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Metabolic Pathways Leading to Mercury Methylation in *Desulfovibrio desulfuricans* LS†

SUNG-CHAN CHOI, THEODORE CHASE, JR., AND RICHARD BARTHA*

Department of Biochemistry and Microbiology, Cook College, Rutgers University, New Brunswick, New Jersey 08903-0231

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The synthesis of methylmercury by *Desulfovibrio desulfuricans* LS was investigated on the basis of ^{14}C incorporation from precursors and the measurement of relevant enzyme activities in cell extracts. The previously observed incorporation of C-3 from serine into methylmercury was confirmed by measurement of relatively high activities of serine hydroxymethyltransferase and other enzymes of this pathway. High rates of label incorporation into methylmercury from $H^{14}COO^-$ and $H^{14}CO_3^-$ prompted the assay of enzymes of the acetyl coenzyme A (CoA) synthase pathway. These enzymes were found to be present but at activity levels much lower than those reported for acetogens. Propyl iodide inhibited methylmercury and acetyl-CoA syntheses to similar extents, and methylmercury synthesis was found to compete with acetyl-CoA synthesis for methyl groups. On the basis of these findings, we propose that in methylmercury synthesis by *D. desulfuricans* LS the methyl group is transferred from CH_3-tetrahydrofolate via methylcobalamin. The methyl group may originate from C-3 of serine or from formate via the acetyl-CoA synthase pathway. These pathways are not unique to *D. desulfuricans* LS, and thus the ability of this bacterium to methylate mercury is most likely associated with the substrate specificity of its enzymes.

Anaerobic aquatic sediments exposed to low-level inputs of inorganic mercury convert it to monomethylmercury, a highly hazardous environmental pollutant prone to biomagnification (16). Studies of the effects of specific inhibitors on such sediments indicated that over 95% of mercury biomethylation is carried out by sulfate-reducing microorganisms (8). Propyl iodide inhibition of biomethylation by *Desulfovibrio desulfuricans* LS and reversal of the inhibition by light indicated that methylation was mediated by a corrinoid compound (2, 4). More efficient incorporation into CH_3Hg^+ of ^{14}C from [3-^{14}C]pyruvate than from [1-^{14}C]pyruvate and very efficient incorporation from [3-^{14}C]serine suggested involvement of the C_1-tetrahydrofolate (THF) pathway. In extracts of this organism, which contain a 40-kDa corrinoid protein, methyl transfer from CH_3-THF to mercury is enzymatically catalyzed (5). The importance of a corrinoid protein as a methyl transfer intermediate in the acetyl coenzyme A (CoA) formation pathway of *Clostridium thermoaceticum* (13) and other anaerobes (28) suggested that enzymes of this pathway are responsible for generation and transfer of the methyl moiety to Hg^{2+} by *D. desulfuricans* LS. The enzymes of this pathway include a methyltransferase and CO dehydrogenase/acetyl-CoA synthase in addition to formate dehydrogenase, the THF pathway enzymes, and the corrinoid protein. We report here on levels of these enzymes in *D. desulfuricans* and discuss their relationship to Hg^{2+} methylation.

MATERIALS AND METHODS

Culture, culture conditions, and extracts. *D. desulfuricans* LS was isolated and maintained as previously described (8). Because the presence of a contaminant at even a low level could cause misleading results, the purity of the culture was checked frequently and rigorously with both microscopic examination and a $FeSO_4$-containing diagnostic medium on which non-sulfate-reducing contaminants are conspicuous by their failure to form black haloes around colonies (26). Cells grown on Postgate's lactate-sulfate medium (26) were harvested, and extracts were prepared as previously described (5). Cell protein was measured by the Bradford method (3).

