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INACTIVATION BY ACETYLENE OF SPINACH NITRATE REDUCTASE

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The molybdoprotein NADH-nitrate reductase (NADH : nitrate oxidoreductase, EC 1.6.6.1) from spinach can be inactivated by acetylene only when the enzyme is in its reduced state. Other gases such as ethylene, carbon monoxide, dinitrogen and others did not alter the enzyme activity. From the two partial activities of nitrate reductase, only the terminal nitrate reductase was impaired by acetylene while the dehydrogenase activity was rather stimulated. Functional dehydrogenase activity was required for inactivation when NADH was the reductant. Dithionite, dithionite + MV or dithionite + FMN were also able to sustain acetylene inactivation, whether or not nitrate reductase was previously depleted of its dehydrogenase activity. However, ascorbate or ascorbate + DCIP did not cooperate with acetylene for inactivating nitrate reductase. Nitrate and the competitive inhibitors with respect to nitrate of nitrate reductase, namely azide, cyanate and carbamyl phosphate, protected nitrate reductase from acetylene inactivation. Cyanide-inactivated nitrate reductase was still sensitive to acetylene, since, once the cyanide-inactivated enzyme was placed under acetylene, no ferricyanide reactivation could be attained. These results suggest that reduced nitrate reductase might bind acetylene at the nitrate active site, where molybdenum is supposed to be implicated, thus impairing the reduction of nitrate.

Introduction

NADH-nitrate reductase (NADH : nitrate oxidoreductase, EC 1.6.6.1) opens the metabolic route of nitrate assimilation by reducing nitrate to nitrite. Subsequently nitrite is converted to ammonia by ferredoxin-nitrite reductase (EC 1.6.6.4) [1,2]. Today it is widely accepted that ammonia is incorporated to carbon skeletons mainly by the combined performance of glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.7.1), resulting into the net production of glutamate from 2-oxoglutarate [3].

Spinach nitrate reductase is a multimeric enzyme of molecular weight 197 000. This protein includes a small electron transport chain with FAD, cytochrome *b*-557 and molybdenum as the main redox components [4]. In addition to the overall reaction, namely reduction of nitrate with NADH, nitrate reductase displays two partial activities: (a) a NADH-dehydrogenase activity, which, with the presumable involvement of FAD and cytochrome *b*-557, reduces a wide variety of electron acceptors, such as cytochrome *c*, DCIP, $K_3Fe(CN)_6$ and different quinones; and (b) the terminal nitrate reductase activity, which catalyzes, without the obligate operation of the NADH-dehydrogenase, the immediate reduction of nitrate to nitrite using different electron donor systems, such as dithionite-reduced MV and dithionite- or light-reduced FMN [4,5]. Molybdenum, apparently held in a special cofactor, is essential for this terminal activity [4]. From the two partial activities, the NADH-dehydrogenase is more sensitive to heating and to mercurial compounds [6].

Most workers in the field attribute a primary role

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; MV, MV$^+$, methyl viologen and its reduced form.

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in the regulation of assimilatory nitrate reduction to nitrate reductase [2,7,8]. At least in green algae, nitrate reductase exhibits two physiologically interconvertible forms: active and inactive [9]. In vitro nitrate reductase, not only from green algae but also from higher plants, can be inactivated slowly by incubation with reductants, such as NAD(P)H or dithionite, and much more rapidly in the presence of either cyanide, hydroxylamine, ferrocytochrome *c* or ADP [6,10–13]. The slow inactivation of nitrate reductase that occurs in the presence of NAD(P)H or dithionite implies that, for short periods of time, the enzyme can exist in a reduced state but fully active [4]. In most cases, and especially when cyanide is the inactivating agent, the inactive form of the enzyme can be reactivated by oxidation with ferricyanide, trivalent manganese or blue light irradiation [14,15].

Nitrogenase, another molybdoprotein, besides its physiological activity of reducing dinitrogen to ammonia, efficiently uses acetylene as substrate with the concomitant release of ethylene. Cyanide and azide can also be reduced by this enzyme [16]. It has been characterized from nitrogenase an iron-molybdenum cofactor that, when properly reduced, can carry out by itself the reduction of acetylene [17,18].

