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The synthesis of acetyl-CoA by *Clostridium thermoaceticum* from carbon dioxide, hydrogen, coenzyme A and methyltetrahydrofolate

Ewa Pezacka and Harland G. Wood

Department of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44016, USA

Abstract. It has been demonstrated that enzymes from *Clostridium thermoaceticum* catalyze the following reaction in which Fd is ferredoxin and CH_3THF is methyltetrahydrofolate.

$$CO_2 + H_2 + CH_3THF + CoASH \xrightarrow{ATP, Fd}$$

$$CH_3\,COSCoA + THF + H_2O.$$

The system involves hydrogenase, CO dehydrogenase, a methyltransferase, a corrinoid enzyme and other unknown components. Hydrogenase catalyzes the reduction of ferredoxin by H_2; CO dehydrogenase then uses the reduced ferredoxin to reduce CO_2 to a one-carbon intermediate that combines with CoASH and with a methyl group originating from CH_3THF to form acetyl-CoA. It is proposed that these reactions are part of the mechanism which enables certain acetogenic autotrophic bacteria to grow on CO_2 and H_2.

Key words: *Clostridium thermoaceticum* – CO_2 utilization – Hydrogenase – Autotrophism – Acetogenic bacteria – Acetyl-CoA synthesis

Studies in 1942 showed that *Clostridium thermoaceticum* ferments glucose with the formation of approximately 3 mol of acetate per mol of glucose, and since then a long series of studies have led to the following concept of the mechanism. The glucose is metabolized to 2 mol of pyruvate, one of which is converted to acetate and CO_2. The CO_2 is reduced to formate which, in turn, is converted to formyl-THF and reduce to CH_3THF. The methyl is then transferred to the cobalt of the corrinoid enzyme. Carbons 2 and 3 of the second mol of pyruvate give rise to another mol of acetate and the third mol of acetate is formed from carbon 1 and the methyl of the corrinoid. The details of these developments have been reviewed recently (Ljungdahl and Wood 1982; Wood et al. 1982; Zeikus 1983) and provide background and references.

Recently, Drake et al. (1981) isolated five fractions from *C. thermoaceticum* which catalyze the most unique portion of this pathway, i.e., formation of [2-^{14}C]acetyl-CoA and [2-^{14}C]acetyl phosphate from $^{14}CH_3THF$ and pyruvate. One of the fractions (F_3) was not resolved and contained four major proteins of which one was shown to be the nickel enzyme, carbon monoxide dehydrogenase (Drake et al. 1980).

Offprint requests to: H. G. Wood

Recently this enzyme has been purified by Ragsdale et al. (1983a) and by Diekert and Ritter (1983). It catalyzes the following reaction.

$$CO + H_2O + 2\,Fd_{ox} \leftrightharpoons CO_2 + 2H^+ + 2\,Fd_{red}. \quad (1)$$

Formate is not utilized by CO dehydrogenase but we have postulated (Drake et al. 1980; Hu et al. 1982) that an enzyme-bound intermediate is formed, which is designated [HCOOH], to indicate there is the equivalent of the addition of H_2O when CO_2 is formed.

Hu et al. (1982) found that CO could serve as the carboxyl donor in place of pyruvate and that only two of the five fractions were required for the formation of acetyl-CoA from CO, CH_3THF and CoA. These were fraction F_2, a methyltransferase, and fraction F_3 which contains the CO dehydrogenase and the corrinoid enzyme (Fig. 1).

It was proposed (Hu et al. 1982; Wood et al. 1982) that both the carboxyl of pyruvate and CO are converted to the same C_1 intermediate via CO dehydrogenase, [HCOOH] in Fig. 1. The methyltransferase catalyzes the transfer of the methyl of CH_3THF to the corrinoid enzyme. Then by a mechanism whose details are not yet understood, the C_1 intermediate combines with the methyl of the corrinoid enzyme and CoASH yielding acetyl-CoA. ATP is the only cofactor required for this conversion. Fraction F_1 is phosphotransacetylase and in its presence, the acetyl-CoA with phosphate is converted to acetyl phosphate. Hu and Wood (1983) have shown that F_3 contains another essential component in addition to the corrinoid enzyme and CO dehydrogenase and this component is designated F_3 ($\times$) in Fig. 1.

With pyruvate as the carboxyl donor, ferredoxin, thiamin pyrophosphate and fraction F_4 are required in addition to CoA, ATP and fractions F_2 and F_3 for formation of acetyl-CoA. F_4 is a thiamin pyrophosphate dependent pyruvate ferredoxin oxidoreductase (Drake et al. 1981) which catalyzes the formation of acetyl-CoA from carbons 2 and 3 simultaneously reducing ferredoxin as illustrated in Fig. 1. The CO_2 from the carboxyl of pyruvate is shown in brackets to indicate that it occurs in a bound, unidentified form. This proposal is based on the observation of Schulman et al. (1973) that when the carboxyl of pyruvate is converted to the carboxyl group of acetyl-CoA, it does not equilibrate completely with $NaHCO_3$-CO_2. That the carboxyl of pyruvate gives rise to CO_2 is evident since the pyruvate:ferredoxin oxidoreductase of F_4 catalyzes a rapid exchange of $^{14}CO_2$ with the carboxyl of pyruvate (Drake et al. 1981).

