Metabolic regulation of β-hydroxybutyrate dehydrogenase in *Acinetobacter calcoaceticus* var. *lwoffi*

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Abstract

Comparison of cell-free extracts of *Acinetobacter* isolates which accumulated PHB, with isolates which did not, revealed the presence of β-hydroxybutyrate dehydrogenase only in PHB accumulating isolates. One of these enzymes had a specific requirement for NAD and was inhibited by high substrate concentrations as well as NADH, acetyl-CoA and oxaloacetic acid.

Introduction

Poly-β-hydroxybutyrate (PHB) is a carbon reserve material accumulated by a number of bacterial species (Dawes and Senior, 1973). During investigations into the mechanism of biological phosphate removal by activated sludge systems, Fuhs and Chen (1975) observed the accumulation of PHB in *Acinetobacter spp.* More recently, *Acinetobacter calcoaceticus* var. *lwoffi* strains isolated from a Johannesburg activated sludge plant, were shown to accumulate PHB under standardised experimental conditions (Lawson and Tonhazy, 1980). This ability was also observed by Lötter with *Acinetobacter* strains isolated from a variety of laboratory-scale activated sludge systems (Lötter *et al.*, 1986).

Synthesis and degradation of PHB have been observed in nutrient removing activated sludge plants (Hart and Melmed, 1982; Lötter, 1987). Investigations into PHB metabolism in a pure culture of *A. calcoaceticus* var. *lwoffi*, revealed uptake of acetate and conversion to PHB under anaerobic conditions, and degradation of the polymer under aerobic conditions.

During studies on the role of the anaerobic zone in biological phosphate removal in activated sludge systems, phosphate removal was shown to correlate with β-hydroxybutyrate dehydrogenase activity (Lötter and van der Merwe, 1987). The enzymatic synthesis and degradation of PHB has been well studied in a number of organisms (Delafield *et al.*, 1965; Senior and Dawes, 1971; Oeding and Schlegel, 1973), and the enzyme β-hydroxybutyrate dehydrogenase has been observed in all organisms which accumulate PHB (Dawes and Senior, 1973). This enzyme has, in fact, only been implicated in the degradation reaction, which oxidises β-hydroxybutyrate to acetoacetic acid, in organisms possessing this pathway (Dawes and Senior, 1973). To date, the presence of this enzyme has not been reported for *Acinetobacter* spp.

Wentzel *et al.* (1986) postulated that variations in the NADH/NAD ratio controlled the mechanism of biological phosphate removal through its control of the synthesis and degradation of PHB. In view of the apparent importance of regulating PHB metabolism in biological phosphate removal, this study was undertaken to ascertain the presence of this enzyme in *Acinetobacter calcoaceticus* var. *lwoffi* strains isolated from an activated sludge plant, and to investigate some aspects of its metabolic control.

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Materials and methods

Strains of *Acinetobacter calcoaceticus* var. *lwoffi*, previously isolated from an activated sludge plant (Lötter and Murphy, 1985), were grown on nutrient agar, supplemented with β-hydroxybutyric acid (Bovre and Henriksen, 1976), and evaluated for PHB accumulation by microscopic examination, after staining with Sudan Black (Gurr, 1973).

The isolates were then grown to stationary phase in a medium containing $(NH_4)_2SO_4$, 2g/ℓ; $MgSO_4$, 0,5 g/ℓ; KH_2PO_4, 0,25 g/ℓ; $CaCl_2.2H_2O$, 0,2 g/ℓ; sodium acetate, 5 g/ℓ (Fuhs and Chen, 1975) and harvested by centrifugation. Cell pellets were resuspended in buffer containing 20 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA pH 7,2 and disrupted by ultrasonication at 4°C (Parker and Weitzman, 1970). The cell-free extract was obtained by centrifugation at 35 000 g for 15 min at 6°C. Protein was determined by the dye-binding method of Bradford (1976).

