# THE SYNTHESIS OF ONE-CARBON UNITS FROM $CO_2$ VIA A NEW FERREDOXIN DEPENDENT MONOCARBOXYLIC ACID CYCLE *

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## 1. Introduction

$CO_2$ was shown to be the predominant precursor of one-carbon units in *Clostridium kluyveri* [1, 2]. For this $CO_2$ assimilation, three mechanisms can be envisaged: (1) a direct $CO_2$ reduction to formate, formally a reversal of one of the known formate dehydrogenases [3–6], followed by formate activation to formyl tetrahydrofolate; (2) a direct $CO_2$ reduction to formyl tetrahydrofolate without free formate as an intermediate, formally a reversal of a formyl tetrahydrofolate dehydrogenase [7]; and (3) an indirect $CO_2$ reduction via reductive carboxylation of acetyl CoA to pyruvate [8], pyruvate cleavage to acetyl CoA and formate and formate fixation into formyl tetrahydrofolate.

In this communication data are presented indicating that the anabolic $CO_2$ reduction and fixation into the one-carbon units is effected solely via mechanism (3): this new ferredoxin dependent pyruvate synthase–pyruvate formate lyase cycle is tentatively named reductive monocarboxylic acid cycle.

## 2. Methods

All experiments were carried out with freshly harvested cells grown on ethanol-acetate-bicarbonate media [9], which were supplemented with 5 mM sodium formate where indicated. Cell-free lysates and ferredoxin were prepared as described [10]. Dowex-2-acetate treated lysates were obtained by anaerobically passing up to 10 ml crude lysate through 2 g moist weight of the resin packed in a 1 cm ∅ column equilibrated with 25 mM tris acetate pH 7.5 containing 25 mM mercaptoethanol. DEAE cellulose treated lysates were made similarly.

All assays were carried out at 37° in 22 ml Thunberg tubes after repeated evacuation and refilling with the desired gas. Radioactivity was determined in a Packard Liquid Scintillation Spectrometer 3380 using Bray scintillator [11].

## 3. Results

### 3.1. *Formate dehydrogenase (mechanism 1)*

If cell-free lysates of *C. kluyveri* were incubated under $H_2$ (= reduced ferredoxin) with or without NADH or NADPH regenerating systems [10, 12] no $^{14}CO_2$ reduction to $^{14}$C-formate could be detected. Neither did the same extracts catalyze the reverse reaction under Ar with or without $NAD^+$ or $NADP^+$ regenerating systems. As the sensitivities of the analytical methods employed [13] (tables 1 and 3) would easily allow the determination of less than 10 nmoles, it is concluded that cell-free lysates are devoid of a formate dehydrogenase.

### 3.2. *Formyl tetrahydrofolate dehydrogenase (mechanism 2)*

In the presence of tetrahydrofolate and the same

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Table 1
$^{14}$C-Formate formation from $^{14}CO_2$ and $^{14}CO_2$ formation from $^{14}$C-formate in cell-free lysates of *Clostridium kluyveri.*

| System | $^{14}$C-Formate (nmoles/ 30 min) | $^{14}CO_2$ (nmoles/ 30 min) |
|---|---|---|
| Complete | 63.2 | 110 |
| minus acetyl phosphate | $<0.5$ | $<1$ |
| plus glyoxylate | $<0.5$ | $<4$ |
| minus acetyl phosphate, plus pyruvate | 50.0 | 250 |

$^{14}$C-Formate formation from $^{14}CO_2$: tris-acetate pH 7.5, 100 mM; glutathione red. 2.5 mM; acetyl phosphate K, Li, 25 mM; coenzyme A, 0.5 mM; $K_2{}^{14}CO_3$, 5 mM (200,000 dpm/μmole); 16 mg crude lysate protein; water to 1 ml; gas phase: hydrogen. Additions: sodium glyoxylate, 2 mM; sodium pyruvate, 5 mM; $^{14}$C-formate was determined as described in table 3.
$^{14}CO_2$ formation from $^{14}$C-formate: tris-acetate pH 7.5, 100 mM; glutathione red., 2.5 mM; acetyl phosphate K, Li, 25 mM; coenzyme A, 0.5 mM; $NAD(P)^+$, 1 mM; $^{14}$C-formate, 2 mM (200,000 dpm/μmole); 16 mg crude lysate protein; water to 1 ml; gas phase: argon; Additions: sodium glyoxylate, 2 mM; sodium pyruvate 5 mM. Assay mixtures – with 0.1 ml 1 N KOH in the side arm – were stopped by injection of 0.5 ml 2 M citrate buffer pH 5.4 and shaken for another 45 min at 37° to effect complete absorption of formed $^{14}CO_2$ by the side arm KOH, aliquots of which were counted in a 4% (w/v) Aerosil Bray scintillator [14].

