide; FDH, formate dehydrogenase; FGSH, *S*-formylglutathione; GSH, glutathione; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; HCHO, formaldehyde; $HCOO^-$, formate; HCO_3^-, bicarbonate; HMGSH, *S*-(hydroxymethyl)glutathione; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; 3-PGA, 3-phosphoglycerate; Rubisco, ribulose-1,5-bisphosphate carboxylase; RuDP, ribulose 1,5-diphosphate.

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biological formaldehyde oxidation (*14*). The properties of a few bacterial (*15*) or lower eucaryotic (*18*) GSH-FDH mutants support the concept that this enzyme functions in either formaldehyde oxidation or assimilation.

The identification of the *Rhodobacter sphaeroides adhI*−*cycI* operon (*16*) has prompted an investigation of the potential physiological role(s) of its gene products, GSH-FDH and isocytochrome c_2 respectively, in formaldehyde metabolism. In various yeasts (*25*), methanoarchae (*26*), and Gram-positive and Gram-negative eubacteria (*27*, *28*), formaldehyde is generated when either methylated compounds or methanol is used as a carbon or energy source. Depending on the organism and the availability of other carbon sources, formaldehyde can be assimilated by one of several pathways or dissimilated (*25*−*28*). In the following experiments, we took advantage of the fact that *R. sphaeroides* is a facultative phototroph and tested the role of GSH-FDH under photosynthetic (where formaldehyde is produced when methanol serves as a sole carbon source; *29*, *30*) and aerobic respiratory conditions (where methanol can be cometabolized if another carbon source is provided; *29*).

Our data suggest that GSH-FDH serves a central role in formaldehyde assimilation by photosynthetic cells and its dissimilation under aerobic respiratory conditions. Changes in enzyme levels, the accumulation of formaldehyde oxidation products, and the growth phenotypes of wild-type and mutant cells suggest that a GSH-FDH-dependent pathway produces CO_2 that can be ultimately assimilated by the Calvin−Benson−Bassham cycle under photosynthetic conditions. Under aerobic respiratory conditions, GSH-FDH activity during cometabolism of formaldehyde appears to contribute to its dissimilation. From these results, we propose how GSH-FDH acts in formaldehyde dissimilation under aerobic conditions and its assimilation when photosynthetic cells generate all their carbon from methanol oxidation. In addition, we provide a working model for how isocytochrome c_2, the second product of the *adhI*−*cycI* operon (*16*), could function under these conditions.

EXPERIMENTAL PROCEDURES

Strains, Growth Media, and Genetic Techniques. *R. sphaeroides* cells were grown in Sistrom's succinate-based medium at 32 °C (*31*). To assess formaldehyde metabolism in *R. sphaeroides* under aerobic conditions, 100 mM methanol was added to this succinate-based minimal medium. Photosynthetic growth with 100 mM methanol as a sole carbon source used the same basal salts medium lacking succinate and amino acids, with the addition of 80 mM dimethyl sulfoxide (*32*). Mutants of wild-type strain 2.4.1 lacking either GSH-FDH (*adhI* gene product; *16*) or isocytochrome c_2 (*cycI* gene product; *33*), ADHI2 and CYCI1, respectively, were generated using previously described suicide plasmids as the source of mutant alleles. When necessary, *R. sphaeroides* cells were grown in medium supplemented with kanamycin (25 mg/mL) or spectinomycin (25 mg/mL).

Extract Preparation and Enzyme Assays. GSH-FDH and formate dehydrogenase specific activities were measured in crude cell extracts. Cells were harvested by centrifugation (5000*g* for 15 min at 4 °C), washed in 100 mM sodium phosphate buffer (pH 7.6) containing 5 mM EDTA (*34*), and stored at −20 °C until enzyme assays were performed. Thawed cells were suspended in 5 mL of the above buffer and lysed on ice using a Branson sonicator (3.5 min at 50% duty cycle). DNase was added to a final concentration of 5 mg/mL and the lysate was incubated on ice for 30 min. After cell debris was removed by centrifugation (10000*g* for 15 min), the supernatant was used as a crude cell extract. Protein concentrations were determined by a modification of the Folin−phenol method (*35*) with bovine serum albumin as a standard.

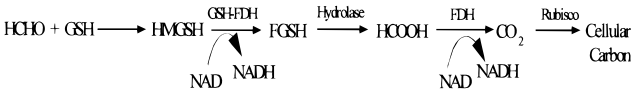
GSH-FDH activity was measured using HMGSH and NAD^+ as substrates (*20*). Formate dehydrogenase activity was measured under identical conditions except that formate (final concentration 1 mM) was used as a reductant. In both cases, enzyme activity was measured by the time- and reductant-dependent formation of NADH (increase in absorbance at 340 nm) at room temperature in an SLM DW2000 spectrophotometer. One unit of GSH-FDH or formate dehydrogenase activity is the amount of enzyme required to reduce 1 mmol of NAD^+/min.

