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THE INHIBITION OF THE *NEUROSPORA CRASSA* NITRATE REDUCTASE COMPLEX BY METAL-BINDING AGENTS

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SUMMARY

1. The *Neurospora crassa* nitrate reductase complex is sensitive to inhibition by the metal-binding agents cyanide, sulfide and thiourea. The degree of sensitivity and the inhibitory pattern produced by these inhibitors is dependent on the oxidation state of the enzyme. When oxidized, the nitrate reductase is less sensitive to the inhibitors, and their action is competitive with respect to nitrate. However, if the enzyme is reduced by preincubation with NADPH and FAD, its sensitivity to these particular metal-binding agents is increased and nitrate can no longer competitively alleviate their action.

2. Artificial electron acceptors for the diaphorase activity of the nitrate reductase complex can, in varying degrees, effect a reversal of this non-competitive inhibition of the reduced enzyme.

3. Presumably, the molybdenum moiety of the nitrate reductase complex has a greater affinity for the metal-binding agents when it is reduced. The phenomenon could serve to regulate the activity of the nitrate reductase complex.

INTRODUCTION

The nitrate reductase complex from *Neurospora crassa* is a soluble electron transport system, using electrons sequentially transferred from NADPH *via* enzyme-bound FAD, cytochrome b_{557} and molybdenum moieties to reduce nitrate for assimilatory purposes. The complex also exhibits a diaphorase-like activity by reducing artificial electron acceptors, such as ferricyanide. This diaphorase activity is relatively insensitive to metal-binding agents, indicating the artificial acceptors interact with the electron transfer sequence somewhere before molybdenum. On the other hand, inhibition of the overall NADPH:nitrate reductase activity by various metal-binding agents such as cyanide has been employed as an indirect means of establishing the role of molybdenum in this enzyme[1–3]. In addition to more direct procedures, this method has also been used in recent studies of nitrate reductases from other orga-

Abrevation: DCIP, 2,6-dichlorophenylindophenol.

nisms[4–9] to confirm the presence of molybdenum in these proteins. Certain of these latter investigations[6,8,9] have indicated that cyanide interacts with a reduced form of the nitrate reductase since preincubation of cyanide, enzyme and reduced pyridine nucleotide electron donor gave maximal inhibition. The inhibition was non-competitive with respect to nitrate, but could be prevented in the presence of this electron acceptor. Ferricyanide but not nitrate could reverse the inhibition, prompting the suggestion that this artificial electron acceptor oxidized the reduced enzyme form and thereby elicited reversal[9]. These observations may have physiological significance since both the *Chlorella vulgaris*[10,11] and the *Chlamydomonas reinhardti*[12] nitrate reductases can occur in an inactive form in crude extracts. The inactive enzyme from both algae can be reactivated by oxidation with ferricyanide. A regulatory scheme postulating an inactive, reduced enzyme form *vs* an active, oxidized form has been suggested based on these results[9,10,12].

This report deals with similar inhibition studies on the *Neurospora* enzyme and shows that the inhibition by certain metal-binding agents, *i.e.* cyanide, sulfide and thiourea, is competitive with nitrate in the absence of any preincubation with an electron donor, but non-competitive following preincubation of enzyme, inhibitor and NADPH. This latter type of inhibition can be reversed by ferricyanide, 2,6-dichlorophenolindophenol (DCIP) or mammalian cytochrome *c*. The mechanism of this reversal is best explained as the oxidation of the electron donor in the presence of these artificial electron acceptors *via* the diaphorase activity of the nitrate reductase complex, thus returning the enzyme to the oxidized state in which it is less sensitive to metal-binding agents and inhibition by metal-binding agents is competitive with nitrate.