Incorporation of ^{14}C into methylmercury. [^{14}C]formate (specific activity, 52 mCi/mmol) and $H^{14}CO_3^-$ (specific activity, 9.2 mCi/mmol) were obtained from Amersham Inc. (Arlington Heights, Ill.). ^{14}C incorporation from formate was measured with cells growing on Postgate's medium C (26), modified by replacement of lactate with formate (50 mM). During exponential growth, 18.27 μCi of [^{14}C]formate and 0.092 mM $HgCl_2$ were added simultaneously. After 2 days at 37°C, the methylmercury produced was extracted (22) and quantified by gas chromatography (4). Incorporation of ^{14}C was measured by liquid scintillation counting of subsamples of the methylmercury extract. ^{14}C incorporation from $H^{14}CO_3^-$ was carried out similarly, by addition of $H^{14}CO_3^-$ and $HgCl_2$ to cells growing on lactate-sulfate medium.

Enzyme assays. The enzymes assayed, the reactions they catalyze in vivo, and the assay methods used are summarized in Table 1. All assays, other than those for hydrogenase, CO dehydrogenase, and acetyl-CoA synthase, were carried out in nitrogen-sparged solutions under a nitrogen atmosphere.

Hydrogenase was assayed by monitoring the reduction of methyl viologen (MeV; ε_{603} = 11.3 mM^{-1} cm^{-1}) (12) in a semi-micro cuvette tightly closed with a 3-mm silicon septum. The reaction mixture contained 20 mM MeV and 20 mM 2-mercaptoethanol in 40 mM 3-(4-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.0. This mixture was sparged, via needles inserted through the septum, for 10 min with N_2 (control) or H_2 (assay; solubility of H_2 in H_2O at 37°C, 0.794 mM). After equilibration at 37°C for 5 min, cell extract (0.1 mg of protein per ml of reaction mixture) was added and the A_{603} was monitored, the endogenous rate with N_2 being subtracted from the rate with H_2. Reduction of 2 μmol of MeV equals

* Corresponding author. Phone: (908) 932-9739. Fax: (908) 932-8965.

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TABLE 1. Physiological reactions, assay methods, and references for various enzyme activities measured in *D. desulfuricans* LS extracts

Enzyme and reaction catalyzed[a]	Assay method	Reference
Hydrogenase		12
H_2 + 2 cytochrome $c_3 \leftrightarrow$ 2 cytochrome c_{3red} + $2H^+$	H_2 + 2MeV $\rightarrow 2H^+$ + $2MeV_{red}$	
Carbon monoxide dehydrogenase		29
CO + 2Fd + $H_2O \leftrightarrow HCO_3^-$ + $2Fd_{red}$ + H^+	CO + 2MeV + $H_2O \rightarrow HCO_3^-$ + $2MeV_{red}$ + H^+	
Serine hydroxymethyltransferase		35
Serine + THF $\leftrightarrow$ glycine + methylene-THF	Serine + THF $\rightarrow$ glycine + methylene-THF; methylene-THF + $NADP^+ \rightarrow$ methenyl-THF + NADPH + H^+	
Formate dehydrogenase		34
$HCOO^-$ + 2 cytochrome $c_3 \rightarrow CO_2$ + 2 cytochrome c_{3red} + H^+	$HCOO^-$ + 2MeV $\rightarrow HCO_3^-$ + $2MeV_{red}$	
N^{10}-formyl-THF synthetase		27
$HCOO^-$ + THF + ATP $\rightarrow$ 10-formyl-THF + ADP + P_i	$HCOO^-$ + THF + ATP $\rightarrow$ 10-formyl-THF + ADP + P_i 10-Formyl-THF + $H^+ \rightarrow$ methenyl-THF + H_2O	
N^5,N^{10}-methenyl-THF cyclohydrolase		21
10-Formyl-THF + $H^+ \leftrightarrow$ methenyl-THF + H_2O	Methenyl-THF + $H_2O \rightarrow$ 10-formyl-THF + H^+	
N^5,N^{10}-methylene-THF dehydrogenase		21
Methylene-THF + $NADP^+ \leftrightarrow$ methenyl-THF + NADPH	Methylene-THF + $NADP^+ \leftrightarrow$ methenyl-THF + NADPH	
N^5,N^{10}-methylene-THF reductase		6
Methylene-THF + $2Fd_{red}$ + $2H^+ \leftrightarrow$ methyl-THF + 2Fd	Methyl-THF + 2MeV $\rightarrow$ methylene-THF + $2MeV_{red}$ + $2H^+$	
Acetyl-CoA synthase		31
CH_3-THF + CO + CoA-SH $\leftrightarrow CH_3COSCoA$ + THF	[^{14}C]CH_3-THF + CO + CoA-SH $\rightarrow$ [^{14}C]$CH_3COSCoA$ + THF	