Extracts were prepared for ribulose-1,5-bisphosphate carboxylase (Rubisco) enzyme assays using published procedures (*36*). One unit of Rubisco activity is the amount of enzyme required to fix 1 nmol of CO_2/min.

^{13}C NMR Spectroscopy. Cells were harvested by centrifugation (5000*g* for 15 min at 4 °C), washed once in 0.15 M phosphate buffer (pH 7.1), and suspended in the same buffer at $\sim 10^{11}$ cells/mL. Samples were prepared for ^{13}C NMR spectroscopy by mixing these concentrated cell suspensions (2.5 mL) and D_2O (0.3 mL). At time zero, [^{13}C]-formaldehyde or [^{13}C]formate was added to a final concentration of 10 mM. For whole-cell analysis of photosynthetic formaldehyde metabolism, concentrated cell suspensions were placed in 10 mm NMR tubes and degassed using a stream of N_2, and the tubes were sealed with rubber stoppers prior to addition of [^{13}C]formaldehyde with a syringe. Aerobic formaldehyde metabolism was analyzed by continuously bubbling concentrated cell suspensions with 69% N_2, 30% O_2, and 1% CO_2 in the presence of antifoam A spray (Dow Corning). In this case, aliquots were removed for NMR analysis at the indicated time points following the addition of ^{13}C-labeled compounds.

Decoupled ^{13}C NMR spectra were acquired at 100.6 MHz using a sweep width of 25252.525 Hz with a deuterium lock on a Bruker Instruments DMX-400 Avance console, 9.4 T wide-bore magnet, NMR spectrophotometer at National Magnetic Resonance Facility at University of Wisconsin—Madison. The pulse duration was 15 μs with a relaxation time of 5 s. The spectra were Fourier-transformed with 5 Hz of line broadening; data sizes were 16 384 and 32 768 before and after transformation, respectively. Peak assignments were aided by an internal reference provided by tetramethylsilane (TMS; 0 ppm) in a capillary present in the NMR tube. To assign peaks, control spectra were generated with either commercially available standards or synthetic material (data not shown). Control experiments showed that none of the presumed formaldehyde oxidation products were formed when [^{13}C]formaldehyde was added to either sterile culture medium or the phosphate buffer used to suspend cells for NMR spectroscopy (data not shown).

Table 1: Enzyme Specific Activities under Different Growth Conditions[a]



energy generation	carbon source(s)	GSH-FDH[b]	formate dehydrogenase[c]	Rubisco[d]
photosynthesis	succinate	47 (10)	0.3 (0.1)	7 (2)
	methanol	483 (95)	8.0 (1.5)	44 (10)
respiration	succinate	21 (4)	0.9 (0.2)	<0.1
	succinate + methanol	149 (30)	7.0 (1.5)	<0.1

[a] Data are averages of assays of at least three independent cultures. Numbers in parentheses represent error between independent cultures. The presumed GSH-FDH-dependent formaldehyde oxidation and assimilation pathway is depicted above the data (see text). Abbreviations: HCHO, formaldehyde; GSH, glutathione; HMGSH, *S*-(hydroxymethyl)glutathione; *S*-formylGSH, *S*-formylglutathione; $HCOO^-$; formate; CO_2, carbon dioxide; FDH, formate dehydrogenase; NAD/NADH, pyridine nucleotide coenzymes. [b] GSH-FDH activity in micromoles of NAD reduced per minute per milligram protein. [c] Formate dehydrogenase activity in micromoles of NAD reduced per minute per milligram protein. [d] Rubisco activity in nanomoles of CO_2 fixed per minute per milligram protein.

RESULTS

Photosynthetic Formaldehyde Oxidation Can Generate Carbon Dioxide via a GSH-FDH-Dependent Pathway. To gain insight into the role of *R. sphaeroides* GSH-FDH, cells were grown using methanol or succinate as a sole photosynthetic carbon source. This was an excellent starting point to assess the role of GSH-FDH since previous work had implicated formaldehyde as a byproduct of photosynthetic methanol oxidation by related species of purple photosynthetic bacteria (*29*, *30*). Under the conditions we used, methanol utilization by photosynthetic cultures of *R. sphaeroides* was reasonably efficient since growth yields with 100 mM methanol as a sole carbon source [final density ~(4−8) × 10^8 cfu/mL] were only slightly lower that when the four-carbon organic acid succinate (35 mM) was the sole carbon source added to this medium [final density ~(2−3) × 10^9 cfu/mL]. The contribution of GSH-FDH to formaldehyde oxidation under these conditions was investigated by several independent means because other potential routes for one-carbon assimilation could exist in *R. sphaeroides* (*37*, *38*).

