Chirality of the Hydrogen Transfer to NAD
Catalyzed by (3R)Hydroxybutyrate Dehydrogenase from Pseudomonas lemoignei

Miguel A. Alizade and Karl Gaede

Instituto Venezolano de Investigaciones Científicas, IVIC, Departamento de Bioquímica, Caracas

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Chirality, Hydrogen Transfer, (3R) Hydroxybutyrate Dehydrogenase

The chirality of the hydrogen transfer from (3R)-hydroxybutyrate to NAD catalyzed by (3R)-hydroxybutyrate dehydrogenase (E.C. 1.1.1.53, 3-3-hydroxybutyrate : NAD oxidoreductase) from Pseudomonas lemoignei was investigated. [4-3H]NAD was enzymatically reduced to [4-3H]NADH with (3R, 3S)-[3-3H]hydroxybutyrate and (3R)-hydroxybutyrate dehydrogenase. From these experiments it can be concluded that (3R)hydroxybutyrate dehydrogenase from P. lemoignei should be classified as an B or (S) type dehydrogenase.

The hydrogen transfer from the substrate to the coenzyme, and vice versa, catalyzed by pyridine nucleotide-dependent oxidoreductases proceeds stereospecifically. According to their ability to catalyze hydrogen transfer to the pro(R) or pro(S) position of the C-4 prochiral center of the nicotinamide ring of the coenzyme, dehydrogenases have been classified as oxidoreductases of the A or B-type.

A number of simple tentative rules have been correlated to the observed stereochemistry of hydrogen transfer to the coenzyme catalyzed by pyridine nucleotide-linked oxidoreductases with their inducible or constitutive nature and chemical substrate structure. One rule predicted that "The overwhelming majority of NAD or NADP-linked dehydrogenase utilizing primary or secondary non-steroid alcohols or amines, which are not phosphorylated are of the A-type". To date, more than 35 pyridine nucleotide oxidoreductases fitting into this category have been investigated and only 1 exception to the aformentioned rule was found. This exception is represented by the constitutive, NAD-linked B-type (R)hydroxybutyrate dehydrogenase from Rhodopseudomonas sphaeroides. To elucidate if this exception to the proposed rule holds also true for another (R)hydroxybutyrate dehydrogenase isolated from a different bacterial genus, we decided to investigate the chirality of the constitutive NAD-linked (R)hydroxybutyrate dehydrogenase from Pseudomonas lemoignei.

[4-3H]NAD was enzymatically reduced to [4-3H]NADH with non-labelled (3R)hydroxybutyrate and (3R)hydroxybutyrate dehydrogenase from P. lemoignei. The chirality at the C-4 position of the produced [4-3H]NADH was analyzed by transfer of the hydrogen located at the B or (S) position to (S)glutamate with 2-oxoglutarate and (S)glutamate dehydrogenase, a B-type oxidoreductase. From Table I one can ascertain that less than 2% of the label originally located at the (4S) position of the generated [4-3H]NADH is transferable to 2-oxoglutarate by the reaction catalyzed by (S)glutamate dehydrogenase, remaining instead more than 90% of the label attached to the concomitantly produced NAD. Hence the label of the generated [4-3H]NADH must be located at the (4R) position of the nicotinamide ring proving therefore that the hydride transferred from non-labelled (3R)hydroxybutyrate to [4-3H]NAD catalyzed by (R)hydroxybutyrate dehydrogenase from Pseudomonas lemoignei must have entered the (4S) position of the produced (4R) [4-3H]NADH. These results were confirmed in the experimental set-up described in Table II. Non-labelled NAD was enzymatically reduced to [4-3H]NAD with (3R, 3S)-[3-3H]hydroxybutyrate and (3R)hydroxybutyrate dehydrogenase.

