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STEREOCHEMISTRY OF THE HYDROGEN TRANSFER TO NAD CATALYZED BY D-GALACTOSE DEHYDROGENASE FROM *PSEUDOMONAS FLUORESCENS*

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Summary

The stereochemistry of the hydrogen transfer to NAD catalyzed by D-galactose dehydrogenase (E.C. 1.1.1.48) from *P. fluorescens* was investigated. The label at C-1 of D-[1-^{3}H]galactose was enzymatically transferred to NAD and the resulting [4-^{3}H]NADH was isolated and its stereochemistry at C-4 investigated. It was found that the label was exclusively located at the 4(S) position in NADH which calls for classification as a B-enzyme. This result was confirmed by an alternate approach in which [4-^{3}H]NAD was reduced by D-galactose as catalyzed by D-galactose dehydrogenase. The stereochemistry at C-4 of the nicotinamide ring would then have to be opposite to that in the first experiment. As expected, the label was now exclusively located in the 4(R) position, again confirming the B-classification of the enzyme.

In an extension of the rules formulated by Vennesland et al. [1] and Bentley [2,3] as to the correlation of certain substrate characteristics with the stereospecificity of the corresponding enzyme, we have recently formulated a set of additional rules linking substrate structure, the inducible or constitutive nature of the enzyme and its specificity for the coenzyme with the stereochemistry of the enzyme catalyzed transfer of hydrogen to the coenzyme [11]. One of our rules predicted that "all inducible NAD linked dehydrogenases from the *Pseudomonas* genus are of the B-type" [4]. Another rule stated that in addition, "all dehydrogenases transferring hydrogen to both coenzymes, NAD and NADP, are of the B-type." As long as the enzyme activity can be kinetically followed no other limitations were imposed.

It was interesting to see what an inducible enzyme of the *Pseudomonas* genus, not specific for NAD but able to work with NAD and NADP, would do in respect to the stereospecificity of hydrogen transfer. One might postulate that the enzyme should again be of the B-type. No inducible dehydrogenase

which lacks specificity in regard to the coenzyme has ever been investigated from *Pseudomonas* in regard to the stereospecificity of the hydrogen transfer to the coenzyme. Recently the purification and description of the kinetic properties of D-galactose dehydrogenase (E.C. 1.1.1.48) from *P. fluorescens* was reported [6]. Similar to D-galactose dehydrogenase from *P. saccharophila* [7] the enzyme isolated from *P. fluorescens* [6] is induced in the presence of D-galactose as sole source of carbon in the media [5—7]. However, the enzyme from *P. saccharophila* is specific for NAD [7]; whereas, the one isolated from *P. fluorescens* can utilize both NAD and NADP [6].

Materials and Methods

D-Galactose dehydrogenase (D-galactose : NAD 1-oxidoreductase, EC 1.1.1.48) from *P. fluorescens*, (S)glutamate dehydrogenase (EC 1.4.1.3) from beef liver, and (S)lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle were purchased from Boehringer Mannheim Corporation. NAD, (S) lactic acid and D-galactose were obtained from Sigma Chemical Co., and (S)glutamic acid from The Matheson Co. The labeled substrates D-[1-^{3}H]galactose with a specific radioactivity of 5.9 Ci/mol and [4-^{3}H]NAD with a specific radioactivity of 50 Ci/mol were purchased from New England Nuclear and the Radiochemical Centre, respectively.

Stereochemistry of the enzymic hydrogen transfer from D-galactose to [4-^{3}H]NAD

0.80 μmol [4-^{3}H]NAD with a specific radioactivity of $1.1 \cdot 10^8$ dpm/μmol were enzymatically reduced to [4-^{3}H]NADH with 110 μmol of unlabeled D-galactose in 3.0 ml of 0.1 M Tris · HCl at pH 9.1 and 30°C. The reaction was started with 0.1 unit D-galactose dehydrogenase. After incubation for 40 min, 0.77 μmol of NADH were isolated and the stereochemistry at C-4 determined. Table I summarizes the results.

Stereochemistry of the enzymic hydrogen transfer from D-[1-^{3}H]galactose to NAD

0.0072 μmol D-[1-^{3}H]galactose with a specific radioactivity of $1.30 \cdot 10^{10}$ dpm/μmol was diluted with 0.6 μmol of unlabeled D-galactose and the

TABLE I

STEREOCHEMISTRY OF THE ENZYMIC HYDROGEN TRANSFER FROM D-GALACTOSE TO [4-^{3}H]NAD CATALYZED BY D-GALACTOSE DEHYDROGENASE

Specific radioactivities (dpm/μmol):			
[4-^{3}H]NAD[1]	[4-^{3}H]NADH[1]	(S)glutamate[2]	[4-^{3}H]NADH[3]
$1.1 \cdot 10^8$	$1.09 \cdot 10^8$	$7.8 \cdot 10^5$	$1.12 \cdot 10^8$

The specific radioactivities of Table I refer to the following steps in the overall reaction scheme:

[4-^{3}H]NAD[1] + D-galactose $\overset{\text{GalDH}}{\rightleftharpoons}$ D-galactonate + [4-^{3}H]NADH[1]

[4-^{3}H]NADH[1] + α-ketoglutarate + NH_3 $\overset{\text{(S)GDH}}{\rightleftharpoons}$ (S)glutamate[2] + [4-^{3}H]NAD

