Carbon monoxide fixation into the carboxyl group of acetyl coenzyme A during autotrophic growth of *Methanobacterium*

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1. INTRODUCTION

Methanobacterium thermoautotrophicum is a methane-forming archaebacterium which grows on H_2 and CO_2 as sole energy and carbon sources [1,2]. This autotrophic organism does not assimilate CO_2 via the Calvin cycle (for literature see [3,4]). Acetyl CoA rather than 3-phosphoglycerate appears as the earliest detectable CO_2 fixation product [3,5]. The operation of a reductive tricarboxylic acid cycle like that of the green sulfur bacteria [6,7] has also been excluded as the mechanism of acetyl CoA synthesis [8]. All available data have suggested instead a total synthesis of acetyl CoA via one-carbon intermediates [3–5].

It has been recently suggested [9,10] that acetyl CoA synthesis in autotrophic methanogens could be mechanistically related to acetate formation from 2 CO_2 in acetogenic bacteria. In the latter group of eubacteria experiments with ^{14}CO have shown that the carboxyl group of acetate is derived from CO, which itself is probably formed from CO_2 via direct reduction [9,11]. To test for this possibility we grew *M. thermoautotrophicum* on 80% H_2/20% CO_2 in the presence of 5% ^{14}CO and then investigated the incorporation of ^{14}C into cellular compounds. We found that ^{14}CO was specifically incorporated into carbon positions which are biosynthetically derived from the carboxyl group of acetyl CoA. Under the above conditions, about 15% of the acetyl CoA was synthesized from 1 CO_2 (methyl group) and 1 CO (carboxyl group). Also, an exchange of unlabeled CO with $^{14}CO_2$ was observed.

2. MATERIALS AND METHODS

Growth: *M. thermoautotrophicum* (Marburg strain), was grown at 65°C on 40 ml mineral medium in a pressurized closed vessel with 1.1 l gas phase (80% H_2/20% CO_2/0.1% H_2S) at 1 bar overpressure [12]. Just before inoculation with 5% preculture, 50 ml ^{14}CO were added with a syringe. ^{14}CO was prepared from ^{14}C-formate as in [13], and unlabeled CO was added as carrier to give a specific radioactivity of 49000 dpm/μmol CO. Samples (1 ml) of the culture were periodically withdrawn for determination of cell density and radiocarbon incorporation. The gas phase was simultaneously sampled for gaschromatographic analysis of CO, CO_2, and CH_4 (0.4 ml) and for determination of radioactivity in CO_2 (1 ml). At an optical density of $\Delta A_{578} = 1.4$, the culture was harvested by centrifugation. The cells were then washed and fractionated as in [7], and the radioactivity in each fraction was determined. From the protein and cell wall fraction L-alanine was isolated; its specific radioactivity was measured and the label distribution in each individual carbon atom was determined by complete chemical degradation.

The fixation of $^{14}CO_2$ into CO was studied under identical conditions, except that CO_2, rather than CO was labeled with ^{14}C (final specific radioactivity 112000 dpm/μmol total CO_2). At the beginning and at the end of the experiment, 40 ml gas samples were transferred into an evacuated 60-ml stoppered vial containing 5 ml of 5 M KOH, and $^{14}CO_2$ was quantitatively absorbed by 3 h shaking. The remaining gas phase, which contain-

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ed ^{14}CO, $^{14}CH_4$ and H_2 was quantitatively transferred to another evacuated anaerobic vial, in which ^{14}CO was enzymatically oxidized to $^{14}CO_2$ with partially purified CO dehydrogenase from *Clostridium thermoaceticum* as in [11]. The oxidation of CO was followed gaschromatographically. The $^{14}CO_2$ formed from ^{14}CO was trapped in 1 M NaOH, and radioactivity was determined after flushing the trap with CH_4 to remove any dissolved $^{14}CH_4$.

Analytical methods: Alanine was determined enzymatically [5]. After conversion to L-lactate it was oxidatively decarboxylated with bichromate, yielding CO_2 (C-1) and acetate (C-2 and C-3). The acetate was then isolated and subjected to Schmidt degradation [7,14]. Authentic ^{14}C-labeled standards were identically treated in parallel. $^{14}CO_2$ was absorbed in 1 M NaOH. Radioactivity was determined by liquid scintillation counting in Aqualuma® cocktail, using [^{14}C]toluene as the internal standard. When total ^{14}C-uptake into all cellular fractions was to be determined, the washed cells were solubilized prior to counting by incubation at 50°C in 0.2 ml 1 M NaOH. CO, CO_2 and CH_4 were determined gaschromatographically [15]. Cell growth was determined by measuring the optical density in a cuvette (d = 1 cm) at 578 nm [12].

3. RESULTS

M. thermoautotrophicum was grown on an 80% H_2/20% CO_2 gas mixture at 1 bar overpressure, in the presence of 5% (v/v) ^{14}CO (49000 dpm/μmol). The cells assimilated ^{14}C from ^{14}CO under these conditions and grew exponentially with a doubling time of 140 min.

Radiocarbon uptake paralleled growth, with a final fixation of 1.5 μmol ^{14}CO/mg cells (dry wt), which corresponds to an incorporation rate of about 15 nmol.min^{-1}.mg protein^{-1}. During the same experiment, about 1.5 μmol ^{14}CO/mg cells were oxidized to $^{14}CO_2$. The final specific radioactivity of CO_2 in the gas phase was 220 dpm/μmol.

