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

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INTRODUCTION

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.

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-Hydroxyisobutyrate 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 \( A_{280} \) on a Gilford 240 spectrophotometer, and was initiated by the addition of enzyme (50 μl). One unit of enzyme activity is defined as the amount of enzyme which catalyses conversion of 1 μmol of substrate into product/min at 25 °C.

Electrophoresis

Polyacrylamide-gel electrophoresis was performed as described by Reid & Bieleski (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/HC1 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-hydroxyisobutyrate or 3-hydroxyisobutyrate for activity staining (25 °C, approx. 2 h).

**Results of enzyme activity selectively**

(c) at BCL buffer, 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. sphaeroides* is unstable in dilute solution at 37 °C. Ca\(^{2+}\) and, to a lesser extent, Mn\(^{2+}\) 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 μg 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-Ca\(^{2+}\) 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-Mn\(^{2+}\) had a similar, albeit less marked, protective effect on HBDH activity: 30 min incubation at 37 °C in buffer containing 1 mM-MnCl\(_2\) 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 μg/ml with 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-CaCl\(_2\) resulted in substantial retention of both HBDH (75%) and HIBDH (95%) activities. Since the undialysed enzyme contains approx. 173 mm-(NH\(_4\))\(_2\)SO\(_4\), the inference was that (NH\(_4\))\(_2\)SO\(_4\) might be protective to the HIBDH activity. This conclusion is supported by the finding (Fig. 2) that addition of 200 mm-(NH\(_4\))\(_2\)SO\(_4\) 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-CaCl\(_2\) and 200 mM-(NH\(_4\))\(_2\)SO\(_4\) 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 reappearance of activity on storage at 4 °C or on precipitation with saturated NaCl or (NH\(_4\))\(_2\)SO\(_4\) (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 CaCl\(_2\) (1 mM) or (NH\(_4\))\(_2\)SO\(_4\) (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-(NH\(_4\))\(_2\)SO\(_4\)] had a pH optimum at

**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-(NH\(_4\))\(_2\)SO\(_4\)/1 mm-CaCl\(_2\), pH 7. The HBDH activity peak 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%.

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3-Hydroxyisobutyrate dehydrogenase

For details, see the Experimental section. Fractions were assayed for $A_{280}$ (A), HBDH activity (○) and HIBDH activity (□).

**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 (○) or at 37°C (△), or at 37°C in 10 mM-sodium phosphate buffer, pH 7, containing 1 mM-CaCl$_2$ (□) or 200 mM-(NH$_4$)$_2$SO$_4$ (○) or 1 mM-CaCl$_2$ + 200 mM-(NH$_4$)$_2$SO$_4$ (△). 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-(NH$_4$)$_2$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-(NH$_4$)$_2$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 BHDH or HIBDH as opposed to unmodified commercial enzyme are denoted as †P < 0.01 and ††P < 0.001.

<table>
<thead>
<tr>
<th>Addition</th>
<th>3-Hydroxybutyrate and/or 3-hydroxyisobutyrate (umol/2 h per g wet wt.)</th>
<th>Acetoacetate (and other metabolites) (umol/2 h per g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Commercial enzyme</td>
<td>HBDH</td>
</tr>
<tr>
<td>None</td>
<td>0.64±0.09 (24)</td>
<td>0.56±0.006 (12)</td>
</tr>
<tr>
<td>3 mm-Leucine</td>
<td>1.44±0.14 (12)**</td>
<td>1.36±0.19 (6)**</td>
</tr>
<tr>
<td>3 mm-Valine</td>
<td>1.51±0.10 (12)**</td>
<td>0.68±0.06 (6)†</td>
</tr>
</tbody>
</table>

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.

REFERENCES


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