First publ. in: FEMS Microbiology Letters 177 (1999), pp. 1-6

Net synthesis of acetate from CO_2 by *Eubacterium acidaminophilum* through the glycine reductase pathway

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Abstract

Eubacterium acidaminophilum combines the oxidation of amino acids such as alanine or valine with the reduction of glycine to acetate in a two-substrate fermentation (Stickland reaction). In the absence of glycine, dense cell suspensions oxidized alanine or valine only to a small extent, with limited production of hydrogen and acetate. Experiments with ^{14}C-labeled carbonate revealed that acetate was formed under these conditions by net reduction of CO_2/HCO_3^-; ^{14}C-labeled formate was formed as an intermediate. *E. acidaminophilum* did not grow with hydrogen plus CO_2; dense cell suspensions under H_2/CO_2 produced only very small amounts (<0.5 mM) of acetate. There was no activity of carbon monoxide dehydrogenase, indicating that the glycine pathway was used for acetate synthesis. The results are explained on the basis of biochemical and energetic considerations.

Keywords: Glycine reduction; Acetogenesis; CO_2 fixation; *Eubacterium acidaminophilum*

1. Introduction

Among the bacteria catalyzing the so-called Stickland fermentation of amino acids [1,2], *Eubacterium acidaminophilum* is perhaps the most versatile one. It can either carry out the complete combined fermentation of e.g. alanine plus glycine to acetate plus ammonia plus CO_2, or convert alanine to acetate plus CO_2 plus hydrogen if a hydrogen-scavenging partner is present. It can also grow by reduction of glycine to acetate plus ammonia, or of betaine to acetate and trimethylamine, with hydrogen as electron donor [3]. These activities require sufficient amounts of selenium in the growth medium because the enzymes involved contain at least one selenoprotein [4,5]. The reduction of glycine through this pathway proceeds via acetyl phosphate and allows ATP synthesis by substrate-level phosphorylation which is quite unusual in a reductive fermentation pathway.

E. acidaminophilum can also dismutate glycine as sole substrate to acetate, ammonia, and CO_2, and contains all enzymes involved in glycine oxidation through glycine decarboxylation and the tetrahydrofolate pathway [3]. So far, it has never been shown that this organisms could also catalyze a net synthesis of acetate from CO_2, using the same pathway in the opposite direction. During studies on the energetic limitations of syntrophic amino acid fermenta-

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tion with this organism, we observed that the fermentation equations did not balance as expected, and could explain this only by a net synthesis of acetate through CO_2 reduction. Here we document this new metabolic capacity of *E. acidaminophilum* which represents to our knowledge the third known pathway through which acetate can be formed anaerobically from CO_2.

2. Materials and methods

2.1. Strains source and cultivation

E. acidaminophilum strain al-2 (DSM 3953, ATCC 49065) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Cells were grown in a fresh water mineral medium containing 1 μM Na_2SeO_3 as described [3].

2.2. Experiments with cell suspensions

Cells were harvested in the late exponential growth phase by centrifugation in 125 ml infusion bottles for 30 min at $3000 \times g$ at 4°C. The pellet was washed with 50 mM potassium phosphate buffer, pH 7.0, containing 2.5 mM dithioerythritol as reducing agent. Cells were resuspended in the same buffer to a final optical density of $OD_{578} = 15$. Dense suspensions (4 ml) were incubated in 8 ml serum bottles, with either 10 mM alanine or 10 mM valine as substrate. Samples were taken through the butyl rubber stoppers with Unimetrics microliter syringes, and deep-frozen at −18°C. Possible dependence of substrate turnover on CO_2 availability was checked in either CO_2-free assays that had been gassed repeatedly with CO_2-free N_2, or in replicates containing 30 mM $NaHCO_3$ supplied from a 0.5 M stock solution that was kept under CO_2 gas atmosphere. Acetate formation from H_2/CO_2 (80/20) was checked with similar washed cell suspensions that were incubated under this gas mixture at 30°C. Controls were run under N_2/CO_2 (80/20).

2.3. Labeling experiments

Assays analogous to those described above were performed with either 5 mM alanine or 5 mM valine as substrate in the presence of 20 mM $NaHCO_3$ which received in addition 10 μl $Na_2\ ^{14}CO_3$ per 1 ml cell suspension (= 250 kBq), corresponding to a specific labeling of 12.5 kBq per μmol total CO_2/HCO_3^-. Samples were taken and deep-frozen as described above. After centrifugation, the supernatant was separated by high-pressure liquid chromatography (Beckman System Gold) on an ion exchange column (Sykam Aminex HPX-87 H) with 5 mM H_2SO_4 as eluent, 0.4 ml per min. The chromatographic conditions were set to allow quantitative separation of CO_2, formate, acetate, and higher fatty acids. Quantification of radiolabeled compounds was performed with a RAMONA-5 on-line liquid scintillation detector system (Raytest, Straubenhardt, Germany) equipped with a 400 μl glass scintillator flow cell that had been calibrated before with [^{14}C]acetate standards (1–150 kBq per ml).

