Purification and Properties of β -Hydroxybutyrate Dehydrogenase from *Mycobacterium phlei* ATCC354

By K. R. DHARIWAL AND T. A. VENKITASUBRAMANIAN

Department of Biochemistry, Vallabhbhai Patel Chest Institute,
University of Delhi, Delhi 110007, India

(Received 14 June 1977; revised 13 September 1977)

β -Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was purified 145-fold from *Mycobacterium phlei* ATCC354 by ammonium sulphate fractionation and DEAE-cellulose chromatography. The pH optima for oxidation and reduction reactions were 8.4 and 6.8 respectively. The purified enzyme was specific for NAD, NADH, acetoacetate and D(-)- β -hydroxybutyrate. K_m values for DL- β -hydroxybutyrate and NAD were 7.4 mM and 0.66 mM respectively. The enzyme was inactivated by mercurial thiol inhibitors and by heat, but could be protected by NADH, Ca^{2+} and partially by Mn^{2+} . The enzyme did not require metal ions and was insensitive to EDTA, glutathione, dithiothreitol, β -mercaptoethanol and cysteine.

INTRODUCTION

Intracellular depolymerization of poly- β -hydroxybutyrate leads to the formation of β -hydroxybutyrate, which is further metabolized by a soluble NAD-dependent enzyme, D(-)- β -hydroxybutyrate dehydrogenase (EC 1.1.1.30; D-3-hydroxybutyrate dehydrogenase:NAD⁺ oxidoreductase). The enzyme has been reported in several bacteria which form the β -hydroxybutyric acid polymer as an intracellular reserve material (Shuster & Doudoroff, 1962). The enzyme occurs in rat liver mitochondria (Lehninger, Sudduth & Wise, 1960) where it is possibly involved in fatty acid oxidation and in an electron shuttle mechanism across the mitochondrial membrane (Devlin & Bedell, 1960).

We have attempted to isolate poly- β -hydroxybutyrate and have investigated potential biosynthetic and catabolic pathways of this polymer in mycobacteria. Screening of various species of mycobacteria for the degradative enzyme, β -hydroxybutyrate dehydrogenase, revealed that the enzyme is present only in *Mycobacterium phlei* (unpublished results). This prompted us to purify and study the properties of this enzyme from *M. phlei*.

METHODS

Organism, media and culture conditions. *Mycobacterium phlei* ATCC354 was grown in the medium of Youmans & Karlson (1947) except that magnesium citrate was replaced by citric acid and MgCO_3 . Cells were grown at 37 °C without shaking and collected after 5 days by filtration or centrifugation and washed with cold distilled water.

Preparation of cell-free extract. A suspension of bacteria (30 % wet wt/vol.) was made in Tris/HCl buffer (0.05 M, pH 7.4) and ultrasonically treated for 3 min in a MSE 100 W ultrasonic disintegrator at a frequency of 20 kHz. The sonicated material was centrifuged at 10000 g for 30 min to obtain a cell-free extract.

Enzyme assay. The activity of β -hydroxybutyrate dehydrogenase was determined by the method of Bergmeyer (1974). The reaction system contained, in 3 ml, the following reagents (μmol): Tris/HCl buffer pH 8.0, 100; NAD, 1.5; DL- β -hydroxybutyrate, 15; and enzyme. To determine the effect of pH on the reduction of acetoacetate, the reaction system contained, in 3 ml, the following reagents (μmol): buffer,

100; NADH, 1.0; acetoacetate, 15; and enzyme. The reactions were followed by measuring the change in absorbance at 340 nm.

Purification of the enzyme. Ammonium sulphate fractionation of the cell-free extract was carried out in the usual manner, collecting the protein which precipitated between 65 and 95 % saturation. The precipitate was dissolved in a small volume of 0.05 M-Tris/HCl buffer (pH 7.4) and dialysed at 4 °C against the same buffer for about 16 h with at least three changes of fresh buffer. This fraction was further purified by chromatography on DEAE-cellulose. Elution from the column was with a linear gradient of KCl (0.05 to 0.5 M) in 0.05 M-Tris/HCl buffer (pH 7.4) at a flow rate of 25 ml h⁻¹; 2.5 ml fractions were collected.

Polyacrylamide disc gel electrophoresis was performed by the method of Davis (1964). Protein was estimated by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

β -Hydroxybutyrate dehydrogenase eluted from DEAE-cellulose as a single peak. Attempts to purify the enzyme further by chromatography on Sephadex G-100 or G-200 were unsuccessful. The most active fraction obtained from the DEAE-cellulose column was 145-fold purified over the crude extract (Table 1). Polyacrylamide disc gel electrophoresis of the purified enzyme revealed three bands.