METHODS

Preparation and assay of the enzyme

Partially purified preparations of NADPH:nitrate reductase were obtained from *Neurospora crassa* strain 5297a. Mycelia were grown on Fries media containing NH_4^+ as the sole nitrogen source and then induced for maximal nitrate reductase activity by two consecutive 3 h exposures to fresh Fries media having sodium nitrate as the only nitrogen source[13]. Crude extracts were prepared as described previously[13], and then adjusted to pH 5.0–5.2 by the dropwise addition of 1 M acetic acid. Following clarification by centrifugation, the supernatant solution was treated with $(NH_4)_2SO_4$ to a final concentration of 50% saturation, and the resultant precipitate collected by centrifugation and dissolved in 0.1 M potassium phosphate buffer (pH 7.3) containing 5 mM cysteine·HCl and 0.5 mM EDTA. This $(NH_4)_2SO_4$ precipitate was then dialyzed against 35% saturated $(NH_4)_2SO_4$–0.1 M potassium phosphate buffer (pH 7.3) *plus* cysteine and EDTA for 4 h, clarified by centrifugation and redialyzed 12 h against 40% saturated $(NH_4)_2SO_4$ in phosphate buffer to yield the 35–40% $(NH_4)_2SO_4$ precipitate, as described earlier[3]. This precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 6.0) containing 20% glycerol, 5 mM cysteine·HCl and 0.5 mM EDTA, and stored at −15 °C until used. These partially purified nitrate reductase preparations had specific activities of at least 200 nmoles NO_2^- formed/min per mg protein.

Nitrate reductase activities were measured as described by Garrett and Nason[3].

Analysis of data

The double reciprocal plots of the Lineweaver–Burk type were drawn according to coordinates obtained from analysis of the data by the least squares method using a Wang Series 370 Electronic Calculator. Correlation coefficients for the data were greater than 99%.

RESULTS

Competitive and non-competitive inhibition of nitrate reductase by cyanide, sulfide and thiourea

In our routine assays for nitrate reductase activity, reagents have always been added in the following order: buffer, nitrate, FAD, enzyme, then NADPH to initiate the reaction. In inhibition studies, the inhibitor is added after FAD and before enzyme. Under such conditions and without any preincubation, cyanide, sulfide and thiourea exhibit typical competitive inhibition with respect to nitrate, as evidenced in Figs 1A and 1C. The K_i values for cyanide, sulfide and thiourea are 19, 23 and 420 μM, respectively.

In contrast to these observations, when buffer, FAD, enzyme and NADPH (all of the components of the reaction mixture except nitrate) were preincubated with cyanide, sulfide or thiourea for 5 min at 0 °C, non-competitive inhibition resulted, as shown by Figs 1B and 1D. Assays were performed by following the sequence of 5 min preincubation at 0 °C, brief incubation at 30 °C to warm the sample, followed by addition of nitrate to initiate the reaction. In the absence of inhibitors, preincubation with NADPH had no effect. In addition to altering the inhibition pattern, preincubation in the presence of reductant increased the sensitivity of the nitrate reductase to the metal-binding agents, as evidenced by the K_i values of 5.6, 19 and 107 μM for cyanide, sulfide and thiourea, respectively.

Different batches of partially purified enzyme were used in these experiments, and the observed K_m values for nitrate varied from 0.2–0.4 mM. Preincubation had no effect on the variation; the K_m remained constant for any given enzyme preparation. Approximately a 2-fold variation in K_i values occurred also, but the K_i values for the enzyme preincubated with NADPH were consistently much lower than the K_i values observed in the competitive case. The order of sensitivity, cyanide $>$ sulfide $>$ thiourea, was consistent throughout, in both the competitive and non-competitive situations.

Prevention and reversal of the non-competitive inhibition of nitrate reductase

Table I illustrates some of the properties of the non-competitive inhibition of the nitrate reductase by cyanide, sulfide and thiourea. In all cases, this inhibition is obtained only if both NADPH and FAD are present during preincubation of the enzyme with the inhibitor. Further, if nitrate is also present, no inhibition results. Obviously, nitrate can only prevent this inhibition; it cannot reverse when added later. Note that only one-tenth of usual substrate amounts of NADPH are necessary in the preincubation mixture to achieve inhibition.