The strain giving the highest specific activity (NWO1) was used for further studies. The selected isolate was grown to stationary phase in the medium described previously. Cells were harvested as before and the cell-free extract subjected to ammonium sulphate treatment. The protein obtained by precipitation between 0,45 g/mℓ and 0,65 g/mℓ ammonium sulphate, was dissolved in 100 mM Tris buffer, pH 8,5 and dialysed againsts the same buffer. The extracts were subjected to gel electrophoresis according to Laemmli (1970).

The cell extracts were diluted with 0,05 M phosphate buffer, pH 7,0 to give a protein concentration of 24 mg/mℓ. The diluted solution was filtered through a 0,45 μm Millex H4 filter unit. The filtrate was further diluted with sample buffer (0,05 M Tris-HCl, pH 6,8) and boiled for 3 to 4 min. The density was increased by the addition of a few crystals of sucrose.

Eighty μg protein per channel was applied and 20 μℓ of bromophenol blue solution (2 mg/mℓ ethanol) was added as a tracking dye. Samples were run on 12,6 and 7% gels. A potential of 60 V was applied until the sample had moved through the stacking gel. Thereafter 220 V was applied until the marker front had migrated to 10 mm from the bottom end of the gel. The temperature was kept constant at 10°C.

Gels were fixed overnight in 40% (v/v) methanol which contained 10% acetic acid, before staining with a modification of the enzymatic staining technique described by Gabriel (1971).

The β-hydroxybutyrate dehydrogenase activity was determined on the cell-free extracts. The activity was determined by measuring the appearance of NADH at 340 nm with β-hydroxybutyric acid as substrate (Williamson and Mellanby, 1974). One activity unit was defined as the amount of enzyme

which was necessary to cause the absorbance at 340 nm to increase by one unit. The reaction mixture consisted of β-hydroxybutyrate, NAD and Tris-HCl buffer at pH 8,5.

In order to investigate the possible effect of certain metabolites on the activity of the enzyme, a number of modifications were made to the reaction mixture. The β-hydroxybutyrate concentration was varied between 0 and 150 μM and NAD from 5 to 20 mM. The specificity for NAD was determined by replacing NAD with NADP in the reaction mixture.

The reverse reaction, namely the reduction of acetoacetate to β-hydroxybutyrate was measured by monitoring the disappearance of NAD at 340 nm. The acetoacetate for this reaction was synthesised as described by Procos (1961). NADH at concentrations between 5 and 15 mM was added to the reaction mixture to determine the effect of this product on the reaction. The reaction was carried out at different acetoacetate concentrations.

The effect of acetyl-CoA and oxaloacetic acid on the enzyme activity, was determined by adding these metabolites at concentrations of 0 to 20 mM to the reaction mixture. The reaction was also followed in the presence of the metabolites at different concentrations of β-hydroxybutyrate.

The staining solution had the following composition:

1,0 mℓ each of 1 M β-hydroxybutyric acid
NAD (10 mg/mℓ)
0,1 M NaCl
5 nM $MgCl_2$
2,5 mℓ each of 0,05 M Tris-HCl buffer, pH 7,4
Nitroblue tetrazolium (1 mg/mℓ)
0,25 mℓ phenazine metosulphate (1 mg/mℓ).

The gels were incubated in the staining solution at 30°C for 30 min in the dark. After colour development, the gels were rinsed sequentially in water and 7,5% acetic acid.

The efficiency of the staining technique was tested by analysing β-hydroxybutyrate dehydrogenase from *Rhodopseudomonas sphaeroides* under the same electrophoretic conditions, whereafter the gels were stained.

Results and discussion

The results of the screening of *Acinetobacter* isolates for PHB accumulation and enzyme activity are given in Table 1.

