THE STEREOCHEMICAL COURSE OF THE HYDROGEN TRANSFER TO NAD, CATALYZED BY BACTERIAL GLUCOSE DEHYDROGENASE AND HYDROGENASE OF *ALCALIGENES EUTROPHUS* H 16

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Received 20 December 1976

1. Introduction

Glucose dehydrogenase from *Bacillus* species [1] and soluble hydrogenase from *Alcaligenes eutrophus* H 16 [2,3] which became recently available may be of interest for several practical aspects. Glucose dehydrogenase from *Bacillus subtilis* is much more active than the enzyme enriched from bovine liver [4].

In contrast to other hydrogenases such as those from Clostridia [5] hydrogenase from *Alcaligenes eutrophus* H 16 catalyzes the reaction

$$H_2 + NAD^+ \rightleftharpoons NADH + H^+$$

without additional cofactors or electron carriers. In the last years many pyridine nucleotide depending enzymes have been characterized with regard to their stereochemistry of hydrogen transferred to the coenzyme and some tentative rules were first formulated by Vennesland et al. [6] and later by others [7]. In the relative long list of the stereochemistry of pyridine nucleotide dependent dehydrogenases [8] so far only one example of a hydrogenase is described.

Bone [9] reduced NAD^+ with tritium-labeled hydrogen gas by hydrogenase from *Pseudomonas ruhlandii*. Tritium was observed in malate when the resulting [4-^{3}H]NADH was incubated with malate dehydrogenase and oxaloacetate. No tritium could be detected when the [4-^{3}H]NADH was used to transform α-ketoglutarate with glutamate dehydrogenase to glutamate. Therefore S-stereospecificity has been assumed. As only qualitative statements were made by Bone [9] we assume that noticeable hydrogen-exchange took place between the gas phase and the protons of the water.

For the glucose dehydrogenase from bovine liver S-stereospecificity was found [10]. We determined the stereospecificity of the above mentioned enzymes.

2. Materials and methods

Alcohol dehydrogenase (EC 1.1.1.1), glucose dehydrogenase (EC 1.1.1.47), glutamate dehydrogenase (EC 1.4.1.3) and NAD^+ grade I (obtained from Boehringer Mannheim GmbH) were used without further purification. Hydrogenase (EC 1.12.1.2) was purified from *Alcaligenes eutrophus* according to Schneider et al. [3]. Omitting the last purification step we obtained a specific activity of 18 U/mg.

2.1. *Preparation of 4 R [4-^{3}H] NADH and 4 S [4-^{3}H] NADH*

Pyrophosphate buffer, 5.0 ml, 0.1 M, pH 8.8 (containing 85 μmol glycine and 31 μmol semicarbazide) 45 μmol NAD^+, 15 μmol glutathione, 4.5 U alcohol dehydrogenase and 10 μl [1-^{3}H]ethanol (specific radioactivity 3.25×10^6 dpm/μmol) were incubated for 5 h at room temperature. The reaction was stopped by heating and the mixture was separated on a DEAE–Sephadex column. The [4-^{3}H]NADH was eluted with a 0.01–0.4 M $KHCO_3$-gradient, desalted by ultrafiltration and lyophilized (specific radioactivity 4.2×10^5 dpm/μmol).

The S-enantiomer of [4-^{3}H]NADH was obtained by the incubation of 54 μmol D-[1-^{3}H]glucose (specific radioactivity 4.4×10^6 dpm/μmol), 16 μmoles NAD^+

and 3 U of glucose dehydrogenase from *Bacillus subtilis* in 5.0 ml 0.05 M Tris–HCl, pH 8.2, at room temperature. After 150 min the reaction was stopped and [4-^{3}H]NADH was isolated as described above. The 4 S [4-^{3}H]NADH contained 6.6 × 10^5 dpm ^{3}H/μmol.

