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A Glutathione-dependent Formaldehyde-activating Enzyme (Gfa) from *Paracoccus denitrificans* Detected and Purified via Two-dimensional Proton Exchange NMR Spectroscopy*[S]

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The formation of *S*-hydroxymethylglutathione from formaldehyde and glutathione is a central reaction in the consumption of the cytotoxin formaldehyde in some methylotrophic bacteria as well as in many other organisms. We describe here the discovery of an enzyme from *Paracoccus denitrificans* that accelerates this spontaneous condensation reaction. The rates of *S*-hydroxymethylglutathione formation and cleavage were determined under equilibrium conditions via two-dimensional proton exchange NMR spectroscopy. The pseudo first order rate constants k_1* were estimated from the temperature dependence of the reaction and the signal to noise ratio of the uncatalyzed reaction. At 303 K and pH 6.0 k_1* was found to be 0.02 s^{-1} for the spontaneous reaction. A 10-fold increase of the rate constant was observed upon addition of cell extract from *P. denitrificans* grown in the presence of methanol corresponding to a specific activity of 35 units mg^{-1}. Extracts of cells grown in the presence of succinate revealed a lower specific activity of 11 units mg^{-1}. The enzyme catalyzing the conversion of formaldehyde and glutathione was purified and named glutathione-dependent formaldehyde-activating enzyme (Gfa). The gene *gfa* is located directly upstream of the gene for glutathione-dependent formaldehyde dehydrogenase, which catalyzes the subsequent oxidation of *S*-hydroxymethylglutathione. Putative proteins with sequence identity to Gfa from *P. denitrificans* are present also in *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*, and *Mesorhizobium loti*.

Formaldehyde is a highly toxic compound due to nonspecific reactivity with proteins and nucleic acids (1). It is liberated as a result of demethylation reactions in mammals (2) or from environmental sources (3), and it is a central intermediate upon growth of methylotrophic bacteria on one-carbon substrates like methanol or methane (4). The most widespread enzymatic system for the conversion of formaldehyde is the glutathione (GSH)[1]-linked oxidation pathway, which has been found in bacteria, mammals, and plants. In autotrophic methylotrophic bacteria like *Paracoccus denitrificans* and *Rhodobacter sphaeroides* as well as methylotrophic yeasts, it is involved in the complete oxidation of methanol to carbon dioxide (5–8). In higher organisms, as well as non-methylotrophic bacteria, such as *Escherichia coli*, glutathione-linked formaldehyde oxidation serves to detoxify the one-carbon unit (9, 10).

The glutathione-dependent formaldehyde conversion to formate starts with the adduct formation, formaldehyde reacts with the SH group of glutathione producing *S*-hydroxymethylglutathione (Reaction 1) (11). This reaction is considered to proceed *in vivo* uncatalyzed by a specific enzyme (6, 7, 10, 11). The product of this reaction, *S*-hydroxymethylglutathione, is oxidized by glutathione-dependent formaldehyde dehydrogenase (GS-FDH) (Reaction 2), which belongs to the class III alcohol dehydrogenases and has been characterized from various organisms (6, 7, 9, 12). The enzyme has been shown to be induced upon formaldehyde stress in different microorganisms (10, 13). In the subsequent enzymatic reaction, *S*-formylglutathione hydrolase (FGH) regenerates glutathione and forms formate (Reaction 3) (14), which can be further oxidized to carbon dioxide.

$$GSH + HCHO \rightleftharpoons GSCH_2OH$$

REACTION 1

$$GSCH_2OH + NAD^+ \rightleftharpoons GSCHO + NADH + H^+ \text{ (GS-FDH)}$$

REACTION 2

$$GSCHO + H_2O \rightleftharpoons GSH + HCOOH \text{ (FGH)}$$

REACTION 3

In this study, we investigated whether the condensation of formaldehyde and glutathione (Reaction 1) proceeds indeed only non-enzymatically *in vivo*. We have chosen *P. denitrificans* as a model organism, since it is a facultative methylotroph and converts high amounts of formaldehyde during energy metabolism upon

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[1] The abbreviations used are: GSH, glutathione; Gfa, glutathione-dependent formaldehyde-activating enzyme; GS-FDH (or GD-FALDH), glutathione-dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; Fae, tetrahydromethanopterin-dependent formaldehyde-activating enzyme; EXSY, proton exchange NMR spectroscopy.