One indication that GSH-FDH was involved in formaldehyde oxidation was the ~10-fold increase in its specific activity in cells using methanol as a sole photosynthetic carbon source (Table 1). This level of GSH-FDH activity, ~483 μmol min^{-1} (mg of protein)$^{-1}$, meets or exceeds that of enzymes known to be involved in carbon assimilation by purple bacteria (see below). Previous experiments have shown that extracts from a defined GSH-FDH mutant lack detectable GSH-dependent formaldehyde oxidation (*16*). Thus, the GSH-FDH activity we measured in photosynthetic cells using succinate as a sole photosynthetic carbon source [~47 μmol min^{-1} (mg of protein)$^{-1}$] most likely represented basal *adhI* expression.

Of equal significance, the inability of a GSH-FDH mutant to use methanol as a sole photosynthetic carbon source (Table 2) demonstrated a key role for this enzyme under these conditions. The ability of a mutant lacking the other product of the *adhI*−*cycI* operon, isocytochrome c_2, to use methanol as a sole photosynthetic carbon source (Table 2) demonstrated that the behavior of the GSH-FDH mutant is not due to polarity of this mutation on downstream genes. Reasons why cells lacking isocytochrome c_2 can use methanol as a sole photosynthetic carbon source will be presented in the discussion.

Table 2: Growth of *R. sphaeroides* Strains

strain	relevant genotype	photosynthetic methanol utilization[a]	aerobic methanol resistance[b]	source
2.4.1	wild type	+	+	lab strain
ADHI2	2.4.1; Δ*adhI*::Ω*spc*	−	−	this work
CYCI1	2.4.1; Δ*cycI*::Ω*spc*	+	+	this work
CfxAB	2.4.1; ΔcfxA::*kan*, ΔcfxB::Ω*spc*	−	+	*32*

[a] Growth in medium containing 100 mM methanol and 0.625% (w/v) DMSO. [b] Growth in medium containing 34 mM succinate and 100 mM methanol.

To further analyze how formaldehyde or the products of GSH-FDH activity were metabolized during photosynthetic methanol utilization, similar extracts were assayed for enzymes known to participate in one-carbon metabolism in other microbes (*25*−*28*). The use of methanol as a sole photosynthetic carbon source increased the specific activity of an NAD-linked formate dehydrogenase ~15-fold [specific activity ~8 μmol min^{-1} (mg of protein)$^{-1}$] compared to phototrophic cells grown in a succinate-based medium (Table 1). In contrast, enzymes characteristic of other formaldehyde assimilatory pathways (hydroxypyruvate reductase, 5,10-methylenetetrahydrofolate reductase, serine transaminase) were either not detectable or present at levels several orders of magnitude below GSH-FDH or formate dehydrogenase when cells use methanol as a sole photosynthetic carbon source (data not shown). One interpretation of the relative amount and increases in GSH-FDH and formate dehydrogenase specific activities in methanol-grown cells is that both enzymes are important for generating reducing power in the form of NADH when this is the sole photosynthetic carbon source. If this were true, a significant fraction of the formaldehyde generated from photosynthetic methanol oxidation would be converted to FGSH by GSH-FDH, converted to formate by a *S*-FGSH hydrolase (*14*, *27*), and subsequently oxidized to carbon dioxide by formate dehydrogenase.

To examine this possibility, NMR spectroscopy was used to trace the metabolic fate of ^{13}C-labeled compounds added to concentrated suspensions of photosynthetic cells that were previously grown using methanol as a sole carbon source. To increase our chances of detecting products of formaldehyde oxidation, the substrate of GSH-FDH activity, [^{13}C]-formaldehyde, was added to concentrated cell suspensions.

Within 30 min after [^{13}C]formaldehyde was added (Figure 1, top), there was detectable accumulation of 5,10-methylenetetrahydrofolate (5,10-CH_2-THF; 66.6 ppm), *S*-(hydroxymethyl)glutathione (HMGSH; 65.2 ppm), and formate ($HCOO^-$; 171.1 ppm). The accumulation of formate soon after [^{13}C]formaldehyde was added was consistent with its sequential oxidation by GSH-FDH and formate dehydrogenase. Control experiments with crude extracts from analogous cultures are consistent with this proposition since the addition of glutathione is required for the accumulation of

