3-Hydroxyisobutyrate dehydrogenase, an impurity in commercial 3-hydroxybutyrate dehydrogenase

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The enzymic determination of D-3-hydroxybutyrate and acetoacetate normally involves the use of 3-hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30) of bacterial origin. We show that HBDH from *Rhodopseudomonas spheroides* (BCL, grade II) contains a 3-hydroxyisobutyrate dehydrogenase (HIBDH) activity: activity with 3-hydroxyisobutyrate as substrate was > 10% of that with 3-hydroxybutyrate. However, HBDH could be prepared essentially free of HIBDH activity by incubation at 37 °C in the presence of 1 mM-CaCl₂, to produce an enzyme preparation that may be used for the specific determination of 3-hydroxybutyrate. Use of the purified enzyme preparations indicated that a major product of valine metabolism in hemidiaphragms from 40 h-starved rats was 3-hydroxyisobutyrate rather than 3-hydroxybutyrate.

INTRODUCTION

The reaction catalysed by 3-hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.30) provides the basis for an enzymic method for determination of the ketone bodies D-3-hydroxybutyrate and acetoacetate (Mellanby & Williamson, 1974). Unlike mammalian HBDH, which is associated with the particulate cell fraction and is difficult to solubilize, the enzyme from *Rhodopseudomonas spheroides* is readily solubilized and may be obtained in purified form from commercial sources (Bergmeyer *et al.*, 1974). In our laboratories HBDH from *R. spheroides* (grade II; Boehringer) is used routinely for the determination of 3-hydroxybutyrate and acetoacetate in tissue extracts.

The metabolism of 3-methyl-2-oxobutanoate (the 2-oxo acid analogue of valine) by isolated rat hepatocytes leads to the accumulation of a product that behaves as a reductive substrate for the reaction catalysed by commercial HBDH (Lund, 1981). Similarly, valine metabolism by hemidiaphragms from 40 h-starved rats apparently produces large amounts of 3-hydroxybutyrate, as determined with commercial HBDH (Caldecourt, 1985). These findings are anomalous, in view of the known degradative pathways for valine in liver and skeletal muscle. Whereas acetoacetate and 3-hydroxybutyrate are products of leucine metabolism, valine is metabolized via conversion into succinyl-CoA, and theoretically should not give rise to the ketone bodies. 3-Hydroxyisobutyrate is an intermediate in the pathway of valine oxidation, being produced from 3-hydroxyisobutyryl-CoA in the 3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4) reaction and converted into methylmalonate semialdehyde in the 3-hydroxyisobutyrate dehydrogenase (HIBDH; EC 1.1.1.31) reaction, and has been demonstrated to be a major product of valine metabolism in muscle *in vitro* (Spydevold & Hokland, 1981; Wagenmakers *et al.*, 1985; Lee & Davis, 1986; see Palmer *et al.*, 1985). In the present paper we demonstrate that commercial HBDH will react with both 3-hydroxy-

butyrate and 3-hydroxyisobutyrate, and that the endogenous activity with the latter substrate is sufficient to account for the apparent production of 3-hydroxybutyrate from valine and its 2-oxo acid derivative. A method is described whereby HBDH may be prepared free of HIBDH by selective inactivation of HIBDH, yielding a preparation which is suitable for specific determination of 3-hydroxybutyrate and acetoacetate in mixtures of these ketone bodies together with 3-hydroxyisobutyrate.

EXPERIMENTAL

Materials

HBDH from *Rhodopseudomonas spheroides* (grade II) was obtained from Boehringer Corp. Ltd. as a suspension (5 mg of protein/ml) in 3.2 M-(NH₄)₂SO₄, approx. pH 6. Unless otherwise stated, the enzyme was dialysed at 2 °C for 1 h against a 100-fold excess of 10 mM-sodium phosphate buffer, pH 6.8, and diluted to a protein concentration of 270 µg/ml in 10 mM-sodium phosphate buffer, pH 6.8, or 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-CaCl₂, 10 mM-MnCl₂, 200 mM-(NH₄)₂SO₄, or 1 mM-CaCl₂ + 200 mM-(NH₄)₂SO₄. Inactivation of component HBDH and HIBDH activities was achieved by incubation at 37 °C for the times and other conditions specified in the text. The enzyme preparations were normally stopped at 0–4 °C.

DL-3-Hydroxyisobutyrate was prepared by the method of Robinson & Coon (1963) and its purity was confirmed by n.m.r. DL-3-Hydroxybutyrate was purchased from Sigma Chemical Co., and Sephadex G-200 (superfine grade) from Pharmacia.

