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EXPERIMENTAL ARTICLES

Enzymes of the Citramalate Cycle in *Rhodospirillum rubrum*

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Abstract—*Rhodospirillum rubrum* is among the bacteria that can assimilate acetate in the absence of isocitrate lyase, the key enzyme of glyoxylate shunt. Previously we have suggested the functioning of a new anaplerotic cycle of acetate assimilation in this bacterium: citramalate cycle, where acetyl-CoA is oxidized to glyoxylate. This work has demonstrated the presence of all the key enzymes of this cycle in *R. rubrum* extracts: citramalate synthase catalyzing condensation of acetyl-CoA and pyruvate with the formation of citramalate, mesaconase forming mesaconate from L-citramalate, and the enzymes catalyzing transformation of propionyl-CoA + glyoxylate 3-methylmalyl-CoA ⟷ mesaconyl-CoA. At the same time, *R. rubrum* synthesizes crotonyl-CoA carboxylase/reductase, which is the key enzyme of ethylmalonyl-CoA pathway discovered recently in *Rhodobacter sphaeroides.* Physiological differences between the citramalate cycle and the ethylmalonyl-CoA pathway are discussed.

Key words: carbon metabolism, glyoxylate cycle, citramalate cycle, acetate.

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The glyoxylate cycle discovered by Kornberg and Krebs [1] enables bacteria to utilize acetate and metabolically related compounds as a sole growth substrate (Fig. 1A). In the glyoxylate cycle, the key enzymes of which are isocitrate lyase and malate synthase, acetyl-CoA is oxidized to glyoxylate. Further condensation of glyoxylate with another molecule of acetyl-CoA results in the formation of malate, and oxidation of the latter results in regeneration of oxaloacetate, which is an acceptor of acetyl-CoA in the tricarboxylic acid cycle (TCA cycle). Thus, the glyoxylate cycle fills up the pool of TCA cycle intermediates consumed for the biosynthesis of cell components.

However, there is a large group of organisms that can grow on acetate or substrates metabolized through acetate in the absence of isocitrate lyase. Some representatives of the alphaproteobacteria *Rhodospirillum rubrum, Rhodobacter sphaeroides, Phaeospirillum fulvum, Paracoccus versutus*, and *Methylobacterium extorquens* belong to this group [2–5]. It also includes some streptomycetes [6].

The mechanism of acetate assimilation in these microorganisms has remained obscure for a long time. However, recently different groups of researchers have suggested several pathways of acetate utilization. For example, we have suggested the functioning in *R. rubrum* of a citramalate cycle (CM cycle) [7–10], where acetyl-CoA is oxidized to glyoxylate in the following reaction sequence: acetyl-CoA + pyruvate ⟶ citramalate ⟶ mesaconate ⟶ mesaconyl-CoA ⟶ 3-methylmalyl-CoA ⟶ propionyl-CoA + glyoxylate (Fig. 1B). Regeneration of pyruvate, which is an acceptor of acetyl-CoA in the citramalate synthase reaction, involves propionyl-CoA carboxylase. For *M. extorquens*, the group of Lidstrom [11] suggested another pathway of acetate assimilation, including the enzymes and intermediate compounds of the pathways of poly-β-hydroxybutyrate (PHB) biosynthesis, valine degradation, and biosynthesis of polyketide antibiotics. Finally, an ethylmalonyl-CoA pathway (EM pathway) of acetate assimilation has been recently demonstrated in *Rb. sphaeroides*. Besides unique reactions, this pathway includes a series of enzymes of both the CM cycle and the pathway suggested for *Methylobacterium* (Fig. 1B) [12, 13]. In the EM pathway, acetoacetyl-CoA formed by condensation of two acetyl-CoA molecules is converted into crotonyl-CoA, which is then carboxylated with the involvement of crotonyl-CoA carboxylase/reductase to form ethylmalonyl-CoA. The latter is isomerized to methylsuccinyl-CoA, and oxidized to mesaconyl-CoA. Mesaconyl-CoA is transformed into 3-methylmalyl-CoA, and further to propionyl-CoA and glyoxylate. Condensation of acetyl-CoA and glyoxylate results in the formation of malate, and carboxylation of propionyl-CoA results in the formation of succinyl-CoA.

Our suggestion about the functioning of the CM cycle in *R. rubrum* is based, first of all, on the results of the study of cell suspensions in vivo [7–10]. Although the possibility of acetate and pyruvate conversion into glyoxylate and propionate with citramalate and mesaconate as intermediates was also demonstrated in vitro

