Biochemical Origins of Lactaldehyde and Hydroxyacetone in *Methanocaldococcus jannaschii*†

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ABSTRACT: The biochemical routes for the metabolism of methylglyoxal and the formation of lactaldehyde and hydroxyacetone in *Methanocaldococcus jannaschii* have been established. The addition of methylglyoxal and NADH, NADPH, $F_{420}H_2$, or DTT to a *M. jannaschii* cell extract stimulated the production of both lactaldehyde and hydroxyacetone. Using appropriately labeled NADH, NADPH, and $F_{420}H_2$, hydride transfer was only observed from $F_{420}H_2$ to lactaldehyde. It was shown that cell extracts of this Archaea readily catalyzed the $F_{420}H_2$-dependent reduction of methylglyoxal to lactaldehyde, a precursor of the lactate found in coenzyme F_{420}. This conversion was established by measuring the incorporation of deuterium from (5*RS*)[5-$^2H_1$]$F_{420}H_2$ into the C-2 position of the formed lactaldehyde. In vivo generated (5*R*)[5-$^2H_1$]$F_{420}H_2$ was also found to incorporate deuterium into lactaldehyde. The experimental data indicated that the *pro-R* hydrogen of $F_{420}H_2$ was transferred during the reduction. The stereochemistry of this transfer was opposite from that observed for all other known enzyme-catalyzed hydride-transfer reactions involving F_{420}. [1,3,3,3-$^2H_4$]-Methylglyoxal was incorporated into lactaldehyde and hydroxyacetone as an intact unit during this reduction with the occurrence of some deuterium exchange. The exchange observed during this incorporation into lactaldehyde was substantially more than the exchange observed during the incorporation into the hydroxyacetone. The hydroxyacetone was derived directly from methylglyoxal, with the hydrogen for the reduction being derived from water. Hydroxyacetone was also readily formed by the condensation of pyruvate with formaldehyde. The product of the MJ0663 gene was shown to catalyze this condensation reaction.

Methylglyoxal has recently been shown to function as a central metabolite in the biosynthesis of aromatic amino acids in the methanogenic Archaea *Methanocaldococcus jannaschii* (*1, 2*). Methylglyoxal was also proposed to be a precursor to L-lactaldehyde, the precursor of the lactate moiety present in coenzyme F_{420} (*3*). Methylglyoxal was found to occur at a concentration of 0.07 mM in cell extracts of this methanogen, and its concentration increased to 2.1 mM after the cell extracts were incubated with 10 mM fructose-1,6-bisphosphate. It was proposed that the methylglyoxal arose both chemically and enzymatically from glyceraldehyde-3-P and dihydroxyacetone-P generated by the aldolase-catalyzed cleavage of the fructose-1,6-bisP (*1*). Hydroxyacetone and hydroxyacetone-P were also found in cell extracts of this methanogen at a concentration of 5 and 9 μM, respectively (*2*). The simplest route to lactaldehyde or hydroxyacetone would be the NAD(P)H-dependent reduction of either the C-2 or the C-1 carbonyl of methylglyoxal, respectively. Here, I show that neither lactaldehyde nor hydroxyacetone arose by the direct hydride transfer from a pyridine nucleotide to methylglyoxal. The reaction generating L-lactaldehyde in *M. jannaschii* in fact involves a hydride-mediated $F_{420}H_2$-dependent reduction of the C-2 of methylglyoxal. The data indicate that the C-5 *pro-R* hydrogen of $F_{420}H_2$ is transferred in the reduction of methylglyoxal to lactaldehyde. The stereochemistry of this transfer was opposite from that observed for all other known enzyme-catalyzed hydride-transfer reactions involving $F_{420}H_2$. The biosynthesis of hydroxyacetone can proceed by either a $F_{420}H_2$-dependent reduction at C-1 of methylglyoxal that does not involve a hydride transfer to methylglyoxal or the condensation of formaldehyde with pyruvate catalyzed by the product of MJ0663 gene.

MATERIALS AND METHODS

Chemicals. Pyruvate, formaldehyde, NAD, NADP, NADH, NADPH, *O*-(4-nitrobenzyl)hydroxylamine·HCl, NaB^2H_4, ^2H_2O (99.9 atom % D), and methylglyoxal (40% solution in water) were obtained from Sigma-Aldrich. [U-$^{13}C_3$]-Pyruvate and $^2H_2$-paraformaldehyde were obtained from Cambridge Isotopes. F_{420} was extracted from *M. jannaschii* cells (*4*) and purified as described below.

Synthesis of (4RS)-[4-$^2H_1$]-NADH and (4RS)-[4-$^2H_1$]-NADPH. NAD or NADP (5 mg) was dissolved in 0.4 mL of ^2H_2O, and solid $NaHCO_3$ (<0.5 mg) was added to produce a pH ~7 solution. The samples were then degassed by sparging with argon gas, and 5 mg of sodium dithionite was added to produce a yellow solution. The samples were left at room temperature, and portions were removed as a function of time and diluted in water. Their absorbance spectra was recorded to follow the course of the reduction.

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The absorbance at 340 nm was found to continue to increase in the reaction mixture for about 2 h and to give a final A_{340}/A_{260} ratio of 0.35, indicating that more than 80% of the samples had been reduced at C-4 of the nicotinamide ring. All visible yellow color was gone at this time. The UV spectral data also indicated the absence of C-2 and C-6 reduced species that occur with borohydride reductions (*5*). Because this reduction was not stereospecific, it was expected that both the C-4 *pro-R* and *pro-S* deuteriums of both [4-$^2H_1$]-NADH and [4-$^2H_1$]-NADPH were each present in equal amounts. ES−mass spectral analysis showed that each molecule had incorporated one atom of deuterium to an extent of >98%. ES−MS data were obtained by direct infusion of the sample diluted in water using a MicroMass 3200 Q trap LC−mass spectrometer. NAD solutions (~ 0.5 μg/mL) showed $MH^+ = m/z$ 664.5 and $MNa^+ = m/z$ 686.4 in positive-ion mode and $(M - H)^- = m/z$ 662.5 in negative-ion mode. NADH showed $(M + 2Na)^+ = m/z$ 710.3 and $(M + 3Na)^+ = m/z$ 731.8 in positive-ion mode and $M^- = m/z$ 664.1 in negative-ion mode. (4*RS*)-[4-$^2H_1$]-NADH showed $(M + 2Na)^+ = m/z$ 711.3 and $(M + 3Na)^+ = m/z$ 733.3 in positive-ion mode and $(M - H + Na)^- = m/z$ 687.1 in negative-ion mode. The NADPH showed $(M + 4Na)^+ = m/z$ 834.2 in positive-ion mode. Thus, the pyridine nucleotides reduced under conditions where one hydrogen was incorporated increased by m/z 1 and those where a deuterium was incorporated increased by m/z 2. The dominance of the sodiated ions in the synthetic compounds is a result of the presence of sodium in the samples from the sodium dithionite.