TABLE 1
PHB ACCUMULATION AND β-HYDROXYBUTYRATE DEHYDROGENASE ACTIVITY IN *A. CALCOACETICUS* VAR. *LWOFFI* ISOLATES

Isolate	PHB accumulation	Specific enzyme activity AU/mg protein
NWO1	+	1,71
NWO2	–	Nil
NWO3	–	Nil
NWO4	+	0,02
NWO5	–	Nil
NWO6	+	0,05
NWO7	+	0,23
NWO8	+	0,72
NWO9	+	0,06
NWO10	+	0,46

AU: Absorbance units

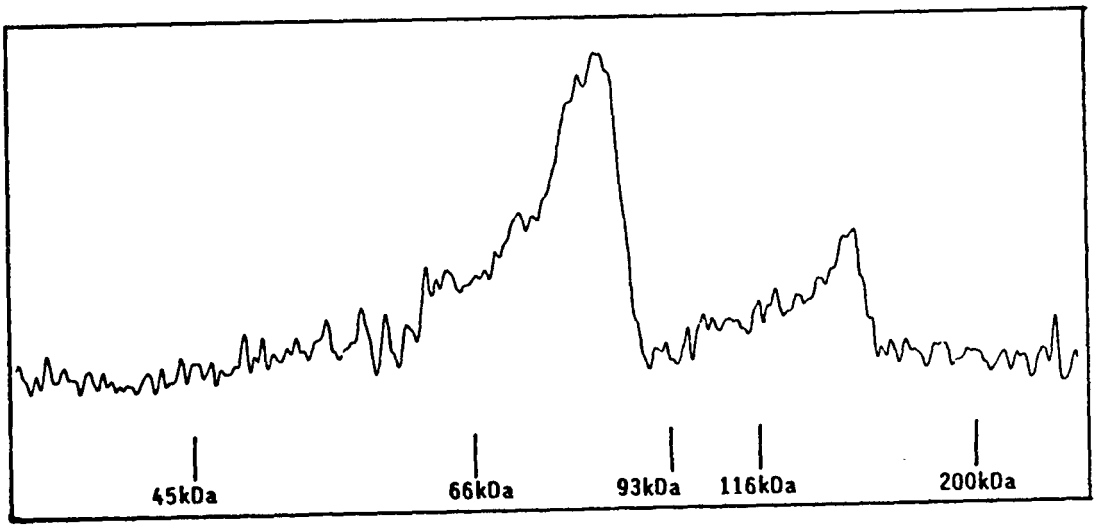

Figure 1
Densitogram of electrophoresis pattern of protein extract on 7% gel showing two peaks visualised with β-hydroxybutyric acid and NAD.

TABLE 2
ENRICHMENT TABLE FOR THE ISOLATION OF β-HYDROXYBUTYRATE DEHYDROGENASE

	Step	Volume (m)	Total protein (mg)	Total activity (ncat)	Specific activity (ncat/mg)	Purification (-times)
1	Crude extract	40	24	31,2	1,3	1
2	0 – 45% $(NH_4)_2 SO_4$ supernatant	39	10,9	18,8	1,7	1,3
3	0 – 65% $(NH_4)_2 SO_4$ precipitate	5	6,72	52,3	7,8	6,0

All the cell-free extracts from isolates which accumulated PHB, contained β-hydroxybutyrate dehydrogenase activity, in common with other bacteria, which have been shown to accumulate PHB (Dawes and Senior, 1973). Isolates which did not accumulate PHB under the experimental conditions, which were previously used to evaluate this characteristic (Bovre and Henriksen, 1976), did show β-hydroxybutyrate dehydrogenase activity.

The ability to accumulate PHB has previously been observed to occur in all *Acinetobacter* isolates from an activated sludge plant (Lawson and Tonhazy, 1980; Lötter *et al.*, 1986). *Acinetobacter* strains which have the ability to accumulate PHB should dominate the population in the plant, due to the advantage conferred on them by the presence of stored substrate under anaerobic conditions.

The purification obtained with the isolation procedure described, is given in Table 2.

Enzymatic staining of the gels revealed one band on the 12,6% gel and two bands on the 7,0% gel (see Fig. 1). Densitrometry measurements of the activity on the gel revealed more than 90% of the activity to be contained in the larger band. This extract was considered suitable for initial metabolic studies.