The experiments described in this article show that spinach nitrate reductase is unable to reduce acetylene to ethylene. However, acetylene inactivates nitrate reductase when the active form of the enzyme is maintained in its reduced state with an appropriate reductant. Nitrate and several competitive inhibitors with respect to nitrate of nitrate reductase protect the enzyme from acetylene inactivation. Cyanide-inactivated nitrate reductase is still more sensitive to acetylene. A special link between acetylene and the molybdenum domain of the enzyme, stronger than that formed by cyanide, might be postulated to explain this acetylene blocking of the nitrate reductase activity.

Methods

Materials. Cytochrome *c*, pyridine nucleotides, flavin nucleotides, Tris, EDTA, dithioerythritol, *p*-hydroxymercuribenzoate and Blue Sepharose CL-6B were purchased from Sigma. Methyl viologen and bovine serum albumin were obtained from Serva. $(NH_4)_2SO_4$, dithionite, ascorbic acid, 2,6-dichlorophenolindophenol, ferricyanide, cyanate, cyanide, azide and carbamyl phosphate were obtained from Merck. Sephadex G-25 was from Pharmacia. Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad. Streptomycin sulphate was from Cia. Española de Penicilina. Acetylene and other gases were purchased from Sociedad Española de Oxígeno. All other reagents used were of the purest grade available.

Plant material and enzyme purification. Field-grown spinach (*Spinacia oleracea* L.) plants were used as nitrate reductase source. The same day in which the plants were harvested, leaves were excised, washed with running tap water and either used for the purification of nitrate reductase or kept at −20°C until use.

Nitrate reductase was purified as described by Notton et al. [19,20] with some modifications. Because of their protective effects on nitrate reductase activity [21], 0.1 mM dithioerythritol, 0.1 mM EDTA and 20 μM FAD were included in the buffers throughout the purification procedure, which consisted basically of the following steps: (1) preparation of the crude extract; (2) treatment with streptomycin sulphate; (3) precipitation with 50% saturation $(NH_4)_2SO_4$; (4) chromatography on hydroxyapatite and (5) affinity chromatography on Blue Sepharose CL-6B. Nitrate reductase was eluted from the Blue Sepharose with 25 mM phosphate buffer (pH 6.8)/0.5 M KCl/0.2 mM NADH (cf. Ref. 20). For the subsequent studies, the active fractions from step 5 were pooled, concentrated by precipitation with $(NH_4)_2SO_4$ (50% saturation), and then desalted with Sephadex G-25 equilibrated with 0.1 M phosphate buffer (pH 7.5).

Enzyme assays. NADH-nitrate reductase was determined either by nitrite formation or by NADH oxidation [21]. Nitrite formation was measured, after 5 min incubation at 30°C, in an assay mixture containing 0.1 M Tris-HCl buffer (pH 7.5)/10 mM KNO_3/0.3 mM NADH/the adequate amount of enzyme in a final volume of 1.0 ml. NADH oxidation was followed spectrophotometrically at 340 nm in a similar reaction mixture, but with 0.1 mM NADH and a total volume of 3.0 ml.

$FMNH_2$-nitrate reductase and $MV^{\cdot}$-nitrate reductase were estimated by nitrite formation like NADH-nitrate reductase, but using as electron donor systems: 0.1 mM FMN reduced with 0.1 ml $Na_2S_2O_4$

(8 mg/ml in 0.1 M $NaHCO_3$) or 0.1 mM MV reduced with 0.1 ml $Na_2S_2O_4$ solution, respectively [22,23]. Reactions were stopped by vigorous shaking in a cyclomixer until complete autoxidation of the dithionite present.

NADH-dehydrogenase activity was determined spectrophotometrically by measuring the initial rate of the reduction of cytochrome *c* at 550 nm [24]. The assay mixture contained, in 3.0 ml, 0.1 M Tris-HCl buffer (pH 7.5)/60 μM cytochrome *c*/0.1 mM NADH/the adequate amount of enzyme.

Enzymatic activity units are expressed as μmol substrate transformed/min.

Analytical methods. Protein was determined according to the method of Lowry et al. [25] as modified by Bailey [26], using bovine serum albumin as standard.