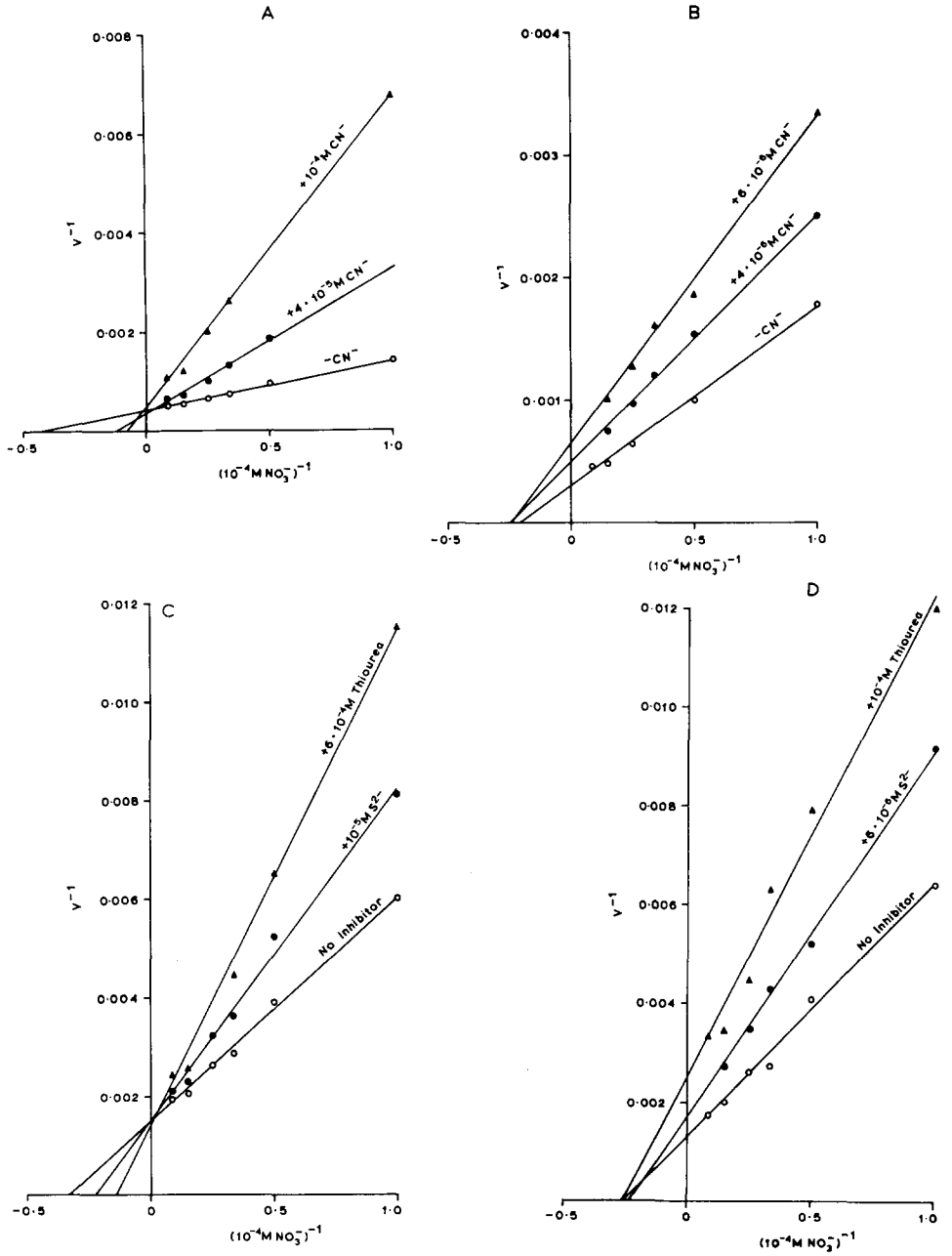

Fig. 1. Competitive and non-competitive inhibition of oxidized and reduced forms of *N. crassa* nitrate reductase, respectively, by cyanide, sulfide and thiourea. In A and C, the standard assay protocol (see text) was followed in performing nitrate saturation curves in the absence or presence of fixed amounts of the indicated inhibitor. In B and D, all ingredients of the reaction mixture except nitrate were preincubated 5 min at 0 °C with or without inhibitor prior to assaying the response to varying nitrate concentrations. The velocity, v, is given as nmoles of NO_3^- formed/min, and 150–180 μg protein (35–40% $(NH_4)_2SO_4$ precipitate fraction) were used per reaction mixture. In A, C, and D, the reaction period was 1 min; in B, 2 min.