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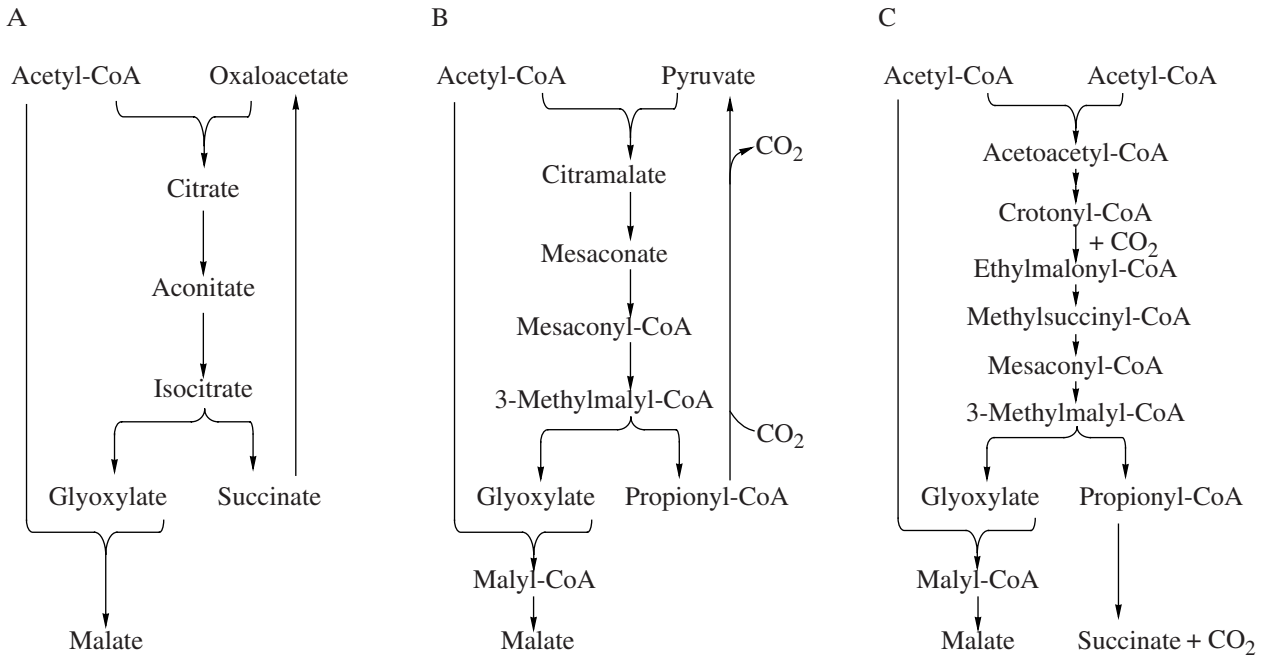

Fig. 1. Schematic representations of glyoxylate cycle (A), citramalate cycle (B), and ethylmalonyl-CoA pathway (C). Note that the formation of malate from acetyl-CoA and glyoxylate in isocitrate lyase-minus bacteria occurs not directly but with the involvement of malyl-CoA lyase and malyl-CoA-hydrolyzing enzyme [17].

[7, 14], a detailed study of reactions of citramalate formation and further conversion into propionyl-CoA and glyoxylate was not carried out.

The goal of this work was to study the key enzymes responsible for oxidation of acetyl-CoA into glyoxylate in *R. rubrum* and the possibility of functioning in *R. rubrum* of other pathways of acetate assimilation suggested recently.

MATERIALS AND METHODS

Bacteria and cultivation conditions. The object of study was the strain *Rhodospirillum rubrum* 1R from the collection of the Department of Microbiology, Moscow State University. The culture was grown under photoheterotrophic conditions in the Ormerod's medium as described [7].

Preparation of cell extracts. Cells were destroyed with a French press under anaerobic conditions [15].

Enzymes activities were determined at room temperature. The reaction mixture contained 0.5–1.5 mg protein/ml.

The activities of pyruvate kinase, pyruvate:phosphate dikinase, pyruvate:water dikinase, and pyruvate carboxylase were determined as described previously [16]. Propionyl-CoA-carboxylase was measured radiochemically by propionyl-CoA-dependent fixation of CO_2 [10].

Phosphoenolpyruvate (PEP) carboxylase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxytransphosphorylase activities were measured spectrophotometrically by NADH oxidation in the presence of malate dehydrogenase in the reaction mixture of the following composition: Tris–HCl (pH 7.8), 50 mM; phosphoenolpyruvate (PEP), 6 mM; $NaHCO_3$, 15 mM; NADH, 0.6 mM; $MnCl_2$, 4 mM; DTT, 5 mM; malate dehydrogenase, 1 U/ml; and the extract. At the measurement of PEP carboxykinase or PEP carboxytransphosphorylase, the mixture contained 5 mM of GDP (ADP) or 2 mM of KH_2PO_4, respectively.

The activity of citramalate synthase was determined colorimetrically by (1) pyruvate-dependent formation of CoA from acetyl-CoA (with DTNB) and (2) decrease of pyruvate depending on the presence of acetyl-CoA (with phenyl hydrazine) [15].

Mesaconase (mesaconate hydratase) was measured spectrophotometrically by mesaconate formation from citramalate at a wavelength of 250 nm (ε_{250} = 2.26 mM cm^{-1}) in the reaction mixture of the following composition: Tris–HCl (pH 7.8), 50 mM; (L)- or (D)-citramalate, 20 mM; and the extract. The reaction product was identified as mesaconate by HPLC in an Aminex column [15]. In the reverse direction, the reaction was measured in the following mixture: Tris–HCl (pH 7.8), 50 mM; mesaconate (or citraconate), 20 mM; and the extract.