Recently, Drake (1980) has reported that cell-free extracts from *C. thermoaceticum* contain hydrogenase. This enzyme

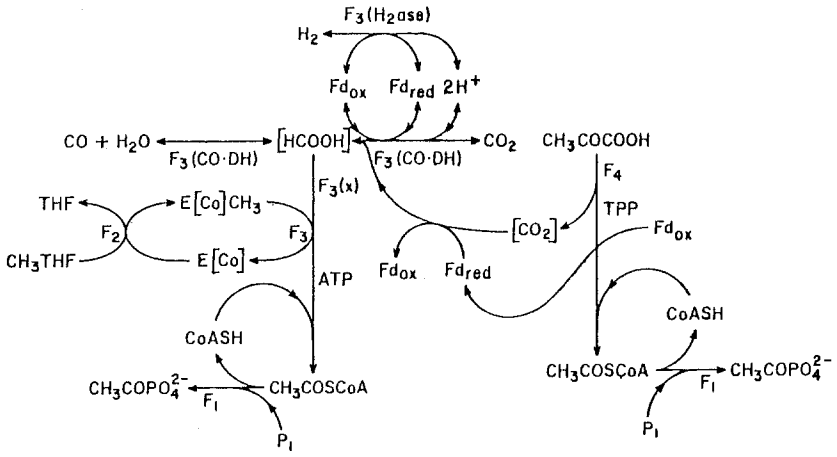

Fig. 1
The pathway of formation of acetyl-CoA and acetyl phosphate from CH_3THF with either CO or CO_2 and H_2 or pyruvate as the source of the carboxyl group. *THF* is tetrahydrofolate, F_1 is phosphotransacetylase; F_2, methyltransferase, F_3 contains CO dehydrogenase (CO · DH) and a corrinoid enzyme E[Co]; F_4 is a pyruvate ferredoxin oxidoreductase; *Fd* is ferredoxin. *H_2ase* is hydrogenase; *TPP* is thiamin pyrophosphate and F_3 (×) is an, as yet, unidentified enzyme

had not been observed previously in this bacterium, apparently because proper precautions were not taken to exclude oxygen to which it is extremely sensitive. We report here that the hydrogenase is present in fraction F_3 and reduces ferredoxin. When pyruvate is oxidized to acetyl-CoA, the reduced ferredoxin that is generated is coupled by the CO dehydrogenase to the reduction of $[CO_2]$ to the C_1 intermediate [HCOOH], see Fig. 1. Accordingly, in the presence of hydrogenase, H_2 should be capable of replacing pyruvate as the electron donor for generation of reduced ferredoxin and for conversion of CO_2 to [HCOOH] by CO dehydrogenase. This sequence is illustrated at the top of Fig. 1 and involves the following reactions:

$$2\,Fd_{ox} + H_2 \xrightarrow{\text{Hydrogenase}} 2H^+ + 2\,Fd_{red} \quad (2)$$

$$2H^+ + CO_2 + 2\,Fd_{red} \xrightarrow{\text{CO dehydrogenase}} [HCOOH] + 2\,Fd_{ox} \quad (3)$$

$$[HCOOH] + CoASH + CH_3THF \xrightarrow[F_3x,\ \text{corrinoid enzyme}]{\text{ATP, transmethylase}} CH_3COSCoA + THF + H_2 \quad (4)$$

$$\text{Sum: } CO_2 + H_2 + CoASH + CH_3THF \rightarrow CH_3COSCoA + THF + H_2O. \quad (5)$$

We show here that this sequence is catalyzed by enzymes from *C. thermoaceticum* and report on some of the properties of this system.

Materials and methods

Organism and purification of enzymes. Culture of *Clostridium thermoaceticum* and purification of enzymes were performed as previously outlined (Drake et al. 1980, 1981; Hu et al. 1982). In addition, the F_3 component was dialyzed in an Amicon apparatus with a UM 10 membrane against 30 mM potassium phosphate buffer, pH 6.8, containing 10 mM dithioerythritol. Fraction F_2 was pure as judged by polyacrylamide gel electrophoresis, whereas the F_3 component had 4 major protein bands on polyacrylamide gel electrophoresis.

