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Ribose Biosynthesis and Evidence for an Alternative First Step in the Common Aromatic Amino Acid Pathway in *Methanococcus maripaludis*

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An acetate-requiring mutant of *Methanococcus maripaludis* allowed efficient labeling of riboses following growth in minimal medium supplemented with [2-^{13}C]acetate. Nuclear magnetic resonance and mass spectroscopic analysis of purified cytidine and uridine demonstrated that the C-1′ of the ribose was about 67% enriched for ^{13}C. This value was inconsistent with the formation of erythrose 4-phosphate (E4P) exclusively by the carboxylation of a triose. Instead, these results suggest that either (i) E4P is formed by both the nonoxidative pentose phosphate and triose carboxylation pathways or (ii) E4P is formed exclusively by the nonoxidative pentose phosphate pathway and is not a precursor of aromatic amino acids.

The pentose phosphate pathway provides pentoses for nucleosides and erythrose 4-phosphate (E4P) for aromatic amino acids (AroAAs) (8, 21). In most methanogens, pentoses are formed by the oxidative pentose phosphate pathway by oxidative decarboxylation of hexoses (2, 4–6). Because extracts of *Methanococcus maripaludis* contained high activities for transketolase and transaldolase and lacked detectable glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities, Yu et al. proposed that this organism formed pentoses by a nonoxidative pentose phosphate (NOPP) pathway (Fig. 1a) (22). Subsequent labeling studies with *Methanococcus voltae* and *Methanococcus jannaschii* confirmed some aspects of this proposed pathway (2). However, in the methanococci as well as in *Methanobacterium thermoautotrophicum*, *Methanosphaera stadtmaniae*, *Methanobrevibacter smithii*, and *Methanospirillum hungatei*, phenylalanine and tyrosine were preferentially enriched at the C-7 atom (and not at both the C-7 and C-8 atoms) following labeling with [1-^{13}C]pyruvate (2, 5). This pattern was not consistent with the formation of these amino acids by E4P formed by the NOPP. Thus, it was proposed that E4P was formed by carboxylation of a 3-carbon sugar and not by the NOPP pathway (Fig. 1b) (2, 5). However, there is an alternative explanation for these results. E4P may not be a precursor of AroAA biosynthesis in methanogens. This alternative can be examined in methanococci, where the enrichment of ribose is expected to be affected by the removal of E4P for AroAA biosynthesis. Thus, the labeling patterns of ribose were determined in cells of *M. maripaludis* grown on [2-^{13}C]acetate.

MATERIALS AND METHODS

Strain and growth conditions. For the labeling experiment, all glassware was washed overnight in 10% H_2SO_4 and rinsed in deionized and distilled water. *M. maripaludis* JJ12, an acetate-requiring mutant of strain JJ1 (12), was grown in mineral medium supplemented with 1.36 g of $CH_3COONa \cdot 3H_2O$ per liter. Cultures (5 ml) were inoculated with fewer than 2.5×10^7 cells to avoid selection for revertants and were grown under 276-kPa H_2:CO_2 (80:20, vol/vol) at 37°C as described previously (19), except that the vitamin solution was omitted. Following overnight growth, these cultures were inoculated into 100 ml of medium containing 0.73 g of $^{13}CH_3COONa$ per liter. Cultures (100 ml) were incubated in 1-liter Wheaton (Millville, N.J.) bottles under 138-kPa H_2:CO_2 with the $NaHCO_3$ concentration reduced to 2 g per liter. When the culture absorbance at 600 nm was approximately 1, cells from four bottles were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C and were stored at −20°C.