[a] The subscript $_{red}$ signifies reduced.

oxidation of 1 μmol of H_2. Similar procedures, with the appropriate substrate being substituted for H_2, were followed for the assay of CO dehydrogenase (solubility of CO at 37°C, 0.707 mM) (29), formate dehydrogenase (34), and methylene-THF dehydrogenase (21).

Serine hydroxymethyltransferase was monitored by the appearance of NADPH (ε_{340} = 6.22 mM^{-1} cm^{-1}) when the 5,10-methylene-THF was oxidized to methenyl-THF in the presence of 5,10-methylene-THF dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) (35).

10-Formyl-THF synthetase was assayed by conversion of the product to 5,10-methenyl-THF (ε_{350} = 24.9 mM^{-1} cm^{-1}) upon addition of acid (27). 5,10-Methenyl-THF cyclohydrolase, which physiologically catalyzes the cyclization of 10-formyl-THF to 5,10-methenyl-THF, was assayed in the reverse direction, by observing the decrease in A_{350} (21). The substrate 5,10-methenyl-THF was prepared from 5-formyl-THF (Fluka Chemie AG, Buchs, Switzerland) by incubation in 0.1 M HCl for 4 h (32).

5,10-Methylene-THF dehydrogenase activity was measured by oxidation of 5,10-methylene-THF with reduction of $NADP^+$ to NADPH. 5,10-Methylene-THF was prepared by the nonenzymatic reaction of HCHO with THF (17).

Acetyl-CoA synthase activity was measured by transfer of $^{14}CH_3$ from CH_3-THF to acetyl-CoA. The reaction mixture consisted of 10 μmol of 2-mercaptoethanol, 1 μmol of CoA-SH, and cell extract (0.35 mg of protein) in 0.5 ml of 50 mM MOPS buffer, pH 7.0, in a 1.5-ml glass vial capped with a Teflon-lined serum stopper. The vial (at 37°C) was flushed with H_2 for 5 min and then with CO for 5 min; on occasion, MeV (10 μM) was added at the beginning of H_2 flushing. The reaction was initiated by the addition of 10 nmol of $^{14}CH_3$-THF (specific activity, 55 mCi/mmol; Amersham), and the mixture was incubated at 37°C. The reaction was stopped at various times (0 to 30 min) by heating at 95°C for 5 min. Subsequently, precipitated protein was removed by centrifugation, and when necessary, samples were frozen and stored at −20°C until analysis. The $^{14}CH_3COSCoA$ produced was separated from $^{14}CH_3$-THF by high-performance liquid chromatography using a Partisphere 5-μm-particle-size C_{18} column (Whatman Inc., Clifton, N.J.) connected to a 254-nm detector. Samples were thawed, carrier acetyl-CoA (5 μl, 0.5 μmol) was added to a 50-μl aliquot, and the aliquot was injected onto the column via a 20-μl injection loop. The column was eluted with 13% methanol in 10 mM potassium phosphate buffer, pH 5.5, at a flow rate of 1.0 ml/min. Under these conditions the CoA-SH, acetyl-CoA, and CH_3-THF standards eluted at 4.5, 7.2, and 11.0 min, respectively. The acetyl-CoA peak (about 1 ml) was collected, and its radioactivity was measured.

