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EXPERIMENTAL ARTICLES

A Study of the Mechanism of Acetate Assimilation in Purple Nonsulfur Bacteria Lacking the Glyoxylate Shunt: Acetate Assimilation in *Rhodobacter sphaeroides*

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Abstract—The mechanism of acetate assimilation in the purple nonsulfur bacterium *Rhodobacter sphaeroides*, which lacks the glyoxylate shunt, has been studied. It has been found that the growth of this bacterium in batch and continuous cultures and the assimilation of acetate in cell suspensions are not stimulated by bicarbonate. The consumption of acetate is accompanied by the excretion of glyoxylate and pyruvate into the medium, stimulated by glyoxylate and pyruvate, and inhibited by citramalate. The respiration of cells in the presence of acetate is stimulated by glyoxylate, pyruvate, citramalate, and mesaconate. These data suggest that the citramalate cycle may function in *Rba. sphaeroides* in the form of an anaplerotic pathway instead of the glyoxylate shunt. At the same time, the low ratio of fixation rates for bicarbonate and acetate exhibited by the *Rba. sphaeroides* cells (approximately 0.1), as well as the absence of the stimulatory effect of acetate on the fixation of bicarbonate in the presence of the Calvin cycle inhibitor iodoacetate, suggests that pyruvate synthase is not involved in acetate assimilation in the bacterium *Rba. sphaeroides.*

Key words: *Rhodobacter sphaeroides*, citramalate pathway, glyoxylate shunt, acetate.

In most bacteria, acetate is assimilated via the tricarboxylic acid (TCA) cycle. Many C_4 acids in this cycle are expended for biosynthetic purposes and are replenished via the glyoxylate cycle [1], which is an anaplerotic pathway producing malate (an intermediate of the TCA cycle) from two molecules of acetyl-CoA. The key enzymes involved in the glyoxylate cycle are isocitrate lyase, which splits isocitrate into glyoxylate and succinate, and malate synthase, which synthesizes malate from glyoxylate and acetyl-CoA. However, there is a large group of microorganisms (from various taxa) that do not possess isocitrate lyase (ICL^- phenotype) but can grow on acetate, using it as the sole source of carbon and energy. For instance, the purple nonsulfur bacteria *Rhodospirillum rubrum, Rhodobacter sphaeroides*, and *Phaespirillum fulvum* [2, 3], as well as some methylotrophic bacteria with the serine pathway, such as *Methylobacterium extorquens* AM1 [4, 5], have this property.

We have recently suggested a new pathway involving acetate oxidation to glyoxylate for the bacterium *Rsp. rubrum*, which is called the citramalate cycle, (figure) [6–9]. The major intermediate of this cycle, citramalate, is produced by the condensation of pyruvate and acetyl-CoA. Through a number of sequential reactions, citramalate is converted into propionyl-CoA and glyoxylate. The latter condenses with another acetyl-CoA molecule, forming malate in a reaction catalyzed by malate synthase. Malate enters the TCA cycle, thereby replenishing the intermediates of this cycle that have been consumed for biosynthetic purposes. Pyruvate, which serves as the acetyl-CoA acceptor in a reaction catalyzed by citramalate synthase, is replenished through the carboxylation of propionyl-CoA. This carboxylation leads to the formation of methylmalonyl-CoA, which transforms into succinate. The latter is converted to oxaloacetate in the TCA cycle. The oxaloacetate is then decarboxylated, forming phosphoenolpyruvate (PEP). The conversion of PEP to pyruvate completes the citramalate cycle (figure). The stoichiometry of this cycle is as follows: acetate $\longrightarrow$ glyoxylate + 4[H]. Acetate assimilation in *Rsp. rubrum* takes place only in the presence of high concentrations of bicarbonate, due to the reaction of propionyl-CoA carboxylation in the citramalate cycle. CO_2 dependence can be considered as an attribute of the citramalate cycle.