2.4. Chemical determinations

Acetate and isobutyrate were quantified either by HPLC (see above) or by gas chromatography [6]. Alanine was determined enzymatically [7]. Cell growth was measured via optical density at 578 nm wavelength. Protein was quantified by the Bradford method [8]. Hydrogen was quantified by gas chromatography with a Carlo Erba GC 6000 gas chromatograph equipped with a thermal conductivity detector. The gas sample was run on a Carbosieve SII column (Supelco) 60–80 mesh. At 120°C nitrogen gas was used as carrier, the thermal conductivity detector was run at 130°C, filament temperature 350°C. The system was calibrated with defined H_2/N_2 mixtures at 0.1–2%.

Carbon monoxide dehydrogenase was assayed after [9]; benzyl viologen-dependent hydrogenase in an analogous manner [10].

2.5. Chemicals and gases

All chemicals were of the highest purity that is commercially available, and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Eastman Kodak (Rochester, NY, USA), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Pharmacia (Freiburg, Germany), Serva

(Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany), and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

3. Results

3.1. Experiments in dense suspensions

E. acidaminophilum grew with glycine as sole substrate with a doubling time of 1.16 h ($\mu = 0.597\ h^{-1}$) if selenite was included in the medium at 10^{-6} M concentration. Other amino acids such as alanine or valine had been reported to be degraded by the pure culture only if e.g. glycine was provided as a hydrogen-scavenging cosubstrate. In experiments to elucidate the energetic limits of this kind of fermentation in the absence of a hydrogen-scavenging cosubstrate, we observed the following substrate turnover balances: Resting cell suspensions produced from 10 mM alanine, 3 mM acetate plus 0.4 mM H_2 (= 1000 Pa in the headspace) within 24 h; from 10 mM valine, 4.5 mM isobutyrate, 3–3.5 mM acetate and 0.8 mM H_2 (= 2.000 Pa H_2) were produced in the same time (Figs. 1 and 2). Similar fermentation balances had been reported in the original description of this bacterium [3]. The comparably high background acetate formation in the absence of valine may be due to endogenous acetate formation from storage compounds. Obviously, acetate was synthesized from CO_2 which was the only electron acceptor available in the medium. In order to examine this hypothesis, we followed the same reactions in the presence and absence of HCO_3^-/CO_2 in the medium. The rate of acetate formation in the presence of CO_2/HCO_3^- was about twice as high as in its absence (results not shown); obviously, the endogenous CO_2 production from alanine oxidation could not cover the CO_2 need sufficiently.

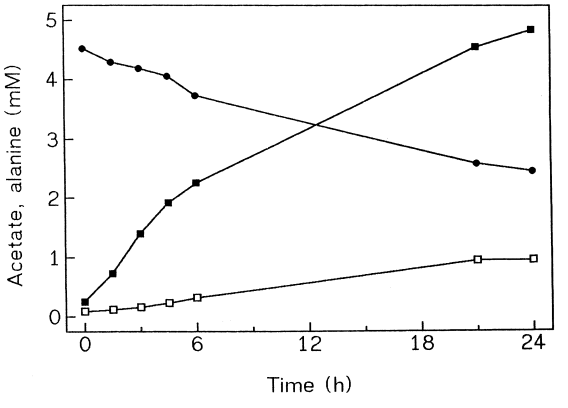

Fig. 1. Degradation of alanine (●) by dense cell suspensions of *E. acidaminophilum*. Formation of acetate in the presence (■) and in the absence (□) of alanine.

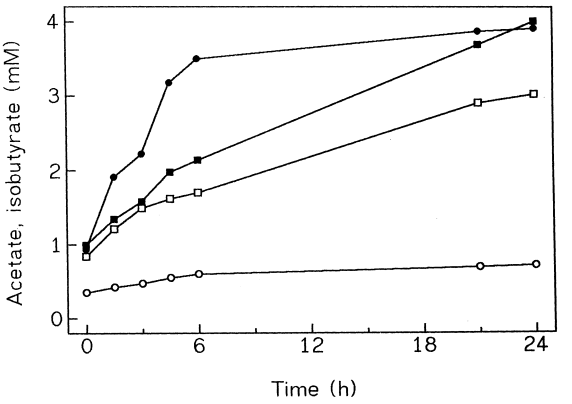

Fig. 2. Degradation of valine by dense cell suspensions of *E. acidaminophilum*. Formation of acetate (■) and isobutyrate (●) in the presence of valine. Open symbols: formation of acetate (□) and isobutyrate (○) in the absence of valine.