TABLE I

INHIBITION OF THE NITRATE REDUCTASE COMPLEX BY PREINCUBATION WITH CYANIDE, SULFIDE OR THIOUREA IN THE PRESENCE OF NADPH AND FAD

Aliquots of the partially purified nitrate reductase were preincubated for 5 min at 0 °C with and without inhibitor, NADPH, FAD and/or nitrate. These preincubation mixtures (0.35 ml final volume) all contained 153 μg partially purified enzyme and 30 μmoles phosphate buffer (pH 7.5). In addition, the following amounts of substances were included, where indicated: NADPH, 10 nmoles; FAD, 5 nmoles; NO_3^-, 10 μmoles; and CN^-, 6 nmoles, S^{2-}, 20 nmoles; or thiourea, 300 nmoles. Following preincubation, the mixtures were warmed to 30 °C, and 0.05 ml 2 mM NADPH and 0.1 ml 0.1 M NO_3^- were added to initiate the assay for NADPH:nitrate reductase activity.

Preincubation conditions	*NADPH:nitrate reductase activity (nmoles NO_2^- formed/min)*
NADPH, FAD	38.7
NADPH, FAD, CN^-	3.9
FAD, CN^-	33.8
NADPH, FAD, CN^-, NO_3^-	35.2
NADPH, CN^-	29.4
NADPH, FAD	31.8
NADPH, FAD, thiourea	4.4
FAD, thiourea	30.9
NADPH, FAD, thiourea, NO_3^-	32.4
NADPH, thiourea	29.4
NADPH, FAD	39.2
NADPH, FAD, S^{2-}	4.9
FAD, S^{2-}	36.8
NADPH, FAD, S^{2-}, NO_3^-	39.2
NADPH, S^{2-}	30.4

TABLE II

NADPH-DEPENDENT INHIBITION OF THE NITRATE REDUCTASE COMPLEX BY CYANIDE, SULFIDE AND THIOUREA—REVERSAL BY ARTIFICIAL ELECTRON ACCEPTORS

Preincubation mixtures (0.35 ml final volume) contained 139 μg partially purified nitrate reductase, 10 nmoles NADPH, 5 nmoles FAD, 30 μmoles phosphate buffer (pH 7.5), and, where indicated, 6 nmoles of CN^-, 20 nmoles of S^{2-} or 300 nmoles of thiourea. Following preincubation for 5 min at 0 °C, the mixtures were warmed to 30 °C. At this point, NADPH:nitrate reductase activity was assayed by adding 0.05 ml 2 mM NADPH and 0.1 ml of 0.1 M NO_3^-; or, alternatively, either ferricyanide, cytochrome *c*, or 2,6-dichlorophenolindophenol was added as indicated, and the mixture was incubated briefly at 30 °C in order to elicit reversal of inhibition. Then, NADPH and nitrate were added to assay enzymatic activity.

Preincubation and treatment	*NADPH:nitrate reductase activity (nmoles NO_2^- formed/min)*		
	CN^- expt	*S^{2-} expt*	*Thiourea expt*
Minus inhibitor	63.7	59.8	60.8
Plus inhibitor (CN^-, S^{2-} or thiourea)	4.1	7.2	4.3
Reversal			
Plus inhibitor, *plus* $Fe(CN)_6^{-3}$, 60 nmoles	53.4	31.8	19.1
Plus inhibitor, *plus* cytochrome *c*, 0.4 mg	24.0	24.5	10.3
Plus inhibitor, *plus* 2,6-dichlorophenolindophenol, 38 nmoles	21.6	33.3	11.8