Nitrite was measured by the diazo-coupling colorimetric assay of Snell and Snell [27].

Spectrophotometric enzyme assays were carried out in a Aminco Chance DW-2 spectrophotometer, and colorimetric measurements in a Pye Unicam SP600 ultraviolet spectrophotometer.

Results

Fig. 1 shows that the activity of nitrate reductase, measured by oxidation of NADH with saturating concentrations of nitrate, was not altered when acetylene was added either simultaneously with nitrate or during turnover. In contrast, if the enzyme in the presence of NADH was maintained for several minutes under acetylene atmosphere, previously to the addition of nitrate, the nitrate-dependent NADH oxidation was greatly impaired. No enzymatic oxidation of NADH was observed due to only the presence of acetylene.

Fig. 2 shows the time course of acetylene inactivation of the NADH-nitrate reductase. The enzyme was preincubated either in the presence or absence of NADH, under acetylene or air at atmospheric pressure. The enzyme inactivation was achieved only when both acetylene and NADH were simultaneously present. In Table I it is shown that, from a variety of different gases, including air, N_2, O_2, CO_2, CO, and C_2H_4, only acetylene caused a significant inactivation of nitrate reductase. Since the slight inactivation observed under N_2 or O_2 was rather similar to that

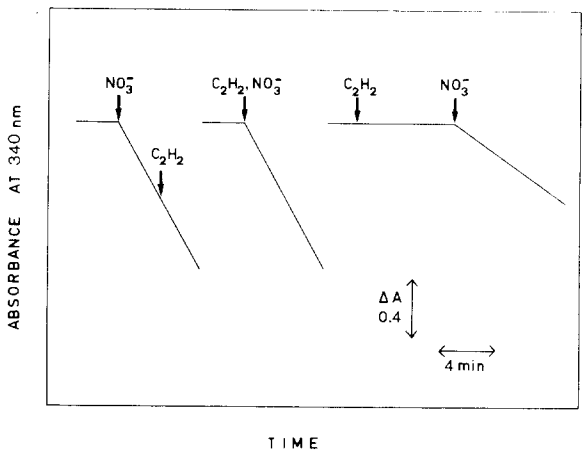

Fig. 1. Effect of acetylene on the NADH-nitrate reductase activity. Initially, the reaction mixture contained, in 2.5 ml, 0.1 M Tris-HCl buffer (pH 7.5)/0.3 mM NADH/nitrate reductase (0.11 mg). Where indicated, a highly concentrated KNO_3 solution was added to obtain a final concentration of 10 mM NO_3^-, and/or the gas phase was changed from air to acetylene.

obtained under air, air atmosphere was selected as control for the subsequent acetylene experiments.

Table II shows that, besides NADH, other low

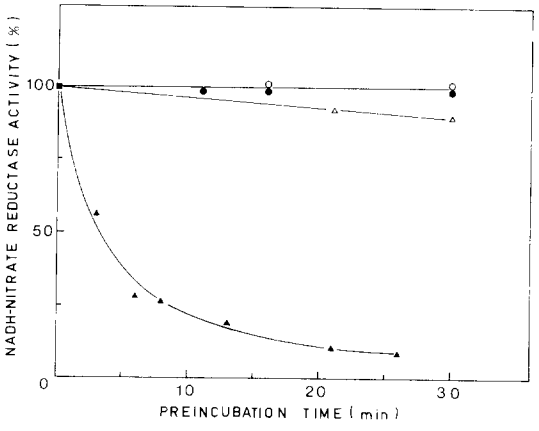

Fig. 2. Time course of acetylene inactivation of NADH-nitrate reductase. Nitrate reductase (0.13 mg) was preincubated at 4°C in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) with air (○——○); acetylene (●——●); air and 0.6 mM NADH (△——△); or acetylene and 0.6 mM NADH (▲——▲). At the coresponding times, 0.1-ml aliquots from the different preincubation mixtures were taken out for measuring NADH-nitrate reductase activity by NADH oxidation as described in the Methods. 100% activity corresponds to 0.14 units per ml preincubation mixture.