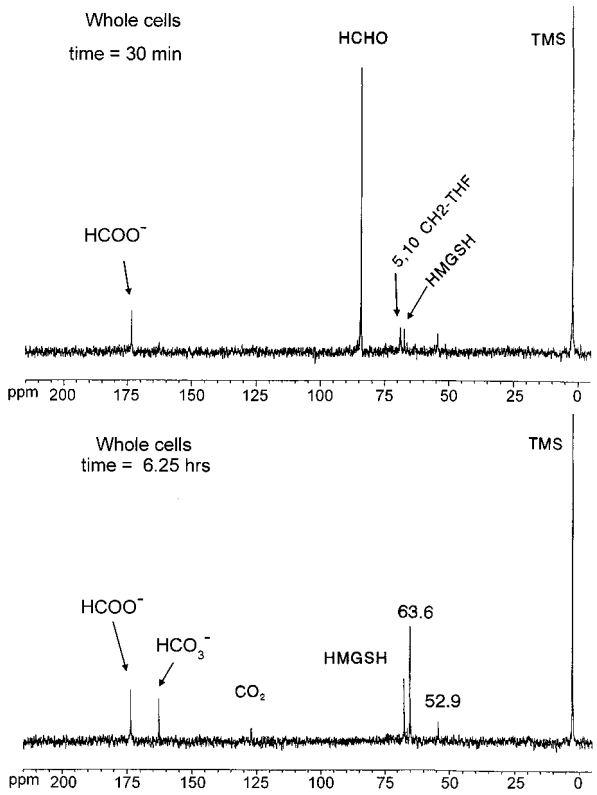


FIGURE 1: Metabolic fate of formaldehyde produced by methanol oxidation under photosynthetic conditions. Metabolism of ^{13}C-labeled formaldehyde was followed after its addition to concentrated suspensions of cells grown photosynthetically using methanol as a sole carbon source. (Top) spectra collected 30 min after the addition of ^{13}C-labeled formaldehyde; (bottom) spectra collected 6.25 h after the addition of ^{13}C-labeled formaldehyde. All assigned peaks were normalized using tetramethylsilane (TMS) as an internal standard. Abbreviations: HCHO, formaldehyde; $HCOO^-$, formate; HMGSH, *S*-(hydroxymethyl)glutathione; 5,10 CH_2-THF, 5,10−methylene-tetrahydrofolate; HCO_3^-, bicarbonate; CO_2, carbon dioxide.

these compounds from [^{13}C]formaldehyde (data not shown). Because the signal strength from ^{13}C-labeled atoms is not equivalent, we are unable to use relative peak areas to assess the fraction of the formaldehyde that resides in different compounds.

One additional compound (chemical shift of 52.9 ppm) was accumulated to detectable levels within 30 min after the addition of [^{13}C]formaldehyde to concentrated cell suspensions (Figure 1, top). NMR analysis of control compounds showed that this 52.9 ppm signal was not any of the common intermediates in central carbon metabolism we tested (acetate, pyruvate, oxaloacetate, glycerate, glycerol, glycerol 3-phosphate, plus many other amino acids and nucleosides) or a product of formaldehyde assimilation by other known pathways (serine, choline, betaine, etc.). For these reasons, we have been unable to assess the source, significance, and abundance of this compound relative to other products of formaldehyde metabolism (see below).

Using NMR spectroscopy, we were also able to monitor the time-dependent loss of formaldehyde and a change in the pattern of ^{13}C-labeled compounds accumulated by concentrated cell suspensions. Some 6.25 h after [^{13}C]-formaldehyde was added (Figure 1, bottom), the majority of it was depleted and the accumulated products included HMGSH, formate, bicarbonate (HCO_3^-; 160.2 ppm), carbon dioxide (CO_2; 124.7 ppm), the previously described 52.9 ppm species, and another unknown compound (63.6 ppm). The time-dependent accumulation of bicarbonate and carbon dioxide at the apparent expense of [^{13}C]formaldehyde was consistent with the coupled use of GSH-FDH and formate dehydrogenase in formaldehyde oxidation by these concentrated cell suspensions. Control experiments showed that the accumulation of formate from [^{13}C]formaldehyde was not seen using suspensions of photosynthetic cells grown in the presence of succinate (data not shown). This failure to detect significant metabolic conversion of formaldehyde in succinate-grown photosynthetic cultures presumably reflects the low levels of GSH-FDH and formate dehydrogenase in these cells (Table 1). In contrast, the GSH-FDH [483 μmol min^{-1} (mg of protein)$^{-1}$] and formate dehydrogenase [8 μmol min^{-1} mg protein) specific activities measured in photosynthetic cells grown with methanol as a sole carbon source (Table 1) exceed what would be needed for the $\sim 3 \times 10^{11}$ cells we used (∼45−50 mg of protein; *39*) to deplete the 30 μmol of [^{13}C]formaldehyde we added to these concentrated cell suspensions.