Isolation of [^{13}C]acetate-labeled nucleosides. Cells were fractionated by a modification of the procedure of Roberts et al. (15) as described previously (18). The cell pellet was resuspended in 4 ml of ice-cold 5% trichloroacetic acid and incubated on ice for 30 min. The pellet was collected by centrifugation (at 8,000 × *g* for 20 min at 20°C), resuspended in 4 ml of 75% ethanol, and incubated at 45°C for 15 min. The pellet was collected as above, resuspended in 4 ml of ether-ethanol-water (4:3:1), and incubated at 45°C for 15 min. Following centrifugation, the pellet was resuspended in 4 ml of ice-cold 5% trichloroacetic acid and heated in a boiling-water bath for 30 min. After centrifugation and removal of the pellet, the pH of the supernatant was adjusted to 5.0 to 5.5 with 1 M $(NH_4)_2CO_3$, pH 9.9, and 1.75 M acetic acid. P1 nuclease (350 U/ml; Sigma Chemical Co., St. Louis, Mo.) at 25 μl per ml of reaction mixture was added, and the solution was incubated for 6 h at 37°C. After adjusting the pH to 9 with 1 M $(NH_4)_2CO_3$, pH 9.9, and adding 1 mM (each) $ZnCl_2$ and $MgCl_2$, alkaline phosphatase (Type VII-L bovine intestinal, in 3 M NaCl; Sigma) was added to a final concentration of 53 U/ml, and the solution was incubated for 15 h at 37°C. The resulting solution of nucleosides was acidified to pH 4 with glacial acetic acid and passed through a column of DEAE Sephadex A-25 (6 by 270 mm; Pharmacia, Uppsala, Sweden) equilibrated with water. Fractions (5 ml) containing a detectable absorbance at 260 nm were lyophilized. Nucleosides were separated by high-performance liquid chromatography at 0.5 ml/min and 30°C) on an Econosphere C_{18} reversed-phase column (250 mm by 4.6 mm [inside diameter]; 5 μm; Alltech Associates, Inc., Deerfield, Ill.) in 0.1 M trimethylammonium acetate buffer with 1% acetonitrile, pH 7.1 (10). Fractions (0.2 ml) containing cytidine (retention time, 9.9 min) and uridine (retention time, 12.8 min) were collected and lyophilized. Approximately 0.37 mg of cytidine and 0.42 mg of uridine were obtained.

NMR. ^{1}H nuclear magnetic resonance (NMR) data were acquired in solutions of D_2O at 20°C on a Bruker AMX400 spectrometer (400.13 MHz). The suppression of HDO signal was achieved by a presaturation method with a low-power pulse of 1 s. All data were obtained with a spectral width of 4.5 kHz, a relaxation delay time of 10 s, and an acquisition time of 1 s. For cytidine and uridine, 128 and 256 scans, respectively, were performed. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate via the HDO resonance frequency at 4.81 ppm at 20°C. ^{13}C NMR data were acquired under the same conditions at 100.61 MHz except that the spectral width was 9.5 kHz, the relaxation delay time was 20 s, the acquisition time was 500 ms, and 2,048 scans were performed. ^{13}C chemical shifts were externally referenced to tetramethylsilane via the $CHCl_3$ resonance frequency at 77.0 ppm at 20°C.

Mass spectroscopy. Electrospray mass spectra were acquired by using a Micromass Quattro II (Beverly, Mass.) triple quadrupole mass spectrometer equipped with an electrospray source. The source temperature was set to 80°C, and the ion source capillary and cone voltages were optimized to 2,650 and 13 V, respectively. All mass spectra were recorded in multichannel analysis mode, and each represented the summation of 10 individual scans. Nucleoside samples were diluted in 10 mM ammonium acetate-methanol (95:5, vol/vol) to a final concentration of approximately 30 pmol μl^{-1} and were delivered via a syringe infusion pump (model 200; KD Scientific, Boston Mass.) at a flow rate of 10 $\mu l\ min^{-1}$ into

