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INTERCONVERSION OF NITRATE REDUCTASE FROM *ANKISTRODESMUS BRAUNII* RELATED TO REDOX CHANGES

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Reversible inactivation of homogeneous nitrate reductase (NAD(P)H: nitrate oxidoreductase, EC 1.6.6.2) from the green alga *Ankistrodesmus braunii* has been carried out by aerobic incubation of the enzyme with reduced pyridine nucleotide. The involvement of superoxide radicals in the inactivation process is inferred from the fact that it does not take place in the absence of oxygen or in the presence of superoxide dismutase. On the other hand, cyanide also causes the inactivation of the enzyme under reducing conditions. The inactivation of *A. braunii* nitrate reductase takes place in two steps; the first is the one-electron reduction of the enzyme probably involving the molybdenum centers, and the second, and rate-limiting step, results from the interaction of the reduced enzyme with a nucleophylic agent such as superoxide or cyanide. The mean potential value, at pH 7.5, of the inactivation process, measured by reductive titration with dithionite in the presence of cyanide, was −50 mV. Inactive nitrate reductase, previously dialyzed to remove the inactivating agents, can be immediately reactivated by treatment with ferricyanide in a process requiring the removal of only one electron. This process showed a mean potential value, measured by oxidative titration with ferricyanide, of +230 mV at pH 7.5, independent of the system used to inactivate the enzyme.

Introduction

Assimilatory nitrate reductase (NAD(P)H : nitrate oxidoreductase, EC 1.6.6.2) from the green alga *Ankistrodesmuc braunii* can be purified to homogeneity by a simple method which includes as the main step an affinity chromatography on Blue-Sepharose [1]. The native enzyme, M_r = 467 400, is composed of eight subunits of 58 000 each, including four molecules of FAD, four *b*-type cytochromes and two atoms of molybdenum as prosthetic groups [2]. Molybdenum in nitrate reductase has been proposed as forming part of a cofactor which appears to be common to all molybdoenzymes with the sole exception of nitrogenase [3,4]. In addition, two enzymatic activities which participate sequentially in the transfer of electrons from NAD(P)H to nitrate are present in the nitrate reductase complex, the first is a FAD-dependent NAD(P)H-diaphorase and the second is the molybdoprotein terminal nitrate reductase [2].

Although the assimilation of nitrate by photosynthetic microorganisms appears to be primarily regulated at the nitrate uptake level [5–7], the nitrate reductase activity seems to be another key point of control for this pathway [8,9]. Nitrate reductase from the green algae has been proposed to exist in two metabolic interconvertible forms, one oxidized-active and the other reduced-inactive [8,9]. The reversible inactivation of nitrate reductase can be carried out either in vivo by addition of ammonia to the cell cultures [10] or in vitro by incubation of the enzyme solution with NAD(P)H, alone [11] or in the presence of ADP [12] or cyanide [13,14]. In all cases, the original activity of the enzyme can be

immediately recovered by treatment with ferricyanide [11–14].

Losada et al. [9] have proposed that the ammonia-promoted in vivo inactivation of nitrate reductase in green algae is due to the uncoupling by ammonia of the photosynthetic phosphorylation, which increases the intracellular levels of NAD(P)H and ADP which in turn inactivate the terminal moiety of the nitrate reductase complex. By contrast, the hypothesis of Lorimer et al. [15] suggests that the ammonia-promoted in vivo inactivation of nitrate reductase in *Chlorella vulgaris* involves the formation of a complex of the reduced enzyme with firmly bound cyanide.

In this paper we have studied the in vitro interconversion of nitrate reductase from *A. braunii* using homogeneous preparations of enzyme. The inactivation of nitrate reductase requires an one-electron reduction (E'_0, pH 7.5 = –50 mV) of the enzyme molecule, and its further interaction with superoxide or cyanide. The oxidative titration of inactive nitrate reductase shows that the original activity is recovered parallel to the removing of one electron per enzyme molecule (E'_0, pH = 7.5 = +230 mV). Molybdenum seems to be the prosthetic group involved in this redox interconversion of the nitrate reductase activity.

Materials and Methods

Chemicals. FAD, Tris, EDTA, Blue-Sepharose Cl-6B and superoxide dismutase were purchased from Sigma (St. Louis, U.S.A.). NADH and NADPH were from Boehringer (Mannheim, F.R.G.). Methyl viologen was from Serva (Heidelberg, F.R.G.). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). All other chemicals were reagent grade and used as supplied.

Growth of cells. Ankistrodesmus braunii strain 202-7c from Göttingen University's culture collection was grown on 8 mM KNO_3 as described by Kessler et al. [16], except that silicone was added as an antifoaming agent. After three days of growth the cells were harvested by centrifugation, washed, and stored at –20°C until use, usually within one month, without significant loss of nitrate reductase activity.

Purification of nitrate reductase. The enzyme was purified from the crude cell extract by a procedure which included as main steps: streptomycin sulfate fractionation and affinity chromatography on Blue-Sepharose, according to De la Rosa et al. [1]. The resulting purified nitrate reductase preparations supposed a 1200-fold purification with an overall yield of 60%, and had a specific activity of approx. 40 units/mg protein.

