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An NADP-Linked Acetoacetyl CoA Reductase from *Zoogloea ramigera*

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Abstract. *Zoogloea ramigera* I-16-M was found to contain two stereospecific acetoacetyl CoA reductases; one was $NADP^+$-linked and D(–)-β-hydroxybutyryl CoA specific and the other was NAD^+-linked and L(+)-isomer specific. The $NADP^+$-linked enzyme, purified approximately 150-fold, had a pH optimum for the reduction of acetoacetyl CoA at 8.1, but no definite pH optimum for the oxidation of β-hydroxybutyryl CoA. The apparent Michaelis constants for acetoacetyl CoA and NADPH were 8.3 and 21 μM, respectively. The enzyme was markedly inhibited by acetoacetyl CoA at concentrations higher than 10 μM.

The incorporation of [1-^{14}C]acetyl CoA into poly-β-hydroxybutyrate (PHB) by bacterial crude extract (containing β-ketothiolase, acetoacetyl CoA reductases, enoyl CoA hydratases and PHB synthases) or by a system reconstituted from purified preparations of β-ketothiolase, acetoacetyl CoA reductase and PHB synthase, was observed only in the presence of NADPH, but not NADH. Among various enzymes involved in PHB metabolism, only the specific activity of glucose 6-phosphate dehydrogenase was elevated 5-fold within 2 h after the addition of glucose to the cells grown in the basal medium.

These findings suggest that, in *Z. ramigera* I-16-M, acetoacetyl CoA is directly reduced to D(–)-β-hydroxybutyryl CoA by the $NADP^+$-dependent reductase, and PHB synthesis is at least partially controled by NADPH availability through glucose 6-phosphate dehydrogenase.

Key words: Poly-β-hydroxybutyrate – PHB – Acetoacetyl CoA reductase – *Zoogloea ramigera*.

Poly-β-hydroxybutyrate (PHB) is a unique intracellular lipid reserve accumulated by many different types of bacteria (Stanier et al., 1959; Merrick and Doudoroff, 1961; Sierra and Gibbons, 1962; Crabtree et al., 1965; Stokes and Powers, 1967; Lin and Sadoff, 1968; Senior and Dawes, 1973). PHB synthase, which catalyzes the final stage of PHB synthesis, incorporates exclusively D(–)-β-hydroxybutyryl CoA into a PHB primer (Merrick and Doudoroff, 1961; Griebel et al., 1968; Fukui et al., 1976).

As to the synthesis of D(–)-β-hydroxybutyryl CoA, two different pathways have been suggested: (a) in *Rhodospirillum rubrum* (Moskowitz and Merrick, 1969), acetoacetyl CoA is first reduced to L(+)-β-hydroxybutyryl CoA by an NAD-linked dehydrogenase, and then converted successively into D(–)-β-hydroxybutyryl CoA through the activity of two stereospecific enoyl CoA hydratases; and (b) in *Azotobacter beijerinckii* (Ritchie et al., 1971), acetoacetyl CoA is directly reduced to D(–)-β-hydroxybutyryl CoA by an NADP-linked reductase [D(–)-β-hydroxybutyryl CoA-NADP oxidoreductase, EC 1.1.1.36].

During our studies on the metabolism of PHB in *Zoogloea ramigera* I-16-M (Fukui et al., 1976), we observed the presence of two stereospecific acetoacetyl CoA reductases; one reductase was NADP-linked and the other one NAD-linked. Apparently, only the former enzyme participates in the formation of D(–)-β-hydroxybutyryl CoA required for PHB synthesis in *Z. ramigera* I-16-M. This paper describes the partial purification as well as a number of characteristics of the NADP-linked reductase.

Non-Standard Abbreviation. PHB = poly-β-hydroxybutyrate

MATERIALS AND METHODS

Culture and Extraction of Cells. *Zoogloea ramigera* I-16-M (ATCC 19623) was grown at 30° C on a shaker in a medium (500 ml in 2000-ml *Sakaguchi* flasks) consisting of 0.5% casamino acids, 0.5% yeast extract, 0.2% K_2HPO_4, and 0.1% KH_2PO_4 (Fukui et al., 1976). This basal medium contained on the average about 0.01% carbohydrate (as glucose), which was derived for the most part from yeast extract preparations, and was enough to induce

floc formation to a certain extent. Furthermore, in contrast to PHB synthase (Fukui et al., 1976), the total activity of acetoacetyl CoA reductase in the bacterium was not significantly affected by the degree of flocculation. Therefore, the growth medium for enzyme purification was not fortified with glucose.