Mesaconate-CoA ligase and succinate-CoA ligase were measured by CoA decrease in the following mixture: Tris–HCl (pH 8.0), 100 mM; $MgCl_2$, 2 mM; ATP, 5 mM; CoA, 0.8 mM; mesaconate (succinate), 5 mM; and the extract (0.1–0.6 mg protein/ml). Samples (100 μl) were transferred into 1 ml of 0.5 mM DTNB in

Enzymes involved in acetate assimilation by *R. rubrum* cells grown in the medium with acetate or malate (in nmol min^{-1} (mg protein)$^{-1}$)

Enzyme	Growth conditions	
	Acetate + $NaHCO_3$	Malate
Citramalate synthase		
by pyruvate consumption	16	17
by CoA formation	58	32
Mesaconase		
mesaconate formation	350	20
mesaconate consumption	230	ND*
Mesaconate-CoA ligase	11	ND*
Succinate-CoA ligase	130	ND*
Condensation of propionyl-CoA and glyoxylate	540	165
3-Methylmalyl-CoA lyase	75	ND*
Malyl-CoA lyase	140	ND*
Propionyl-CoA carboxylase	120	70
Pyruvate carboxylase	65	52
Phosphoenolpyruvate carboxylase	17	10
Phosphoenolpyruvate carboxykinase		
ADP-depndent	<5	<5
GDP-dependent	200	90
Phosphoenolpyruvate carboxy-transphosphorylase	<5	<5
Pyruvate-water dikinase	<2	<2
Pyruvate-phosphate dikinase	8	3
Pyruvate kinase	48	55
Crotonyl-CoA carboxylase/reductase	37	42

* ND, not determined.

100 mM Tris–HCl (pH 8.0). CoA concentration was measured at 412 nm after incubation for 1 min at room temperature.

The activities of enzymatic condensation of propionyl-CoA and glyoxylate were determined by glyoxylate decrease depending on the presence of propionyl-CoA. Glyoxylate concentration was determined by the reaction with phenyl hydrazine ($\varepsilon_{324} = 17.0\ mM^{-1}\ cm^{-1}$) [15].

3-Methylmalyl-CoA lyase and malyl-CoA lyase were measured with phenyl hydrazine by 3-methylmalyl-CoA (malyl-CoA)-dependent formation of glyoxylate at 324 nm in the following reaction mixture: MOPS–KOH (pH 7.7), 200 mM; phenylhydrzine, 3.5 mM; $MgCl_2$, 10 mM; 3-methylmalyl-CoA (or 0.1 mM malyl-CoA), 0.1 mM; and the extract. Besides, the possibility of glyoxylate formation was studied in samples with the reaction mixture containing, instead of 3-methylmalyl-CoA: 3-methylmalate, 2 mM, ATP, 5 mM, and CoA, 2 mM; succinyl-CoA, 0.5 mM; mesaconate, 10 mM, ATP, 5 mM, and CoA, 2 mM; mesaconate, 10 mM, and succinyl-CoA, 0.5 mM.

The activity of crotonyl-CoA carboxylase/reductase was determined radiochemically by crotonyl-CoA-dependent fixation of [^{14}C]bicarbonate [13].

The products of condensation of [^{14}C]acetyl-CoA with pyruvate and [^{14}C]propionyl-CoA with glyoxylate were analyzed as described [15].

Protein assay. Protein was assayed according to Bradford, using bovine serum albumin as a control.

The synthesis of CoA esters. U-[^{14}C]Acetyl-CoA and 2-[^{14}C]propionyl-CoA were synthesized enzymatically [15]. Acetyl-CoA, propionyl-CoA, crotonyl-CoA, and succinyl-CoA were synthesized from respective anhydrides according to [18].

RESULTS

Condensation of pyruvate and acetyl-CoA. The presence of citramalate synthase (the enzyme catalyzing condensation of acetyl-CoA and pyruvate) in the extracts of *R. rubrum* cells, was evidenced by acetyl-CoA-dependent consumption of pyruvate, and stimulation by the latter of CoA release from acetyl-CoA (Table). The difference in the activities obtained by two different methods may be due to the high K_M value of the enzyme with respect to pyruvate (3.2 mM, determined by CoA formation). Pyruvate concentration in the measurements by CoA release was much higher (20 mM) than the in the measurements by pyruvate consumption (8 mM). The activity was somewhat higher in the acetate-grown cells as compared with malate culture. The reaction depended on the presence of Mn^{2+}; only 30% activity was observed in the presence of Mg^{2+} ions.

