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Induction of Glutathione-Dependent Formaldehyde Dehydrogenase Activity in *Escherichia coli* and *Hemophilus influenza*

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We have examined the induction of glutathione-dependent formaldehyde dehydrogenase (GS-FDH) activity in *Escherichia coli* and *Hemophilus influenza*. Formaldehyde was found to induce enzyme activity in both *E. coli* and *H. influenza* at concentrations between 0.6 and 20 ppm. Higher formaldehyde concentrations were toxic. Methanol concentrations up to 20% (200,000 ppm) and sodium formate concentrations up to 2% (20,000 ppm) gave negligible amounts of induction. The basic mechanism of induction was probed by inducing GS-FDH activity in the presence of rifampicin to inhibit RNA synthesis or chloramphenicol to inhibit protein synthesis. Both reagents inhibited GS-FDH induction, demonstrating that regulation occurs at the level of transcription. These results indicate that at least one function of GS-FDH in Gram-negative bacteria is to detoxify exogenous formaldehyde encountered in their environment and that GS-FDH inducibility may be a common feature of Gram-negative bacteria.

The glutathione-dependent formaldehyde dehydrogenases (GS-FDHs) are ubiquitous enzymes found in plants, animals, and bacteria (1-5). The finding that the mammalian GS-FDHs are homologous to the mammalian Class III alcohol dehydrogenases (3,6) demonstrated that the GS-FDHs are members of the superfamily of zinc containing alcohol dehydrogenases. GS-FDH catalyzes the first of two enzyme catalyzed reactions involved in the oxidation of highly toxic formaldehyde to formic acid.

$$GSH + CH_2O \rightleftharpoons GS\text{-}CH_2\text{-}OH + NAD^+ \overset{GS\text{-}FDH}{\rightleftharpoons}$$
$$GS\text{-}CHO + NADH + H_2O \rightleftharpoons GSH + HCOOH$$

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The true substrate for this enzyme is hydroxymethyl glutathione, the adduct formed (without enzyme catalysis) between formaldehyde and the SH group of glutathione. The highly conserved nature of this enzyme and its widespread distribution indicates that it represents the predecessor to the entire family of dimeric Zn ADHs found in plants and animals (4,5,7).

In methylotrophic bacteria GS-FDHs are involved in the oxidation of methanol and other one-carbon (C_1) generating metabolites (8). A related trimeric cofactor dependent FDH, which uses a sulfhydril containing cofactor other than glutathione, has been identified in some gram-positive bacteria when grown C_1 substrates (9-11), and this cofactor has recently been identified as mycothiol (1-O-(2′-[N-acetyl-L-cysteinyl]-amido-2′-deoxy-α-D-glucopyranosyl)-D-myo-inositol) (12). A related cofactor independent formaldehyde dehydrogenase has been characterized from *Pseudomonas putida* (13,14). GS-FDH activity is found in the Gram-negative methylotroph *Paracoccus denitrificans* upon growth on C_1 substrates but not upon growth on succinate (15). Bacterial GS-FDHs are also found in non-methylotrophic bacteria, such as *E. coli* (4, 16), and the presence of high levels of plasmid encoded GS-FDH activity is associated with resistance to the biocidal effects of formaldehyde in several enterobacteria (including *E. coli*) (17-19) and in yeast (20,21).

The observation of GS-FDH activity in *Paracoccus denitrificans* only upon growth on C_1 substrates (15) indicates that this enzymes activity is inducible in some of the organisms in which it is found. To further test this possibility we have examined the levels of GS-FDH activity in *E. coli* and *H. influenza* as a function of added formaldehyde, time, and transcription (rifampicin) or translation (chloramphenicol) inhibitors. To determine if other C_1 compounds could induce GS-FDH activity we also tested methanol and formic acid as inducers of GS-FDH activity. The results of these studies are presented below.