Measurement of HBDH and HIBDH activities

The reaction mixtures contained 100 µmol of Tris/HCl buffer, pH 8.5, 1 µmol of NAD⁺ and either 10 µmol of DL-3-hydroxybutyrate (sodium salt) or 10 µmol of

Abbreviations used: HBDH, 3-hydroxybutyrate dehydrogenase; HIBDH, 3-hydroxyisobutyrate dehydrogenase.

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DL-3-hydroxyisobutyrate (sodium salt) in a total volume of 3.1 ml. The reaction was monitored at 25 °C as the change in A_{340} on a Gilford 240 spectrophotometer, and was initiated by the addition of enzyme (50 μ l). One unit of enzyme activity is defined as the amount of enzyme which catalyses conversion of 1 μ mol of substrate into product/min at 25 °C.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed as described by Reid & Bielecki (1968). The running and stacking gels were 10% (w/v) and 5% (w/v) acrylamide respectively. The gels were stained with Coomassie Brilliant Blue R-250 (Meyer & Lamberts, 1965) or were overlaid with 2% (w/v) agarose in 25 ml of 50 mM-Tris/HCl buffer, pH 8.5, containing 20 mg of NAD⁺, 10 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 4 mg of phenazine methosulphate and 6 mg of 3-hydroxybutyrate or 3-hydroxyisobutyrate for activity staining (25 °C, approx. 2 h).

Gel filtration on Sephadex G-200

Undialysed enzyme (0.9 ml; 5.4 mg of protein) was applied to a Sephadex G-200 column (1.5 cm $\times$ 29 cm) equilibrated at 2 °C with 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-CaCl₂ and 200 mM-(NH₄)₂SO₄. The columns were eluted with the same buffer, and fractions (3.5 ml) were assayed for A_{280} and HBDH and HIBDH activities

Metabolism of valine or leucine by isolated rat hemidiaphragms

Male Wistar rats (100–120 g) were maintained at approx. 17 °C on a 12 h-light/12 h-dark cycle (light from 08:00 h) and were starved for 40 h before the start of the experiments, at 11:00–13:00 h. Hemidiaphragms were preincubated (30 min, 37 °C) in substrate-free Krebs–Ringer bicarbonate buffer, pH 7.4, and subsequently incubated (2 h, 37 °C) in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 3 mM-leucine, 3 mM-valine or no added substrate as described previously (Palmer *et al.*, 1983, 1984a,b). Media were gassed with O₂/CO₂ (19:1). After 2 h, the media were acidified with HClO₄ and 3-hydroxybutyrate and acetoacetate were determined in KOH-neutralized HClO₄ extracts by the method of Mellanby & Williamson (1974) by using (a) the unmodified BCL grade II enzyme, or (b) dialysed enzyme incubated at 37 °C for 15 min in the presence of 1 mM-CaCl₂ to inactivate HIBDH activity selectively, or (c) dialysed enzyme incubated at 37 °C for 45 min in the presence of 200 mM-(NH₄)₂SO₄ to inactivate HBDH activity selectively (see below for details). Results are expressed as means $\pm$ S.E.M., with the numbers of observations in parentheses. Statistical significance of difference was assessed by Student's *t* test for unpaired data.

RESULTS

Commercial HBDH contains HIBDH activity

At pH 8.5 and 25 °C, the activity of commercial HBDH with DL-3-hydroxyisobutyrate as co-substrate with NAD⁺ was 12.3% of that with DL-3-hydroxybutyrate as co-substrate. There was no activity with either 3-methyl-2-oxobutanoate or 4-methyl-2-oxopentanoate as co-substrate. Polyacrylamide-gel electrophoresis

under non-dissociating conditions indicated that the commercial enzyme preparation contained six major protein bands. The major protein band (accounting for approx. 44% of total protein, by densitometric analysis) possessed HBDH activity, as assessed by a specific stain with 3-hydroxybutyrate as substrate. It was not possible to detect the HIBDH activity with an activity stain with 3-hydroxyisobutyrate as substrate, presumably because the activity was below the limits of detection of the assay system.

Gel filtration on Sephadex G-200

The elution profile for dialysed commercial HBDH on Sephadex G-200 is shown in Fig. 1. The column was equilibrated in 10 mM-sodium phosphate buffer/200 mM-(NH₄)₂SO₄/1 mM-CaCl₂, pH 7. The HBDH activity peak was coincident with the protein (A_{280}) peak, whereas the HIBDH activity was consistently eluted at a fractionally higher elution volume. Recovery after gel filtration of the HBDH and HIBDH activities was 100%.

