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Pathway of carbon dioxide reduction to acetate without a net energy requirement in *Clostridium purinolyticum*

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1. INTRODUCTION

One of the first experiments performed with radioactive carbon (^{11}C) clearly indicated that acetate can be synthesized from CO_2 by the purine-fermenting anaerobe *Clostridium acidiurici* when it grows on a reduced substrate such as hypoxanthine [1]. Evidence for this ability of *C. acidiurici* and *C. cylindrosporum* was obtained by the use of $^{13}CO_2$. Mass analysis indicated that about 9% of the acetate formed were doubly labelled [2].

The pathway of acetate synthesis from CO_2 as studied in *C. acidiurici* involves two CO_2-fixing reactions catalyzed by formate dehydrogenase (CO_2 reductase) [3] and glycine synthase [4] which are interconnected by enzymes acting on tetrahydrofolate (FH_4) derivatives [5]. Acetate is formed from glycine via serine and pyruvate as indicated by enzymic and labelling data [4,6].

A second pathway has been shown to operate in the saccharolytic and autotrophic anaerobes *C. thermoaceticum, C. formicoaceticum, C. aceticum, Acetobacterium woodii* [7] and *C. thermoautotrophicum* (J. Wiegel, personal communication). Both pathways seem to imply the same set of initial reactions which involve the ATP-requiring activation of formate by formyl-FH_4 synthetase. However, they definitely differ in the last steps. In organisms such as *C. thermoaceticum* acetate is formed from methyl-FH_4 via a corrinoid [7] and a formyl intermediate derived from carbon monoxide (CO) or pyruvate [8]. For this group of organisms the importance of CO and nickel has been emphasized [9,10], whereas for *C. acidiurici* and *C. cylindrosporum* CO is without any effect [9].

C. purinolyticum is a newly isolated species which depends on selenium compounds for growth on purines and forms acetate from CO_2 [11]. As in *C. acidiurici* and *C. cylindrosporum* both formate and glycine are intermediates of purine breakdown. However, the labelling data indicate that glycine is directly reduced to acetate during growth of *C. purinolyticum* on glycine [12], thereby ruling out serine and pyruvate as intermediates of acetate formation.

In this communication we present data obtained for *C. purinolyticum* that (i) CO_2 is reduced to formate and formate is converted to acetate, (ii) an active ATP-forming glycine reductase is constitutively present in cell extracts, (iii) CO is inert for cells of this bacterium, and (iv) the corrinoid content is low compared with *C. formicoaceticum*. A pathway for CO_2 reduction to acetate is proposed which differs from those known by introducing the ATP-forming selenium-dependent glycine reductase as catalyst in the final step of acetate formation. The energetic advantage of this modification is discussed.

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2. MATERIALS AND METHODS

C. purinolyticum (DSM1384) was grown on adenine or glycine as substrate as described [11,12] except that 10^{-7} M $NiCl_2$ was added. *C. acidiurici* (DSM604) and *C. cylindrosporum* (DSM605) were cultured on hypoxanthine [13], *C. formicoaceticum* (DSM92) on the substrates indicated in a complex medium [14]. Determination of acetate, formate, glycine, adenine, and whole cell protein were as described before [11,12]; CO was assayed according to [15]. Preparation and protein determination of cell-free extracts were carried out as reported previously [14]. CO_2 reductase activity was measured [16] in warburg vessels using a modified assay; the test system contained in a total volume of 2 ml: 100 mM potassium phosphate buffer (pH 7.5), 10 mM methyl viologen, 5 mM dithioerythritol, 30 mM $KHCO_3$, 0.75 mM $NaH^{14}CO_3$ (0.4 μCi), 0.1 mM $NADH_2$, 2.5 U of purified hydrogenase from *Alcaligenes eutrophus*, and extract (200 μl). In some experiments methyl viologen was reduced by 15 mM sodium dithionite instead of hydrogenase in connection with hydrogen and $NADH_2$. CO_2 fixation metabolites were separated and determined by isoionic-exchange chromatography [17]. Radioactivity was measured using 9 ml Quickszint 212 (Koch-Light Laboratories Ltd., Colnbrook, England) and 1 ml sample material. CO dehydrogenase activity was determined according to [9], however, the assay contained 100 mM potassium phosphate buffer (pH 7.3) and 4 mM methyl viologen. Glycine reductase activity was assayed as described before [12]. Quantification of the amount of B_{12} was carried out according to [18] using a B_{12}-methionine-auxotrophic mutant of *Escherichia coli* (ATCC10799, DSM1595, NCIB8134) as test organism. One unit of enzyme activity was defined as 1 μmol substrate transformed or product formed per min at 37°C.