Enzymatic assays. Nitrate reductase activity was determined at 30°C colorimetrically by measuring the nitrite formation. The standard assay system contained, in a final volume of 1 ml, 100 μmol Tris-HCl buffer (pH 7.5), 10 μmol KNO_3 and either 0.3 μmol NADH for NADH-nitrate reductase activity, or 0.15 μmol methyl viologen and 4.6 μmol $Na_2S_2O_4$ for reduced methyl viologen-nitrate reductase activity. The reaction was started by addition of the enzyme and stopped after 5 min by rapid oxidation of the electron donor system in a Vortex mixer.

Analytical methods. Protein was estimated by the method of Bailey [17], with bovine serum albumin as standard. Absorbance of the standards at 279 nm was measured to determine their concentration ($\epsilon^{1\%}_{279nm} = 6.7$). Nitrite was estimated as described by Snell and Snell [18].

Potentiometric titrations. Redox titrations of the inactivation and reactivation processes were carried out at 30°C under anaerobic conditions in a cylindrical, thermostatized 6 ml cell equipped with a magnetic stirrer. The reaction mixtures were deaerated by flushing with nitrogen for 20 min before starting the titrations, and nitrogen was also blown onto the surface of the mixture during the process. The oxidation-reduction potential was measured with a Metrohm pH-meter (model E-512) equipped with a combined Pt-Ag/AgCl electrode previously calibrated against a saturated quinhydrone solution at pH 7.0. The following redox mediators were used at a final concentration of 25 μM each: 1,4-naphthoquinone (E'_0, pH 7 = +36 mV), methylene blue (E'_0, pH 7 = +11 mV), indigo-tetrasulfonate (E'_0, pH 7 = –46 mV) and 2-hydroxy-1,4-naphthoquinone (E'_0, pH 7 = –139 mV) for reductive titration; and 2,6-diclorophenol-indophenol (E'_0, pH 7 = +217 mV), 1,2-naphthoquinone (E'_0, pH 7 = +143 mV), *N*-methylphenazinium methosulfate (E'_0, pH 7 = +80 mV), and methylene blue for oxidative titration. Sodium dithionite and potassium ferricyanide, at 50 mM each, were used to poise the redox potential of the reaction mixtures.

Results and Discussion

Inactivation of nitrate reductase from A. braunii by aerobic incubation with NADH

Purified nitrate reductase was inactivated up to 90% after 60 min of aerobic incubation with NADH (Fig. 1). If the incubation was performed under an inert atmosphere of argon the enzymatic activity stayed at a high level all along. These results indicate that this in vitro inactivation of nitrate reductase is not due to a simple reduction of the enzyme molecule, since the process requires the simultaneous presence of a reductant and oxygen.

A similar requirement for oxygen has been reported for the redox inactivation of nitrate reductase from *Chlorella fusca* [19] and other enzymes such as nitrite reductase [20], xanthine dehydrogenase [21] and sulfite reductase [22]. They are all flavoproteins in which the reduced flavin can react with oxygen

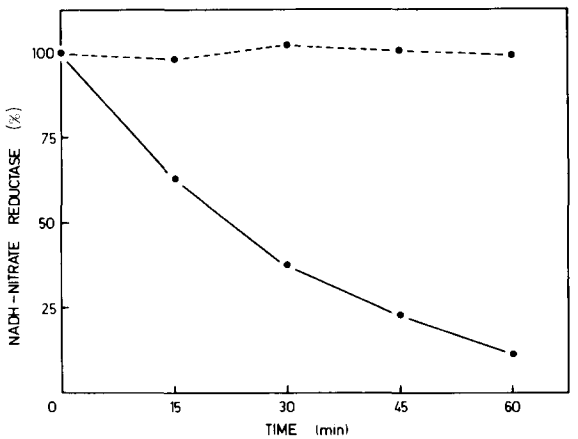

Fig. 1. Effect of anaerobiosis on the inactivation of *A. braunii* nitrate reductase by incubation with NADH. Aliquots of a purified nitrate reductase preparation (10 μg protein) were incubated at 0°C in a final volume of 1 ml with 100 mM Tris-HCl buffer (pH 7.5)/10 mM $MgCl_2$/0.6 mM NADH. When anaerobiosis was required, the incubation system, before addition of NADH, was evacuated at 0°C with a vacuum pump for 3 min in order to remove the dissolved air, refilled with argon to restore the atmospheric pressure, and kept 30 s with stirring. This process was repeated three times. The NADH solution was submitted to the same process. At the indicated times, NADH-nitrate reductase activity was measured by adding 0.05 ml of each incubation system to the reagents of the standard assay. ●- - - - -●, argon; ●———●, air.

