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, Departemento de Bioquímica, Caracas

(Z. Naturforsch. **32 c**, 874–876 [1977]; received May 6/July 11, 1977)

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:

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

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

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

Requests for reprints should be sent to Dr. Miguel Alizade, IVIC, CBB, Apartado 1827, *Caracas-101*, Venezuela.

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:

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

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

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

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.

Work was supported by the Regional Program of Scientific and Technological Development of the Organization of the American States (OAS) and the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT), Venezuela.

[1] B. Vennesland, Topics in Current Chemistry, **Vol. 48,** pp. 39–45, Springer Verlag, Berlin, New York 1974.
[2] G. Popjack, The Enzymes (P. D. Boyer, ed.), **Vol. 2,** pp. 115–215 [1970].
[3] R. Bentley, Molecular Assymetry in Biology, **Vol. 2,** pp. 1–89, Academic Press, New York 1970.
[4] M. A. Alizade and K. Brendel, Naturwissenschaften **7,** 346–347 [1975].
[5] A. Kraus and H. Simon, Hydrogen Transfer Processes, **Vol. 2** (E. Buncel, and C. C. Lee, eds.), Elsevier, Amsterdam 1976.
[6] N. Savage and M. J. Weight, Biochim. Biophys. Acta **159,** 401–403 [1968].
[7] E. P. Delafield, K. E. Cooksey, and M. Doudoroff, J. Biol. Chem. **240,** 4023–4028 [1965].
[8] H. Bush, R. B. Hurlbert, and V. B. Potter, J. Biol. Chem. **196,** 717–721 [1952].
[9] H. U. Bergmeyer, K. Gawehn, H. Klotzsch, H. A. Krebs, and D. H. Williamson, Biochem. J. **102,** 423–431 [1967].
[10] D. H. Williamson and J. Mellanby, Methods of Enzymatic Analysis (H. U. Bergmeyer, ed.), 2nd ed. pp. 1836–1837, Academic Press, New York 1974.
[11] E. Silverstein, Anal. Biochem. **12,** 199–212 [1965].
[12] M. A. Alizade, R. Bressler, and K. Brendel, Biochim. Biophys. Acta **364,** 204–208 [1974].
[13] I. Gutmann and W. Wahlefeld, in loc. cit. 10, 1464–1468.