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MATERIALS AND METHODS

Bacterial strains and reagents. *E. coli* DH5α, JM109, XL1Blue, were from Dr. Gautham Chaudhuri (Meharry Medical College), *E. coli* strain K12 was obtained from Dr. Mark Levitch (Meharry Medical College), and *H. influenza* Rd was obtained from Dr. Andrew Plaut (Tufts University Medical School). *E. coli* was grown in Lauria Broth and *H. influenza* in brain heart infusion (BHI) supplemented with hemin (10 μg/ml) and NAD^+ (10 μg/ml) at 37°C. Formaldehyde was obtained as a 20% solution in sealed ampules (Ladd Research Industries, Burlington VT).

GS-FDH activity as a function of added formaldehyde, methanol, and sodium formate. GS-FDH activity was measured after exposure of *E. coli* strain DH5α to formaldehyde, as well as methanol and sodium formate, the more reduced and oxidized metabolites of formaldehyde. Saturated overnight cultures of *E. coli* strain DH5α were diluted 1 to 10 into 2.5 ml fresh LB media containing various amounts of formaldehyde, methanol, or sodium formate. After incubation for 30 min at 37 °C the cultures were placed on ice for 15 min. The absorbance at 600 nm was measured to determine the extent of bacterial growth, 1 ml aliquotes were removed, the cells pelleted by centrifugation, and the pellets stored at − 80 °C. Cell pellets were resuspended in 300 μl lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% sucrose, 0.1 mg/ml lysozyme), place on ice for 15 min, and then sonicated. Cell debris was removed by centrifugation, and GS-FDH activity in the supernatants assayed as described below.

Time course of induction as a function of added formaldehyde. Induction was studied as a function both of added formaldehyde and time in *E. coli* strains DH5α, JM109, and XL1Blue as well as in *H. influenza* Rd. LB or BHI cultures prepared with varying amounts of formaldehyde (0-200 ppm) were inoculated 1 to 100 from saturated overnight cultures. At varying time intervals 1 ml aliquotes were removed, and placed on ice in eppendorf tubes. Cells were collected by centrifugation and stored frozen at −80 °C. Cells were lysed by treatment with 300 μl lysis buffer followed by sonication, centrifugation to remove cell debris, and the supernatants assayed for GS-FDH activity.

Effects of added rifampicin or chloramphenicol on GS-FDH induction in E. coli. To determine if the induction of GS-FDH activity occurred at the level of transcription, translation, or via post-translational modification, the effect of rifampicin (transcription inhibitor) and chloramphenicol (translation inhibitor) were investigated. These experiments were performed by diluting a saturated overnight culture of *E. coli* strain K12 1 to 10 into fresh culture with or without 6.3 ppm formaldehyde and with or without either 150 μg/ml rifampicin or 20 μg/ml chloramphenicol. As a control the same experiment was performed for β-galactosidase induction by 0.5 mM IPTG. The β-galactosidase control necessitated the use of *E. coli* strain K12 for this experiment. After 30 minutes aliquotes were removed, supernatants prepared as described above, and assayed as described below for GS-FDH activity, or as described below to determine the β-galactosidase activity. These experiments were performed in triplicate.

GS-FDH assays. Extracts were assayed for GS-FDH activity by adding 100 μl of the extract to 900 μl assay reagent and following the time course of NADH formation at 340 nm. The assay reagent was prepared so that the concentration of reagents in the final assay mixture was 90 mM sodium phosphate (pH 8.0), 4.8 mM NAD^+, 1.0 mM formaldehyde, and 1.0 mM glutathione. The rate of NADH formation was calculated from the rate of absorbance change at 340 nm using ϵ^{340}=6220 M^{-1} cm^{-1}.

β-Galactosidase assays. β-Galactosidase assays were performed using *o*-nitrophenyl β-galactoside (ONPG) as described in Miller (22). 200 μl of bacterial suspension was transferred to an eppendorf tube containing 1 drop of $CHCl_3$ and 5 μl 10% SDS and the mixture vortexed. To this was added 0.8 ml of 8 mg/ml ONPG in "Z-buffer" (100 mM sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol, pH 7.0). After 45 min the reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3, and the absorbance read at 420 nm. Blanks for rifampicin were included to account for the significant absorbance of rifampicin at 420 nm.

