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REGULATION OF NITRATE REDUCTASE ACTIVITY BY NADH AND CYANIDE

LARRY P. SOLOMONSON

Forschungsstelle Vennesland der Max-Planck-Gesellschaft, Harnackstr. 23, 1 Berlin 33 (Germany)

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SUMMARY

NADH:nitrate oxidoreductase (EC 1.6.6.1) of *Chlorella vulgaris* is regulated by oxidation–reduction reactions. With a highly purified preparation of nitrate reductase, a second factor such as cyanide is required in addition to a reductant such as NADH to effect a rapid, reversible conversion of the active form to the inactive form. NADH and cyanide are the most effective combination yet tested, either reagent being effective at concentrations equivalent to the concentration of enzyme. Other reductants such as NADPH or dithionite may be substituted for NADH; and ferrocyanide, sulfide or hydroxylamine may be substituted for cyanide but higher concentrations of these reagents are required to effect a rapid inactivation of the enzyme. The inactive form of the enzyme is rapidly converted to the active form by the addition of ferricyanide. Regulation occurs on the nitrate-reducing moiety. Diaphorase activity is the same in the active and inactive forms. The conversion of the active form to the inactive form is prevented by nitrate, nitrite or flavins. No detectable changes in the absorption spectrum between 240 and 650 nm, or in molecular size, are associated with the inactivation–activation reaction. Both forms of the enzyme are stable after removal of effectors by gel filtration. No correlation was found between the state of activation and the state of oxidation of the cytochrome *b* associated with nitrate reductase.

INTRODUCTION

Previous reports from this laboratory [1, 2] together with reports from Losada and coworkers [3, 4] suggest that NADH:nitrate reductase (EC 1.6.6.1) is regulated by oxidation–reduction reactions. Vennesland and Jetschmann [5] reported that the nitrate reductase of *Chlorella vulgaris* [6, 7] can exist in an inactive form which was slowly converted to an active form under a variety of conditions. Losada et al. [8] also reported that the nitrate reductase of *Chlorella fusca* could be converted to an inactive form if ammonia is added to actively growing cells shortly before harvesting. The conversion of the inactive form to the active form was later found to be greatly enhanced by the addition of the artificial oxidant ferricyanide [1, 3].

Reduced pyridine nucleotides [2, 4], as well as a low molecular weight fraction separated from crude extracts [2], were found to cause a reversible conversion of the active form to the inactive form. It was also shown by Solomonson and Vennesland

[9], as well as by Relimpio et al. [10] and by Garrett and Greenbaum [11], that cyanide is a much more effective inhibitor of nitrate reductase in the presence of reduced pyridine nucleotide. This cyanide inhibition was also rapidly reversed by the addition of ferricyanide [2].

Two components of NADH:nitrate reductase may be functionally distinguished, namely a nitrate-reducing component and an NADH-oxidizing or "diaphorase" component. The latter component catalyzes the reduction of electron acceptors such as cytochrome *c*, dichlorophenolindophenol or ferricyanide by NADH and may act rather independently of the nitrate-reducing component [9]. The nitrate-reducing component may be studied separately from the diaphorase component by substituting certain artificial electron donors such as reduced viologen dyes for NADH. This component, in contrast to the NADH:nitrate reductase and diaphorase activities, is relatively insensitive to reagents which react with sulfhydryl groups [3, 12].

We have also previously reported [9] that the nitrate reductase of *C. vulgaris* is closely associated with a b-type cytochrome with spectral properties identical to the cytochrome *b* associated with the NADPH:nitrate reductase of *Neurospora crassa* [13].

The present study will focus on the conditions for, and the characteristics of, the reversible inactivation of a highly purified nitrate reductase of *C. vulgaris*.

METHODS

Preparation of cell extracts

Sonicated extracts of *C. vulgaris* Beijerinck [6, 7] were prepared from cells which had been grown under continuous light with nitrate as the only nitrogen source and CO_2 as the only carbon source as previously described [9]. After removal of large particles and unbroken cells by centrifugation, the sonicated extracts were stored frozen at −20 °C.