RESULTS AND DISCUSSION

Incorporation of radiolabeled carbon into methylmercury. Label from $H^{14}COO^-$ was incorporated into CH_3Hg^+ with at least 50% retention of specific activity (Table 2). The initial specific activity of $H^{14}COO^-$ is a maximum estimate, since there was also a rapid exchange with $H^{12}CO_3^-$ already formed (15); thus, the retention of specific activity may have been even greater. Incorporation of label from $H^{14}CO_3^-$ into CH_3Hg^+ was lower and probably occurred via exchange with $HCOO^-$. The high retention of specific activity of label from formate, like that from serine, indicates that the methyl group is derived from the C_1-THF pathway. Label from the C-3 of pyruvate could be incorporated into CH_3Hg^+ via serine, as previously

TABLE 2. ^{14}C incorporation into methylmercury from various substrates by *D. desulfuricans* LS[a]

Labeled substrate	Sp act (mCi/mmol) of:		% Retention of sp act
	Substrate	Methylmercury	
[1-^{14}C]pyruvate	9.2	1.0	10.9
[3-^{14}C]pyruvate	17.2	3.6	20.9
[3-^{14}C]serine	56.0	53.2	95.0
$H^{14}COO^-$	0.018[b]	0.009	50.0
$H^{14}CO_3^-$	9.2	0.33	3.6

[a] Data for labeled pyruvate and serine are from Berman et al. (2).
[b] The ^{14}C added (original specific activity, 52 mCi/mmol) was diluted by unlabeled formate in the medium.

TABLE 3. Levels of certain enzymes in *D. desulfuricans* LS extracts

Enzyme	Maximum sp act (U[a])	Apparent K_m[b] (mM)	Levels in acetogens (U[a])	
			C. thermoaceticum[c]	*C. thermoautotrophicum*[d]
Hydrogenase	0.081	ND[e]	2.2[f]	0.16
Carbon monoxide dehydrogenase	0.178	ND	13.0[g]	3.8
Serine hydroxymethyltransferase	0.042	0.116 (serine)	ND	ND
Formate dehydrogenase	0.004	0.675 ($HCOO^-$)	0.48	0.072
Formyl-THF synthetase	0.002	0.271 ($HCOO^-$)	12.5	11.1
Methenyl-THF cyclohydrolase	0	ND	1.3	0.63
Methylene-THF dehydrogenase	0.011	0.290 (CH_2=THF)	1.8	0.82
Methylene-THF reductase	0.029	0.342 (CH_3-THF)	0.58	ND

[a] U, micromoles of product formed or substrate used per minute per milligram of cell protein (V_{max} at saturation with the substrate shown in parentheses in the next column).
[b] The apparent K_ms were determined for the substrate shown in parentheses. Other substrates were at saturating levels.
[c] Data are from Andreesen et al. (1), except as otherwise indicated.
[d] Data are from Clark et al. (7). Shown are activities of cells grown on fructose.
[e] ND, not determined.
[f] Data are from Drake (10).
[g] Data are from Ragsdale et al. (29).

suggested (2), or via reversal of the acetyl-CoA synthase reaction (33).

Levels of enzymes of the acetyl-CoA pathway. The levels of enzymes of the acetyl-CoA pathway found in extracts of *D. desulfuricans* LS are shown in Table 3, along with levels of the enzymes in extracts of two acetogens. The levels of activity we observed for enzymes of the THF pathway were much lower (20- to 6,000-fold) than the ones reported for acetogens. Either their importance in *Desulfovibrio* metabolism is small, supplying methyl groups for biosynthesis and for the minor production of methane from pyruvate reported in sulfate-reducing bacteria (25, 33), or our assay conditions were not even close to optimal. Dramatic improvement in activity of crude extracts has been achieved in the course of studies on *C. thermoaceticum* (31). Similarly, much higher activities of enzymes of the C_1-THF pathway in *Desulfobacterium autotrophicum* were observed when the tetraglutamyl tetrahydropterin found in this organism was used instead of THF (20). The enzyme activities of the acetyl-CoA pathway in *D. desulfuricans* may undergo a similar upward revision when the cofactor requirements are fully understood and the assay conditions are optimized.

