CO_2-REDUCTION TO FORMATE BY NADPH. THE INITIAL STEP IN THE TOTAL SYNTHESIS OF ACETATE FROM CO_2 IN *CLOSTRIDIUM THERMOACETICUM** †

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1. Introduction

The reduction of CO_2 to formate is an important process for the fixation of CO_2 either in the anabolism or the catabolism of many clostridia [1]. In the anabolism of *C. kluyveri* [2, 3] and of *C. pasteurianum* [4] CO_2 conversion to formate is a key step in the formation of the S-methyl group of methionine and the C_2 and C_8 positions of the purines. In the hexose catabolism of *C. thermoaceticum* [5] and of *C. formicoaceticum* [6], and in the purine catabolism of *C. acidiurici* and *C. cylindrosporum* [6] formate formation from CO_2 is the initial reaction in the total synthesis of acetate from CO_2.

For the anabolic reduction of CO_2, two mechanisms have been demonstrated: CO_2 is converted to formate with reduced ferredoxin as electron donor in *C. pasteurianum* [7] by a direct (acceptor-independent) reduction, and in *C. kluyveri* [8] by an indirect (acetyl-CoA dependent) reduction.

For the catabolic reduction of CO_2, mechanisms have not been elucidated. In *C. thermoaceticum* [9] formate oxidation to CO_2 with $NADP^+$ has been demonstrated, which, however, could not be reversed. Only with CO_2 concentrations higher than 100 mM a slight reversal might have been observed when the process was coupled to the formyltetrahydrofolate synthetase reaction [10]. The suggested dehydrogenase in CO_2 reduction therefore remained doubtful.

In this communication evidence is presented that, with the appropriate NADPH regenerating system, cell-free lysates of *C. thermoaceticum* readily catalyze a direct reduction of CO_2 to formate.

2. Materials and methods

Frozen cells of *C. thermoaceticum* grown on glucose [11] were a gift of Dr. Ljungdahl. *C. pasteurianum* ATCC 6013 was grown on the glucose/NH_4^+ medium as described by Lovenberg et al. [12].

Ferredoxin was prepared from *C. pasteurianum* (Fd_{past}) by the method of Mortenson [13]. Hydrogenase was partially purified from *C. pasteurianum* by anaerobically acidifying lysates (50 mg protein/ml) with 1 N acetic acid to pH 4.9 and subsequently heating to 60° for 25 min. After cooling and centrifuging at 40,000 *g* for 30 min, the supernatant containing the hydrogenase (5–10 I.U./mg protein) was adjusted to pH 8.0 with 1 N Tris base.

Crude lysates (25 mg protein/ml) of *C. thermoaceticum* were obtained by incubating 2 g of frozen cells in 6 ml of 100 mM Tris-acetate pH 8.5 for 30 min at 37° under hydrogen in the presence of 100 I.U. of *C. pasteurianum* hydrogenase with 5 mg of lysozyme and 0.5 mg of DNAase and by then centrifuging at 40,000 *g* for 30 min.

Nucleotide-free lysates were made by anaerobically passing 5 ml of freshly prepared crude lysate through

* In this paper no distinctions will be made between CO_2, HCO_3^- or CO_3^{2-}.

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a mixture of 2 g of Dowex-2-acetate and 200 mg of HCl- and EDTA-pretreated charcoal, which had been thoroughly evacuated and gassed with H_2 before being packed into a column of 1 cm diameter.

Ferredoxin- and nucleotide-free lysates were obtained similarly. The column was first filled with the Dowex-2-acetate/charcoal mixture as described and then layered with 1.5 ml of DEAE-cellulose. The column was equilibrated with 50 mM Tris-acetate pH 7.5 containing 50 mM mercaptoethanol.

All assays were carried out at 55° in 22 ml Thunberg tubes or in 1 ml anaerobic cuvettes after repeated evacuation and refilling with the desired gas. [^{14}C]Formate was determined after separation by isoionic column chromatography [14]. Ferredoxin reduction was measured by hydrogen formation using hydrogenase as the indicator enzyme; the hydrogen formed was quantitated gas-chromatographically [15]. Methyl viologen reduction was followed photometrically at 600 nm, NAD(P) reduction at 366 nm. Detailed procedures are given in the legends to tables and figures.

3. Results

NADH, NADPH or reduced ferredoxin have to be considered as possible physiological electron donors for a reduction of CO_2 to formate. Experimentally these should be used in the form of regenerating systems (RS) rather than in substrate quantities for two reasons: i) formate formation from CO_2 (CO_2/formate, E'_0 = –420 mV) with reduced pyridine nucleotides (NAD (P)$^+$/NAD (P)H, E'_0 –320 mV) is thermodynamically very unfavorable; ii) it is practically impossible to work with substrate amounts of an autoxidizable electron donor protein such as ferredoxin. With the thermophile *C. thermoaceticum* in whose lysates most enzymes have their optimum around 55° and are essentially inactive around 35°, the commercially available NAD (P)H-regenerating systems cannot be used due to the heat lability of the component enzymes, i.e., galactose dehydrogenase of the NADH-RS and glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase of the NADPH-RS. Therefore, regenerating systems functioning at 55° had to be developed first.