Preparation of nitrate reductase

The purification procedure previously described [9] has been modified. The first $(NH_4)_2SO_4$ fractionation was omitted; and the enzyme was precipitated directly from the crude extract with protamine sulfate, fractionation being achieved as previously described. The final, red-brown, sticky precipitate was extracted overnight at 4 °C with 0.1 M phosphate buffer (pH 7.2), 0.15 mM dithioerythritol, 0.1 mM EDTA and 0.03 mM chloramphenicol. After centrifugation, the nitrate reductase was fully activated by the addition of $K_3[Fe(CN)_6]$ to a final concentration of 1 mM ferricyanide. After incubation for 30 min at 0 °C, the extract was fractionated with $(NH_4)_2SO_4$. The nitrate reductase activity was collected in the 25–40% $(NH_4)_2SO_4$ fraction. The precipitate was resuspended in a small volume of 0.07 M phosphate buffer (pH 7.0), 0.15 mM dithioerythritol, 0.1 mM EDTA, and 0.03 mM chloramphenicol and applied to a Sephadex G-200 column (5 cm × 50 cm) equilibrated with the same buffer. The peak fractions from the G-200 fractionation were combined and sedimented with $(NH_4)_2SO_4$ at 45% saturation. The sediment was resuspended in the starting buffer for the DEAE column. Buffers for the DEAE fractionation were prepared from equimolar mixtures of KH_2PO_4 and K_2HPO_4, and always contained 0.15 mM dithioerythritol and 0.1 mM EDTA. The starting buffer contained

25 mM phosphate. The sample was first passed through a Sephadex G-25 column equilibrated with starting buffer to remove $(NH_4)_2SO_4$, then applied to a DE-52 (Whatman) column (2.5 cm × 15 cm) and washed with about 200 ml of starting buffer. The enzyme was then eluted with a continuous gradient made from 300 ml of starting buffer and 300 ml of buffer containing 0.25 M phosphate. The peak fractions were combined, sedimented with $(NH_4)_2SO_4$ as above and resuspended in a small volume of the same buffer as was used for the first G-200 fractionation. The sample was applied to a second G-200 column (1.5 cm × 85 cm) and eluted with the same buffer. The peak fractions were pooled, 0.5 vol. of glycerol added (33% glycerol final) and aliquots stored at −20 °C. The final, purified enzyme was stable and generally had a specific activity at 20 °C of 21–22 units per mg protein. The overall yield was 25–30%. For some of the tests, enzyme after the first G-200 fractionation was used. This enzyme normally had a specific activity of 9–10 units per mg protein.

Inactivation experiments

Except where otherwise noted, in all of these experiments, the enzyme was incubated at 20 °C with the reagents and small aliquots withdrawn at intervals and transferred to the assay mixture. Control experiments have shown that these reagents do not interfere with the assay. This is partly due to the large dilution of reagents and partly due to the fact that nitrate, which is present in the assay system, prevents further inactivation but does not cause any appreciable reactivation over the time course of the assay. The ferredoxin:NADP reductase (EC 1.6.99.4) used in one of the experiments was prepared from spinach according to the method of Gewitz and Völker [14]. The ratio of absorbance at 456 to 275 nm was 0.110. The flavin concentration was estimated from the absorbance at 456 nm using $\varepsilon_{mM} = 10.3$ at this wavelength [15].

Enzyme assays and protein determinations

All enzyme assays were done at 20 °C. NADH:nitrate reductase and NADH:cytochrome *c* reductase were assayed as previously described [9]. The assay mixture for reduced methyl viologen:nitrate reductase consisted of 0.1 M phosphate buffer, (pH 7.6), 0.01 M KNO_3, 5.75 mM $Na_2S_2O_4$, 12 mM $NaHCO_3$ and 0.39 mM methyl viologen. The reaction was started by addition of enzyme and was stopped after 3 min by rapid oxidation of the electron donor system on a Vortex mixer. The nitrite formed was measured with sulfanilamide and *N*-(napthyl)ethylene diamine hydrochloride [16]. One unit of NADH:nitrate reductase is that amount which catalyzes the oxidation of one micromole of NADH per minute by nitrate, under the conditions of the assay. One unit of cytochrome *c* reductase is that amount which catalyzes the reduction of one micromole of cytochrome *c* by NADH per minute under the specified conditions but after correction to maximum velocity at a saturating concentration of cytochrome *c* [9]. One unit of methyl viologen:nitrate reductase is that amount which catalyzes the reduction of one micromole of nitrate by reduced methyl viologen per minute under the conditions of the assay.

