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ACTIVATION OF *THALASSIOSIRA PSEUDONANA* NADH: NITRATE REDUCTASE

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Key Word Index—*Thalassiosira pseudonana*; diatom; nitrate reductase; NADH; cysteine; EDTA; enzyme activation; blue-Sepharose.

Abstract—NADH: nitrate reductase (NR) has been isolated in both active and inactive states, and both could be purified using blue-Sepharose. The state of activation of the enzyme depended on the presence or absence of agents such as cysteine or EDTA during the assay. When NR was assayed, the addition of activator before NADH led to maximum activity. Therefore, the reduced NR appeared to be inactivated during the assay in the absence of activator. Inactivation may have occurred via a mechanism similar to the inactivation of lipoamide dehydrogenase by trace metals, such as Cu^{2+}. The activation of NR by cysteine or EDTA was interpreted as protection of the reduced enzyme due to chelation of trace metals in the assay solution by the activators.

INTRODUCTION

Nitrate reductase (EC 1.6.6.1) (NR) is generally recognized as the catalyst for the initial step of nitrate assimilation, where nitrate is reduced to nitrite with a pyridine nucleotide as electron donor. The pyridine nucleotide-linked NRs have been isolated and studied from fungi, algae and higher plants [1]. The NR from the centric diatom *Thalassiosira pseudonana* has been obtained in a purified form and the regulation and properties have been described in detail [2]. *T. pseudonana* NR uses NADH as electron donor and is believed to use FAD, cytochrome *b* and metal(s) as electron carriers. This algal NR is asymmetric in shape with a MW of 330 000. The *T. pseudonana* NR is similar to that which has been purified in homogeneous form from *Chlorella vulgaris* [3]. A comparison of properties has been presented previously by Amy and Garrett [2]. Although the physical and enzymatic properties of these two algal NRs are similar, the *in vivo* regulation of the enzyme activities may differ. Both NRs are rapidly inactivated when ammonia is added to cells growing on nitrate. The *C. vulgaris* NR is isolated with bound cyanide and can be activated by treatment with ferricyanide [4]. The *T. pseudonana* NR is only slightly activated by ferricyanide treatment [2]. Since these two NRs have similar NADH dehydrogenase activities [2], the absence of significant ferricyanide activation of the *T. pseudonana* NR suggests the enzyme was not isolated with bound cyanide. Other algal NRs appear to divide into two classes: ferricyanide-activated forms and forms not activated by ferricyanide [5]. Although the presence or absence of ferricyanide activation does not establish the presence or absence of cyanide regulation *in vivo*, it appears that not all algal NRs are isolated in cyanide-inhibited forms and that some algal NRs may be regulated by a mechanism other than *in vivo* cyanide inhibition.

Alternative mechanisms for the regulation of NR have been hypothesized. Losada and coworkers suggested that the regulation of NR is via a physiological interconversion of two NR forms [6]. The oxidized NR is viewed as the active form and the reduced enzyme as inactive. Another means for NR inactivation is suggested to occur by enzymatic alteration of NR by a cellular component specific for NR [1]. This mechanism has been suggested for NRs from higher plants and fungi but not for algae. A form of activation has been described for NRs from *Neurospora crassa* [7] and higher plants [8]. In these studies, the NR can be inactivated by dialysis or gel filtration and reactivated by addition of a variety of agents including amino acids, thiols and EDTA [7, 8]. Ketchum *et al.* found that dialysis of *N. crassa* NR resulted in an EDTA-dependent form of the enzyme [7]. When the EDTA was replaced by amino acids, the properties of the NR were markedly affected, which suggested that the binding of these agents to NR could be the basis of a regulatory mechanism [7]. The studies with higher plant NRs have led to the suggestion that the inactivation by removal of EDTA or cysteine and reactivation by these agents may be related to heavy metal binding to the NR [8]. This reversible inactivation and activation are suggested to result from the physical state of the enzyme generated by the isolation procedure [8]. This protection-by-chelator mechanism suggested for higher plant NRs was rejected by Ketchum *et al.* [7], who favor a direct physical contact of the activators with *N. crassa* NR as the mechanism of this regulation of activity.

