Plant Physiol. (1988) 87, 143–147
0032-0889/88/87/0143/05/$01.00/0

# Plasmalemma Redox Activity in the Diatom *Thalassiosira*[1]

## A POSSIBLE ROLE FOR NITRATE REDUCTASE

Received for publication June 29, 1987 and in revised form January 26, 1988

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### ABSTRACT

**Plasmalemma redox activity in the diatom *Thalassiosira* is competitively inhibited by antiserum prepared against algal nitrate reductase (NR), and fluorescent labeling experiments reveal the binding of NR antiserum to the cell surface. Furthermore, the external electron acceptor Cu bathophenanthroline disulfonate causes immediate inhibition of intracellular primary amine production. A model is proposed in which plasmalemma-bound nitrate reductase reduces extracellular electron acceptors and intracellular nitrate and also acts as a trans-plasmalemma proton pump.**

A considerable body of literature has accumulated in the past 10 to 15 years concerning the redox systems of higher plants and animals (6, 7). Plasmalemma redox enzymes can transfer electrons from an internal or, in some cases, an external reductant to artificial electron acceptors such as DCPIP,[3] ferricyanide, and Cyt *c*. Often, transplasmalemma electron transport appears to be accompanied by proton extrusion from the cell (16, 22, 24). Based on the latter observation, it has been proposed that the plasmalemma redox enzyme(s) may act as an alternate proton pump and form the energetic basis for active transport of nutrients across the plasmalemma (7).

Enzymically mediated redox activity at the plasmalemma of eukaryotic phytoplankton has recently been demonstrated in our laboratory (12, 13). Redox activity in phytoplankton exhibits saturation kinetics (half-saturation constants in the range 2 to 20 $\mu$M for Cu [II]·phen derivatives) is inhibited by mild heat treatment and membrane-impermeable thiol binding reagents, and the enzyme active site has a standard reduction potential within the range $-0.1$ to $+0.1$ V. We noted (12) that the properties of the redox enzyme were similar to those of NR (EC 1.6.6.2); however, we did not provide any evidence to support the contention that NR was mediating the surface redox activity. In addition to its nominal function, *i.e.* the enzymic reduction of $NO_3^-$, NR also possesses a partial NAD(P)H-Cyt *c* reductase (diaphorase) activity through the flavoprotein subunit of the enzyme (11). NR has generally been considered a soluble enzyme. However, recent immunochemical studies of marine dinoflagellates and diatoms indicate that NR occurs in the plasmalemma as well as intracellularly (2). We have investigated in more detail the hypothesis that NR occurs in the plasmalemma of eukaryotic phytoplankton and that it is responsible, at least in part, for the observed surface redox activity in these organisms.

## MATERIALS AND METHODS

Cell culture techniques and conditions were as described previously (12). Cell cultures used were *Thalassiosira weissflogii* Grunow (clonal designation Actin) and *T. pseudonana* (Hust.) Hasle (3-H). Experimental incubations were carried out at 22°C under room lighting. Surface redox activity was assayed by following the reduction of Cu(II)(phen)$_2$ or Cu(II)(BPDS)$_2$ as previously described (12). Crude antiserum prepared against purified *Chlorella* NR by the method of Funkhouser and Ramadoss (8) was kindly supplied by Edward Funkhouser, Texas A & M University. The crude sheep antiserum was purified by immunoaffinity chromatography. Rabbit anti-sheep immunoglobulin (purchased from Boehringer-Mannheim) was covalently attached to CNBr-activated Sepharose 4B, and the sheep antiserum was purified according to instructions from the manufacturer (Pharmacia). The purified antiserum was passed through a Sephadex G-25 column, and the collected preparation was used without further purification. In redox inhibition experiments with anti-NR, 25 $\mu$L crude antiserum or 75 $\mu$L purified antiserum was added to 1.5 mL culture and incubated for 15 min before commencing the assay measurements. Previous studies using this antiserum (8) showed that purified anti-NR gave only one precipitin band in Ouchterlony assays with *Chlorella* whole cell extracts and that the band showed identity with that obtained with purified NR. Further tests of cross-reactivity of the crude and purified anti-NR with the diaphorase moiety of cellular redox enzymes were made using a range of redox enzymes purchased from Sigma; these were lipoamide dehydrogenase (type V from Torula yeast), glutathione reductase (type III from baker's yeast), Cyt *c* reductase (type I from porcine heart), and malic dehydrogenase (from porcine heart mitochondria). Only the diaphorase activity of lipoamide dehydrogenase was inhibited by anti-NR, and this inhibition was strictly non-competitive (data not shown) and thus was not due to anti-NR binding at the metal reduction site of the enzyme.