Isolation of M. jannaschii F_{420} and Synthesis of (5RS)-[5-$^2H_1$]$F_{420}H_2$. Frozen *M. jannaschii* cells (*4*) (1.4 g) were suspended in 3 mL of water, and 6 mL of methanol was added. The sample was heated at 100 °C for 5 min and centrifuged (14000*g* for 5 min). Extraction of the pellet was repeated with 70% methanol (3 mL), and the combined extracts were concentrated to 0.5 mL by evaporation with a stream of nitrogen as the samples were heated. The resulting sample was placed on a DEAE-Sephadex column (1.5 × 10 cm) and eluted with a linear gradient of NH_4HCO_3 from 0 to 2 M (total volume of 400 mL). Fractions containing the single eluting F_{420} peak, identified by its 420 nm absorbance and strong fluorescence, were combined, and NH_4HCO_3 was evaporated with a steam of nitrogen gas. The sample was dissolved in 100 μL of ^2H_2O to produce a 4.0 mM solution of the F_{420} (*6*), from which the different labeled samples of F_{420} were prepared. For the preparation of (5*RS*)-[5-$^2H_1$]$F_{420}H_2$, a 20 μL portion of this solution was placed in a vial flushed with argon and 0.1 mg of [$^2H_4$]$NaBH_4$ was added. After 5 min at room temperature, the yellow color of oxidized F_{420} was gone. After an additional 30 min, anaerobic 1 M HCl was added to pH of 4.0 to destroy the borohydride, and the sample was adjusted back to pH 7.0 with anaerobic 1 N NaOH and evaporated to dryness with a stream of nitrogen gas. To this sample, under argon, was added 100 μL of cell extract and 10 μL of 0.1 M methylglyoxal, and the sample was incubated as described below.

In Vivo Synthesis of (5R)-[5-$^2H_1$]$F_{420}H_2$. For the preparation of [5-$^2H_1$]F_{420}, 20 μL of the above 4 mM solution of F_{420} isolated from *M. jannaschii* was reduced with borodeuteride as described above but under air and in the presence of 0.3 mM riboflavin. The riboflavin catalyzed the rapid air oxidation of the reduced (5*RS*)-[5-$^2H_1$]$F_{420}H_2$ (*7*) to [5-$^2H_1$]F_{420}, which is reduced back to (5*RS*)-[5-$^2H_1$]$F_{420}H_2$ by the borodeuteride. After each cycle, the extent of deuteration of the F_{420} would thereby increase. After 1 h at room temperature, the borodeuteride was decomposed as described above and the sample was evaporated to dryness to remove the ^2H_2O. Control experiments under these same conditions using Fo produced [5-$^2H_1$]-Fo containing 80 atom % deuterium. To the evaporated sample was added 100 μL of cell extract, and the sample was incubated under hydrogen (30 psi) for 2 min at 70 °C. This initial incubation under hydrogen is expected to reduce [5-$^2H_1$]F_{420} to (5*R*)-[5-$^2H_1$]$F_{420}H_2$ catalyzed by the stereospecific F_{420}-dependent dehydrogenase (*8*). The product will contain the same enrichment of deuterium as present in [5-$^2H_1$]F_{420}. The sample was then flushed with argon, mixed with 10 μL of 0.1 M methylglyoxal, incubated for 15 min at 70 °C, and worked up as described below.

Synthesis and Analysis of [2-$^2H_1$]-Lactaldehyde and [1,3,3,3-$^2H_4$]-Lactaldehyde. [2-$^2H_1$]-D,L-Lactaldehyde was prepared by the reduction of pyruvic aldehyde dimethyl acetal with sodium borodeuteride as described in the first step of a previously described synthesis (*3*). For the synthesis of [1,3,3,3-$^2H_4$]-lactaldehyde, the pyruvic aldehyde hydrogens of pyruvic aldehyde dimethyl acetal were exchange-labeled in ^2H_2O containing 0.4 M NaO^2H for 2 h at room temperature. The produced [1,3,3,3-$^2H_4$]-pyruvic aldehyde dimethyl acetal was then reduced with sodium borohydride. The remaining steps were the same as previously described (*3*).

Synthesis and Analysis of [1,3,3,3-$^2H_4$]-Methylglyoxal. [$^2H_6$]-Acetone (48 μL) was added to 350 μL of ^2H_2O in a vial containing a magnetic stirring bar. A total of 111 mg of SeO_2 was added, and the vial was sealed under argon and stirred at 60 °C for 3 h (*9*). After the first minute of the reaction, the clear colorless sample turned red as Se precipitated. The sample was filtered to remove the larger pieces of Se and then worked up by passing the sample through a Dowex 1-8X acetate column to remove the selenite. In one procedure, all of the steps after the separation of the Se were performed with water as the solvent, and in the other procedure, ^2H_2O was used as the solvent. The methylglyoxal was purified by preparative thin-layer chromatography (TLC) with methyl acetate as the eluting solvent. In this solvent, the R_f of methylglyoxal was 0.53. The di-*O*-(4-nitrobenzyl)-hydroxylamine derivative was prepared as described below and analyzed by direct insertion EI−mass spectrometry (70 eV) using a VG-70SE mass spectrometer. The mass spectra of the unlabeled methylglyoxal showed an intense molecular ion at m/z 372 with an intense fragment ion at M − 152 (*p*-nitrobenzyl-O). The ratios of the abundances for the M^+, $M^+ + 1$, and $M^+ + 2$ were 100, 19, and 3.1%, respectively.

The distribution of deuterium in the labeled sample of methylglyoxal, worked up, derivatized in water, and measured from the M^+ of the derivative, was 2.6% $^2H_1$, 11.9% $^2H_2$, 27.1% $^2H_3$, and 58.3% $^2H_4$. The labeled methylglyoxal sample, worked up in ^2H_2O and derivatized in ^2H_2O, was 6.7% $^2H_2$, 29.2% $^2H_3$, and 64.2% $^2H_4$, indicating that little exchange of the labeled methylglyoxal occurred during sample workup or derivative formation.

Cell Extracts of M. jannaschii. Cell extracts of *M. jannaschii* were prepared by sonication under argon, stored as previously described under anaerobic conditions at

−20 °C, and had ~30 mg/mL protein (*1*). The buffer used in the extraction was 50 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/K^+,[1] 10 mM $MgCl_2$, and 20 mM D,L-dithiothreitol (DTT) at pH 7.5 kept under argon.