Cells were harvested at the late exponential growth phase (30° C, 20 h) by centrifugation at 10000 × g for 10 min at 0–4° C, washed once with cold 10 mM Tris-HCl (pH 7.5) containing 10% glycerol, and then resuspended in the same buffer. The cells were then disrupted in a sonic disintegrator (Fukui et al., 1976), or with 0.1 mm Pyrex beads in a Vibrogen Cell Mill (Edmund Bühler, Tübingen, Germany) with running tap water as the coolant, and centrifuged at 700 × g for 10 min; the 700 × g supernatant fraction was then centrifuged at 10000 × g for 10 min. The 700 × g supernatant fraction contained both soluble and particulate PHB synthases (Fukui et al., 1976), acetoacetyl CoA reductase, enoyl CoA hydratase, β-ketothiolase and other enzymes. The 10000 × g supernatant fraction (crude extract), which contained all these enzymes except for the particulate PHB synthase, was used for the enzyme purification.

Enzyme Assays. Acetoacetyl CoA reductase was assayed in both forward and reverse directions. (a) *Reduction of acetoacetyl CoA.* The reaction mixture (a final volume of 0.8 ml) contained 0.016 μmol of acetoacetyl CoA, 0.1 μmol of NADH or NADPH, and 100 μmol of Tris-HCl (pH 8.0). The reaction was initiated by the addition of enzyme, and the rate of NAD^+ or $NADP^+$ formation was measured at 340 nm (25° C) with a Shimazu recordings spectrophotometer, model UV-200. (b) *Oxidation of β-hydroxybutyryl CoA.* The assay mixture (a final volume of 0.8 ml) contained 0.1 μmol of DL-β-hydroxybutyryl CoA, 0.1 μmol of NAD^+ or $NADP^+$, 100 μmol of glycine-NaOH buffer (pH 9.0), and enzyme. The formation of NADH or NADPH was measured at 340 nm as above.

Enoyl CoA hydratase was assayed by measuring the breakdown of crotonyl CoA at 263 nm according to the method of Stern (1955a). *β-Ketothiolase* was assayed, according to Stern (1955b), by measuring the decrease of acetoacetyl CoA at 305 nm. *PHB synthase* was assayed, as described previously (Fukui et al., 1976), by measuring the incorporation of DL-[^{14}C]β-hydroxybutyryl CoA into PHB.

β-Hydroxybutyric acid dimer esterase: The reaction mixture (a final volume of 1.0 ml) contained 1.7 μmol of DL-β-hydroxybutyric acid dimer, 100 μmol of Tris-HCl (pH 8.0) and enzyme. The reaction was carried out at 30° C for 60 min, and terminated by heating at 100° C for 3 min, followed by centrifugation at 1000 × g for 10 min. D(−)-β-Hydroxybutyrate formed in the supernatant fraction was assayed spectrophotometrically as described by Williamson et al. (1962).

D(−)-β-Hydroxybutyrate dehydrogenase was assayed spectrophotometrically as described by Bergmeyer et al. (1967).

Isocitrate dehydrogenase (Cleland et al., 1969), *glucose 6-phosphate dehydrogenase* (Kirkman, 1959), and *hexokinase* (Joshi and Jagannathan, 1966) were assayed as described elsewhere.

One unit of enzyme is defined as the amount of enzyme needed to catalyze the reaction of 1 μmol of substrate per min under the assay conditions. Protein was determined either with Folin-Ciocalteu reagent, or from the level of absorbance at 280 nm as described by Layne (1957).