Among the non-THF enzymes of the pathway, the CO dehydrogenase and formate dehydrogenase activities were about 5% of those reported for *Clostridium thermoautotrophicum* (7) and 10- to 100-fold lower than those reported for acetate-oxidizing sulfate reducers (33). CO dehydrogenase has long been known in *D. desulfuricans* (40). *Desulfovibrio vulgaris* Madison was shown to produce small amounts of CO from lactate or pyruvate, to consume it by the end of the fermentation, and to grow on CO at low concentrations as the electron donor, with acetate as a carbon source (23). However, when *D. vulgaris* Marburg, *Desulfobulbus propionicus* MUD, and *Desulfotomaculum thermobenzoicum* TSB were screened, only *D. thermobenzoicum* TSB showed CO dehydrogenase activity (38).

We found that the activity of serine hydroxymethyltransferase in *D. desulfuricans* LS was relatively high, 0.042 μmol min^{-1} mg of cell $protein^{-1}$. Comparable values were reported for *Escherichia coli* (36).

We did not detect any 5,10-methenyl-THF cyclohydrolase activity in *D. desulfuricans* LS extracts. It is possible that this organism contains this enzyme activity at levels below the detection limit under the assay conditions used or that the organism does not need the activity, since the reaction can also proceed nonenzymatically.

The 5,10-methylene-THF dehydrogenase of *D. desulfuricans* LS used $NADP^+$ as the substrate in preference to NAD^+ (data not shown), as is the case in most acetogenic organisms, except *Clostridium formicoaceticum* and *Acetobacterium woodii*, in which the dehydrogenases use NAD^+ (28).

Acetyl-CoA synthase. We did not detect any acetyl-CoA synthase activity under an atmosphere of 100% CO or at a CO:H_2 ratio of 1:1. However, incubation under H_2 followed by CO, for 5 min each, allowed acetyl-CoA synthesis by the cell extract (Table 4). In the acetyl-CoA synthase system of acetogens, reduction of the corrinoid/FeS protein to the Co^{1+} level is required for it to accept a methyl group from CH_3-THF (30). The proximate reductant is presumably ferredoxin, reduced by either pyruvate:ferredoxin oxidoreductase or H_2. In the present assay, we avoided pyruvate because of the possibility of product inhibition of the acetyl-CoA synthase reaction by acetyl-CoA from the pyruvate:ferredoxin oxidoreductase reaction. Instead, we used H_2 as the reductant. However, the hydrogenases of sulfate-reducing bacteria are inhibited by CO (24), and thus we observed activity only when we reduced the system with H_2 before adding the substrate CO. The need for reduction of the corrinoid protein was supported by the 4.6-fold enhancement of the acetyl-CoA synthase reaction by reduced MeV supplied as an artificial analog of ferredoxin (Table 4). Use of ferredoxin itself, which increases the rate of acetyl-CoA synthesis from CH_3-THF and CO by purified enzymes from *C. thermoaceticum* (31) and is a fourfold-better electron acceptor than MeV for *C. thermoaceticum* CO dehydrogenase (29), might have stimulated the reaction further. While the highest rate of acetyl-CoA synthesis observed by us was 100-fold less than calculated rates of acetate formation

TABLE 4. Effects of MeV, propyl iodide, mercuric ion, and *p*-hydroxymercuribenzoate on acetyl-CoA synthase activity of cell extract of *D. desulfuricans* LS

Treatment	Concn (mM)	Sp act (nmol min^{-1} mg of $protein^{-1}$)	Relative %
H_2, then CO	0.71 (CO)	0.180	100
+ methyl viologen	0.01	0.830	461
+ propyl iodide	0.5	0.005	2.8
+ $HgCl_2$	0.7	0.029	16.1
+ *p*-hydroxymercuribenzoate	1.0	0.084	46.7