3. RESULTS

3.1. Reversibility of formate dehydrogenase reaction

Cell-free extracts of *C. purinolyticum* catalyzed the reduction of CO_2 to formate. Specific activities determined for extracts of cells grown on adenine or glycine were 0.009 and 0.006 U/mg of protein, respectively, at pH 7 which is optimal for the formate oxidation reaction [11]. Formate formation was linear with time up to 10 min and with protein up to 5 mg. Using sodium dithionite instead of the enzymatic reduction of methyl viologen, the CO_2 reductase activity was inhibited by 66%. Formate formation followed Michaelis–Menten kinetics. Plots of 1/v vs. 1/S were linear. Half-maximal velocity was obtained with 2.4 mM HCO_3^- (adenine as growth substrate) and 3.8 mM HCO_3^- (glycine as growth substrate), respectively. The attempt to use ferredoxin- and nucleotide-free extracts according to Thauer [3,16] failed.

3.2. Formation of acetate from formate by whole cells

Adenine- and glycine media were supplemented with increasing concentrations of sodium formate (0–150 mM) and subsequently inoculated with *C. purinolyticum.* After reaching the stationary growth phase the amounts of acetate, adenine, formate and glycine were determined. Neither an inhibition nor a stimulation of growth by formate could be observed. Growing on glycine *C. purinolyticum* transformed in all cases the added formate to acetate and CO_2 (Table 1; only the data with a supplementation of 150 mM formate are given). Using adenine as growth substrate only small

Table 1

Fermentation of glycine in the presence of 150 mM formate by *C. purinolyticum*

Substrates (mM)		Not transformed (mM)	Acetate produced (mM)	Acetate expected [a] (mM)
Glycine:	100	0	111.5	75
Adenine:	1	0		1.25
Formate:	150	7.1		35.7
total			111.5	112

[a] Basis for the theoretical calculation of acetate formation was the fermentation balances for adenine and glycine [11,12] and the assumption that 1 acetate and 2 CO_2 could be formed from 4 formate.