Cells were prepared for fluorescence microscopy by treatment with purified anti-NR as described above. After incubation, the cells were collected by centrifugation and washed twice with fresh medium. A 1 mL aliquot was then treated with 20 $\mu$L rabbit anti-sheep FITC conjugate (Boehringer-Mannheim) for 15 min and washed as above. Cell surface fluorescence was recorded using a Zeiss microscope with epi-illumination fluorescence accessories. A 580 nm LP filter was inserted in the camera tube to remove cell autofluorescence due to Chl.

Intracellular primary amine concentration was determined using fluorescamine according to the method of Clark (5). Nitro-

[1] Supported in part by National Science Foundation grant 8317532-OCE and ONR grant N00014-86-K-0325.

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[3] Abbreviations: DCPIP, dichlorophenolindophenol; NR, nitrate reductase; phen, 1,10-phenanthroline; BPDS, bathophenanthroline disulfonate.

gen-limited ($NO_3^-$-grown) cells were resuspended at a cell density of $10^4$ cells $mL^{-1}$ in fresh $NO_3^-$-containing medium at time zero. For primary amine measurements, a 5 mL aliquot was removed, and the cells were solubilized by addition of 0.1% Triton X-100 (v/v). Cell debris was removed by centrifugation and the supernatant assayed for primary amine concentration. Supernatant from culture aliquots not treated with Triton X-100 was used as a blank.

## RESULTS

Plasmalemma redox activity in *T. weissflogii* and *T. pseudonana* was inhibited by additions of anti-NR (Fig. 1). Preimmunization serum was without effect. The observed high initial reduction rate (lasting 10–15 min), which is nonenzymic and mediated by cell wall components (12), was unaffected by anti-NR as expected. Inhibition of the steady reduction rate by anti-NR was competitive, with a 1:60 dilution causing $K_s$ for $Cu(BPDS)_2$ reduction to increase from about 20 $\mu$M to about 45 $\mu$M; $V_{max}$ was unaffected (Fig. 2). Purified anti-NR displayed identical effects to the crude anti-serum (not shown). The purified anti-NR has been shown previously (8) to inhibit all activities associated with the complete NR complex in Chlorella: NADH:NR, NADH:Cyt *c* reductase, and methyl viologen:NR. As metal complexes are reduced on the external plasmalemma surface in *Thalassiosira* (12), the competitive nature of anti-NR inhibition indicates that the antibody and Cu(II) complex are binding to the same surface site. Unlike previous studies of soluble NR, the diaphorase activity of the putative plasmalemma NR was not nutritionally repressed by ammonium (data not shown).

