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STUDIES ON THE IN VITRO INACTIVATION OF THE *NEUROSPORA CRASSA* ASSIMILATORY NITRITE REDUCTASE IN THE PRESENCE OF REDUCED PYRIDINE NUCLEOTIDES PLUS FLAVIN

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Summary

In vitro inactivation of *Neurospora crassa* nitrite reductase (NAD(P)H: nitrite oxidoreductase, EC 1.6.6.4) can be obtained by preincubation of the enzyme with reduced pyridine nucleotide plus FAD.

The presence of nitrite or hydroxylamine, electron acceptors for the *N. crassa* nitrite reductase, or cyanide, sulfite or arsenite, competitive inhibitors with respect to nitrite of this enzyme, protects the enzyme against this inactivation.

Anaerobic experiments reveal that oxygen is required in order to obtain complete inactivation of nitrite reductase by preincubation with reduced pyridine nucleotide plus FAD. Also, inactivation is prevented if catalase is included in the preincubation mixture.

The presence of hydrogen peroxide in the preincubation mixture increases the sensitivity of nitrite reductase to the in vitro FAD-dependent NAD(P)H inactivation.

Neither electron acceptors, competitive inhibitors nor catalase, agents which protect the enzyme against the FAD-dependent NAD(P)H inactivation, can reverse this process once it has occurred.

Introduction

The assimilatory reduction of nitrate to ammonia occurs in bacteria, fungi, algae and higher plants via a metabolic pathway consisting of two separate enzymatic steps: The $2e^-$ reduction of nitrate to nitrite, catalyzed by nitrate reductase; followed by the $6e^-$ reduction of nitrite to ammonia, catalyzed by nitrite reductase.

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Losada's laboratory [1] has shown that the nitrate reductases from bacteria, fungi, algae and higher plants are inactivated in vitro upon preincubation with electron donors, e.g. NADH or dithionite plus methylviologen. Such inactivated enzymes can then be reverted to an active state by oxidation; ferricyanide is the agent generally used to demonstrate this reversibility. From these observations and complementary in vivo studies, Losada has postulated that these nitrate reductases are subject to metabolic interconversion between an active, oxidized form and an inactive, reduced form through oxidation-reduction reactions.

At present, evidence for interconversion of nitrite reductase between an active and an inactive form has only been reported for bacteria, using partially purified enzyme preparations [2,3].

In light of these reports, it was very interesting to determine whether the *Neurospora crassa* nitrite reductase (NAD(P)H: nitrite oxidoreductase, EC 1.6.6.4) shows some change in activity upon preincubation with reductants.

The assimilatory nitrite reductase of the fungus *N. crassa* is an FAD-dependent metallo-protein (M_r 300 000) which catalyzes the direct and stoichiometric reduction of nitrite to ammonia, using either NADPH or NADH as electron donor [4,5].

The present paper shows the in vitro inactivation of *N. crassa* nitrite reductase activity by the combined action of reduced pyridine nucleotides, the electron donors for the enzyme, and FAD, the indispensable cofactor for the functionality of this protein. However, oxygen is required in order to obtain a complete inactivation and thus, this inactivation is presumably peroxide mediated. In addition, the protection of the enzyme against this inactivation by electron acceptors, catalase and other compounds is also reported. Preliminary accounts of these results have been presented [5—7].

Methods and Materials

Preparation and assay of the enzyme

Partially purified preparations of NAD(P)H:nitrite reductase were obtained from *N. crassa* (wild type 5297a). Large-scale growth, enzyme induction and crude-extract preparation was carried out as described previously [4,5].

The nitrite reductase purification has as principal steps: 35—70% ammonium sulfate fractionation; polyethyleneglycol treatment to remove nucleic acids; and adsorption to a DEAE-cellulose column and elution with a linear phosphate gradient (10—500 mM, pH 7.5). Pooled DEAE eluates were precipitated by addition of solid ammonium sulfate to give 60% saturation, as described earlier [5]. This precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.5) and stored at −70° C until used. These partially purified nitrite reductase preparations had specific activities of at least 245 nmol of NADPH oxidized/min per mg protein.

NAD(P)H:nitrite reductase activities were measured by following the rate of nitrite-dependent pyridine nucleotide oxidation at 340 nm in a Cary 14 Spectrophotometer. The 1.0-ml-total reaction mixture contained potassium phosphate buffer (pH 7.5), 100 μmol; FAD, 10 nmol; sodium nitrite, 1 μmol; NAD(P)H, 0.2 μmol; and adequate amounts of enzyme to catalyze a change of

0.02—0.30 absorbance units per min. The assay was performed at 25°C in cells of 1-cm path length.