Our current report on *T. pseudonana* NR arises from the observation that this NR is also dependent on activators. We have purified the NR using affinity chromatography on blue-Sepharose and studied activation of the NR in both crude extracts and purified preparations.

RESULTS

Activation of NR

In a previous study of *T. pseudonana* NR, the enzyme was extracted and maintained in buffers to which 1 mM 2-mercaptoethanol and 1 mM EDTA had been added [2]. Our initial extractions of *T. pseudonana* were done with cysteine and EDTA added to the buffer. However, if the extract was assayed after storage in the freezer, maximum activity was found when cysteine was added to the assay mixture. Cysteine appeared to be effecting the NR activity during the assay and appeared not to be required during the storage of the extract. A similar result had been shown for partially purified higher plant NR, which had been assayed after storage in the freezer [9]. In order to investigate the dependence of the crude extract NR activity on cysteine and EDTA, the *T. pseudonana* cells were disrupted in buffer with and without addition of the agents. In Table 1, the assays of crude extract NR activity are shown. Very low activity was found when cells were extracted and assayed in the absence of cysteine and EDTA. However, when this same extract was assayed in the presence of either cysteine, EDTA, or both, the NR activity was substantially increased. When cells were extracted in buffers containing either cysteine, EDTA or both, the addition of cysteine or EDTA to the assay was not required for the demonstration of a high NR activity. The combination of extraction and assay with both cysteine and EDTA gave maximum NR activity. These results appear to support the concept that cysteine and EDTA exert their influence on NR during the assay, but also reveal that crude extract NR is largely inactive in the absence of these agents. For many higher plants, the presence of cysteine in the extraction buffer is essential for obtaining maximum NR activity and the addition of 1 mM EDTA to the extraction buffer is recommended [10]. However, when the leaves of spinach or the cotyledons of squash are used, highly active and stable NR can be extracted and assayed in the absence of cysteine and EDTA [11]. Thus, *T. pseudonana* NR appears to be dependent on the presence of the apparent activators, cysteine and EDTA, while higher plant NR appears to be active in the absence of these agents, in some cases. To further establish that the activation of NR by cysteine and EDTA was in the assay, a crude extract of *T. pseudonana*, which had been prepared in a buffer containing both cysteine and EDTA, was treated on Sephadex G-25. When the gel-filtered NR was assayed in the absence of cysteine, low or negligible activity could be demonstrated, while the presence of cysteine in the assay restored the activity (data not shown).

Table 1. Effect of activators on extraction and activity of nitrate reductase

Extraction media	Additions to the assay: None	5 mM Cysteine	5 mM EDTA	Both
Buffer*	1†	16	16	17
Buffer + 5 mM cysteine	11	17	18	19
Buffer + 5 mM EDTA	12	15	19	19
Buffer + 5 mM both	17	19	20	20

* Buffer 0.2M KPi (pH 7.8), 10% glycerol.

† Equals 13 nmol NO_2^- formed/min/l. cells. A culture of *T. pseudonana* was divided into 4 equal portions. NR was extracted and a crude extract was prepared as described in Experimental. NR was assayed by the colorimetric determination of nitrite.

Table 2. Comparison of *T. pseudonana* nitrate reductase activators

Activator (5 mM)	Activity (nmol NO_2^- formed/min)	Stimulation (Fold)
No addition	0.5	1
L-Cysteine	2.8	5.6
L-Cysteic acid	1.0	2
L-Cysteine methyl ester	2.8	5.6
2-Mercaptoethylamine	2.8	5.6
Glutamic acid	0.7	1
Glutathione (reduced)	1.9	3.8

A crude extract was prepared without cysteine or EDTA. NR was assayed by the colorimetric determination of nitrite.