Other Assays. Enzymatic assays of *acetoacetyl CoA* (Senior and Dawes, 1973), *acetyl CoA* (Stadtman, 1957), and *crotonyl CoA* (Stern, 1955a) were performed as described elsewhere. *β-Hydroxybutyryl CoA* was assayed from the level of absorbance at 260 nm ($\varepsilon = 16.4$) (Morris and Redfearn, 1969). *PHB content* was assayed, after digesting other cellular materials with Na hypochlorite, from the level of absorbance at 235 nm of crotonic acid, produced by heating PHB with H_2SO_4 at 100° C for 10 min (Fukui et al., 1976). *Glucose* was assayed as described by Park and Johnson (1949).

Preparations. D(−)-β-Hydroxybutyric acid was prepared by alkaline hydrolysis of PHB, purified from PHB granules of *Z. ramigera* I-16-M (Fukui et al., 1976). *Acetoacetyl CoA* was prepared by treating CoA with diketene according to Ritchie et al. (1971), and purified on a DEAE-cellulose column at pH 4.5 by the method of Moffatt and Khorana (1961). *Acetyl CoA* (both radioactive and non-radioactive) and *crotonyl CoA* were prepared by reacting CoA with acetic anhydride and crotonyl anhydride, respectively, according to Simon and Shemin (1953), and purified by paper chromatography (Flavin, 1963).

D- and DL-β-Hydroxybutyryl CoA's (both radioactive and non-radioactive) were prepared from the corresponding free acids by the mixed anhydride method of Wieland and Rueff (1953), and purified by paper chromatography (Flavin, 1963). [β-^{14}C]L(+)-β-Hydroxybutyryl CoA was prepared, according to Wieland and Rueff (1953), by reacting the radioactive acid with CoA; [β-^{14}C]L(+)-β-hydroxybutyric acid was prepared from the radioactive DL-racemic acid by enzymatic decomposition of its D-isomer, as described previously (Fukui et al., 1976). L(+)-β-Hydroxybutyryl CoA (non-radioactive) was prepared from DL-β-hydroxybutyryl CoA through the selective removal of the D(−)-isomer utilizing PHB synthase. The reaction mixture (1 ml) contained DL-β-hydroxybutyryl CoA (1 μmol), PHB synthase from *Z. ramigera* I-16-M (790 μg, 1.3 units) (Fukui et al., 1976), and potassium phosphate (pH 7.0, 100 μmol). After incubation at 30° C for 1 h, the reaction mixture was centrifuged at 15000 × g for 10 min; in a typical experiment, the supernatant solution contained 0.49 μmol of L(+)-β-hydroxybutyryl CoA and practically no D(−)-isomer.

The dimeric ester of DL-β-hydroxybutyric acid was prepared as described by Olsen et al. (1965).

Purification of the Enzyme. All procedures and centrifugations of the enzyme purification were performed at 0–4° C.

Ammonium sulfate fractionation: Crude extract (60 ml) was brought to 30% saturation with solid ammonium sulfate; the solution was stirred for 20 min and then centrifuged at 10000 × g for 20 min. More solid ammonium sulfate was added to the supernatant solution in order to reach 80% saturation. After stirring for 20 min, the precipitate was collected by centrifugation, dissolved in a minimum volume of 50 mM potassium phosphate (pH 7.0), and dialyzed against the same buffer (2000 ml) overnight.

Hydroxylapatite chromatography: The dialyzed enzyme solution (15 ml) from the previous step was diluted with 9 volumes of distilled water, and applied to a column (3 × 10 cm) of hydroxylapatite (Main et al., 1959) preequilibrated with 5 mM potassium phosphate (pH 7.0). A 500-ml linear gradient of potassium phosphate, 5 mM to 200 mM (pH 7.0), was used to elute the enzyme. Fractions of 7 ml were collected into tubes containing 3 ml of 1 M potassium phosphate (pH 8.0), since this enzyme was rather labile when left standing at a low level of ionic strength.

Sephadex G-200 chromatography: To the pooled active fractions from the previous step, solid ammonium sulfate was added to 80% saturation. After stirring for 10 min, the mixture was centrifuged at 10000 × g for 20 min. The precipitate was dissolved in 10 ml of 0.5 M potassium phosphate (pH 8.0) and then placed on a column (4 × 62 cm) of Sephadex G-200 pre-equilibrated with the same buffer. Fractions of 7 ml were collected and those with high specific activity were pooled.