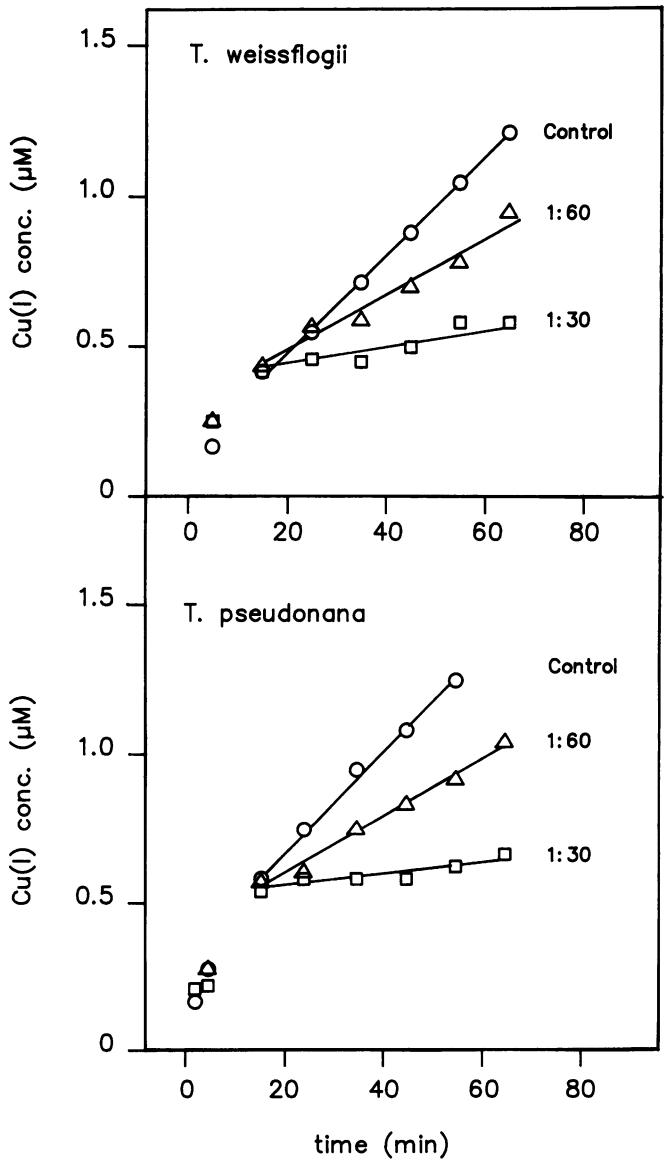


FIG. 1. Effect of anti-NR (1:60 and 1:30 dilutions) on plasmalemma reduction of $Cu(phen)_2$ (10 $\mu$M) by *T. weissflogii* and *T. pseudonana*.

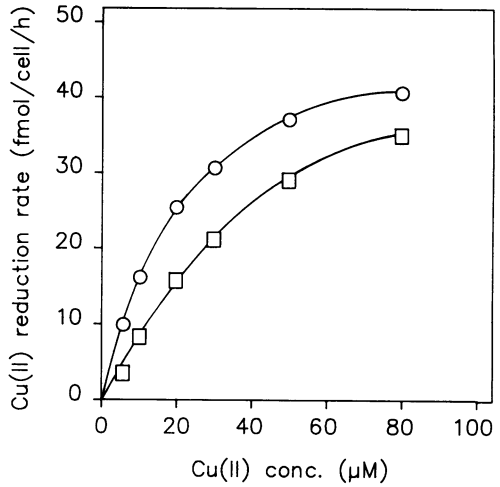


FIG. 2. Effect of anti-NR on $Cu(BPDS)_2$ reduction kinetics in *T. weissflogii*; control (○), + anti-NR (1:60) (□). Data from one set of experiments. Duplicate analyses were not performed due to the scarcity of anti-NR. Previous studies have shown good precision (SE<5%) in all experiments (12).

Binding of anti-NR to the cell surface was visualized by addition of anti-sheep FITC conjugate to the treated cell cultures. The surface of *T. weisflogii* was covered by diffuse fluorescence indicating the presence of NR in the plasmalemma (Fig. 3). Cells treated with anti-sheep FITC conjugate, with and without preimmunization serum, did not exhibit uniform diffuse labeling of the cell surface; microscopic examination showed no cell outline, only a few fluorescent spots. Similar observations of other species of eukaryotic phytoplankton were recently reported (2).

To further test the hypothesis that plasmalemma redox activity was mediated by nitrate reductase, the effect of an external electron acceptor, $Cu(BPDS)_2$, on internal nitrogen reduction was determined. If the diaphorase reduction site of the putative NR is exposed to the external milieu, then external electron acceptors should intercept electrons from NR and therefore affect the rate of internal nitrogen reduction and assimilation. Because the cells were grown on $NO_3^-$ as sole nitrogen source, nitrogen reduction could be followed by monitoring the production of intracellular primary amines. In untreated cultures, primary amine concentration showed a rapid increase within the first 1 to 2 h followed by a slight decrease over the next 18 to 20 h (Fig. 4). This response has been demonstrated previously for N-limited *T. weissflogii* (27) and is due to the rapid synthesis of amino acids during an initial phase of 1 to 2 h in which protein synthesis is slow or absent. The conversion of free amino acids to proteins after the initial lag phase gives an apparent decrease in primary amine concentration because the analytical sensitivity for proteins using fluorescamine is much lower than that for amino acids. Exogenous $Cu(BPDS)_2$ displayed a concentration-dependent effect on intracellular primary amine production. At concentrations of 0.5 and 1.0 $\mu$M, a small effect was elicited within the first 2 h of incubation, but subsequently these treatments were identical to the control. Higher concentrations of $Cu(BPDS)_2$ caused more severe inhibition of primary amine production, with the 10 $\mu$M