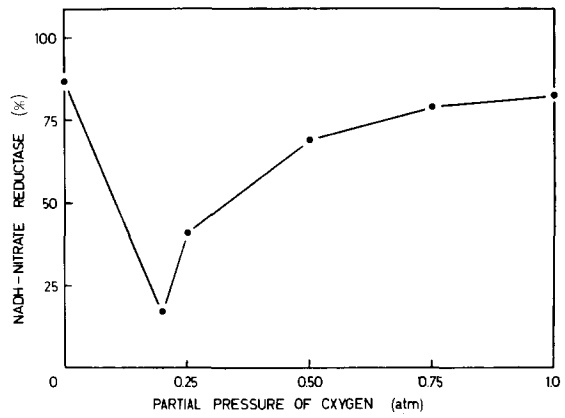

Fig. 2. Effect of the partial pressure of oxygen on the inactivation of nitrate reductase by NADH. 1-ml samples containing 12 μg purified nitrate reductase each, 20 μM FAD and 10 mM $MgCl_2$ in 100 mM Tris-HCl buffer (pH 7.5), were evacuated at 0°C with a vacuum pump. The atmospheric pressure was restored with an oxygen/argon mixture at the ratio indicated in the figure for each sample (oxygen pressure of 1 atm was pure oxygen). This process was repeated three times, and afterwards 20 μl of an anaerobic 30 mM NADH solution were added to each sample. After 45 min at 0°C, NADH-nitrate reductate activity was measured.

and produce hydrogen peroxide, superoxide, hydroxyl radicals and so on [23].

Fig. 2 shows the results obtained after incubation of nitrate reductase with NADH under different partial pressures of oxygen. These data indicate that the inactivation of the enzyme depends on the oxygen concentration, being maximal when the incubation was performed under air. The inactivation of nitrate reductase gradually decreases as the oxygen tension is increased, being negligible under an atmosphere of pure oxygen. In all cases the inactivated enzyme may recover its original activity by oxidation with ferricyanide (data not shown).

NADH-dependent inactivation of nitrate reductase. Protection by dithionite or superoxide dismutase and stimulation by FAD

Dithionite, a strong reducing agent which removes the dissolved oxygen in the aerobic reaction mixture of nitrate reductase with NADH, prevented the inactivation of the enzyme. On the other hand, when superoxide dismutase was included in the incubation system, nitrate reductase was also completely pro-

TABLE I

INACTIVATION OF *A. BRAUNII* NITRATE REDUCTASE BY NADH. STIMULATORY EFFECT OF FAD AND PROTECTION BY DITHIONITE OR SUPEROXIDE DISMUTASE

Aliquots of purified nitrate reductase (15 μg protein) were incubated in a final volume of 1 ml with 100 mM Tris-HCl buffer (pH 7.5) and, where indicated, 0.6 mM NADH, 0.2 mM FAD, 20 mM sodium dithionite and 15 000 units superoxide dismutase. After 30 min at 0°C, NADH-nitrate reductase activity was measured by adding 0.05 ml of each incubation system to the reagents of the standard assay.

Reaction mixture	NADH-nitrate reductase (%)
Enzyme	100
Enzyme + FAD	93
Enzyme + NADH	44
Enzyme + NADH + FAD	23
Enzyme + $Na_2S_2O_4$	99
Enzyme + FAD + $Na_2S_2O_4$	97
Enzyme + NADH + FAD + $Na_2S_2O_4$	112
Enzyme + superoxide dismutase	102
Enzyme + NADH + FAD + superoxide dismutase	98

tected against inactivation by NADH, indicating that superoxide (O_2^-) is involved in the inactivation mechanism (Table I). Catalase and some scavengers of hydroxyl radicals ($OH^\cdot$), such as mannitol or ethanol, did not protect nitrate reductase against its inactivation by aerobic incubation with NADH (data not shown).

TABLE II

REQUIREMENT OF THE DIAPHORASE ACTIVITY OF THE NITRATE REDUCTASE COMPLEX FOR THE NADH-DEPENDENT ENZYME INACTIVATION

Four 1-ml samples containing 7 μg purified nitrate reductase, 2 μmol EDTA and 0.1 mmol Tris-HCl buffer (pH 7.5) were used. Two of them were incubated at 45°C during 15 min (enzyme without diaphorase activity), while the other two were kept at 0°C (enzyme with active diaphorase). Afterwards, 20 μl of a 30 mM NADH solution were added where indicated. After 60 min at 0°C, reduced methyl viologen-nitrate reductase activity was measured by adding 0.05 ml of each reaction mixture to the reagents of the standard assay.

Reaction mixture	reduced methyl viologen-nitrate reductase (%)
Enzyme (inactive diaphorase)	100
Enzyme (active diaphorase)	102
Enzyme (inactive diaphorase) + NADH	85
Enzyme (active diaphorase) + NADH	32

The addition of FAD, prosthetic group of *A. braunii* nitrate reductase [1,2], to the incubation mixture enhanced the inactivating effect of NADH on nitrate reductase (Table I). Furthermore, the FAD-dependent diaphorase activity of the enzyme complex was required for the NADH-induced inactivation of reduced methyl viologen-nitrate reductase (Table II). These observations can be explained assuming that the diaphorase activity is necessary for the transfer of electrons from NADH towards the regulatory center and/or for the FAD-mediated superoxide production, since flavin is an effective generator of oxygen radicals either in flavoproteins with dehydrogenase activity or in systems where free flavin is reduced in the presence of oxygen [23].

