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Role of carbon monoxide dehydrogenase in the autotrophic pathway used by acetogenic bacteria

(*Clostridium thermoaceticum*/corrinoids/tetrahydrofolate/CO_2 fixation/acetate synthesis)

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ABSTRACT **Anaerobic acetogenic bacteria utilize a pathway of autotrophic growth that differs from any previously described. One part of the pathway involves the reduction of CO_2 to formate and its subsequent conversion to the methyl moiety of methyltetrahydrofolate. The second part involves the formation of a one-carbon intermediate from CO, CO_2 and H_2, or the carboxyl of pyruvate and combination of the intermediate with CoA and methyltetrahydrofolate mediated by a corrinoid enzyme to yield acetyl-CoA. Our studies have been concerned with this latter portion of the pathway and we have proposed that a one-carbon intermediate is formed via carbon monoxide dehydrogenase. It remained possible, however, that the function of the CO dehydrogenase is to reduce the cobalt of the corrinoid enzyme to Co^+, which is required for it to act as a methyl acceptor, and that the dehydrogenase is not involved directly in the formation of a C_1 intermediate. All the enzymes required for the synthesis of acetyl-CoA from CO and methyltetrahydrofolate or from methyltetrahydrofolate and the carboxyl of pyruvate have now been purified. With these purified enzymes, it has been possible to show that CO dehydrogenase is essential for acetyl-CoA synthesis with CO as the substrate under conditions in which the cobalt of the corrinoid is reduced by other means. In addition, using pyruvate ferredoxin oxidoreductase, it has been shown that a $^{14}C_1$–CO dehydrogenase complex is formed from [1-^{14}C]pyruvate. Furthermore, [1-^{14}C]acetyl-CoA was synthesized using the $^{14}C_1$–CO dehydrogenase complex. Thus the evidence appears conclusive that CO dehydrogenase has a direct role in the formation of the carboxyl of acetyl-CoA.**

Recently, a pathway of autotrophic growth was discovered that is used by anaerobic acetogenic bacteria—i.e., bacteria that produce acetate as the major end product of fermentation (1, 2). It differs from other known pathways of metabolism of one-carbon compounds and was found through studies of *Clostridium thermoaceticum* and other bacteria that form 3 mol of acetate per mol of glucose. These bacteria convert carbohydrates to pyruvate and then to acetyl-CoA and CO_2, and CO_2 acts as the electron acceptor in the fermentation. One CO_2 is reduced to formate and it, with utilization of one ATP, is converted to formyltetrahydrofolate, which is reduced by a series of steps to methyltetrahydrofolate. The methyltetrahydrofolate then combines with the second CO_2 and CoASH, thus forming a third acetyl-CoA. The overall conversion is illustrated in reactions **1–6** (THF, tetrahydrofolate; CH_3THF, methyltetrahydrofolate).

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \rightarrow 2\text{CH}_3\text{COCOOH} + 4e^- + 4\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O} \quad [1]$$

$$2\text{CH}_3\text{COCOOH} + 2\text{CoASH} \rightarrow 2\text{CH}_3\text{COSCoA} + 2\text{CO}_2 + 4e^- + 4\text{H}^+ \quad [2]$$

$$\text{ATP} + \text{CO}_2 + \text{THF} + 6\text{H}^+ + 6e^- \rightarrow \text{CH}_3\text{THF} + \text{ADP} + \text{P}_i + \text{H}_2\text{O} \quad [3]$$

$$2\text{H}^+ + 2e^- + \text{CO}_2 + \text{CH}_3\text{THF} + \text{CoASH} \rightarrow \text{CH}_3\text{COCoA} + \text{THF} + \text{H}_2\text{O} \quad [4]$$

$$3\text{CH}_3\text{COSCoA} + 3\text{P}_i + 3\text{ADP} \rightarrow 3\text{CH}_3\text{COOH} + 3\text{ATP} + 3\text{CoASH} \quad [5]$$

$$\text{Sum: Glucose} + 4\text{ADP} + 4\text{P}_i \rightarrow 3\text{CH}_3\text{COOH} + 4\text{ATP} + 4\text{H}_2\text{O} \quad [6]$$

The unique portions of this sequence are reactions **3** and **4** by which the third mol of acetyl-CoA is formed.