Stability at 37 °C

Crystalline HBDH from *R. spheroides* is unstable in dilute solution at 37 °C. Ca²⁺ and, to a lesser extent, Mn²⁺ protect the enzyme against this inactivation (Bergmeyer *et al.*, 1967). The HBDH and HIBDH activities of the commercial enzyme (after dialysis at 4 °C against, and subsequent dilution to 270 μ g of protein/ml with, 10 mM-sodium phosphate buffer, pH 7) were stable at 0 °C, but rapidly lost activity on incubation at 37 °C (Fig. 2). If 1 mM-Ca²⁺ was included in the incubation medium, the activity with 3-hydroxybutyrate was selectively retained: approx. 75% of HBDH activity remained after 30 min incubation at 37 °C, whereas no activity with 3-hydroxyisobutyrate remained. The inclusion of 10 mM-Mn²⁺ had a similar, albeit less marked, protective effect on HBDH activity: 30 min incubation at 37 °C in buffer containing 1 mM-MnCl₂ resulted in retention of 46% of HBDH activity and total loss of HIBDH activity (results not shown).

Incubation at 37 °C of undialysed enzyme diluted to a protein concentration of 270 μ g/ml with 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-CaCl₂ resulted in substantial retention of both HBDH (75%) and HIBDH (95%) activities. Since the undialysed enzyme contains approx. 173 mM-(NH₄)₂SO₄, the inference was that (NH₄)₂SO₄ might be protective to the HIBDH activity. This conclusion is supported by the finding (Fig. 2) that addition of 200 mM-(NH₄)₂SO₄ to the incubation buffer after dialysis selectively protected the HIBDH activity against inactivation at 37 °C. Under these circumstances > 97% of HBDH activity was lost. Addition of both 1 mM-CaCl₂ and 200 mM-(NH₄)₂SO₄ to the incubation buffer after dialysis predictably protected both HBDH and HIBDH activities at 37 °C.

Inactivation at 37 °C of HBDH and HIBDH activities was irreversible, in that there was no re-appearance of activity on storage at 4 °C or on precipitation with satd. (NH₄)₂SO₄ (final concn. 3.2 M). Enzyme activity measured at pH 8.5 with either 3-hydroxybutyrate or 3-hydroxyisobutyrate as substrate was not affected by addition of CaCl₂ (1 mM) or (NH₄)₂SO₄ (200 mM) to the assay system.

The HIBDH activity [prepared essentially free of HBDH activity by incubation for 60 min at 37 °C in the presence of 200 mM-(NH₄)₂SO₄] had a pH optimum at

Fig. 1. Gel filtration of commercial enzyme in Sephadex G-200

For details, see the Experimental section. Fractions were assayed for A_{280} (Δ), HBDH activity ($\circ$) and HIBDH activity ($\square$).

Fig. 2. Thermal inactivation of the HBDH and HIBDH activities of the commercial enzyme

Commercial enzyme was incubated (for details, see the Experimental section) in 10 mM-sodium phosphate buffer, pH 7, at 0 °C ($\circ$) or at 37 °C (Δ), or at 37 °C in 10 mM-sodium phosphate buffer, pH 7, containing 1 mM- CaCl_2 ($\square$) or 200 mM- $(\text{NH}_4)_2\text{SO}_4$ ($\bullet$) or 1 mM- CaCl_2 + 200 mM- $(\text{NH}_4)_2\text{SO}_4$ ($\blacktriangle$). At time intervals samples of these incubation media were assayed for (a) HBDH and (b) HIBDH activities. Activity is expressed as a percentage of initial activity.

approx. pH 8.6 and apparent K_m values for DL-3-hydroxyisobutyrate and NAD^+ of 0.1 mM and 0.022 mM respectively. The pH optimum of the HBDH activity prepared free of HIBDH activity by thermal inactivation (15 min at 37 °C in the presence of 1 mM- CaCl_2) was approx. pH 9.