Initial reaction rates for the enzyme were first determined at a β-hydroxybutyrate concentration range of 50 to 150 μM at different co-factor concentrations. The dependence of the enzyme activity on substrate concentration at different co-factor concentrations, is shown in Fig. 2.

Substrate inhibition was observed at β-hydroxybutyrate concentrations above 100 mM, as is clearly shown in the Lineweaver-Burk manipulation of the data obtained at 1 co-factor concentration (Fig. 3).

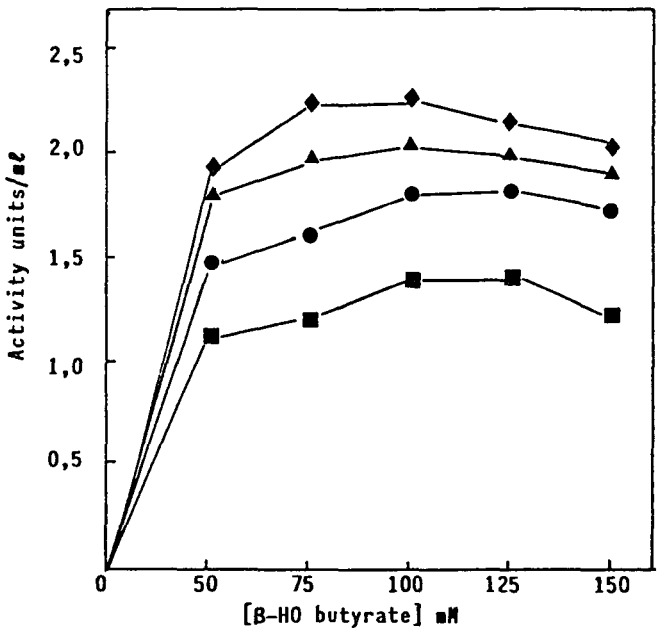

Figure 2
The effects of β-hydroxybutyrate and NAD concentrations on β-hydroxybutyrate dehydrogenase activity.

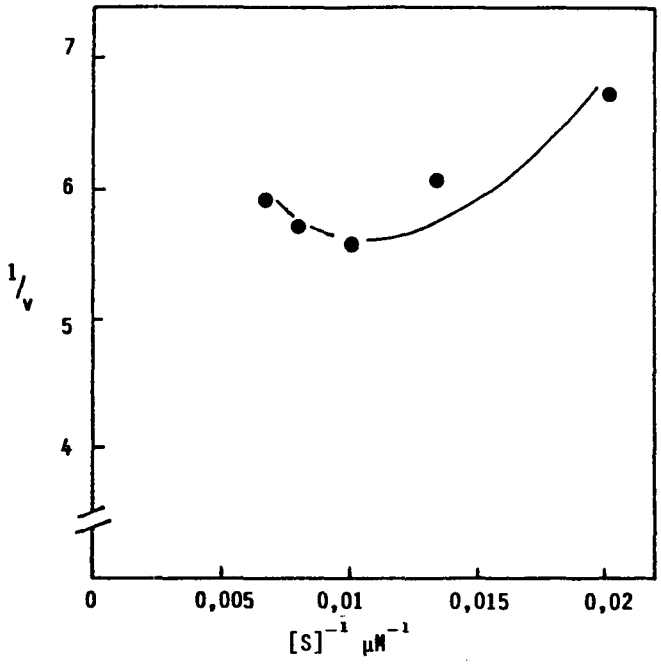

Figure 3
Lineweaver-Burk manipulation of data obtained with 100 μM β-hydroxybutyrate and 15 nM NAD as co-factor.