Hydrogenase was purified from the crude F_1 fraction previously (Drake et al. 1981) designated cF_1. The brown solution was applied to a column of DE-52 cellulose (2.8 × 2.0 cm) and elution was performed stepwise, under anaerobic conditions, with Tris buffer, 20 mM, pH 7.4, containing the following concentrations and volumes of $MgCl_2$: 0.00 M, 150 ml; 0.065 M, 25 ml; 0.09 M, 25 ml and 0.40 M, 25 ml. Maximum amounts of hydrogenase were found in a brown-colored solution obtained by elution with 0.065 M $MgCl_2$. The eluate was subjected to a heat treatment under H_2 at 70° C for 5 min and the mixture was centrifuged under H_2. The supernatant solution was chromatographed on a Sephacryl S-300 to separate the ferredoxin from the hydrogenase. The fractions which contained the hydrogenase activity were pooled and applied to a DE-22 cellulose column (1.8 × 2.5 cm) equilibrated with 0.02 M Tris-HCl, pH 7.4, and washed with this buffer and then eluted with the buffer containing 0.065 M $MgCl_2$. This hydrogenase preparation was free of ferredoxin as judged by the requirement of ferredoxin for the reduction of metronidazol with H_2. It was stored anaerobically for several months in 25% glycerol at −20° C with a 20% to 30% loss in activity.

Ferredoxin of *C. thermoaceticum* was purified as described earlier (Drake et al. 1981). The ratio of A_{390} : A_{280} was 0.68. The ferredoxin gave a single band on polyacrylamide gel electrophoresis and gel electrofocusing (Wrigley 1971) when visualized with the highly sensitive silver nitrate stain (Morrissey 1981). The absorption spectrum was used as a qualitative estimation of the extent of oxidation and reduction of ferredoxin.

Assay of formation of acetyl-CoA. Reactions were performed anaerobically in serum-stoppered vials at 55° C as described elsewhere (Drake et al. 1981). The gas phase was H_2 (chemically pure grade, 100%); the gas and the liquid phases were equilibrated by periodic shaking in a water bath. After the equilibration, the protein components were injected into the vial, and the reaction was initiated by injecting the $^{14}CH_3THF$ or $NAH^{14}CO_3$. Progress of the reaction was measured by determining the amount of ^{14}C present in the acetyl-CoA. The reaction were terminated with 0.4 ml of 2.2 N $HClO_4$. When $^{14}CH_3THF$ was the substrate, 0.25 ml of 0.1 M NaOH were added to adjust the pH to 2.5–3.0 and the mixture was chromatographed on Dowex 50. During this chromatography, the acetyl-CoA hydrolyzes to acetate which is separated from the unreacted $^{14}CH_3THF$ which remains tightly bound on the resin (Drake et al. 1981). When $NaH^{14}CO_3$ was used, the reaction was terminated with $HClO_4$ as above and CO_2 was passed through the solution to

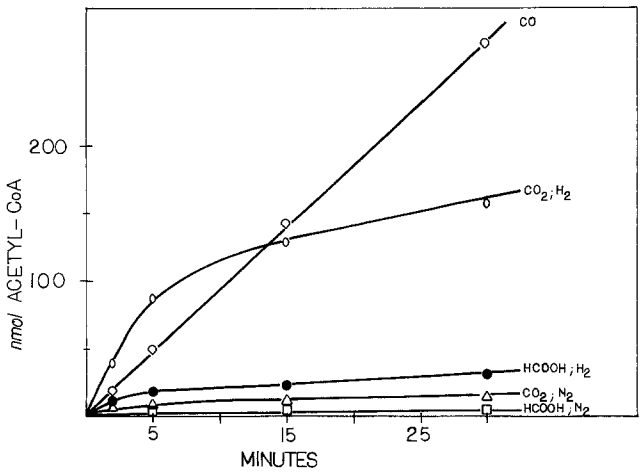

Fig. 2. Formation of acetyl-CoA with ^{14}CO, or $NaH^{14}CO_3$ and H_2 or $H^{14}COOH$ and H_2 as the sources of C_1 for synthesis of acetyl-CoA. CO: Assay conditions were as described in Materials and methods and legend of Table 2 with the following concentrations of protein fractions; F_2, 19 μg and F_3, 180 μg. Ferredoxin was omitted and the gas phase was ^{14}CO (20,000 cpm/μmol). CO_2, H_2: Assay conditions were as with CO except F_2 was 25 μg; F_3 was 220 μg and ferredoxin (25 μg) was included. $NaH^{14}CO_3$; 50 μmol (280,000 cpm) was the C_1 donor and the gas phase was 100% H_2. HCOOH, H_2: Conditions as with CO_2, H_2 except $NaH^{14}CO_3$ was replaced with $H^{14}COOH$, 50 μmol (280,000). CO_2, N_2: Conditions as with CO_2, H_2 except the gas phase was 100% N_2. HCOOH, N_2: Conditions as with HCOOH, H_2 except the gas phase was 100% N_2

Table 1. Activity of enzymes and concentration of coenzymes in fraction F_3[a]

Enzymes	Specific activity (U/mg protein)	Total activity (units)
CO dehydrogenase	4.89	117
Formate dehydrogenase	12.50	300
Hydrogenase	5.34	128
Coenzymes	**Specific content (nmol/mg protein)**	**Total content (nmol)**
Corrinoids	1.96	47.0
Flavin	0.94	22.0

[a] 50 g of centrifuged cell were used in this preparation. The cell-free extract contained 1.23 μmol/mg protein of the protein-bound corrinoids. The protein concentration was 4.8 mg/ml and total volume 5 ml. Units are in μmol/min

remove $^{14}CO_2$. This solution was then adjusted to ~pH 7 by addition of 0.25 ml of 2 M KOH and the solution was chromatographed on Dowex 50 as described above.