2.2. *Enzymatic reduction of α-ketoglutarate with [4-^{3}H]NADH obtained from D-[1-^{3}H]glucose and glucose dehydrogenase*

In 1.0 ml 0.05 M phosphate buffer, pH 7.0, 36 U glutamate dehydrogenase, 0.28 μmol [4-^{3}H]NADH with a total radioactivity of 1.86 × 10^5 dpm and 1.0 μmol α-ketoglutarate were incubated. The reaction course was followed at 334 nm and stopped after 30 min.

The experiments with hydrogenase are described in table 1.

3. Results

Nearly no tritium was found in the volatile fraction after incubation of 4 R [4-^{3}H]NADH with hydrogenase as shown in the table exp. 1 and 2.

Since the question arose whether an exchange takes place under these conditions we incubated a similar amount of NAD^+ together with 4 R [4-^{3}H]NADH. After 1 h about 4% tritium was volatile (exp. 3).

To prove that 4 R [4-^{3}H]NADH was still reactive after the incubation with hydrogenase acetaldehyde 50 μmol, 5.5 mg glutathione and alcohol dehydrogenase (4.5 U) were added to the reaction mixture of exp. 1. After 30 min 90% of the tritium was volatile as can be explained by the formation of tritium-labeled ethanol.

When [4-^{3}H]NADH obtained according to the reaction

$$[1\text{-}^3\text{H}]\text{glucose} + \text{NAD}^+ \xrightleftharpoons[\text{dehydrogenase}]{\text{glucose}}$$

4 S [4-^{3}H]NADH + gluconate (1)

was incubated with hydrogenase and NAD^+ 96% of the tritium was volatile after 1 h as shown in exp. 4. This reveals that by reaction (1) 4 S [4-^{3}H]NADH has been formed which reacts with hydrogenase as follows:

$$4\text{ S }[4\text{-}^3\text{H}]\text{NADH} + \text{NAD}^+ + \text{HOH} \xrightleftharpoons{\text{hydrogenase}}$$

$$^3\text{HOH} + \text{NADH} + \text{NAD}^+ \quad (2)$$

However if the NAD^+ is omitted and a hydrogen atmosphere is used the exchange proceeds much slower. Even after 10 h, still 37% of the tritium of 4 S [4-^{3}H] NADH is not exchanged. This shows that hydrogenase in the reduced state catalyses only a slow exchange of NADH with protons of the medium.

In order to prove the stereochemical course of the glucose dehydrogenase independently we used the [4-^{3}H]NADH obtained according reaction (1) for the reduction of α-ketoglutarate, by another reaction. The glutamate formed contained 95% of the tritium originally present in [4-^{3}H]NADH.

These results reveal an unequivocal stereospecificity of the hydrogen-transfer to NAD^+ catalyzed by hydrogenase and glucose dehydrogenase from *Bacillus subtilis*. Both enzymes show S-specificity.

Table 1

Exp. No.	[4-^{3}H]NADH (μmol)	Chirality	Hydrogenase (mU)	Gas atmosphere	NAD (μmol)	Percent tritium	
						Volatile with H_2O	Not volatile
1	13.0	R	36	H_2	0	0.3	99
2	4.2	R	724	H_2	0	0.7	99
3	0.60	R	180	N_2	0.42	4.0	96
4	0.25	S	180	N_2	0.21	91.5	8.5
5[a]	0.56	S	180	H_2	0	62.0	37[a]

[a] Reaction time 10 h

A total volume of 2.54 ml contained 125 μmol Tris–HCl buffer, pH 8.2 and the components as presented for the different experiments. Specific radioactivities of the [4-^{3}H]NADH 4.2 × 10^5 dpm/μmol and 6.6 × 10^5 dpm/μmol. Reaction time 1 h, temperature 33°C.

Acknowledgements

We wish to thank Professor H. Schlegel and Drs H. Brinkmann and K. Schneider for generous gifts of *Alcaligenes eutrophus* as well as for the preprint of the procedure for the hydrogenase purification and Dr F. P. Schmidtchen for 4 R [4-^{3}H]NADH. Financial support was given by Bundesministerium für Forschung und Technologie.

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