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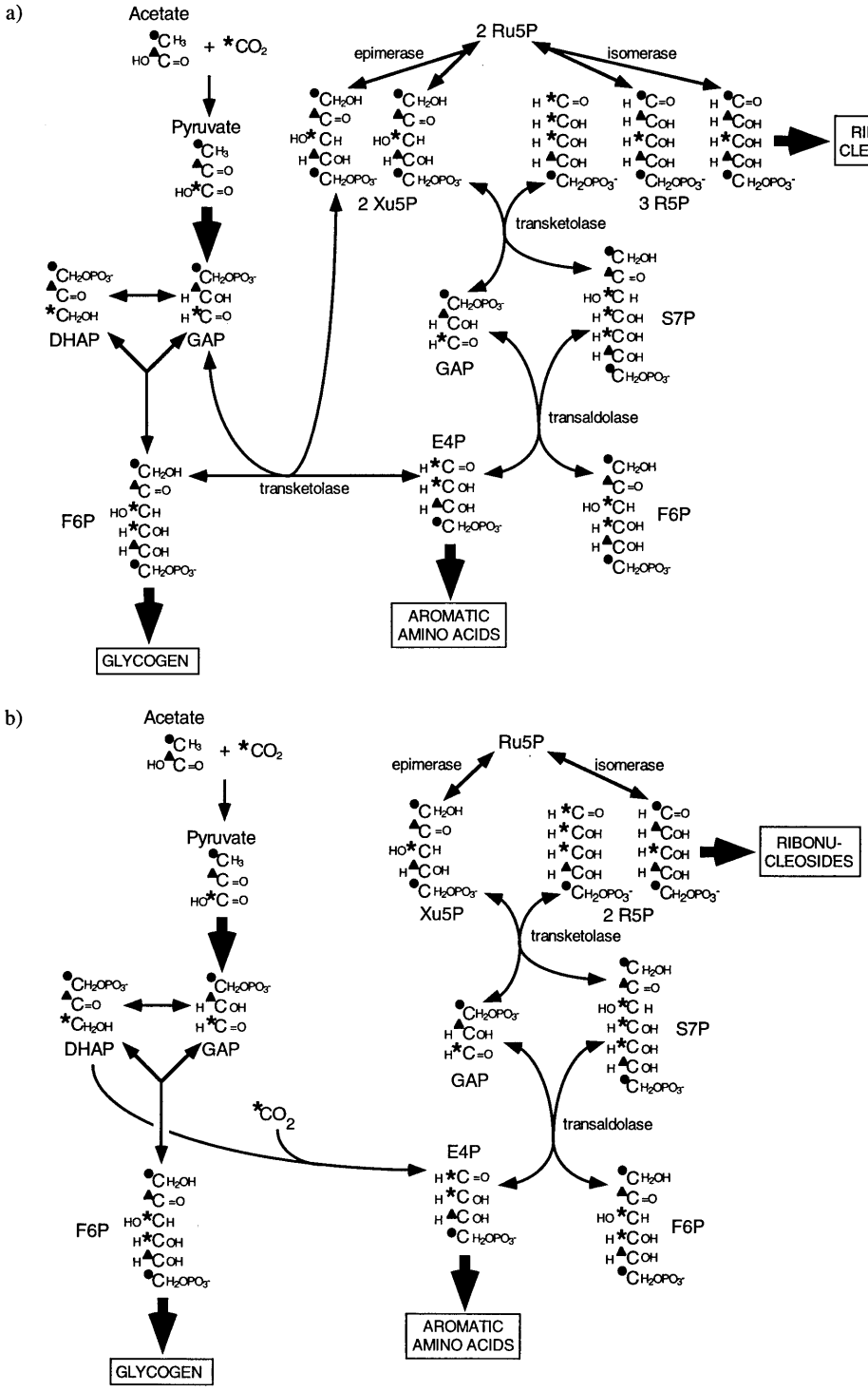

FIG. 1. The NOPP pathway in methanococci. Large arrows indicate multiple steps. DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; F6P, fructose-6-phosphate; Xu5P, xylulose 5-phosphate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate. Carbon labels: ●, C-2 of acetate; ▲, C-1 of acetate; ★, CO_2. (a) The pathway and the expected labeling patterns in *M. maripaludis* (22). Consumption of E4P for biosynthesis would increase the amount of ribose 5-phosphate formed via xylulose 5-phosphate. (b) Modification of the pathway as proposed by Choquet et al. (2). E4P is formed by carboxylation of a triose such as dihydroxyacetone phosphate, and the fructose-6-phosphate-dependent transketolase reaction is absent. Although the labeling pattern of E4P is unchanged, 50% of the ribose 5-phosphate is now formed via xylulose 5-phosphate, and the labeling pattern of ribose is not affected by the consumption of E4P for AroAA biosynthesis.