The aim of this work was to investigate the possibility of the involvement of the citramalate cycle in acetate assimilation in the ICL-negative *Rba. sphaeroides.*

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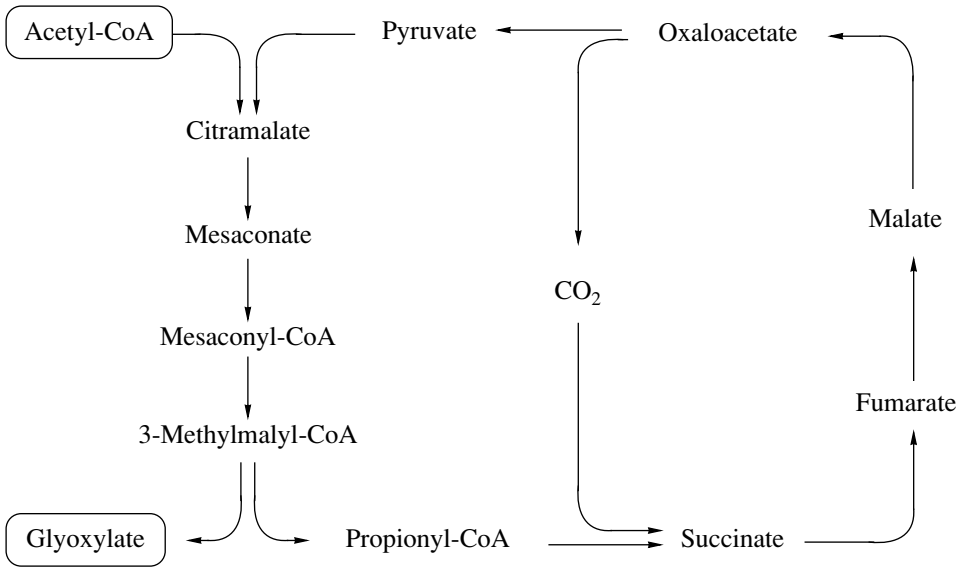

The citramalate cycle involved in the assimilation of acetate by *Rsp. rubrum* cells [6–9].

This bacterium, like *Rsp. rubrum*, is a member of the α subgroup of proteobacteria, although these two bacteria belong to different orders (*Rhodobacterales* and *Rhodospirillales*, respectively) [10].

MATERIALS AND METHODS

The experiments were carried out with three bacteria, *Rhodobacter sphaeroides* 2R, *Rhodospirillum rubrum* 1R, and *Rhodopseudomonas palustris* 1K, obtained from the culture collection of the Department of Microbiology at Moscow State University. These bacterial strains were grown photoheterotrophically at 30°C in an Ormerod medium containing malate and acetate (1 g/l) according to the method described in [8]. Continuous cultivation in a turbidostat mode was performed in a 0.5-l photobioreactor at a cell density of 0.23–0.34 mg of dry wt./ml and illumination of 60 W/m^2 [11]. Batch cultivation was performed in 500-ml flasks filled with the medium and closed with ground glass stoppers under 20-W/m^2 illumination. Cells for the cell suspension experiments were grown to the early exponential phase, collected by centrifugation, washed in a basal mineral medium, and resuspended at a density of 0.1–0.2 mg of protein/ml.

Experiments with [^{14}C]-labeled substrates were carried out in syringes under 30-W/m^2 illumination. The reaction was started by adding, unless stated otherwise, 5 mM $NaH^{14}CO_3$ (0.04 MBq) or 5 mM [2-^{14}C]acetate (0.02 MBq) and stopped, at regular intervals, by filtering 1 ml of the cell suspension through 0.45-μm nitrocellulose filters. The filters, with adsorbed cells, were then dried, and their radioactivity was counted in an LKB RacBeta model 1127 liquid scintillation counter.

In order to study the excretion of keto acids, the cell suspensions were incubated at 30°C for 2 h in syringes under 30-W/m^2 illumination in the presence of 1 g/l acetate and, when required, inhibitors. The excreted keto acids were analyzed in the form of 2,4-dinitrophenylhydrazones [12] by thin-layer chromatography (TLC) in an *n*-butanol–ethanol–0.5 N NH_4OH (7 : 1 : 2) mixture.

Cell respiration was measured polarographically using a 1-ml Clark-type oxygen electrode and cell suspensions containing 0.05–0.1 mg of protein/ml.