TABLE I

EFFECT OF DIFFERENT GASES ON THE NADH-NITRATE REDUCTASE ACTIVITY

Nitrate reductase (1 mg) was preincubated at 4°C in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) with 0.6 mM NADH, under the indicated gas phases. After 30 min preincubation NADH-nitrate reductase activity was measured on aliquots from the preincubation mixtures as in Fig. 2. Activities are expressed as percentages of the control (enzyme, air) at zero time. 100% activity corresponds to 0.4 units per ml preincubation mixture.

Preincubation system	NADH-nitrate reductase activity (%)
Enzyme, air	92
Enzyme, NADH, air	84
Enzyme, NADH, C_2H_2	8
Enzyme, NADH, C_2H_4	90
Enzyme, NADH, N_2	78
Enzyme, NADH, O_2	64
Enzyme, NADH, CO_2	92
Enzyme, NADH, CO	70

potential reducing systems like NADPH, dithionite, dithionite + MV or dithionite + FMN were equally effective in sustaining this acetylene inactivation, while ascorbate or ascorbate + DCIP did not cooperate with acetylene in the inactivation of the nitrate reductase.

It was interesting to know which of the two partial activities of the enzyme was affected by this gas. Table III shows that the terminal activity, assayed either with reduced FMN or reduced MV as electron donors, was greatly diminished by acetylene, whilst NADH-dehydrogenase activity was rather stimulated. Fig. 3 shows that, as the enzyme was gradually depleted of its dehydrogenase activity by increasing heating times, the terminal nitrate reductase became progressively more resistant to the acetylene inactivation, provided that NADH was the corresponding reductant. Table IV shows that acetylene in the presence of dithionite generated the same degree of inactivation of the terminal nitrate reductase whether or not the enzyme was deprived by heat treatment of its dehydrogenase activity.

Since nitrate reduction is closely related with the terminal activity and acetylene interaction takes place precisely on this terminal activity, it was interesting

TABLE II

EFFECT OF DIFFERENT REDUCING SYSTEMS IN SUSTAINING ACETYLENE INACTIVATION OF NADH-NITRATE REDUCTASE

Nitrate reductase (0.26 mg) was preincubated at 4°C in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) and, where indicated, 0.6 mM NADH, 0.6 mM NADPH, 4.6 mM $Na_2S_2O_4$, 0.1 mM FMN, 0.1 mM MV, 4 mM ascorbate, and 10 μM DCIP were added. After 20 min under air or acetylene atmosphere, 0.1-ml aliquots were taken out from the preincubation mixtures for measuring NADH-nitrate reductase activity, determined by NO_2^- appearance. Activities are expressed as percentages of the corresponding controls at zero time. 100% activity corresponds to 0.14 units per ml preincubation mixtures.

Preincubation system		
Liquid phase	Gas phase	
	Air	Acetylene
NADH-nitrate reductase activity (%)		
Enzyme	98	97
Enzyme, NADH	83	8
Enzyme, NADPH	95	7
Enzyme, $S_2O_4^{2-}$	77	1
Enzyme, $S_2O_4^{2-}$, FMN	96	2
Enzyme, $S_2O_4^{2-}$, MV	98	1
Enzyme, ascorbate	92	77
Enzyme, ascorbate, DCIP	98	88

TABLE III

EFFECT OF ACETYLENE ON THE PARTIAL ACTIVITIES OF NITRATE REDUCTASE

Nitrate reductase (0.33 mg) was preincubated at 4°C in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) with or without 0.6 mM NADH under air or acetylene atmosphere. After 30 min preincubation 0.1-ml aliquots from the preincubation mixtures were taken out for measuring the different enzymatic activities (mU · ml^{-1})

Preincubation system	$FMNH_2$-nitrate reductase	MV· nitrate reductase	NADH-dehydrogenase
Enzyme, air	175	173	300
Enzyme, NADH, air	169	171	463
Enzyme, NADH, C_2H_2	30	26	617