Protein was measured by a modified method of Lowry [17], after precipitation and washing once with 5% trichloroacetic acid at 0 °C. The trichloroacetic acid precipitation was necessary to eliminate the interference by dithioerythritol of the

protein determination. In all cases bovine serum albumin was used as the standard and was treated in the same manner.

RESULTS

Reversibility and localization of the inactivation reaction

With a purified preparation of nitrate reductase, no significant inactivation is caused by NADH alone or by cyanide alone. When, however, both substances are present, a rapid and fully reversible inactivation of NADH:nitrate reductase and reduced methyl viologen:nitrate reductase activities is observed. NADH:cytochrome *c* reductase activity remains essentially constant under all conditions. These results, illustrated in Fig. 1, indicate that the regulatory site of nitrate reductase is localized on the nitrate-reducing component of the enzyme, in agreement with previous experiments with crude enzyme [1, 2]. It is also apparent that two components are required for the reversible inactivation of nitrate reductase, a reductant such as NADH and a second substance such as cyanide. Previous experiments using crude enzyme were not clear on this point. A slow, but appreciable, inactivation by NADH or NADPH was always observed so that the effect of added cyanide (or other substances) could not be properly evaluated.

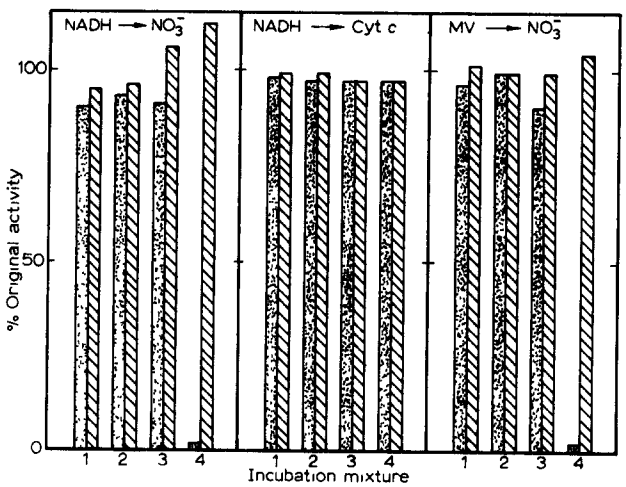


Fig. 1. Reversibility and localization of the inactivation reaction. Incubation mixtures contained 0.05 M sodium, potassium phosphate buffer (pH 7.6) with the following additions: 1, none, 2; 10 μM HCN; 3, 100 μM NADH; 4, 10 μM HCN and 100 μM NADH. These mixtures were equilibrated at 20 °C before starting the reaction by addition of enzyme to a final concentration of 0.92 unit NADH: nitrate reductase per ml. At $t = 5$ min, an aliquot was transferred to the respective enzyme assay mixture and assayed as described in Methods. These activities are represented by the stippled bars and are expressed as percent of the original activity. Just after withdrawing the aliquot at $t = 5$ min, ferricyanide was added to a final concentration of 400 μM and buffer adjusted to 0.1 M phosphate (pH 6 8). After an additional 5-min incubation at 20 °C, an aliquot was withdrawn for the enzyme assays as described above. The enzyme activities after ferricyanide activation are represented by the cross-hatched bars. Initial activities were: 10.1 units NADH:nitrate reductase/mg protein, 76 units NADH:cytochrome *c* reductase per mg and 19 5 units methyl viologen:nitrate reductase per mg. Cyanide ($pK_a = 9.2$), which was added as KCN will be present as HCN at pH 7.6.

In preparing enzyme which will not be inactivated by NADH or NADPH alone, we have found it necessary to fully activate the enzyme (by the addition of ferricyanide) at an early stage of the enzyme preparation. The enzyme remains in the active form after removal of excess ferricyanide. If instead the enzyme is activated by ferricyanide after the final purification step and excess reagent removed by passage through a Sephadex G-25 column, NADH or NADPH alone will then partially convert the enzyme to the inactive form. These results could be explained by the presence of a small amount of bound ferrocyanide or cyanide remaining after activation of the enzyme by ferricyanide.