Other assays. The carbon monoxide dehydrogenase was assayed spectrophotometrically by following the reduction of the methyl viologen at 578 nm ($\varepsilon_{578} = 9.7 \times 10^4\ M^{-1}\ cm^{-1}$) (Drake et al. 1980). Hydrogenase, likewise, was monitored by reduction of methyl viologen (Drake 1980), with a mixture containing 50 mM Tris-HCl, pH 8.8, 5 mM dithiothreitol and 2 mM methyl viologen, and with a gas phase of 100% H_2. Formate dehydrogenase was assayed with either NADP ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) or methyl viologen ($\varepsilon_{600} = 1.13 \times 10^4\ M^{-1}\ cm^{-1}$) as electron acceptors (Andreesen and Ljungdahl 1974; Ljungdahl and Andreesen 1975). Ferredoxin was measured by H_2-hydrogenase-dependent reduction of metronidazole (Chen and Blanchard 1979). Absorption at 580 nm ($\varepsilon = 10.13 \times 10^3\ M^{-1}\ cm^{-1}$) was used to determine the amount of dicyanocorrinoid present in fraction F_3. Flavin concentration was calculated from absorption at 450 nm in the oxidized and reduced states after acid liberation of coenzymes from fraction F_3 ($\varepsilon = 11.5 \times 10^3\ M^{-1}\ cm^{-1}$) (Meyer 1982). Protein was measured by the micro method of Bradford (1976). The synthesis and purification of $^{14}CH_3THF$ was performed by a modification of the methods of Blair and Saunders (1970) and Parker et al. (1971) as described elsewhere (Drake et al. 1981). The $[^{14}C]HCO_3^-$ was from New England Nuclear Corp. (Boston, MA, USA). $[^3H]$CoASH was from Amersham Corporation (Arlington Heights, IL, USA). All reagents utilized were of the highest purity available.

Table 2. The requirements for the conversion of $NaHCO_3$, H_2, CH_3THF and CoASH to acetyl-CoA[a]

Reaction mixture	Acetyl-CoA (nmol)	Relative activity (%)
Complete	209	100
Minus F_2	31	15
Minus F_3	10	5
Minus Fd	21	10
Minus H_2 (argon)	15	7

[a] In all experiments, fraction F_3, after the DEAE Bio-Gel A column was dialyzed anaerobically for removal of NaCl, a potential inhibitor of hydrogenase (Drake 1980). In each case, the solution (550 μl) contained (in micromol): potassium phosphate buffer (pH 6.0), 50; coenzyme A, 1; ATP, 2; $NaHCO_3$, 50; dithioerythreitol, 5; Fe $(NH_4)_2(SO_4)_2$, 0.6; $^{14}CH_3THF$, 1 (77,400 cpm/μmol); in a 4 ml vial. The atmosphere was 100% H_2 and the temperature was 55° C. The reaction was started by injection of $^{14}CH_3THF$ (10 μl) and was stopped after 30 min by injection of 200 μl of 2 N $HClO_4$. F_2: 20 μg; F_3: 210 μg; Fd: 25 μg

Results

Conversion of CO_2, H_2, CH_3THF and CoASH to Acetyl-CoA. We have found that in addition to the CO dehydrogenase. Fraction F_3 contains hydrogenase, fromate dehydrogenase, corrinoid and flavin as shown in Table 1. In contrast to Drake (1980), who reported that ferredoxin is not reduced by the hydrogenase of *Clostridium thermoaceticum*, we find it is reduced by our hydrogenase preparation as well as by fraction F_3. Experiments relative to reactions 2–5 are shown in Table 2. It is seen that fraction F_2 (transmethylase) and F_3, which contains the corrinoid enzyme, hydrogenase, and at least one other unknown enzyme are required as well as ferredoxin. The flavins in fraction F_3 apparently are not effective as electron acceptors for hydrogenase in this sequence since the yield of acetyl-CoA was low when ferredoxin was omitted.

