STEREOSPECIFICITY OF HYDROGEN REMOVAL FROM PYRIDINE NUCLEOTIDE: THE REACTIONS CATALYZED BY NITRATE REDUCTASE AND BY XANTHINE OXIDASE

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1. Introduction

The generalization has been made that pyridine nucleotide-linked dehydrogenases that catalyze the same reaction show the same stereospecificity for hydrogen removal from the reduced 4-position of the nicotinamide moiety of the pyridine nucleotide [1–3]. The present study presents evidence that the NAD(P): nitrate oxidoreductases constitute an exception to this rule. Davies et al. [1] have reported that NADPH: nitrate oxidoreductase (EC 1.6.6.3) from the yeast *Candida utilis* catalyzes the removal of hydrogen from the B (or pro S) position of the NADPH. The present study describes experiments which show that the NADH: nitrate oxidoreductase (EC 1.6.6.2) of *Chlorella vulgaris* uses the opposite A (or pro R) position. Evidence will also be presented that xanthine oxidase from bovine milk has B stereospecificity for NADH when either nitrate or oxygen is used as the oxidant.

2. Materials and methods

Alcohol dehydrogenase (EC 1.1.1.1) from yeast, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle, and xanthine oxidase (EC 1.2.3.2) from bovine milk were purchased from Boehringer. A partially purified sample of NADH: nitrate oxidoreductase (EC 1.6.6.1) from *Chlorella vulgaris* (10 units per mg) was a gift from Dr L. P. Solomonson.

4-[^{3}H] nicotinamide adenine dinucleotide, hereafter called [^{3}H] NAD^+, (50 mCi/mmol), was obtained from Amersham Buchler, Braunschweig. Radioactivity measurements were made with a Nuclear Chicago ISOCAP/300 Scintillation Counter. Up to 0.5 ml of aqueous sample was added to 15 ml of scintillation fluid prepared by dissolving 4 g of 2,5-diphenyloxazole (PPO) in 400 ml of ethanol and 600 ml of toluene. The efficiency of counting was about 15%.

A-labelled [^{3}H] NADH, i.e. 4R-4[^{3}H] NADH, was prepared by incubating at 25°C in a vol of 1 ml: [^{3}H] NAD^+ (0.1 μCi, 6 μmol), glyceraldehyde 3-phosphate (40 μmol), $NaHAsO_4$ (5 μmol), Tris–HCl buffer, pH 8.0 (100 μmol), and glyceraldehyde 3-phosphate dehydrogenase (70 units). After 10 min, the reaction was complete, the reaction mixture was diluted tenfold with water, and the [^{3}H] NADH was isolated by chromatography on a Whatman DE-52 DEAE-cellulose column (0.9 cm × 6 cm), as described by Davies et al. [1]. B-labelled [^{3}H] NADH, i.e. 4S-4-[^{3}H] NADH, was obtained by incubating at 25°C in a vol of 1 ml: [^{3}H] NAD^+ (0.1 μCi, 6 μmol), ethanol (35 mmol), semicarbazide (1 mmol), Tris–HCl buffer, pH 8.0 (100 μmol) and alcohol dehydrogenase (300 units). After 5 min the reaction was complete, and the [^{3}H] NADH was isolated as previously described.

For determination of the stereospecificity of the nitrate reductase from *Chlorella vulgaris*, the reaction mixtures contained, in a volume of 1 ml: A- or B-labelled [^{3}H] NADH (4 nCi, 0.2 μmol), KNO_3 (10 μmol), Tris–HCl buffer, pH 8.0 (100 μmol), and nitrate reductase (0.5 units). When the stereospecificity of the diaphorase activity was tested, cytochrome *c* (0.5 μmol) or 2,6-dichlorophenolindophenol (1 μmol) were substituted for the KNO_3. After complete oxidation of the NADH (2 or 3 min), the reaction mixture was diluted tenfold with water, and the

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solution was passed through a column (0.9 cm × 6 cm) of Whatman DE-52 DEAE-cellulose, equilibrated with 50 mM Tris–HCl buffer, pH 8.0 [1]. Fractions of 2 ml were collected and their absorbance at 260 nm and radioactivity were measured.

Experiments with xanthine oxidase were carried out in Warburg vessels at 25°C. The reaction mixtures contained, in a volume of 1.5 ml: A- or B-labelled [^{3}H] NADH (4 nCi, 0.2 μmol), KNO_3 (20 μmol), Tris–HCl buffer, pH 8.0 (100 μmol) and xanthine oxidase (1.2 units). The vessels were flushed with argon for 10 min, and the reactions were started by adding the KNO_3 and the [^{3}H] NADH from a side arm. After 30 min, the NADH was completely oxidized, and the products were examined as previously described. When oxygen was employed as the oxidant, the reactions were carried out in air, and the KNO_3 was omitted. The xanthine oxidase preparation catalyzed the oxidation of NADH by nitrate or by oxygen at about the same rates.