NADPH or dithionite also cause a reversible inactivation when substituted for NADH. All of these reagents also cause a rapid reduction of the cytochrome *b* associated with nitrate reductase. Only in the case of NADH, however, is the cytochrome *b* rapidly reoxidized by nitrate, which is consistant with NADH being the preferred electron donor for the enzymic reduction of nitrate.

Ferrocyanide, sulfide or hydroxylamine may be substituted for cyanide but higher concentrations are required and, in the case of sulfide and hydroxylamine, complete reversibility is difficult to demonstrate suggesting that some irreversible inactivation may have occurred. The nitrile, cyanoacetic acid had no inactivating effect up to a concentration of 1 mM.

NADH and cyanide have been the most effective combination yet tested. Therefore, these two reagents have been used for most of our studies of the inactivation reaction.

Transfer of reducing equivalents to the regulatory site

The experiment illustrated in Fig. 2 was designed to test whether the reducing equivalents for the inactivation reaction are being donated via the diaphorase component of the enzyme or whether there is perhaps a separate site for NADH (or NADPH) on the nitrate-reducing moiety. Reagents such as *N*-ethylmaleimide or 5,5-dithiobis(2-nitrobenzoic acid) which react with thiol groups, inhibit the diaphorase activity but have little or no effect on the nitrate-reducing activity of the enzyme (when reduced methyl viologen is the electron donor). In the experiment of Fig. 2, the enzyme was preincubated with *N*-ethylmaleimide which, in the absence of NADH, largely inhibited NADH:cytochrome *c* reductase activity while having no effect on the reduced methyl viologen:nitrate reductase activity. Since *N*-ethylmaleimide reacts with sulfhydryl groups to form a stable thiol ether bond, the inhibition is irreversible. NADH prevents the inhibition by *N*-ethylmaleimide.

If the reducing equivalents for the inactivation reaction are being donated via the diaphorase component of the enzyme, the inactivation should then be prevented if the enzyme is preincubated with *N*-ethylmaleimide which largely "turns off" the diaphorase component. This effect can indeed be demonstrated if a limiting concentration of NADH is used. With higher concentrations of NADH, there are apparently enough reducing equivalents transferred to the regulatory site so that little or no effect is seen under the conditions used for the experiment described in Fig. 2. *N*-Ethylmaleimide also prevents the inactivation with limiting concentrations of NADPH suggesting that both NADH and NADPH react at the same site. No effect of *N*-ethylmaleimide on the reactivation of methyl viologen:nitrate reductase activity by ferricyanide was observed. An unequivocal demonstration that reducing equivalents

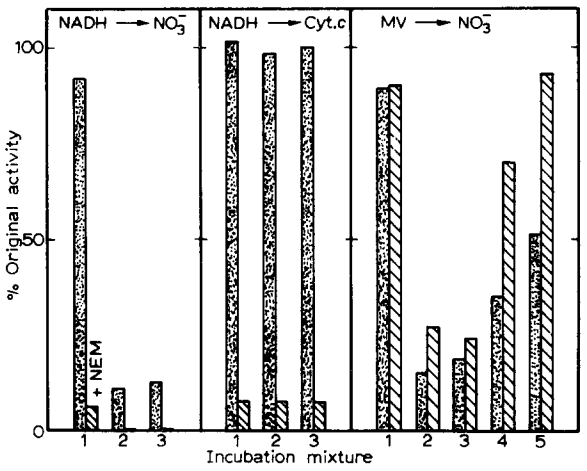


Fig. 2. Prevention of inactivation by *N*-ethylmaleimide. Inactivation mixtures contained 0.05 M sodium, potassium phosphate buffer (pH 7.6), 0.2 mM HCN plus (cross-hatched bars) and minus (stippled bars) 5 mM *N*-ethylmaleimide with the following additions: 1, none; 2, 0.8 μM NADH added at $t = 3$ min; 3, 5.0 μM NADPH added at $t = 3$ min; 4, 0.16 μM NADH added at $t = 3$ min; 5, 1.0 μM NADPH added at $t = 3$ min. The mixtures (without NADH or NADPH) were equilibrated at 20 °C before starting the reaction by the addition of enzyme to a final concentration of 0.92 unit NADH:nitrate reductase per ml. After a total incubation time of 6 min, an aliquot was transferred to the respective enzyme assay mixture and assayed as described in Methods. The initial enzyme activities were the same as for Fig. 1.

to the regulatory site are being transferred via the diaphorase component awaits a physical separation of the diaphorase component from the nitrate-reducing component.

