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

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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. 1.1.1.30, D-3-hydroxybutyrate : NAD oxidoreductase) from *Pseudomonas lemoignei* was investigated. [4-^{3}H]NAD was enzymatically reduced to (4R) [4-^{3}H]NADH with (3RS) hydroxybutyrate. This observation was confirmed since NAD could be reduced to (4S) [4-^{3}H] NADH with (3RS) [3-^{3}H]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 [1–3].

A number of simple tentative rules have been proposed to correlate 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 [1–4]. One rule predicted [4] that "The overwhelming majority of NAD or NADP-linked dehydrogenases 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 afore mentioned rule was found [1–5]. This exception is represented by the constitutive, NAD-linked B-type (R) hydroxybutyrate dehydrogenase from *Rhodopseudomonas sphaeroides* [6]. 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-^{3}H]NAD was enzymatically reduced to [4-^{3}H]NADH with non-labelled (3R) hydroxybutyrate and (3R) hydroxybutyrate dehydrogenase from *P. lemoignei*. The chirality at the C-4 position of the produced [4-^{3}H]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-^{3}H]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-^{3}H]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-^{3}H]NAD catalyzed by (R) hydroxybutyrate dehydrogenase from *Pseudomonas lemoignei* must have entered the (4S) position of the produced (4R) [4-^{3}H]NADH. These results were confirmed in the experimental set-up described in Table II. Non-labelled NAD was enzymatically reduced to [4-^{3}H]NADH with (3R, 3S) [3-^{3}H]hydroxybutyrate and (3R) hydroxybutyrate

Table I. Stereochemistry of the hydrogen transfer from (3R) hydroxybutyrate to [4-^{3}H]NAD catalyzed by (3R) hydroxybutyrate dehydrogenase from *P. lemoignei*. 2.1 μmol [4-^{3}H]NAD were enzymatically reduced to (4R) [4-^{3}H] NADH with 12 μmol (3RS) hydroxybutyrate and 0.2 U (3R) hydroxybutyrate dehydrogenase from *P. lemoignei* in a total volumen of 3.0 ml of hydrazine Tris buffer pH 8.5. After 30 min incubation at 30 °C 1.9 μmol [4-^{3}H]NADH were isolated as described in the Methods Section.

Specific radioactivities * [dpm/mol]			
[4-^{3}H]NAD[a]	[4-^{3}H]NADH[a]	(S) glutamate[b]	[4-^{3}H]NADH[c]
2.1×10^6	2.2×10^6	3.8×10^4	1.9×10^6
1.9×10^6	1.6×10^6	3.1×10^4	1.8×10^6

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

$$\text{[4-}^3\text{H]NAD}^a + \text{(3R) hydroxybutyrate} \underset{\text{HBDH}}{\rightleftharpoons} \text{acetoacetate} + \text{[4-}^3\text{H]NADH}^a$$

$$\text{[4-}^3\text{H]NADH}^a + \text{2-oxoglutarate} \underset{\text{GDH}}{\rightleftharpoons} \text{(S) glutamate}^b + \text{[4-}^3\text{H]NAD}^c$$

$$\text{[4-}^3\text{H]NAD}^c + \text{(S) lactate} \underset{\text{LDH}}{\rightleftharpoons} \text{pyruvate} + \text{[4-}^3\text{H]NADH}^c.$$

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Abbreviations: HBDH, (3R) hydroxybutyrate dehydrogenase E.C. 1.1.1.30); 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-^{3}H]hydroxybutyrate to NAD catalyzed by (3R) hydroxybutyrate dehydrogenase from *P. lemoignei*. 1.8 μmol NAD were enzymatically reduced to (4S) [4-^{3}H]NADH with 8.5 μmol (3RS) [3-^{3}H]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 (4S) [4-^{3}H]NADH were isolated.

Specific radioactivities * [dpm/mol]			
(3RS) [3-^{3}H] Hydroxybutyrate	[4-^{3}H]NADH[a]	(S) Glutamate[b]	NADH[c]
2.2×10^7	2.0×10^7	2.0×10^7	4.8×10^4
2.2×10^7	1.9×10^7	2.1×10^7	3.2×10^5

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

NAD[a] + (3RS) [3-^{3}H]hydroxybutyrate
$\xrightleftharpoons{\text{HBDH}}$ acetoacetate + [4-^{3}H]NADH[a]

[4-^{3}H]NADH[a] + 2-oxoglutarate + NH_3
$\xrightleftharpoons{\text{GDH}}$ (S) glutamate[b] + [4-^{3}H]NAD[c]

[4-^{3}H]NAD[c] + (S) lactate
$\xrightleftharpoons{\text{LDH}}$ pyruvate + [4-^{3}H]NADH[c].

dehydrogenase from *P. lemoignei*. As expected, more than 90% of the label located at the (4S) position of the generated [4-^{3}H]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 spheroides* [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" [3], 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 spheroides*, (3R) hydroxybutyrate, NAD and lactate dehydrogenase from rabbit muscle, were obtained from Sigma. [4-^{3}H]NAD with a specific radioactivity of 50 Ci/mol and NaB^3H_4 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-^{3}H]hydroxybutyric acid: 100 μmol ethylacetoacetate dissolved in 2.5 ml 0.01 M $K_4P_2O_7\cdot3H_2O$ – HCl buffer pH 9.5 were reduced to (3RS) [3-^{3}H]hydroxybutyrate ethyl ester with successive additions in 30 min intervals of: first, 2 μmol non-labelled NaBH, second, 160 μmol $NaBH_4$ with a specific radioactivity of 150 Ci/mol, and third, 400 μmol non-labelled $NaBH_4$. Thereafter, the mixture was incubated for 24 hours at room temperature with 1 ml 40% HBr, concentrated twice in vacuo with 2 ml H_2O, and finally poured on a 1×30 cm Dowex-1X8, 200 – 400 mesh anion exchange column in its formate form. After washing the column with 300 ml water, the (3RS) [3-^{3}H]hydroxybutyric acid was eluted with a linear gradient of 250 ml water and 250 ml 1 N formic acid [8]. The fractions containing (3RS) hydroxybutyric acid were concentraetd in vacuo, resuspended and concentrated twice with 2 ml water; 82 μmol (3RS) [3-^{3}H]hydroxybutyric acid with a specific radioactivity of 10 Ci/mol were recovered. The specific radioactivity of the (R) enantiomer was determinated incubating 0.5 μmol (3RS) [3-^{3}H]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-^{3}H]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 mM NH_4HCO_3, which displaced NAD, and elution with 10 – 15 ml 0.2 M NH_4HCO_3 [11].

Analysis of the chirality of [4-^{3}H]NADH

The ^{3}H content of the B-position of 0.30 μmol [4-^{3}H]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 NH_4HCO_3 at a pH of 7 and 25 °C. After the reaction had reached equilibrium, the enzyme was deactivated by heating for 1 min at 90 °C. 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 [13]. The generated NADH was isolated as already described [11] and its specific radioactivity determined.

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