Specific radioactivities * [dpm/mol]

<table>
<thead>
<tr>
<th>[4-3H]NADa</th>
<th>[4-3H]NADHb</th>
<th>(S)glutamatec</th>
<th>[4-3H]NADC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1×10⁶</td>
<td>2.2×10⁶</td>
<td>3.8×10⁴</td>
<td>1.9×10⁶</td>
</tr>
<tr>
<td>1.9×10⁶</td>
<td>1.6×10⁶</td>
<td>3.1×10⁴</td>
<td>1.8×10⁶</td>
</tr>
</tbody>
</table>

* The specific radioactivities of Table I refer to the following steps:

- [4-3H]NADa + (3R)hydroxybutyrate
- acetoacetate + [4-3H]NADHb
- [4-3H]NADHb + 2-oxoglutarate
- (S)glutamatec + [4-3H]NADC
- [4-3H]NADC + (S)lactate
- pyruvate + [4-3H]NADc

Abbreviations: HBHDH, (3R)hydroxybutyrate dehydrogenase (E.C. 1.1.1.53); GDH, (S)glutamate dehydrogenase (E.C. 1.4.1.3); LDH, (S)lactate dehydrogenase (E.C. 1.1.1.27).
Table II. Stereochemistry of the hydrogen transfer from (3RS) [3-3H] hydroxybutyrate to NAD catalyzed by (3R) hydroxybutyrate dehydrogenase from *P. lemoignei*. 1.8 µmol NAD were enzymatically reduced to (4S) [4-3H]NADH with 8.5 µmol (3RS) [3-3H]hydroxybutyrate and 0.2 U (3R) hydroxybutyrate dehydrogenase from *P. lemoignei* in 3.0 ml hydrazine Tris buffer pH 8.5. After 30 min incubation at 30 °C, 1.7 µmol (S) lactate and 4 U (S) lactate dehydrogenase from rabbit muscle, were added. After another 30 min incubation, the NADH was enzymatically reduced to (S) glutamate with 8.5 µmol (S) lactate and 4 U (S) lactate dehydrogenase from rabbit.

<table>
<thead>
<tr>
<th>Specific radioactivities * [dpm/µmol]</th>
<th>Hydroxybutyrate</th>
<th>Glutamate</th>
<th>NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 × 10^7</td>
<td>2.0 × 10^7</td>
<td>2.0 × 10^7</td>
<td>4.8 × 10^4</td>
</tr>
<tr>
<td>2.2 × 10^7</td>
<td>1.9 × 10^7</td>
<td>2.1 × 10^7</td>
<td>3.2 × 10^4</td>
</tr>
</tbody>
</table>

* The specific radioactivities of Table II refer to the following steps:

\[
\begin{align*}
\text{NAD}^a & \xrightarrow{\text{H}^+} \text{acetoacetate} + (3R) [3-3H] \text{hydroxybutyrate} \\
\text{acetoacetate} & \xrightarrow{(3R) [4-3H] \text{NADH}^b} \text{2-oxoglutarate} + \text{NH}_3^+ \\
\text{2-oxoglutarate} & \xrightarrow{(S) \text{glutamate}^c} (3R) [4-3H] \text{NADH}^d \\
\text{2-oxoglutarate} & \xrightarrow{(S) \text{lactate}^e} (3R) \text{NAD}^f \\
\text{NAD}^f & \xrightarrow{\text{pyruvate}^g} (3R) \text{NADH}^h.
\end{align*}
\]

dehydrogenase from *P. lemoignei*. As expected, more than 90% of the label located at the (3S) position of the generated [4-3H]NADH can now be transferred to (S) glutamate by the reaction catalyzed by (S) glutamate dehydrogenase, remaining less than 2% of it attached to the concomitantly produced NAD. This outcome allows the classification of (3R) hydroxybutyrate dehydrogenase from *P. lemoignei* as an B or (S) type dehydrogenase.

Presently constitutive, NAD-linked (3R) hydroxybutyrate dehydrogenase isolated from *Rhodopseudomonas sphaeroides* 6 and *P. lemoignei*, both have been classified as B-type enzymes, supporting Bentley’s rule in the sense that “the chirality of an enzyme reaction is independent of the source of the enzyme” 5, and contradicting the tentative proposal that constitutive NAD or NADP-linked oxidoreductases — acting on non-phosphorylated alcohols or amines — should be of the A-type 4.