This autotrophic synthesis of acetate from CO_2 by heterotrophs has been the subject of numerous studies [for review, see Ljungdahl and Wood (3), Wood *et al.* (2), and Zeikus (4)]. This type of mechanism enables certain bacteria to grow on CO_2 and H_2. With the enzyme hydrogenase, these bacteria generate electrons ($H_2 \rightarrow 2H^+ + 2e$) to reduce CO_2 to methyltetrahydrofolate and form acetyl-CoA. The acetyl-CoA then serves as the building material for their anabolic processes.

Although it was suggested long ago that the fermentation of glucose by *C. thermoaceticum* involves an autotrophic-like pathway (5) and information has been accumulating toward understanding the mechanism (2, 3), it is the most recent developments that have more firmly established this pathway. Drake *et al.* (6) have isolated five fractions that catalyze the synthesis of acetylphosphate from pyruvate and methyltetrahydrofolate. Four are homogeneous: methyltransferase, pyruvate ferredoxin oxidoreductase, ferredoxin, and phosphotransacetylase; the fifth fraction, F_3, contains four unidentified proteins. It was known from investigations by Diekert and Thauer (7) that *C. thermoaceticum* contains CO dehydrogenase, and this enzyme was found to be present in fraction F_3 (8). Carbon monoxide dehydrogenase catalyzes reaction **7**.

$$\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2e \quad [7]$$

This discovery was followed by a surprising development. It

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was found (1) that methyltransferase in combination with fraction F_3 catalyzes reaction **8**.

$$CO + CH_3THF + CoASH \rightarrow CH_3COSCoA + THF \quad [8]$$

Kerby and Zeikus (9) then demonstrated that *C. thermoaceticum* can be grown on CO or CO_2 and H_2 as sources of carbon and energy, thus demonstrating that *C. thermoaceticum* has the properties of a facultative chemoautotroph. The electrons required for the reductive processes of growth on CO are generated in the CO dehydrogenase reaction.

CO dehydrogenase has recently been purified to homogeneity by Ragsdale *et al.* (10, 11). They showed that it contains Ni, Zn, and iron-sulfur clusters and have presented evidence (12) based on EPR spectroscopic data that a nickel–carbon complex is formed in the reaction of CO dehydrogenase with either CO_2 or CO. We have proposed the hypothesis that the CO dehydrogenase has a central role not only in the growth on CO but also in growth on CO_2 and H_2 or pyruvate (Fig. 1). All three substrates are proposed to yield an as yet unidentified C_1 intermediate that is complexed with CO dehydrogenase and is the precursor of the carboxyl of acetyl-CoA. After formation of the CO dehydrogenase–C_1 complex, the conversion to $CH_3COSCoA$ is considered to be identical from all three sources.

The scheme of Fig. 1 is based on the following recent findings. Pezacka and Wood (13) have demonstrated the synthesis of acetyl-CoA from CO_2 and H_2 by enzymes purified from *C. thermoaceticum* including hydrogenase. The hydrogenase generates reduced ferredoxin and, with the reduced ferredoxin as an electron donor, the CO_2, together with methyltetrahydrofolate and CoASH, is converted to acetyl-CoA as shown in Fig. 1. Hu *et al.* (14) have purified the corrinoid enzyme and methylated it using methyltetrahydrofolate and methyltransferase. With the methylated corrinoid enzyme, synthesis of acetyl-CoA occurs in the absence of the methyltransferase in accord with the scheme of Fig. 1 (14). Very recently (15), a protein was purified that has been designated F_x, because its function is unknown. It is now possible to synthesize acetyl-CoA from CO, CoASH, and methyltetrahydrofolate using entirely purified enzymes—i.e., with methyltransferase, the corrinoid enzyme, CO dehydrogenase, and protein F_x. Furthermore, the conversion using pyruvate and methyltetrahydrofolate can be accomplished with the above enzymes plus pyruvate ferredoxin oxidoreductase, thiamin pyrophosphate, and ferredoxin.