The condensation product was identified by HPLC with an Aminex column separating organic acids. Incubation of [^{14}C]acetyl-CoA and pyruvate resulted in the

formation of [^{14}C]citramalate (Fig. 2). At the same time, the analysis of reaction products with a RP-C_{18} column separating CoA esters revealed no citramalyl-CoA (data not shown). Thus, condensation of acetyl-CoA and pyruvate in the extracts of *R. rubrum* cells seems to be catalyzed by citramalate synthase that performs the following reaction: acetyl-CoA + pyruvate ⟶ citramalate + CoA. However, our results do not completely exclude the possibility of intermediate formation of citramalyl-CoA, which is further degraded to citramalate and CoA, as has been shown for malate formation from acetyl-CoA and glyoxylate in *Rb. capsulatus*: acetyl-CoA + glyoxylate ⟶ malyl-CoA ⟶ malate + CoA [17].

Conversion of citramalate to mesaconate. *R. rubrum* was shown to possess mesaconase, catalyzing the reaction of citramalate ⟷ mesaconate + H_2O. Its activity is higher in the extracts of cells grown on acetate (Table). The enzyme is specific to (L)-citramalate: the rate of dehydration of (D)-citramalate was below 10% of that for the (L) isomer. The reaction can be measured in the reverse direction with mesaconate as a substrate, but not with citraconate (the product of (D)-citramalate dehydration).

Formation of mesaconyl-CoA. The extracts of *R. rubrum* were shown to possess the activity of mesaconate-CoA ligase forming mesaconyl-CoA from mesaconate, Mg^{2+}-ATP and CoA. However, the measured activity was low, compared with the activity of succinate-CoA ligase from the TCA cycle (table).

It is interesting that free mesaconate was one of the products of [^{14}C]propionyl-CoA and glyoxylate condensation (see below). Its formation was readily observed during the analysis of nonhydrolyzed samples by HPLC in an Aminex column. The mesaconate peak was observed in addition to the peak of CoA esters (mainly mesaconyl-CoA) and propionate during the experiment in the presence of a large amount of the extract (2 mg/ml) after 30-min incubation (data not shown).

Condensation of propionyl-CoA and glyoxylate and formation of 3-methylmalyl-CoA and mesaconyl-CoA. The extracts of *R. rubrum* cells catalyze propionyl-CoA and glyoxylate condensation as measured by glyoxylate consumption in the presence of propionyl-CoA (Table). Condensation products are CoA esters, because glyoxylate does not stimulate CoA release from propionyl-CoA (data not shown). The activity was much higher in the extracts of acetate-grown cells. The reaction was completely dependent on the presence of Mn^{2+} ions in the mixture. With Mg^{2+} used instead of Mn^{2+}, the reaction activity decreased by 30%.

The propionyl-CoA and glyoxylate condensation products were analyzed by HPLC in a column RP-C_{18}. In the presence of glyoxylate, [^{14}C]propionyl-CoA generated two products identified as 3-methylmalyl-CoA and mesaconyl-CoA (Fig. 3). To verify the identification of the condensation products, samples were exposed to alkaline hydrolysis and applied to an Aminex column. Besides [^{14}C]propionate, the samples were shown to contain [^{14}C]mesaconate and [^{14}C]3-methylmalate (Fig. 4). The maximal formation of 3-methylmalyl-CoA was observed in the samples after incubation for 15 min. On exhaustion of propionyl-CoA, the amount of 3-methylmalyl-CoA decreased, while the peak of mesaconyl-CoA increased (Figs. 3, 4). At the increase of extract amount in the mixture from 0.2 to 2 mg protein/ml, mesaconyl-CoA became the main condensation product already after 5 min of incubation (data not shown). Thus, the extracts of *R. rubrum* cells grown on acetate showed the following reaction sequence: propionyl-CoA + glyoxylate ⟶ 3-methylmalyl-CoA ⟶ mesaconyl-CoA.

As CoA esters are not hydrolyzed in these reactions, they are entirely reversible.

3-Methylmalyl-CoA lyase reaction may also be measured spectrophotometrically by formation of glyoxylate from 3-methylmalyl-CoA (Table). Besides, *R. rubrum* growing on acetate synthesizes malyl-CoA lyase (Table). Glyoxylate formation was not observed in the variants with 3-methylmalate + ATP + CoA, 3-methylmalate + succinyl-CoA, mesaconate + ATP + CoA, or mesaconate + succinyl-CoA used instead of 3-methylmalyl-CoA.

Enzymes participating in the oxidation of propionyl-CoA to pyruvate. The enzyme activities of the enzymes involved in pyruvate regeneration are presented in the Table. Participation of propionyl-CoA carboxylase reaction in acetate assimilation has been proved previously in our experiments in vivo [9, 10]. The reaction product is (*S*)-methylmalonyl-CoA. Its further metabolism is associated with racemization into (*R*)-enantiomer and transformation into succinyl-CoA catalyzed by B_{12}-dependent mutase. The presence of respective enzymes was shown for *R. rubrum* [19]. Further oxidation of succinyl-CoA to oxaloacetate is associated with TCA cycle reactions [7, 8]. Pyruvate regeneration from oxaloacetate is possible with the involvement of GDP-dependent phosphoenolpyruvate carboxykinase and pyruvate kinase (table).