The ^{14}CO-labeled cells were fractionated into cell walls, nucleic acids, protein, and lipids, all of which contained ^{14}C. L-alanine was isolated from a hydrolysate of the protein fraction, and was determined to have a specific radioactivity of 7360 dpm/μmol. This value corresponds to 15% of the specific radioactivity of ^{14}CO. Upon degradation of the alanine (after its conversion to lactate), more than 95% of the radioactivity was found in C-2. The carboxyl group (C-1) and methyl group (C-3) were virtually unlabeled (table 1).

Table 1

Distribution of ^{14}C in alanine, which was isolated from *Methanobacterium thermoautotrophicum* after autotrophic growth on $H_2/CO_2/^{14}CO$. The specific radioactivity of the isolated alanine was 7360 dpm/μmol

C-atom of alanine	dpm/μmol	% label in individual carbon atom
C-1	< 120	< 2
C-2	7160	97
C-3	160	2

The above growth experiment was then repeated with the ^{14}C label in CO_2 instead of in CO. The bacteria were grown on 80% H_2/20% $^{14}CO_2$ (112000 dpm/μmol CO_2) at 1 bar overpressure in the presence of 5% (v/v) ^{12}CO. An incorporation of ^{14}C into the CO pool occurred: the specific radioactivity of CO increased from < 10 dpm/μmol to 530 dpm/μmol. About 0.5 μmol ^{14}CO was formed or labeled by exchange from $^{14}CO_2$/mg dry wt of cells.

4. DISCUSSION

In *M. thermoautotrophicum*, pyruvate is formed from acetyl CoA via reductive carboxylation. C-2 of pyruvate (≙alanine) is derived from the carboxyl group of acetyl CoA [5]. In this communication we show that only C-2 of alanine, as opposed to C-1 and C-3, became labeled when the cells were grown in the presence of ^{14}CO. This finding shows that CO is specifically incorporated into the carboxyl group of acetyl CoA. It also shows that CO is not incorporated via CO_2 since the carboxyl of pyruvate, which is derived from CO_2, was not labeled. Also, free formate appears not to be an intermediate since it is not assimilated by *M. thermoautotrophicum* in significant amounts (unpublished). In [10] it was reported that cell extracts of

H_2/CO_2-grown *Methanosarcina barkeri* mediated acetate formation from ^{14}CO (1 nmol.min^{-1}.mg protein^{-1}), but these authors did not demonstrate specific incorporation into the carboxyl group.

Our data suggest that in *Methanobacterium*, acetyl CoA is formed in a carbonylation reaction mechanistically related to the chemical synthesis of acetic acid from methanol and CO [11,16]. The CO required for the carbonylation of a methyl-intermediate could be synthesized from CO_2 via direct reduction (fig.1). This proposal is supported by the observation that *M. thermoautotrophicum* contains an active CO dehydrogenase even when grown in the absence of CO [15], and that during growth on $^{14}CO_2$ in the presence of ^{12}CO, significant amounts of ^{14}CO were formed.

It is indicated in fig.1 that bound rather than free CO is an intermediate in acetyl CoA formation from 2 CO_2. If free CO were the intermediate, one would expect the specific radioactivity of the acetyl CoA carboxyl group to approach that of the large pool of CO in the gas phase above the culture. We found instead that only 15% of the acetyl CoA carboxyl groups was derived from CO in the gas phase. This result indicates that bound CO, derived from CO_2 and in partial equilibrium with free gaseous CO, is the actual intermediate.

The standard redox potential ($E^{\circ\prime}$) of the CO_2/CO couple is -567 mV [11]. If bound CO is the product of CO_2 reduction, the redox potential (E') becomes, however, considerably more positive, making a H_2-dependent reduction of CO_2 to CO thermodynamically feasible. The observed high in vivo rate of exchange between $^{14}CO_2$ and ^{12}CO indeed shows that this interconversion is possible. The pathway depicted in fig.1 could thus account for autotrophic CO_2 fixation via one-carbon intermediates to give acetyl CoA in *Methanobacterium*.

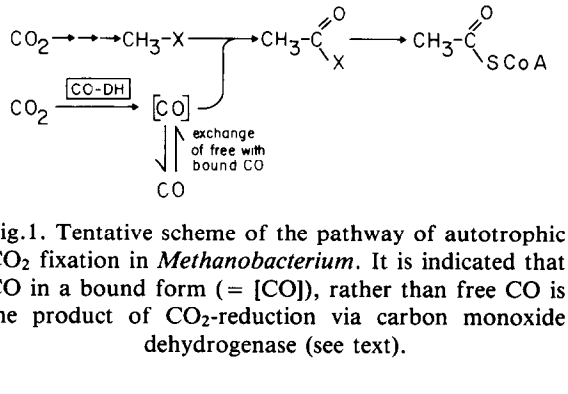

Fig.1. Tentative scheme of the pathway of autotrophic CO_2 fixation in *Methanobacterium*. It is indicated that CO in a bound form (= [CO]), rather than free CO is the product of CO_2-reduction via carbon monoxide dehydrogenase (see text).

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