3.1. *Regenerating systems functioning at 55°*

3.1.1. Reduced ferredoxin regenerating system (Fd_{red}-RS)

Under an atmosphere of hydrogen hydrogenase (2.5 I.U.) and ferredoxin (0.25 mg protein/ml), both isolated from *C. pasteurianum,* were found to constitute an effective Fd_{red}-RS stable at 55°.

3.1.2. Reduced methyl viologen regenerating system (MV_{red}-RS)

This system was obtained by replacing ferredoxin in the Fd_{red}-RS by 10 mM methyl viologen.

3.1.3. NADH regenerating system (NADH-RS)

C. thermoaceticum lysates were observed to catalyze the reduction of NAD^+ ($[S]_{0.5\ V}$ = 0.5 mM) with reduced ferredoxin at 55° (35 I.U./g protein). This reaction coupled to the Fd_{red}-RS formed the NADH-RS. NADH was used in 1 mM concentrations.

3.1.4. NADPH regenerating system (NADPH-RS)

C. thermoaceticum lysates were found to also catalyze the reduction of $NADP^+$ ($[S]_{0.5\ V}$ = 0.14 mM) with reduced ferredoxin at 55° (150 I.U./mg protein). This process in combination with the Fd_{red}-RS constituted an effective NADPH-RS. $NADP^+$ was added in 0.25 mM concentrations. The ferredoxin-$NADP^+$ reductase of this system was active only in the presence of small concentrations of either NADH (0.1 mM) as in other clostridia [15] or of methyl viologen (table 1). 2.5 μM methyl viologen was routinely used for the activation, since at this low concentration the dye was not effective as a reductant of CO_2. In order to ensure comparable conditions 2.5 μM methyl viologen was also included in the assay mixtures, when electron donors other than NADPH were tested. It should be pointed out that in the MV_{red}-RS methyl viologen was used in 4000-fold concentrations.

3.2. *CO_2 reduction to formate*

Using the regenerating systems described above, nucleotide- and ferredoxin-free lysates of *C. thermoaceticum* were found to reduce CO_2 to formate with NADPH as electron donor. Neither reduced ferredoxin nor NADH could substitute for NADPH. In high con-

Table 1
An NADPH-regenerating system functioning at 55° (hydrogenase of *C. pasteurianum* and ferredoxin-$NADP^+$ reductase of *C. thermoaceticum*).

Additions	$NADP^+$ reduction with H_2 (nmoles/min/mg protein)
None	< 5
NADH	< 5
MV	19
Fd	6
Fd + NADH	150
Fd + MV	110
Fd + NADH–H_2	< 5
Fd + MV–H_2	< 5

Basic system: imidazol acetate pH 7.0, 100 mM; mercaptoethanol, 25 mM; nucleotide- and ferredoxin-free lysate of *C. thermoaceticum* (containing hydrogenase of *C. pasteurianum*, 0.5 I.U.), 1 mg protein; $NADP^+$, 2.5 mM; water to 1 ml; gas phase: H_2. Additions: Fd_{past}, 0.25 mg protein; NADH, 0.1 mM; methyl viologen (MV), 2.5 μM.

centrations reduced methyl viologen ($[S]_{0.5\ V} = 0.5$ mM) was an effective but artificial reductant (table 2).

A net synthesis of formate rather than a CO_2-formate exchange reaction was measured with the isotope method employed since similar results were obtained using the enzymatic formate determination as described by Rabinowitz and Pricer [16].

The NADPH-dependent formate formation from CO_2 was linear with time up to 10 min and with protein up to 7.5 mg/ml. The pH optimum tested in Tris-acetate, imidazol acetate or potassium phosphate was broad with a maximum at pH 7. Half maximal velocities were obtained with 0.02 mM NADPH (fig. 1A) and 11 mM CO_2 (fig. 1B).

The cofactor requirement for the CO_2 reduction to formate was identical to that observed for the reverse reaction (table 2). In neither direction had ATP, CoA or acetyl-CoA any effect. Since also pyruvate formate lyase activity could not be detected even in the presence of ferredoxin and S-adenosylmethionine [4], the indirect CO_2 reduction as found in *C. kluyveri* [8] can be excluded. Rather, a direct mechanism similar to that first demonstrated in *C. pasteurianum* [7] is operative.

With the cell-free lysates routinely prepared from frozen cells (one year old) relatively low CO_2 reducing activities (5–10 I.U./g protein) were obtained. Using extracts prepared from freshly harvested cells 10 times higher velocities were observed, which should be sufficient to account for the proposed role of the enzyme in the total synthesis of acetate from Co_2 (fig. 2).

Table 2
Cofactor requirement for the reversible CO_2-reduction to formate in *C. thermoaceticum*.