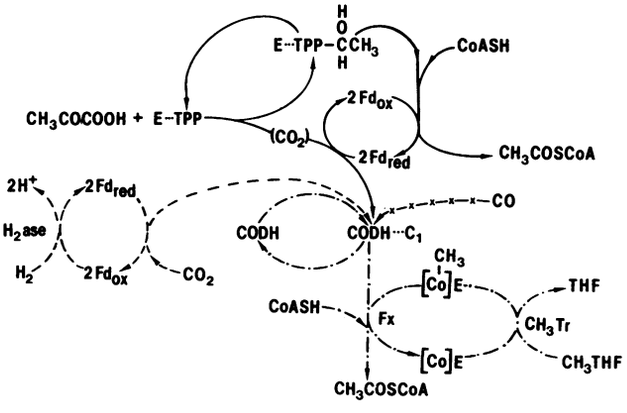

FIG. 1. Converging pathways to CO dehydrogenase (CODH) of pyruvate (indicated by ——), of CO (indicated by ×-×-), and of CO_2 and H_2 (indicated by ------) followed by an identical pathway with methyltetrahydrofolate (CH_3THF) and CoASH to acetyl-CoA (indicated by –·–·). CODH–C_1 = unidentified C_1 intermediate(s) complexed with CO dehydrogenase, (CO_2) is a bound form of CO_2 formed from one carboxyl of pyruvate, CH_3Tr = methyltransferase. E-TPP-$CHOHCH_3$ = the hydroxyethyl complex with E-TPP (E-TPP = pyruvate ferredoxin oxidoreductase), H_2ase = hydrogenase, F_x = required protein with unknown function.

We have proposed (6) that, when pyruvate is the substrate, hydroxyethylthiamin pyrophosphate is formed as an intermediate from carbons 2 and 3 (Fig. 1) and then is oxidized to acetyl-CoA with reduction of ferredoxin (reaction **9**) (Fd_{ox} and Fd_{red}, oxidized and reduced ferredoxin).

$$CH_3COCOOH + 2Fd_{ox} + CoASH \rightarrow 2H^+ + 2Fd_{red} + (CO_2) + CH_3COSCoA \quad [9]$$

With the reduced ferredoxin in combination with CO dehydrogenase, the (CO_2) is converted to the C_1 intermediate complexed with CO dehydrogenase (CODH-C_1 of Fig. 1). (CO_2) is used in Fig. 1 and reaction **9** because Schulman *et al.* (16) have found with methyltetrahydrofolate and CoASH that the C-1 of pyruvate is converted to the carboxyl of acetyl-CoA without equilibration with CO_2. Furthermore, the rate of the conversion was unaffected when the concentration of CO_2/HCO_3^- was <1 μM. These results show that the carboxyl of pyruvate is converted to acetyl-CoA without passage through free CO_2. Nevertheless, pyruvate ferredoxin oxidoreductase does catalyze rapid exchange of CO_2 with the carboxyl of pyruvate (6).