The effect of cyanide concentration

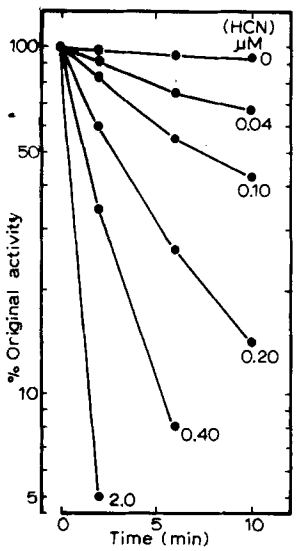
Fig. 3 shows the effect on the rate of inactivation of varying the concentration of cyanide in the presence of a high concentration of NADH. The enzyme concentration used in this experiment was 0.077 μM using a provisional turnover number of 11 200 [9] based on cytochrome *b* content. As can be seen from Fig. 3, the effective concentration of cyanide is of about the same magnitude. Extrapolation of the initial rates of inactivation shown in Fig. 3 to 50% yields a value for the half-time, τ, for each concentration of cyanide used. The order, n, of the reaction may then be calculated from the relationship [18]:

$$n = 1 + \frac{\log \tau_1 - \log \tau_2}{\log c_2 - \log c_1}$$

where τ is the half-time of inactivation, as defined above, at a given cyanide concentration, c. From this relationship a value of 2.0 was calculated for the order of the inactivation reaction with cyanide in the presence of a saturating concentration of NADH. Thus, one molecule of cyanide reacts with one molecule of enzyme.

The effect of reduced pyridine nucleotide concentration

Figs 4a and 4b show the effective concentration of NADH and NADPH,



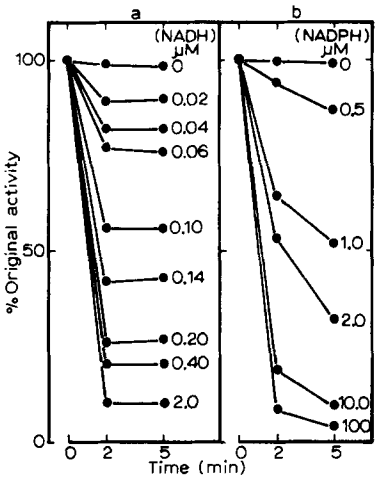


Fig. 3. Effect of cyanide concentration on the rate of inactivation in the presence of excess NADH. Incubation mixtures containing 0.05 M sodium, potassium phosphate buffer (pH 7.6), 0.2 mM NADH and the indicated concentrations of cyanide were equilibrated at 20 °C before starting the reaction by addition of enzyme to a concentration of 0.86 unit NADH:nitrate reductase per ml. Aliquots were withdrawn at the indicated times and immediately transferred to the NADH:nitrate reductase assay mixture. The initial specific activity was 10.1 units NADH:nitrate reductase per mg protein.

Fig. 4. (a) Effect of NADH concentration on the extent of inactivation in the presence of excess cyanide. (b) Effect of NADPH concentration on the rate of inactivation in the presence of excess cyanide. Incubation mixtures containing 0.05 M sodium, potassium phosphate buffer (pH 7.6), 0.2 mM HCN and the indicated concentrations of NADH or NADPH were equilibrated at 20 °C before starting the reaction by the addition of enzyme to a concentration of 1.05 units of NADH:nitrate reductase per ml. Aliquots were transferred to the enzyme assay mixture at the indicated times. The initial specific activity was 19.5 units NADH:nitrate reductase per mg protein.

respectively, for the inactivation of nitrate reductase in the presence of excess cyanide. The inactivation reaction with limiting concentrations of NADH reaches equilibrium within 2 min under these conditions and remains constant. The inactivation reaction with limiting concentrations of NADPH in the presence of excess cyanide is considerably slower and relatively higher concentrations are required.