The cultures were checked for possible contaminations with homoacetogenic bacteria. These tests as well as checks for carbon monoxide dehydrogenase activity were always negative.

3.2. Labeling experiments

Suspension experiments in the presence of radiolabeled carbonate documented that there was indeed a significant incorporation of CO_2/HCO_3^- into acetate. As shown in Fig. 3, $^{14}CO_2$-carbon was incorporated into acetate at a high rate in the presence of alanine as electron donor; the rate of incorporation was far lower in the absence of alanine. Formate was identified transiently as a labeled side product which disappeared later during the incubation experiment. Similar results were obtained with valine as electron donor: Labeled acetate was formed at a high rate in the presence of valine, and formate was again formed as a labeled transient coproduct (Fig. 4). The specific radioactivity of the formed acetate was

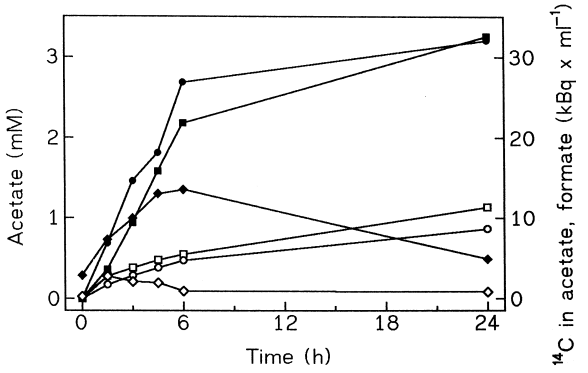

Fig. 3. Formation of [^{14}C]acetate and [^{14}C]formate from [^{14}C]Na_2CO_3 by dense suspensions of *E. acidaminophilum* with alanine as electron donor. Filled symbols: (■) acetate, (●) ^{14}C label in acetate, (♦) ^{14}C label in formate in the presence of 5 mM alanine; open symbols: (□) acetate, (○) ^{14}C label in acetate, (◇) ^{14}C label in formate in the absence of alanine.

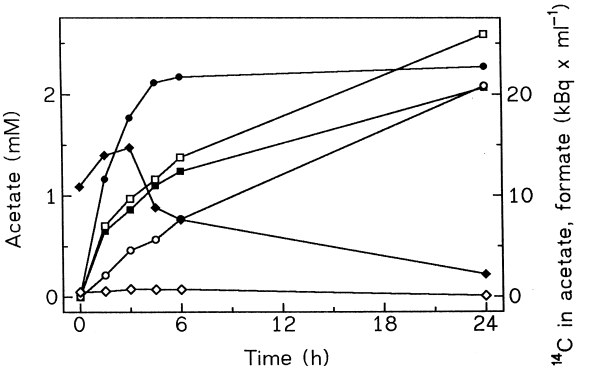

Fig. 4. Formation of [^{14}C]acetate and [^{14}C]formate from [^{14}C]Na_2CO_3 by dense suspensions of *E. acidaminophilum* with valine as electron donor. Filled symbols: (■) acetate, (●) ^{14}C label in acetate, (♦) ^{14}C label in formate in the presence of 5 mM valine; open symbols: (□) acetate, (○) ^{14}C label in acetate, (◇) ^{14}C label in formate in the absence of valine.

in both cases slightly lower than that of the CO_2/HCO_3^- used, indicating that the total CO_2 pool was diluted by endogenous CO_2 from amino acid oxidation (Table 1).

3.3. Lithotrophic acetate formation

In growth experiments, we did not observe growth with H_2/CO_2 in the absence of organic electron donors, as also observed before [3]. Although there was sufficient hydrogenase activity in glycine-grown cells (0.6 μmol H_2 per min and mg protein), dense cell suspensions under H_2/CO_2 formed acetate only at a low rate (0.62 nmol acetate per min and mg protein) which was about twice as high as the endogenous acetate production under N_2/CO_2 (0.29 nmol per min and mg protein). Also these findings confirm earlier observations [3].