**Experimental Section**

(3R)hydroxybutyrate dehydrogenase (EC 1.1.1.30) from *Pseudomonas lemoignei* and *Rhodopseudomonas sphaeroides*, (3R)hydroxybutyrate, NAD and lactate dehydrogenase from rabbit muscle, were obtained from Sigma. [4-3H]NAD with a specific radioactivity of 50 Ci/mol and NaBH₄ with a specific radioactivity of 150 Ci/mol were purchased from New England Nuclear.

Four mg (3R)hydroxybutyrate dehydrogenase from *P. lemoignei* with a specific activity of 10 U/mg were further purified to a final specific activity of 35 U/mg by chromatography at 5 °C on a 2 × 6 cm DEAE-cellulose column in the phosphate form as described by Delafield et al. 7.

Synthesis of (3RS)[3-3H]hydroxybutyric acid: 100 µmol ethylacetocetate dissolved in 2.5 ml 0.01 M K₂P₂O₇·3H₂O – HCl buffer pH 9.5 were reduced to (3RS)[3-3H]hydroxybutyrate ethyl ester with successive additions in 30 min intervals of: first, 2 µmol non-labelled NaBH₄, second, 160 µmol NaBH₄ with a specific radioactivity of 150 Ci/mol, and third, 400 µmol non-labelled NaBH₄. Thereafter, the mixture was incubated for 24 hours at room temperature with 1 ml 40% HBr, concentrated twice in vacuo with 2 ml H₂O, and finally poured on a 1 x 30 cm Dowex-1X8, 200 – 400 mesh anion exchange column in its formate form. After washing the column with 300 ml water, the (3RS) [3-3H]hydroxybutyric acid was eluted with a linear gradient of 250 ml water and 250 ml 1 M formic acid 8. The fractions containing (3RS) hydroxybutyric acid were concentrated in vacuo, resuspended and concentrated twice with 2 ml water; 82 µmol (3RS) [3-3H]hydroxybutyric acid with a specific radioactivity of 10 Ci/mol were recovered. The specific radioactivity of the (R) enantiomer was determined incubating 0.5 µmol (3RS) [3-3H]hydroxybutyric acid with 2.0 µmol NAD and 0.2 U (3R) hydroxybutyrate dehydrogenase from *Rhodopseudomonas sphaeroides* in 3.0 ml 0.1 M hydrazine Tris buffer pH 8.5 for 30 min at 30 °C 9,10.

The generated NADH was isolated and its specific radioactivity determined as described in the Method Section.

**Isolation of [4-3H]NADH**

NADH was isolated by chromatography on a 1 × 5 cm DEAE-cellulose anion exchange column in the bicarbonate form, washing with 100 ml 3.5 M NH₄HCO₃, which displaced NAD, and elution with 10 – 15 ml 0.2 M NH₄HCO₃ 11.

**Analysis of the chirality of [4-3H]NADH**

The ³H content of the B-position of 0.30 µCi [4-3H]NADH was transferred to (S) glutamate with 2.0 µmol 2-oxoglutarate and 3 U (S) glutamate dehydrogenase from beef liver — a B type oxidoreductase 1-3 — in 1 ml of 1 M NH₄HCO₃ at a pH of 7 and 25 ºC. After the reaction had reached equilibrium, the enzyme was deactivated by heating for 1 min at 90 ºC. The (S) glutamate formed in a 0.2 ml aliquot was diluted with 2.8 mmol non-labelled (S) glutamate and recrystallized to constant specific radioactivity three times from water 12. In another 0.5 ml aliquot the specific radioactivity of the concomitantly produced NAD was determined by its reduction to NADH with 5 µmol (S) lactate and 4 U (S) lactate dehydrogenase from rabbit.
muscle in 1 ml of glycine/hydrazine/NaOH buffer pH 9.0. The generated NADH was isolated as already described and its specific radioactivity determined.

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4 M. A. Alizade and K. Brendel, Naturwissenschaften 7, 346—347 [1975].
7 E. P. Delafield, K. E. Cooksey, and M. Doudoroff, J. Biol. Chem. 240, 4023—4028 [1965].