FERREDOXIN DEPENDENT CO_2 REDUCTION TO FORMATE IN CLOSTRIDIUM PASTEURIANUM

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Summary: Cell-free lysates of Clostridium pasteurianum catalyzed the reduction of CO_2 to formate. Only reduced ferredoxin could serve as electron donor. NADH or NADPH were totally inactive. Substrates other than CO_2 and ferredoxin were not required for the activity. The reaction was reversible.

The reduction of CO_2 to formate is an essential process both in the catabolism and the anabolism of many strict anaerobes. Formate is the key intermediate in the synthesis from CO_2 of acetate or methane (1), and of C_1-unit positions such as the S-methyl group of methionine (2,3). Recently, formate formation from CO_2 was shown in Clostridium kluyveri to proceed via a ferredoxin dependent monocarboxylic cycle with pyruvate synthase and pyruvate formate lyase as the component enzymes (4,5). This mechanism is in line with the view that CO_2 reduction is always indirect, requiring a low molecular weight CO_2 acceptor (6).

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In this communication evidence is presented indicating that cell-free lysates of Clostridium pasteurianum convert CO_2 to formate and that in this reaction only CO_2 and reduced ferredoxin are required as substrates. These findings demonstrate that CO_2 reduction can also be direct, i.e. independent of a CO_2 acceptor.

Materials and methods

Enzymes, coenzymes and substrates were obtained from Boehringer, Mannheim; methyl viologen (MV) and benzyl viologen (BV) from Serva, Heidelberg. C. pasteurianum ATCC 6013 was grown on glucose/NH_4^+ media as described by Lovenberg et al. (7).

Preparation of cell-free extracts: Crude lysates were obtained from frozen cells by lysozyme and DNase treatment (8). Ferredoxin (Fd) was prepared from C. pasteurianum by the method of Mortenson (9). Nucleotide-free lysates were made by anaerobically passing 10 ml of freshly prepared crude lysate through a mixture of 2 g of Dowex-2-acetate and 100 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 used was filled first with the Dowex-2-acetate/charcoal mixture described and then on top of it with 1.5 ml DEAE-cellulose. The columns were equilibrated with 100 mM Tris·acetate pH 8.0 containing 50 mM mercaptoethanol.

Enzyme assays: All assays were carried out at 37° in 22 ml Thunberg tubes after repeated evacuation and refilling

with the desired gas. ^{14}C-formate was determined after separation by isoionic column chromatography (10), ^{12}C-formate by coupling with endogenous formyltetrahydrofolate synthetase (11). $^{14}CO_2$ was quantitated after absorption in KOH (12), H_2 by gas-chromatography (8). Detailed procedures are given in the legends to tables or figures.

Regenerating systems (RS): NADH-RS: galactose, 20 mM; galactose dehydrogenase (EC 1.1.1.48), 1 U; NADH, 2.5 mM. NADPH-RS: glucose-6-phosphate, 40 mM; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1 U; $NADP^+$, 0.5 mM.

Results

Crude lysates were found to reduce CO_2 to formate under a H_2 atmosphere. Conversely, under an Ar atmosphere, the same lysates catalyzed the dehydrogenation of formate to stoichiometric amounts of CO_2 and H_2. Both reactions could also be observed in nucleotide-free lysates (Fig. 1). This clearly demonstrated that neither ATP, CoA, acetyl CoA nor any other nucleotide coenzyme were required for the reaction. Reduction and oxidation were both proportional to protein concentration up to 15 mg/ml and linear with time up to 30 minutes. At 10 mM concentration saturation was obtained both for formate and carbonate.

In the nucleotide-free lysates pyruvate was neither synthesized from CO_2 nor degraded to yield CO_2. These findings exclude pyruvate as an intermediate of the reaction.

In both directions the activity had a pH-optimum at pH 7. It appeared to be independent of divalent cations, as EDTA, NaF or Mg^{2+} had no effect. The reaction was most ac-

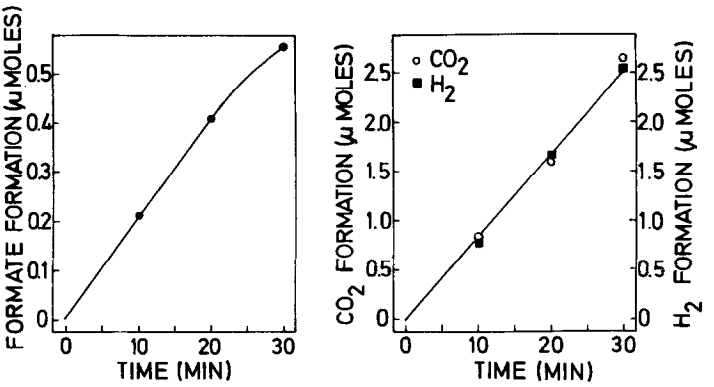

Fig. 1 Kinetics of the reversible CO_2 reduction to formate in cell-free extracts of Clostridium pasteurianum.

Formate formation from CO_2: Tris·acetate pH 7.5, 100 mM; mercaptoethanol, 25 mM; potassium C-14-carbonate, 10 mM (200.000 dpm/µmole); 9 mg nucleotide-free lysate protein; water to 1 ml; gas phase: hydrogen. 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 (isoionic chromatography) (10). C-14-formate was recovered in fractions 9-12 and counted in Bray scintillator (13).