4. Discussion

In the present communication, we document that *E. acidaminophilum* can catalyze a net synthesis of acetate from CO_2. Occurrence of this capacity among non-homoacetogenic bacteria has been documented before for *Clostridium acidiurici* and *C. cylindrosporum* [11], and for *C. purinolyticum* [12]. With that, the glycine pathway represents the third way through which acetate can be synthesized by anaerobic bacteria. So far, acetate synthesis through the carbon monoxide dehydrogenase pathway (Acetyl-CoA or Wood-pathway) has been described which is used by homoacetogenic, methanogenic, and several sulfate-reducing bacteria in their assimilatory metabolism [13], and the reversed tricarboxylic acid cycle that is applied for autotrophic cell carbon synthesis by green phototrophic bacteria and few sulfate reducers [13].

Only the homoacetogens are able to couple lithotrophic acetate formation from hydrogen and CO_2 with energy conservation and growth [14,15]; the other organisms mentioned depend on other energy sources, and may excrete acetate only in traces during cell matter synthesis.

Table 1
Incorporation of ^{14}C label from [^{14}C]$NaHCO_3$ (specific radioactivity 12.5 kBq μmol^{-1}) into acetate

Electron donor	Acetate formed (μmol)	Specific radioactivity in acetate (kBq μmol^{-1})
Alanine (5 mM)	3.26	9.85
Valine (5 mM)	2.07	11.0

In the present study, we observed net acetate synthesis from CO_2 by dense cell suspensions of *E. acidaminophilum* with alanine or valine as electron donor. Alanine fermentation under these conditions should follow the equation

$$\text{Alanine} + 2H_2O \rightarrow \text{acetate}^- + NH_4^+ + CO_2 + 2H_2 \quad \Delta G_0' = +2.7 \text{ kJ mol}^{-1} \quad (1)$$

This reaction was partly combined with acetogenic CO_2 reduction, according to

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \quad \Delta G_0' = -95 \text{ kJ mol}^{-1} \quad (2)$$

The overall reaction is exergonic

$$2 \text{ Alanine} + 2 H_2O \rightarrow 3 \text{ Acetate}^- + 2 NH_4^+ + H^+ \quad \Delta G_0' = -92 \text{ kJ mol}^{-1} \quad (3)$$

and should allow ATP synthesis and growth since synthesis of one ATP requires a $\Delta G_0'$ of at least -70 kJ per reaction run [16]. However, we found neither complete substrate turnover nor active growth under these conditions, and there was also no lithotrophic growth on the basis of reaction 2, and only very slow (cometabolic?) acetate formation from H_2 and CO_2.

The biochemical pathway used for glycine dismutation to CO_2 and acetate has been worked out in detail in the past [3], and is probably used as well in the reductive direction, as suggested before for some non-homoacetogenic clostridia [11,12]: CO_2 is reduced via formate, ATP-dependent linkage to tetrahydrofolate, and subsequent reduction to methylene tetrahydrofolate. The methylene derivative is reductively carboxylated and aminated to form glycine which is subsequently reduced to acetate, releasing ammonia. This step also releases one ATP in a substrate-level phosphorylation reaction, thus recovering the ATP invested before. All reactions, except for the one catalyzed by glycine reductase, are known to be reversible, with NADPH as electron donor. From this point of view, it is not surprising that the overall reaction chain can operate to form acetate from CO_2, but the overall process does not provide for net ATP formation. This explains why growth of *E. acidaminophilum* with H_2+CO_2 was never observed, and acetate formation from H_2+CO_2 was found only as a cometabolic side activity. With that, this bacterium differs from the homoacetogenic bacteria which appear to conserve a fraction of an ATP either in the methylene tetrahydrofolate reductase reaction [14] or through a sodium ion-translocating methyl transferase reaction [17].

The observed excretion of radiolabeled formate during acetate formation from alanine or valine with $^{14}CO_2$ indicates that the ATP level in the cells under these conditions is probably insufficient to initiate the ATP-dependent formate activation. Obviously, the electron flow through the reductive reaction chain is impeded, and this exergonic branch of the metabolism (reaction 2) can be employed only to a limited extent.

In our experiments (alanine 10 mM, NH_4^+ 5 mM), the reaction stopped when acetate reached 3 mM concentration and hydrogen 0.01 atm, corresponding to a remnant energy in the system (according to reaction 1) in the range of -20 kJ mol^{-1}. This is the minimum amount of energy needed to maintain viability with a fully charged cytoplasmic membrane [18], and is the same energy level where also the syntrophically fermenting bacteria find their energetic limits. Thus, although basically possible, the glycine pathway is not an efficient alternative to the carbon monoxide dehydrogenase pathway for a net acetate synthesis.

Acknowledgements

This study was financed in part by funds of the University of Constance and of the Fonds der Chemischen Industrie, Frankfurt/M.

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