Protein determination

The biuret method described by Layne [8] was used with bovine serum albumin as the standard.

Results and Discussion

Inactivation of nitrite reductase

Table I shows that nitrite reductase activity from *N. crassa* assayed either with NADPH or NADH as electron donor is inactivated by preincubation of the enzyme for 5—10 min at room temperature with reduced pyridine nucleotide plus FAD. It is interesting to emphasize in this table that FAD is required in the preincubation mixtures in order to obtain full inactivation of the enzyme. When the enzyme is preincubated with reduced pyridine nucleotide alone, some inactivation is obtained. In this case, it is possible that a little endogenous FAD accompanied the enzyme preparation. If an oxidized form of the pyridine nucleotide is used in the preincubation mixture instead of the reduced one, the enzyme is unaffected. This shows the necessity of reducing power in the preincubation mixture in order to observe inactivation of the nitrite reductase. Similar results to those shown in Table I were obtained when an enzymatic preparation desalted by passage through a Sephadex G-25 column was used. This experiment was done in order to avoid the possible effect, in the nitrite reductase inactivation, of NH_4^+ which is the end product of nitrite reduction and which accompanied the $(NH_4)_2SO_4$-precipitated enzyme fraction.

Concentrations of 2 mM reduced pyridine nucleotide and 10 μM FAD in the preincubation mixture are optimal to obtain a complete inactivation of the enzyme after 10 min of incubation. The inactivation of nitrite reductase is independent of pH in the range between 6 and 9.

TABLE I

FAD—DEPENDENT NAD(P)H INACTIVATION OF *N. CRASSA* NITRITE REDUCTASE

Aliquots of the partially purified nitrite reductase preparation (4 mg protein) were preincubated in a final volume of 0.25 ml with 50 μmol potassium phosphate buffer (pH 7.5) and where indicated, NAD(P)H or $NAD(P)^+$, 0.5 μmol and FAD, 25 nmol. After 10 min at room temperature, NAD(P)H:nitrite reductase activity was measured by adding 0.02 ml of each preincubation system to the reagents of the standard assay (see Methods and Materials).

Preincubation system	Nitrite-dependent NAD(P)H oxidation (nmol/min)	
	NADPH	NADH
Enzyme	30.0	22.6
+ FAD	30.5	21.0
+ NADPH	15.3	12.4
+ NADPH + FAD	3.8	2.6
+ $NADP^+$ + FAD	29.0	20.5
+ NADH	16.1	11.4
+ NADH + FAD	3.2	3.2
+ NAD^+ + FAD	28.8	21.3

Protection of N. crassa nitrite reductase against FAD-dependent NAD(P)H inactivation

If an electron acceptor for the nitrite reductase of this fungus, either nitrite or hydroxylamine, is included in the preincubation mixture, the enzyme is markedly protected against inactivation (Table II). In order for this protection to be successful, the substrate concentration must be high enough to oxidize all of the reduced pyridine nucleotide in the preincubation mixture.

On the basis of these results, it became interesting to determine whether competitive inhibitors can protect the enzyme against this inactivation.

Lineweaver-Burk plots show (Fig. 1) that cyanide, sulfite and arsenite are competitive inhibitors with respect to nitrite of the *N. crassa* nitrite reductase. The corresponding K_i values for these inhibitors are 0.2 μM, 0.25 mM and 1.2 mM, respectively. These competitive inhibitors cannot accept electrons from the enzyme. The presence of one of these competitive inhibitors in the preincubation mixture at concentrations 10-fold their respective K_i values completely prevents inactivation of the enzyme, as shown in Table II. This protection is obtained independent of the reduced pyridine nucleotide used in the preincubation mixture. Presumably, binding of either a substrate or a competitive inhibitor to the active site prevents the modification of nitrite reductase produced by its preincubation in the presence of NAD(P)H and FAD, and thereby protects it from inactivation. However, none of these compounds can reverse the effect of inactivation. These results are very similar to those reported by Kemp and Atkinson [2], who observed the NADH:nitrite reductase from *Escherichia coli* was inactivated in vitro by preincubation with NADH, but the results differ in that the activity of the *N. crassa* nitrite reductase was not enhanced by preincubation with nitrite. These results are also similar to those reported by Vega et al. [3], who have demonstrated that the *Azotobac-*

TABLE II

FAD-DEPENDENT NAD(P)H INACTIVATION OF *N. CRASSA* NITRITE REDUCTASE: PROTECTION BY SUBSTRATES AND COMPETITIVE INHIBITORS

Aliquots of a partially purified nitrite reductase preparation (1 mg protein) were preincubated in a final volume of 0.25 ml with 50 μmol potassium phosphate buffer (pH 7.5) and substrates, competitive inhibitors and cofactors as indicated below. After 10 min at room temperature, NADPH:nitrite reductase activity was measured by adding 0.02 ml of each preincubation system to the reagents of the standard assay. [NAD(P)H] = 2 mM, [FAD] = 10 μM.