Crotonyl-CoA-carboxylase/reductase. Under growth in the medium with acetate as well as in the medium with succinate, *R. rubrum* synthesizes crotonyl-CoA-carboxylase/reductase, which is the key enzyme of the EM pathway of acetate assimilation. The enzyme activity on both media was approximately the same (table).

DISCUSSION

In this work, we have demonstrated the presence of all the enzymes CM cycle in cell extracts of *R. rubrum* (table). *R. rubrum* growth in the medium with acetate with a generation time of 6 h, which corresponds to a specific carbon fixation rate of 170 nmol min^{-1} (mg pro-

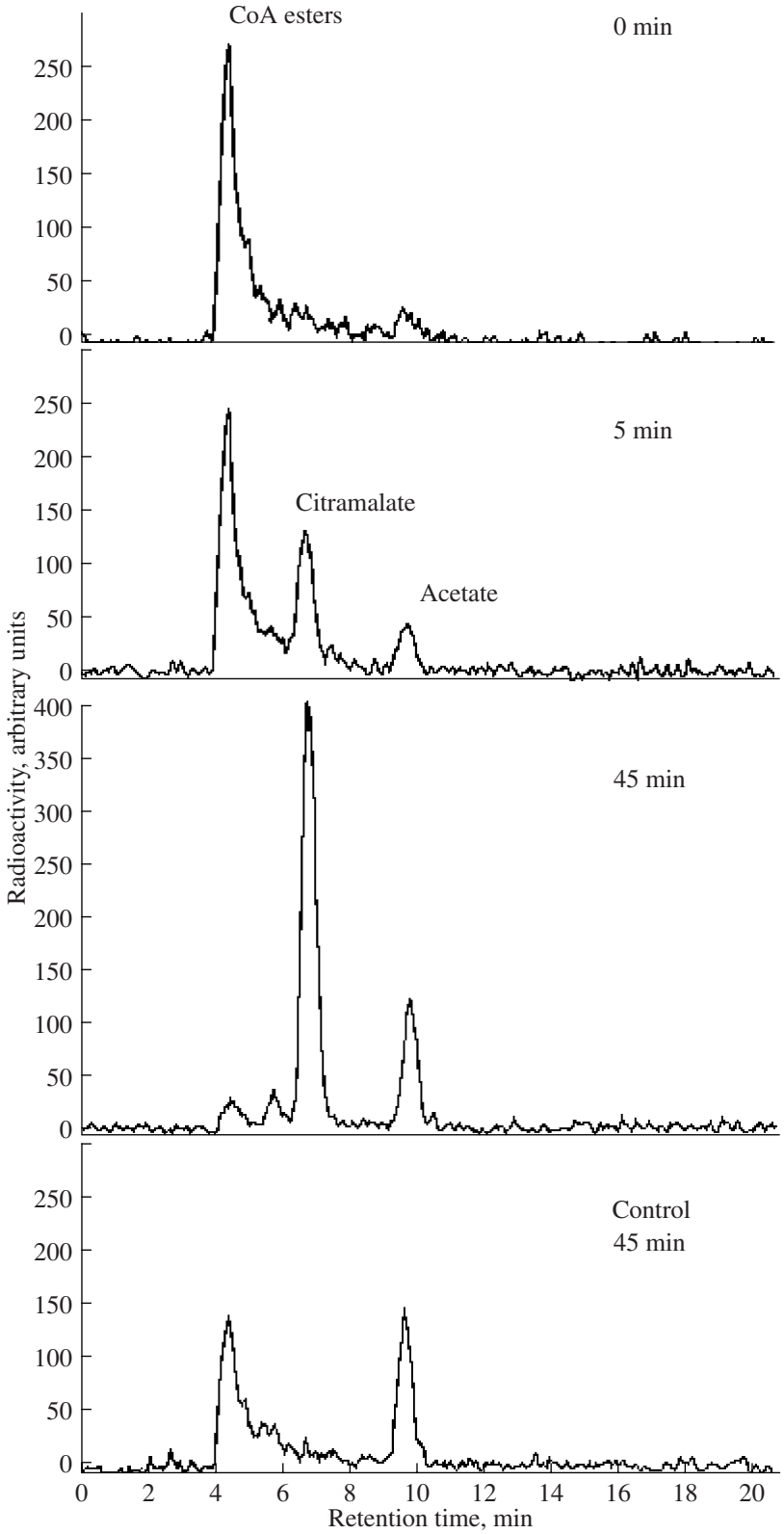

Fig. 2. The [^{14}C]acetyl-CoA and pyruvate condensation products in the extracts of *R. rubrum* cells grown on acetate. Samples were taken after 0, 5, and 45 min of incubation and analyzed in an Aminex column separating organic acids. Protein content in reaction mixture was 2.3 mg/ml. In the control (45 min), reaction mixture contained no pyruvate.

