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THE STEREOSPECIFICITY OF NITRATE REDUCTASE FOR HYDROGEN REMOVAL FROM REDUCED PYRIDINE NUCLEOTIDES

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Summary

The stereospecificity of the hydrogen removal from reduced pyridine nucleotides catalyzed by nitrate reductase (NADH : nitrate oxidoreductase, EC 1.6.6.1, and NAD(P)H : nitrate oxidoreductase, EC 1.6.6.2) was investigated. A high degree of enzyme purification was required to obtain conclusive results. Improvements are described for the purification of nitrate reductase from *Chlorella fusca* and from spinach (*Spinacea oleracea*, L.) leaves. The latter enzyme is shown to contain a cytochrome. With highly purified nitrate reductase preparations from *Cl. fusca*, *Neurospora crassa*, *Rhodotorula glutinis* and spinach leaves the stereospecificity of the reaction was determined to be predominantly of the A-type in all cases.

Introduction

Many generalizations regarding the stereospecificity of reactions catalyzed by pyridine nucleotide dehydrogenases have been proposed [1—5]. The one which states that enzymes which catalyze the same reaction have the same stereospecificity regardless of the source has been shown to be true in practically all the cases where tested. Only the pyridine nucleotides transhydrogenases [6] and, more recently, the nitrate reductases [7] have been reported to constitute exceptions to the general rule.

Davies et al. [3] reported stereospecificity of the B-type for the removal of hydrogen from NADPH catalyzed by the NADPH : nitrate reductase (EC 1.6.6.3) of the yeast *Candida utilis*. Opposite results were obtained for the NADH : nitrate oxidoreductase from the green algae *Chlorella vulgaris* (EC 1.6.6.1) which exhibited A-stereospecificity with respect to NADH, whereas

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bovine liver xanthine oxidase (EC 1.2.3.2) was shown to have B-stereospecificity for NADH for the reaction of reduction of nitrate [7]. The occurrence of these discrepancies and the fact that only a few nitrate reductases had been examined so far for stereospecificity, prompted us to carry out a more extensive study.

Materials and Methods

Alcohol dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1) from yeast, glutamate dehydrogenase (EC 1.4.1.2) from bovine liver, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle, and NAD-kinase (EC 2.7.1.23) from pigeon liver were purchased from Boehringer. The labelled compounds [4-^{3}H]nicotinamide adenine dinucleotide, i.e. [^{3}H]NAD$^+$, with a specific radioactivity of 50 Ci/mol, and D-[1-^{3}H]glucose, 500 Ci/mol, were obtained from Amersham Buchler, Braunschweig. Blue Dextran-Sepharose was prepared as described by Ryan and Vestling [8]. FAD-Sepharose containing 15—20 μmol FAD per gram dried gel, was prepared as described in the booklet Pharmacia Affinity Chromatography for the binding of ligands to CNBr-activated Sepharose 4B.

Preparation of [^{3}H]NADH

A-labelled [^{3}H]NADH, i.e. 4-(*R*)-[4-^{3}H]NADH and B-labelled [^{3}H]NADH, i.e. 4-(*S*)-[4-^{3}H]NADH were prepared as described previously [7]. Alternatively, A-labelled [^{3}H]NADH was prepared by incubating at 30° C in a volume of 1 ml: [^{3}H]NAD$^+$ (0.2 μCi/3μmol), Tris · HCl buffer, pH 8.0 (100 μmol), sodium glutamate (20 μmol), hydrazine sulfate (200 μmol) and glutamate dehydrogenase (1.2 units). After 30 min, the reaction mixture was diluted 10-fold with water and the [^{3}H]NADH was isolated [7].