Recently, Kräutler (17) using methylcobalamin and CO has shown by irradiation with visible light that acetylcobalamin is formed in high yield when the CO pressure is elevated. The reaction is proposed to occur through formation of a CH_3 radical, which is energetically favorable for combination with CO. This finding has raised the possibility that CO may be converted to acetyl-CoA by direct reaction with the methylcorrinoid enzyme. R. K. Thauer has suggested (personal communication) that, when pyruvate or CO is the substrate, the mechanism may not involve formation by CO dehydrogenase of the C_1 intermediate as shown in Fig. 1 but rather that this enzyme may be required for reduction of the cobalt of the corrinoid protein. Hu *et al.* (14) have shown that the cobalt of the corrinoid protein must be reduced to the Co^+ state before it can act as a methyl acceptor and they used either CO, CO dehydrogenase and ferredoxin or pyruvate, pyruvate ferredoxin oxidoreductase, thiamin pyrophosphate, and ferredoxin for this reduction. Therefore, with pyruvate as the substrate, the cobalt would be reduced and CO dehydrogenase would not be required if its only purpose is for the reduction. Consequently, a requirement for CO dehydrogenase with pyruvate as the substrate would indicate that CO dehydrogenase has a function in addition to that of the reduction, in accord with Fig. 1.

We report here an investigation of the requirement for CO dehydrogenase in the synthesis of acetyl-CoA. Our results are in accord with the scheme of Fig. 1.

MATERIALS AND METHODS

Growth of *C. thermoaceticum*, Purification of Enzymes, and Assays. *C. thermoaceticum* was grown as described by Drake *et al.* (6). The purification and assays were as previously outlined: methyltransferase and pyruvate ferredoxin oxidoreductase (6), hydrogenase and ferredoxin (13), and fraction F_x (15). CO dehydrogenase was kindly provided by Steve Ragsdale. All purifications were done in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) at room temperature.

Reactions for acetyl-CoA synthesis were carried out anaerobically in serum-stoppered vials at 55°C as described (6, 13). Progress of the reaction was measured by determining the amount of ^{14}C present in the acetyl-CoA. When [*methyl-*

^{14}C]methyltetrahydrofolate was the substrate, the mixture was chromatographed on Dowex-50, H^+ form. When [1-^{14}C]pyruvate was used, the mixture was chromatographed on Dowex-1 formate (18). Total synthesis of acetyl-CoA (^{14}C labeled and unlabeled) was assayed by the spectrophotometric method of Schulman and Wood (19).

The [^{14}C]methyltetrahydrofolate was from Amersham and the [1-^{14}C]pyruvate was from New England Nuclear. All reagents used were of the highest purity available. Protein was determined as described by Bradford (20).

RESULTS

Requirements for the Conversion of [1-^{14}C]Pyruvate, Methyltetrahydrofolate, and CoASH to [1-^{14}C]Acetyl-CoA. Six proteins are required for the conversion of the C-1 of pyruvate, methyltetrahydrofolate, and CoASH to acetyl-CoA (Table 1). The same is true whether [1-^{14}C]pyruvate or [^{14}C]methyltetrahydrofolate is used to measure the formation of acetyl-CoA. Since the cobalt of the corrinoid enzyme is reduced by the pyruvate system (14), it is clear that the CO dehydrogenase is required for a function over and beyond reduction of the cobalt. It also is shown in Table 1 that FAD can replace ferredoxin as an electron carrier, though not as effectively.

Further evidence that CO dehydrogenase is required with pyruvate as the substrate is given in Table 2. The rate is dependent on the concentration of CO dehydrogenase when the concentration of pyruvate ferredoxin oxidoreductase is held constant and, likewise, when the CO dehydrogenase concentration is held constant, the rate is dependent on the concentration of pyruvate ferredoxin oxidoreductase.

Requirement for CO Dehydrogenase When CO Is the Source of the C-1 of Acetyl-CoA. The determination of this requirement was made with ^{14}CO in the presence of the complete pyruvate system. If the only function of CO dehydrogenase is to reduce the cobalt of the corrinoid protein then, in the presence of an active pyruvate system that reduces the cobalt of the corrinoid, CO dehydrogenase should no longer be required. The results of Table 3 (experiment A) show that CO dehydrogenase is required and experiment B shows that the pyruvate system is active under these conditions.