Chemicals. Casamino acids and yeast extract were obtained from Difco Laboratories; CoA from Kyowa Hakko Co., Tokyo, Japan; NADH and NADPH from Oriental Yeast Co., Tokyo, Japan; crotonyl anhydride from Tokyo Kasei Co., Tokyo, Japan; [β-^{14}C]D-

β-hydroxybutyric acid (15 mCi/mmol) and [β-^{14}C]DL-β-hydroxybutyric acid (4 mCi/mmol) from Radiochemical Center, Amersham, England; [1-^{14}C]acetic anhydride (5 mCi/mmol) from New England Nuclear Corp., Boston, Mass., U.S.A.; and L(+)-β-hydroxyacyl CoA dehydrogenase of pig heart from Boehringer Mannheim, Germany. Other chemicals of reagent grade were obtained commercially.

RESULTS

Two Types of Acetoacetyl CoA Reductases in Bacterial Crude Extract

Table 1 shows that DL-β-hydroxybutyryl CoA could be oxidized by the crude bacterial extract in the presence of either NAD^+ or $NADP^+$. However, L(+)-β-hydroxybutyryl CoA was oxidized much faster in the presence of NAD^+, while D(−)-isomer was oxidized more rapidly in the presence of $NADP^+$. These results indicate the possible presence of two types of acetoacetyl CoA reductases in *Zoogloea ramigera* I-16-M: one was NAD^+-dependent and L(+)-β-hydroxybutyryl CoA specific and the other was $NADP^+$-dependent and D(−)-isomer specific.

Effect of Enoyl CoA Hydratase on Incorporation of [^{14}C]-β-Hydroxybutyryl CoA into PHB

Since the crude extract also contained enoyl CoA hydratase activity (Table 2, see below), the possible presence of a pathway of the *Rhodospirillum rubrum* type (Moskowitz and Merrick, 1969) was investigated.

Table 1. Substrate stereospecificity of acetoacetyl CoA reductase in *Zoogloea ramigera* I-16-M

Substrate	Concentration	Enzyme activity	
		$NADP^+$	NAD^+
	mM	ΔE_{340}/min	
DL-β-Hydroxybutyryl CoA	0.125	0.037	0.015
D(−)-β-Hydroxybutyryl CoA	0.063	0.085	0.002
L(+)-β-Hydroxybutyryl CoA	0.063	0.001	0.016

The crude extract (11 μg) of *Z. ramigera* I-16-M was incubated at pH 9.0 as described in the text

The β-hydroxybutyryl unit was incorporated into PHB, by the 700 × g supernatant fraction [containing both soluble and particulate PHB synthases, acetoacetyl CoA reductase, enoyl CoA hydratase, and β-ketothiolase ("Materials and Methods")], only from [β-^{14}C]D-β-hydroxybutyryl CoA, but not from the L-isomer (Fig. 1). However, a slight incorporation of the radioactivity from the L-isomer was observed when the 700 × g supernatant fraction was incubated with the substrate in the presence of NAD^+ and