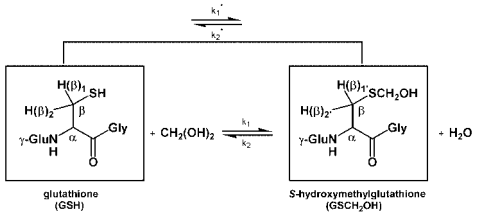

FIG. 1. **Formation of *S*-hydroxymethylglutathione ($GSCH_2OH$) from formaldehyde and GSH (γ-L-glutamyl-L-cysteinyl-glycine).** Two diasteriotopic protons at C_β have different chemical shifts and were used for the analysis of EXSY (see Fig. 2).

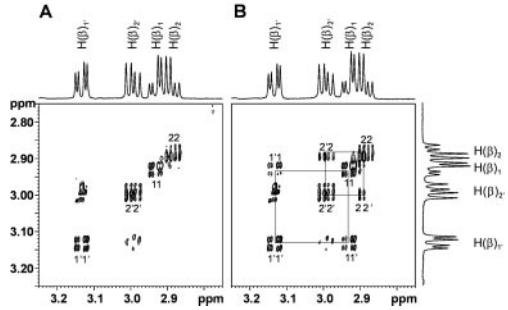

FIG. 2. **Aliphatic region of the one-dimensional and two-dimensional EXSY NMR spectra of the C_β protons of glutathione and *S*-hydroxmethylglutathione at equilibrium without (*A*) and with (*B*) cell extract from *P. denitrificans* at 303 K and pH 6.0.** Two-dimensional ^1H homonuclear EXSY NMR spectra (400 ms mixing time) are shown in the center and one-dimensional proton NMR spectra above and lateral. The signals $H(\beta)_1$ and $H(\beta)_2$ of the one-dimensional spectra are from protons of glutathione and the signals $H(\beta)_{1'}$ and $H(\beta)_{2'}$ from protons of *S*-hydroxymethylglutathione (Fig. 1). Diagonal peaks of the glutathione and *S*-hydroxymethylglutathione protons in chemical exchange are labeled with 11 and 1′1′ or 22 and 2′2′. Off-diagonal cross-peaks arising from chemical exchange are labeled with 1′1 and 11′ or 2′2 and 22′. These exchange cross-peaks are clearly visible in the presence of cell extract of *P. denitrificans* (1.04 mg), whereas in the absence of cell extract they are hardly detectable because of the signal to noise ratio (*B*). The NMR spectra were acquired at a ^1H frequency of 600.13 MHz on a DRX600 spectrometer (Bruker) and processed as described under "Experimental Procedures." The NMR tube (ϕ 5 mm) contained 0.6 ml of a reaction mixture made up of 10.8 mM glutathione, 5 mM formaldehyde, 60 μl of D_2O, 1.04 mg of cell extract protein of methanol grown *P. denitrificans* (*B*) in 120 mM potassium phosphate buffer, pH 6.0.

growth on methanol by glutathione-linked enzymes. Glutathione-dependent formaldehyde dehydrogenase and *S*-formylglutathione hydrolase have been shown to be essential for growth of the autotrophic bacterium in the presence of methanol (6, 14).