The Calvin Cycle Assimilates the Products of GSH-FDH Activity. To assess how the products of GSH-FDH activity might be assimilated, levels of characteristic enzyme activities (*12*) were also examined in cells using methanol as a sole photosynthetic carbon source. One possible route for assimilating formaldehyde oxidation products is the well-studied Calvin−Benson−Bassham cycle that is known to support autotrophic growth of purple photosynthetic bacteria (*37*, *38*). An indication that the Calvin−Benson−Bassham cycle could assimilate the combined products of GSH-FDH and formate dehydrogenase activity was the ∼7-fold increase in ribulose-1,5-bisphosphate carboxylase (Rubisco) specific activity observed in photosynthetic cells using methanol as a sole carbon source (Table 1). This level of Rubisco activity, ∼44 μmol min^{-1} (mg of protein)$^{-1}$, is only ∼2−3-fold lower than that present when *R. sphaeroides* uses CO_2 as a sole photosynthetic carbon source (*32*, *37*, *38*). The inability of a defined Calvin cycle mutant (strain CfxAB; *32*) to use methanol as a sole photosynthetic carbon source also supported the proposal that the Calvin−Benson−Bassham cycle was a significant contributor to assimilating the CO_2 produced under these conditions (Table 2).

By NMR spectroscopy, the expected accumulation of characteristic intermediates of the Calvin−Benson−Bassham cycle was not detected when either [^{13}C]formaldehyde (Figure 1, bottom) or bicarbonate (data not shown) was added to suspensions of cells grown with methanol as a sole photosynthetic carbon source. To independently confirm that methanol-grown photosynthetic cells were capable of CO_2 fixation, we demonstrated the ribulose-1,5-diphosphate-dependent accumulation of [^{13}C]bicarbonate into 3-phosphoglycerate (179.6 ppm; Figure 2) in crude extracts. If the Calvin−Benson−Bassham cycle was a significant contributor to assimilation of the products of GSH-FDH-dependent formaldehyde oxidation, our failure to see the accumulation of known pathway intermediates by NMR spectroscopy in concentrated cell suspensions could simply reflect their rapid turnover.

In considering whether other pathways might contribute significantly to assimilating formaldehyde or the products of GSH-FDH activity when photosynthetic cells use methanol as a sole carbon source, it is relevant to note that known

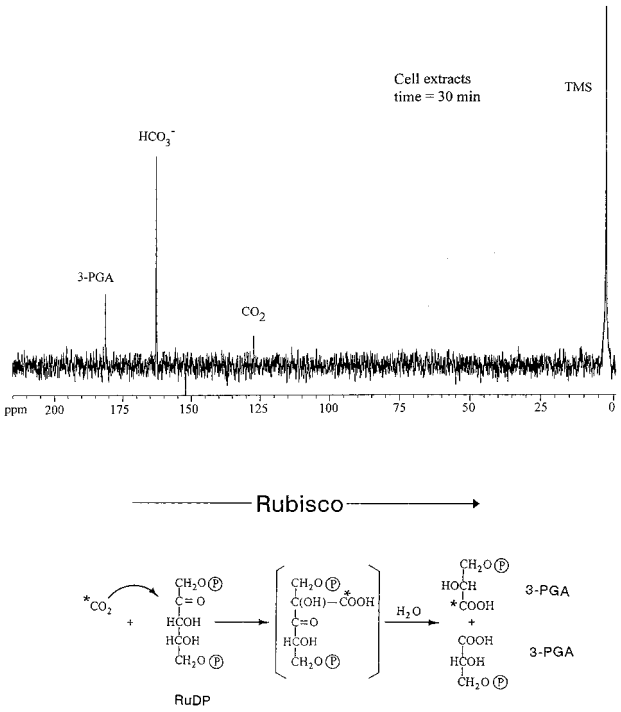


FIGURE 2: 3-Phosphoglycerate formation in methanol-grown cells under photosynthetic conditions. The fixation of carbon dioxide into Calvin−Benson−Bassham pathway intermediates was tested by adding ^{13}C-labeled bicarbonate (HCO_3^-) to extracts of *R. sphaeroides* cells grown photosynthetically on methanol. The formation of 3-phosphoglycerate (3-PGA) is observed following the addition of ribulose 1,5-bisphosphate. All assigned peaks were normalized using tetramethylsilane (TMS) as an internal control. The ribulose-1,5-bisphosphate carboxylase-(Rubisco-) catalyzed reaction is indicated at the bottom; the asterisk indicates the position in 1 mol of 3-PGA that is predicted to be labeled after Rubisco activity. Abbreviations: CO_2, carbon dioxide; RuDP, ribulose 1,5-bisphosphate.

