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Methanococcus jannaschii Generates L-Proline by Cyclization of L-Ornithine

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Cell extracts of *Methanococcus jannaschii* have been shown to readily convert L-ornithine to L-proline. This cyclization reaction proceeds with the loss of only the C-2 nitrogen, as has been documented for ornithine cyclodeaminase (EC 4.3.1.12). Since no gene homologous to that coding for ornithine cyclodeaminase is present in the genome of *M. jannaschii*, these results indicate that proline biosynthesis in *M. jannaschii* is accomplished by a previously unrecognized enzyme.

The established pathway for proline biosynthesis in microorganisms is shown in the upper portion of Fig. 1. The reaction sequence involves (i) the phosphorylation of the δ-carboxyl of L-glutamate to form L-glutamyl-5-P, (ii) the NADH-dependent reduction of L-glutamyl-5-P to glutamic acid-γ-semialdehyde, (iii) the cyclization of glutamate-γ-semialdehyde to Δ^1-pyrroline-5-carboxylic acid, and (iv) the reduction of Δ^1-pyrroline-5-carboxylic acid to L-proline (2, 5, 13). As first pointed out by Selkov et al. for *Methanococcus jannaschii* (10), and later as a general characteristic of the genomes of most of the *Archaea*, genes coding for the three enzymes in this pathway are largely absent in the *Archaea* (4). In contrast, the genes for the biosynthesis of L-ornithine are generally present in all of the archaeal genomes (4). A simple solution to explain the absence of the proline biosynthetic genes in the genomes of some of the *Archaea* is that proline is derived by the cyclization of L-ornithine. This reaction could be accomplished by ornithine cyclodeaminase (EC 4.3.1.12), an enzyme which is presently considered to have a limited distribution among the bacteria (12). Ornithine cyclodeaminase was first isolated from *Clostridium sporogenes,* where it functions as the first step in the anaerobic catabolism of L-ornithine via proline to δ-aminovaleric acid (1). The proposed chemical steps for the mechanism of this enzyme involve the oxidative deamination of the α-amino group of ornithine to 2-oxo-5-aminopentanoic acid, which cyclizes to Δ^1-pyrroline-2-carboxylic acid, which is subsequently reduced to L-proline. The ornithine cyclodeaminase has been shown to contain 1 mol equivalent of bound NAD^+, which is considered to function as a recycling redox carrier in this transformation (7). Mass spectroscopic data showed that [5-^{15}N]ornithine is converted to [^{15}N]proline by ornithine cyclodeaminase, confirming that the initial oxidation of the ornithine is at C-2 (8). Using deuterated ornithine and [^{15}N]ornithine, we have now demonstrated that proline in *M. jannaschii* is derived from ornithine by a mechanism that is analogous to that demonstrated by the ornithine cyclodeaminase. Since there is no enzyme-encoding gene with a sequence homologous to that coding for the ornithine cyclodeaminase present in the *M. jannaschii* genome, we propose that a currently unidentified enzyme, functioning with an analogous mechanism, is involved in proline biosynthesis in *M. jannaschii*.

Preparation and analysis of cell extracts. Cell extracts of *M. jannaschii*, *Methanosarcina thermophila* strain TM-1, and *Methanobacterium thermoautotrophicum* strains ΔH and Marburg were prepared as previously described (16). The protein concentrations of the cell extracts used typically ranged from 7 to 26 mg/ml.

Incubation with substrates. Cell extracts (50 μl) were incubated with millimolar concentrations of the substrates under argon for 2 h at 50°C. L-[2,4,4′-$^2H_3$]glutamic acid and L-[3,3′,4,4′,5′-$^2H_6$]ornithine were obtained from Cambridge Isotope Laboratories, Inc. [5-^{15}N]ornithine was prepared from potassium [^{15}N]-phthalimide by the following series of reactions. Potassium [^{15}N]-phthalimide (98 atom% ^{15}N) was reacted with dibromopropane in acetone to form ^{15}N-labeled *N*-(3-bromopropyl)phthalimide (14), which was condensed with ethyl acetamidocyanoacetate in ethanol in the presence of sodium ethoxide. Acid hydrolysis of the condensation product (6 M HCl, 24 h, 110°C) and separation of the resulting products on a Dowex 50-8X (H^+) column with an HCl gradient resulted in the isolation of chromatographically pure [5-^{15}N]ornithine. Δ^1-Pyrroline-5-carboxylic acid was prepared from the 2,4-dinitrophenylhydrazine derivative as previously described (6).