Localization of the regulatory center of nitrate reductase

The addition of NADH to an aerobic solution of nitrate reductase led to the immediate reduction of enzyme-bound cytochrome *b*-557 [1], and slowly to the enzyme inactivation (Fig. 1). After inactivation has taken place, the solution was filtered through a Sephadex G-25 column (1 × 13 cm) in order to remove the excess of reagents, and nitrate reductase remained inactive but with its heme chromophore oxidized, as proved by spectrophotometric analysis (data not shown). When a small amount of NADH was added to the inactive enzyme solution, the heme group was again immediately reduced just as it occurred in the active enzyme. By contrast, the further addition of an excess of nitrate slowly reoxidized the heme of the inactive enzyme, requiring a few hours for the reaction to be complete, while reoxidation by nitrate of the reduced heme in the active nitrate reductase preparation was complete in a few minutes (Fig. 3). These results indicate that the NADH-inactivated nitrate reductase presents a permanent jam in its electron transport chain which is localized between cytochrome *b*557 and the nitrate-reducing catalytic site.

It is important to emphasize that both nitrate, the physiological electron acceptor of nitrate reductase, or cyanate, a competitive inhibitor with respect to nitrate, protect the *A. braunii* enzyme against inac-

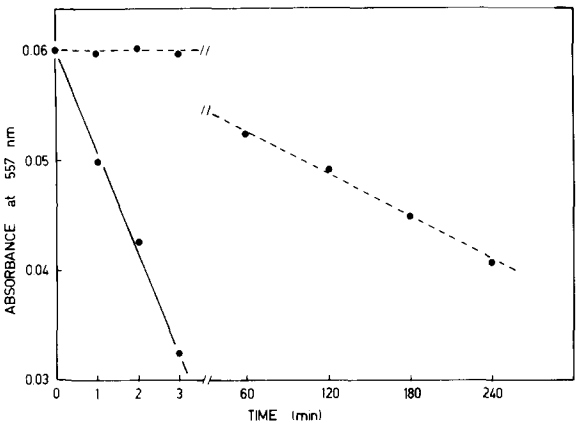

Fig. 3. Reoxidation rate by nitrate of the heme chromophore of the active or inactive enzymes. Two purified nitrate reductase preparations were used in this experiment. The active one was the resulting preparation from the purification procedure. The inactive nitrate reductase was prepared as indicated in Fig. 1 and, after 60 min of incubation, the enzyme solution was filtered through a Sephadex G-25 column to remove the excess of reagents. 0.2 mg of each enzyme preparation in 1 ml 0.1 M potassium phosphate buffer (pH 7.5) were placed in two separate cuvettes. The samples were reduced with a small amount of NADH and then an excess of nitrate was added to both enzyme solutions. Absorbance at 557 nm was measured at the indicated times. •———•, active enzyme; •- - - - -•, inactive enzyme.

tivation by NADH [7]. Therefore, the modification occurring in nitrate reductase which leads to its inactivation is closely related with the active site where nitrate binds to the enzyme and is reduced, i.e., probably in the molybdenum centers. This is in good agreement with the data of Solomonson et al. [24] who found in nitrate reductase from *Chlorella vulgaris* one cyanide-binding site per atom of molybdenum. In other molybdoenzymes, which are inactivated by either cyanide or hydrogen peroxide in the presence of their physiological reductant, molybdenum seems to be involved in the enzyme interconversion [21, 25].

Redox titration of the inactivation process

Homogeneous nitrate reductase from *A. braunii* can be completely inactivated after 15 min of incubation with 4.6 mM dithionite and 2 μM cyanide in a process which does not require a functional diaphorase activity. The addition of ferricyanide to the inactive enzyme led to its immediate reactivation (data not shown).

In order to study the nature of the modification occurring in the protein during the inactivation of *A. braunii* nitrate reductase, a reductive titration of this process was carried out with dithionite in the presence of cyanide. NADH was not used as reductant because: (1) pyridine nucleotide introduces electrons in the enzyme at the beginning of the transport chain, probably at the FAD site, not reflecting the redox potential of the regulatory center but that of the enzyme-bound flavin; and (2) the NADH-induced inactivation of nitrate reductase requires a functional diaphorase activity (see above) which is difficult to maintain during the time necessary for the experiment.

Thus, nitrate reductase was anaerobically incubated with cyanide at different redox potentials that were adjusted by addition of small volumes of a dithionite solution. The inactivation kinetics were followed in each case, obtaining curves that reach a plateau (Fig. 4, inset) from which the redox titration curve was constructed (Fig. 4). Therefore, the nitrate reductase inactivation is a process depending on the redox potential and involving the transfer of only one electron. The 'midpoint potential', at pH 7.5, calculated for the redox change involved in the inactivation process was −50 mV.

Redox titration of the reactivation process

Taking into account that the nitrate reductase activity is usually recovered by treating the inactive enzyme with ferricyanide, the oxidative titration with this oxidant was studied. Thus, nitrate reductase, previously inactivated with dithionite and cyanide and dialyzed to remove the excess of reagent, was incubated at different potentials adjusted with ferricyanide. The reactivation kinetics were followed at each redox potential (Fig. 5, inset), observing that they also reach a plateau that depends on the potential employed. The titration curve obtained in this case (Fig. 5) indicates that the reactivation of nitrate reductase is an oxidative process requiring only one electron. The 'midpoint potential', at pH 7.5, calculated for this redox change was +230 mV. The same value was obtained when the enzyme was previously inactivated by aerobic incubation with NADH, indicating that the reductive modification occurring