To determine *S*-hydroxymethylglutathione formation from formaldehyde and glutathione in *P. denitrificans*, we used proton exchange NMR spectroscopy (15). The method is based on the finding that the protons at the C_β atom of the thiol group of the cysteine part in glutathione and *S*-hydroxymethylglutathione exhibit different chemical shifts and that the saturation transfer kinetics of these protons can be followed by proton exchange NMR spectroscopy (EXSY) (Fig. 1). We used the two-dimensional EXSY approach to detect the activity of an previously unknown enzyme and used it for purification of the enzyme from cell extracts. To our knowledge this is the first time that EXSY has been successfully applied to find a previously unknown enzyme.

EXPERIMENTAL PROCEDURES

NMR Measurements—Rates of *S*-hydroxymethylglutathione formation from formaldehyde and glutathione were determined under equilibrium conditions via EXSY (16). NMR spectra were acquired at a ^1H frequency of 600.13 MHz on a DRX600 spectrometer (Bruker) and processed with the program XWINNMR (Bruker). The assays were performed in NMR tubes (ϕ 5 mm) with 0.6 ml of reaction mixture. Standard assays contained 10.8 mM GSH and 5 mM formaldehyde in 120 mM potassium phosphate buffer pH 6.0 (H_2O/D_2O = 9:1) if not otherwise noted. Exchange rates $v_1 = k_1^*$ [GSH] = $v_2 = k_2^*$ [$GSCH_2OH$] (see Fig. 1) were calculated from the concentrations of GSH and $GSCH_2OH$ in equilibrium which were obtained by integration of one-dimensional spectra yielding the [GSH]/[$GSCH_2OH$] ratio (see Fig. 2). From the ratios, the relative populations p_{GSH} = [GSH]/([GSH] + [$GSCH_2OH$]) and $p_{GSCH2OH}$ = [$GSCH_2OH$]/([GSH] + [$GSCH_2OH$]) were calculated, whereby [GSH] + [$GSCH_2OH$] equals the GSH concentration added. GSH was considered to be fully protonated, since measurements were performed between pH 5.5 and 6.5 and the pK_a of GSH is 9.12. The second order rate constants k_1 and k_2 were defined from k_1^* and k_2^* and the equilibrium concentration of formaldehyde: $v_1 = k_1$[GSH][HCHO] = $v_2 = k_2$[$GSCH_2OH$][H_2O]. [H_2O] was considered to be constant, since measurements were performed in aqueous solution. The exchange rates v_1 and v_2 were calculated from the concentration of GSH, HCHO, and $GSCH_2OH$, and the rate constants k_1 and k_2, which are related to the relative populations p_{GSH} and $p_{GSCH2OH}$, and the peak volumes V_{ij} and the mixing time τ_m by the expression V_{ij} = (exp ($-R\tau_m$))$_{ij}$. For definition of V_{ij} and R, see Ref. 16. The enzyme activities were calculated from the exchange rates v_1 of the $GSCH_2OH$ formation and converted from the unit mM s^{-1} to μmol min^{-1} (=1 unit).

Bacterial Growth and Enzyme Purification—*P. denitrificans* (DSM413), *E. coli* DH5α, and *Methylobacterium extorquens* AM1 were cultivated as described previously (4, 10). For enzyme purification from methanol-grown *P. denitrificans*, 20 g of wet cells were resuspended in 120 mM potassium phosphate buffer and broken by a French press. Purification of Gfa was performed by four chromatographic steps at 4 °C. The soluble fraction of the cell extract was loaded onto a DEAE-Sephacel (Sigma) column equilibrated with 50 mM potassium phosphate, pH 7.0. Protein was eluted with the following gradient steps of NaCl in this buffer: 60 ml of 0 mM, 60 ml of 150 mM, 60 ml of 200 mM, 60 ml of 250 mM, and 60 ml of 500 mM. Gfa was eluted at 150 mM NaCl. Active fractions were diluted 1:2 in 10 mM potassium phosphate buffer, pH 7.0, and loaded onto a hydroxyapatite column (Bio-Rad) equilibrated in the same buffer. Protein was eluted with a stepwise increasing potassium phosphate gradient (10–250 mM in 275 ml). Gfa was recovered in the flow-through of the column, which was subjected to chromatography on Q-Sepharose (Amersham Biosciences, Inc.) in 120 mM potassium phosphate buffer. Protein was eluted with an increasing gradient of NaCl (0–300 mM in 450 ml). Gfa was eluted at 60 mM NaCl. Active fractions were pooled and diluted 1:2 in 50 mM potassium phosphate buffer, pH 7.0, and loaded onto a Mono Q column (Amersham Biosciences, Inc.). Protein was eluted with an increasing gradient of NaCl in this buffer (0–500 mM in 100 ml). Gfa was eluted at 400 mM NaCl. GS-FDH was measured photometrically and purified as described previously (6).