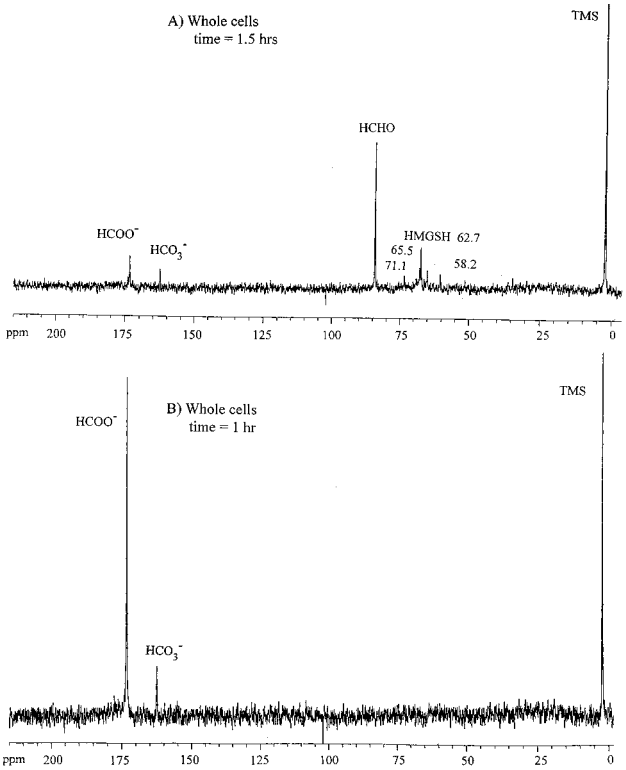


FIGURE 3: Metabolic fate of formaldehyde or formate during aerobic methanol oxidation. The metabolic fate of ^{13}C-labeled one carbon compounds as followed by NMR spectroscopy. (A) ^{13}C-Labeled formaldehyde was added to a concentrated suspension of aerobic cells grown in succinate-based minimal medium supplemented with 100 mM methanol. This spectrum was collected after 1.5 h of incubation. (B) ^{13}C-Labeled formate was added to a concentrated suspension of aerobic cells grown in succinate-based minimal medium supplemented with 100 mM methanol. This spectrum was collected after 1 h. All assigned peaks were normalized using tetramethylsilane (TMS) as an internal control. Abbreviations: HCHO, formaldehyde; $HCOO^-$, formate; HCO_3^-, bicarbonate; HMGSH, *S*-(hydroxymethyl)glutathione.

intermediates of alternative pathways like the serine cycle, the ribulose monophosphate cycle, or the xylulose monophosphate cycle were not detectable when [^{13}C]formaldehyde or [^{13}C]bicarbonate was added either to whole cells or to crude extracts (Figures 1 and 2). While the failure to detect significant levels of enzyme activities or accumulated intermediates cannot be taken as evidence against the function of other pathways, the combined enzymatic, spectroscopic, and genetic evidence suggests that GSH-FDH, subsequent enzymes like formate dehydrogenase, and activities in the Calvin−Benson−Bassham cycle are significant contributors to formaldehyde oxidation and assimilation when photosynthetic cells use methanol as a sole carbon source.

Products of GSH-FDH Activity Are Dissimilated under Aerobic Respiratory Conditions. *R. sphaeroides* and several other species of other purple photosynthetic bacteria are unable to use the formaldehyde generated from methanol oxidation as a sole carbon source under aerobic respiratory conditions (*29*). Several observations suggest that cells generate formaldehyde when methanol and succinate are cometabolized under aerobic respiratory conditions. First, wild-type cells grew aerobically when 100 mM methanol was added to succinate-based minimal medium. However, a GSH-FDH mutant was unable to grow aerobically when methanol was added to a succinate-based minimal medium (Table 2). The behavior of the GSH-FDH mutant implied that this enzyme was required for removal of either methanol, formaldehyde, or some other toxic byproduct under aerobic conditions (see Discussion).

Of equal significance, the levels of GSH-FDH [149 μmol min^{-1} (mg of protein)$^{-1}$] and formate dehydrogenase [7 μmol min^{-1} (mg of protein)$^{-1}$] during respiratory cometabolism of methanol and succinate are close to those measured in photosynthetic cells using methanol as a sole carbon source (483 and [8 μmol min^{-1} (mg of protein)$^{-1}$], respectively; Table 1). The magnitude of the increases in GSH-FDH (~7-fold) and formate dehydrogenase (~8-fold) specific activities seen when methanol was cometabolized with succinate are not surprising if they contribute significantly to formaldehyde oxidation under these conditions (Table 1).

To dissect the metabolic fate of formaldehyde in aerobic respiratory cells, NMR spectroscopy was used to monitor the pattern of accumulated intermediates. Two hours after [^{13}C]formaldehyde was added to concentrated suspensions of cells grown aerobically in a succinate-based minimal medium supplemented with methanol (Figure 3A), one observes the accumulation of HMGSH, formate, bicarbonate and several compounds whose identity we have been unable to determine by previously mentioned criteria (chemical shifts of 71.1, 65.5, 62.7, and 58.2 ppm). The accumulation of HMGSH, formate, and bicarbonate would be expected if GSH-FDH contributed to formaldehyde oxidation when

aerobic cells are grown in methanol-supplemented medium. Control experiments show that the products of combined GSH-FDH and formate dehydrogenase activity (i.e., formate and bicarbonate) were not accumulated to detectable levels when cell suspensions were analyzed that contained basal levels of these two enzymes (i.e., cells grown aerobically in succinate-based medium; data not shown).