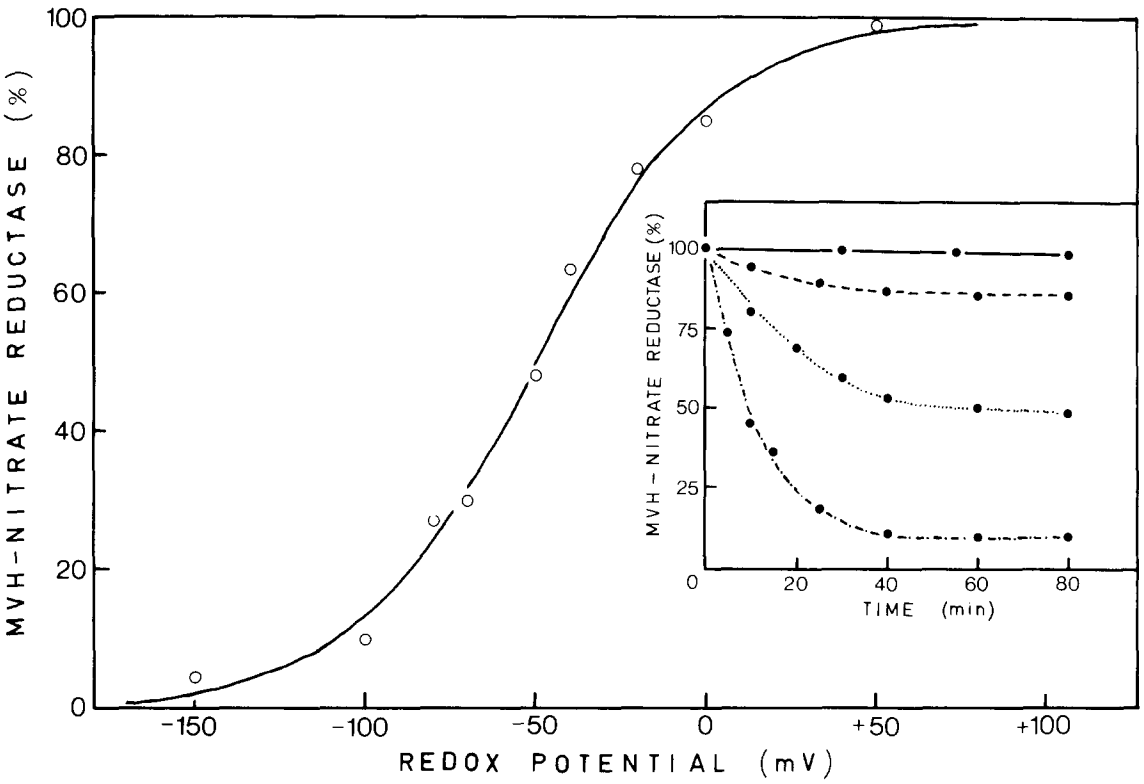

Fig. 4. Reductive titration of the inactivation process of nitrate reductase. The redox potential of a solution containing a mixture of the mediators and 2 μM KCN in 4 ml 50 mM Tris-HCl buffer (pH 7.5), was fitted to the desired value with a 50 mM dithionite solution, and then 14 μg nitrate reductase were added. The reaction mixture was incubated with stirring at 30°C, and 0.2-ml aliquots were taken out at the indicated times with a syringe to assay the reduced methyl viologen-nitrate reductase activity. The experiment was repeated for each potential. The activity (o———o) (E'_0, pH 7.5 = −50 mV) once it became stabilized is plotted vs. the redox potential, and the continuous line represents the theoretical curve supposing n = 1. Inset, the time course for the inactivation reaction at the indicated potentials are shown. E' = +50 mV (•———•); 0 mV (•- - - - -•); −50 mV (•· · · · ·•) and −100 mV (•– · – · –•).

in the enzyme when it is inactivated with dithionite and cyanide is similar to that of the enzyme modified with pyridine nucleotide and superoxide.

It must be noted that the inactive nitrate reductase solutions, after being dialyzed to remove the excess of reagent, presented a redox potential of about +200 mV. This potential is high enough to reoxidize the enzyme, but the reactivation did not occur unless ferricyanide was present in the incubation system (see legend to Fig. 5).

Mechanism proposed for the redox interconversion of A. braunii nitrate reductase

The interconversion of *A. braunni* nitrate reductase has been demonstrated to be due to oxidation-reduction reactions. The inactivation implies two steps: (1) the one-electron reduction of enzyme-bound molybdenum either by pyridine nucleotide or dithionite, and (2) the interaction of the reduced species of nitrate reductase with either superoxide or cyanide, which is probably the rate-limiting step, to yield a permanently inactivated nitrate reductase complex. The reactivation process is a consequence of the reoxidation of the regulatory center of the enzyme by removing one electron, and the simultaneous dissociation of the inactivating nucleophylic agent (Fig. 6). We assume here that molybdenum is as Mo(VI) in the native form of the enzyme, as reported for other nitrate reductases [26].