Preparation of [^{3}H]NADPH

A-labelled [^{3}H]NADPH, i.e. 4-(*R*)-[4-^{3}H]NADPH, was obtained by phosphorylation of [^{3}H]NAD$^+$ with ATP and NAD-kinase, and further reduction of the resulting [^{3}H]NADP$^+$ with glucose 6-phosphate and glucose-6-phosphate dehydrogenase. The initial reaction mixture contained, in a volume of 1 ml: [^{3}H]NAD$^+$ (0.5 μCi/15 μmol), Tris · HCl buffer, pH 8.0 (100 μmol), nicotinamide (8 μmol), ATP (15 μmol), $MgCl_2$ (10 μmol) and NAD kinase (0.15 units). The reaction was carried out at 37° C for 90 min and, after this time, the following compounds were added, and the final volume made up to 3 ml: Tris · HCl buffer, pH 8.0 (200 μmol), glucose 6-phosphate (6 μmol), $MgCl_2$ (10 μmol) and glucose 6-phosphate dehydrogenase (70 units). After 10 min at 30° C, 15 ml of water were added and the solution applied to a Whatman DE-52 DEAE-cellulose column (0.9 × 6 cm) equilibrated with 0.1 M Tris · HCl buffer, pH 8.0. The column was washed first with 20 ml of 0.06 M NaCl/0.1 M Tris · HCl buffer, pH 8.0, then with 6 ml of 0.1 M NaCl/0.1 M Tris · HCl buffer, pH 8.0, and the [^{3}H]NADPH was finally eluted with 0.2 M NaCl/0.1 M Tris · HCl buffer, pH 8.0 (cf. ref. 3). B-labelled [^{3}H]NADPH, i.e. 4-(*S*)-[4-^{3}H]NADPH, was obtained by incubating at 30° C in a volume of 3 ml: D-[1-^{3}H]glucose (190 μCi, 750 μmol), Tris · HCl buffer, pH 8.0 (200 μmol), NADP$^+$ (6 μmol), ATP (10

μmol), $MgCl_2$ (20 μmol), hexokinase (70 units) and glucose 6-phosphate dehydrogenase (70 units). The [^{3}H]NADPH was isolated as described above.

Assay of stereospecificity

For determination of the stereospecificity of the NADH : nitrate reductase from *Cl. fusca* and spinach, the reaction mixture contained, in a volume of 1 ml: A- or B-labelled [^{3}H]NADH (4 nCi, 0.2 μmol), Tris · HCl buffer, pH 8.0 (100 μmol), KNO_3 (10 μmol) and nitrate reductase (0.05 units). After complete oxidation of the NADH (5 min), the reaction mixture was diluted 10-fold with water and processed as previously described [7] to separate water and NAD^+. When the stereospecificity of the NAD(P)H : nitrate reductases from *Rhodotorula glutinis* and *Neurospora crassa* was tested, A- or B-labelled [^{3}H]-NADPH (4.5 nCi, 0.2 μmol) were substituted for the [^{3}H]NADH, and FAD (3 nmol) was included in the reaction mixture; with nitrate reductase, 0.1 units. After complete oxidation of the NADPH the reaction mixture was diluted 10-fold with water, and the solution passed through a column (0.9 × 6 cm) of Whatman DE-52 DEAE-cellulose, equilibrated with 0.1 M Tris · HCl buffer, pH 8.0. The $NADP^+$ was eluted by washing the column with 0.06 M NaCl/0.1 M Tris · HCl, pH 8.0 [3].

Assay of nitrate reductase activity

Nitrate reductase activity was estimated spectrophotometrically by following the nitrate-dependent NAD(P)H oxidation at 334 nm. The reaction mixtures were the same as those used for the determination of stereospecificity. A unit of enzyme is defined as that amount which catalyzes the oxidation of 1 μmol of NADH or NADPH per min at 30° C.

Electrophoresis

Analytical disc gel electrophoresis of nitrate reductases was performed in 5% and 7.5% acrylamide gels. Conditions of the electrophoretic procedure, staining of protein bands and detection of nitrate reductase activity have been described by Solomonson et al. [9].

Purification of nitrate reductases

The NAD(P)H nitrate reductase (EC 1.6.6.2) of the fungus *N. crassa* was prepared by a modification of the procedure of Garrett and Greenbaum [10] and finally purified by FAD-Sepharose affinity chromatography [11]. Extracts of nitrate-induced cells were acidified to pH 5.2 and clarified by centrifugation. The supernatant was treated with 60% $(NH_4)_2SO_4$ saturation and, after centrifugation, the sediment was resuspended in 0.1 M potassium phosphate buffer (pH 7.2) containing 5 mM cysteine and 0.5 mM EDTA. This preparation was passed through a Sephadex G-25 column equilibrated with 10 mM potassium phosphate buffer (pH 6.85) containing 0.1 mM dithioerythritol (buffer A). This protein solution was applied to a Bio-Gel A-1.5 m column (2.5 × 50 cm) and eluted with buffer A. The peak fractions were combined and nitrate reductase was sedimented with $(NH_4)_2SO_4$ (50% saturation). The sediment was redissolved in buffer A and the remaining $(NH_4)_2SO_4$ was removed by filtration through a Sephadex G-25 column. The resulting preparation was applied to a