In order to perform an enzyme assay, the cells were washed in a 50 mM Tris–HCl buffer (pH 7.5) and resuspended in 5 ml of the respective assay buffer. The cells were disrupted either ultrasonically (22 kHz, 3 min, 4°C) or using an X-press at 7000–10000 kg. Unbroken cells and cell debris were removed by centrifugation at 40000 *g* for 20 min (4°C). The enzymes in the supernatant were assayed at room temperature. The assay mixtures contained 0.5–1.5 mg of protein/ml. Enzyme activity was expressed in nmol/(min mg protein).

The activity of isocitrate lyase (EC 4.1.3.1) was measured using phenylhydrazine [13], lactate dehydrogenase [14], or 2,4-dinitrophenylhydrazine [15]. Malate synthase (EC 4.1.3.2) was assayed spectrophotometrically by the glyoxylate-dependent formation of CoA from acetyl-CoA in the presence of 5,5'-dithiobis-(2-nitrobenzoate) [13].

Protein concentration was measured according to the method of Lowry *et al.* using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Growth of *Rba. sphaeroides* on acetate. This bacterium was found to be able to grow photoheterotrophically in the medium in which acetate was the sole

source of carbon and energy. The addition of bicarbonate to the batch culture not only failed to stimulate but even slightly inhibited its growth. Specifically, after 72 h of cultivation, the biomass comprised 1.2 mg of protein/ml in the medium with acetate, while, in the medium with acetate and bicarbonate, it was 0.9 mg of protein/ml. Similar results were obtained when *Rba. sphaeroides* was continuously cultivated in the photobioreactor. In this case, the addition of CO_2 to the argon gassed through the photobioreactor did not stimulate bacterial growth (Table 1). Nevertheless, increasing the argon flow rate through the bioreactor from 20 to 100 ml/min, which enhanced the removal of the CO_2 produced during acetate assimilation, did not diminish the growth of *Rba. sphaeroides*. In contrast, the removal of metabolic CO_2 from the gas phase of the bioreactor slowed down the growth of *Rsp. rubrum*, and the increase in the argon flow rate through the bioreactor to 100 ml/min completely suppressed the growth of this bacterium (Table 1).

The absence of the glyoxylate cycle in *Rba. sphaeroides*. The information on isocitrate lyase activity in *Rba. sphaeroides* is contradictory. For instance, Albers and Gottschalk [2] and Kornberg and Lascelles [3] reported the absence of this enzyme in *Rba. sphaeroides*, whereas Blasco *et al.* [15] detected low isocitrate lyase activity using the colorimetric method with 2,4-dinitrophenylhydrazine. In our studies, we failed to detect isocitrate lyase in *Rba. sphaeroides* 2R cells with the three methods used (see the Materials and Methods section), although isocitrate lyase activity was easily detected in *Rps. palustris* (data not presented), which is an ICL-positive species [16]. In the *Rba. sphaeroides* cells, the activity of the other enzyme involved in the glyoxylate cycle, malate synthase, comprised 23 nmol/(min mg protein). Thus, *Rba. sphaeroides* 2R should be considered as an ICL-negative strain.

Secretion of keto acids by *Rba. sphaeroides* cells. The *Rba. sphaeroides* cells incubated for 2 h in the presence of acetate secreted glyoxylate into the medium (Table 2). After 4 h of incubation, the medium also contained pyruvate. The addition of fluoroacetate, an inhibitor of the aconitase reaction in the TCA cycle, stimulated the secretion of glyoxylate. It should be noted that aconitase is involved not only in the TCA cycle but also in the glyoxylate cycle. For this reason, if the glyoxylate cycle is in operation, fluoroacetate must suppress the production of glyoxylate. The absence of the inhibitory action of fluoroacetate on the secretion of glyoxylate by the *Rba. sphaeroides* cells suggests that the glyoxylate cycle does not function in this bacterium. Presumably, glyoxylate and pyruvate are secreted by the *Rba. sphaeroides* cells incubated in the presence of acetate due to the functioning of the citramalate cycle.