When crude extracts were prepared in the absence of cysteine and EDTA, maximum activation was achieved at 1 mM cysteine in the assay. Other compounds including derivatives of cysteine were found to activate the crude extract NR (see Table 2). For maximum activation the thiol functional group appeared to be required, but cysteic acid was a weak activator. Cysteine methyl ester and the cysteine analogue, 2-mercaptoethylamine, were activators equal to cysteine when compared at concentrations of 5 mM.

To determine if cysteine activation of NR depended on assay conditions, the order of addition of assay components was varied (see Table 3). Maximum activity was obtained when the NR was added to the complete mixture or when NR was mixed with nitrate and cysteine before NADH was added. The lowest

Table 3. Nitrate reductase activity as effected by the order of addition of the assay components

Order of addition	Activity (nmol NADH oxidized/min)
Cys, NO_3^-, NADH, NR	40
NR, NO_3^-, Cys, NADH	40
NR, Cys, NADH, NO_3^-	34
NR, NADH, Cys, NO_3^-	15
NO_3^-, NADH, NR, Cys	13
NO_3^-, NADH, NR (no cys)	10

The concentration of each assay component was: KPi (pH 7.5), 25 mM; NO_3^-, 0.5 mM; NADH, 0.1 mM; and Cys, 5 mM. The change in A_{340} was measured at 22°. The NR was a crude extract preparation.

Table 4. Purification of *T. pseudonana* nitrate reductase *

	Activity (μ mol/ min)	Protein (mg)	Sp. act. (μmol/ min/mg)	Recovery (%)
Crude extract	0.8	8.6	0.09	100
Blue-Sepharose	0.4	0.1	4.0	50

* Average of 18 different extractions.

rates were obtained when NADH was mixed with NR before cysteine addition. The brief incubation of NADH and NR used here, *ca* 10 sec, was sufficient to inhibit the effect of cysteine. Nitrate appeared to exert little influence on the inhibitory capacity of NADH.

Purification of NR

The *T. pseudonana* NR was purified by affinity chromatography on blue-Sepharose. The procedure was developed using NR which had been extracted in the presence of cysteine and EDTA. The crude extract NR was bound to the blue-Sepharose using a batch procedure and, after washing the gel, the NR was eluted with NADH. The results of several purifications are summarized in Table 4. Methyl viologen:NR activity co-eluted with the NADH:NR activity from the blue-Sepharose column. When *T. pseudonana* cells were extracted in buffer not containing cysteine and EDTA, the NR could be purified by the blue-Sepharose procedure and activated after elution (Fig. 1). The recovery of NR in these experiments was lower than the average reported in Table 4. The low recovery may have resulted from exposure of the NR to NADH in the absence of activators, but the inhibitory effect of NADH was diminished at the low temperature of the isolation procedure.

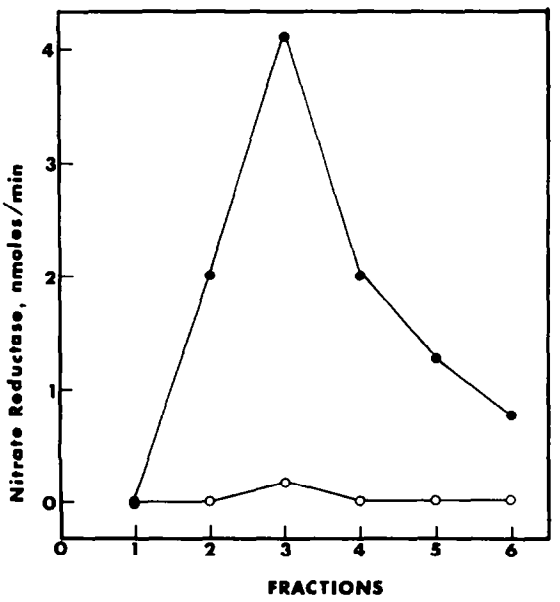

Fig. 1. Blue-Sepharose purification of *T. pseudonana* nitrate reductase. A crude extract was prepared in 0.2 M KPi (pH 7.8), containing 10% glycerol. After NR was bound to the blue-Sepharose, the gel was washed and packed into a column. The NR was eluted with 0.1 mM NADH in the extraction buffer and 3 ml fractions were collected. The NR was assayed both with 5 mM cysteine, ●——● (39% recovery) and without cysteine, O——O (1% recovery). The NR activity was determined by the nitrite assay.