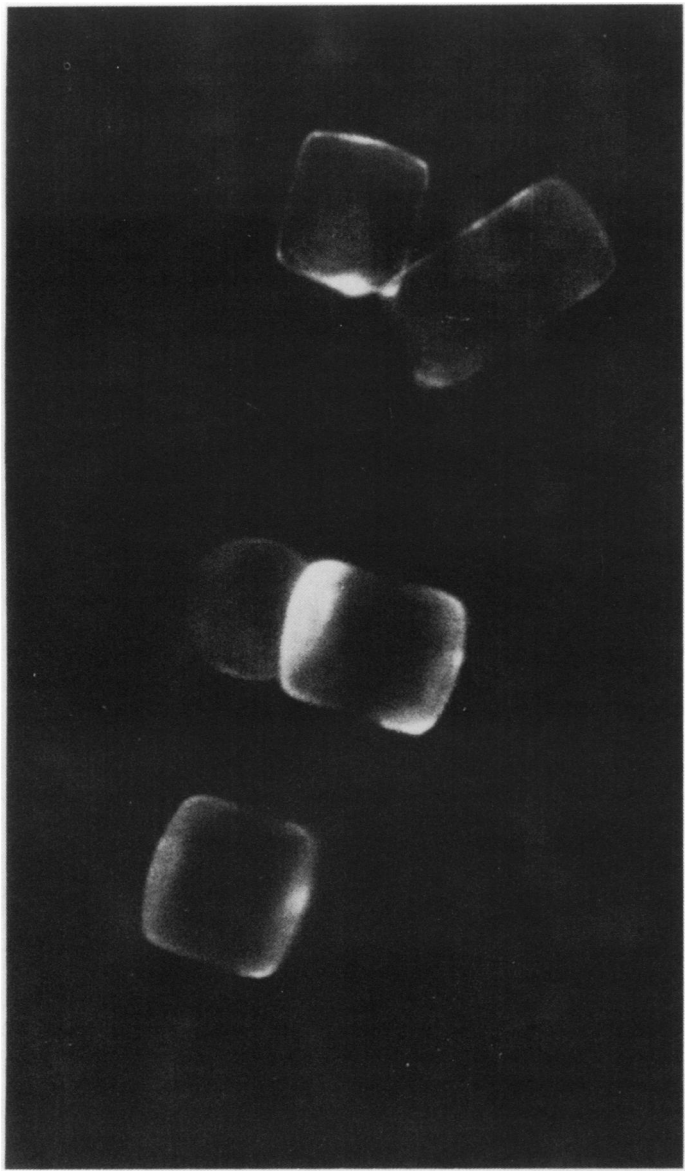

FIG. 3. Surface labeling of *T. weissflogii* by sheep anti-NR and anti-sheep FITC conjugate.

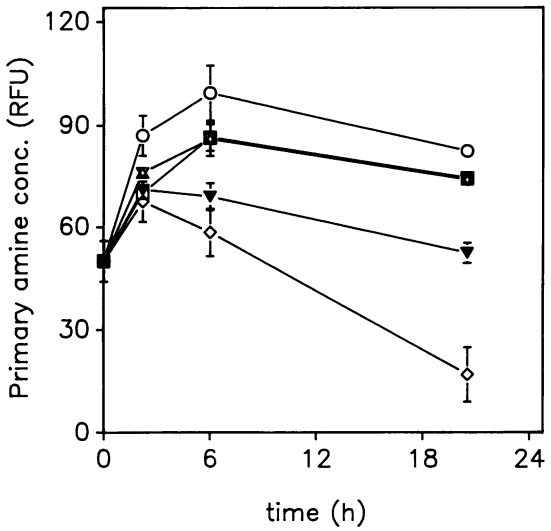


FIG. 4. Effect of $Cu(BPDS)_2$ on intracellular primary amine concentration in *T. weissflogii*, 0 (○), 0.1 (▲), 0.5 (▽), 1.0 (□), 5.0 (▼), 10.0 μM (◇). Data are the mean of triplicate samples. Standard error indicated by bars.

treatment causing inhibition throughout the entire incubation period. Due to the 1:2 metal to ligand stoichiometry, as the concentration of $Cu(BPDS)_2$ is increased (by addition of concentrated Cu + BPDS stock) the free ion activity of Cu(II) in solution actually decreases. Since metal toxicity in phytoplankton is directly related to the free metal ion activity (1, 25), it is concluded that the observed behavior was not due to nonspecific inhibitory effects of Cu on cellular metabolism. Also, $Cu(BPDS)_2$ at concentrations of 10 and 100 μM does not inhibit methylamine uptake (N Price, personal communication). However, $Cu(BPDS)_2$ may have other effects on nitrogen metabolism than simple competition with $NO_3^-$ for reductants; $NO_3^-$ transport or other reduction steps could be affected.