Table 1. Requirements for the formation of labeled acetyl-CoA from [1-^{14}C]pyruvate, methyltetrahydrofolate, and CoA and from pyruvate, [^{14}C]methyltetrahydrofolate, and CoASH

Reaction mixture	[2-^{14}C]Acetyl-CoA formed,* nmol	[1-^{14}C]Acetyl-CoA formed,† nmol
Complete	248	183
Minus CO dehydrogenase or E-TPP or F_x or Fd	0	0
Minus methyltransferase and [Co]E	0	0
Minus Fd, plus FAD	162	98

The complete reaction mixture contained 3.9 units of pyruvate-ferredoxin oxidoreductase thiamin pyrophosphate (E-TPP), 2.8 units of CO dehydrogenase, 1.5 units of methyltransferase, 0.3 unit of corrinoid enzyme ([Co]E), 2 nmol of ferredoxin (Fd), 23 μg of F_x protein, 1.6 μmol of thiamin pyrophosphate, 2 μmol of ATP, 10 mM $MgCl_2$, 2 μmol of CoASH, 5 μmol of dithiothreitol, 0.37 μmol of $Fe(NH_4)_2(SO_4)_2$, 50 μmol of potassium phosphate buffer (pH 6.0), and either 2 μmol of [1-^{14}C]pyruvate (580,000 cpm/μmol) plus 2 μmol of unlabeled methyltetrahydrofolate or 5 μmol of unlabeled pyruvate plus 2 μmol of labeled methyltetrahydrofolate (200,000 cpm/μmol). When ferredoxin was replaced by FAD, the amount was 2.5 μmol. The volume was 650 μl and gas phase was argon. The temperature was 55°C. The reaction was stopped after 15 min by addition of 200 μl of 2 M $HClO_4$. One unit of enzyme is the amount that produces 1 μmol of product/min.

*[2-^{14}C]Acetyl-CoA is formed from [*methyl*-^{14}C]methyltetrahydrofolate.

†[1-^{14}C]Acetyl-CoA is formed from [1-^{14}C]pyruvate.

Table 2. Effect of concentration of pyruvate-ferredoxin oxidoreductase and of CO dehydrogenase on yield of [2-^{14}C]acetyl-CoA from [*methyl*-^{14}C]methyltetrahydrofolate, pyruvate, and CoASH

Pyruvate-ferredoxin oxidoreductase, unit(s)	CO dehydrogenase, unit(s)	[2-^{14}C]Acetyl-CoA formed, nmol
3.9	0.03	0
3.9	0.3	52.0
3.9	2.6	248.0
0.4	2.6	20.0
1.95	2.6	180.0
3.9	2.6	248.0

Conditions were as described in the legend to Table 1. Units are in μmol of product formed per min.

The lower yield of acetyl-CoA in the experiments of Table 3 compared with those of Table 1 is partly due to the fact that less pyruvate ferredoxin oxidoreductase and CO dehydrogenase were used and partly to the fact that, although both pyruvate and CO contribute to C-1 of acetyl-CoA, one of them is unlabeled. (The competition by unlabeled substrate probably reduces the incorporation of ^{14}C from the labeled substrate.)

Evidence for the Formation of a CO Dehydrogenase–C_1 Complex from [1-^{14}C]Pyruvate. In these tests with [1-^{14}C]pyruvate, the gas phase was argon and the corrinoid enzyme, methyltransferase, and methyltetrahydrofolate were omitted to prevent removal of the resulting C_1 intermediate from the CO dehydrogenase through the formation of [1-^{14}C]acetyl-CoA. After termination of the reaction, the mixture was passed through a Sephadex G-50 column to remove unreacted [1-^{14}C]pyruvate. The results of these experiments are given in Table 4. In experiments 1–6, 0.34 nmol of CO dehydrogenase was used and, in experiments 7 and 8, 5.2 nmol. The results show that ^{14}C was bound to protein in the complete mixtures (nos. 1 and 7) and that the amount bound was dependent on the concentration of CO dehydrogenase present. If CO dehydrogenase was omitted (nos. 2 and 8), the amount bound was greatly reduced. If both CoASH and