A Hill plot (Fig. 5) of the data from Fig. 4a and the data of a duplicate experiment yields a complex curve with a maximum slope of 1.6. An s_{50} value of 0.11 μM NADH was obtained from these data which compares with an enzyme concentration of 0.094 μM calculated as described above. Further analysis of these data in conjunction with binding studies and prosthetic group and subunit analysis will be necessary before any firm conclusions can be made concerning "cooperativity". This preliminary data suggests, however, that the reduction phase of the inactivation process is not a simple one-step reduction. Earlier data on the activation reaction with ferricyanide (cf. Fig. 2, ref. 1) indicates this may be true of the activation process also.

Prevention of inactivation by nitrite and flavins

The conversion of the active from of nitrate reductase to the inactive form is

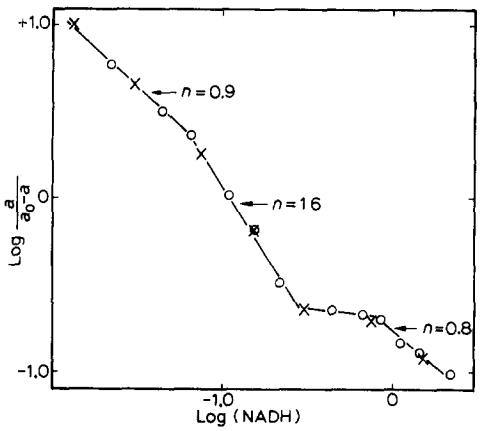
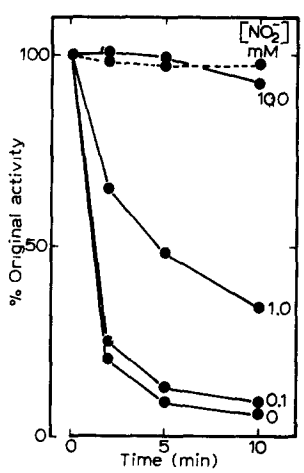


Fig. 5. Hill plot of the data from Fig. 4a (○) and a duplicate experiment (×). a_0 is the average of the activities measured after 2 and 5 min in the absence of NADH. a is the average of the activities measured after 2 and 5 min in the presence of the indicated μmolar concentration of NADH n is the slope of the line.

prevented by nitrate [2]. Nitrite, a competitive inhibitor of nitrate reductase [9], or flavins also prevent the conversion of the active form to the inactive form. These results are illustrated in Figs 6 and 7 which show the effect of varying concentrations



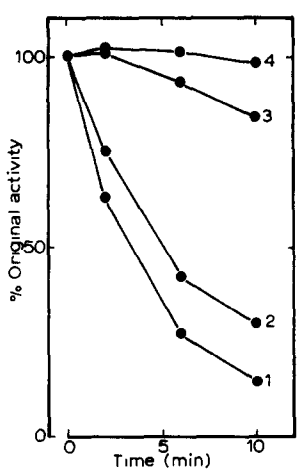


Fig. 6 Prevention of inactivation by nitrite. Incubation mixtures containing 0 05 M phosphate buffer (pH 7.6) 0.2 mM NADH, 1 μM HCN and the indicated concentrations of nitrite were equilibrated at 20 °C before starting the reaction by the addition of enzyme to a concentration of 1.27 units NADH:nitrate reductase per ml. Aliquots were transferred to the enzyme assay mixture at the indicated times. The dashed line indicates the change in activity with time in the presence of phosphate buffer alone. The initial specific activity was 20.3 units NADH:nitrate reductase per mg protein

Fig. 7. Prevention of inactivation by flavins. Incubation mixtures contained 0 05 M phosphate buffer (pH 7.6), 0.2 mM NADH and 0.2 μM HCN with the following additions: 1, none; 2, 1.6 μM ferredoxin:NADP reductase; 3, 16 μM ferredoxin:NADP reductase; 4, 0.2 mM FAD. After equilibration at 20 °C, the reaction was started by the addition of enzyme to a final concentration of 0.87 unit NADH:nitrate reductase per ml. Aliquots were withdrawn at the indicated intervals and assayed for NADH:nitrate reductase activity. The initial specific activity was 9.8 units per mg.

of nitrite (Fig. 6) or flavins (Fig. 7) on the inactivation reaction. Most effective among the flavins tested was the flavoenzyme, ferredoxin:NADP reductase. Free flavins such as FAD, FMN and riboflavin are also effective in preventing the inactivation. Higher concentrations of cyanide can overcome the effect of nitrate [2], nitrite or flavins.