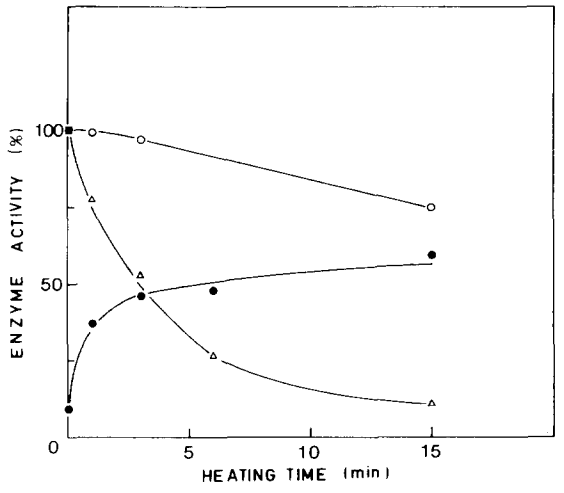

Fig. 3. Requirement of the NADH-dehydrogenase activity for the NADH-dependent acetylene inactivation of nitrate reductase. Incubation mixtures containing 0.09 mg nitrate reductase in 0.5 ml of Tris-HCl buffer (pH 7.5) were heated at 38°C during the indicated times. Aliquots from each incubation mixture were then taken out to measure $MV^{\cdot}$-nitrate reductase (○———○) and NADH-dehydrogenase (△———△) activities, and the remainder of the mixtures were immediately incubated for 9 min at 4°C with 0.24 mM NADH under acetylene atmosphere. After this treatment, $MV^{\cdot}$-nitrate reductase activity (●———●) was again determined on the corresponding aliquots. 100% activity for NADH-dehydrogenase and $MV^{\cdot}$-nitrate reductase of the enzyme before heating corresponded to 0.22 and 0.4 units per ml incubation mixture, respectively.

to study the acetylene inactivation in the presence of nitrate. Fig. 4 shows that the rate of acetylene inactivation was greatly diminished when nitrate was present in the preincubation mixture, whether NADH or dithionite was the reductant. Table V shows that not only nitrate but also cyanate, azide and carbamyl phosphate, competitive inhibitors with respect to nitrate, were also able to protect the enzyme against acetylene effect.

Table VI shows that acetylene-inactivated nitrate reductase was not reactivated by incubation with ferricyanide, whilst cyanide-inactivated enzyme was almost fully reactivated by this oxidant under identical conditions. Furthermore, the acetylene-inactivated enzyme, made free of inactivating agents by filtration on Sephadex G-25, could not be reactivated