RESULTS

In most organisms, the conversion of exogenous or endogenous formaldehyde proceeds by addition to glutathione prior to oxidation by GS-FDH. To address the question of whether an enzyme exists which catalyzes the formation of *S*-hydroxymethylglutathione from formaldehyde and glutathione, we analyzed cell extracts of *P. denitrificans* grown under methylotrophic conditions. The rates of formaldehyde-glutathione condensation were determined by one-dimensional and two-dimensional proton exchange NMR spectroscopy. Recording of the standard spectra was performed at pH 6.0, 303 K (30 °C) and under aerobic conditions, since *P. denitrificans* is an aerobic mesophilic bacterium. To increase the accuracy of the analysis, a product/educt ratio of 1:1 was aspired and achieved by using a ratio of glutathione to formaldehyde of 2:1 (10.8 mM glutathione, 5 mM formaldehyde). This ratio was used throughout this study.

In Fig. 2, the aliphatic regions of a one-dimensional proton NMR spectrum and a two-dimensional ^1H homonuclear EXSY NMR spectrum of glutathione and *S*-hydroxymethylglutathione at equilibrium, in the absence (*A*) and in the presence (*B*) of cell extract from methanol grown *P. denitrificans*, are shown. From the one-dimensional spectra, the relative populations p_{GSH} = 0.52 and $p_{GSCH2OH}$ = 0.48 were obtained by integration of the signals 1 and 1′ (Fig. 2, *A* and *B*). Integration of the signals 2 and 2′ yields the same values for both species. From the two-dimensional spectrum in the presence of cell extract (Fig. 2*B*) the peak volumes of the protons 1 and 2 were obtained and k_1^* = 0.24

TABLE I

Effect of cell extracts of different organisms on the rate of S-hydroxymethylglutathione formation in 120 mM potassium phosphate buffer, pH 6.0, and 303 K (30 °C)

The rates of *S*-hydroxymethylglutathione formation were determined under equilibrium conditions by EXSY and one-dimensional NMR spectroscopy. The experiments were performed in NMR tubes (∅ 5 mm). The 0.6-ml reaction mixture contained 10.8 mM glutathione, 5 mM formaldehyde, 60 μl of D_2O, and 1.04 mg of cell extract protein if not otherwise noted. Where indicated, denatured cell extract protein was applied, which was boiled for 5 min at 95 °C and centrifuged. A unit of enzyme activity was defined as the formation of 1 μmol of *S*-hydroxymethylglutathione from formaldehyde and glutathione per min minus the spontaneous reaction rate without enzyme added. The activity of GS-FDH is given as a control and was measured photometrically with NAD as electron acceptor to exclude an effect of the dehydrogenase on the exchange rates (6). For definition of k_1* and calculation of the activities, see "Experimental Procedures." LB, Luria-Bertani medium; ND = not detectable.