Molecular properties of the active and inactive forms

No significant differences in sedimentation behavior on sucrose gradients or migration on polyacrylamide gel electrophoresis was observed between the active form and the inactive form of the enzyme, indicating no gross changes in molecular size are associated with the activation–inactivation reactions. In both cases the inactivated enzyme remains in the inactive form after separation from the inactivating reagents, NADH and cyanide. Experimental details for the density gradient centrifugation and gel electrophoresis of the purified enzyme will be described elsewhere.

A difference spectrum of the active versus the inactive form of the enzyme showed no significant differences between 240 and 650 nm. For this experiment two samples of purified enzyme containing 21 units of NADH:nitrate reductase per mg protein were taken and one sample inactivated with NADH and cyanide. After incubation for 15 min at 0 °C, the sample treated with NADH and cyanide had less than 2% of the original activity of NADH:nitrate reductase. Both samples were passed over a Sephadex G-25 column equilibrated with 0.05 M phosphate buffer (pH 7.6), 0.15 mM dithioerythritol and 0.1 mM EDTA to remove excess reagents. The enzyme remained in either the active form or the inactive form after passage through the Sephadex column and the inactive form could be fully converted to the active form by the addition of ferricyanide. The absorption spectra and the difference spectrum (0–0.1 scale) were recorded on a Zeiss DMR-21 recording spectrophotometer. Both samples had an absorbance at 412 nm of 0.081 and at 280 nm of 0.198. A conclusion to be drawn from this experiment is that the cytochrome *b* associated with nitrate reductase, although being reduced by all reductants so far shown to be effective as inactivating reagents, does not remain reduced after removal of reagents under conditions where the enzyme remains fully inactive. Thus, no correlation exists between the state of oxidation of the cytochrome *b* and the state of activation of the enzyme.

DISCUSSION

Our studies on a purified nitrate reductase from *C. vulgaris* show quite clearly that two factors are required for the reversible inactivation of nitrate reductase, namely a reductant and a second factor such as cyanide. Further, our results show that both NADH and cyanide are effective at concentrations equivalent to the concentration of enzyme. The sensitivity of the enzyme to cyanide in the presence of excess NADH may, in fact, be adapted for use as a sensitive bioassay for cyanide. We have used this tool together with a chemical assay for cyanide to identify cyanide in cell cultures of *C. vulgaris* at levels expected if it were functioning as a regulator of nitrate reductase activity in these cells (Gewitz, H. S., Lorimer, G., Solomonson, L. P. and Vennesland, B., unpublished). We anticipate that cyanide may be found in other "non-cyanogenic" organisms as well. More data will be required, however, to establish a central role for cyanide in the regulation of nitrate assimilation.

A number of laboratories have reported that reductants increase the sensitivity of nitrate reductase to inhibition by cyanide [9–11] or hydroxylamine [9] and that this inhibition is reversed either by prolonged dialysis [19] or by the addition of an artificial oxidant such as ferricyanide [2]. Our central thesis is that a substance such as cyanide must be present in order for the enzyme to be reduced to an inactive state by a reductant such as NADH, NADPH or dithionite. This inactive form is stable after removal of reagents but may be converted to the active form slowly by oxidation with O_2 (or air) or nitrate or rapidly by the addition of an artificial oxidant such as ferricyanide.

In possible conflict with this thesis is a recent report of Maldonado et al. [4] who in studies with a partially purified nitrate reductase from *C. fusca* showed that ADP together with NADH is required to achieve inactivation of nitrate reductase at a maximal rate. The concentration of ADP required (0.3 mM) and the rate of inactivation (35–75 % inactivation after 30 min) achieved would argue against this being a major regulatory mechanism in vivo. We have not observed any effect of ADP, either alone or in combination with NADH or NADPH, on the rate of inactivation of the nitrate reductase from *C. vulgaris*. In fact, we earlier reported a stimulatory effect of ADP on the rate of activation of nitrate reductase in crude extracts [20]. These apparent discrepancies could be due either to basic differences in the regulatory properties of the nitrate reductases from these two strains of *Chlorella* or, alternatively, to differences in the relative purities of the two enzymes. It should be noted that the present studies were made on enzyme which has a specific activity of 10–20 units/mg protein while the Seville group has reported a maximum specific activity of 0.6 unit/mg for their most highly purified enzyme [3]. Thus the effect of ADP may be indirect. This is not to say the effect of ADP on the inactivation reaction is not of physiological significance but only that we have observed no effect of ADP on our purified enzyme.