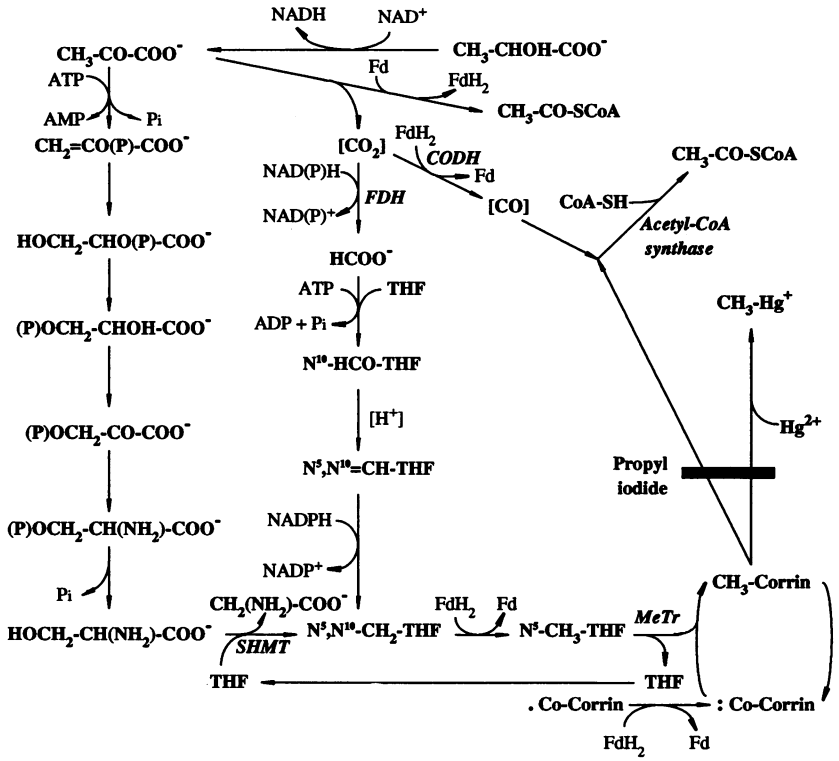

FIG. 1. Proposed metabolic pathway involved in mercury methylation by *D. desulfuricans* LS. The propylation of corrinoid by propyl iodide blocked both acetyl-CoA synthesis and mercury methylation. Fd, ferredoxin; *FDH*, formate dehydrogenase; *CODH*, CO dehydrogenase; *MeTr*, methyltransferase; *SHMT*, serine hydroxymethyltransferase.

through this pathway in *C. thermoaceticum* (66 to 99 nmol min^{-1} mg of cell $protein^{-1}$) (31), it was within an order of magnitude of rates originally observed for in vitro formation of acetate from CH_3-THF and pyruvate with acetogens (8 nmol min^{-1} mg^{-1} with *C. thermoaceticum* extracts [11], 5.4 nmol min^{-1} mg^{-1} with extracts of *C. thermoautotrophicum* grown on fructose [7], and 0.15 nmol min^{-1} mg^{-1} with *Acetobacterium woodii* extracts [37]). Comparable rates for $CH_3COSCoA$ formation from CH_3-THF and CO are not available because studies were done with partially purified fractions rather than crude extract (13), but a rate calculated from observations with purified proteins was 80 nmol min^{-1} mg^{-1} (CO dehydrogenase) or 1.6 nmol min^{-1} mg^{-1} (total protein) if CO dehydrogenase constitutes 2% of the cell protein (31).

Implication of acetyl-CoA pathway in methylmercury synthesis. Propyl iodide, known to inhibit methylmercury formation (2), similarly inhibited acetyl-CoA synthase in our experiments (Table 4), as expected from competition for the corrinoid. We also found mercuric ion to be an effective inhibitor (84% inhibition at 0.7 mM), as expected if it is competing with acetyl-CoA synthase for methyl groups. This is probably not simple inhibition of enzymes with SH groups, since *p*-hydroxymercuribenzoate was a much less effective inhibitor. CO dehydrogenase of *D. desulfuricans* has been reported not to be inhibited by 1 mM Hg^{2+} (41).