Conditions	$10^{-2}\ k_1$*	Activity	Specific activity	Specific activity GS-FDH
	s^{-1}	*units*	*units* mg^{-1}	*units* mg^{-1}
−Protein	2[a]	5		
+Cell extract				
P. denitrificans, grown on methanol	20	41	35	1.1
P. denitrificans, grown on methanol, ½ × protein	11	23	35	1.1
P. denitrificans, denatured	3[a]	6		
P. denitrificans, grown on succinate	8	16	11	<0.04
E. coli DH5α, grown on LB + formaldehyde (250 μM)	5	10	5	0.55
E. coli DH5α, grown on LB	4	9	4	0.05
M. extorguens AM1, grown on methanol	3[a]	6		ND

[a] Estimated from signal to noise ratio.

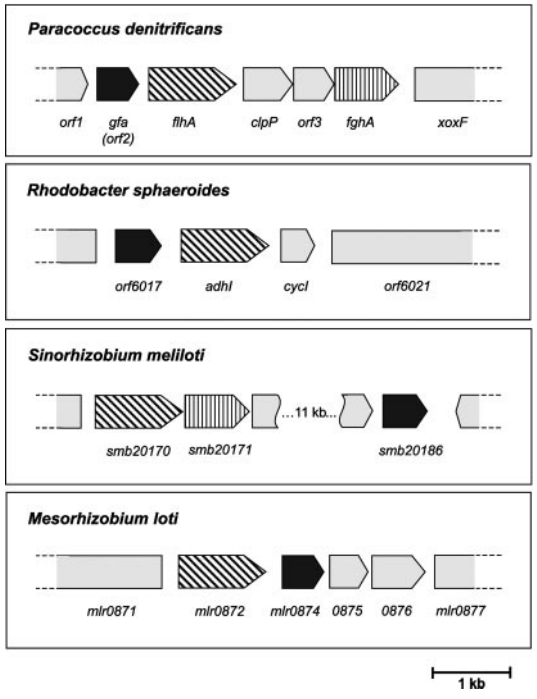

FIG. 3. **Comparison of the genomic arrangement of Gfa from *P. denitrifians* and putative proteins from the complete genomes of *R. sphaeroides*[2] and the symbiotic nitrogen-fixing bacteria *S. meliloti* (19) and *M. loti* (20).** The open reading frames for proteins with sequence identity to each other are marked with the same pattern. In *P. dentitrificans*, *gfa*, formerly *orf2* (6,14), encodes Gfa (this work); *flhA* encodes GS-FDH (or GD-FALDH) (6); and *fghA* encodes FGH (14). In *R. sphaeroides*, *adhI* was shown to encode GS-FDH (7) and is located downstream of an open reading frame with sequence identity to Gfa.[2] Putative proteins with sequence identity to Gfa from *P. denitrificans* could also be found in the currently unfinished genomes of *T. ferrooxidans* and *S. putrefaciens* (not shown).[3]

s^{-1} and k_2* = 0.21 s^{-1} calculated (16). From the data, an exchange rate v_1 was obtained corresponding to 41 units for the rate in the presence of cell extract of methanol grown *P. denitrificans*, which has 35 units mg^{-1} cell extract protein (Table I). Without cell extract (Fig. 2*A*) a spontaneous rate of only 5 units was determined. No increase in the spontaneous rate was observed if supernatant of denatured and centrifuged cell extract from *P. denitrificans* was applied, indicating that the observed activity is the result of enzyme catalysis. Addition of purified GS-FDH from *P. denitrificans*, which oxidizes *S*-hydroxymethylglutathione (Reaction 2), did not result in higher *S*-hydroxymethylglutathione formation from formaldehyde and glutathione (Table I). This shows that the observed acceleration is catalyzed by a separate enzyme distinct from GS-FDH. Analysis of cell extract of *P. denitrificans* grown in the presence of succinate revealed that enzymatic formaldehyde conversion is still clearly detectable with an activity of 11 units mg^{-1} amounting to one-third of the activity in comparison to cells grown in the presence of the one-carbon substrate. Activity of GS-FDH, which was measured as a control enzyme, was not detectable upon growth in the presence of succinate and shows a more pronounced effect of induction (Table I; Ref. 6).