The NR isolated by the blue-Sepharose procedure was of sp. act. greater than that reported by Amy and Garrett [2]. The NR had pH optima of 7.5 for both the NADH: and methyl viologen: NR activities. The NADH:NR activity was inhibited by *p*-hydroxymercuribenzoate, sodium azide and mild heat treatment. When the substrate kinetics were analysed, the fully activated NR had apparent K_ms of 40 μM KNO_3 and 8 μM NADH. The apparent K_m for nitrate was unaltered by changes in the activator concentration. However, the apparent K_m for NADH decreased from 20 to 10 μM when the concentration of cysteine was increased from 0 to 1 mM. The apparent V_{max} in the experiment increased three-fold, when the NR was activated by 1 mM cysteine.

DISCUSSION

Blue-Sepharose has been used as an affinity gel to purify *T. pseudonana* NR. Both the sp. act. and the recovery of the blue-Sepharose NR preparation exceed the previously reported values for NR from *T. pseudonana* [2]. The blue-Sepharose procedure requires only a few hr to complete and provides a highly purified NR, which has not been exposed to prolonged processing. Blue-Sepharose has been used to purify NRs from squash [12], corn [12], spinach [13], *Chlorella variegata* [14] and *Aspergillus nidulans* [15].

Many of the biochemical properties of the blue-Sepharose preparation of *T. pseudonana* NR are similar to values reported previously [2]. The sensitivity of the NR to *p*-hydroxymercuribenzoate, azide and mild heat treatment is consistent with the previous work [2]. The pH optima and the apparent K_m values for NADH and nitrate also compare favorably [2].

A novel feature of this study of *T. pseudonana* NR is the dependence of the enzyme on exogenous activators. Cysteine, cysteine analogues and EDTA were found to activate the NR. If these activators are omitted from the extraction buffer, the crude extract NR activity is dependent on the addition of an activator to the assay. Moreover, NR extracted in the presence of cysteine and EDTA can be rendered activator dependent by gel filtration. The NR can be purified on blue-Sepharose in the inactive form and activated after purification. The dependence of isolated and purified NRs from higher plants and the fungus *N. crassa* on activators, either cysteine or EDTA, has been reported [7–9]. However, the activation of algal NR in the crude extract has been found, in some cases, to occur when ferricyanide was added [1, 4, 5]. For *Chlorella vulgaris* NR, this activation by ferricyanide has been linked to cyanide as a bound inhibitor of the enzyme [4]. Cyanide, which competitively inhibits oxidized NR, binds to and noncompetitively inhibits reduced NR [4]. Thus, the inactivation of NR by cyanide requires a reduced NR and when the oxidant ferricyanide is added, the cyanide inhibition is relieved. Since *T. pseudonana* NR was found to be only slightly activated by ferricyanide [2], we conclude that the inactive form of NR present in crude

extracts of this alga is not the result of inhibition by bound cyanide.