Cloning and Expression of MJ0663-Derived Protein in Escherichia coli. The *M. jannaschii* gene at locus MJ0663 (Swiss-Prot accession number Q58077.1) was amplified by polymerase chain reaction (PCR) from genomic DNA using oligonucleotide primers synthesized by Invitrogen: forward (5′-GGTGGTCATATGGTGGGTGGTAATATTAAATTCT-TAGAAGC-3′) and reverse (5′-GATCGGATCCTTAAATA-TTTGGTTTAGGCAATGGCTCG-3′). PCR was performed as described previously (*10*) using a 55 °C annealing temperature. The primers introduced a *Nde* I and *Bam*H I site at the 5′ and 3′ ends, respectively. The amplified PCR product was purified by QIAQuick spin column (Invitrogen), digested with restriction enzymes *Nde* I and *Bam*H I, and then ligated into the compatible sites in plasmid pT7-7 (Novagen) by bacteriophage T4 DNA ligase (Invitrogen) to make the recombinant plasmid pMJ0663. DNA sequences were verified by dye−terminator sequencing at the University of Iowa DNA facility. The resulting plasmid, pMJ0663, was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) cells. The transformed cells were grown in Luria–Bertani medium (200 mL; Difco) supplemented with 100 μg/mL ampicillin at 37 °C with shaking until they reached an absorbance at 600 nm of 1.0. Adding lactose to a final concentration of 28 mM induced the recombinant protein production. After an additional 2 h of incubation, the cells were harvested by centrifugation (4000*g* for 5 min) and frozen at –20 °C. Induction of the desired protein was confirmed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis of total cellular proteins. Proteins were extracted from the *E. coli* proteins as previously described (*11*). Large amounts of the proteins were recombinantly expressed but most were found to be insoluble and not stable at 80 °C, a temperature at which most *M. jannaschii* proteins are stable. As a result, the protein was only subjected to a 58 °C heat treatment to denature the *E. coli* enzymes.

Incubation of M. jannaschii Cell Extracts with Precursors. Anaerobically prepared cell extracts (25–100 μL) were added to 1.5 mL septated vials flushed with argon or H_2 atmosphere, and 5–10 μL amounts of 0.05 or 0.1 M aqueous anaerobic solutions of the substrates were added to give the final stated concentrations. After incubation for the indicated times at 70 °C, 0.4 volume of 0.1 M *O*-(4-nitrobenzyl)hydroxylamine·HCl and 0.8 volume of 1 M NaOAc buffer at pH 4.0 were added and the samples were heated at 100 °C for 15–20 min. Methanol (4.8× the volume of cell extract used) was added, and the samples were centrifuged (14000*g*, 5 min). The clear soluble material was separated from the pellet and concentrated with a stream of nitrogen gas, and the contained individual derivatives were purified by preparative TLC using methylene chloride/methyl acetate (1:1, v/v) as the eluting solvent. In some cases, the derivatives were extracted from the samples with methylene chloride prior to preparative TLC purification. The area of the plate containing the desired derivatives, made visible by exposing the TLC plates to UV light, was scraped from the TLC plate, and the derivative was eluted with methyl acetate. Absorbance at 270 nm of these solutions were used to quantitate the amount of the material recovered using $\varepsilon = 9490\ M^{-1}\ cm^{-1}$. The R_f values of *O*-(4-nitrobenzyl)hydroxylamine and the hydroxyacetone, lactaldehyde, and methylglyoxal *O*-(4-nitrobenzyl)hydroxylamine derivatives were 0.45, 0.56, 0.61, and 0.73, respectively. After evaporation of the methyl acetate and reaction of the residue with 10–20 μL of a tetramethylsilane (TMS) reagent consisting of a (9:3:1, v/v/v) mixture of pyridine, hexamethyldisilazane, and chlorotrimethylsilane, for 10 min at 100 °C, the sample was ready for gas chromatography−mass spectrometry (GC−MS) analysis. GC−MS analyses of the derivatized samples were obtained using a VG-70-SE gas chromatography–mass spectrometer operating at 70 eV and equipped with a RTX-5M5 column (0.32 mm by 30 m) that was programmed from 80 to 280 °C, at 8 °C/min. The retention time for the lactaldehyde and hydroxyacetone derivatives were 16.5 and 17.1 min, respectively. The methylglyoxal derivative did not elute from for the GC column under the conditions used.

RESULTS

Incubation of Cell Extracts with Methylglyoxal and Different Reducing Agents. Cell extracts prepared in extraction buffer in the presence and absence of DTT were incubated with methylglyoxal and different reducing agents to determine which was the most efficient in generating lactaldehyde and hydroxyacetone. The levels of lactaldehyde and hydroxyacetone in the incubated cell extracts were measured from the absorbance of the preparative TLC-purified *O*-(4-nitrobenzyl)hydroxylamine derivatives. The levels of lactaldehyde and hydroxyacetone increased by different amounts depending upon the reducing agent used (Table 1). Of the two the pyridine nucleotides used, NADPH produced a slightly larger increase in the amount of lactaldehyde and hydroxyacetone than NADH. Incubation under hydrogen increased the levels even further. Including 43 mM DTT in the incubation mixture had the largest increase in the total amount of lactaldehyde and hydroxyacetone, with the sum of both products accounting for 92% of the methylglyoxal added. Incubation with $F_{420}H_2$ under argon had the largest increase in the production of lactaldehyde. It is interesting that the amount of hydroxyacetone was more than the total amount of $F_{420}H_2$ added, indicating that either an additional source of reductant was present in the cell extract or another route to hydroxyacetone was occurring.

To test the possible involvement of a direct hydride transfer in the pyridine nucleotide reductions, cell extracts (100 μL) were incubated with methylglyoxal (7.4 mM) and (4*RS*)-[4-$^2H_1$]NADH (2.7 mM) or (4*RS*)-[4-$^2H_1$]NADPH (2.7 mM) under argon for 30 min at 70 °C and the produced lactaldehyde and hydroxyacetone were found to contain no deuterium. Because the deuterated pyridine nucleotide samples each had an equal mixture of the *pro-R* and *pro-S* C-4 deuterated stereoisomers, neither stereoisomer of NADH or NADPH served as a direct hydride donor for the generation of lactaldehyde or hydroxyacetone. To test the other hydride donor coenzyme, coenzyme F_{420}, a cell extract

[1] Abbreviations: LC−MS, liquid chromatography−mass spectrometry; ESI, electrospray ionization; DTT, D,L-dithiothreitol; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid).

Table 1: Levels of Lactaldehyde and Hydroxyacetone Generated in *M. jannaschii* Cell Extracts Incubated with Methylglyoxal with and without Different Reducing Agents

	experiment[a]	lactaldehyde (mM)	hydroxyacetone (mM)
1	control[b]	0.05	0.1
2	+methylglyoxal[b]	0.10	0.2
3	+methylglyoxal + 7.7 mM NADH[b]	0.21	1.6
4	+methylglyoxal + 7.7 mM NADPH[b]	0.35	2.0
5	+methylglyoxal + hydrogen[c]	0.65	5.1
6	+methylglyoxal[d]	0.05	1.8
7	+methylglyoxal + 1.5 mM added $F_{420}H_2$[d]	0.80	3.9
8	+methylglyoxal + 43 mM DTT[d]	0.27	7.7

[a] Cell extracts were incubated with methylglyoxal (8.7 mM) with different reductants, and at the end of the incubations, the *O*-(4-nitrobenzyl)hydroxylamine derivatives were prepared as described in the text. The formed derivatives were separated by TLC and quantitated by absorbance after adjusting for the absorbances from a methylglyoxal blank. All incubations were performed under an argon atmosphere, except for experiment 5, which was performed under hydrogen. [b] Cell extracts (25 μL) were prepared in extraction buffer containing ~10 mM DTT and were incubated for 30 min at 70 °C. [c] The cell extract (25 μL) was incubated for 20 min at 70 °C under hydrogen with 8 mM methylglyoxal. [d] These cell extracts (50 μL) were prepared with extraction buffer without DTT and were incubated for 15 min at 70 °C under argon with 8 mM methylglyoxal.