The influence of temperature and pH upon the rate of *S*-hydroxymethylglutathione formation from formaldehyde and glutathione was analyzed. The rate of the spontaneous reaction *versus* the accelerated rate in the presence of cell extract of methanol-grown *P. denitrificans* was determined between 293 and 333 K (20–60 °C). In both cases, the rate of *S*-hydroxymethylglutathione formation increased about 3-fold when the temperature was raised from 293 K to 303 K (20 and 30 °C). The increase of the spontaneous rate was linear up to 333 K (60 °C), whereas determination of the enzyme-promoted rates, by addition of cell extract, above 323 K (50 °C) was not possible due to protein denaturation. Dependence of the pH on the rate of *S*-hydroxymethylglutathione formation was determined between pH 5.5 and 6.5. The spontaneous rate increased with higher pH; the rate k_1* without cell extract was only 0.03 s^{-1} at pH 5.5 and 0.45 s^{-1} at pH 6.5. In the presence of cell extract from *P. denitrificans* the rate was always higher. At pH values higher than 6.5 the determination was rather difficult due to instability of *S*-hydroxymethylglutathione *in vitro* (17).

The enzyme that catalyzes the formation of *S*-hydroxymethylglutathione, glutathione-dependent formaldehyde-activating enzyme, Gfa, was purified from *P. denitrificans* as described under "Experimental Procedures." The enzyme activity was detected via NMR measurements. After four chromatographic steps, preparations contained only one polypeptide with an apparent molecular mass of 21 kDa, as revealed by SDS-PAGE and exhibited a specific activity of 350 units mg^{-1}. Purification was about 24-fold with a yield of 6%. UV/visible spectroscopy did not reveal the presence of a chromophoric prosthetic group. The N-terminal amino acid sequence of the 21-kDa polypeptide was determined (MVDTSGVKIHPAVDNG; terminal methionine cleaved off to 90%) and matched exactly that predicted for the *orf2* gene product (6, 14). We now assign this gene as *gfa*. Gfa from *P. denitri-*

ficans shows high sequence identity to putative proteins known from the complete genome sequences of the α-proteobacteria *R. sphaeroides* (72%),[2] *Sinorhizobium meliloti* (75%) (19), and *Mesorhizobium loti* (61%) (20). Putative proteins with sequence identities of about 63% could also be identified in the currently unfinished genome sequences of the γ-proteobacteria *Thiobacillus ferrooxidans* and *Shewanella putrefaciens*.[3]

Interestingly, *gfa* from *P. denitrificans* is located directly upstream from *flhA* coding for GS-FDH (or GD-FALDH) (6) (Fig. 3). In *R. sphaeroides* (7)[2] and *T. ferrooxidans*,[3] the same arrangement of genes for the putative glutathione-dependent proteins could be found, whereas in *M. loti* the arrangement of the two genes is inverted (20). In *S. meliloti,* the genes for a putative Gfa and a putative GS-FDH are located about 13 kb apart on the pSymB megaplasmid (Fig. 3). This genome region also includes a putative methanol dehydrogenase structural gene (19). In *S. putrefaciens*, the gene for a protein with sequence identity to Gfa is located directly downstream of a putative iron containing alcohol dehydrogenase.[3] No more additional putative proteins with sequence identity to Gfa from *P. denitrificans* could be identified. Therefore Gfa is not conserved in all organisms that have been shown to contain GS-FDH, *i.e. E. coli* (10).

DISCUSSION

In this study, we detected and purified a novel glutathione-dependent formaldehyde-activating enzyme Gfa from the facultative methylotrophic bacterium *P. denitrificans.* The condensation of formaldehyde and glutathione to *S*-hydroxymethylglutathione is the first step in the widespread glutathione-linked conversion of formaldehyde and was thought to occur without enzymatic catalysis *in vivo*.