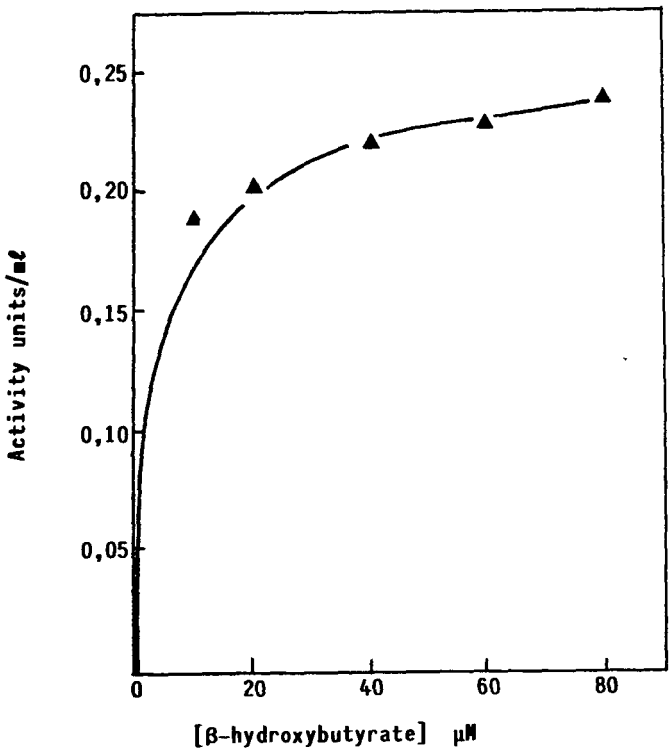

Figure 4
The effect of β-hydroxybutyrate concentration on β-hydroxybutyrate dehydrogenase activity.

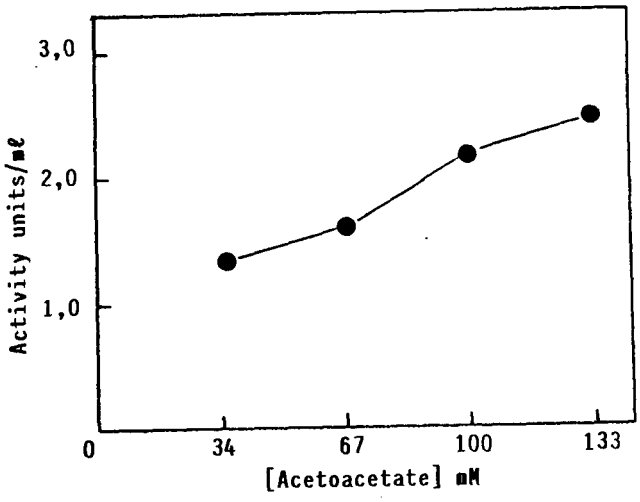

Figure 5
The effect of varying concentrations of acetoacetate on the activity of β-hydroxybutyrate dehydrogenase.

The initial rates were consequently determined over a β-hydroxybutyrate concentration range of 0 to 80 µM. The results are shown in Fig. 4.

In order to determine whether NADP can replace NAD co-factor, the enzyme was incubated with β-hydroxybutyrate at 100 mM and NADP at 15 mM, under the standard test conditions. No activity was observed. The enzyme from *A. calcoaceticus* var. *lwoffi* thus has a specific requirement for NAD, in common with enzymes from *Hydrogenomonas* spp. (Ahrens and Schlegel, 1966), *Rhodopseudomonas sphaeroides* (Bergmeyer *et al.*, 1967) and *Azotobacter beijerinckii* (Dawes and Senior, 1973).

The reverse reaction, namely, the reduction of the acetoacetate to β-hydroxybutyrate, was determined by incubating the enzyme with acetoacetate at a concentration range of 34 to 133 mM, and 15 mM NADH. The curves obtained with different concentrations of acetoacetate and 15 mM NADH are given in Fig. 5.

The catalysis of the reverse reaction has already been observed in other bacteria (Bergmeyer *et al.*, 1976). Although β-hydroxybutyrate dehydrogenase can catalyse the reverse reaction of acetoacetate to β-hydroxybutyrate, it is unlikely that synthesis occurs according to this route. In other organisms which accumulate PHB, this step in the synthesis pathway is catalysed by an acetoacetyl-CoA reductase (Ritchie *et al.*, 1971), an enzyme which has still to be detected in *Acinetobacter*.