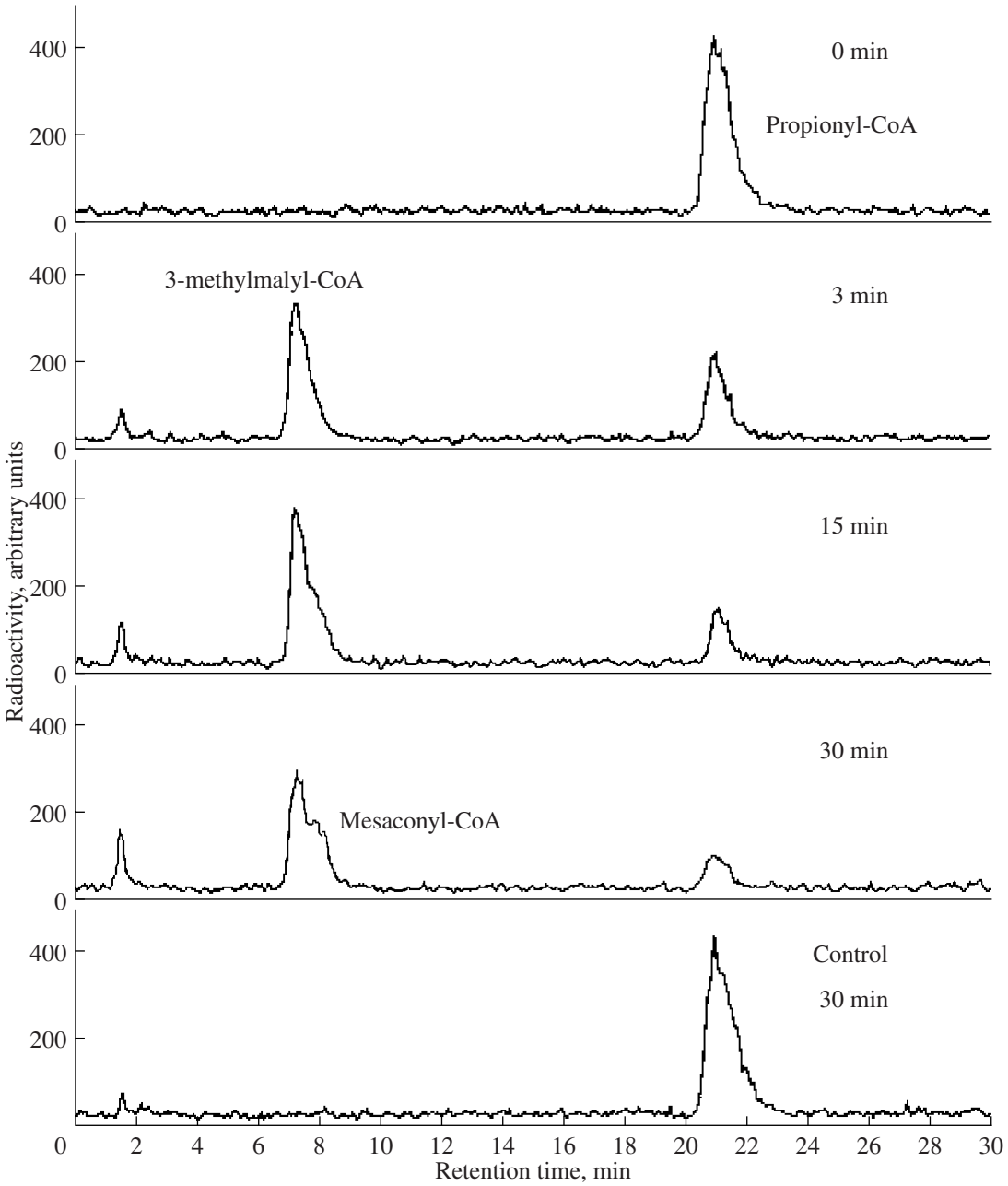

Fig. 3. The [^{14}C]propionyl-CoA and glyoxylate condensation products in the extracts acetate-grown *R. rubrum* cells. Samples were taken after 0, 3, 15, and 30 min of incubation and analyzed in an RP_{18} column separating CoA esters. Protein content in reaction mixture was 0.2 mg/ml. In the control (30 min), reaction mixture contained no glyoxylate.

tein)$^{-1}$. Since one C_4 molecule is formed from two molecules of the C_2-substrate per one turn of the CM cycle, the minimal in vivo specific activity of enzymes in the cycle is 40 nmol min^{-1} (mg protein)$^{-1}$. Since the temperature of bacterial growth (28–30°C) exceeded the assay temperature (room temperature, approx. 20°C), this value decreases to 20–25 nmol min^{-1} (mg protein)$^{-1}$. Thus, the determined activities of the CM cycle enzyme are sufficient for explanation of the observed growth rate (table). These activities are higher in acetate-grown cells comparing with the cells grown on the medium with malate.