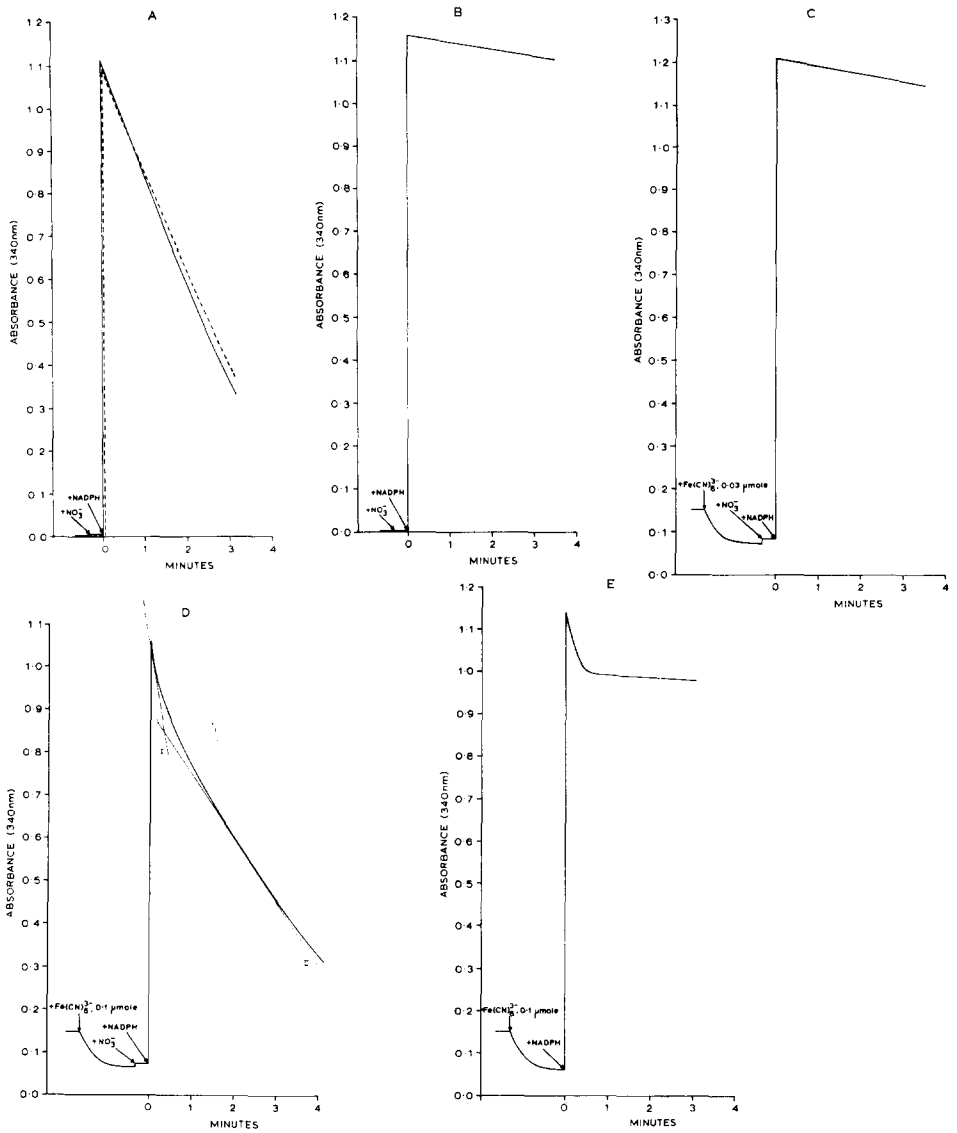

Fig. 2. The role of the diaphorase activity of the *N. crassa* nitrate reductase complex in the ferricyanide reversal of the NADPH-dependent inhibition by cyanide. In this experiment, NADPH oxidation was measured spectrophotometrically using a Cary Model 14 Spectrophotometer. (A) No preincubation with NADPH. Control (———): 65 μmoles phosphate buffer (pH 7.3), 5 nmoles FAD and 70 μg enzyme preparation in a volume of 0.8 ml were placed in both sample and reference cuvettes. The absorbance was constant. Then, 10 μmoles nitrate in 0.1 ml was added to each, with no resultant absorbance change. Then, 0.1 ml water was added to the reference cuvette and 0.2 μmole NADPH (in 0.1 ml) to the sample, and the resultant decrease in absorbance was monitored as a measure of the nitratedependent NADPH oxidation activity. *Plus* cyanide (— — —): Same as control, except 10 nmoles of cyanide were present throughout. (B –E) Preincubation with NADPH and cyanide. (B) The phosphate buffer, FAD, enzyme and cyanide were preincubated 5 min at 0 °C with 20 nmoles of NADPH (final volume = 0.8 ml). No absorbance change was observed upon addition of nitrate. Then water was added to the reference and 0.2 μmole NADPH to the sample cuvette, and the resultant absorbance changes followed. (C) Prenicuba-