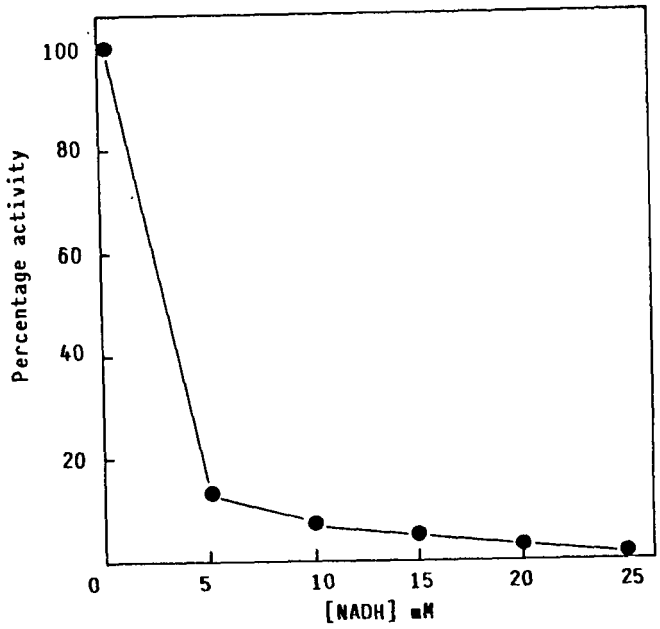

Figure 6
The effect of various concentrations of NADH on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate.

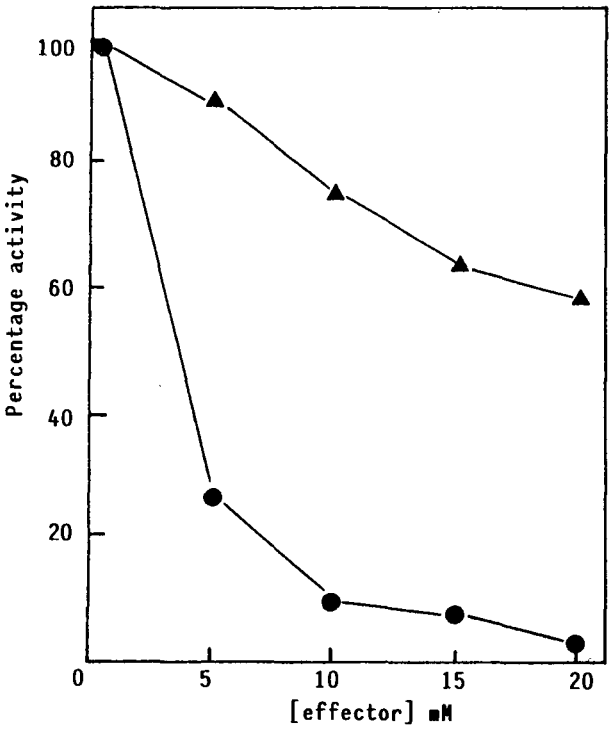

Figure 7
The effect of acetyl - CoA ▲ – ▲; and oxaloacetate ● – ●; on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate.

The effect of NADH on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate is shown in Fig. 6. The reduced form of the nicotinamide has a strong inhibitory effect on the enzyme.

The metabolites oxaloacetate and acetyl-CoA, both have an inhibitory effect on the oxidation of β-hydroxybutyrate by the enzyme (Fig. 7).

Conclusions

One of the enzymes involved in PHB metabolism has been extracted from an *Acinetobacter calcoaceticus* var. *lwoffi* isolated from an activated sludge plant. The enzyme, β-hydroxybutyrate dehydrogenase is inhibited by NADH, acetyl-CoA and oxaloacetate, metabolites which have previously been shown to inhibit this enzyme in other bacteria (Bergmeyer *et al.*, 1967; Oeding and Schlegel, 1973; Dawes and Senior, 1973).

These findings provide direct support for the biochemical model of Wentzel *et al.* (1986), in that these authors postulated metabolic feedback control as the regulatory mechanism which provided *Acinetobacter* with a means to proliferate under alternating anaerobic/anoxic/aerobic conditions.

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