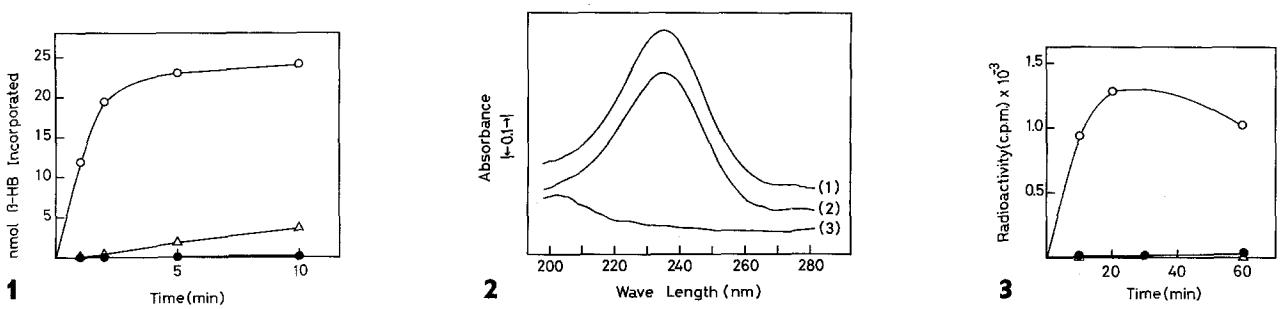

Fig. 1. Incorporation of [β-^{14}C]D- or [β-^{14}C]L-β-hydroxybutyryl CoA into PHB by 700 × g supernatant fraction. The reaction mixture (50 μl) contained potassium (7.5 μmol, pH 7.0), reduced glutathione (0.5 μmol), 700 × g supernatant fraction of bacterial extract (70 μg of protein), and [β-^{14}C]D-β-hydroxybutyryl CoA (0.05 μmol, 1.71×10^4 cpm) (○——○), or [β-^{14}C]L-β-hydroxybutyryl CoA (0.05 μmol, 2.18×10^4 cpm) (●——●), or [β-^{14}C]L-β-hydroxybutyryl CoA (0.05 μmol, 2.18×10^4 cpm), NAD^+ (0.05 μmol) and NADPH (0.05 μmol) (△——△). The reaction was carried out at 30° C and terminated by the addition of 0.5% trichloroacetic acid (0.1 ml). The radioactivity incorporated into PHB fraction was determined using the PHB synthase assay method

Fig. 2. PHB synthesis by crude bacterial extract. The reaction mixture (250 μl) contained potassium phosphate (50 μmol, pH 7.0), reduced glutathione (2.5 μmol), crude extract (150 μg of protein) and CoA derivative and/or pyridine nucleotides. The reaction was carried out at 30° C for 30 min, and terminated by the addition of 5% trichloroacetic acid (0.2 ml), followed by a sodium hypochlorite solution (0.5 ml, about 10% as active chlorine). After incubation at 30° C for 1 h, PHB was extracted with chloroform (1 ml), evaporated to dryness, mixed with concentrated H_2SO_4 (2.5 ml), and heated in a boiling water bath for 10 min. After cooling, absorption spectra were examined. (*1*) D-β-hydroxybutyryl CoA, 0.1 μmol; (*2*) crotonyl CoA, 0.2 μmol, NAD^+, 0.25 μmol, and NADPH, 0.25 μmol; and (*3*) crotonyl CoA, 0.2 μmol

Fig. 3. Effect of NADPH and NADH on incorporation of [1-^{14}C]acetyl CoA into PHB by crude extract. The reaction mixture (50 μl) contained potassium phosphate (7.5 μmol, pH 7.0), reduced glutathione (0.25 μmol), [1-^{14}C]acetyl CoA (0.1 μmol, 4.2×10^4 cpm), NADPH or NADH (0.05 μmol) and 700 × g supernatant fraction of bacterial extract (77 μg of protein). The reaction was carried out at 30° C, and terminated by the addition of 5% trichloroacetic acid (0.1 ml), and PHB was extracted with chloroform and counted (Fukui et al., 1976). ○ NADPH; ● NADH; △ no addition of nucleotide