Table II presents the results of studies where reversal of the non-competitive inhibition was tried using the artificial electron acceptors ferricyanide, DCIP and mammalian cytochrome *c*. In these experiments, preincubated mixtures of enzyme, NADPH, FAD and inhibitor were treated briefly with the electron acceptors prior to conducting nitrate reductase assays on the samples. In all cases, some reversal of inhibition was achieved. Thiourea-inhibited, reduced enzyme was least susceptible to reversal by any oxidant. Ferricyanide proved to be the superior agent for reversal of cyanide inhibition, as reported for the *Chlorella* enzyme[9]; but DCIP and cytochrome *c* also had significant effects here. DCIP was slightly better than ferricyanide in reversing the sulfide inhibition of the reduced nitrate reductase complex.

As indicated above, the reversal by ferricyanide of cyanide inhibition of the reduced enzyme (*i.e.* enzyme preincubated with reduced pyridine nucleotide) has been reported previously for *Chlorella pyrenoidosa*[9]. The phenomenon has been implicated as an analog of a possible regulatory process for controlling nitrate reductase activity, with ferricyanide acting as an oxidant of the reduced, inactive enzyme to generate the oxidized, active form. However, the role of ferricyanide in such *in vitro* systems is perhaps best explained through its function as a substrate of the diaphorase moiety of the nitrate reductase complex, leading to the oxidation of NAD(P)H, as demonstrated in Fig. 2. Fig. 2A demonstrates the effect of 10^{-5} M cyanide on NADPH:nitrate reductase activity as measured by the decrease in absorbance at 340 nm due to the nitrate-dependent oxidation of NADPH. Since no preincubation was done, little inhibition is obtained at this cyanide concentration (ΔA/min, *minus* cyanide = 0.262; ΔA/min, *plus* cyanide = 0.241) [J. M. Vega and M. Losada (personal communication) have indicated that the slight curvature in the "*plus* cyanide" rate may be indicative of a gradual progression toward the non-competitive type of inhibition]. Fig. 2B depicts the inhibition obtained when the enzyme is preincubated 5 min at 0 °C with 10^{-5} M cyanide, FAD and low levels of NADPH, prior to measuring nitrate-dependent NADPH oxidation (ΔA/min = 0.017). Such preincubated mixtures can be treated with ferricyanide to elicit reversal of the inhibition, as demonstrated above in Table II. The addition of insufficient ferricyanide to oxidize all the NADPH present in the preincubation gives essentially no reversal, because the trace amounts of residual NADPH keep the enzyme reduced (Fig. 2C, ΔA/min = 0.018). However, provided enough ferricyanide is added to oxidize all the NADPH in the preincubation mixture, the inhibition is reversed (Fig. 2D, ΔA/min (Phase II) = 0.160). Note that the rate of decrease in absorbance at 340 nm following addition of substrate amounts of NADPH exhibits two phases. The initial, more rapid Phase I is due to the oxidation of NADPH by the excess ferricyanide remaining, while the slower Phase II can be attributed to nitrate-dependent NADPH oxidation. This explanation is supported by a comparison of Fig. 2D with Fig. 2E, where the same experiment was done except no nitrate was added. Obviously, if nitrite for-

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tion as in B was done, but 30 nmoles ferricyanide were added to the preincubated mixture in the sample cuvette, and the ensuing absorbance changes monitored. When no fuhrter change occurred, nitrate was added to both cuvettes, followed by 0.2 μmole NADPH (to sample) and water (to reference). (D) The protocol of C was followed except the amount of ferricyanide added was increased to 100 nmoles. (E) The D experiment was repeated except no nitrate was ever added. Consequently, the decrease in absorbance at 340 nm is only due to ferricyanide-dependent NADPH oxidation (diaphorase activity).

mation instead of nitrate-dependent NADPH oxidation had been employed to measure nitrate reductase activity, high levels of ferricyanide would have given an apparently poor reversal of the inhibited nitrate reductase because the electrons from NADPH preferentially reduce ferricyanide instead of nitrate.