Table 3. Evidence that CO dehydrogenase is essential for the formation of labeled acetyl-CoA from [1-^{14}C]pyruvate and from ^{14}CO in the presence of pyruvate

Time, min	Exp. A: [1-^{14}C]Acetyl-CoA formed,* nmol		Exp. B: [1-^{14}C]Acetyl-CoA formed,† nmol	
	Complete	Minus CO dehydrogenase	Complete	Minus CO dehydrogenase
5	ND	ND	39	0
10	25	1.0	58	0.6
20	56	1.8	80	1.2

Conditions were as described in Table 1 except that the gas phase was CO and the following amounts were used: in experiment A, 1.0 unit of E-TPP, 0.8 unit of CO dehydrogenase, 1.8 units of methyltransferase, 1.7 units of [Co]E, 2.6 nmol of ferredoxin, 23 μg of F_x protein, 2.5 μmol of pyruvate, and 2 μmol of methyltetrahydrofolate and the gas phase (4.5 ml) was ^{14}CO (102,000 cpm/μmol); in experiment B, 1.5 unit of E-TPP, 0.7 unit of CO dehydrogenase, 2.5 units of methyltransferase, 0.9 unit of [Co]E, 2 nmol of ferredoxin, 17 μg of F_x protein, 2.5 μmol of [1-^{14}C]pyruvate (580,000 cpm/μmol), and 2 μmol of methyltetrahydrofolate. Abbreviations are as in Table 1.

*From ^{14}CO.

†From [1-^{14}C]pyruvate.

Table 4. Evidence that a CO dehydrogenase–$^{14}C_1$ complex is formed from [1-^{14}C]pyruvate

Exp.	Reaction mixture	^{14}C-bound protein, nmol	$^{14}CO_2$, nmol	Acetyl-CoA formed, nmol
	CO dehydrogenase = 0.34 nmol			
1	Complete	0.31	720	320
2	Minus CODH	0.03	800	390
3	Minus Fd and CoASH	0.00	0	0
4	Minus CoASH	0.09	1.5	0.0
5	Minus CoASH, plus Fd_{red}	0.16	1.4	0.0
6	Minus CODH and CoASH, plus Fd_{red}	0.02	1.1	0.0
	CO dehydrogenase = 5.2 nmol			
7	Complete	4.7	640	390
8	Minus CODH	0.05	780	420

Complete reaction mixtures (750 μl) contained either 0.34 nmol (experiments 1–6) or 5.2 nmol (experiments 7 and 8) of CO dehydrogenase [assuming M_r = 420,000 (11)], 0.84 nmol of E-TPP [assuming M_r = 255,000 (6)], 2 nmol of ferredoxin, 1.6 μmol of thiamin pyrophosphate, 1 μmol of CoASH, 2 μmol of [1-^{14}C]pyruvate (580,000 cpm/μmol), 10 mM $MgCl_2$, 5 μmol of dithiothreitol, 0.37 μmol of $Fe(NH_4)_2(SO_4)_2$, and 50 μmol of potassium phosphate buffer (pH 6.0). In experiments 5 and 6, reduced ferredoxin (Fd_{red}) was generated from 1.5 nmol of ferredoxin by using H_2 and hydrogenase. The time of reaction was 10 min and the temperature was 55°C, the gas phase was argon. ^{14}CO was formed as follows: experiment 1, 130 nmol; experiment 2, 82 nmol; experiments 3–6, none. It was not determined in experiments 7 and 8. Determinations of CO_2 and CO were as described by Hu *et al.* (1). Abbreviations are as in Table 1.