Our results suggesting that reducing equivalents for the inactivation reaction are donated via the diaphorase moiety of the enzyme are in agreement with the results of Moreno et al. [3] and of Garrett and Greenbaum [11]. The latter authors found that FAD together with NADPH increases the sensitivity of the NADPH:nitrate reductase of *Neurospora* to inhibition by cyanide, sulfide and thiourea. The reduction of nitrate or diaphorase electron acceptors by NADPH also required added FAD. The inhibition of the *Neurospora* enzyme by cyanide, sulfide or hydroxyurea in combination with NADPH and FAD was partially reversed by diaphorase reagents, especially ferricyanide. Garrett and Greenbaum [11] attributed this reversal to the oxidation of NADPH; the enzyme then being auto-oxidized. Our results would not support this interpretation. The inactive form of the enzyme from *C. vulgaris* is stable after removal of inactivating reagents by gel filtration and no changes in the difference spectrum attributable either to reduced pyridine nucleotide or to reduced cytochrome *b* were observed. Moreno et al. [3] reported an experiment similar to the experiment described in Fig. 2 which also suggested that reducing equivalents for the inactivation reaction were donated via the diaphorase moiety.

Whether the effect of cyanide is due to a direct reaction or interaction of cyanide with the molybdenum of nitrate reductase has not been firmly established. Nicholas and Nason [21] early presented evidence that the NADPH:nitrate reductase of *Neurospora* could be inactivated by dialysis against cyanide. After removal of the

cyanide by dialysis, the enzyme could be specifically reactivated by addition of molybdate. These results were taken as evidence that cyanide specifically complexed with the molybdo moiety of the enzyme. These results, however, have not been confirmed in other laboratories. Notton and Hewitt [19], in fact, recently demonstrated with a nitrate reductase from spinach that molybdenum is not removed from the enzyme by cyanide nor is it incorporated into the enzyme during the reversal of cyanide inhibition.

Cyanide also inhibits other molybdo-enzymes [22–24] but in the case of xanthine oxidase from milk this has been shown to be due to the reaction of cyanide with a persulfide group of the enzyme with the resultant formation of free thiocyanate. The enzyme, freed of cyanide and thiocyanate could be largely reactivated by reaction with sulfide with an accompanying incorporation of sulfide sulfur into the protein [23]. This is unlikely to be the case with nitrate reductase, however, since the enzyme is rapidly and fully reactivated by reaction with ferricyanide alone, even after removal of low molecular weight substances by passage through a Sephadex G-25 column. EPR studies on other molybdo-enzymes have suggested that cyanide may interact directly with the molybdo moiety [22, 24]. In the case of sulfite oxidase [24] which is also associated with a cytochrome *b* [25], cyanide has no effect on the oxidized enzyme but irreversibly inactivates the enzyme which has been reduced by sulfite. No attempt was apparently made in these studies to reverse the inactivation by an artificial oxidant such as ferricyanide.

Stiefel [26] has recently proposed a model for nitrate reduction in which Mo(IV) is oxidized to Mo(VI) in reducing nitrate to nitrite. Mo(VI) would then presumably be re-reduced to Mo(IV) by the electron donor. If this model is correct, then a possible explanation for the inactivation reaction would be that a substance which complexes with the molybdo-moiety may alter the properties of the molybdo-center in such a way that it could be reduced by electron donors to a stable Mo(III) state which would not participate in O-atom transfer reactions [27], and thus would not be able to reduce nitrate. The prevention of inactivation by nitrate and competitive inhibitors of nitrate which would presumably stabilize the Mo(IV) or Mo(VI) state would be consistant with this hypothesis. Also, as pointed out by Stiefel [26], the acidity of coordinated complexes of molybdenum may differ greatly depending on the oxidation state of the molybdenum. This could account for the pronounced pH effect on the rate of activation of nitrate reductase noted earlier by Vennesland and Jetschmann [5]. Further studies, especially with EPR, are obviously needed to determine the oxidation state of molybdenum in the active and inactive states.

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