Gfa is not the first example of a protein that catalyzes the condensation of formaldehyde and a cofactor to form an adduct in the process of energy metabolism. It was recently shown that the methylotrophic proteobacterium *M. extorquens* AM1 possesses a tetrahydromethanopterin-linked formaldehyde-activating enzyme, Fae, which catalyzes the condensation of formaldehyde and tetrahydromethanopterin producing methylene tetrahydromethanopterin (22). Fae is present in all heterotrophic methylotrophic proteobacteria we tested that contain tetrahydromethanopterin-dependent enzymes.[4] Both formaldehyde-converting enzymes, Gfa and Fae, are composed of one type of subunit of about 20 kDa and lack a chromophoric prosthetic group. In addition, both enzymes are encoded next to genes for enzymes involved in further oxidation of the cofactor-bound one-carbon unit to carbon dioxide (6, 22). The primary sequences of Gfa and Fae do not reveal any sequence identity to each other and have obviously evolved independently, which is not too surprising, since the cofactors are very different, and binding of formaldehyde occurs either to the sulfur atom of glutathione or the N^5,N^{10} nitrogen atoms of tetrahydromethanopterin.

Tetrahydromethanopterin-dependent enzymes are restricted to methylotrophic proteobacteria and methanogenic archaea, whereas the glutathione-linked formaldehyde dehydrogenase is widespread in procarya and eucarya (6, 7, 9, 12). Nevertheless, the presence of Gfa appears to be limited. It might be that Gfa is present only in organisms that produce and consume large amounts of intracellular formaldehyde, whereas the spontaneous formation of *S*-hydroxymethylglutathione would be sufficient for detoxification of exogenous formaldehyde, which may occur in the environment. In this respect it is interesting to discuss the bacteria that contain a Gfa homolog. Methanol consumption of the nitrogen-fixing bacteria *S. meliloti* and *M. loti* appears likely, since they contain open reading frames for putative proteins with high sequence identity to Gfa as well as putative proteins for *S*-hydroxymethylglutathione oxidation and methanol dehydrogenase structural genes (19, 20). A functional active Gfa homolog could also be expected in *R. sphaeroides* where the role of glutathione-linked formaldehyde dehydrogenase has been shown under both photosynthetic and aerobic respiratory conditions (8). *S. putrefaciens* is able to grow anaerobically in the presence of formate and proposed to form free formaldehyde intracellulary (21). A *Thiobacillus* species, *Thiobacillus thioparus*, also forms formaldehyde upon growth on methyl mercaptan (18). The same might be true for *T. ferrooxidans*, which possesses putative proteins for Gfa and glutathione-linked formaldehyde dehydrogenase.

We cannot rule out that another glutathione-linked formaldehyde-activating enzyme might have evolved that is shared by other organisms. We observed a slight increase in *S*-hydroxymethylglutathione formation in cell extracts of *E. coli*, which was, however, not induced by formaldehyde stress like GS-FDH so that the presence of glutathione-linked formaldehyde activation could not be demonstrated.

At present it is not clear whether Gfa serves solely as an enzyme or can also serve as a formaldehyde scavenger to prevent unspecific binding of the toxin. In this respect, it is interesting to note that in *P. denitrificans*, Gfa activity could also be detected in cells grown in the absence of methanol, whereas activity of GS-FDH is not detectable under these growth conditions. Therefore it is likely that the corresponding genes are under the control of different promotors.

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[2] Sequence data was obtained from the Oak Ridge National Laboratory webpage at genome.ornl.gov/microbial/rsph/.

[3] Preliminary sequence data was obtained from The Institute for Genomic Research website at www.tigr.org.

[4] M. Goenrich and J. A. Vorholt, unpublished results.

ACCELERATED PUBLICATION:

A Glutathione-dependent Formaldehyde-activating Enzyme (Gfa) from *Paracoccus denitrificans* Detected and Purified via Two-dimensional Proton Exchange NMR Spectroscopy

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