Metabolism of valine and leucine by hemidiaphragms from 40 h-starved rats

Hemidiaphragms from 40 h-starved rats provided with either leucine or valine appeared to produce a metabolite capable of acting as a reducing agent in the reaction catalysed by the commercial enzyme preparation (Table 1). The metabolite(s) produced by hemidiaphragms provided with valine include 3-hydroxyisobutyrate, as it did not react with HBDH prepared free of HIBDH activity by thermal inactivation at 37 °C in the presence of 1 mM- CaCl_2 , but reacted to some degree with HIBDH

prepared free of HBDH by incubation of the impure preparation at 37 °C in the presence of 200 mM- $(\text{NH}_4)_2\text{SO}_4$. Provision of leucine, but not valine, led to production of acetoacetate (measured with commercial HBDH). It was possible to confirm the identity of this metabolite as acetoacetate on the grounds that it was not reactive with HIBDH [prepared free of HBDH activity by thermal inactivation in the presence of 200 mM- $(\text{NH}_4)_2\text{SO}_4$]. With purified HIBDH there remains significant production of a metabolite that generates NAD^+ from NADH in the assay system. The identity of this metabolite(s) is as yet unknown.

DISCUSSION

The significant activity of commercial HBDH with 3-hydroxyisobutyrate as substrate imposes a major

Table 1. Apparent production of 3-hydroxybutyrate and acetoacetate by hemidiaphragms from 40 h-starved rats

Metabolite production was determined by using (a) the unmodified Boehringer grade II enzyme, or (b) dialysed enzyme incubated for 15 min at 37 °C in the presence of 1 mM-CaCl₂ to inactivate HIBDH selectively (referred to as 'HBDH'), or (c) dialysed enzyme incubated for 60 min at 37 °C in the presence of 200 mM-(NH₄)₂SO₄ to inactivate HBDH selectively (referred to as 'HIBDH'). Statistically significant effects of leucine or valine addition are denoted as **P* < 0.01 and ***P* < 0.001. Statistically significant effects of using HBDH or HIBDH as opposed to unmodified commercial enzyme are denoted as †*P* < 0.01 and ††*P* < 0.001.

Addition	3-Hydroxybutyrate and/or 3-hydroxyisobutyrate (μmol/2 h per g wet wt.)			Acetoacetate (and other metabolites) (μmol/2 h per g wet wt.)	
	Commercial enzyme	HBDH	HIBDH	Commercial enzyme	HIBDH
None	0.64 ± 0.09 (24)	0.56 ± 0.006 (12)	0.26 ± 0.009 (12)††	0.83 ± 0.13 (12)	0.76 ± 0.10 (12)
3 mM-Leucine	1.44 ± 0.14 (12)**	1.36 ± 0.19 (6)**	0.24 ± 0.07 (6)††	1.56 ± 0.17 (6)*	0.75 ± 0.06 (6)††
3 mM-Valine	1.51 ± 0.10 (12)**	0.68 ± 0.06 (6)†	0.52 ± 0.17 (5)*††	0.77 ± 0.13 (6)	0.75 ± 0.006 (6)

constraint on its use for the specific enzymic determination of 3-hydroxybutyrate. Valine metabolism in muscle both *in vivo* (see Palmer *et al.*, 1985) and *in vitro* (Table 1, see also Lund, 1981; Wagenmakers *et al.*, 1985; Lee & Davis, 1986) may produce 3-hydroxyisobutyrate, and to eliminate interference from 3-hydroxyisobutyrate it is therefore necessary to use an HBDH preparation free of HBDH activity in the enzymic determination of 3-hydroxybutyrate. This preparation is readily achieved by selective inactivation at 37 °C of HIBDH in the presence of 1 mM-CaCl₂. The similarities of the *M_r* values of the HBDH and HIBDH activities of *R. spheroides* precludes their separation by gel filtration. Several companies market bacterial HBDH of varying degrees of purity, and the extent, if any, to which these other commercial preparations are contaminated with HIBDH activity is not known. However, given the possible importance of 3-hydroxyisobutyrate as a product of valine metabolism, particularly in the starved state (see Palmer *et al.*, 1985), it is clear that, whatever the source of the enzyme, inactivation of the contaminating HIBDH activity is a necessary precaution before use of the enzyme to determine 3-hydroxybutyrate in blood and tissue extracts.

HIBDH may be prepared largely free (> 97%) of HBDH activity in activation at 37 °C in the presence of (NH₄)₂SO₄, but, since up to 3% of initial HBDH activity invariably remains after inactivation, constituting a major contaminant to the HIBDH activity, it is questionable whether this method can be used to yield a preparation suitable for the specific determination of 3-hydroxyisobutyrate.

We thank Andrew Worrall for undertaking n.m.r. analysis of 3-hydroxyisobutyrate, and Linda Readings for expert technical assistance. The work was supported by a grant from the Wellcome Trust.

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Received 12 September 1986/17 October 1986; accepted 29 October 1986