Properties of β -hydroxybutyrate dehydrogenase

Stability of the enzyme. The partially purified enzyme lost less than 15 % activity in 20 days at -20 °C. A similar behaviour has been reported for this enzyme from *Pseudomonas lemoignei* (Delafield, Cooksey & Doudoroff, 1965) and *Rhodopseudomonas spheroides* (Bergmeyer, Gawehn & Klotzsch, 1967) but the enzyme from *Rhodospirillum rubrum* (Shuster & Doudoroff, 1962) was cold labile.

Effect of enzyme concentration. Under the experimental conditions employed, the enzyme reaction was linear for the first 3 min. The initial reaction rate increased linearly with increasing concentration of enzyme up to the amount giving a ΔE_{340} value of 0.030 in the first minute.

Effect of pH. The oxidation reaction was maximum at pH 8.4; the rate decreased rapidly above pH 9.0 and below pH 7.6 (Fig. 1*a*). The reduction reaction with acetoacetate as substrate was maximum between pH 6.4 and 7.0; the rate declined slowly at alkaline pH (Fig. 1*b*). These pH optima are similar to β -hydroxybutyrate dehydrogenases purified from other bacteria (Shuster & Doudoroff, 1962; Delafield *et al.*, 1965; Bergmeyer *et al.*, 1967; Robert, Alexander & Louis, 1973).

Substrate specificity. The enzyme was specific for NAD, NADH, DL- β -hydroxybutyrate and acetoacetate. It was specific for the D(-) stereoisomer as it did not oxidize L- β -hydroxybutyrate even when present at 10 mM. NADP and NADPH could not replace NAD and NADH. DL-Malate, DL-lactate, D-gluconate, pyruvate and oxaloacetate were not substrates for the enzyme.

Kinetics. Using double-reciprocal plots in the usual manner, the K_m values of the enzyme for DL- β -hydroxybutyrate and NAD were calculated as 7.4 mM and 0.66 mM respectively. These values were higher than those reported for other bacteria for these compounds (Bergmeyer *et al.*, 1967; Senior & Dawes, 1973).

Thermostability. The enzyme, at 0.24 mg protein ml⁻¹, lost 70 % of its activity after 15 min at 37 °C. Inactivation was dependent on the protein concentration; preparations containing 2.4 mg protein ml⁻¹ lost little activity at 37 °C. Loss of activity at 37 °C also occurred with a preparation from *Rhodops. spheroides* (Bergmeyer *et al.*, 1967). The enzyme was not stabilized at 37 °C by its substrates, acetoacetate and DL- β -hydroxybutyrate. Cysteine and β -mercaptoethanol were also without effect. Of a number of cations, Ca²⁺ was the best stabilizer of enzyme activity at 37 °C, followed by Mn²⁺. There was no protection by Na⁺, Mg²⁺ and Zn²⁺. Of various nucleotides, NADH markedly protected the enzyme at 37 °C but other nicotinamide adenine dinucleotides were almost ineffective.

Table 1. Purification of β -hydroxybutyrate dehydrogenase from *M. phlei* ATCC354

Cell-free extract was prepared from surface-grown cells of *M. phlei* ATCC354, suspended in 0.05 M-Tris/HCl buffer (pH 7.4) and disrupted ultrasonically. Enzyme was purified by ammonium sulphate fractionation and DEAE-cellulose column chromatography.

Purification steps	Protein (mg)	Total units	Specific activity*	Purification (fold increase)	Yield (%)
Cell-free extract	2600	250	0.096	—	100
65 to 95 % saturated (NH ₄) ₂ SO ₄ fraction	168	153	0.91	9.4	61
DEAE-cellulose (fraction no. 35)	6	84	14.0	145	34

* Results expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Fig. 1. Effect of pH on (a) the oxidation of β -hydroxybutyrate and (b) the reduction of acetoacetate. Citrate, phosphate, Tris/HCl and glycine/NaOH buffers (0.1 M) covering the pH ranges of 4 to 6, 6.2 to 7.2, 7.4 to 9.0 and 9.5 to 10.0, respectively, were used. The assay systems were otherwise as described in Methods.

Effect of various reagents. By preincubating the enzyme with various reagents for 5 min, the enzyme was found to be extremely sensitive to *p*-chloromercuribenzoate and HgCl_2 , but was unaffected by dithiothreitol, β -mercaptoethanol, glutathione or cysteine up to 33 mM. NADH and Ca^{2+} , which stabilized the enzyme at 37 °C, also protected the enzyme against inactivation by the two thiol reagents. Other nucleotides and Mn^{2+} , Mg^{2+} , Zn^{2+} or Na^+ were ineffective in this capacity.

