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

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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 (GS)$^1$-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 formaldehyde 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 hydratase (FGH) regenerates glutathione and forms formate (Reaction 3) (14), which can be further oxidized to carbon dioxide.

\[
\text{GSH} + \text{HCHO} \rightarrow \text{GSCH}_2\text{OH}
\]

**REACTION 1**

\[
\text{GSCH}_2\text{OH} + \text{NAD}^+ \rightarrow \text{GSCHO} + \text{NADH} + \text{H}^+ \quad (\text{GS-FDH})
\]

**REACTION 2**

\[
\text{GSCHO} + \text{H}_2\text{O} \rightarrow \text{GSH} + \text{HCOOH} \quad (\text{FGH})
\]

**REACTION 3**

In this study, we investigated whether the condensation of formaldehyde and glutathione (Reaction 1) proceeds indeed only non-enzymatically in vitro. 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 hydratase; Fae, tetrahydromethanopterin-dependent formaldehyde-activating enzyme; EXSY, proton exchange NMR spectroscopy.
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 \textit{P. denitrificans}, we used proton exchange NMR spectroscopy (15). The method is based on the finding that the protons at the C4 atom have different chemical shifts and were used for the analysis of EXSY (see Fig. 2).

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**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 (Ø 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 and broken by a French press. 

Formaldehyde-glutathione condensation was studied in aqueous solution. The rates of formaldehyde-glutathione condensation were determined by one-dimensional and two-dimensional proton 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.

**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 \textit{P. denitrificans} grown under methylo trophic 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 \textit{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 the one-dimensional proton NMR spectrum and a two-dimensional 1H homonuclear EXSY NMR spectrum of glutathione and S-hydroxymethylglutathione at equilibrium without (A) and with (B) cell extract from \textit{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(β), and H(β), of the one-dimensional spectra are from protons of glutathione and the signals H(β), and H(β), of protons of S-hydroxymethylglutathione (Fig. 1). Diagonal peaks of the glutathione and S-hydroxymethylglutathione protons in chemical exchange are labeled with 11 and 1′ or 22 and 2′. Off-diagonal cross-peaks arising from chemical exchange are labeled with 1′1′ or 2′2′. These exchange cross-peaks are clearly visible in the presence of cell extract of \textit{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 previously (6).

**Fig. 2.** Aliphatic region of the one-dimensional and two-dimensional EXSY NMR spectra of the C4 protons of glutathione and S-hydroxymethylglutathione at equilibrium without (A) and with (B) cell extract from \textit{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(β), and H(β), of the one-dimensional spectra are from protons of glutathione and the signals H(β), and H(β), of protons of S-hydroxymethylglutathione (Fig. 1). Diagonal peaks of the glutathione and S-hydroxymethylglutathione protons in chemical exchange are labeled with 11 and 1′ or 22 and 2′. Off-diagonal cross-peaks arising from chemical exchange are labeled with 1′1′ or 2′2′. These exchange cross-peaks are clearly visible in the presence of cell extract of \textit{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 previously (6).

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In Fig. 2, the aliphatic regions of the 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 \textit{P. denitrificans}, are shown. From the one-dimensional spectra, the relative populations \( p_{\text{GSH}} = 0.52 \) and \( p_{\text{GSH2OH}} = 0.48 \) were obtained by integration of the signals 1 and 1′ (Fig. 2A 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. 2B) the peak volumes of the protons 1 and 2 were obtained and \( k^* = 0.24 \).
The rates of S-hydroxymethylglutathione formation were determined under equilibrium conditions by ESSY and one-dimensional NMR spectroscopy. The experiments were performed in NMR tubes (ø 5 mm). The 0.6-ml reaction mixture contained 10.8 mM glutathione, 5 mM formaldehyde, 80 µl of D₂O, 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 $k_1$, calculation of the activities, see “Experimental Procedures.” LB, Luria-Bertani medium; ND = not detectable.

### 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)**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$10^{-3} k_1$</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s⁻¹</td>
<td>units</td>
<td>units mg⁻¹</td>
<td>units mg⁻¹</td>
</tr>
<tr>
<td>−Protein</td>
<td>2⁺</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Cell extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. denitrificans</em>, grown on methanol</td>
<td>20</td>
<td>41</td>
<td>35</td>
<td>1.1</td>
</tr>
<tr>
<td><em>P. denitrificans</em>, grown on methanol, 1/2 × protein</td>
<td>11</td>
<td>23</td>
<td>35</td>
<td>1.1</td>
</tr>
<tr>
<td><em>P. denitrificans</em>, denatured</td>
<td>3⁺</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. denitrificans</em>, grown on succinate</td>
<td>8</td>
<td>16</td>
<td>11</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td><em>E. coli</em> DH5a, grown on LB + formaldehyde (250 µM)</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>0.55</td>
</tr>
<tr>
<td><em>E. coli</em> DH5a, grown on LB</td>
<td>4</td>
<td>9</td>
<td>4</td>
<td>0.05</td>
</tr>
<tr>
<td><em>M. extorguens</em> AM1, grown on methanol</td>
<td>3⁺</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Estimated from signal to noise ratio.

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. 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).
ficans shows high sequence identity to putative proteins known from the complete genome sequences of the α-proteobacteria R. sphaeroides (72%), Sinothrichobium 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 ferroxoxidans 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. ferroxoxidans,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 methyloptroph 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 methyloptrophic 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 methyloptroph proteobacteria we tested that contain tetrahydromethanopterin-dependent enzymes.4 Both formaldehyde-convert 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 N5,N10 nitrogen atoms of tetrahydromethanopterin.

Tetrahydromethanopterin-dependent enzymes are restricted to methyloptroph proteobacteria and methanogenic archaea, whereas the glutathione-linked formaldehyde dehydrogenase is widespread in procarys 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-hydroxymethyl-glutathione 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 intracellularly (21). A Thiobacillus species, Thiobacillus thioparus, also forms formaldehyde upon growth on methyl mercaptan (18). The same might be true for T. ferroxoxidans, 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 promoters.

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ACCELERATED PUBLICATION:

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