was incubated under hydrogen with (5*RS*)-[5-$^2H_1$]$F_{420}H_2$ and methylglyoxal. The produced lactaldehyde contained 48% of the molecules with a single deuterium, and the produced hydroxyacetone contained no deuterium (experiment 1 in Table 2). The presence of 52% of the molecules with no deuterium can be explained by the use of unlabeled $F_{420}H_2$ produced by the F_{420}-dependent hydrogenase and the presence some unlabeled lactaldehyde present in the extract. Incubation of a cell extract under argon with in vivo generated (5*R*)-[5-$^2H_1$]$F_{420}H_2$ and methylglyoxal produced lactaldehyde containing 30% of the molecules with a single deuterium and hydroxyacetone with no deuterium (experiment 2 in Table 2). Because the deuterium in the lactaldehyde in both experiments 1 and 2 was contained in both the *m*/*z* 117 and 253 fragment ions of the lactaldehyde derivative, then all of the deuterium was incorporated at C-2 (parts A and B of Figure 1). The occurrence of this electron impact induced rearrangement, and fragmentations required to generate the *m*/*z* 253 fragment ion were established from the mass spectra of the synthetic [2-^{2}H]-lactaldehyde derivative that showed 100% of the deuterium residing in both the *m*/*z* 117 and 253 fragment ions. Incubation of cell extract prepared with no DTT with 39 mM lactaldehyde for 15 min at 70 °C in air produced 1.4 mM of methylglyoxal, and 24 mM lactaldehyde was recovered from the incubation. This production of methylglyoxal was not observed when the incubation was performed under argon. This observation confirms the reversal of the $F_{420}H_2$ reduction of methylglyoxal reaction, likely made possible by the air oxidation of the formed $F_{420}H_2$.

Incubation of cell extracts containing 50% ^2H_2O with methylglyoxal under hydrogen led to the incorporation of the label into both lactaldehyde and hydroxyacetone each to different extents (experiment 3 in Table 2). Examination of the labeling in the fragments of the lactaldehyde derivative show that they are consistent with some deuterium residing at each of the positions of the lactaldehyde. The *m*/*z* 253 fragment ion that contains the C-2 hydrogen as well as the C-1 hydrogen has a deuterium distribution indicating that about 5% deuterium resided on C-2 and 5% deuterium resided on C-1. This result indicates that little deuterium was incorporated at C-2 during the reduction of methylglyoxal to lactaldehyde in this experiment. From the measured abundance of deuterium in the *m*/*z* 103 fragment ion of the hydroxyacetone derivative, most of the deuterium was incorporated at C-1. Thus, the hydrogen and deuterium from water were incorporated at the carbonyl group being reduced. Control experiments showed that neither lactaldehyde or hydroxyacetone exchanged their hydrogens with ^2H_2O either in cell extracts containing 50% deuterated water in extraction buffer under the incubation conditions used or when heated for 10 min at 100 °C (data not shown). Thus, the exchange likely occurred with methylglyoxal during its enzymatic reduction. Likewise, incubation of cell extracts with 10 mM hydroxyacetone produced no detectable lactaldehyde, and incubation with lactaldehyde produced no increase in the amount of hydroxyacetone present. These observations indicated the lack of a chemical (*12*) or enzymatic isomerization of hydroxyacetone to lactaldehyde.

Incubation of the cell extract with [1,3,3,3-$^2H_4$]-methylglyoxal under hydrogen produced labeled lactaldehyde and hydroxyacetone containing a distribution of deuterium similar to that present in the methylglyoxal used in the incubation. This showed that the methylglyoxal was incorporated as a intact unit (experiment 4 in Table 2). The total extent of enrichment in the produced lactaldehyde was less than the hydroxyacetone, and each was less than that observed in the methylglyoxal used. This is apparent by the decrease in the ratio of molecules with three compared to four deuterium atoms (experiment 4 in Table 2). This can be explained by a partial enzyme-catalyzed exchange during their reduction. This also explains the presence of molecules arising from methylglyoxal in deuterated water having more than one deuterium (experiment 3 in Table 2).

Testing Alternate Routes for Methylglyoxal Formation and Metabolism. Incubation of a cell extract with pyruvate and [$^2H_2$]-formaldehyde under argon produced labeled hydroxyacetone with two deuteriums at C-1 (experiment 1 in Table 3). The fact that the deuteriums were contained at C-1 was confirmed by the presence of the *m*/*z* 103 fragment ion containing two deuterium atoms. This ion had less deuterium than measured from the molecular ion because of some hydrogen scrambling either before or during the fragmentation. Also, incubation of the cell extract with methylglyoxal and [$^2H_2$]-formaldehyde produced hydroxyacetone containing two deuterium atoms at C-1 (experiment 2 in Table 3). This can be explained by the oxidation of the methylglyoxal to pyruvate and the condensation of the C-2 and C-3 $^{13}C_2$ unit of pyruvate with formaldehyde catalyzed by the MJ0663 encoded enzyme (see below). To confirm this, a cell extract was incubated with [U-$^{13}C_3$]-pyruvate and formaldehyde under argon and the generated hydroxyacetone had 35% of the molecules containing a $^{13}C_2$ unit as measured from the $M^+ - 15$ ion at *m*/*z* 281 containing all of the hydroxyacetone carbons. Because the *m*/*z* 103 fragment ion contained no excess ^{13}C, then the $^{13}C_2$ unit was at C-2 and C-3 as expected from the proposed mechanism of this reaction (Figure 2).

Table 2: Distribution and Extent of Deuterium Incorporation from Deuterated Labeled Precursors into Lactaldehyde and Hydroxyacetone by Cell Extracts of *M. jannaschii*

		lactaldehyde						hydroxyacetone					
			distribution of $^2H^b$ (%)						distribution of $^2H^b$ (%)				
	experiment[a]	ion (*m/z*)	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$	ion (*m/z*)	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$
1	methylglyoxal + 0.46 mM[c]	281	52	48	0.0			281	100				
	(5*RS*)-[5-$^2H_1$]$F_{420}H_2$[d] + H_2	117	54	46	0.0								
		253	52	48	0.0								
2	methylglyoxal + 0.46 mM[c]	281	70	30	0.0			281	100				
	(5*R*)-[5-$^2H_1$]$F_{420}H_2$[e] + H_2 and then argon		70	30	0.0								
			70	30	0.0								
3	methylglyoxal + 50% ^2H_2O + H_2	281	79.9	15.7	3.3	1.0		281	74.2	23.7	2.1	0.0	
		117	81.5	16.0	2.4			103	79	21			
		253	88.5	8.6	2.9								
4	[1,3,3,3-$^2H_4$]-methylglyoxal[f] + H_2	281	45.1	4.5	13	26.4	11	281	0.5	3.2	15.3	37.2	43.7
		253	46.8	53.2									