TABLE 1

Effect of Methanol, Formaldehyde, or Sodium Formate on *E. coli* DH5α Growth (A^{600}) and GS-FDH Activity

% MeOH	A^{600}	Activity (μM/min)
	Methanol	
20	0.86	0.10
6.3	1.13	0.27
2.0	1.16	0.23
0.63	1.13	0.18
0.20	1.18	0.34
ppm CH_2O	A^{600}	Activity (μM/min)
	Formaldehyde	
630	0.95	0.19
200	1.00	0.12
63	1.05	0.10
20	1.17	3.36
6.3	1.17	3.59
%HCOONa	A^{600}	Activity (μM/min)
	Formate	
2.0	0.87	0.03
0.63	1.00	0.24
0.20	1.07	0.25
0.063	1.15	0.33
0.020	1.15	0.32

RESULTS

GS-FDH induction in E. coli strain DH5α by added formaldehyde, methanol, and sodium formate. Table 1 summarizes the effect of added formaldehyde, methanol, and sodium formate on the growth and GS-FDH activity of *E. coli* DH5α. These results demonstrate inhibition of bacterial growth by high concentrations of all three compounds. At the lower concentrations of all three compounds the bacteria are seen to grow to an A^{600} of 1.15. The GS-FDH activity of cultures grown in the presence of formaldehyde increases about 10 fold, whereas methanol and sodium formate have no detectible effect.

Time course of GS-FDH induction in E. coli strain XL1Blue and H. influenza Rd as a function of added formaldehyde. Figure 1 summarizes the effect of varying amounts of formaldehyde on the time course GS-FDH activity of *E. coli* strain XL1Blue and *H. in-*

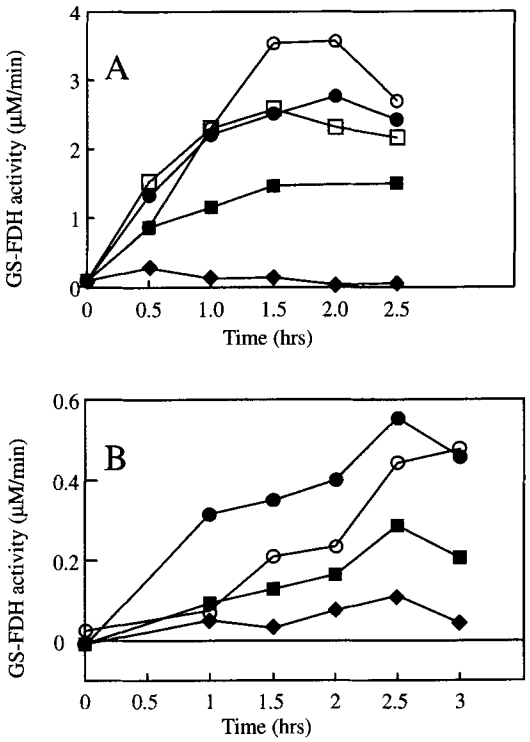

FIG. 1. Time course of GS-FDH induction in (A) *E. coli* strain XL1Blue and (B) *H. influenza* Rd as a function of the following concentrations of added formaldehyde: ■, 0.63 ppm; □, 2.0 ppm; ●, 6.3 ppm; ○, 20 ppm; and ◆, 63 ppm.

fluenza Rd. Similar results were also obtained for *E. coli* strains DH5α and JM109.

Effects of rifampicin or chloramphenicol of GS-FDH induction in E. coli strain K12. Figure 2 summarizes the effect of added rifampicin or chloramphenicol on GS-FDH induction in *E. coli* stain K12, and the control experiment of the effect added rifampicin or chloramphenicol on β-galactosidase induction by IPTG. Both GS-FDH and β-galactosidase induction are inhibited by the addition of rifampicin or chloramphenicol.