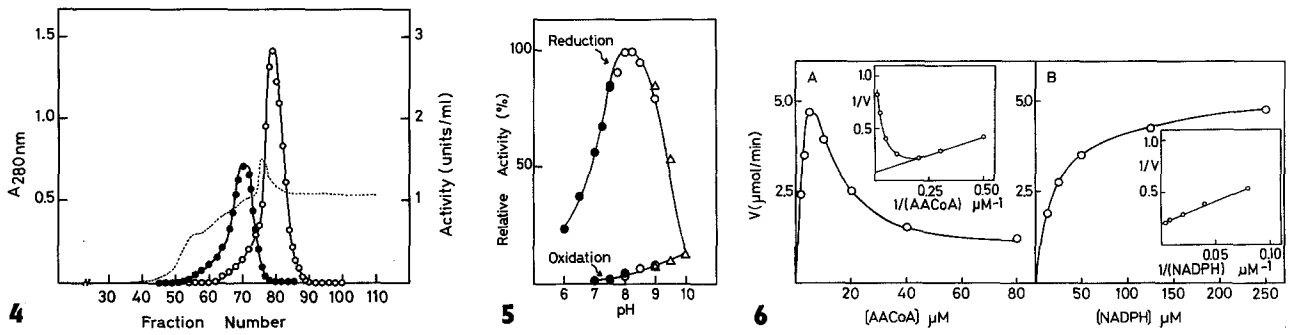

Fig. 4. Elution of acetoacetyl CoA reductase from a Shephadex G-200 column. The combined active fractions from a hydroxylapatite column were precipitated by 80% saturation of $[NH_4]_2SO_4$, dissolved in 10 ml of 0.5 M potassium phosphate, and applied to a column (4 × 62 cm) of Sephadex G-200 equilibrated with the same buffer. The enzyme was eluted with the same buffer at a flow rate of 14 ml per hour, and 7-ml fractions were collected. ○ acetoacetyl CoA reductase; ● β-ketothiolase; ---- protein

Fig. 5. pH Activity curves of NADP-linked acetoacetyl CoA reductase. Reduction of acetoacetyl CoA and oxidation of β-hydroxybutyryl CoA were assayed in 0.125 mM buffers. ● phosphate; ○ Tris-HCl; △ glycine-NaOH

Fig. 6A and B. Effect of acetoacetyl CoA and NADPH concentrations on activity of acetoacetyl CoA reductase and determination of K_m values. (A) The experiments were performed in the presence of 0.1 μmol of NADPH and increasing concentrations of acetoacetyl CoA with the purified enzyme (1.3 μg, 0.01 unit) under standard assay conditions. (B) The experiments were performed in the presence of 0.016 μmol of acetoacetyl CoA and increasing concentrations of NADPH with the purified enzyme (1.3 μg, 0.01 unit) under standard assay conditions

Table 2. Purification of NADP-linked acetoacetyl CoA reductase from *Z. ramigera* I-16-M

Step	Acetoacetyl CoA reductase				β-Ketothiolase	Enoyl CoA hydratase
	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Activity ratio (NADPH/NADH)	Total activity (units)	
1. Crude extract	780	39	0.05	0.6	460	260
2. $[NH_4]_2SO_4$, 30–80%	490	77	0.16	1.5	130	180
3. Hydroxylapatite	47	60	1.3	72	9.4	120
4. Sephadex G-200	6.9	54	7.7	155	0.2	0.016

The preparation was started with 16 g (wet) of *Z. ramigera* I-16-M cells

NADPH. These results indicate that L-β-hydroxybutyryl CoA was not converted to its D-isomer, the substrate for PHB synthase, through the action of enoyl CoA hydratases, but that the L-isomer could be oxidized by an NAD^+-linked dehydrogenase to acetoacetyl CoA, and then reduced to the D-isomer by an NADPH-dependent reductase.

Formation of PHB from β-Hydroxybutyryl CoA or Crotonyl CoA in Crude Bacterial Extract

PHB formation was observed when the 10000 × g supernatant fraction [containing acetoacetyl CoA reductases, enoyl CoA hydratases and soluble PHB synthase ("Materials and Methods")] was incubated either with D-β-hydroxybutyryl CoA [Fig. 2 (1)] or with crotonyl CoA in the presence of NAD^+ and NADPH [Fig. 2 (2)]. However, PHB was not formed when the crude extract was incubated with crotonyl CoA alone [Fig. 2 (3)].

These results further indicate that no conversion of L-β-hydroxybutyryl CoA to its D-isomer via crotonyl CoA occurred during the incorporation of β-hydroxybutyryl unit into PHB by the crude bacterial extract.