## DISCUSSION

The presence of NR in the plasmalemma of the marine diatom *Thalassiosira* and its possible role as a plasmalemma redox enzyme are indicated by: (a) immunofluorescent labeling of the cell surface by anti-NR, (b) competitive inhibition of plasmalemma redox activity by anti-NR, and (c) inhibition of intracellular primary amine production by the impermeable electron acceptor $Cu(BPDS)_2$.

The localization of NR in unicellular algae and higher plants has been the subject of some contention. Traditionally, NR has been regarded as a soluble cytoplasmic enzyme based mainly on the fact that NR could be easily isolated and purified from the high-speed supernatant of mechanically disrupted whole cell preparations. This supposition, however, does not preclude the additional presence of NR in the plasmalemma or other cellular membranes. For example, in some more detailed studies of unicellular algae where both dissolved and particulate fractions have been analyzed, significant NR activity has been found in the particulate fraction (10, 19). Furthermore, in green algae it appears that there is a considerable quantity of NR associated with the pyrenoid (14). Cells from other phyla also have NR bound or closely associated with the plasmalemma. These include the fungus *Neurospora crassa* (20) and certain species of denitrifying bacteria, *e.g. Paracoccus denitrificans* (26). The possible occurrence of NR in the plasmalemma of eukaryotic phytoplankton, then, is not without biological precedent. In fact, it has been suggested (23) that the accessibility of artificial electron acceptors to the reduction site of the NAD(P)H:diaphorase moiety of NR indicates that NR is probably membrane bound or complexed within the cell.

Clearly, one major difference between the putative plasmalemma NR and the cytoplasmic NR that is assayed using traditional techniques is that the plasmalemma reductase is not nutritionally repressed by ammonium. While this indicates that the two enzymes are not identical, it does not necessarily imply that the plasmalemma redox enzyme is not NR or a NR subunit. It is possible that the plasmalemma and cytoplasmic forms of NR are regulated separately and that the NAD(P)H:diaphorase subunit of the plasmalemma NR (that which is assayed in the experiments described above) is synthesized and maintained in the plasmalemma even in the presence of ammonium. Possible support for this proposition is provided by the observation of Funkhouser and Ramadoss (8), who noted that ammonium-grown *Chlorella* cells contain proteins that cross-react with anti-NR; we believe these might be constitutively produced NR subunits. It is pertinent to note that even when cytoplasmic NR is repressed by ammonium, complete repression does not usually occur and there remains a basal level of NR activity (15, 17, 21). It is also