Evidence that formate dehydrogenase is not envolved in the conversion of CO_2 to C-1 of Acetyl-CoA. CO, CH_3THF and CoASH are converted to acetyl-CoA by fractions F_2 and F_3 in the absence of ferredoxin but $[^{14}C]$formate is not (Drake et

Table 3. Conversion of $H^{14}COOH$ to $^{14}CO_2$ and [^{14}C]acetyl-CoA, and of $NaH^{14}CO_3$ to $H^{14}COOH$ and [^{14}C]acetyl-CoA by fractions F_2 and F_3

	Substrate	A $H^{14}COOH$		B $NaH^{14}CO_3$	
	Products	^{14}C acetyl-CoA (nmol)	$^{14}CO_2$ (nmol)	^{14}C acetyl-CoA (nmol)	$H^{14}COOH$ (nmol)
Reaction mixture	Complete	20	490	150	0
	+ NADP	80	900	180	90
	+ NADPH	76	840	200	730

Experiment A: Assay conditions were as described in the legend of Table 2 and in Materials and methods: $H^{14}COOH$, 20 μmol (113,000 cpm); NADP, 2 μmol; $NADPH_2$, 2 μmol; F_2, 30 μg; F_3, 350 μg; ferredoxin, 25 μg; the atmosphere was H_2, incubation time, 30 min. Experiment B: As above except $NaH^{14}CO_3$, 20 μmol (138,000 cpm) in place of $H^{14}COOH$

Table 4. Effect of inhibitors of formate dehydrogenase on the formation of acetyl-CoA by fractions F_2 and F_3 from $^{14}CH_3THF$, CoASH with CO or $NaHCO_3$ and H_2 or HCOOH and H_2 as the source of C_1

Reaction mixture[a]	Formate dehydrogenase (U/mg of F_3)	CO dehydrogenase (U/mg of F_3)	Source of C-1		
			CO	$NaHCO_3/H_2$	$HCOOH/H_2$
			Acetyl-CoA formed		
			(nmol)	(nmol)	(nmol)
Complete	10.25	5.25	230	142	22
+NADP	12.10	5.00	240	180	94
+NADP+EDTA	3.8	6.24	220	180	20
+EDTA	0	6.24	290	200	20
+Hypophosphite	0	4.32	190	142	20
+NADP+hypophosphite	0	4.00	200	140	20

[a] Conditions were as described in the legend to Table 2. F_3, 230 μg; F_2, 20 μg; Fd, 15 μg; $^{14}CH_3THF$, 1 μmol (140,000 cpm). The gas phase was CO when CO was the C_1 donor, otherwise H_2. $NaHCO_3$ and formate were 20 μmol. The inhibitors EDTA and sodium hypophosphite were at 100 mM and 5 mM respectively. NADP was 2 mM. The activity of CO dehydrogenase and formate dehydrogenase were measured as described in Materials and methods.

al. 1981). However, in the presence of H_2 and ferredoxin, [^{14}C]acetyl-CoA is formed from $H^{14}COOH$, CH_3THF and CoASH but as shown in Fig. 2, the yield is much lower than from ^{14}CO or $NaH^{14}CO_3$ and H_2. With formate as a substrate, H_2 is absolutely essential. The initial rate with CO_2 and H_2 was more rapid than with CO. The decrease in rate with CO_2 and H_2 was due to inactivation of the hydrogenase. Following addition of hydrogenase at 5 min in a separate test, there was an increase in rate comparable to that of the first 5 min. Schmidt degradation (Phares 1951) showed the ^{14}C is confined to C-1.

The observation that ^{14}C from formate is incorporated into acetyl-CoA raises the questions whether the formate is converted directly or indirectly to the carboxyl of acetyl-CoA and also whether formate dehydrogenase is involved in the conversion of either formate or CO_2 and H_2 to the C-1 of acetyl-CoA. Formate dehydrogenase, which catalyzes reaction 6 (Andreesen and Ljungdahl 1974; Ljungdahl and Andreesen 1975; Thauer 1972) has recently been purified from *C. thermoaceticum* by Yamamoto et al. (1983).

$$HCOOH + NADP \leftrightharpoons CO_2 + NADPH + H^+. \quad (6)$$

In Table 3, data are shown that were obtained when NADP and NADPH were added which are required for formate dehydrogenase activity. Although CO_2 was formed from $H^{14}COOH$ in the absence of NADP or NADPH, the conversion was increased when either was added and this increase was accompanied by an increased yield of acetyl-CoA. With $^{14}CO_2$ as the labeled substrate, formate was formed in the presence of either NADP or NADPH but not in their absence. When they were present, there was some increases in the yield of acetyl-CoA from HCOOH but the amount was much less than that obtained with $NaHCO_3$. The results shown are representative of several experiments.

EDTA (Andreesen and Ljungdahl 1974) and hypophosphite (Pines 1958) are inhibitors of formate dehydrogenase. The effects of these compounds on the formation of acetyl-CoA are shown in Table 4. In these experiments, with CO or $NaHCO_3$ and H_2 or formate and H_2 as the C_1 donors, the activity of formate dehydrogenase and CO dehydrogenase were determined as well as the yield of acetyl-CoA. When hypophosphite was present, there was complete inhibition of formate dehydrogenase even in the presence of NADP. Hypophosphite caused about 20% inhibition of CO dehydrogenase. The CO dehydrogenase was not inhibited by EDTA; in fact, some increase in activity was observed. The results show clearly that formation of acetyl-CoA occurred whether or not formate dehydrogenase was active. Thus, formate dehydrogenase is not involved in the conversion of CO or $NaHCO_3$ and H_2 or formate and H_2 to the C-1 of acetyl-CoA. In the absence of EDTA, the yield of acetyl-CoA