Incorporation of [1-^{14}C]Acetyl CoA into PHB by Crude Extract in the Presence of NADH or NADPH

As shown in Figure 3, the incorporation of [1-^{14}C]-acetyl CoA into PHB by the 700 × g supernatant fraction was much higher in the presence of NADPH

Table 3. Enzymatic synthesis of poly-β-hydroxybutyrate in a reconstituted system

System	Incorporation of [1-^{14}C]acetyl CoA into PHB (cpm)
1. Complete	3130
2. − NADPH	10
3. − NADPH + NADH	11
4. − PHB synthase	15
5. − Acetoacetyl CoA reductase	10
6. − β-Ketothiolase	9

The reaction mixture (0.2 ml) contained potassium phosphate (pH 7.5, 30 μmol), reduced glutathione (1 μmol), [1-^{14}C]acetyl CoA (2.9×10^4 cpm, 0.05 μmol), NADPH or NADH (0.1 μmol), purified granular PHB synthase (Fukui et al., 1976) (4 μg, 0.02 unit), purified β-ketothiolase (Nishimura et al., 1977) (1 μg, 0.08 unit), and purified acetoacetyl CoA reductase (6.5 μg, 0.05 unit). The radioactivity of PHB fraction was counted as described in the text

than in the presence of NADH. Therefore, in *Z. ramigera* I-16-M, acetoacetyl CoA, the condensation product of acetyl CoA by β-ketothiolase, seemed to be reduced directly to D(−)-β-hydroxybutyryl CoA with NADPH as the electron donor, prior to its incorporation into PHB.

Purified Acetoacetyl CoA Reductase

Results of a partial purification of the enzyme are summarized in Table 2. Although the total activity of the $NADP^+$-linked reductase in the crude extract was relatively smaller than that of β-ketothiolase and enoyl CoA hydratase, the final preparation from a Sephadex G-200 column (Fig. 4) represented a 150-fold purification, and contained only trace activities of β-ketothiolase, enoyl CoA hydratase, and the NAD^+-linked reductase.

The pH optimum for the reduction of acetoacetyl CoA of the purified enzyme was 8.1. In contrast, the rate of oxidation of β-hydroxybutyryl CoA was much lower than that of the reduction reaction at pH 7.0–10.1, showing no definite pH optimum (Fig. 5).

Substrate-saturation curves were shown in Figure 6. From the Lineweaver-Burk plots, the K_m values of the purified enzyme for acetoacetyl CoA and NADPH were estimated to be 8.3 μM and 21 μM, respectively. The enzyme activity was markedly inhibited by acetoacetyl CoA at concentrations higher than 10 μM.

PHB Synthesis in a Reconstituted System

When purified preparations of the granular PHB synthase (Fukui et al., 1976), β-ketothiolase (Nishimura et al., 1977) and the reductase were incubated with [1-^{14}C]acetyl CoA and NADPH, the incorporation of the radioactivity into PHB proceeded linearly for about 10 min (Fig. 7). Under these conditions, NADH could not replace NADPH as in the case of Figure 3, and the omission of any of three enzymes resulted in a marked reduction of PHB synthesis (Table 3).

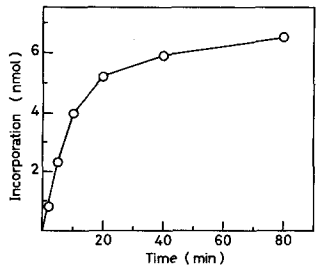

Fig. 7
Incorporation of [1-^{14}C]-acetyl CoA into PHB in a reconstituted system. Experimental conditions were described in Table 3