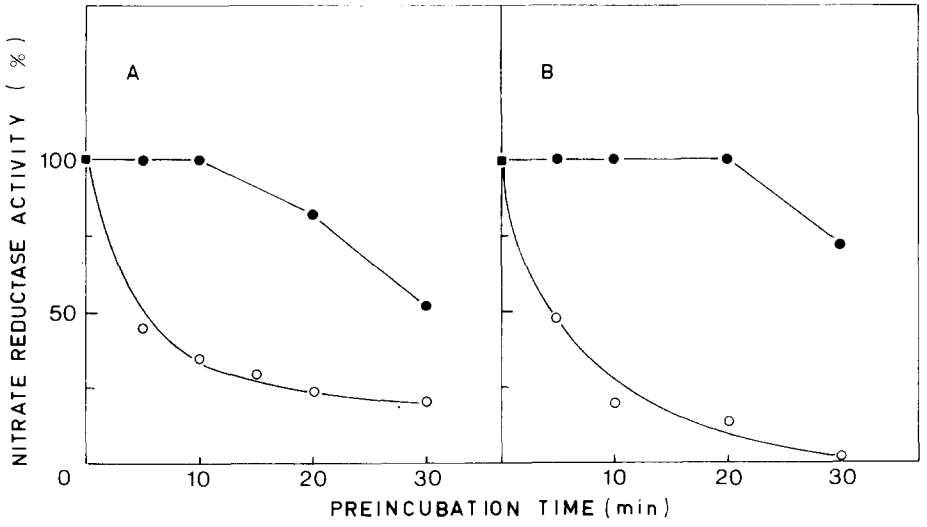

Fig. 4. Protection by nitrate of the acetylene inactivation of nitrate reductase. Nitrate reductase (0.2 mg) was preincubated at 4°C in 2.0 ml of 0.1 M Tris-HCl buffer (pH 7.5) under acetylene atmosphere, either in the absence (○———○) or presence (●———●) of 0.1 M KNO_3, with 0.6 mM NADH (part A) or 4.6 mM $Na_2S_2O_4$ (part B). At the indicated times, 0.1-ml aliquots from the corresponding preincubation mixtures were taken out to measure NADH-nitrate reductase (part A) or $MV^{\cdot}$-nitrate reductase (part B) as described in the Methods. These measurements were corrected for NO_2^- formed in those preincubation mixtures that contained KNO_3. 100% activity for NADH-nitrate reductase and $MV^{\cdot}$-nitrate reductase corresponds to 0.18 and 0.31 units · ml^{-1} preincubation mixture.

TABLE IV

DITHIONITE-DEPENDENT ACETYLENE INACTIVATION OF NITRATE REDUCTASE DEPRIVED OF NADH-DEHYDROGENASE ACTIVITY

Nitrate reductase was maintained at 4°C or heated at 38°C for 15 min under similar conditions as in Fig. 3. Subsequently, the preincubation mixtures were kept for 9 min in the presence of 0.3 mM NADH or 4.6 mM $Na_2S_2O_4$, under acetylene or air, as indicated. After this time, 0.1-ml aliquots from the corresponding preincubation mixtures were taken out for measuring $MV^{\cdot}$-nitrate reductase. Activities are expressed as percentages of the control at the time of adding the reductants. 100% activity corresponds to 0.34 units · ml^{-1} preincubation mixture.

Preincubation system	$MV^{\cdot}$-nitrate reductase activity (%)
Enzyme, air	100
Enzyme, NADH, C_2H_2	10
Enzyme, $S_2O_4^{2-}$, C_2H_2	10
Heated enzyme, air	90
Heated enzyme, NADH, C_2H_2	47
Heated enzyme, $S_2O_4^{2-}$, C_2H_2	10

TABLE V

PROTECTION BY AZIDE, CARBAMYL PHOSPHATE AND CYANATE OF THE ACETYLENE INACTIVATION OF NITRATE REDUCTASE

Nitrate reductase (0.04 mg) was preincubated at 4°C for 11 min in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5)/ 0.24 mM NADH/20 μM azide/0.2 mM carbamyl phosphate, or 50 μM cyanate, under acetylene or air, as indicated. After this time, NADH-nitrate reductase activity was measured by NADH oxidation, using 0.05-ml aliquots from the corresponding preincubation mixtures. Activities are expressed as percentages of the control at zero time. 100% activity corresponds to 0.1 units per ml preincubation mixture.

Preincubation system	NADH-nitrate reductase activity (%)
Enzyme, air	100
Enzyme, NADH, air	93
Enzyme, NADH, C_2H_2	18
Enzyme, NADH, azide, C_2H_2	36
Enzyme, NADH, carbamyl phosphate, C_2H_2	53
Enzyme, NADH, cyanate, C_2H_2	76

TABLE VI

FERRICYANIDE EFFECT ON NITRATE REDUCTASE PREVIOUSLY INACTIVATED EITHER WITH ACETYLENE, CYANIDE, OR CYANIDE AND ACETYLENE

Nitrate reductase (2.3 mg) was preincubated at 4°C in 1.5 ml 0.1 M Tris-HCl buffer (pH 7.5)/0.18 mM NADH/20 μM KCN, under acetylene or air, as indicated. Treatments with KCN lasted 5 min, and all others 40 min. Dialyses of the pertinent mixtures were performed on Sephadex G-25 after the times mentioned above. After the corresponding treatments, 0.05-ml aliquots were taken out from the preincubation mixtures and assayed for $MV^{\cdot}$-nitrate reductase, with and without previous incubation for 3 min with 0.4 mM $K_3Fe(CN)_6$. Activities are expressed as percentages of the control at zero time. 100% activity corresponds to 0.32 units · mg^{-1} protein.