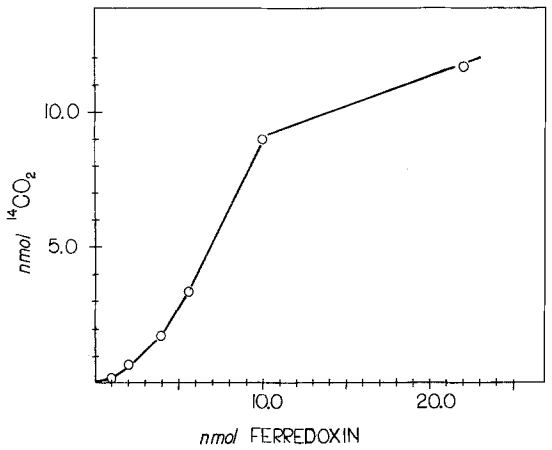

Fig. 3. Oxidation of $H^{14}COOH$ by ferredoxin and formation of $^{14}CO_2$. Stoppered vials containing 0.5 ml 50 mM Tris-HCl buffer, pH 7.4, 5.0 µM dithiothreitol and 50 µmol $H^{14}COOH$ (1,200,000 cpm/l µmol) were flushed with argon several times before the ferredoxin was added. The time was 30 min, and the temperature was 55° C. To terminate the reaction, sulfuric acid was added. The vials containing the reaction mixture were attached to a train consisting of four test tubes (1.5 by 10 cm) containing 5 ml of hyamine hydroxide each. Nitrogen gas was admitted which passed through the reaction mixture and train. The hyamine hydroxide was assayed for $^{14}CO_2$

Table 5. V_{max} and K_m of substrates in the synthesis of acetyl-CoA by fractions F_3 and F_2 from *Clostridium thermoaceticum*

No.	Variable substrate	K_m (mM)	V_{max} (µmol/ min)
1	^{14}CO	2.8	0.050
2	H_2	1.7	0.034
3	$^{14}CH_3THF$ (CO as C_1)	40.0	0.050
		0.47	
4	$^{14}CH_3THF$ (CO_2 as C_1)	34.0	0.045
		0.55	
5	[^{3}H]CoASH (CO as C_1)	4.7	0.045
6	[^{3}H]CoASH (CO_2 as C_1)	3.8	0.040

Assay conditions were as described in the Materials and methods and legend of Table 2 using 210 µg of F_3, 25 µg of F_2, 20 µg of Fd. Experiment 1: The amount of ^{14}CO (210,000 cpm/20 µmol) in the gas phase was varied. Experiment 2: The amount of H_2 in the gas phase was varied. The $NaH^{14}CO_3$ (280,000 cpm) was 40 mM. Experiments 3 and 4: $^{14}CH_3$-THF (150,000 cpm/µmol) was 1 to 20 mM. The gas phase was CO or H_2 with $NaH^{14}CO_3$. Experiments 5 and 6: Varied concentration of [^{3}H]CoA (140,000 cpm/µmol) with CO or $NaHCO_3$ as in Experiment 3 and 4. The concentration of H_2 and CO in the liquid phase of reaction were calculated from standard solubility tables

from formate increased when NADP was added. This result is in conformity with the results shown in Table 3 and indicates that the increased formation of acetyl-CoA may result from utilization of the CO_2 formed from formate by formate dehydrogenase.

We have observed that ferredoxin is slowly reduced when it is incubated with formate under anaerobic conditions. This reduction of ferredoxin by formate involves oxidation of formate to CO_2 as shown in Fig. 3 and most likely is the source of CO_2 that is formed from formate (Table 3) in the absence of NADP and NADPH when formate dehydrogenase is inactive.

Kinetics of the synthesis of acetyl-CoA with variable concentrations of CO, H_2, CH_3THF and CoASH. K_m values of the individual substrates and the V_{max} as determined from the rate of overall synthesis of acetyl-CoA are shown in Table 5. The V_{max} values were approximately the same with each substrate, which is to be expected since the substrates were all present in excess and the overall synthesis is determined by the same rate-limiting reaction. Except for hydrogenase, K_m values are not known for the individual reactions of the system. Drake (1980) found the K_m for H_2 with hydrogenase of *C. thermoaceticum* to be 0.17 mM with methylviologen as the acceptor and 0.08 mM with benzylviologen. These K_m's were measured at pH 8.5 and 50° C whereas, in our study, at pH 6.0 and temperature 55° C, the K_m was 1.7 mM. Kleiner and Burris (1970) found that the K_m for H_2 was 10 times higher at pH 7 than at pH 8 with hydrogenase from *C. pasteurianum*. Thus, the higher K_m which we observe in the overall system may be due to the differences in pH, i.e., 6 compared to 8.5. The K_m's for CoASH and also for CH_3THF were about the same when assayed with either CO or CO_2 as C_1 donor. These findings are in accord with the scheme shown in Fig. 1 since these substrates involve identical reactions with either CO or CO_2 as the C_1 donor. It is noteworthy that two