This mechanism is compatible with those proposed

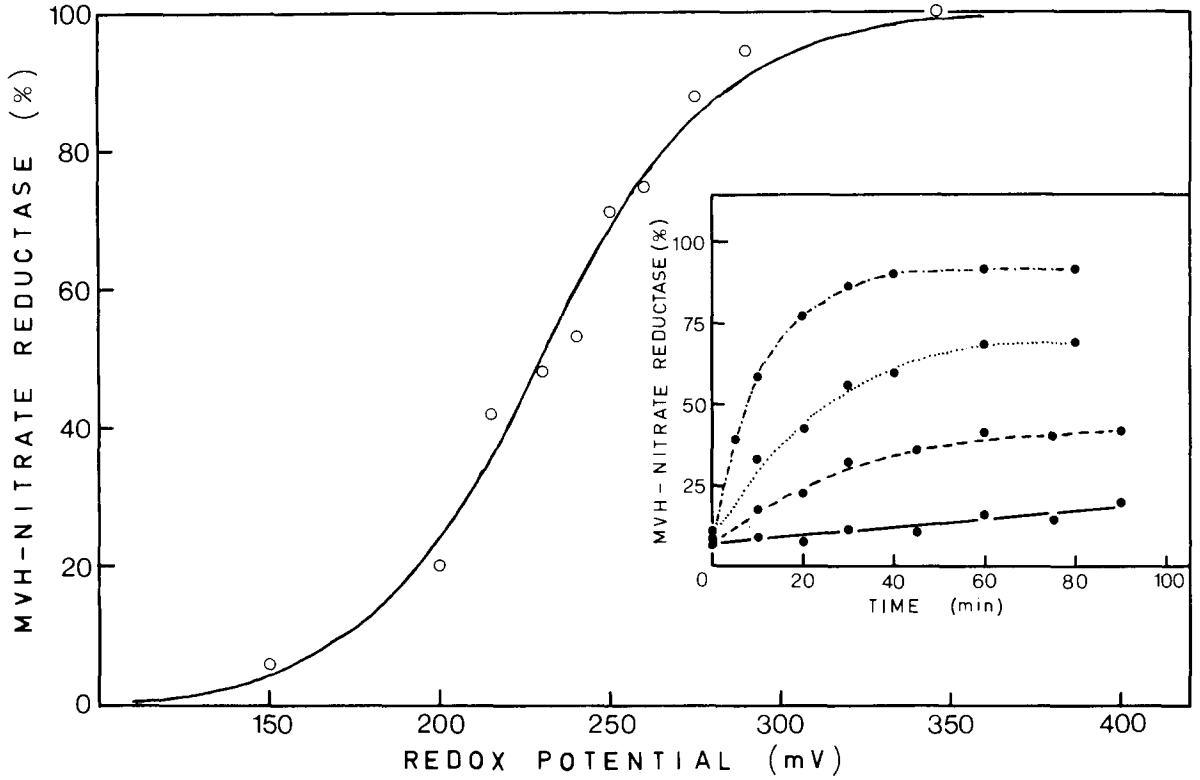

Fig. 5. Oxidative titration of the reactivation process of inactive nitrate reductase. The enzyme was previously inactivated by incubation with 4.6 mM dithionite and 3 μM cyanide for 20 min at 30°C, which were removed after inactivation by dialysis against 50 mM Tris-HCl buffer (pH 7.5) for 4 h. The redox potential of a solution containing the mediators in 4 ml of 50 mM Tris-HCl buffer (pH 7.5) was poised at approx. −150 mV with 50 mM dithionite, then 13 μg of inactive nitrate reductase were added with a syringe and the redox potential was fitted to the desired value with 50 mM ferricyanide. The reaction solution was incubated with stirring at 30°C and 0.2-ml aliquots were taken out at the indicated times to assay the reduced methyl viologen-nitrate reductase activity. The experiment was repeated for each potential. The activity (○) (E'_0, pH 7.5 = +230 mV) once it became stabilized is plotted vs. the redox potential, and the continuous line represents the theoretical curve supposing $n = 1$. Inset, the time course for the reactivation reaction at the indicated potentials are shown. E' = 290 mV (•– · – · –•); 250 mV (•· · · · · ·•); 215 mV (•- - - - -•) and 200 mV (•———•). Similar results were obtained when the enzyme was inactivated by aerobic incubation with NADH (data not shown).

by Vennesland and coworkers [13,15] in which cyanide binds molybdenum of nitrate reductase from *C. vulgaris*, previously reduced by NADH, and by Hewitt et al. [27] who recently suggested that the inactive form of nitrate reductase from higher plants is a stable complex of Mo(IV)-CN^-. However, it contradicts those proposed by Chaparro et al. [19] and Coughlan et al. [28] who suggested that the inactive form of nitrate reductase is an 'over-reduced' species of the enzyme. Thus, the latter authors assume that the inactivation by cyanide of several molybdoenzymes results from overreduction of the molybdenum centers until the level of Mo(III) or Mo(II) [28]. Besides, the inactivation does not occur by reduction of a disulfide bridge of the protein, as previously reported [29].