Preincubation system	NADPH:nitrite reductase (relative enzymatic activity)*
Enzyme	100
+ NADPH + FAD	8.3
+ NO_2^- (0.4 mM)	5.5
+ NO_2^- (2 mM)	90.0
+ NH_2OH (4 mM)	4.8
+ NH_2OH (20 mM)	74.0
+ SO_3^{2-} (2 mM)	97.0
+ CN^- (2 μM)	91.5
+ AsO_2^- (10 mM)	74.0

* 100% activity equals 29.7 nmol NADPH oxidized per min.

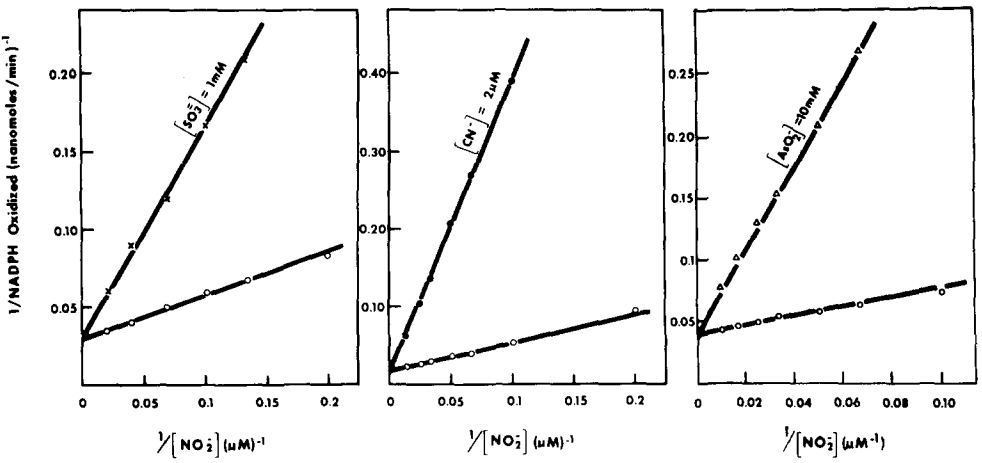

Fig. 1. Competitive inhibition by sulfite (a), cyanide (b), or arsenite (c) with respect to nitrite of *N. crassa* nitrite reductase. Experimental conditions were as in the standard assay for NADPH:nitrite reductase activity, except that the concentration of nitrite was varied as indicated. ○———○, control (without inhibitor); X———X, plus sulfite (1 mM); ●———●, plus cyanide (2 μM); △———△, plus arsenite (10 mM). The amount of enzyme preparation used per assay was 0.15 mg.

ter chroococcum NADH:nitrite reductase can be inactivated in vitro by preincubation of the enzyme with NADH. However, in this latter case, nitrite not only specifically protected against the loss in activity but also reversed this inactivation.

Although NAD(P)H is effective, dithionite did not cause the inactivation of the nitrite reductase, either in the presence or absence of FAD. An explanation of this anomaly is that the sulfite formed upon dithionite oxidation protects the enzyme by virtue of its ability to bind at the active site. An alternative or parallel explanation is that dithionite addition renders the preincubation mixture essentially anaerobic and thereby prevents inactivation.

Effect of anaerobiosis and protection by catalase in the FAD-dependent NAD(P)H inactivation of N. crassa nitrite reductase

The results shown in Table III indicate that oxygen is required in order to obtain a complete inactivation of the enzyme. This requirement suggested the possibility that superoxide or peroxide might be involved in the inactivation process. Consequently, the effects of superoxide dismutase and catalase on the inactivation process were examined. The results shown in Table III indicate that when catalase is included in the preincubation mixture, the nitrite reductase is protected against inactivation, indicating that the FAD-dependent NAD(P)H inactivation of the nitrite reductase probably occurs as a result of the formation of peroxide, perhaps via an interaction between reduced flavin and oxygen. Superoxide dismutase had no effect. Peroxide formation is an intermediate step in the nitrite reductase inactivation mechanism and not the final one, because catalase cannot reverse the inactivation once it has occurred.