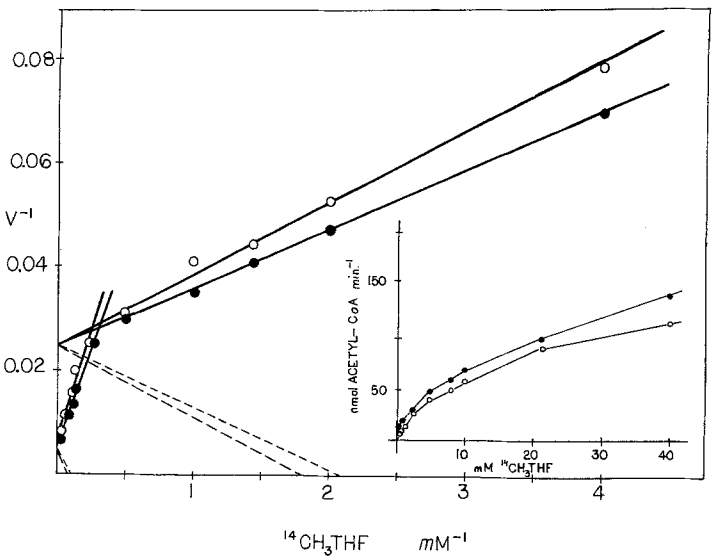

Fig. 4. Reciprocal plots of acetyl-CoA formation with CO or $NaHCO_3$ and H_2 as C_1 donors with variable concentrations of CH_3THF (150,000 cpm/µmol). V is nmol of acetyl-CoA synthesized per min (●——●) with CO as the gas phase, (○——○) with $NaHCO_3$, 10 µM and gas phase H_2. See Table 5 for K_m values. The inset shows the rate of acetyl-CoA formation with variation of the concentration of CH_3THF

K_m values were obtained with CH_3THF (Fig. 4). This observation will be considered in the Discussion.

It is known that CO is a competitive inhibitor of hydrogenase (Erbes and Burns 1978; Kleiner and Burris 1970). CO, likewise, was a competitive inhibitor against H_2 when the synthesis of acetyl-CoA was used as the measure (Fig. 5). The K_i for CO was 8.4 µM.

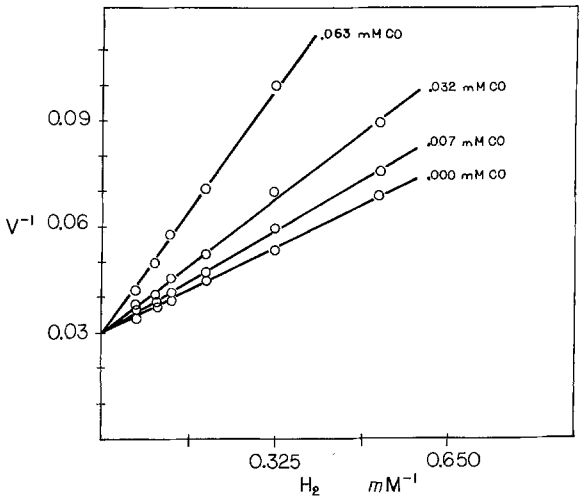

Fig. 5. Competitive inhibition by CO of the synthesis of acetyl-CoA from $NaHCO_3$, H_2, CH_3THF and CoASH. Velocity, V, is nmol acetyl-CoA formed per min and was measured at times not greater than 5 min. The concentrations of H_2 and CO are mM as calculated from standard solubility tables. The conditions were as described in the legend of Table 2

Discussion

We have previously demonstrated that acetyl-CoA is synthesized from CO, CoASH and CH_3THF by enzymes from *Clostridium thermoaceticum* (Hu et al. 1982). We now show that enzymes from this organism catalyze the synthesis of acetyl-CoA from CO_2, H_2, CoASH and CH_3THF. Furthermore, Kerby and Zeikus (1983) have shown that *C. thermoaceticum* has the ability to grow with CO or CO_2 and H_2 as energy sources and thus have demonstrated that *C. thermoaceticum* is a facultative autotroph. The complete pathway of acetate synthesis involves, in addition to the reactions shown in Fig. 1, the reduction of CO_2 to formate which is then converted to the CH_3 group of CH_3THF. This portion of the pathway has been well established through the studies of Ljungdahl and others with *C. thermoaceticum* and *C. formicoaceticum* (see Ljungdahl and Wood 1982; Zeikus 1983 for reference). The present studies lend further support to our speculation (Wood et al. 1982) that all acetogenic organisms that grow anaerobically using Co or CO_2 and H_2 may utilize the same pathway for synthesis of acetate as does *C. thermoaceticum*.