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

TABLE II

STEREOCHEMISTRY OF THE ENZYMIC HYDROGEN TRANSFER FROM d-[1-^{3}H]GALACTOSE TO NAD CATALYZED BY D-GALACTOSE DEHYDROGENASE

Specific radioactivities (dpm/μmol):			
D-[1-^{3}H]galactose[1]	[4-^{3}H]NADH[1]	(S)[2-^{3}H]glutamate[2]	NADH[3]
$1.5 \cdot 10^8$	$1.4 \cdot 10^8$	$1.5 \cdot 10^8$	$1.1 \cdot 10^6$

The specific radioactivities of Table II refer to the following steps in the overall reaction scheme:

$$\text{D-[1-}^3\text{H]galactose}^1 + \text{NAD} \overset{\text{GalDH}}{\rightleftharpoons} \text{D-galactonate} + \text{[4-}^3\text{H]NADH}^1$$

$$\text{[4-}^3\text{H]NADH}^1 + \alpha\text{-ketoglutarate} + \text{NH}_3 \overset{\text{(S)GDH}}{\rightleftharpoons} \text{(S)[2-}^3\text{H]glutamate}^2 + \text{NAD}$$

$$\text{NAD} + \text{(S)lactate} \overset{\text{(S)LDH}}{\rightleftharpoons} \text{NADH}^3 + \text{pyruvate}$$

hydrogen content at C_1 enzymatically transferred to NADH with 27 μmol of NAD. The reaction was performed at 30°C in 3.0 ml of 0.1 M Tris · HCl buffer of pH 9.1 using 0.1 units D-galactose dehydrogenase. After 40 min 0.58 μmol [4-^{3}H]NADH were isolated and the stereochemistry at C-4 determined. Table II summarizes the results.

Isolation of NADH

The NADH produced was isolated by placing the media onto a 1 × 5 cm DEAE-cellulose ion exchange column in the bicarbonate form. Elution with 100 ml of 3.5 mM NH_4HCO_3 displaced D-galactose and NAD, while the NADH was eluted with 10—15 ml of 0.2 M NH_4CO_3 [8]. This latter eluate was concentrated in vacuo at 40°C, taken up in 2—3 ml water and enzymatically determined with 1.2 μmol of sodium pyruvate and 6 units of (S)lactate dehydrogenase per ml in 1 M NH_4HCO_3 (pH 7 at 25°C) [9].

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

The ^{3}H content of the (S)position at C_4 (0.35 to 0.40 μmol [4-^{3}H]NADH) was transferred to (S)glutamate with 1.8 μmol α-ketoglutarate and 3 units (S)glutamate dehydrogenase (a B-enzyme) in 1 ml of 1 M NH_4HCO_3 at pH 7 and 25°C. After the reaction had reached equilibrium which took less than 5 min, the enzyme was deactivated by heating for 1 min at 90°C, and the incubation mixture divided into two parts in order to separately determine the specific radioactivities of the concommitantly produced (S)glutamate and NAD. The specific radioactivity of the (S)glutamate produced was determined by diluting it with 2.8 mmol of unlabeled (S)glutamic acid and recrystallization from water three times to constant specific radioactivity. In a second experiment, to another aliquot of the original solution containing 0.2 to 0.3 μmol NAD, was added 25 μmol of non-labeled (S)lactate, and the NAD reduced to NADH with 6 units (S)lactate dehydrogenase (an A-enzyme) in 0.5 M glycine/hydrazine buffer of pH 9.5 [10]. The NADH produced was isolated as described above.

Results and Discussion

The results summarized in Tables I and II show that when hydrogen from D-galactose is transferred to [4-^{3}H]NAD the label is located at the 4-(R) posi-

tion of the NADH produced. Since the transferred hydrogen must be located at the opposite or 4-(S) side of the NADH formed, it can be concluded that D-galactose dehydrogenase from *P. fluorescens* should be classified as a B-type enzyme in regard to the stereochemistry of the hydrogen transfer to NAD. This conclusion is supported by the results summarized in Table II in which the hydrogen transfer from D-[1-^{3}H]galactose to NAD was further investigated. It was found that in this case the transferred label is exclusively located at the 4(S) position of the isolated NADH. This result is in agreement with the above conclusion that D-galactose dehydrogenase from *P. fluorescens* is a B-enzyme. In addition, both Tables I and II also show that similar to most NAD(P) linked dehydrogenases investigated until now [2,3], the hydrogen transfer to the coenzyme is direct and does not involve the protium of the surrounding media.

Other inducible dehydrogenases in the genus Pseudomonas which are specifically linked to NAD have also been shown to be B-enzymes, such as 3α, 3β, and 17β steroid dehydrogenases (EC 1.1.1.50, 1.1.1.51 and 1.1.1.63) from *P. testosteroni* [2,3] and (R)carnitine dehydrogenase (EC 1.1.1.108) from *P. aeruginosa* [4]. We have advanced the thesis that all inducible NAD linked dehydrogenases in the genus *Pseudomonas* are B-enzymes [4]. The results presented in this communication indicate that this rule may be expanded to all inducible dehydrogenases of the Pseudomonas genus regardless of their coenzyme specificity.

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