FAD-Sepharose column (1 × 6 cm) equilibrated with buffer A and, after washing of the column with the same buffer nitrate reductase was eluted with a linear gradient solution between 0 and 1 M KCl in buffer A. The yield of nitrate reductase obtained at the affinity chromatography step was of 35%. The activity of the partially purified enzyme in the peak fractions was between 15 and 20 units per mg protein which represented an overall purification of about 1000-fold.

Electrophoretically homogenous NAD(P)H : nitrate reductase (EC 1.6.6.2) from the yeast *Rh. glutinis* was prepared as described elsewhere [12] (520-fold purification), and had a specific activity of 76 units per mg protein, with NADPH as electron donor.

NADH : nitrate reductase (EC 1.6.6.1) from *Cl. fusca* was a gift of Dr. W.G. Zumft. The enzyme had been prepared as follows: (1) preparation of the cell-free extract, (2) adsorption on a DEAE-cellulose column and elution with a continuous salt gradient (0.045—0.3 M NaCl), (3) precipitation with 40% ammonium sulfate, and (4) chromatography on a Sepharose 4B column. The resulting enzyme preparation had a specific activity of 0.2 units per mg protein (25-fold purification). Where indicated, the enzyme was further purified by affinity chromatography on Blue Dextran-Sepharose by applying the above preparation to a column of this material (1 × 6 cm) which had been previously equilibrated with 10 mM Tris · HCl buffer (pH 8.0) containing 20 μM FAD. The column was then eluted with the same buffer supplemented with 100 μM NADH. The inclusion of this step resulted in an extra purification of 40-fold with 48% recovery of enzyme activity. The resulting partially purified preparation of nitrate reductase had a specific activity of 8 units per mg protein.

NADH : nitrate reductase (EC 1.6.6.1) from spinach leaves was purified as previously described [13,14]. Where indicated, the enzyme was subjected to affinity chromatography on Blue Dextran-Sepharose. For this purpose, the calcium phosphate gel eluate (specific activity 0.4 units per mg protein) was passed through a Sephadex G-25 column equilibrated with 20 mM Tris · HCl buffer (pH 7.5) containing 0.1 mM DTE and 0.1 mM EDTA, and applied to a Blue Dextran-Sepharose column (2 × 10 cm) equilibrated with the same buffer. After washing with the equilibration buffer, nitrate reductase was eluted with 50 μM NADH in the same buffer. This step (42% recovery of nitrate reductase) resulted in a further purification of 45-fold (overall purification of 1600-fold) yielding an enzyme preparation with a specific activity of 22 units per mg protein. Analytical polyacrylamide gel electrophoresis of this enzyme preparation showed the presence of three different protein bands. The thickest and most darkly staining zone which represented the great majority of the total protein was coincident with nitrate reductase activity, whereas the other two fainter protein bands did not.

Spectroscopic examination of this preparation of nitrate reductase from spinach leaves, after concentration with 70% ammonium sulfate showed that it contained a cytochrome (oxidized form) which was immediately reduced after addition of NADH or dithionite, and rapidly re-oxidized on addition of nitrate. The absorption spectra before and after reduction with NADH are shown in Fig. 1. The spectra corresponded to a cytochrome of the *b* type, with maxima at 557, 527 and 423 nm for the reduced enzyme and 412 nm for the oxidized.