Acetate assimilation by *Rba. sphaeroides* cells. As in the case of *Rsp. rubrum*, the bacterium *Rba. sphaeroides* can assimilate acetate in light under anaerobic conditions (Table 3), the acetate assimilation rate being considerably higher in *Rba. sphaeroides* than in *Rsp. rubrum* cells (150–300 and 15–30 nmol/(min mg protein), respectively) (Table 3 and [8]). At the same time, in contrast to the case of *Rsp. rubrum*, CO_2 does not stimulate acetate assimilation in the bacterium *Rba. sphaeroides*, although glyoxylate and pyruvate do stimulate this assimilation. Glyoxylate, as the substrate of the malate synthase reaction, provides for the utilization of additional acetyl-CoA in the TCA cycle. The stimulatory action of pyruvate may result from its

Table 1. The specific growth rate μ (h^{-1}) of *Rba. sphaeroides* and *Rsp. rubrum* when cultivated continuously in the medium containing acetate as a function of the argon gassing rate through the photobioreactor and the concentration of CO_2 in the gas phase

Gas composition and gassing rate	*Rba. sphaeroides*	*Rsp. rubrum*
95% Ar + 5% CO_2 (20 ml/min)	0.112	0.084
100% Ar, (20 ml/min)	0.084	0.044
100% Ar, 100 ml/min (100 ml/min)	0.101	0.023

Table 2. The secretion of glyoxylate by *Rba. sphaeroides* cells incubated anaerobically in light

Compounds present in the medium	Glyoxylate secretion, nmol/(h mg protein)
Acetate	76.0
Acetate + fluoroacetate	160.0
Acetate + CO_2	64.0
Acetate + fluoroacetate + CO_2	158.0

Note: The concentrations of acetate, bicarbonate, and fluoroacetate in the medium were 5, 5, and 1 mM, respectively. The incubation time was 2 h.

Table 3. The effect of various compounds on the assimilation of acetate in *Rba. sphaeroides* cells

Compounds present in the medium	Acetate assimilation, nmol/(min mg protein)
Acetate	248.0
Acetate + bicarbonate	226.0
Acetate + glyoxylate	334.0
Acetate + pyruvate	298.0
Acetate + citramalate	155.0
Acetate + mesaconate	219.0
Acetate + fluoroacetate	122.0
Acetate + malonate	46.0

Note: The concentration of fluoroacetate in the medium was 1 mM. The concentration of all the other compounds was 5 mM.

Table 4. The rate of oxygen consumption by *Rba. sphaeroides* cells

Compounds present in the medium	Oxygen consumption, nmol/(min mg protein)
Endogenous respiration	5.0
Acetate	29.0
Acetate + bicarbonate	30.0
Glyoxylate	20.0
Pyruvate	22.0
Acetate + pyruvate	38.0
Acetate + glyoxylate	48.0
Acetate + citramalate	36.0
Acetate + mesaconate	35.0
Citramalate	14.0
Mesaconate	12.0

Note: The concentration of all the compounds in the medium was 5 mM.

Table 5. The assimilation of bicarbonate in *Rba. sphaeroides* cells

Compounds present in the medium	Bicarbonate assimilation, nmol/(min mg protein)
Bicarbonate	28.0
Bicarbonate + acetate	32.0
Bicarbonate + H_2	62.0
Bicarbonate + H_2 + acetate	20.0
Bicarbonate + iodoacetate	7.0
Bicarbonate + H_2 + iodoacetate	3.0
Bicarbonate + H_2 + iodoacetate + acetate	3.0
Bicarbonate + H_2 + iodoacetate + propionate	16.0

Note: The concentrations of bicarbonate, acetate, propionate, and iodoacetate in the medium were 5, 5, 5, and 5×10^{-2} mM, respectively.

involvement in metabolic processes in the role of the acetyl-CoA acceptor in the citramalate cycle. In comparison to fluoroacetate, which inhibited acetate assimilation by approximately 50% (Table 3), malonate (the inhibitor of succinate dehydrogenase in the TCA cycle) at a concentration of 5 mM suppressed acetate assimilation by more than 80%. This result may be due to the fact that succinate dehydrogenase is involved in both the TCA and citramalate cycles (figure). Citramalate substantially inhibited the assimilation of [2-^{14}C]acetate. It should be noted that earlier data showing similar findings for *Rsp. rubrum* were interpreted as a dilution of the radioactive label due to the assimilation of citramalate, instead of acetate, in the TCA cycle [6, 8, 9].