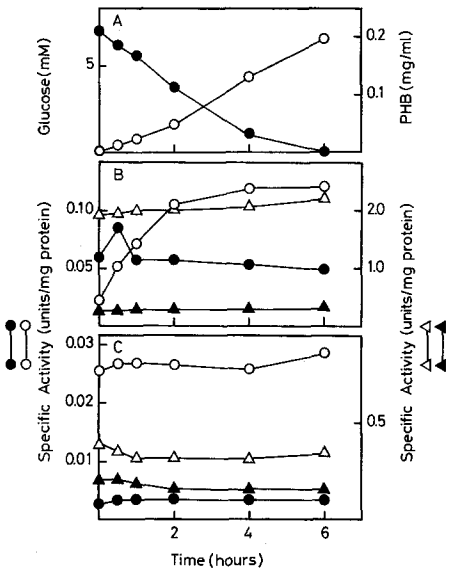

Fig. 8 A–C. Effect of glucose addition on bacterial enzyme activities. Cells were grown in the basal medium at 30° C for 20 h, harvested, and resuspended in 50 mM potassium phosphate (pH 7.0) containing 2 mM $[NH_4]_2SO_4$ and 1 mM $MgSO_4$. After adjusting the cell density to 7.5 at 650 nm, aliquots (1 ml) of cell suspensions were incubated in the presence of 7 mM glucose at 30° C. At intervals, the reaction mixture were centrifuged at $2000 \times g$ for 10 min, and glucose in the suspernatant fraction and PHB in the precipitates were assayed. Other aliquots were sonicated for 5 min and centrifuged at $15000 \times g$ for 10 min; PHB synthase activity in the sonicate and other enzyme activities in the supernatant fractions were determined. (A) ○ PHB; ● glucose. (B) ○ glucose 6-phosphate dehydrogenase; ● PHB synthase; △ β-ketothiolase; ▲ NADP-linked acetoacetyl CoA reductase. (C) ○ β-hydroxybutyrate dehydrogenase; ● β-hydroxybutyrate dimer esterase; △ NADP-linked isocitrate dehydrogenase; ▲ hexokinase

Effect of Glucose Addition on Enzyme Activities

As shown in Figure 8A, when glucose was added to the cells grown in the basal medium, PHB increased linearly with time, reaching its maximum after 6 h of glucose addition with a concomitant decrease in glucose concentration. Among the various enzymes involved in PHB synthesis, only the specific activity of glucose 6-phosphate dehydrogenase was rapidly elevated to about a 5-fold increase within 2 h after glucose addition (Fig. 8B). On the other hand, except for a transient, slight increase in the PHB synthase activity (Fig. 8B), activities of β-ketothiolase, acetoacetyl CoA reductase, β-hydroxybutyrate dehydrogenase, β-hydroxybutyrate dimer esterase, $NADP^+$-linked isocitrate dehydrogenase and hexokinase were not significantly affected by glucose (Fig. 8B and C).

DISCUSSION

A pathway of the *Azotobacter beijerinckii* type (Ritchie et al., 1971), but not that of the *Rhodospirillum rubrum* type (Moskowitz and Merrick, 1969), appears to be responsible for the formation of PHB from acetyl CoA in *Zoogloea ramigera* I-16-M; acetoacetyl CoA, formed from acetyl CoA by the action of β-ketothiolase, is directly reduced to D(-)-β-hydroxybutyryl CoA by an NADPH-linked reductase, and then incorporated into PHB by PHB synthase. Although there was some NADH-linked reductase activity, and considerable enoyl CoA hydratase activity in the bacterial crude extract, the incorporation of [1-^{14}C]-acetyl CoA into PHB was more than 100-fold faster in the presence of NADPH than in the presence of NADH (Fig. 3). Therefore, there was no indication of the conversion of L(+)-β-hydroxybutyryl CoA, a possible reduction product of acetoacetyl CoA by an NADH-linked reductase (Table 1), to the D(-)-isomer by L- and D-specific enoyl CoA hydratases during the formation of PHB from acetyl CoA in this microorganism.

The nature of a 150-fold purified NADPH-linked reductase (Table 2), its strict dependence on NADPH (Table 3) and pH activity curves (Fig. 5), also suggests a direct reduction of acetoacetyl CoA by the enzyme. In contrast to a similar enzyme from *Azotobacter beijerinckii* (Ritchie et al., 1971), the reductase from *Z. ramigera* I-16-M was not very active in the oxidation of β-hydroxybutyryl CoA, showing no apparent pH optimum. PHB synthesis from acetyl CoA in a system reconstituted of purified enzyme preparations (Fig. 7 and Table 3) also supported the presence of a pathway of the *Azotobacter beijerinckii* type.

A marked increase in the activity of glucose 6-phosphate dehydrogenase, by the addition of glucose to the growth medium, prior to the maximum formation of PHB (Fig. 8), also emphasized the principal role of the NADPH-linked acetoacetyl CoA reductase in PHB synthesis in this bacterium. Physiological roles of NADH-linked acetoacetyl CoA reductase and enoyl CoA hydratases in lipid metabolism in *Z. ramigera* remain to be investigated.

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