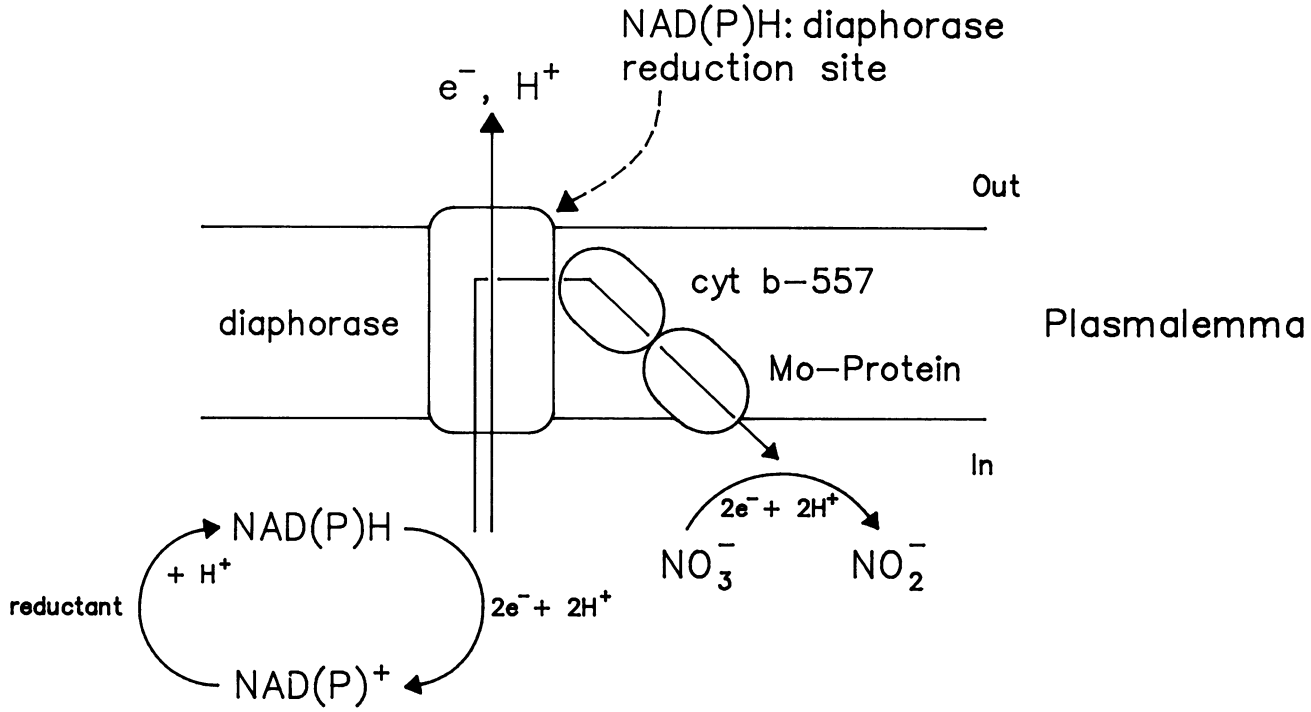


FIG. 5. Proposed model for plasmalemma-bound NR. The NAD(P)H:diaphorase subunit functions as a transmembrane reductase for external electron acceptors and as a proton pump. The Cyt *b*-557 and the molybdoprotein subunits, which may not always be present, loop back to the inside of the plasmalemma to catalyze $NO_3^-$ reduction. The exact proton balance in such a system depends on the nature of the initial $NAD(P)^+$ reductant and on the possible back transport of $H^+$ across the plasmalemma.

evident that the NAD(P)H:diaphorase partial activity is not repressed as rapidly or to the same extent as the complete NAD(P)H:NR activity (15).

To explain our observation of plasmalemma redox activity in the diatom *Thalassiosira*, a model for plasmalemma bound NR is proposed (Fig. 5). In this model, the NAD(P)H:diaphorase subunit spans the plasmalemma from inside to outside, and the Cyt *b*-557 and molybdoprotein subunits loop back to the internal plasmalemma surface. This orientation of NR in the plasmalemma facilitates internal reduction of nitrate and external reduction of artificial electron acceptors. The relative position of the Cyt *c* (and DCPIP, ferricyanide, etc.) reduction site within the complete NR molecule is the same as previously hypothesized (11). With this proposed orientation of NR, the diaphorase subunit functions as a proton pump whose activity is coupled to that of the reductase. There are many reports of redox enzyme-linked proton extrusion in higher plant and animal cells (16, 22, 24), and preliminary experiments indicate a similar mechanism may also operate in *Thalassiosira* (data not shown). It is possible that the energy associated with the increase in membrane potential formed by transmembrane proton movement could be linked, either directly or indirectly, with the uptake of nitrate or other nutrient ions. This mechanism of NR-mediated proton transport is similar to that which is believed to facilitate nitrate transport across the inner cell membrane of the bacterium *Escherichia coli* (9). With this proposed orientation of NR, external electron acceptors will intercept electrons from NR and therefore inhibit the intracellular reduction and assimilation of nitrate. (Inhibition of $NO_3^-$ transport is also possible if it is linked to the proton pumping of the diaphorase subunit.) Pistorius *et al.* (18) observed ferricyanide inhibition of $NO_3^-$ utilization in *Chlorella*. They proposed that the inhibition was due to binding of free $CN^-$ to NR. In view of similar results reported here, using a different external electron acceptor, $Cu(BPDS)_2$, one may speculate that the inhibition of $NO_3^-$ utilization in *Chlorella* may have been due to electron interception from plasmalemma NR by ferricyanide.

A specific plasmalemma NADPH:ferric chelate reductase, which is essential for iron uptake, is induced in iron-starved root cells of higher plants (3, 22). It is thus interesting to note that NR has been demonstrated to reduce iron siderophores, and it has been suggested that NR may be involved in iron assimilation by root cells (4).

While the data presented in this paper do not categorically demonstrate that plasmalemma redox activity in the diatom *Thalassiosira* is mediated by NR, we believe the data do provide good evidence to support this hypothesis. Clearly there are differences between the putative plasmalemma NR and soluble NR, the most obvious being the lack of ammonium repression of enzyme activity. Final proof awaits the isolation and characterization of the plasmalemma redox enzyme(s) of eukaryotic phytoplankton.

*Acknowledgments*—Neil Price and Mark Gerath are thanked for helpful comments.

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