TABLE III

FAD-DEPENDENT NADPH* INACTIVATION OF *N. CRASSA* NITRITE REDUCTASE: EFFECT OF ANAEROBIOSIS AND CATALASE

Aliquots of a partially purified nitrite reductase (0.85 mg of protein) were preincubated in a final volume of 2 ml with 40 μmol potassium phosphate buffer (pH 7.5), and where indicated NADPH, 0.4 μmol; FAD, 2 nmol; catalase (3.4 mg of protein), 1330 units and superoxide dismutase (0.6 mg of protein), 2000 units. After 10 min at room temperature, NADPH:nitrite reductase activity was measured by adding 0.2 ml of each preincubation system to the reagents of the standard assay.

Preincubation system	NADPH-nitrite reductase (nmol NADPH oxidized per min)
Enzyme	58.0
**Enzyme	57.4
Enzyme + NADPH + FAD	5.6
**Enzyme + NADPH + FAD	30.0
Enzyme + NADPH + FAD + catalase	46.7
Enzyme + NADPH + FAD + superoxide dismutase	10.7
Enzyme + NADPH + FAD + catalase + superoxide dismutase	47.6

* Similar results were obtained when NADH was used in place of NADPH in the preincubation systems.

** The preincubation systems, before the addition of the enzyme were evacuated, at room temperature, with a vacuum pump for 5 min in order to remove the dissolved air, refilled with nitrogen gas to restore atmospheric pressure and kept 5 min. This process was repeated three times. The enzymatic preparation was submitted to the same process, but at 0° C.

Effect of hydrogen peroxide on the FAD-dependent NAD(P)H inactivation of N. crassa nitrite reductase

In confirmation of the above, it can be seen that the nitrite reductase was inactivated by preincubation for 10 min at room temperature with 10 mM hydrogen peroxide alone (Table IV). 1 mM of hydrogen peroxide did not have any effect under this condition. The presence of 1 mM hydrogen peroxide in the

TABLE IV

EFFECT OF HYDROGEN PEROXIDE IN THE PREINCUBATION OF *N. CRASSA* NITRITE REDUCTASE WITH LOW CONCENTRATIONS OF NADPH PLUS FAD

Aliquots of a partially purified nitrite reductase preparation (0.64 mg of protein) were preincubated in a final volume of 0.25 ml with 50 μmol potassium phosphate buffer (pH 7.5) and where indicated, FAD, 2.5 nmole; NADPH, 5 nmol and hydrogen peroxide, 0.25 μmol (or like indicated below). After 5 min at room temperature. NADPH:nitrite reductase activity was measured by adding 0.02 ml of each preincubation system to the reagents of the standard assay.

Preincubation system	NADPH:nitrite reductase (nmol NADPH oxidized per min)
Enzyme	42.0
+ NADPH + FAD	37.6
+ H_2O_2	35.5
+ FAD + H_2O_2	40.3
+ NADPH + H_2O_2	38.8
+ NADPH + FAD + H_2O_2	9.5
+ H_2O_2 (10 mM)	11.2

preincubation mixture of the nitrite reductase with 20 μM reduced pyridine nucleotide (an amount which cannot produce inactivation of nitrite reductase) plus FAD, produced a very rapid inactivation of the nitrite reductase activity. The results may reflect the fact that the enzyme is much more susceptible to peroxide if it is in a reduced state.

In vitro inactivation of the nitrate-assimilating enzymes (i.e. nitrate and nitrite reductase) from other organisms by preincubation with reduced pyridine nucleotide has been reported [2,9—12]. In these organisms, this transformation is reversible, i.e. in the presence of oxidants, the enzyme again becomes active [3,9,13]. The physiological significance of this interconversion by oxidation-reduction reactions in *Chlorella fusca* [12,14], *Chlamydomonas reinhardii* [11,15] and *Chlorella vulgaris* [10,13,16] has been discussed. The results reported in this paper indicate that the FAD-dependent NAD(P)H inactivation of *N. crassa* nitrite reductase is peroxide mediated and therefore is not a consequence of a simple reduction of the enzyme. On the basis of this observation, it becomes important to determine whether oxygen generally plays a role in in vitro inactivation studies of this type. At present there is no evidence that the *N. crassa* nitrite reductase is inactivated in vivo.

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