CO_2 and H_2 formation from formate: Tris·acetate pH 7.5, 100 mM; mercaptoethanol, 25 mM; C-14-formate, 10 mM (200.000 dpm/µmole); 9 mg nucleotide-free lysate protein; water to 1 ml; gas phase: argon. The assay mixtures - with 0.2 N KOH in the side arm - were freeze-stopped; 2 ml of the gas phase were taken with a gas-tight syringe for gas chromatographic determination of H_2 formation (8); then 0.5 ml of a 2 M citrate buffer pH 5.4 were injected; the mixtures were shaken for another 45 minutes at room temperature to effect complete absorption of formed C-14-CO_2 by the side arm KOH, aliquots of which were counted in Aerosil Bray scintillator (12,13).

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tive in acetate buffer, while sulfate and phosphate buffers were less favorable. Chloride was strongly inhibitory.

The conversion of CO_2 to formate required reduced ferredoxin as the reductant; NADH or NADPH were totally inactive. Ferredoxin (E_o' = -420 mV) could be replaced to varying degrees by methyl viologen (E_o' = -440 mV) and by benzyl viologen (E_o' = -359 mV) (tab. 1).

Table 1. Ferredoxin dependence of the reversible CO_2 reduction to formate

$CO_2 \longrightarrow$ Formate		Formate $\longrightarrow CO_2$	
Electron donor	formate formed (nmoles/20 min)	Electron acceptor	CO_2 formed (nmoles/20 min)
none	40	none	< 100
NADH-RS	40	NAD^+	< 100
NADPH-RS	40	$NADP^+$	< 100
Fd_{red}	990	Fd_{ox}	3.400
MV_{red}	410	MV_{ox}	2.800
BV_{red}	276	BV_{ox}	3.800

Formate formation from CO_2: Tris·acetate pH 7.5, 100 mM; mercaptoethanol, 25 mM; potassium C-14-carbonate, 10 mM (200.000 dpm/µmole), 12 mg ferredoxin- and nucleotide-free lysate protein; and where indicated, NADH-RS, NADPH-RS, Fd 0.3 mg protein, MV 10 mM, BV 10 mM; water to 1 ml; gas phase: hydrogen. C-14-formate was determined as described in figure 1.

CO_2 formation from formate: Tris·acetate pH 7.5, 100 mM; mercaptoethanol, 25 mM; C-14-formate, 10 mM (200.000 dpm/µmole); 12 mg ferredoxin- and nucleotide-free lysate protein; and where indicated, NAD^+ 2.5 mM, $NADP^+$ 2.5 mM, Fd 0.3 mg protein, MV 10 mM, BV 10 mM. C-14-CO_2 was determined as described in figure 1.

The reserve reaction, formate dehydrogenation to CO_2 and H_2, was found to have the same specificity. Ferredoxin rather than a pyridine nucleotide was operative as the oxidant. It could be replaced by both methyl and benzyl viologen (tab. 1).

The reduction of CO_2 to formate could be coupled with the endogenous formyltetrahydrofolate synthetase of the ex-

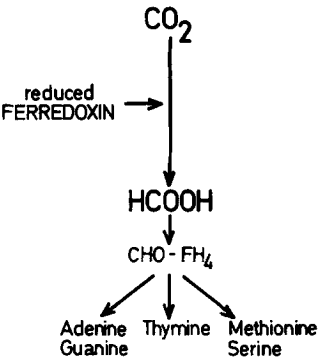

Fig. 2 The CO_2 reductase pathway of one-carbon unit formation from CO_2.

tracts; in the presence of ATP, tetrahydrofolate and Mg^{2+} formyltetrahydrofolate was formed from CO_2 and H_2. This coupled process was proportional to protein concentration and nearly linear with time.

The CO_2 reductase activity was very sensitive to oxygen; O_2 inactivated extracts could partially be restored by preincubation with mercaptoethanol.

Discussion

In this communication evidence is presented indicating that CO_2 can be reduced to formate directly without the participation of a carbon compound functioning as a CO_2 acceptor. Thus the long held view that CO_2 reduction is always indirect, i.e. acceptor dependent, need no longer be maintained.

The reverse reaction, formate dehydrogenation to CO_2, has been demonstrated in many organisms. NAD^+ dependent formate dehydrogenases were found in plants (14), liver (15),

and a photosynthetic bacterium (16); a $NADP^+$ specific enzyme has been partially purified from C. thermoaceticum (17); cytochrome b or c requiring enzymes were detected in Escherichia coli (18) and Desulfovibrio vulgaris (19), respectively. Ferredoxin is the electron acceptor for the enzyme from Methanobacillus omelianskii (20) and from C. acidi urici (21). In no case was the reaction shown to be reversible.

In C. kluyveri (3) and in C. pasteurianum (22) glycine is synthesized from threonine via threonine aldolase rather than from serine via serine aldolase. Consequently, C_1-units such as the positions 2 and 8 of the purines and the S-methyl group of methionine are not formed from serine; instead they are derived from formate via formyltetrahydrofolate synthetase.

In C. kluyveri, which grows on ethanol, acetate and bicarbonate as the sole carbon and energy sources, formate was shown to be synthesized from CO_2 by a ferredoxin dependent reductive carboxylation of acetyl CoA to pyruvate followed by pyruvate cleavage to acetyl CoA and formate (4). The enzymatic data presented in this note indicate that in the saccharolytic C. pasteurianum formate can be formed by a direct, ferredoxin dependent reduction of CO_2 (Fig. 2). CO_2 and reduced ferredoxin are both generated in the pyruvate dehydrogenase reaction.

It cannot be excluded, however, that formate may also be formed from pyruvate via pyruvate formate lyase. This enzyme could not be demonstrated in the extracts used; as it is known to be very labile, its presence cannot definitely be ruled out.

In view of its physiological function the enyzme catalyzing CO_2 reduction to formate in C. pasteurianum is tentatively named CO_2 reductase.

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