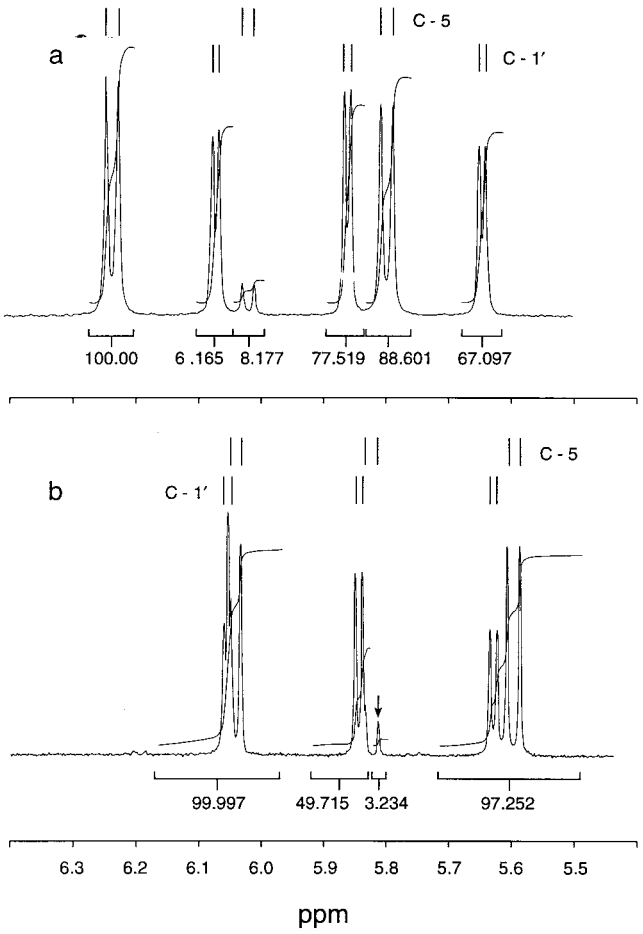

FIG. 2. Proton NMR spectra for the H atoms at C-1′ and C-5 of cytidine and uridine from *M. maripaludis* JJ12 following growth on [2-^{13}C]acetate. The integrals of the peaks are given below the spectra. (a) Cytidine. The $J_{H\text{-}C}$ coupling constants for the C-1′ and C-5 H atoms were 168 and 172 Hz, respectively. For calculation of the enrichment of cytidine, see Results. (b) Uridine. The $J_{H\text{-}C}$ coupling constants for the C-1′ and C-5 H atoms were 170 and 176 Hz, respectively. The arrow indicates one of the ^{12}C peaks for C-5, while the other ^{12}C peak overlaps the ^{12}C peaks for C-1′. For calculation of the ^{13}C enrichment, it was assumed that the total signal (99.997 + 49.715 + 3.234 + 97.252 = 250.198) could be divided equally between C-1′ and C-5. The ^{13}C enrichment of C-5 was calculated as 125.099 − (3.234 × 2)/125.099. The ^{13}C enrichment of C-1′, uncorrected for the maximal enrichment, was calculated as 125.099 − 49.715 + 3.234/125.099.

the ion source through a six-valve Rheodyne injector (Cotati, Calif.) using a 100-μl sample loop.

RESULTS AND DISCUSSION

An acetate auxotroph of *M. maripaludis*, strain JJ12, allowed efficient incorporation of ^{13}C-labeled acetate. In preliminary experiments, wild-type *M. maripaludis*, strain JJ1, was grown in mineral medium with [2-^{13}C]acetate. NMR analysis of the isolated alanine revealed only a 27% enrichment in the β carbon, indicating that most of the carbon was derived from autotrophic CO_2 fixation (data not shown). In contrast, the acetate auxotroph *M. maripaludis* JJ12 incorporated acetate much more efficiently. Following growth with [2-^{13}C]acetate, the enrichment at the C-5 of cytidine, which is derived entirely from the C-2 of acetate, was 95.8% (Fig. 2a). Although the complexity of the spectrum made quantitation difficult, the C-5′ of cytidine was also highly enriched (data not shown).

The extent of labeling at the C-1′ of cytidine and uridine in *M. maripaludis* was not consistent with the proposal that E4P was formed entirely by carboxylation of a 3-carbon intermediate of gluconeogenesis (2). In this scenario, 50% of label at the C-1′ should be derived from the C-2 of acetate (Fig. 1b). Instead, the enrichment observed was 63.8% (Fig. 2a). After correcting for a maximum enrichment of 95.8%, the amount of carbon obtained from the C-2 of acetate was 66.6%. In addition, no label was detected in the C-2′, C-3′, C-4′, and C-6 carbons (data not shown), indicating that scrambling of the isotope did not occur (20). Although the spectra of the C-5 and C-1′ H atoms of uridine overlapped, it was possible to estimate the enrichment by assuming that both H atoms contributed equally to the sum of the integrals of the observed peaks (Fig. 2b). After correcting for the maximal enrichment of 94.8% obtained from the C-5 position, a similar enrichment value for the C-1′, 66.3%, was obtained (Fig. 2b). ^{13}C NMR confirmed this value, and the enrichment of the C-1′ of uridine observed by this method was 65.3% (data not shown). Lastly, the isotopically enriched uridine was examined by electrospray mass spectroscopy. The intensities of the observed peaks, in arbitrary units, at molecular masses of 245, 246, 247, and 248 Da were 0 (not detected), 4.29, 11.7, and 18.7, respectively. After correction for the natural abundance of ^{13}C, ^{17}O, and ^{15}N, the relative abundances of the unenriched, +1-mass-unit, +2-mass-units, and +3-mass-units uridine were calculated to be 0, 13.1, 34.1, and 52.9%, respectively. This pattern was consistent with the enrichment determined by NMR. For instance, if the maximum enrichment by [2-^{13}C]acetate in any position was 95% and the specific enrichment of the C-1′ position was 67%, the expected abundances at +2 and +3 mass units were 39 and 57%, respectively, which were close to the measured values. In contrast, if the specific enrichment of the C-1′ position was 50%, the expected abundances of the same peaks would be 52 and 43%, respectively, very different from the measured values.