Consistent with the above-mentioned role of GSH-FDH in aerobic formaldehyde dissimilation, we observed accumulation of bicarbonate when ^{13}C-labeled formate was added to concentrated cell suspensions of cells grown in methanol-supplemented medium (Figure 3B). In contrast to the situation when photosynthetic cells use methanol as a sole carbon source (see above), we were unable to see conversion of [^{13}C]bicarbonate to known Calvin−Benson−Bassham cycle intermediates using extracts from aerobic cells grown in methanol-supplemented medium (data not shown). One possible explanation for the apparent inability of aerobic cells that were grown in methanol-supplemented media to assimilate [^{13}C]bicarbonate was the lack of detectable Rubisco activity (Table 1). Dissimilation of formaldehyde under these conditions would also explain why Calvin cycle mutants grow when methanol is cometabolized by aerobic respiratory cultures (Table 2). When data from enzymatic, spectroscopic, and phenotypic assays are considered together, it appears that GSH-FDH activity aids the formation of formate and carbon dioxide as major products of formaldehyde oxidation when methanol is cometabolized by aerobic respiratory cultures.

DISCUSSION

It should not be surprising to find that GSH-FDH activity is widely conserved when one considers the multiple biotic and abiotic sources of a potentially lethal compound like formaldehyde. Of the various proteins capable of oxidizing formaldehyde, the widespread occurrence of GSH-FDH enzymes and their high activity with physiologically relevant substrates like HMGSH has been taken as evidence for their universal function in its biological detoxification or assimilation (*14*). Our data implicate GSH-FDH in generating reducing power in the form of NADH when formaldehyde is generated from methanol under both respiratory and photosynthetic conditions. It also suggests that the products of GSH-FDH activity can differ depending on whether this compound is the only available carbon source. The conclusions of our studies and their ramifications for future analysis of formaldehyde oxidation by GSH-FDH-dependent pathways in bacteria, microbes and higher eucaryotes are summarized below.

Role of GSH-FDH in Formaldehyde Assimilation. It can be difficult to decipher how GSH-FDH-dependent pathways oxidize exogenous formaldehyde since most cells are killed by levels of this compound needed for it to serve as a sole carbon source in batch culture (*40*). In this study, we capitalized on previous suggestions that purple phototrophs like *R. sphaeroides* generated formaldehyde when methanol was a sole photosynthetic carbon source (*29*, *30*). This allowed us to examine the role of GSH-FDH when formaldehyde is formed during cometabolism (respiration) or generated as a sole source of carbon (photosynthesis).

Several pieces of data suggest that GSH-FDH plays a central role in assimilating the formaldehyde produced when photosynthetic cells use methanol as a sole carbon source. The inability of GSH-FDH mutants to use methanol as a sole photosynthetic carbon source, the levels of GSH-FDH and other enzyme activities, and the conversion of ^{13}C-labeled compounds into formate and carbon dioxide suggest that these growth conditions lead to significant carbon flux through GSH-FDH and formate dehydrogenase. If this were the case, then GSH-FDH-dependent formaldehyde oxidation under photosynthetic conditions by *R. sphaeroides* uses enzymes, intermediates, and products similar to those employed by methanol-utilizing bacteria, lower eucaryotic microbes, plants, and animals (*12*, *41*−*43*).

Another simple interpretation of our data is that the Calvin−Benson−Bassham cycle plays an important role in assimilating the carbon dioxide generated from photosynthetic formaldehyde oxidation by the GSH-FDH-dependent pathway. For example, we showed that a defined Calvin cycle mutant is unable to use methanol as a sole photosynthetic carbon source. Methanol-grown cells also contain levels of Rubisco, a key enzyme of this pathway, which approximate those present under autotrophic conditions (*32*, *37*, *38*). In purple bacteria like *R. sphaeroides*, the Calvin−Benson−Bassham cycle is required for cells to use carbon dioxide as a sole carbon source under autotrophic conditions (*37*, *38*). However, the Calvin−Benson−Bassham cycle has an additional role in recycling reducing power under photosynthetic conditions in this bacterium (*32*, *37*, *38*). For example, the mutant we used (CfxAB) is unable to use a reduced carbon source (malate or butyrate) under photosynthetic conditions unless an external electron acceptor, such as dimethyl sulfoxide, is present (*32*, *37*, *38*). It appears that photosynthetic use of a reduced carbon source like methanol generates excess reductant since growth also requires the addition of an electron sink like dimethyl sulfoxide. While other routes for formaldehyde oxidation or carbon dioxide assimilation cannot be unambiguously ruled out at this time (*44*), our combined data suggest that the Calvin−Benson−Bassham cycle is a contributor to assimilating the products of GSH-FDH-dependent formaldehyde oxidation under photosynthetic conditions.