*After incubation, a portion of the reaction mixture was chromatographed to remove unreacted [1-^{14}C]pyruvate on a Sephadex G-50 column (35 cm × 0.7 cm) equilibrated with 40 mM 2-[*N*-morpholino]ethanesulfonic acid buffer, pH 6.0/5 mM dithiothreitol. All filtrations were done in an anaerobic chamber.

ferredoxin were omitted, there was no reaction of the pyruvate, as shown by the fact that no $^{14}CO_2$, ^{14}CO, or acetyl-CoA was formed and no ^{14}C was bound to protein (no. 3). This experiment served as a control and shows that the procedures effectively separated the [1-^{14}C]pyruvate from the proteins.

On the molar basis, the amount of $^{14}CO_2$ formed in experiments 1, 2, 7, and 8 was much greater than that of acetyl-CoA. These results indicated that some compound, in addition to acetyl-CoA, was formed from carbons 3 and 2 of the pyruvate. Acetaldehyde was determined in experiments 7 and 8 by using alcohol dehydrogenase. It was found that 180 nmol of acetaldehyde was formed in experiment 7 and 210 in experiment 8, making the total recovery of acetyl-CoA plus acetaldehyde 89% and 81%, respectively, of the CO_2. Some acetylmethylcarbinol may also be formed from pyruvate.

It seemed likely that the protein-bound ^{14}C might be as $^{14}CO_2$ or ^{14}CO and tests were done by passage of CO_2 or CO through the filtrate of experiment 7 at 55°C. There was no loss of ^{14}C with CO. With CO_2, 20%, 33%, and 50% of the protein-bound ^{14}C was removed and recovered in a hyamine hydroxide (New England Nuclear) train after 1, 2, and 3 hr, respectively. When the filtrate was treated with $HClO_4$ (0.2 M) and flushed with CO_2 for 30 min, 94% of the ^{14}C was recovered in the hyamine hydroxide.

In addition, the convertibility to [1-^{14}C]acetyl-CoA of the protein-bound ^{14}C was determined. For this purpose, methyltetrahydrofolate, CoASH, methyltransferase, corrinoid enzyme, and protein F_x were added to a portion of the filtrate of experiment 7 with argon as the gas phase. Only 10% of the protein-bound ^{14}C was converted to acetyl-CoA. It was suspected that the low conversion was due to the inactivation of CO dehydrogenase during formation of the C_1 complex and the Sephadex G-50 filtration. If so, the ^{14}C bound to the inactive CO dehydrogenase would not be converted to acetyl-CoA. A control was set up identical to experiment 7 except that ferredoxin was omitted to prevent formation of protein-bound ^{14}C from the [1-^{14}C]pyruvate. In this case, [^{14}C]methyltetrahydrofolate was used and the gas phase was CO; thus, the conversion of methyltetrahydrofolate to [2-^{14}C]acetyl-CoA was the measure of the activity of the control. A third assay of acetyl-CoA synthesis from [^{14}C]methyltetrahydrofolate and CO was done with an amount of CO dehydrogenase equivalent to that of experiment 7 and the control but the CO dehydrogenase was used directly without incubation with pyruvate ferredoxin oxidoreductase, pyruvate, ferredoxin, and CoASH and it was not subjected to gel filtration. It was found that the conversion of [^{14}C]methyltetrahydrofolate to acetyl-CoA in the control was only 20% of that observed with untreated CO dehydrogenase. Therefore, a correction for the inactivation in experiment 7 similar to that observed in the control indicates that 50% of the protein-bound ^{14}C would have been converted to acetyl-CoA if inactivation had not occurred.

DISCUSSION

The principal objective of this investigation was to determine whether, in this newly recognized chemoautotrophic pathway of acetogenic bacteria, CO dehydrogenase plays a central role through formation of a C_1 intermediate (as proposed in Fig. 1) or functions to reduce the cobalt of the corrinoid enzyme so that it can act as a methyl acceptor. The availability of a system consisting entirely of purified enzymes that catalyzes the synthesis of acetyl-CoA from methyltetrahydrofolate and CoA with CO, CO_2 and H_2, or pyruvate has made it possible to investigate this question directly. The results are in accord with the scheme of Fig. 1; CO dehydrogenase is required even under conditions in which the cobalt of the corrinoid enzyme is reduced. The CO dehydrogenase, of course, may also serve in maintaining the cobalt of the corrinoid reduced so that it is active as a methyl carrier.