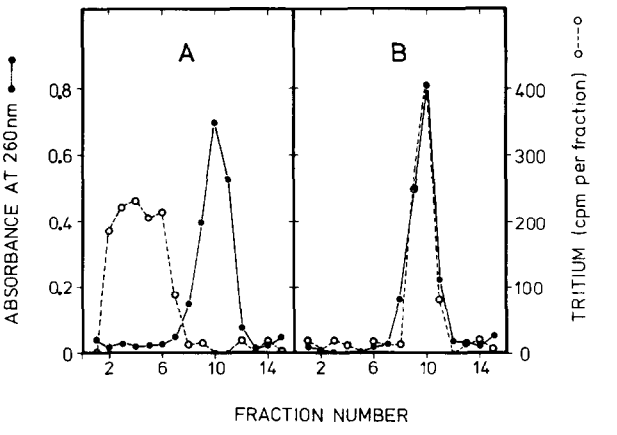

Fig.1. Determination of stereospecificity of *Chlorella vulgaris* nitrate reductase. A: A-labelled [^{3}H]NADH was used as substrate; B: the substrate was the B-labelled [^{3}H]NADH. Other experimental conditions are described under Materials and methods.

3. Results and discussion

When NADH is oxidized enzymatically by nitrate, the hydrogen atom which is removed from the NADH, appears in the water. Passage of the reaction mixture through the Whatman DE-52 DEAE-cellulose column results in clear separation of the water, which emerges first, and the NAD^+ which is eluted later, and is located by absorbance measurements at 260 nm. Results of experiments in which tritium-labelled NADH was oxidized by nitrate in the presence of nitrate reductase of *Chlorella vulgaris*, are shown in fig.1. When A-labelled [^{3}H] NADH was employed, the tritium was removed from the NAD^+ formed, and appeared in the water, whereas it was retained in the NAD^+ formed when B-labelled NADH was oxidized. The nitrate reductase of *Chlorella vulgaris* is clearly specific for the A position of the reduced pyridine nucleotide.

Like other nitrate reductases of the same type [4], this enzyme also has diaphorase activity; that is, it catalyzes the oxidation of NADH when a variety of oxidants, such as cytochrome *c* or ferricyanide or various redox dyes are substituted for nitrate [5]. In an experiment in which cytochrome *c* was employed as the oxidant, 93% of the tritium remained in the NAD^+ formed on oxidation of B-labelled [^{3}H] NADH. In a similar experiment with dichlorophenolindophenol as oxidant, 100% of the tritium remained in the NAD^+ formed from B-labelled [^{3}H] NADH. Thus, the diaphorase activity of the enzyme has the same A stereospecificity for the NADH as the nitrate reductase activity, in keeping with the assumption that the same enzyme reaction center activates the NADH, whether the oxidant is nitrate or cytochrome *c* or a dye.

Like nitrate reductase, xanthine oxidase is a molybdoflavoprotein; and it has, in fact, NADH: nitrate oxidoreductase activity, though the turnover number of the reaction is relatively low [6]. The stereospecificity of xanthine oxidase for NADH was determined with nitrate, and with oxygen as oxidants. The results, summarized in table 1, show that this enzyme removes most of the tritium from the B-labelled [^{3}H] NADH, but not from the A-labelled [^{3}H] NADH, whether nitrate or oxygen was employed to oxidize the NADH. Thus xanthine oxidase has B-stereospecificity for NADH. A previous, preliminary report that this enzyme is not stereospecific for NADH [7] can be attributed to the fact that a mixed reaction was probably obtained in the previous experiments, in which NADH was oxidized in the presence of oxygen and a dye, added to increase the slow reaction rate in oxygen.

The stereospecificity of the nitrate reductase of the yeast *Candida utilis* has been shown to be B [1].

Table 1
Stereospecificity of bovine milk xanthine oxidase for hydrogen removal from NADH

Electron acceptor	Radioactivity of NAD^+ (% of total)	
	Substrate: A-[^{3}H]NADH	Substrate: B-[^{3}H]NADH
Nitrate	98.3	1.6
Oxygen	98.1	6.1

For experimental conditions see Materials and methods.

This is in contrast to the results here found for the *Chlorella* enzyme, and shows that nitrate reductases must be divided in two groups of opposite stereospecificity. All of the pyridine nucleotide: nitrate oxidoreductases examined to date have been found to be molybdoflavoproteins, but some of these enzymes (such as the one from *Neurospora* [8] or the one from *Chlorella* [5]) contain heme in addition, whereas others (such as xanthine oxidase [6]) apparently contain non-heme iron [9]. In the case of the *Aspergillus* enzyme, the presence or absence of heme has been disputed [9,10]. Perhaps *Aspergillus* contains two different nitrate reductases. A more extensive, comparative study of these enzymes is required to determine whether the stereospecificity can be corelated with other properties of the enzyme, such, for example, as the presence of heme or of non-heme iron or with any other property which can be measured.

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