GSH-FDH Plays a Role in Aerobic Formaldehyde Dissimilation. When methanol is added to aerobic cultures growing in a succinate-based minimal medium, our data suggest that GSH-FDH is involved in formaldehyde dissimilation. One likely reason why the products of aerobic formaldehyde oxidation are not assimilated is the well-known inability of wild-type *R. sphaeroides* to transcribe genes for enzymes like Rubisco in the presence of oxygen (*45*).

Other Functions Required for Formaldehyde Oxidation by GSH-FDH Activity. Our working hypothesis predicts that *S*-formylglutathione hydrolase and formate dehydrogenase are also required for GSH-FDH to function when formaldehyde is produced during photosynthetic methanol utilization. However, these activities do not appear to be cotranscribed with the GSH-FDH structural gene (*adhI*) since previous studies identified only one downstream gene (*cycI*) that encodes a *c*-type cytochrome, isocytochrome c_2 (*16*). Now that tentative functions for GSH-FDH activity are emerging, it is logical to ask about the role of isocytochrome c_2 in formaldehyde metabolism. GSH-FDH (cytoplasm) and isocytochrome c_2 (periplasm) are found in different intracellular compartments of this Gram-negative bacterium (*16*),

so it seems unlikely that these two proteins are direct redox partners. However, most eubacteria use a periplasmic methanol dehydrogenase to generate formaldehyde from methanol oxidation (*46*). Thus, our working model considers isocytochrome c_2 as a mobile electron carrier from a presumed periplasmic methanol dehydrogenase to membrane-bound redox enzymes (*47*). We know isocytochrome c_2 can substitute for cytochrome c_2 in electron transfer between cytochrome bc_1 and reaction center complexes *in vivo* (*34*) and *in vitro* (*48*), so function of cytochrome c_2 (or some other mobile electron carrier; *39*, *47*) presumably explains why placing a *cycI* mutation in an otherwise wild-type strain does not abolish photosynthetic methanol utilization. In contrast, cells lacking both cytochrome c_2 and isocytochrome c_2 are unable to grow via photosynthesis even in succinate-based minimal medium (*33*). Thus, the failure of this mutant to use methanol as a sole photosynthetic carbon source most likely reflects a general inability to reduce light-oxidized reaction center complexes in the absence of these two periplasmic electron carriers.

Additional Biological Consequences of Formaldehyde Oxidation. The widespread occurrence of GSH-FDH in nonautotrophic organisms suggests that it also has another important and conserved biological function. A role of *R. sphaeroides* GSH-FDH in preventing the accumulation of toxic levels of formaldehyde is illustrated by the observation that methanol kills cells lacking this enzyme. It appears that methanol sensitivity of a GSH-FDH mutant reflects formaldehyde toxicity since *R. sphaeroides* strains that cannot oxidize methanol are viable under these conditions (*49*). When one considers that formaldehyde induces transcription of the GSH-FDH gene (Barber and Donohue, unpublished results), it seems likely that the basal levels of this enzyme in succinate-based minimal medium prevent the accumulation of this reactive and toxic compound in wild-type cells. Unfortunately, the sensitivity of the GSH-FDH mutant to formaldehyde and the levels of this compound needed to trace metabolism by NMR spectroscopy precluded us from testing if this toxin was metabolized by other routes in cells lacking this enzyme. In addition, the inability of wild-type cells to utilize formate as a sole carbon and energy source under photosynthetic conditions (*50*) prevented us from asking if a GSH-FDH mutant was able to use one-carbon compounds, which are predicted to enter the pathway downstream of this enzyme.

The requirement for an external electron acceptor to support growth during use of methanol as a sole photosynthetic carbon source also illustrates that considerable carbon flux through GSH-FDH can alter other metabolic functions. When one considers the substrates and products of our proposed pathway for formaldehyde oxidation, high carbon flux through GSH-FDH could potentially alter one-carbon availability, the redox state of the pyridine nucleotide pool, and the equilibrium between oxidized and reduced GSH.

In summary, we have described how GSH-FDH functions in formaldehyde oxidation and assimilation by the facultative phototroph *R. sphaeroides.* Our data indicate that the most likely role of GSH-FDH during photosynthetic methanol utilization is formaldehyde oxidation via a scheme similar to that used by several aerobic microbes (*12*, *25*). Under aerobic respiratory conditions, *R. sphaeroides* GSH-FDH appears to generate reducing power in the form of NADH during formaldehyde dissimilation. If one considers that GSH-FDH enzymes are commonly found in other nonautotrophs, it seems likely that aerobic formaldehyde dissimilation by *R. sphaeroides* is reminiscent of what occurs in a wide variety of bacterial and eukaryotic cells. In the future, it will be interesting to see if the wide distribution of GSH-FDH reflects their common role in formaldehyde dissimilation, to identify other gene products required to remove this potentially lethal compound by a GSH-FDH-dependent route, and to see if this enzyme plays a similar role during metabolism of other methylated compounds that generate formaldehyde.

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