The results with [1-^{14}C]pyruvate given in Table 4 provide further evidence that CO dehydrogenase has a direct role in forming the C_1 intermediate. ^{14}C from [1-^{14}C]pyruvate was found bound to protein and the amount bound was proportional to the amount of CO dehydrogenase present (experiments 1 and 7) and in the absence of CO dehydrogenase very little ^{14}C was bound (experiments 2 and 8). The decreased binding in the absence of CO dehydrogenase clearly was not caused by a decrease in the breakdown of pyruvate because the formation of unlabeled acetyl-CoA, $^{14}CO_2$, and ^{14}CO was about the same in the presence and absence of CO dehydrogenase. When ferredoxin and CoASH were omitted, the cleavage of [1-^{14}C]pyruvate to acetyl-CoA and CO_2 did not occur and no $^{14}CO_2$, ^{14}CO, acetyl-CoA or protein-bound ^{14}C was observed (experiment 3). When CoASH was omitted, some protein-bound ^{14}C was observed (experiment 4); this may be because the ferredoxin as isolated is partly reduced (13) and can serve in a reduction required for formation of the $^{14}C_1$–CO dehydrogenase complex. This suggestion is supported by experiment 5. When the ferredoxin was reduced with H_2 and hydrogenase, the yield of protein-bound ^{14}C in the absence of CoASH was about 52% of that found with the complete system.

The bound ^{14}C appears to be as CO_2. When the protein was denatured with $HClO_4$ and CO_2 was passed through the solution for 30 min, about 94% of the ^{14}C was recovered in hyamine hydroxide. Since the presence of reduced ferredoxin improved the yield of protein-bound ^{14}C (Table 4, compare experiment 5 with experiment 4), we considered it

likely that the carboxyl of pyruvate was reduced to the level of CO by CO dehydrogenase during the interaction with pyruvate ferredoxin oxidoreductase. However, with ^{14}CO, we found no indication of formation of a stable C_1 complex. These results indicate that the binding of the carboxyl group of pyruvate to CO dehydrogenase is different than that of CO.

The ^{14}C bound to CO dehydrogenase is convertible to acetyl-CoA following addition of methyltetrahydrofolate, methyltransferase, corrinoid enzyme, protein F_x, and CoASH, although in low yield. Evidence is presented showing that the low yield is because the CO dehydrogenase (or some other factor) is partially inactivated during separation of the $^{14}C_1$–CO dehydrogenase complex from the excess [1-^{14}C]pyruvate. Using a correction for the inactivation, we estimate that approximately 50% of the ^{14}C bound to CO dehydrogenase would have been converted to acetyl-CoA if there had been no inactivation.

There is much about the scheme shown in Fig. 1 that remains uncertain. We have no idea where and how protein F_x functions, what the C_1 intermediate(s) is, and how the methyl of the corrinoid enzyme is joined with the C_1 intermediate and CoASH to form acetyl-CoA. The situation is better for the portion of the pathway that involves the synthesis of methyltetrahydrofolate from CO_2 and tetrahydrofolate. The enzymes for this synthesis have been isolated and the mechanisms have been established in studies by Ljungdahl and his collaborators and by others [see the review by Ljungdahl and Wood (3), the recent publications by Yamamato *et al.* (21) and Clark and Ljungdahl (22), and the recent reveiw by Ljungdahl (23)]. Sihce all the enzymes have been purified that catalyze the reactions of Fig. 1, we now are in a favorable position to elucidate the detailed mechanism of the reactions of this portion of the pathway.

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