Citramalate synthesis in *R. rubrum* most probably involves citramalate synthase without intermediate formation of citramalyl-CoA. The synthesized citramalate theoretically may be both an (L)- and a (D)-stereoisomer. (D)-citramalate synthase has been found in *Methanococcus jannaschii* and the gene encoding this enzyme is known as well [20]. However, BLAST search has not revealed its homologues in the genome

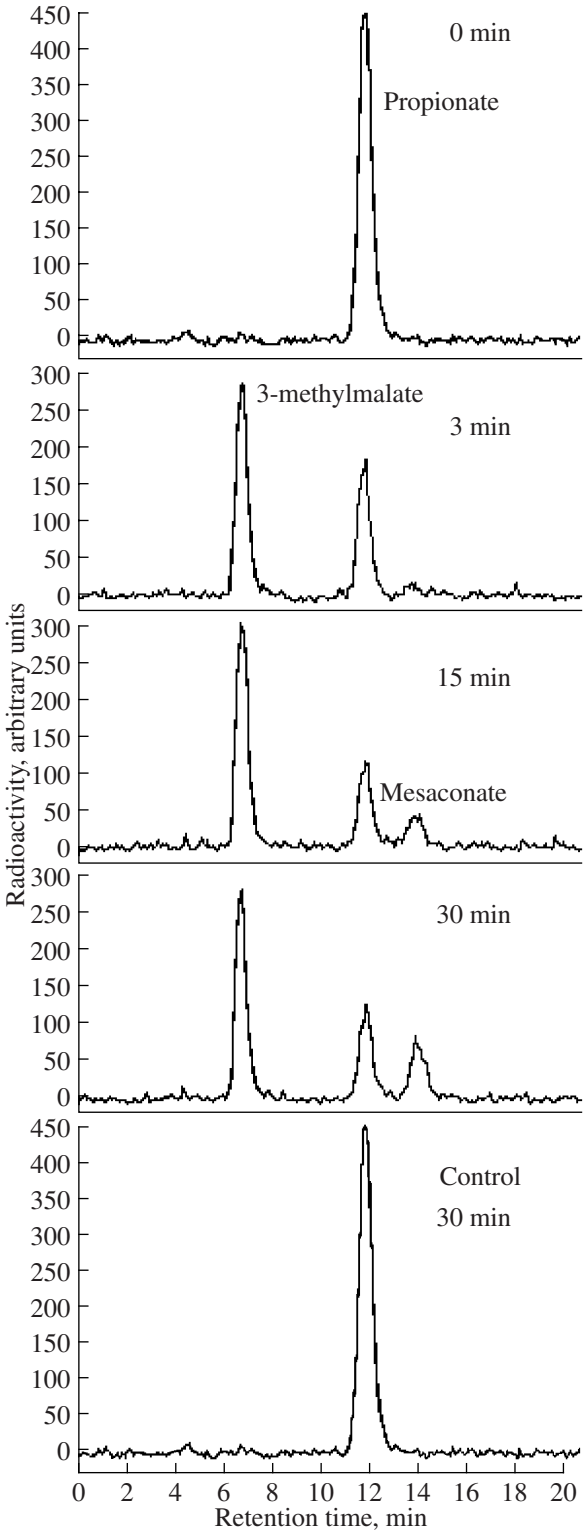

Fig. 4. The [^{14}C]propionyl-CoA and glyoxylate condensation products in the extracts of *R. rubrum* cells grown on acetate. Samples were taken after 0, 3, 15, and 30 min of incubation, hydrolyzed with 1 M KOH (85°C, 1 h), and analyzed in an Aminex column separating organic acids. Protein content in reaction mixture was 0.2 mg/ml. In the control (30 min), reaction mixture contained no glyoxylate.

of *R. rubrum*. The genes encoding (L)-citramalate synthase, which seems to be present in *R. rubrum*, have not been identified up to now; thus, the respective search in the genome is impossible. It is possible, that specific reactions of the CM cycle (citramalate synthase and mesaconase reactions) may be catalyzed by promiscuous enzymes. The BLAST search allows unambiguous identification of only two of the key enzymes of the CM cycle: mesaconyl-CoA hydratase and 3-methylmalyl-CoA lyase, which have been purified recently from the extracts of *Rb. sphaeroides* [17, 21]. They correspond to proteins Rru_A1201 and Rru_A0217 in the genome of *R. rubrum*. Judging by the presence of both malyl-CoA lyase and 3-methylmalyl-CoA lyase activities in *R. rubrum* and by the similarity of the corresponding enzyme with the enzyme from *Rb. capsulatus* [17], malate formation from acetyl-CoA and glyoxylate in *R. rubrum* involves not malate synthase but malyl-CoA lyase/malyl-CoA hydrolyzing enzyme, as it has been shown for *Rb. capsulatus* [17].

Mesaconate activation in *R. rubrum* may be coupled with ATP hydrolysis (table). It is interesting to note that the key enzymes responsible for propionyl-CoA oxidation to pyruvate, i.e. propionyl-CoA carboxylase and phosphoenolpyruvate carboxylase (responsible for decarboxylation reactions in vivo), have the highest activities among the studied carboxylases and are maximal under growth on the medium with acetate (table). The activity of enzymes catalyzing in vivo carboxylation of C_3-substrates (phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxytransphosphorylase, pyruvate carboxylase) was noticeably lower and weakly regulated by growth substrates (table). Among the enzymes responsible for pyruvate and phosphoenolpyruvate interconversion, the highest activity was demonstrated for pyruvate kinase (table), catalyzing the irreversible reaction of pyruvate formation, i.e. working in the direction typical of the CM cycle.