The potentiometric experiments presented in this paper indicate that the 'midpoint potential' of the inactivation process (reductive titration) is significantly lower than that of the reactivation process (oxidative titration). This apparent controversy could be due to two alternative reasons: (1) the redox groups titrated in both cases are different; or (2) only one group is titrated, but during the inactivation

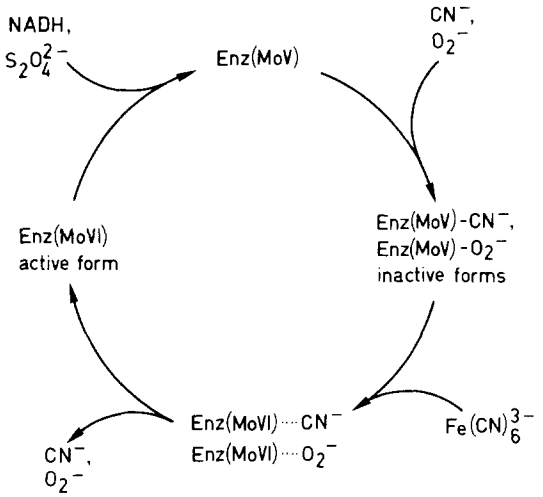

Fig. 6. Mechanism proposed for the redox interconversion of *A. braunii* nitrate reductase. Enz, enzyme.

it is modified and, consequently, its midpoint potential is changed. In the first hypothesis, the reductant (dithionite) would be unable (or would do it very slowly) to reduce directly enzyme-bound molybdenum and the electrons are introduced in the enzyme through its cytochrome *b*-557, whose redox potential (E'_0, pH 7.0 = –73 mV [1]) is similar to that obtained for the inactivation process (E'_0, pH 7.5 = –50 mV (Fig. 4). Once the heme group is reduced, the electrons would pass to the molybdenum centers inside the enzyme and then cyanide would form a stable complex with the reduced metal. The potential of +230 mV obtained for the reactivation process would be that of the couple Mo(V)-CN^-/Mo(VI), CN^-. It is interesting to mention here that Vincent [30] has reported a midpoint potential of +220 mV for the molybdenum prosthetic group of native respiratory nitrate reductase from *Escherichia coli.*

The second hypothesis assumes that dithionite donates electrons directly to molybdenum, reducing it from Mo(VI) to Mo(V) with a midpoint potential of –50 mV. The subsequent binding of cyanide (or superoxide when the enzyme is inactivated by NADH in air) causes a raising of the Mo(V)/Mo(VI) potential to +230 mV, so explaining the higher (more positive) potential obtained by oxidative titration with ferricyanide.

Cammack et al. [31] have reported that in the flavomolybdoprotein xanthine oxidase, dithionite appears to slowly reduce the molybdenum centers and reacts with the enzyme predominantly at the flavin site. Besides, the reduction of xanthine dehydrogenase, other flavomolybdoprotein, by excess of dithionite occurs in several phases, being very slow with the latter one, that involves molybdenum [32, 33]. Molybdoprotein from nitrogenase has also been reported to be very sluggishly reduced by dithionite [34]. These data uphold our first hypothesis in which cytochrome *b*-557 is the entry port for electrons from dithionite to molybdenum. Moreover, Jacob and Orme-Johnson [26] have detected that the reduction of heme and the production of species ascribable to Mo(V) occur at similar redox potentials in *Neurospora crassa* nitrate reductase.

On the other hand, the redox potential of molybdenum in xanthine oxidase is more negative in the desulfo (inactive) form than in the functional one [31]. Recently Barber et al. [35] have reported a similar shift of the molybdenum potential in the native and inactive forms of xanthine dehydrogenase. Besides, Cammack et al. [31] have demonstrated that the selective binding of an anion by Mo(V) bound to xanthine oxidase causes an increase of the Mo(V)/Mo(VI) potential. According with these observations, the binding of cyanide or superoxide to Mo(V) of *A. braunii* nitrate reductase would shift the Mo(V)/Mo(VI) potential from –50 mV to +230 mV.

At this point, it must be noted that the results of the reductive titration presented in Fig. 4 show that the proportion of the active and inactive forms of nitrate reductase becomes constant at each redox potential. The 'midpoint potential' of –50 mV obtained for this process suggests that the heme prosthetic group of the enzyme, although not involved directly in the inactivation of nitrate reductase, serves as the entry port for electrons towards molybdenum. The results of Fig. 5, which also show an equilibrium situation at each redox potential, and the mean value of +230 mV obtained for the reactivation process indicate that the titrated couple is Mo(V)-CN^-/Mo(VI), CN^-. From this point of view, the first of our hypotheses seems to be more credible than the second one.

It is remarkable that the potential of Mo(V)/Mo(VI) varies by over half a volt in different enzymes, being –355 mV, pH 8.2, in xanthine oxidase [31];

−357 mV, pH 7.8, in xanthine dehydrogenase [35]; +117 mV, pH 7.0, in sulfite oxidase [36]; or +220 mV, pH 7.14, in dissimilatory nitrate reductase [30]. At the present moment there is not enough information about the structural and binding aspects of the molybdenum environment to determine the redox potentials and the functional form of *A. braunii* nitrate reductase.