[a] Cell extracts (50 μL) were incubated 15 min at 70 °C with methylglyoxal (9.1 mM) and different reductants, and at the end of the incubations, the *O*-(4-nitrobenzyl)hydroxylamine derivatives were prepared as described in the text. [b] The atom percent deuterium distribution was calculated from the measured isotopic abundances after correcting for the natural isotopic abundances measured from unlabeled samples. The unlabeled samples contained the following isotopic abundances for the ion, ion + *m/z* 1, ion + *m/z* 2, and ion + *m/z* 3. Lactaldehyde *m/z* 281 ($M^+ - 15$), 100, 17.2, 4.6; *m/z* 253 ($M^+ - 43$), 100, 21.9, 4.5. Hydroxyacetone *m/z* 281 ($M^+ - 15$), 100, 19.7, 5.5, 0.6. [c] The concentration of the F_{420} is measured by absorbance (A_{420}) at the end of the experiment after the complete air oxidation of all of the $F_{420}H_2$ to F_{420}. The concentration is the sum of the F_{420} present in the extract (0.11 mM) and that added (0.35 mM). The F_{420} used was obtained from *M. jannaschii* and thus was the same as that found in these cells (*6*). [d] Deuterated F_{420} is expected to contained an equal mixture of the *pro-R* and *pro-S* isomers because it was prepared by chemical reduction. The extent of deuteration was greater than 98% based on liquid chromatography−mass spectrometry (LC−MS). [e] (5*R*)-[5-$^2H_1$]$F_{420}H_2$ was generated in vivo with the F_{420}-dependent dehydrogenase present in the extract from the added [5-$^2H_1$]F_{420}. This reduction was apparent by the loss of the F_{420} color during the incubation under hydrogen. [f] The deuterium distribution in the methylglyoxal used, measured from the M^+ of the derivative, was 2.6% $^2H_1$, 11.9% $^2H_2$, 27.1% $^2H_3$, and 58.3% $^2H_4$.

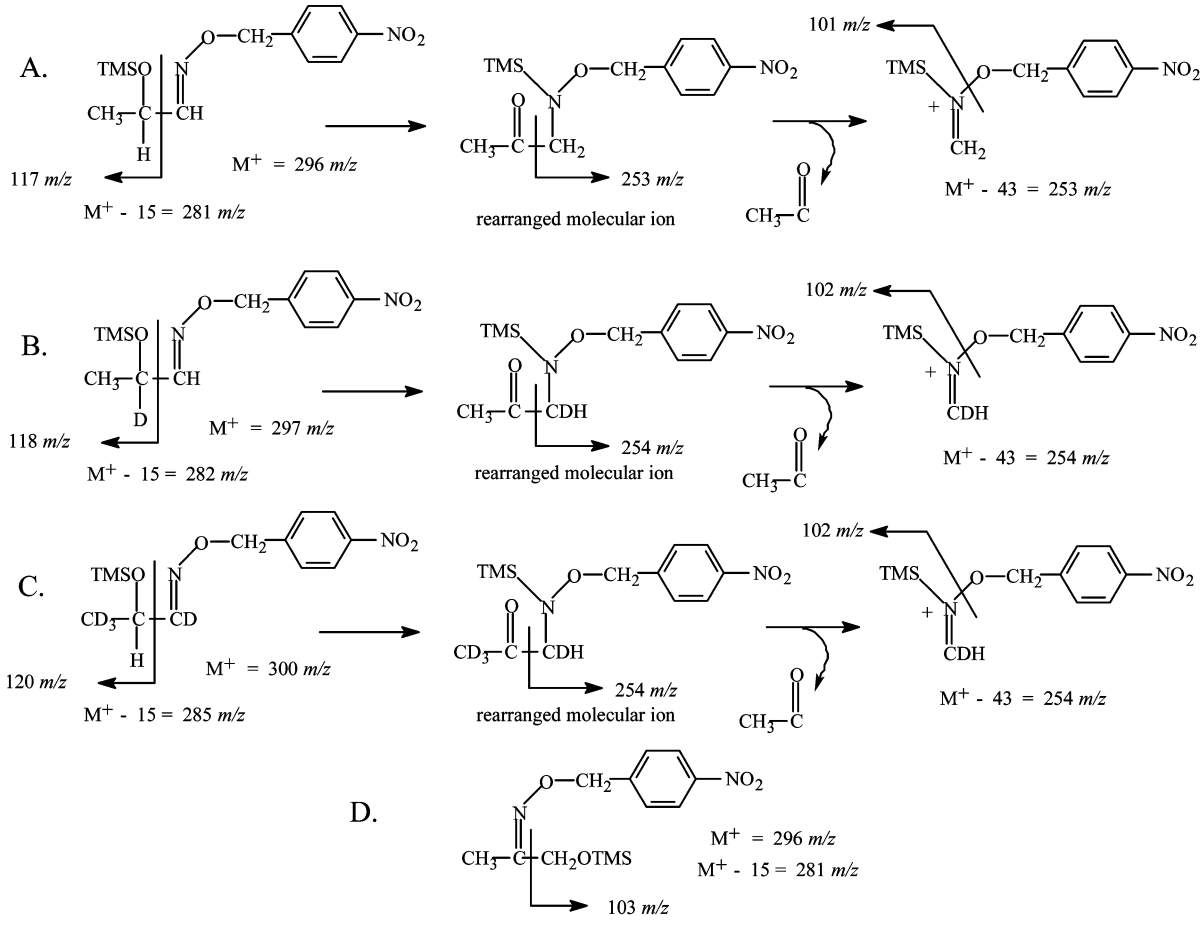

FIGURE 1: Structures and fragmentation of the derivatives used in this study. (A) Fragmentation of the lactaldehyde derivative. (B) Fragmentation of [2-^2H]-lactaldehyde. (C) Fragmentation of the [1,3,3,3-$^2H_4$]-lactaldehyde derivative. (D) Fragmentation of the hydroxyacetone derivative.