The data obtained in our experiments in vivo with *R. rubrum* cell suspensions are in agreement with the functioning of the CM cycle [7–10]. Citramalate has also been shown as one of the first products of acetate fixation in *R. rubrum* [22, 23].

The CM cycle is considered as one of the possible pathways of acetate assimilation in the hyperthermophilic archaeon *Pyrobaculum islandicum* [24]. Moreover, in *Chloroflexus aurantiacus*, the reactions similar to the reactions of the CM cycle, function in the reverse direction for assimilation of glyoxylate formed during autotrophic CO_2 fixation [25].

R. rubrum synthesizes crotonyl-CoA carboxylase/reductase (Table), which is the key enzyme of the EM pathway of acetate assimilation. Besides, mesaconyl-CoA hydratase, malyl-CoA/3-methylmalyl-CoA lyase, and propionyl-CoA carboxylase demonstrated here are also enzymes of the EM pathway [12, 13]. The genes encoding its key enzymes are present in the genome of *R. rubrum* [12]. Moreover, *R. rubrum* groups

with alphaproteobacteria, which demonstrate a correlation of the presence of the crotonyl-CoA carboxylase/reductase gene and a number of other genes with the absence of isocitrate lyase [12]. These facts indicate the possibility of the functioning of the EM pathway. Here emerges the question as to which of the two pathways of acetate assimilation actually functions in this bacterium.

The CM cycle and the EM pathway differ in the degree of CO_2 participation in their reactions (Fig. 1). In the CM cycle, CO_2 plays a catalytic role and its functioning must not result in any noticeable fixation of bicarbonate [7, 9]. It corresponds to the observed effect of CO_2 on acetate metabolism in *R. rubrum*. On the contrary, the ratio of CO_2 fixation to acetate fixation in the EM pathway is 2 : 3. Thus, CO_2 fixation must be observed during the acetate assimilation through the EM pathway. However, stimulation of acetate metabolism by bicarbonate is observed only in *R. rubrum* but not in *Rb. sphaeroides*. Moreover, the rate of acetate fixation in both bacteria exceeds the rate of CO_2 fixation by an order of magnitude [7, 9, 26].

When discussing the possibility of the CM cycle functioning in *Rb. sphaeroides*, we have suggested the following hypothesis explaining the differences in the effect of CO_2 on the metabolism of these two bacteria: because of the higher rate of acetate oxidation, *Rb. sphaeroides* is able to accumulate much larger amount of CO_2 inside the cells and hold it more effectively due to a mucous capsule (which is absent in *R. rubrum*) [15]. This hypothesis can be used for the consideration of the possibility of the EM pathway functioning in *Rb. sphaeroides*: if endogenous CO_2 is mainly utilized, the effect of the addition of exogenous bicarbonate (as well its fixation) may be rather insignificant. It settles the contradiction associated with the presence of carboxylation steps and the absence of CO_2 fixation in the acetate assimilation pathway. Moreover, it also explains the high percentage of PHB accumulation in *Rb. sphaeroides* under growth on acetate [27]: if acetate fixation is directly connected with fixation of the bicarbonate formed in the TCA cycle, there is a possibility of formation of the excess of reducing equivalents, which is utilized for PHB synthesis.

R. rubrum also synthesizes PHB when growing on acetate but in much lower amounts than *Rb. sphaeroides* [27]. However, the effect of CO_2 on acetate fixation in *R. rubrum* is clearly apparent [7–10]. It implies the requirement of exogenous CO_2; accordingly, the functioning of a pathway associated with fixation of considerable amounts of CO_2 must be accompanied by noticeable fixation of bicarbonate, which is however not observed. Besides, the activity of crotonyl-CoA carboxylase/reductase in *R. rubrum* is low (ca. 40 nmol min^{-1} (mg protein)$^{-1}$) and remains the same in acetate and malate-grown cultures (table). It differentiates *R. rubrum* from *Rb. sphaeroides*, where the enzyme activity is much higher (1100 nmol min^{-1} (mg protein)$^{-1}$) in acetate-grown cells and is down-regulated by more than an order of magnitude in cells grown with succinate [13]. Besides, *R. rubrum* synthesizes mesaconase, which is most active under growth on acetate. Our attempts to measure this enzyme in *Rb. sphaeroides* were unsuccessful (Berg and Ivanovsky; unpublished data). Thus, these data are evidence of a possible difference between the mechanisms of acetate assimilation in *R. rubrum* and *Rb. sphaeroides*.

One might suppose that *R. rubrum* switches over from one pathway of acetate assimilation to another or uses both of them depending on growth conditions. Citramalate and crotonyl-CoA can also be utilized for the biosynthesis of amino acids and PHB, respectively. Accordingly, preference may be given to one pathway or another, depending on the physiological status of a cell, presence of bicarbonate in the medium, and other factors.

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