Table 2
Pyruvate synthase activity in cell-free extracts of *Clostridium kluyveri.*

| Protein | System | 1-$^{14}$C-Pyruvate (nmoles/10 min) |
|---|---|---|
| Dowex-2-acetate lysate | Complete | 850 |
| | minus acetyl phosphate | 10 |
| | minus coenzyme A | 32 |
| | plus glyoxylate | 10 |
| DEAE-lysate | Complete | 45 |
| | plus ferredoxin | 350 |

Complete: tris acetate pH 7.5, 100 mM; glutathione red., 2.5 mM; acetylphosphate K, Li, 25 mM; coenzyme A, 0.5 mM; $K_2{}^{14}CO_3$, 10 mM (200,000 dpm/μmole); phosphotransacetylase, 0.5 U; monosodium glutamate, 20 mM; glutamate pyruvate transaminase, 5 U; 4 mg protein of lysates which were pretreated with avidin (1 U/4 mg/30 min/0°) in order to block the pyruvate carboxylase activity of the extracts; water to 1 ml; gas phase: hydrogen. Additions: partially purified *C. kluyveri* ferredoxin, 0.9 mg protein; sodium glyoxylate, 2 mM; Assay mixtures were acidified by injection of 0.1 ml 25% TCA and at 0° repeatedly evacuated and filled with $^{12}CO_2$. Aliquots were then counted. The $^{14}CO_2$ fixation product was identified as alanine by electrophoresis in $HCOOH$-$CH_3COOH$-$H_2O$ (25:75:1000) (1600 V/35 cm; 50 mA; 2 hr).

reductants ($ferredoxin_{red.}$, NADH, NADPH) as in the tests for formate dehydrogenase no formyl tetrahydrofolate formation from $CO_2$ could be demonstrated with or without ATP, indicating the absence of measurable amounts (20 nmoles/30 min) of the tested activity.

### 3.3. *Pyruvate synthase – pyruvate formate lyase – formyl tetrahydrofolate synthetase (mechanism 3)*

Cell-free extracts of *C. kluyveri* were found to reduce $CO_2$ to formate under a $H_2$ atmosphere in the presence of an acetyl CoA regenerating system (table 1). The reaction could be inhibited by glyoxylate. Conversely, under an Ar atmosphere, the same extracts catalyzed the dehydrogenation of formate to $CO_2$ when an acetyl CoA regenerating system was added. Again the process could be blocked by glyoxylate. These findings suggested that the $CO_2$ reduction to formate was effected by the combination of pyruvate synthase and pyruvate formate lyase.

In both directions, acetyl CoA could be replaced by pyruvate, indicating that $^{14}CO_2$ conversion to $^{14}$C-formate and the reverse reaction occurred via exchange reactions (see below).

*Pyruvate synthase:* pyruvate formation from acetyl phosphate and $CO_2$ was dependent on coenzyme A, required ferredoxin as the reductant and was inhibited by glyoxylate (table 2). The reaction was linear with time and proportional to protein concentration up to 10 mg/ml.

*Pyruvate formate lyase:* formate formation from pyruvate was also dependent on coenzyme A, which could not be replaced by phosphate (table 3). The reaction could also be demonstrated by coupling in the presence of ATP and $Mg^{2+}$ with the endogenous formyl tetrahydrofolate activity of the lysates. The reaction was unaffected by glyoxylate, nearly linear with time and proportional to protein concentration up to 16 mg/ml. Besides this pyruvate formate lyase, which also catalyzed a rapid exchange of $^{14}$C-formate

Table 3

Pyruvate formate lyase activity in cell-free lysates of *Clostridium kluyveri.*

| Protein | System | $^{14}$C-Formate (nmoles/10 min) |
|---|---|---|
| Dowex-2-acetate lysate | Complete | 380 |
| | minus coenzyme A | 20 |
| | minus coenzyme A, plus $P_i$ | 20 |

Complete: tris acetate pH 7.5, 100 mM; glutathione red., 2.5 mM; coenzyme A, 0.5 mM; sodium arsenate, 5 mM; sodium glyoxylate, 2 mM; 1-$^{14}$C-pyruvate *, 2 mM (200,000 dpm/μmole); phosphotransacetylase, 1 U; 16 mg protein; water to 1 ml; gas phase: hydrogen. Additions: $KH_2PO_4$ ($P_i$), 25 mM; Assay mixtures were heat stopped (100°/30 sec); pyruvate was converted to alanine by addition of 0.1 ml 1 M glutamate and 0.4 ml of an aqueous solution of 1 U of glutamate pyruvate transaminase and by further incubation at 37° for 30 min; this mixture was then acidified with 0.5 ml 5% TCA, supplemented with 0.5 ml acetone and centrifuged; 1.25 ml of the supernatant was chromatographed on 1.5 g Dowex-2-formate columns (0.5 cm Ø) with 0.4 N HCOOH, as has been described [13]. This method allows the rapid separation of $^{14}CO_2$ fixation metabolites such as alanine, aspartate, lactate and pyruvate.