Testing Homologues of Acetolactate Synthases for the Production of Hydroxyacetone from Pyruvate and Formaldehyde. M. jannaschii encodes three enzymes homologous to acetolactate synthase. The MJ0256 homologue was shown

Table 3: Distribution and Extent of Deuterium Incorporation into Hydroxyacetone from Pyruvate and Deuterated Formaldehyde by Cell Extracts

		hydroxyacetone			
			distribution of ^2H		
	experiment[a]	ion[b] (*m/z*)	$^2H_0$	$^2H_1$	$^2H_2$
1	10 mM pyruvate, 33 mM	281	1.0	3.5	95.5[c]
	$[^2H_2]$-H_2CO + argon	103	3.6	38	58
2	42 mM $[^2H_2]$-H_2CO, 8.5	281	66.1	1.4	32.5
	mM methylglyoxal	103	78	13	8[c]
3	10 mM pyruvate, 33 mM	281	13.2	0.0	86.8
	$[^2H_2]$-H_2CO	103	7.8	35.5	53.8[c]

[a] Experiments 1 and 2 were conducted with 50 μL of *M. jannaschii* cell extract and were incubated with the substrates for 30 min at 70 °C under argon. Experiment 3 was conducted with 50 μL of heat-treated (58 °C) *E. coli* extract containing the MJ0663-derived enzyme, and the sample was incubated with the substrates for 15 min at 58 °C. In each case, the hydroxyacetone derivative was prepared as described in the text. The *O*-(4-nitrobenzyl)hydroxylamine derivative of formaldehyde recovered from these experiments was completely deuterated. [b] The *m/z* 281 ion contains all of the hydrogens of the original hydroxyacetone, and the *m/z* 103 ion contains C-1 (Figure 1). [c] The loss of the high abundance of fragments with two deuteriums is likely due to hydrogen scrambling.

to be sulfopyruvate decarboxylase (*11*); thus, the other two homologues MJ0663 and MJ0277 were tested for their ability to condense pyruvate with formaldehyde to form hydroxyacetone. Incubation of only the *E. coli* cell extract containing the recombinant MJ0663-derived enzyme with pyruvate and ^2H_2CO catalyzed the formation of hydroxyacetone, which contained two deuterium atoms at C-1 based on the isotopic signature of the *m/z* 103 ion (experiment 3 in Table 3). The control containing only the plasmid did not generate hydroxyacetone.

DISCUSSION

Origin of Lactaldehyde. The data presented here support the idea that L-lactaldehyde originates from a $F_{420}H_2$-dependent reduction of methylglyoxal in *M. jannaschii*. Because L-lactaldehyde is a precursor to L-lactate (*3*) and L-lactate is a precursor to coenzyme F_{420} (*13*), this means that coenzyme F_{420} is required to generate the L-lactate used for its own biosynthesis. This same situation also occurs in the biosynthesis of thiamine-PP, where D-1-deoxyxylulose-5-phosphate synthase, a thiamine-PP-dependent enzyme, catalyzed the first step, leading to the biosynthesis of the thiazole moiety of the thiamine (*14, 15*).

Stereospecificity of Hydride Transfer from $F_{420}H_2$ in the Generation of Lactaldehyde. Three lines of evidence indicate that the enzyme involved in the reduction methylglyoxal to lactaldehyde catalyzed the transfer of the *pro-R* hydrogen of $F_{420}H_2$ to methylglyoxal. First, no deuterium was transferred from (4*RS*)-[4-$^2H_1$]-NADPH into L-lactaldehyde. Deuteride from (4*RS*)-[4-$^2H_1$]-NADPH would have been transferred from (4*RS*)-[4-$^2H_1$]-NADPH to coenzyme F_{420} by $F_{420}H_2$:$NADP^+$ oxidoreductase (product of the MJ1501-derived protein), which specifically transfers the *pro-S* C-4 hydrogen of NADPH to the *si*-face of C-5 of the F_{420} (*16*) to generate *pro-S*-labeled $F_{420}H_2$ as demonstrated in the *Methanococcus vannielii* enzyme (*17*). If the same *pro-S* hydrogen transferred to $F_{420}H_2$ was then transferred to methylglyoxal, we should have seen deuterium incorporated at C-2 of the L-lactaldehyde. This stereospecificity of hydride transfer from $F_{420}H_2$ is known to occur in all of the 11 known F_{420}-dependent enzymes (*16, 18*), despite the fact that there is no chemical reason for there being a preference for which hydrogen is transferred in $F_{420}H_2$-dependent reductions. We would expect the enzymes catalyzing these hydride-transfer reactions from $F_{420}H_2$ to use equally the *pro-R* and *pro-S* hydrogens as occurs with the pyridine nucleotide-dependent enzymes (*19*). If the $F_{420}H_2$:$NADP^+$ oxidoreductase was responsible for the formation of $F_{420}H_2$ in *M. jannaschii*, then we must conclude that the stereospecificity of the hydride transfer in the F_{420}-dependent methylglyoxal reductase is opposite from that observed in all of the other known F_{420}-dependent enzymes because we observed no labeled lactaldehyde.

Second, evidence for this opposite stereochemistry of the reductase comes from the experiment on the incorporation of deuterium from deuterated water during the reduction of methylglyoxal incubated with cell extracts under hydrogen. Here, the observed extent of labeling at C-2 (~5%) was much less that the expected 50%, and multiple sites in the molecule are labeled because molecules containing up to three deuterium atoms were detected. Examination of the labeling in the fragments of the lactaldehyde derivative show that they had labeling patterns consistent with some labeling occurring at each of the positions of the lactaldehyde. The *m/z* 253 fragment ion that contains the C-2 hydrogen as well as the C-1 hydrogen has a deuterium distribution, indicating that about 5% deuterium resided on C-2 and 5% deuterium resided on C-1. This labeling at C-2 and C-1 is required because of the presence of about 4% of the ions containing two deuterium atoms. This result indicates that little deuterium was incorporated at C-2 during the reduction of methylglyoxal to lactaldehyde by cell extracts containing deuterated water (experiment 3 in Table 2). This can be explained by the incorporation of deuterium onto the *si*-face of C-5 of F_{420} during its catalytic reduction with the F_{420}-dependent hydrogenase followed by transfer of the hydride from the *pro-R* of the reduced F_{420} to the C-2 of the methylglyoxal. However, because some deuterium was incorporated at C-2 of the lactaldehyde, then the *re*-face of the reduced F_{420} must have also become labeled. This can be explained if one or more other reactions can also use the *pro-R* hydrogen of $F_{420}H_2$. Only when the resulting C-5 deuterated coenzyme is reduced by the F_{420}-dependent hydrogenase would deuterium be incorporated.

In addition to a F_{420}-dependent hydrogenase, *M. jannaschii* as well as most methanogens contain a two-component enzyme system composed of H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) and a F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) that when working together can catalyze the reduction of coenzyme F_{420} (*20*). The operation of either of these enzyme systems will produce $F_{420}H_2$ and could also explain why we observed the stimulation of methylglyoxal reduction to L-lactaldehyde and hydroxyacetone in cell extracts incubated under hydrogen. In either case, the hydrogen for the reduction of F_{420} would arise from water, consistent with the old observation that no carbon-bonded hydrogens are transferred to methane during its formation (*21*). This occurs despite the fact that two of the reactions required for methane formation depend upon F_{420}-dependent enzymatic reactions, where the $F_{420}H_2$-derived hydride is retained in the transfer.