On the basis of inhibition by propyl iodide of the synthesis from CH_3-THF of both acetyl-CoA and CH_3Hg^+, the inhibition of acetyl-CoA synthesis by Hg^{2+}, and the presence of the enzymes of the acetyl-CoA pathway, we propose that CH_3Hg^+ is formed by this pathway (Fig. 1). The CH_3 group of CH_3-THF can arise from formate, from C-3 of serine, or from C-3 of pyruvate via serine (2) and be transferred by a methyltransferase to cobalt in a corrinoid protein, the last intermediate before acetyl-CoA synthase in acetyl-CoA synthesis. Alternatively, the CH_3 group could arise from C-3 of pyruvate by its oxidation to acetyl-CoA, cleavage of acetyl-CoA by CO dehydrogenase, and transfer to the corrinoid protein.

Many sulfate-reducing bacteria utilize the acetyl-CoA pathway in their metabolism (33, 39). Among them are *Desulfovibrio baarsii*, *Desulfotomaculum acetoxidans*, *Desulfobacterium autotrophicum*, *Desulfococcus multivorans*, and *Desulfosarcina variabilis*. Most of these strains use the acetyl-CoA pathway in reverse, for oxidation of acetate. Only in *D. baarsii* has it been firmly demonstrated that the pathway operates in the direction of acetyl-CoA synthesis (14). On the basis of the 16S rRNA sequence, this bacterium has been proposed to be only distantly related to other *Desulfovibrio* species (9). The higher retention of ^{14}C specific activity in CH_3Hg^+ from $H^{14}COO^-$ and [3-^{14}C]serine than from [3-^{14}C]pyruvate suggests that in *D. desulfuricans* LS the pathway is operating in the direction of acetyl-CoA synthesis. If the methyl corrinoid were formed from pyruvate via reversal of the acetyl-CoA synthase reaction (see Fig. 1), then [3-^{14}C]pyruvate would yield $^{14}CH_3Hg^+$ at a high specific activity.

Two other pathways which could lead to mercury methylation are dimethyl sulfide production (1a, 18) and utilization (36a) and methionine biosynthesis (19). Dimethyl sulfide and methanethiol were formed from methyl groups of methoxylated aromatic substrates by transfer to sulfide, the aromatic nucleus being converted quantitatively to acetate (1a). This involves transfer of the methyl group between electron-rich oxygen and sulfur atoms, in contrast to the transfer to Hg^{2+}

studied here. We detected no production of dimethyl sulfide by *D. desulfuricans* LS (data not shown). A report (36a) appearing after this paper was originally submitted described utilization of methanethiol and dimethyl sulfide by sulfate-reducing bacteria related to the genus *Desulfotomaculum*. The methyl groups were converted quantitatively to CO_2. No relationship to the acetogenic pathway or transfer of methyl groups to other acceptors was indicated.

In *Neurospora crassa*, mercury methylation was stimulated by the addition of L-cysteine or DL-homocysteine but was inhibited by the addition of L-methionine (19). From this evidence it was suggested that mercury methylation could be a side reaction of methionine biosynthesis. We found no effect on mercury methylation of inclusion of L-methionine (up to 5 mM) in cultures of *D. desulfuricans* LS (data not shown). On the basis of these results, we discounted participation of the dimethyl sulfide and methionine pathways in mercury methylation by *D. desulfuricans* LS.

The question remains: why is mercury methylation a feature of only some sulfate reducers, with 100- to 1,000-fold-lower levels of enzymes of the acetyl-CoA pathway than acetogens, methanogens, and acetate-oxidizing sulfate reducers? Presumably the answer is that either only these few sulfate reducers possess a specific enzyme required for methyl transfer to mercury or only their version of an enzyme of more general occurrence is capable of recognizing Hg^{2+} as an alternate substrate.

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