* A 0.1 M solution of sodium pyruvate in 0.1 N HCl was kept at minus 20° and thawed immediately before use [15].

Table 4

Activities of enzymes of, or related to, the monocarboxylic acid cycle of one-carbon unit formation from $CO_2$ in *Clostridium kluyveri*

| Enzymes | Lysates of cells from standard media | Lysates of cells from formate supplemented media |
|---|---|---|
| | (U/g protein *) | |
| Pyruvate synthase | 18.7 | 15.5 |
| Pyruvate formate lyase | 6.9 | 0.1 |
| Formyl tetrahydrofolate synthetase | 33.9 | 34.9 |

* 1 U = 1 μmole/min.

Assays: pyruvate synthase; table 2; 4 mg crude lysate protein; pyruvate formate lyase: table 3; 8 mg crude lysate protein; formyl tetrahydrofolate synthetase; tris acetate pH 7.5, 100 mM; ATP, 5 mM; $MgCl_2$, 6 mM; *d,l*-tetrahydrofolate, 2 mM; sodium formate, 20 mM; 2-mercaptoethanol, 20 mM; 0.2 mg crude lysate protein; water to 1 ml; gas phase: argon. Assay mixtures were stopped by addition of 2 ml 0.36 N HCl, after another 15 min the extinction at 350 nm was read against the appropriate blank [16].

into pyruvate, a competing ferredoxin dependent pyruvate dehydrogenase and an interfering pyruvate/$CO_2$ exchange activity was present in the lysates [12]. Both were routinely blocked by the addition of glyoxylate.

*Formyl tetrahydrofolate synthetase:* The formation of formyl tetrahydrofolate from formate and tetrahydrofolate required ATP and $Mg^{2+}$ and was proportional to time and protein concentration within the limits tested (table 4).

### 3.4. *The pyruvate synthase – pyruvate formate lyase cycle of* $CO_2$ *reduction*

Pyruvate synthase and formyl tetrahydrofolate synthetase activities were the same in lysates of cells from formate supplemented and from unsupplemented media, while the pyruvate formate lyase activity was reduced to below 3% in formate grown cells (table 4). This decrease of pyruvate formate lyase activity by formate *in vivo* was suggested by the observation that $^{14}$C-formate was not incorporated into pyruvate by cells growing on formate supplemented media [1, 2]. The absence of an active pyruvate formate lyase in formate grown cells, which may be due to genetic or chemical modification control of the enzyme, together with the finding that formate substitutes for $CO_2$ in one-carbon unit synthesis [1, 2] is taken as evidence that the pyruvate synthase–pyruvate formate lyase cycle is the physiological pathway for $CO_2$ reduction and fixation into the one-carbon units, (fig. 1).

## 4. Discussion

The reduction of $CO_2$ to $C_1$-units is an essential process both in the catabolism and anabolism of many strict anaerobes [17]; its mechanism has remained unknown.

In this note, for the first time, conclusive evidence is presented demonstrating that anabolic $CO_2$ reduction to $C_1$-units is effected via a ferredoxin dependent pyruvate synthase–pyruvate formate lyase cycle, followed by the formyl tetrahydrofolate synthetase

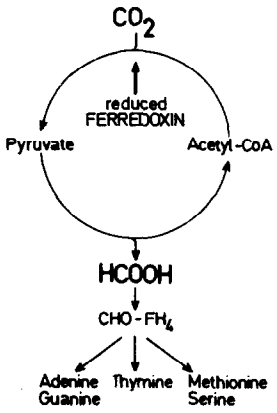


Fig. 1. The reductive monocarboxylic acid cycle of one-carbon unit formation from $CO_2$.

reaction. The cycle is tentatively named "reductive monocarboxylic acid cycle" in analogy to the naming of other $CO_2$ reducing and fixing cycles such as the reductive tricarboxylic acid cycle [18] or the reductive pentose phosphate cycle [19]. The results presented substantiate the view that $CO_2$ reduction is always indirect, requiring a $CO_2$ acceptor. Whether catabolic $CO_2$ reduction to acetate or methane is also indirect and occurs via the monocarboxylic acid cycle remains to be shown.

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