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References

1 De la Rosa, M.A., Diez, J., Vega, J.M. and Losada, M. (1980) Eur. J. Biochem. 106, 249–256
2 De la Rosa, M.A., Vega, J.M. and Zumft, W.G. (1981) J. Biol. Chem. 256, 5814–5819
3 Johnson, J.L. (1980) in Molybdenum and Molybdenum-containing Enzymes (Coughlan, M., ed.), pp. 345–383, Pergamon Press, Oxford
4 Johnson, J.L., Hainline, B.E. and Rajagopalan, K.V. (1980) J. Biol. Chem. 255, 1783–1786
5 Pistorius, E.K., Funkhouser, E.A. and Voss, H. (1978) Planta 141, 279–282
6 Tischner, R. and Lorenzen, H. (1979) Planta 146, 287–292
7 Vega, J.M., Florencio, F.J. and De la Rosa, M.A. (1981) in Proceedings of the 5th International Congress on Photosynthesis, in the press
8 Vennesland, B. and Guerrero, M.G. (1979) in Encyclopedia of Plant Physiology, New Series (Gibbs, M. and Latzko, E., eds.), Vol. 6, pp. 445–456, Springer-Verlag, Berlin
9 Losada, M., Guerrero, M.G. and Vega, J.M. (1981) in Biochemistry and Physiology of Nitrogen and Sulfur Metabolism (Trebst, A. and Bothe, H., eds.), pp. 30–63, Springer-Verlag, Berlin
10 Losada, M., Paneque, A., Aparicio, P.J., Vega, J.M., Cárdenas, J. and Herrera, J. (1970) Biochem. Biophys. Res. Commun. 38, 1009–1015
11 Gómez-Moreno, C., Aparicio, P.J., Palacián, E. and Losada, M. (1972) FEBS Lett. 26, 11–14
12 Maldonado, J.M., Herrera, J., Paneque, A. and Losada, M. (1973) Biochem. Biophys. Res. Commun. 51, 27–33
13 Gewitz, H.S., Lorimer, G.H., Solomonson, L.P. and Vennesland, B. (1974) Nature 249, 79–81
14 Vega, J.M., Herrera, J., Relimpio, A. and Aparicio, P.J. (1972) Physiol. Vég. 10, 637–652
15 Lorimer, G.H., Gewitz, H.S., Völker, W., Solomonson, L.P. and Vennesland, B. (1974) J. Biol. Chem. 249, 6074–6079
16 Kessler, E., Langner, W., Ludewig, I. and Wiechmann, H. (1963) Studies on Microalgae and Photosynthetic Bacteria, pp. 7–20, Jap. Soc. Plant Physiol., Tokyo
17 Bailey, J.L. (1967) Techniques in Protein Chemistry, 2nd edn., p. 340, Elsevier, Amsterdam
18 Snell, F.D. and Snell, C.T. (1949) Colorimetric Methods of Analysis, p. 804, D. Van Nostrand Company, New York
19 Chaparro, A., De la Rosa, M.A. and Vega, J.M. (1979) Z. Pflanzenphysiol. 95, 77–85
20 Vega, J.M., Greenbaum, P. and Garrett, R.H. (1975) Biochim. Biophys. Acta 377, 251–257
21 Betcher-Lange, S.L., Coughlan, M.P. and Rajagopalan, K.V. (1979) J. Biol. Chem. 254, 8825–8829
22 Cohen, H.J. and Fridovich, I. (1971) J. Biol. Chem. 246, 367–373
23 Massey, V., Strickland, S., Mayhew, S.G., Howell, L.G., Engel, P.C., Matthews, R.G., Schuman, M. and Sullivan, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 891–897
24 Solomonson, L.P., Lorimer, G.H., Hall, R.L., Borchers, R. and Bailey, J.L. (1975) J. Biol. Chem. 250, 4120–4127
25 Cohen, H.J., Fridovich, I. and Rajagopalan, K.V. (1971) J. Biol. Chem. 246, 374–382
26 Jacob, G.S. and Orme-Johnson, W.H. (1980) in Molybdenum and Molybdenum-containing Enzymes (Coughlan, M., ed.), pp. 327–344. Pergamon Press, Oxford, New York
27 Hewitt, E.J., Notton, B.A. and Garner, C.D. (1979) Biochem. Soc. Trans. 7, 629–633
28 Coughlan, M.P., Johnson, J.L. and Rajagopalan, K.V. (1980) J. Biol. Chem. 255, 2694–2699
29 Gómez-Moreno, C. and Palacián, E. (1974) Arch. Biochem. Biophys. 160, 269–273
30 Vincent, S.P. (1979) Biochem. J. 177, 757–759
31 Cammack, R., Barber, M.J. and Bray, R.C. (1976) Biochem. J. 157, 469–478
32 Ní Fhaoláin, I. and Coughlan, M.P. (1979) Proc. Royal Irish Academy 79, 111–122
33 Bray, R.C., Barber, M.J., Lowe, D.J., Fox, R. and Cammack, R. (1975) FEBS Meet. Proc. 40, 150–172
34 Zumft, W.G., Mortenson, L.E. and Palmer, G. (1974) Eur. J. Biochem. 46, 525–535
35 Barber, M.J., Coughlan, M.P., Kanda, M. and Rajagopalan, K.V. (1980) Arch. Biochem. Biophys. 201, 468–475
36 Cramer, S.P., Gray, H.B., Scott, N.S., Barber, M. and Rajagopalan, K.V. (1980) in Molybdenum Chemistry of Biological Significance (Newton, W.E. and Otsuka, S., eds.), pp. 157–168, Plenum Press, New York, London