The central feature of the pathway used by *C. thermoaceticum* with pyruvate, CO or CO_2 and H_2 as the substrates is the formation by CO dehydrogenase of the C_1 intermediate designated as [HCOOH] in Fig. 1. With CO_2, a low-potential reductant is required which is provided by H_2 via the hydrogenase reaction. Ferredoxin is the electron carrier; the flavin present in fraction F_3 did not replace its function. With pyruvate, the reduced ferredoxin is generated by the pyruvate ferredoxin oxidoreductase during the formation of acetyl-CoA from carbons 2 and 3. It is used by the CO dehydrogenase for formation of the C_1 intermediate from the CO_2 arising from the carboxyl group of pyruvate. The CO_2 apparently, at least in part, is in a bound form shown as [CO_2] in Fig. 1. Thus, all these fermentations converge at CO dehydrogenase (see Fig. 1). Eden and Fuchs (1982) have indicated that we have hypothesized that the total synthesis of acetyl-CoA from CO_2 involves carboxylation or transformylation of the pyruvate carboxyl to methyltetrahydrofolate. We are not proponents of this view. With pyruvate as the source of C_1, as noted above, the C-1 of pyruvate is somehow converted to the C-1 of acetyl-CoA without complete equilibration with $CO_2/NaHCO_3$ but we do not consider CO_2 per se enters the C-1 of acetyl-CoA via pyruvate.

Although we show the C_1 intermediate as [HCOOH], it is clear that formate is not an intermediate and that formate dehydrogenase plays no role in the portion of the pathway shown in Fig. 1. When the activity of formate dehydrogenase of fraction F_3 was completely inhibited by a high concentration of EDTA (Andreesen and Ljungdahl 1974; Ljungdahl and Andreesen 1975) or by sodium hypophosphite, a structural analogue of formate and a competitive inhibitor (Pines 1958), the CO dehydrogenase was not inhibited and the conversion of CO_2 and H_2 or CO to acetyl-CoA remained active (Table 4). However, in the presence of H_2, some ^{14}C from [^{14}C]formate was observed in acetyl-CoA (Table 3). This conversion apparently occurs indirectly via formation of $^{14}CO_2$ from the $H^{14}COOH$. There is a non-enzymatic reduction of ferredoxin by formate during which CO_2 is generated (Fig. 3). This probably is the route by which ^{14}C from formate is converted to the C-1 of acetyl-CoA in the absence of activity by formate dehydrogenase.

Ljungdahl and coworkers (Elliott and Ljungdahl 1982; Yang et al. 1977) have reported that *C. thermoaceticum* contains two kinds of ferredoxin designated as Fd I and Fd II. Both have similar molecular weights and oxidation-reduction potentials. However, they differ in the number of [Fe_4-S_4] clusters. Moreover, the Fd II is significantly more stable than Fd I. Ferredoxin II could be stored, even aerobically, for 2 to 3 months in 50 % glycerol at −20° C. Our native Fd, purified as described earlier (Drake et al. 1981), is very stable and is active in the synthesis of acetate after storage for 2 months at −20° C in 10 % glycerol. On the basis of this observation, it is probable that our preparation is ferredoxin II which contains two [Fe_4-S_4] clusters.

Ragsdale et al. (1983b) recently have purified CO dehydrogenase from *Acetobacterium woodii* and compared its properties with the enzyme from *C. thermoaceticum*. Both enzymes have an $(\alpha\beta)_3$ structure of M_r 440,000 and 460,000 respectively which dissociate to dimeric $\alpha\beta$ forms. They both contain Ni, Fe, Zn and labile sulfide. The enzyme from *C. thermoaceticum* has 6 Ni, 32 Fe, 3 Zn and 42 labile sulfides (Ragsdale et al. 1983b). With both enzymes, rubredoxin was found to be the most reactive acceptor of electrons from CO. However, on the basis of thermodynamic considerations, they predict that reduced ferredoxin is more favorable for the reverse reaction, i.e., the reduction of CO_2 to CO. The nickel as well as the iron-sulfur clusters was found to be redox-active. On the basis of EPR spectroscopy, they propose a nickel III-carbon species is formed (Ragsdale et al. 1982); it may be the C_1 intermediate in the reaction.

We have found that irrespective of whether CO or CO_2 and H_2 are the cosubstrates, both high and low K_m values are observed for CH_3THF, Fig. 5 and Table 5. The two different K_m's may result from utilization of CH_3THF by two forms of an enzyme. For example, the methyl corrinoid enzyme may form a complex with the unidentified enzyme, F_3x and catalyze the addition of the C_1 intermediate to the methyl group during the formation of acetyl-CoA. This complex may have a lower K_m for transfer from the CH_3THF than do the dissociated components. On the other hand, the two K_m's may result from an allosteric effect of the *dl*-CH_3THF.

S.-I. Hu, in our laboratory, has recently isolated from *C. thermoaceticum* the corrinoid enzyme component of the system catalyzing the synthesis of acetyl-CoA. It has a molecular weight of less than 80,000 (Hu and Wood 1983). Welty and Wood (1978) previously isolated a protein from *C. thermoaceticum* which had the properties of a corrinoid and had a molecular weight of 158,000. This protein may have been a complexed form of the corrinoid enzyme which Hu has isolated.

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