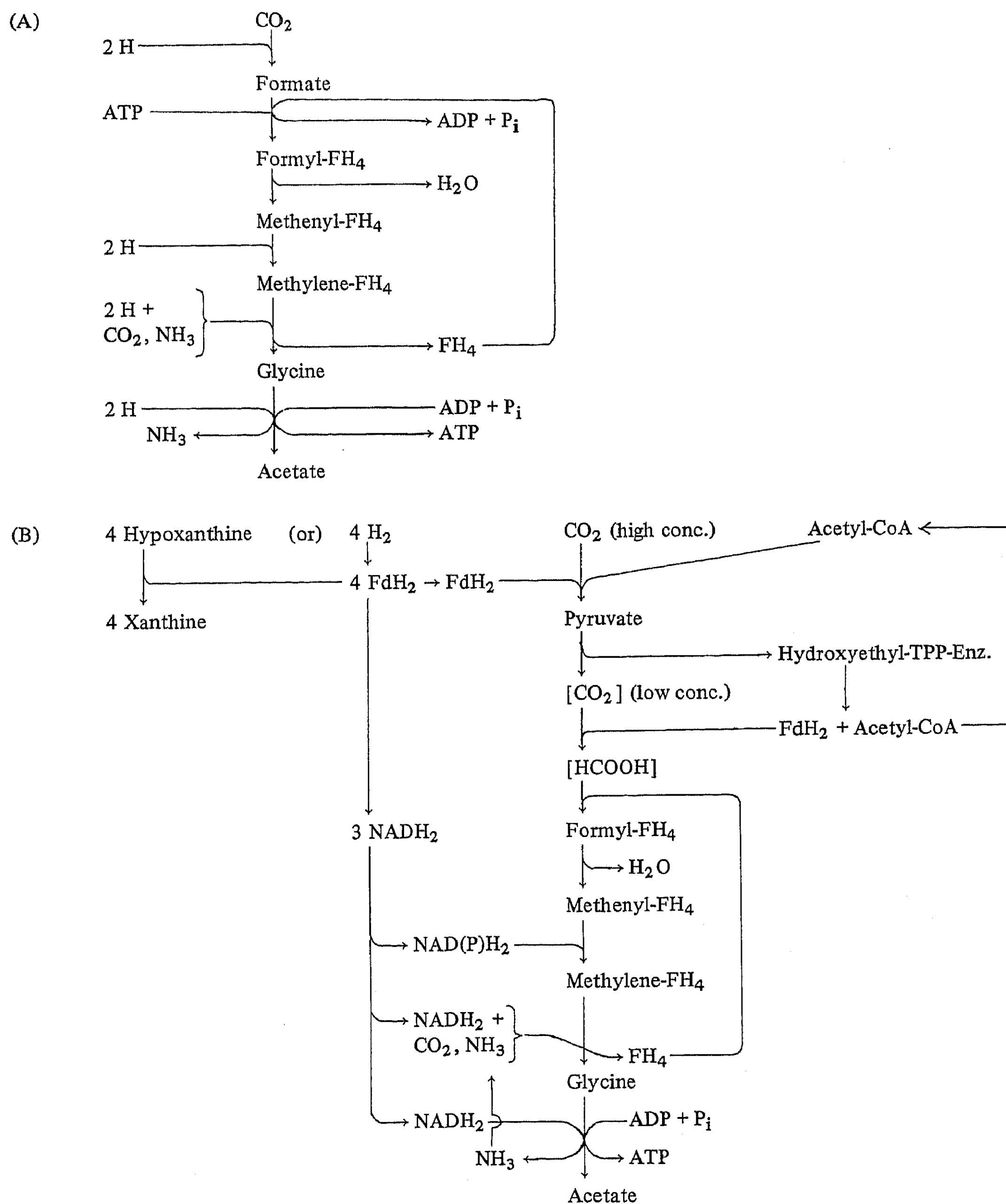


Fig. 1. The glycine reductase pathway of acetate formation from CO_2. (A) Energy-neutral variant; (B) inclusion of proposals of Wood and associates [8,34] into variant A), thereby yielding net ATP. 2 H, reducing equivalents; FH_4, tetrahydrofolate; TPP-Enz, enzyme bound thiamine pyrophosphate; [CO_2], [HCOOH], bound form of CO_2 and formate, respectively.

amounts of formate were transformed to acetate. Formate mostly remained unchanged. This might be due to the fact that formate is a major fermentation product during growth on adenine [11] but not on glycine [12]. In contrast to results obtained with *C. sticklandii* [19] the use of cysteine/H_2S as

reducing agents instead of thioglycolate did not affect the acetate formation from formate by *C. purinolyticum.*

3.3. Presence of ATP-forming glycine reductase

Extracts of adenine-grown cells performed a glycine-dependent ATP formation of 0.028 U/mg of protein using dithiothreitol as electron donor. This corresponds to two thirds the activity obtained with glycine-grown cells of *C. purinolyticum* [12].

3.4. Effect of CO

Cell-free extracts of *C. purinolyticum* showed a low CO dehydrogenase activity of 0.007 U/mg of protein when cells were grown on adenine, but no CO oxidation could be observed by intact cells. CO had no stimulatory or inhibitory effect on growth up to a concentration of 5% in the gas phase as tested. No CO dehydrogenase activity was observed with extracts of glycine-grown cells.

3.5. B_{12} content

C. purinolyticum contained only small amounts of B_{12}. Concentrations of 0.055 and 0.047 μg/mg of protein were determined in cells after growth on adenine and glycine, respectively. The B_{12} content of hypoxanthine-grown cells of *C. acidiurici* and *C. cylindrosporum* was 0.025 and 0.027 μg/mg of protein, respectively. In contrast to these organisms *C. formicoaceticum* contained as much as 2.47 μg/mg of protein when cultured on lactate. The comparative values after growth of *C. formicoaceticum* on fumarate, methanol, ethanol, glycerol and fructose were 1.85, 1.56, 1.40, 0.25, and 0.15 μg/mg of protein, respectively.