Effect of EDTA and metal ions. EDTA inhibits the enzymes from *Rhodosp. rubrum* (Shuster & Doudoroff, 1962) and *P. lemoignei* (Delafield *et al.*, 1965) but not if the enzyme is preincubated with NAD or NADH, Mg^{2+} , Ca^{2+} or Mn^{2+} . The enzyme from *M. phlei*, however, was not inactivated over 5 min incubation with EDTA up to 33 mM. This is similar to the enzyme from *Rhodops. spheroides* (Bergmeyer *et al.*, 1967).

The enzyme of *M. phlei* was not affected by Na^+ , K^+ , NH_4^+ , Zn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Mg^{2+} , Ca^{2+} , Mn^{2+} and Fe^{2+} up to 33 mM. The enzymes isolated from *Rhodosp. rubrum* (Shuster & Doudoroff, 1962), *P. lemoignei* (Delafield *et al.*, 1965) and *Rhodops. spheroides* (Bergmeyer *et al.*, 1967) also did not require metal ions.

The results indicate that the enzyme from *M. phlei* differs from that of *Rhodosp. rubrum* and *P. lemoignei* in that it is insensitive to EDTA and resembles the enzyme from *Rhodops. spheroides* in being sensitive to mercurial thiol reagents and to incubation at 37 °C.

The inhibition by low concentrations of thiol reagents suggests that thiol groups may be involved in the reaction catalysed by the dehydrogenase. The sensitive thiol groups react

extremely rapidly as compared, for example, with the rate of combination of *p*-chloro-mercuribenzoate with the thiol groups thought to be involved in the active centre of lactate dehydrogenase (Dube, Roholt & Pressman, 1963).

The protection of the enzyme by NADH from the action of mercurials is a further indication that a thiol group(s) may be present in the active centre of this enzyme, because thiols include among their functions the binding of nucleotides to proteins (Bergmeyer *et al.*, 1967). Alternatively, the instability of the enzyme may be the result of its dissociation to constituent subunits; this may be prevented by the presence of bivalent cations or by the binding of nicotinamide adenine dinucleotides (Bergmeyer *et al.*, 1967). The marked protective action of Ca^{2+} ions against heat inactivation suggests that protein conformation may be an important factor, since Ca^{2+} ions prevent the denaturation of a number of enzymes by maintaining their tertiary structure (Okunuki, 1961).

K. R. Dhariwal is grateful to the Council of Scientific and Industrial Research, New Delhi for the award of a Senior Research Fellowship. This investigation was supported in part by funds from the Indian Council of Medical Research, New Delhi and the University Grants Commission, New Delhi.

REFERENCES

- BERGMEYER, H. U. (1974). 3-Hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*. In *Methods of Enzymatic Analysis* 1, 475. Edited by H. U. Bergmeyer. New York: Academic Press.
- BERGMEYER, H. U., GAWEHN, K. & KLOTZSCH, H. (1967). Purification and properties of crystalline 3-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*. *Biochemical Journal* **102**, 423–431.
- DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.
- DELAFIELD, F. P., COOKSEY, K. R. & DOUDOROFF, M. (1965). β -Hydroxybutyric acid dehydrogenase and dimer hydrolase of *Pseudomonas lemoignei*. *Journal of Biological Chemistry* **240**, 4023–4028.
- DEVLIN, T. M. & BEDELL, B. H. (1960). Effect of acetoacetate on the oxidation of reduced diphosphopyridine nucleotide by rat liver mitochondria. *Journal of Biological Chemistry* **235**, 2134–2139.
- DUBE, S. K., ROHOLT, O. & PRESSMAN, D. (1963). The chemical nature of the enzyme site of rabbit muscle lactic acid dehydrogenase. *Journal of Biological Chemistry* **238**, 613–617.
- LEHNINGER, A. L., SUDDUTH, H. C. & WISE, J. B. (1960). D- β -Hydroxybutyric acid dehydrogenase of mitochondria. *Journal of Biological Chemistry* **235**, 2450–2455.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- OKUNUKI, K. (1961). Denaturation and inactivation of enzyme proteins. *Advances in Enzymology* **23**, 29–82.
- ROBERT, H., ALEXANDER, P. & LOUIS, H. B. (1973). Model discrimination and nonlinear parameter estimation in the analysis of the mechanism of action of β -hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides*. *Biochimica et biophysica acta* **321**, 1–26.
- SENIOR, P. J. & DAWES, E. A. (1973). The regulation of poly- β -hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochemical Journal* **134**, 225–238.
- SHUSTER, C. W. & DOUDOROFF, M. (1962). A cold-sensitive D(-)- β -hydroxybutyric acid dehydrogenase from *Rhodospirillum rubrum*. *Journal of Biological Chemistry* **237**, 603–607.
- YOUSMANS, G. P. & KARLSON, A. G. (1947). Streptomycin sensitivity of tubercle bacilli and the development of resistance to streptomycin *in vivo*. *American Review of Tuberculosis* **55**, 529–535.