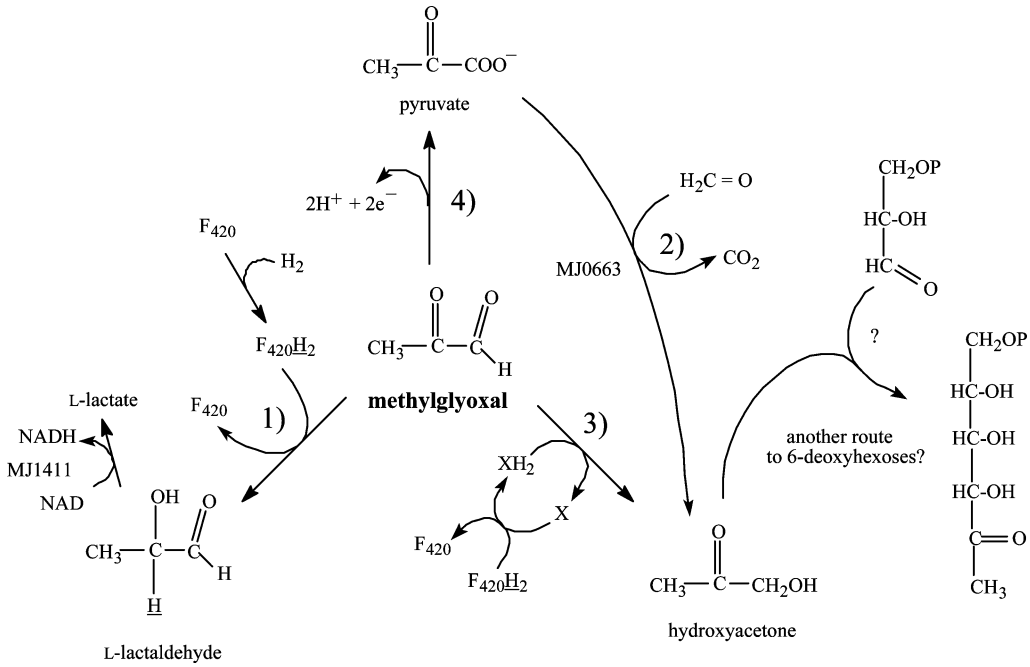

FIGURE 2: Summary of the methylglyoxal metabolism observed in *M. jannaschii*.

Third, in vivo generated (5*R*)-[5-$^2H_1$]$F_{420}H_2$ produced by reduction of [5-$^2H_1$]F_{420} also incorporated deuterium into lactaldehyde (experiment 2 in Table 2). This is only consistent with the *pro-R* hydrogen being transferred.

Search for the Enzyme Catalyzing the Reduction of Methylglyoxal to Lactaldehyde. An important question is: What are the enzymes carrying out these reductions? Because no F_{420}-binding motifs have ever been identified in any of the F_{420}-dependent enzymes, this cannot be used to search for the possible reductases. On the basis of the observed chemistry of the enzyme-catalyzed reduction of the methylglyoxal to L-lactaldehyde, a possible choice for the enzyme would be the F_{420}-dependent secondary alcohol dehydrogenase, which is known to readily reduce acetone to 2-propanol (*22, 23*). This enzyme is a member of the bacterial luciferase family that contains FMN- and F_{420}-dependent oxidoreductases that use a diverse group of substrates (*23*). Only one member of this group, methylenetetrahydromethanopterin reductase (Mer), encoded by the MJ1534 gene, is present in *M. jannaschii*. This enzyme, however, is eliminated from consideration because it has the opposite stereochemistry of reduction (*24*). Therefore, the identification of this reductase will require the isolation and sequencing of the enzyme.

It is also possible that lactaldehyde could be generated in some methanogens by the isomerization of hydroxyacetone that, as we have shown here, can be produced from the condensation of pyruvate with formaldehyde. This idea is stimulated by the observation that the lactaldehyde dehydrogenase, encoded by the MJ1411 gene, is adjacent to the MJ0663 gene in the methanogens *Methanosarcina acetivorans*, *Methanosarcina mazei*, and *Methanosarcinia barkeri*. The activity of such an isomerase in *M. jannaschii* is not supported by the data presented here.

Search for the Enzyme Catalyzing the Reduction of Methylglyoxal to Hydroxyacetone. The data also clearly show that no enzyme in *M. jannaschii* catalyzes the direct transfer of a hydride from $F_{420}H_2$ or NAD(P)H to methylglyoxal because no deuterium was incorporated into hydroxyacetone from these deuterated coenzymes. This is surprising because enzymes abound that are known to catalyze the NADH- or NADPH-dependent reduction of methylglyoxal. These include an aldo−keto reductase (COG0667) present in *E. coli* (*25, 26*) and a methylglyoxal reductase from yeasts (*27*). Other enzymes known to chemically transform methylglyoxal by its reduction include the NADPH-dependent methylglyoxal reductases from mold (*28*) and yeast (*29*), each producing lactaldehyde as the product, as well as the short-chain alcohol dehydrogenase from *Pyrococcus* that readily carries out the NADPH-dependent reduction of methylglyoxal (*30*). Methylglyoxal can also be reduced to hydroxyacetone by a aldehyde reductase/dehydrogenase present in *E. coli* (*31*). None of these enzymes are present in the methanogen as confirmed by the data presented here, showing that the methylglyoxal is not reduced by a hydride-mediated transfer from a reduced pyridine nucleotide, as well as by the absence of genes for these enzymes in the currently sequenced methanogen genomes. Other possible enzymes for the reduction include members of the type A flavoproteins that include the members of the recently identified group of enzymes with $F_{420}H_2$ oxidase (FprA) activity (*18, 32*). In *M. jannaschii*, these include MJ0748, MJ0732, and MJ0534. Each of these enzymes, except MJ0534, harbors the binuclear iron-binding center, is thus likely to be involved in O_2 reduction, and is thus not likely involved in methylglyoxal reduction. The MJ0534 gene product, however, does not contain the essential protein ligands for this iron-binding center and could in fact use methylglyoxal as the terminal electron acceptor. With such an enzyme, no direct hydride transfer to the substrate would occur because of its exchange with the water protons from the flavin. Thus, this enzyme must be tested for its ability to catalyze the reduction of methylglyoxal.

A large proportion of the above-described enzymatic reactions with methylglyoxal serving as a substrate has generally been considered to be involved in the removal of toxic methylglyoxal from cells. Many enzymes are known