4. DISCUSSION

C. purinolyticum performs a net synthesis of acetate when grown on reduced purines such as adenine and hypoxanthine [11] or on glycine in the presence of a molar excess of formate. Using the latter conditions a Stickland-type of reaction can be excluded. In *C. sticklandii* [19], *C. thermoaceticum* [20], and *C. formicoaceticum* [21] [^{14}C]formate is converted to $^{14}CO_2$ and doubly labelled acetate. Even after growth of *C. purinolyticum* on glycine without formate supplementation the label of $^{14}CO_2$ is found to some extent in both carbons of acetate [12] indicating that all the enzymes necessary for synthesis of acetate from CO_2 are constitutively formed.

A reduction of CO_2 to formate is catalyzed by only a few bacterial formate dehydrogenases. The enzyme of *C. pasteurianum* [22] is involved in the synthesis of methylene–FH_4 required for anabolic reactions, whereas in *C. thermoaceticum* [3], *C. formicoaceticum* [23], and *C. acidiurici* [16] the sequence is leading on to acetate. The involvement of trace elements such as molybdenum [22], selenium, and tungsten [24] is a unique characteristic of these formate dehydrogenases.

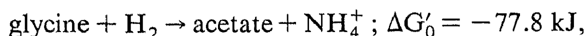
CO_2 is the active substrate species for the formate dehydrogenase of *C. pasteurianum* [25]. The K_s-values for bicarbonate are 11 mM in *C. thermoaceticum* [3] and 8 mM in *C. acidiurici* [16]. These values compare favourably with that obtained for *C. purinolyticum*—3 mM—and might explain the general bicarbonate requirement observed for growth of these bacteria on reduced substrates.

In all three purinolytic species the B_{12} content was quite low even under conditions optimal for acetate synthesis from CO_2. However, the B_{12} content of *C. formicoaceticum* was at least one or two orders of magnitude higher using the same assay system. The values determined for *C. formicoaceticum* corresponded well with those obtained by a different method and also compared favourably with those for *C. thermoaceticum* and *A. woodii* [26]. Therefore, corrinoids seem to play no major role in acetate synthesis by purinolytic species.

CO was totally inert for intact cells of *C. purinolyticum* as observed for *C. acidiurici* and *C. cylindrosporum* [9]. However, a very low CO-dependent methyl viologen reduction was observed in extracts of cells grown on adenine. The absence in glycine-grown cells might indicate that the low CO dehydrogenase activity is a side reaction of a xanthine dehydrogenase-type of enzyme. Both enzymes can occur as molybdoiron-sulphur flavoproteins [27,28]. The latter enzyme is also known for

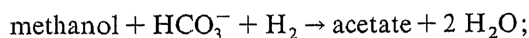
its broad substrate spectrum.

The presence of a glycine reductase in *C. purinolyticum* grown on adenine points to a modification of the pathway of acetate formation as proposed for *C. acidiurici* [4]. After growth on glycine the enzymes serine hydroxymethyltransferase and serine dehydratase are present in *C. purinolyticum* [12] although at much lower activities than in *C. acidiurici* and *C. cylindrosporum* [5]. The labelling data strongly indicate only a biosynthetic function for these enzymes, whereas acetate is formed directly from glycine by glycine reductase [12]. The latter reaction is strongly exergonic [29]:

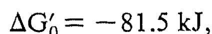


$$\text{glycine} + H_2 \rightarrow \text{acetate} + NH_4^+ ;\ \Delta G_0' = -77.8 \text{ kJ},$$

as is both the acetate formation from the methanol derivative methyl–FH_4:

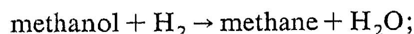


$$\text{methanol} + HCO_3^- + H_2 \rightarrow \text{acetate} + 2\ H_2O;$$



$$\Delta G_0' = -81.5 \text{ kJ},$$

and the final step in methane formation:



$$\text{methanol} + H_2 \rightarrow \text{methane} + H_2O;$$

$$\Delta G_0' = -112.5 \text{ kJ}.$$

Therefore, energy conservation by glycine reductase as observed in extracts of *C. purinolyticum* is thermodynamically quite feasible. If in vivo 1 mol of ATP is formed per mol of glycine reduced as reported for *C. sticklandii* [30], the ATP expenditure during the activation of formate by formyl–FH_4 synthetase would be compensated. Therefore, the pathway of acetate synthesis from CO_2 as proposed in Fig. 1A is the first which is balanced with respect to energy expenditure. This is not the case for a glycine–serine–pyruvate interconversion [4] since the synthesis of the donor methylene–FH_4 from CO_2 requires ATP. In addition, more enzymic reactions are involved which is not economical. However, the glycine reductase pathway requires the availability of selenium which is needed for the synthesis of three enzymes, glycine reductase [30], formate dehydrogenase [13], and xanthine dehydrogenase [24].

A new aspect is introduced by a recent paper [8]. By analogy with methanogenic bacteria [31] both CO_2 and formate might not occur as free intermediates in the acetate synthesis and both formate dehydrogenase and formyl–FH_4 synthetase might be bypassed. Instead, pyruvate will yield by action of a type of pyruvate: ferredoxin oxidoreductase a bound form of CO_2([CO_2]) which will be reduced to a bound formate ([HCOOH]) by one of the proteins present in fraction F_3 [8]. The formate intermediate will then form formyl–FH_4 directly. This possibility strongly implies that [CO_2] has to be more energy-rich than CO_2 like the carboxybiotin in transcarboxylase [32] and oxaloacetate decarboxylase [33], for the reduction of HCO_3^- by H_2 to formate has only a $\Delta G_0'$ of -1.3 kJ [29], whereas the formation of formyl–FH_4 from formate requires 23.4 kJ/mol which generally is compensated by ATP hydrolysis. Therefore, the reduction of [CO_2] to [HCOOH] has to occur at higher energy level which will effectively reduce the exchange between free CO_2 and formate and the bound intermediates. This might offer an explanation for the low activity of CO_2 reduction to formate as generally observed. However, due to its absence in *C. purinolyticum* a CO dehydrogenase [8] cannot catalyze the reduction of [CO_2] to [HCOOH].

The initial steps as proposed by Wood and associates [8] might also occur in heterotrophic and autotrophic acetogenic species such as *C. purinolyticum* and *C. aceticum* when growing on C_2 and C_1 substrates (Fig. 1B). This would only require the additional formation of pyruvate from acetyl-CoA, CO_2 and reduced ferredoxin as it has to occur in any case for biosynthetic reactions [34]. Pyruvate synthase reaction can be catalyzed by pyruvate: ferredoxin oxidoreductase of *C. acidiurici* [35] and reduced ferredoxin can be supplied by xanthine dehydrogenase [36]. The formed pyruvate will then yield [CO_2] and regenerate acetyl-CoA and reduced ferredoxin. The endergonic nature of the conversion of CO_2 to [CO_2] via pyruvate requires at least a gradient in their concentrations as depicted in Fig. 1B. In accordance with this proposal are the facts that CO_2 (or bicarbonate) is generally required for growth, and the K_s-values for a CO_2 reduction to formate are rather high, as discussed above. The high concentration of acetate necessary to initiate growth of certain acetogenic bacteria [37] might be explained by the central catalytic function of acetyl-CoA as indicated in

Fig. 1B. Despite the proposed direct transfer of [HCOOH] to FH_4 [34] the formyl–FH_4 synthetase might still be operative in this reaction, since in *C. cylindrosporum* the enzyme reacts also with carbamoyl phosphate, an analogue of formyl phosphate [38], a candidate of the proposed formyl intermediate [8,34].

Net ATP synthesis during acetate formation from CO_2 is possible by action of the scheme in Fig. 1B, even without the formation of an electrochemical proton gradient as proposed for different anaerobic electron-transferring systems [39].

Under any conditions the formation of a proton gradient by efflux of the product [40] e.g. acetic acid, might additionally contribute to the energy balance.

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