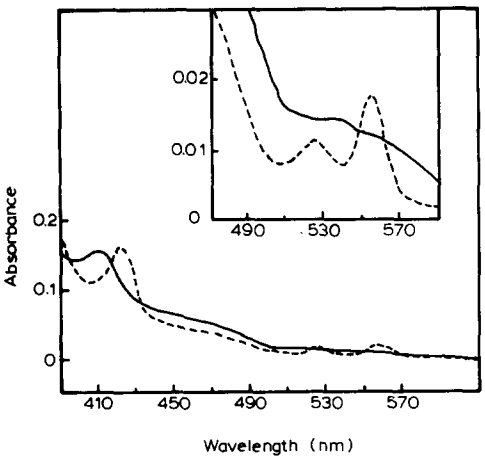

Fig. 1. Absorption spectra of purified NADH : nitrate reductase from spinach leaves. The solution contained 0.12 mg protein per ml 50 mM Tris · HCl buffer (pH 7.5). ———, no additions; - - - - - -, after addition of a small amount of solid NADH. Other experimental conditions are described under Materials and Methods.

These band maxima are identical to those reported for the cytochrome b_{557} present in the nitrate reductase of *N. crassa* [15,16] and *Cl. vulgaris* [9,17]. To the best of our knowledge, this is the first evidence of the presence of a cytochrome in the nitrate reductase of a higher plant.

Results

The general procedure here employed involved the enzymatic oxidation by nitrate of a reduced pyridine nucleotide labelled with tritium specifically in either the A- or the B-4′ position of the nicotinamide moiety of the pyridine nucleotide. The hydrogen which is removed from the pyridine nucleotide on oxidation by nitrate appears in the water. Passage of the sample through a DEAE-cellulose column results in a separation of the water of the reaction mixture, which emerges first, and the oxidized pyridine nucleotide, which is eluted later and located by absorbance measurements at 260 nm. A detailed description of results with nitrate reductase of *Cl. vulgaris* has been given previously [7]. For brevity, the results here tabulated show only the tritium in the oxidized pyridine nucleotide, expressed as percent of the total tritium in the pyridine nucleotide plus water. Thus, the tritium found in the water can be calculated by subtracting the given values from 100. The oxidation of the pyridine nucleotide was always run to completion, so there was no reduced pyridine nucleotide remaining. The results should therefore not be influenced by any isotope rate effects. In each case, duplicate, separate experiments were performed, one with A-labelled reduced pyridine nucleotide, the other with B-labelled reduced pyridine nucleotide. The sum of these results should be 100, if conditions are identical. Deviations from 100 reflect experimental errors. In each case, the authenticity of the labelling was checked by oxidizing the labelled reference compound with an enzyme of known, previously determined stereospecificity. Samples which gave results deviating by more than 5% from

the expected values were rejected. (The reduced pyridine nucleotides have a tendency to auto-oxidize on storage, and the hydrogen removed on auto-oxidation would not be removed stereospecifically. The use of old reference compounds must therefore be avoided). Nitrate reductase often require added flavin. Flavin can interact chemically with reduced pyridine nucleotide, but such interaction was minimal under the conditions here employed. Flavin concentrations were as low as they could be, but still allow a rapid enzyme reaction. Use of enzyme preparations with oxidase activity for reduced pyridine nucleotides was also avoided. Thus, all possible precautions were taken to avoid the occurrence of side reactions with an apparent racemizing effect.

Stereospecificity of hydrogen removal from NADH by NADH : nitrate reductases

Typical results obtained with NADH : nitrate reductase preparations from *Cl. fusca* and from spinach leaves are shown in Table I. These results were selected to illustrate the marked increase in stereospecificity which was obtained when the enzyme preparations were purified on a Blue Dextran-Sepharose column. Prior to this treatment the reactions did not occur with a high level of stereospecificity, though there was a preference for the A side. After the treatment, both enzyme reactions showed A stereospecificity. There was also a marked increase in specific activity achieved by this one purification step.

Experiments were also carried out with partially purified nitrate reductases from young leaves of wheat and corn. These results (not shown) also indicated a low level of sterospecificity, with a preference for the A side, much like the results with spinach enzyme prior to Blue Dextran-Sepharose treatment. We did not succeed in getting enzyme of high specific activity from these sources, and therefore regard these results as inconclusive.