CO_2 ⟶	Formate	Formate ⟶	CO_2
Electron donor	Formate formed (nmoles/10 min/ 5 mg protein)	Electron acceptor	Formate oxidized (nmoles/10 min/ 5 mg protein)
--	23	--	0
NADH-RS	25	NAD^+	< 10
NADPH-RS	280	$NADP^+$	395
Fd_{red}-RS	23	Fd_{ox}	< 10
MV_{red}-RS	1550	MV_{ox}	2500

CO_2 reduction to formate: imidazol acetate pH 7.0, 100 mM; mercaptoethanol, 25 mM; methyl viologen, 2.5 μM; nucleotide- and ferredoxin-free lysate (containing hydrogenase of *C. pasteurianum* 2.5 I.U.), 5 mg protein; potassium [^{14}C]carbonate (40,000 dpm/μmole), 30 mM; water to 1 ml; gas phase: hydrogen; temperature: 55°. Regenerating Systems (RS), see text. Formate determination, see fig. 1. *Formate oxidation to CO_2:* imidazol acetate pH 7.0, 100 mM; mercaptoethanol, 25 mM; nucleotide- and ferredoxin-free lysate (containing hydrogenase of *C. pasteurianum*, 0.5 I.U.), 1 mg protein; sodium formate, 50 mM; water to 1 ml; gas phase: argon; temperature: 55°; electron acceptors as indicated: NAD^+, 1 mM; $NADP^+$, 1 mM; ferredoxin, 0.25 mg protein; methyl viologen (MV), 10 mM.

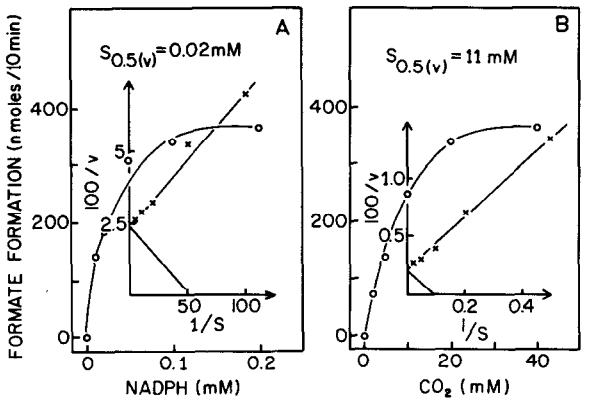

Fig. 1. CO_2 reduction to formate in nucleotide-free lysates of *C. thermoaceticum*. A) $[S]_{0.5\ V}$ of NADPH. B) $[S]_{0.5\ V}$ of CO_2. Assay: imidazol acetate pH 7.0, 100 mM; mercaptoethanol, 25 mM; nucleotide-free lysate of *C. thermoaceticum* (containing hydrogenase of *C. pasteurianum*, 2.5 I.U.), 5 mg protein; Fd_{past}, 0.25 mg; methyl viologen, 2.5 μM; potassium [^{14}C]carbonate (40,000 dpm/μmole), 30 mM or as indicated; $NADP^+$, 0.25 mM or as indicated; water to 1 ml; gas phase; hydrogen; temperature, 55°. The assay mixtures were stopped with 0.5 ml 5% TCA and 0.5 ml acetone and centrifuged; 1.25 ml of the supernatant was chromatographed in 1 ml fractions on 1.5 g Dowex-2-formate columns (0.5 cm diameter) with 0.4 N HCOOH [14]. [^{14}C]Formate was recovered in fractions 9–12.

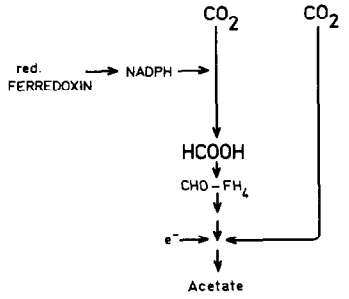

Fig. 2. NADPH dependent CO_2 reduction to formate as the initial step in the total synthesis of acetate from CO_2.

4. Discussion

In this communication evidence is presented for the first time that CO_2 can be reduced to formate with a reduced pyridine nucleotide as the electron donor. The enzyme catalyzing this reaction in *C. thermoaceticum* is similar to the CO_2-reductase of *C. pasteurianum* [7] in that it catalyzes a direct CO_2-reduction, and dissimilar in that NADPH rather than ferredoxin functions as the electron donor.

The NADPH required for the reduction of CO_2 is generated *in vivo* solely via the ferredoxin-$NADP^+$ reductase. This is indicated by the observed specificity of the two electron donating steps in the glucose catabolism of *C. thermoaceticum*. The glyceraldehyde-phosphate dehydrogenase reaction is specific for NAD^+ ($[S]_{0.5\ V}$ = 0.1 mM) and the pyruvate dehydrogenase reaction for ferredoxin ($[S]_{0.5\ V}$ = 50 μg/ml) (unpublished). An $NADP^+$ dependent glucose-6-phosphate dehydrogenase is absent. Thus ultimately both in *C. pasteurianum* and in *C. thermoaceticum* the electrons for the reduction of CO_2 to formate come from ferredoxin.

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