DISCUSSION

The results presented above demonstrate that GS-FDH activity can be induced by exogenously added formaldehyde in several strains of *E. coli,* as well as in *H. influenza* Rd. This demonstrates that the inducibility of this enzyme is a general feature of *E. coli* strains, and possibly Gram-negative bacteria in general. *E. coli* strains resistant to 200 ppm (0.02%) formaldehyde demonstrate a constitutive GS-FDH activity which is unaffected by the addition of formaldehyde (18). Some level of constitutive expression may therefore be a requirement for resistance to high levels of formaldehyde in order to prevent to substantial cellular damage from occurring prior to induction of sufficiently high levels of GS-FDH activity for protection.

Several mechanistic aspects of GS-FDH induction were investigated. It is possible that GS-FDH activity would be induced not by formaldehyde directly, but by its oxidation, reduction, or disproportionation products. A precedent for this possibility is the observation that formaldehyde is the effector for induction of methanol dehydrogenase activity in *Paracoccus denitrificans* (23). Methanol and sodium formate were both tested for their ability to induce GS-FDH activity (Table 1) and were found not to induce GS-FDH activity. These results demonstrate the methanol or formate are not responsible for the observed induction. Induction of GS-FDH activity may occur through several possible mechanisms including regulation of transcription, translation, or mRNA or protein degradation. The effect of rifampicin inhibition of transcription and chloramphenicol inhibition of translation were tested as inhibitors of formaldehyde induced GS-FDH activity. The results from this experiment (Figure 2) demonstrate the both transcription and translation are required for significant induction of GS-FDH activity in *E. coli,* and that regulation occurs at the level of transcription.

The observation of induction of GS-FDH activity in the non-methylotrophic *E. coli* and *H. influenza* by exogenously added formaldehyde demonstrates that at

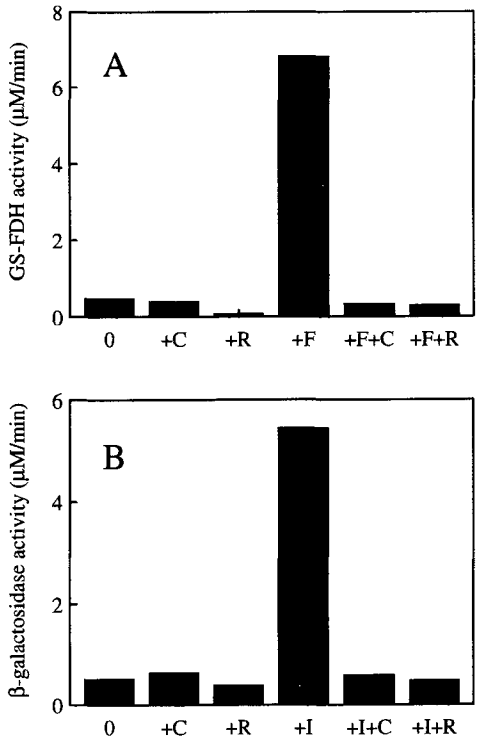

FIG. 2. (A) GS-FDH activity in *E. coli* strain DH5α in the absence (0) or presence (+F) of 6.3 ppm formaldehyde and in the absence (0) or presence of 150 μg/ml rifampicin (+R) or 20 μg/ml chloramphenicol (+C). (B) Control experiment for β-galactosidase induction in the absence (0) or presence (+I) of 0.5 mM IPTG and in the absence or presence of rifampicin or chloramphenicol as in A.

least one function of this enzyme *in vivo* is to detoxify formaldehyde. The Km of the *E. coli* GS-FDH for hydroxymethyl glutathione is 94 μM (4) which corresponds to 3 ppm formaldehyde. This is at the lower end of the concentration range where significant induction is observed in *E. coli* (Figure 1) and it appears that the formaldehyde level at which induction occurs is matched to the kinetic properties of GS-FDH. The GS-FDHs from prokaryotes show higher Km's and kcat's than those from eukaryotes, which indicates a role in formaldehyde detoxification for GS-FDH in prokaryotes and the maintenance of low formaldehyde levels in eukaryotes (24). These observations are all consistent with a role for GS-FDH in the detoxification of exogeneous formaldehyde in *E. coli* and possibly in other non-methylotrophic Gram-negative bacteria such as *H. influenza.*

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