Furthermore, the proposed carboxylation pathway is unlikely because it requires that the cells possess a transketolase which exclusively catalyzes the sedoheptulose 7-phosphate-dependent transketolase reaction and not the fructose-6-phosphate-dependent transketolase reaction. In fact, all transketolases so far described catalyze both reactions (16, 21, 23). Additionally, both transketolase activities have been identified in the archaea *Haloarcula vallismortis* and *Haloferax mediterranei* (14).

The NOPP pathway predicts that 66.7% or more of the carbon at the ribose C-1 will come from the C-2 of acetate (Fig. 1a). If E4P is not diverted from the pathway, exactly 2/3 of the carbon at the C-1 position will come from the C-2 of acetate. This fraction will increase if E4P is removed from the pathway for AroAA biosynthesis. For example, the molar ratio of AroAA to ribose in bacterial cells is about 1:2 (13). If E4P for this amount of AroAA biosynthesis is obtained from the NOPP pathway, then 83% of the C-1 of ribose will be obtained from the C-2 of acetate. In contrast, if E4P is formed by a carboxylation reaction, utilization of E4P for AroAA biosynthesis does not affect the labeling of ribose (Fig. 1b).

Two possibilities might explain the observed nucleoside labeling. One is that E4P is formed by both the NOPP pathway and carboxylation of a triose. The other is that only the NOPP pathway is used for E4P biosynthesis, and E4P is not utilized for AroAA biosynthesis. The second possibility is more plausible. First, if both pathways of E4P production were present, the predicted amount of label in the C-1′ of cytidine and uridine from [2-^{13}C]acetate would be anywhere from 50% to about 83%. Only the second possibility predicts an enrichment

of exactly 66.7%, or close to the actual values found. Second, the first enzyme of the common pathway of AroAA biosynthesis, 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, catalyzes the condensation of E4P and phosphoenolpyruvate (reviewed in reference 9). This activity was not detected in cell extracts of *Methanohalophilus mahii*, whereas shikimate dehydrogenase (step 4) was identified (7). Third, the genes for DAHP synthase and 3-dehydroquinate synthase, the second enzyme in the common AroAA pathway, were also not identified in the genome of *Methanococcus jannaschii* (1, 17). In fact, genes for three enzymes of the common AroAA pathway were not detectable in the *M. jannaschii* genome. In contrast, all the genes for leucine and tryptophan (from chorismate) biosynthesis were identified, and only one gene in each pathway for histidine and lysine biosynthesis was not detected (17). These results suggest that the genes for the first two enzymes of AroAA biosynthesis either are absent or are not recognizable from the sequence.

Finally, the labeling studies of the AroAAs in the methanogens (2, 4–6) are consistent with the well-known common pathway only if E4P is not formed by the NOPP pathway. If E4P is formed by the NOPP pathway, an alternative pathway of AroAA biosynthesis must be present. Speculation on the nature of this pathway is premature, but it could involve condensation of two 3-carbon molecules, followed by carboxylation of a 6-carbon intermediate. Eucarya possess DAHP synthases with broad substrate specificity that utilize 2- to 5-carbon substrates in place of E4P (3). Jensen et al. have proposed an alternative shikimate pathway in plants, the phyto-shikimate pathway, based on the condensation of two 3-carbon substrates (11). However, this pathway is not consistent with the labeling studies of AroAAs in methanogens (2, 4–6).

In conclusion, an expected increase of [2-^{13}C]acetate-derived label in the C-1′ of cytidine and uridine due to the diversion of E4P to AroAA biosynthesis was not observed in *M. maripaludis*. This result, combined with the inability to identify the enzyme activity or the gene for the first step of the classical AroAA biosynthetic pathway in *M. mahii* and *M. jannaschii*, indicates that the methanogens, and perhaps other archaea, may use an alternative first step in AroAA biosynthesis.

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