Treatment	$MV^{\cdot}$-nitrate reductase activity (%)	
	$-K_3Fe(CN)_6$	$+K_3Fe(CN)_6$
Enzyme air	100	70
Enzyme, C_2H_2	74	55
Enzyme, NADH, C_2H_2	2	2
Enzyme, NADH, CN^-, air	5	70
Enzyme, NADH, CN^-, air, then dialyzed	5	54
Enzyme, NADH, C_2H_2, then dialyzed	2	7
Enzyme, NADH, CN^-, air, the dialyzed, then C_2H_2	1	1

by ferricyanide. If acetylene replaced air in the gas phase of the already inactivated nitrate reductase, no ferricyanide reactivation could be attained.

Discussion

Nitrate reductase, unlike nitrogenase, is not able to reduce acetylene to ethylene. However, when nitrate reductase is preincubated with acetylene under reducing conditions, it loses the ability to reduce nitrate to nitrite. This inactivating effect seems to be rather specific for acetylene, since other gases tested did not appreciably modify the enzyme activity. Acetylene, in order to exert its inactivating effect, requires nitrate reductase in its reduced form, independently of the nature of the electron donor.

NADH, NADPH, dithionite, dithionite + MV or dithionite + FMN are equally effective in sustaining acetylene inactivation; however, more positive reductants like ascorbate or ascorbate + DCIP were completely ineffective in this process.

From the two partial activities of nitrate reductase, only the terminal nitrate reductase was impaired by acetylene, while the dehydrogenase was even slightly stimulated. However, when the dehydrogenase activity was thermally destroyed and NADH was the reductant, acetylene inactivation of the terminal nitrate reductase activity was prevented, pressumably due to the fact that NADH could no longer feed electrons to this terminal part of the enzyme. Nevertheless, since the terminal nitrate reductase directly accepts electrons from other reductants, such as dithionite and more readily when dithionite is supplemented with MV or FMN, these electrons donors were able to sustain acetylene inactivation even on the dehydrogenase-depleted enzyme. Hence, dehydrogenase activity appears to be essential for acetylene inactivation only when NADH acts as the corresponding electron donor.

The inactivating site of acetylene in the terminal molybdenum-containing part of the enzyme seems to be closely related with the active site for nitrate. In fact not only nitrate, but also its competitive inhibitors, azide, cyanate and carbamyl phosphate [6,28], effectively protect nitrate reductase from the inactivating effect of acetylene. It is noteworthy that, once acetylene inactivation has taken place, neither nitrate nor its already mentioned competitive inhibitors are able to relieve this inactivation.

Cyanide to exert its inactivating effect requires, as in the case of acetylene, nitrate reductase in its reduced state [6]. It has been suggested, based on electron NMR spectroscopy data, that cyanide-inactivated nitrate reductase has cyanide precisely bound at/or close to the molybdenum domain of the enzyme [29]. Cyanide binds presumably at the nitrate active site, since nitrate and its competitive inhibitors protect the enzyme from cyanide inactivation [28]. The fact that ferricyanide reactivates cyanide-inactivated nitrate reductase may imply the reoxidation of some component of the protein related with the nitrate active site, most likely molybdenum [4,14]. Thus far, cyanide and acetylene inactivations of nitrate reductase look very similar. However, the fact that, in contrast to the cyanide-inactivated nitrate reductase, the acetylene-inactivated enzyme could not be reactivated by ferricyanide implies a significant difference. Moreover, if the cyanide-inactivated nitrate reductase is placed under acetylene atmosphere, it could no longer be reactivated by ferricyanide. Three possible explanations of this result should be put forward: (a) cyanide and acetylene bind different reduced components of the protein, being these assumed components closely related with the nitrate active site; (b) cyanide and/or acetylene bind the same reduced component, but in different manners, making the acetylene link stronger, and (c) acetylene displaces cyanide from its common binding site.

Considering the effective interaction of acetylene with the iron-molybdenum cofactor of nitrogenase [18] and the well documented cyanide inactivation of nitrate reductase [4], the actual candidate for acetylene binding in nitrate reductase might be either molybdenum or some other constituent of the molybdenum domain at the nitrate active site.

A somehow similar inactivating effect created by acetylene on an electron transport chain occurs in denitrifying bacteria. The respiratory dinitrogen production from nitrate and nitrite in these organisms is blocked by acetylene, resulting in the alternative release of N_2O, a possible obligate intermediate in the overall denitrifying reaction [30].

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