Oxygen uptake by *Rba. sphaeroides* cells. These cells, grown anaerobically in light, remain capable of respiration. The respiration of the *Rba. sphaeroides* cells in the presence of acetate was insensitive to CO_2 (Table 4). This finding is in agreement with the data on bacterial growth and acetate assimilation (Tables 1, 3). As in the case of acetate assimilation, cell respiration in the presence of acetate was considerably stimulated by glyoxylate and pyruvate (Tables 1, 4). Citramalate and mesaconate, the putative intermediates of the TCA cycle, also stimulated the respiration of the *Rba. sphaeroides* cells. Consequently, the experimental data on the respiration of *Rba. sphaeroides* cells confirm the absence of the stimulatory effect of CO_2 on acetate assimilation but do not contradict the suggestion that the citramalate cycle is involved in acetate metabolism.

Bicarbonate assimilation in *Rba. sphaeroides* cells. The experiments showed that *Rba. sphaeroides* cells assimilate bicarbonate primarily through the Calvin cycle. Indeed, iodoacetate, the inhibitor of the Calvin cycle, considerably suppressed the assimilation of CO_2 in the presence of endogenous substrates or molecular hydrogen acting as the electron donor (Table 5). It is known that iodoacetate inhibits the Calvin cycle at the glyceraldehyde-3-phosphate dehydrogenase level [17]. In the presence of iodoacetate, acetate did not stimulate the assimilation of CO_2. This result suggested that pyruvate synthase, which carboxylates acetyl-CoA and results in the formation of pyruvate, is not involved in acetate assimilation. In contrast, acetate restored the assimilation of bicarbonate suppressed by iodoacetate in the purple sulfur bacteria *Thiocapsa roseopersicina* and *Ectothiorhodospira shaposhnikovii*, for which the involvement of pyruvate synthase in acetate assimilation has already been demonstrated [18, 19]. A comparison of the data presented in Tables 4 and 5 shows that the CO_2/acetate ratio for *Rba. sphaeroides* did not exceed 0.1, whereas this ratio was 1.0 or even higher for *Tca. roseopersicina* and *Ect. shaposhnikovii* [18, 19]. At the same time, propionate augmented the CO_2 assimilation rate in the presence of iodoacetate, indicating that acetate-grown *Rba. sphaeroides* cells are likely to possess propionyl-CoA carboxylase.

It would be tempting to suggest that oxaloacetate, the acceptor of acetate in the citrate synthase reaction, is synthesized through the carboxylation of PEP, a product of the Calvin cycle. Such a suggestion, however, does not agree with the low CO_2/acetate ratio typical of *Rba. sphaeroides* cells (see above). In other words, the Calvin cycle is unlikely to function as an anaplerotic pathway in *Rba. sphaeroides* cells assimilating acetate.

The experimental data obtained in this work do not allow an unambiguous conclusion on the mechanism of acetate assimilation in *Rba. sphaeroides* to be drawn. Indeed, bicarbonate did not stimulate the growth of this bacterium on acetate, the assimilation of [2-^{14}C]acetate, and cell respiration in the presence of acetate (Tables 1, 3, 4), whereas a stimulatory effect

might indicate the involvement of the citramalate cycle in the assimilation of acetate by *Rsp. rubrum* cells. At the same time, the citramalate cycle may be involved in the assimilation of acetate by *Rba. sphaeroides* cells, as is evident from the following observations: (1) fluoroacetate stimulates the accumulation of glyoxylate in a suspension of *Rba. sphaeroides* cells incubated with acetate (Table 2); (2) pyruvate (the acceptor of acetyl-CoA in the citramalate cycle) stimulates acetate assimilation and cell respiration on acetate (Tables 3, 4); (3) the citramalate cycle intermediates mesaconate and citramalate stimulate the respiration of *Rba. sphaeroides* cells (Table 4); and (4) citramalate strongly inhibits the assimilation of [2-^{14}C]acetate (Table 3).

Further evidence for the involvement of the citramalate cycle in the assimilation of acetate by *Rba. sphaeroides* cells is presented in the accompanying paper.

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