The activation of isolated NR described herein also involves the reduced NR. The order of addition of the components to the assay implies that prior reduction of NR by NADH blocks subsequent activation by EDTA or cysteine. Studies of the flavoprotein lipoamide dehydrogenase may be related to the activation of NR described here. Copper (II) has been implicated in the inactivation of lipoamide dehydrogenase, which can be reversed by cysteine [16, 17]. The effect of copper ions was in the oxidation of sulfhydryl groups to disulfides in the enzyme [18]. The lipoamide dehydrogenase, after copper mediated oxidation, could be reduced to the 'four-electron' form of the active site by electron donors more readily, which resulted in an inactive enzyme [16]. More recent experiments have shown that lipoamide dehydrogenase requires EDTA in the assay and can be inactivated by gel filtration to remove EDTA from the enzyme preparation [8]. Thus, it appears that the flavoprotein portion of NR may be similar in character to lipoamide dehydrogenase and that the activation of the two enzymes by EDTA, cysteine or related compounds may be via protection of the flavin active site from over reduction to the 'four-electron' form [8, 16]. The protection of the enzymes by cysteine or EDTA would appear to result from sequestering the metal contaminants of the assay solution. However, the FAD active site of NR is not as well described as the active site of lipoamide dehydrogenase, although the close association of the FAD with sulfhydryl groups of NR has been reported [19].

For *N. crassa* NR the activation by EDTA was suggested to be via physical contact between the inactive enzyme and the activator and not to be due to the chelation character of the activator [7]. However, transglutaminase has been reported to be inactivated by a copper catalyzed oxidation, which can be reversed with thiol reagents [20]. Similarly, over-reduction of the methylviologen sulfite reductase was suggested as the mechanism of inactivation of this enzyme when thiols or chelators were not present in the assay mixture [21].

Although the mechanism of NR activation by EDTA and thiols has not been established, this activation phenomenon may assist in understanding the regulation of NR. For higher plant NR, we have suggested that the enzyme may exist *in vivo* in a larger protein complex than is currently found for the isolated and purified form [8]. The isolated form of NR may not retain the character and properties of the *in vivo* form, especially with regard to regulatory properties. For *T. pseudonana* NR, no activators appear to be present in crude extracts and the enzyme is largely inactive in the absence of EDTA and cysteine. The disruption of the *T. pseudonana* cells appears to alter the NR from an active state, presumably present in cells growing on nitrate as the sole nitrogen source, to an inactive and activator dependent state.

EXPERIMENTAL

Culture conditions. Bacteria-free cells of *T. pseudonana* were obtained from Professor T. Smayda, University of Rhode Island. Cells were grown and maintained on sterile media f/2-1 prepared according to ref. [22] in aged Narragansett Bay sea water. The algae were maintained in 100 ml subcultures; 200 ml of the culture was used to inoculate 3 l. of media. The cells were gently mixed and bubbled with a steady stream of air. Cells were grown at 20° under continuous fluorescent light supplemented with fluorescent lamps. Cells were harvested by centrifugation and washed once with cold sea water before extraction of NR.

Assays. NR from *T. pseudonana* was assayed at 20° using the colorimetric determination of nitrite as previously described, except that 10 μM FAD was added [12]. NR was also assayed by measuring the decrease in A_{340}. When other activators of NR were tested, the cysteine of the standard assay was omitted and the activator to be tested added. The methylviologen activity of NR was assayed as previously described [9]. Kinetic parameters of the NR were analysed by the direct linear plot using an APL program for location of median intersections. Protein was estimated by the method of ref. [23] or by the method of ref. [24].

Purification of NR. All procedures were done at 0–4°. Cells were suspended in 0.2 M KPi (pH 7.8), 5 mM cysteine, 5 mM EDTA and 10% glycerol. After adding 1 g of insoluble PVP/10 g of cells, the suspension was disrupted at 2.2 kbar with a French pressure cell precooled to 4°. The extract was centrifuged for 5 min at 28 000 g. The supernatant was stirred gently with blue-Sepharose for 30 min. The blue-Sepharose was recovered by filtration and washed with extraction buffer. The blue-Sepharose was then packed into a 0.9 cm dia. column and NR activity was eluted with 0.1 mM NADH in extraction buffer.

Chemicals. Cibacron blue F36A (Polysciences, Inc.) was directly coupled to Sepharose C1 4B (Pharmacia Fine Chemicals) to make blue-Sepharose [12]. All biochemicals were from Sigma. Deionized H_2O was used for preparation of all solns.

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