DISCUSSION

The three metal-binding agents employed in these experiments showed essentially similar effects. Non-competitive inhibition of the nitrate reductase complex could be achieved through their use provided conditions which should give full reduction of the enzyme were met. Namely, both NADPH and FAD were required. Further, the terminal electron acceptor, nitrate, must be absent; otherwise oxidation of the enzyme could occur. The most likely site of action of these inhibitors is the molybdenum component of the nitrate reductase. Presumably then, these metal-binding agents have a greater affinity for the reduced molybdenum species than its oxidized counterpart. It is known that cyanide displays a much greater affinity for Mo(IV) and Mo(V) than for the oxidized Mo(VI) state[14]. Hepatic sulfite oxidase, which is also a molybdohemoprotein, is irreversibly inactivated in the presence of cyanide and sulfite with a concommitant loss of the sulfite-elicited EPR signal attributed to Mo(V)[15]. Vega *et al.*[9] also concluded that the redox state of molybdenum was critical to the cyanide inhibition of the reduced *Chlorella* nitrate reductase. However, in their studies, ferricyanide was unique in its ability to reverse the inhibition, leading them to suggest a direct oxidation of the reduced molybdenum as the mechanism of the reversal.

In the studies reported here, some degree of reversal of the non-competitive inhibition has been achieved with any of the artificial electron acceptors tried, regardless of which metal-binding agent was employed (Table II). Consequently, the phenomenon seems to be of a more general nature, at least for the *Neurospora* enzyme. As such, it seems likely that reversal of the inhibition of the reduced enzyme is achieved by the action of the artificial electron acceptors as substrates of the diaphorase moiety of the nitrate reductase. Since the diaphorase activity is unaffected by these metal-binding agents[3], the acceptors readily oxidize the NADPH in the preincubation mixture. The reduced molybdenum is presumably unstable in the absence of reducing power exerted by excess NADPH and consequently becomes auto-oxidized with concommitant loss in affinity for the metal-binding inhibitors. The results of Fig. 1 reveal that nitrate is a very effective competitor of these inhibitors when the enzyme is oxidized. Therefore, reversal is achieved. The series of experiments presented in Fig. 2 supports these contentions.

The alleviation of inhibition through removal of excess reduced pyridine nucleotide would be more attractive in considerations of the phenomenon as a possible regulatory scheme. Nitrate reductase activity could be regulated through an oxidized, active state *vs* a reduced, inactive state as mediated by two factors: (1) the ultimate synthesis, as a result of nitrate assimilation, of a metabolite with high affinity for the reduced molybdenum cofactor, and (2) changing reduced pyridine nucleotide levels. On the other hand, if the reduced molybdenum–metabolite complex requires a specific oxidant (for which ferricyanide can substitute), the situation

becomes more complex. In addition to the *in vivo* inactivations of the nitrate reductases of *Chlamydomonas*[12] and *Chlorella*[9,10,16,17] mentioned earlier, the *Neurospora* nitrate reductase shows a time-dependent *in vivo* loss in activity[13]. Experiments are now in progress in this laboratory to ascertain if there are significant changes in pyridine nucleotide levels during the time course of induction and decay of *Neurospora* nitrate reductase activity.

Finally, it is interesting to speculate on the competitive inhibition seen with oxidized enzyme *vs* the non-competitive inhibition by metal-binding agents with reduced nitrate reductase. The contrasting patterns are not a property of all metal-binding agents since azide, cyanate and thiocyanate only show competitive inhibition of nitrate reductases[8,9]. The possibility that these latter inhibitors combine more tightly with Mo(VI) than Mo(V) and that nitrate combines only with Mo(VI) is unlikely since the reduced enzyme in the absence of metal-binding agents is not inhibited. Hence, nitrate can combine with the reduced state of the enzyme. Alternatively, application of the term, metal-binding agent, to these inhibitors may be misleading. These substances are all nucleophilic in nature, as befits the term. On the other hand, the substrate, nitrate, is also nucleophilic. It is therefore reasonable to assume other nucleophiles would compete with it for its binding site. The fact that nitrate cannot compete for the reduced molybdenum with cyanide, sulfide or thiourea may indicate that the substrate binding site is distinct from this metal, yet similar in having electrophilic properties.

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