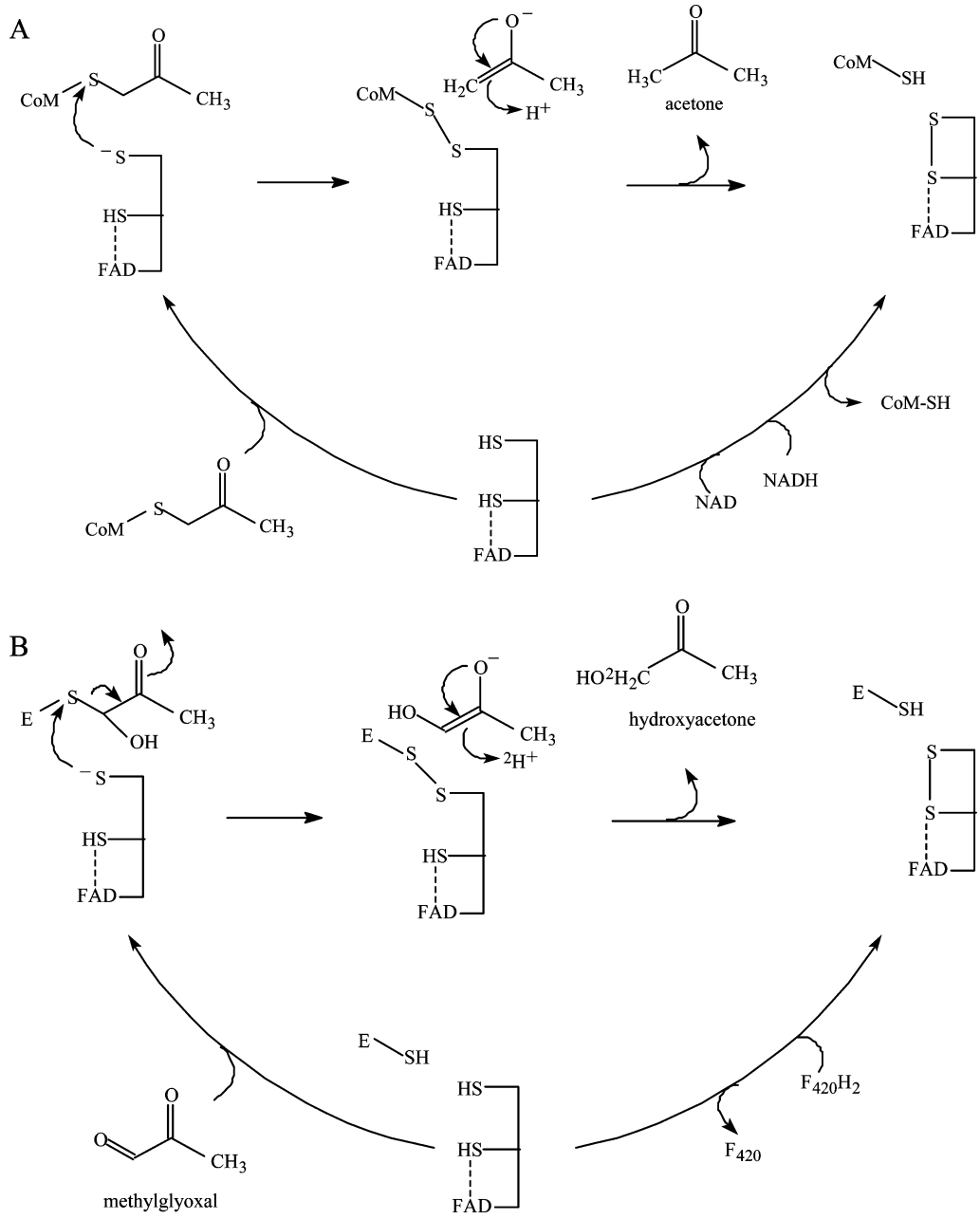

FIGURE 3: (A) Mechanism of 2-ketopropyl-coenzyme M oxidoreductase/carboxylase. (B) Possible mechanism for the methylglyoxal reductase based on that seen in 2-ketopropyl-coenzyme M oxidoreductase/carboxylase.

to metabolize methylglyoxal (*33*) (*34*); however, none of these are known to exist in the methanogens. The canonical and most studied of these biochemical routes for the removal of methylglyoxal from cells is the glutathione-dependent glyoxalase I−II system that consists of two enzymes catalyzing the conversion of methylglyoxal to D-lactate (*35*). The two enzymes functioning in this system are not present in the Archaea, perhaps as a result of the absence of glutathione in the Archaea (*36*). A glyoxalase III enzyme has also been described in *E. coli* (*37*), but its presence in the Archaea cannot be established because its encoding gene has never been identified. I propose that *M. jannaschii* uses a more metabolically efficient method for removing the excess methylglyoxal not used for the biosynthesis of the aromatic amino acids or lactaldehyde. In this system, methylglyoxal is converted into pyruvate and hydroxyacetone that may, in turn, serve as a source of aromatic amino acids. This could be a disproportation reaction, but the labeling data and the fact that the reaction for hydroxyacetone reduction requires hydrogen makes this unlikely.

Possible Mechanism for the Reduction of Methylglyoxal to Hydroxyacetone. Because the reduction of methylglyoxal to hydroxyacetone cannot proceed by either a F_{420} or pyridine nucleotide hydride-transfer reaction, the only logical route is by a flavin- or thiol-dependent reductase. No examples of flavin-dependent aldehyde reductases are known, thus leaving us to consider a thiol-based chemistry. It was readily demonstrated that heating a dilute aqueous solution of methylglyoxal and mercaptoethanol (110 °C for 1.5 h) lead to the formation of hydroxyacetone, indicating that such a reduction can occur chemically (unpublished results). The protein encoded by the MJ0636 gene, annotated as dihydrolipoamide dehydrogenase, is very similar in sequence and predicted structure to the bacterial 2-ketopropyl-coenzyme

M oxidoreductase/carboxylase (*38*) involved in epoxide metabolism (*39*). 2-Ketopropyl-coenzyme M oxidoreductase/carboxylase is proposed to use the reaction mechanism shown in Figure 3A (*40*), which could also be used to catalyze the reduction of an aldehyde. A slight modification of this reaction using the C-1 thiohemiacetal derivative of methylglyoxal from a protein-bound thiol instead of 2-ketopropyl-coenzyme M could result in the NADPH-dependent reduction of methylglyoxal as shown in Figure 3B. We have recombinantly expressed the MJ0636-derived gene product and have demonstrated that it does not catalyze this reduction when incubated with methylglyoxal, NADPH, NADH, coenzyme M, and/or FAD nor does it stimulate methylglyoxal reduction when added to cell extracts (unpublished results). Other enzymes, such as NADH oxidase (MJ0649) and thioredoxin reductase (MJ1536), that also use a reduced pyridine nucleotide to reduce a flavin that in turn reduces a disulfide or sulfinic acid were also found to be unable to catalyze the reduction of methylglyoxal. At present, the enzyme for this reduction is unknown, but on the basis of the data presented here likely uses $F_{420}H_2$ as the reductant without the incorporation of a hydride into the substrate.

Biosynthesis of Hydroxyacetone from Pyruvate. Our discovery that the enzyme derived from the MJ0663 gene catalyzes the condensation of pyruvate with formaldehyde to form hydroxyacetone is a new reaction for an enzyme that is related to acetohydroxy acid synthases (*41*) and may represent a new route for the formation of reduced trioses as shown in Figure 2. *M. jannaschii* has two other genes homologous to acetohydroxy acid synthase, MJ0277 and MJ0256. Acetohydroxy acid synthases have been studied extensively as to their mechanism and phylogeny (*41*). MJ0256 has been demonstrated to encode a sulfopyruvate decarboxylase (*11*). The genes encoding the acetohydroxy acid synthase from several different organisms have been recombinantly expressed and shown to catalyze the expected reaction (*42, 43*). The enzyme from *E. coli* has the ability to react with several different substrates (*44*). Thus, the discovery that the MJ0663-derived enzyme catalyzed the formation of hydroxyacetone is not that unreasonable. If this reaction occurs in vivo, it would represent an alternate system to produce hydroxyacetone, which could be used to generate the deoxysugars for the archaeal aromatic amino acid biosynthesis (Figure 2).

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