Stereospecificity of hydrogen removal from NADPH by NAD(P)H : nitrate reductases

The enzymes from the yeast *Rh. glutinis* and the mold *N. crassa* have a preference for NADPH as a substrate, though they also use NADH. The stereospecificity for hydrogen removal was determined with labelled NADPH, and some typical results are shown in Table II. With the *Rhodotorula* enzyme, which was

TABLE I

STEREOSPECIFICITY OF THE NADH : NITRATE REDUCTASES FROM *CHLORELLA FUSCA* AND SPINACH LEAVES. EFFECT OF THE BLUE DEXTRAN-SEPHAROSE TREATMENT

Source of enzyme	Blue Dextran-Sepharose treatment	Specific activity (units per mg)	Radioactivity of NAD^+ (% of total)	
			With A-[^{3}H]NADH	With B-[^{3}H]NADH
Spinach leaves	—	0.4	31.5	60.0
Chlorella fusca	—	0.2	17.5	90.7
Spinach leaves	+	22.0	5.0	98.5
Chlorella fusca	+	8.0	2.9	100.0

TABLE II

STEREOSPECIFICITY OF THE NAD(P)H - NITRATE REDUCTASES OF *RHODOTORULA GLUTINIS* AND *NEUROSPORA CRASSA*

Source of enzyme	Radioactivity of $NADP^+$ (% of total)	
	With A-[^{3}H]NADPH	With B-[^{3}H]NADPH
Rhodotorula glutinis	4.1	95.5
Neurospora crassa	5.2	79.2
	13.3	91.7

available at a high level of purity [12], the results showed A-stereospecificity for the NADPH. Enzyme preparations of lower specific activity did not show a high level of stereospecificity, however (not shown). The results with *Neurospora* enzyme were not entirely satisfactory. The listed values show the best results we could obtain. There is obviously a preference for the A side, which was not nearly so marked in other experiments with less highly purified preparations.

Discussion

The results reported in the present paper do not support the view that soluble pyridine nucleotide nitrate reductases can be classified into two groups: one with A stereospecificity and the other with B-stereospecificity. Those enzymes which were rather highly purified were all found to have A-stereospecificity (viz. the enzymes from *Cl. vulgaris*, *Cl. fusca*, *Rh. glutinis*, spinach leaves, and probably *N. crassa*). The main difficulty encountered in the present studies was that unless the enzymes were extensively purified, the nitrate reductase stereospecificity determinations gave results which suggested that the reactions were not very stereospecific, though there was generally a preference for the A position. It is conceivable that the purification procedures resulted in selective purification of an A-specific enzyme. It is also conceivable, however, that another pyridine-nucleotide-activating flavoprotein (not a part of the nitrate reductase molecule, but capable of interacting with the flavin of nitrate reductase) was present in the cruder enzyme preparations and interfered with the experiments, or that some other type of interfering reaction occurred. The original experiments of Davies et al. [3] reporting B-stereospecificity for the nitrate reductase of *C. utilis* were carried out with a particulate preparation and it can not be excluded that the oxidation of pyridine nucleotide was coupled to nitrate reduction by a reaction sequence involving several different enzymes. Though the reduction of nitrate catalyzed by xanthine oxidase was found to have B-stereospecificity [7], this reaction is not a major function of the enzyme, which can not be regarded as a "typical" nitrate reductase [18]. That is, it is doubtful whether the enzyme functions physiologically to reduce nitrate. Thus, until it is possible to isolate a purified "typical" pyridine nucleotide nitrate reductase of B-stereospecificity, the question of the occurrence in nature of such an enzyme must be left open.

Solomonson has developed an excellent procedure for the purification of the nitrate rediuctase of *Cl. vulgaris* by affinity chromatography on Blue Dextran-Sepharose [19]. The results presented here show that this type of procedure can be used as an effective purification step for the nitrate reductases of *Cl. fusca* and spinach leaves. The latter enzyme has been shown to have the characteristic cytochrome b_{557} spectrum common to several other nitrate reductases [12]. Blue Dextran-Sepharose has also been applied for the separation of different species of nitrate reductase from rice seedlings [20] and soybean leaves [21]. It can be argued that if two different types of nitrate reductase from the same source can be separated on Blue Dextran-Sepharose, there is all the more reason to believe that the emergence of an enzyme with A-stereospecificity after affinity chromatography indicates that an enzyme of B-stereospecificity was left behind. We have attempted to recover a nitrate reductase with B-stereospecificity from the Blue Dextran-Sepharose columns, without success.

For the present, it is our view that the nitrate reductases can not be regarded as an exception to the generalization that pyridine nucleotide dehydrogenases with the same physiological function have the same stereospecificity for the pyridine nucleotide, regardless of the source of the enzyme.

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