After incubation, 0.1 M HCl in methanol (200 μl) was added, followed by centrifugation (10 min, 14,000 × *g*) to remove the precipitated proteins. The resulting clear liquid was evaporated to dryness with a stream of nitrogen gas, and the free amino acids contained within were converted into the methyl ester trifluoroacetyl derivatives and analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (17). Quantitation of proline and ornithine was determined from the areas of the intensities of their *m*/*z* 166 ions, or the *m*/*z* 167 or *m*/*z* 172 ions for the labeled prolines, using known mixtures of proline and ornithine for calibration. For samples not containing ornithine, the *m*/*z* 211 ion from the β-glutamate present in the *M. jannaschii* cell extracts (9) was used as an internal standard. The establishment of the product of the incubation as L-proline was accomplished by GC-MS of

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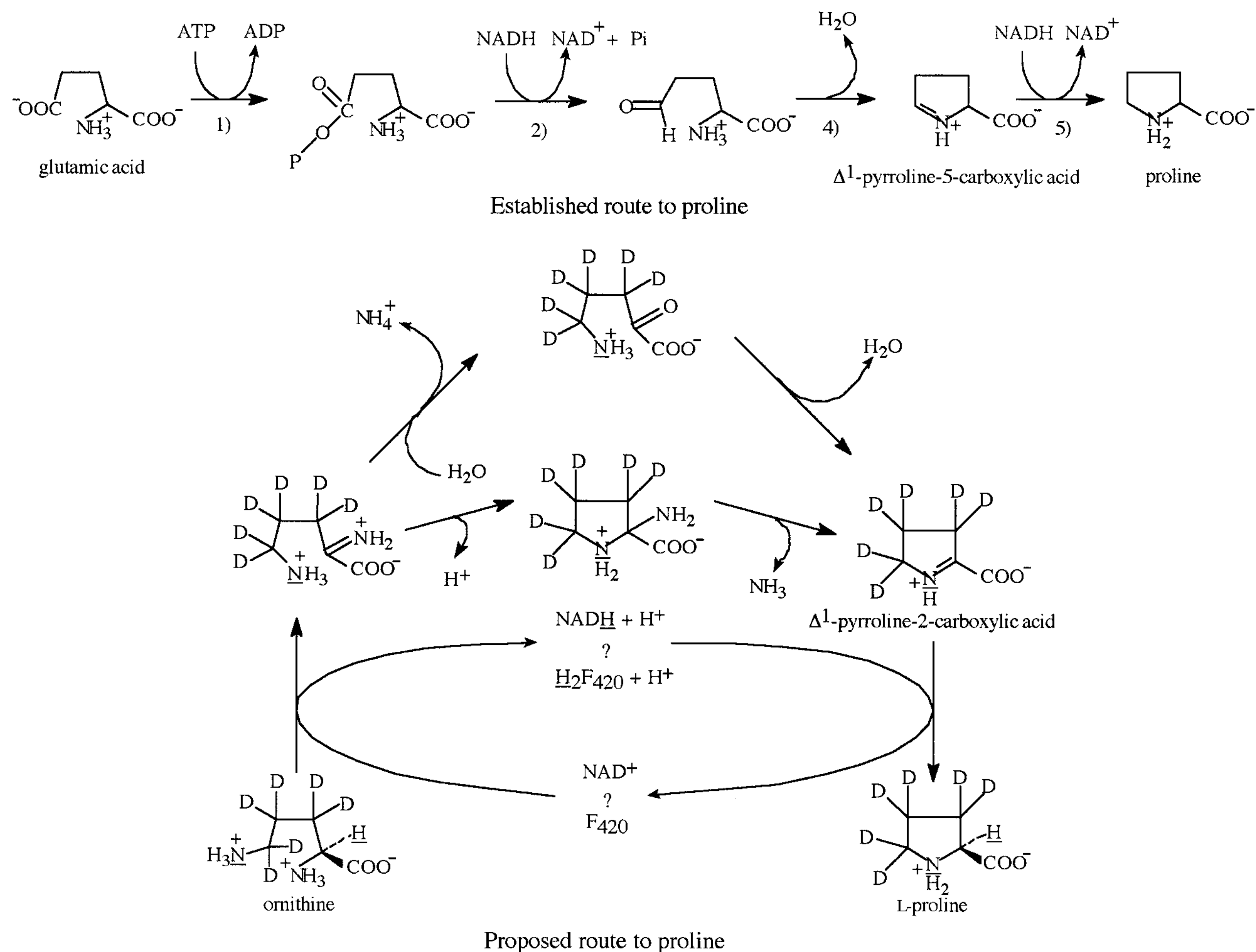

FIG. 1. Established and proposed pathways for proline biosynthesis.

the methyl ester trifluoroacetyl derivative using a type G-TA Chiraldex column as previously described (3).

As can be seen from the data presented in Table 1, incubation of a cell extract of *M. jannaschii* with [2,4,4′-$^2H_3$]glutamic acid, ATP, NADH, and NADPH produced no detectable amount of labeled proline. Likewise, incubation with Δ^1-pyrroline-5-carboxylic acid, NADH, and NADPH at the same concentrations failed to produce any detectable amount of proline (data not shown). Incubation of the cell extract with L-ornithine (9.1 mM) resulted in the production of 0.13 μmol of proline, which corresponded to the conversion of 26% of ornithine to proline. To confirm that ornithine was the sole precursor of the proline, we incubated the cell extracts with L-[3,3′,4,4′,5,5′-$^2H_6$]ornithine and measured the incorporation of six deuteriums in the generated proline, which indicated that the carbon skeleton of the proline was derived from the ornithine as an intact unit. To establish which of the nitrogens was lost in the cyclization, the experiment was repeated with [5-^{15}N]ornithine, and the recovered proline contained 98% ^{15}N. This result showed that the C-2 nitrogen was the one lost in the cyclization. Since the direct displacement of the C-2 amino group by the C-5 amino group is without enzymatic or chemical precedent, the most likely chemical steps for the reaction would involve the oxidation of the C-2 carbon to an imine, intramolecular cyclic addition of the C-5 nitrogen to the C-2 carbon, loss of ammonia, and reduction of the resulting imine, Δ^1-pyrroline-2-carboxylic acid, as shown in lower por-

TABLE 1. Proline formation in cell extracts of *M. jannaschii*

Expt	Precursor(s)	Proline produced (μmol)	% of substrate converted into proline
1	L-[2,4,4′-$^2H_3$]glutamic acid (7.7 mM), ATP (7.7 mM), NADH, and NADPH (7.7 mM)	0.01[a]	<0.024
2	L-Ornithine (9.1 mM)	0.13	26
3	L-[3,3′,4,4′,5,5′-$^2H_6$]ornithine (9.1 mM)	0.19[b]	38
4	[2-^{15}N]ornithine (9.1 mM)	0.19[c]	38
5	L-Ornithine (9.1 mM) and 40% ^2H_2O	0.13[d]	20

[a] The proline contained <0.1% of deuterated prolines. The small amount of unlabeled proline observed resulted from the proline which was present in the cell extract.
[b] All proline molecules contained six deuterium atoms.
[c] The recovered proline had the same ^{15}N abundance as the precursor ornithine.
[d] The recovered proline had no detectable deuterium (<1%).

tion of Fig. 1. Alternately, the imine intermediate generated in the first oxidation could undergo hydrolysis to the keto acid before the cyclization would occur. In this case, elimination of water would produce the Δ^1-pyrroline-2-carboxylic acid. Although not directly confirmed by the data, the likely choice for the coenzyme to be involved in this process would be an enzyme-bound NAD^+ as occurs in ornithine cyclohydrolase. The possible involvement of coenzyme F_{420} must also be considered since coenzyme F_{420} can also effect hydride transfer reactions (15).

Evidence supporting the involvement of either NAD^+ or F_{420} in the reaction comes from the experiment demonstrating that the hydrogen removed during the C-2 oxidation is reincorporated at C-2 in the proline product during the reduction. Thus, the incubation of a cell extract containing 40% deuterated water with ornithine produced proline with no deuterium (Table 1, data for precursor 5). The conclusion from this experiment is that the hydrogen, which is removed from C-2, is not mixed with the solvent and is the same hydrogen that is incorporated in the reduction. These data are consistent with the idea that the enzyme in *M. jannaschii* functions with a mechanism analogous to that of ornithine cyclodeaminase from *C. sporogenes*. Evidence supporting the involvement of NAD^+ was the observation that NADH was found to inhibit the conversion presumably by competing with the required NAD^+ (data not shown).

Similar data were also obtained using cell extracts of *M. thermoautotrophicum* strain ΔH and strain Marburg, indicating that these autotrophs generate their proline by the same mechanism as that found in *M. jannaschii*. Although no gene coding for ornithine cyclodeaminase can be found in the *M. jannaschii* genome, a gene homologous to one coding for *C. sporogenes* ornithine cyclodeaminase is present in the *M. thermoautotrophicum* ΔH (11) and *Methanosarcina barkeri* (http://www.jgi.doe.gov) genomes. Cell extracts of *M. thermophila* strain TM-1, on the other hand, were found to produce no proline from ornithine. The *M. barkeri* genome also has the genes for the biosynthesis of proline from glutamate via Δ^1-pyrroline-5-carboxylic acid. These genomic data indicate that several different routes may be operating in the *Archaea* for the biosynthesis of proline. Although our data cannot rule out the possibility that additional pathways to proline function in vivo, this seems unlikely due to the efficient conversions from ornithine that were observed in vitro.

In total, these observations show that an enzyme with no homology to the known ornithine cyclodeaminases is involved in proline biosynthesis in *M. jannaschii*. We are presently in the process of isolating the enzyme responsible for carrying out the reaction demonstrated here in order to establish the gene required for its production.

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