Evidence for a plasma-membrane-bound nitrate reductase involved in nitrate uptake of Chlorella sorokiniana

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Abstract. Anti-nitrate-reductase (NR) immunoglobulin-G (IgG) fragments inhibited nitrate uptake into Chlorella cells but had no affect on nitrite uptake. Intact anti-NR serum and preimmune IgG fragments had no affect on nitrate uptake. Membrane-associated NR was detected in plasma-membrane (PM) fractions isolated by aqueous two-phase partitioning. The PM-associated NR was not removed by sonicating PM vesicles in 500 mM NaCl and 1 mM ethylenediaminetetraacetic acid and represented up to 0.8% of the total Chlorella NR activity. The PM NR was solubilized by Triton X-100 and inactivated by Chlorella NR antiserum. Plasma-membrane NR was present in ammonium-grown Chlorella cells that completely lacked soluble NR activity. The subunit sizes of the PM and soluble NRs were 60 and 95 kDa, respectively, as determined by sodium-dodecyl-sulfate electrophoresis and western blotting.

Key words: Chlorella – Nitrate reductase (plasma membrane bound) – Nitrate uptake – Plasma membrane

Introduction

Nitrate reductase (NR) and NO₃⁻ transport have several characteristics in common. Induction of each requires NO₃⁻ (Jackson et al. 1973; Oaks and Hirel 1985) and is inhibited by inhibitors of RNA and protein synthesis (Jackson et al. 1973; Klobus et al. 1988). Derepression of the NO₃⁻-uptake system in NH₄⁺-grown Chlorella cells in N-free medium requires protein synthesis (Knobloch 1987). Although the kinetics of NO₃⁻ transport have been extensively characterized (Tischner and Lorenzen 1979; Ullrich 1983; Deane-Drummond 1984; Goyal and Huffaker 1986) an NO₃⁻-transport protein has not been identified from plant roots.

The similarities of induction between NR and NO₃⁻ transport caused Butz and Jackson (1977) to propose that a membrane-associated NR could also function as a transporter for NO₃⁻. Their proposal conflicted with the generally accepted assumption that NR is a soluble cytosolic enzyme in eukaryotic organisms (Oaks and Hirel 1985; Oelmuller et al., 1988). Several reports have suggested that a portion of the total NR was associated with the membranes of various organelles, microbodies (Lips and Avissar 1972), chloroplasts (Kamachi et al. 1987) and the pyrenoid of green algae (Lopez-Ruiz et al. 1985). Evidence for a plasma-membrane (PM)-bound NR in the diatom Thalassio sira was reported by Jones and Morel (1988). Recently, Ward et al. (1988a, b) reported finding an NR associated with a PM fraction isolated from barley and corn roots. In addition, Ward et al. (1988b) found that fragments prepared from anti-soluble NR inhibited NO₃⁻ transport by barley roots.

The purpose of this research was to determine if there is some unity to the above findings by comparing a more divergent species with those already investigated. We show that Chlorella cells also contain an NR associated with a PM fraction and fragments prepared from anti-soluble-NR immunoglobulin G specifically inhibit NO₃⁻ transport by Chlorella cells.
Material and methods

**Plant material.** *Chlorella sorokiniana* (strain 211-8k of the algae collection of the Pflanzenphysiologisches Institut, University of Göttingen, FRG) was used. Algae were cultivated and synchronized as described by Tischner (1976).

**Antiserum preparation.** Antiserum to *Chlorella NR* was prepared as described by Tischner (1984). Immunoglobulin G (IgG) was purified from the serum by protein A-Sepharose chromatography and hydrolyzed with papain (Lifter and Choi 1978). The cleaved IgG fragments were separated from papain by gel filtration on a Sephadex G-150 column, concentrated and used in the uptake experiments.

**Nitrate uptake.** Cells were pretreated with intact anti-NR serum, preimmune IgG fragments or anti-NR IgG fragments (protein (IgG)/chlorophyll ratio = 0.8) in darkness at 39 °C for 3 min prior to nitrate-uptake measurements. Nitrate uptake was determined as described by Tischner and Lorenzen (1979). Cells treated with anti-NR IgG fragments were washed three times with phosphate buffer and a post-wash uptake rate was also determined.

**Plasma-membrane isolation.** *Chlorella* cells were harvested and extracted as described by Tischner (1976). The extraction buffer (buffer 1) was 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol-2-(N-morpholino) ethanesulfonic acid (Tris-Mes; pH 7.8), 250 mM sorbitol, 23 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium molybdate, 5 mM flavine-adenine dinucleotide (FAD), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM leupeptin, 2 mM dithiothreitol (DTT), 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin (BSA). The homogenate was centrifuged at 10000 g for 10 min. The supernatant was centrifuged at 100000 g to obtain a microsome and a soluble fraction.

The microsome fraction was resuspended in 1 mM Tris-Mes (buffer 2) containing 250 mM sorbitol, 1 mM sodium molybdate, 5 mM FAD and partitioned on an aqueous polymer two-phase system as described by Larsson (1985) and Hodges and Mills (1986). The phase system contained 6.5% Dextran T 500, 6.5% polyethylene glycol, 0.33 M sucrose and 4 mM KCl. The contents of the tube were mixed by inversion 40–50 times, and centrifuged in a swinging-bucket rotor at 1200 g for 5 min. The upper phase was collected and partitioned on a fresh lower phase. This was repeated twice to remove all of the chlorophyll-containing material from the upper phase. The first lower phase and the final upper phase (U3) were collected, diluted with buffer 2 lacking sorbitol and centrifuged at 100000 g for 1 h. The pellets were resuspended and again centrifuged at 100000 g for 1 h. The final pellets were suspended in buffer 2 and used for enzyme analysis.

**Salt wash.** The U3 pellet was resuspended in buffer 2 and brought to a final concentration of 500 mM NaCl and 1 mM EDTA in 0.5 ml and placed on ice. After 20 min, the fractions were centrifuged at 100000 g for 1 h. Nitrate-reductase activities were determined in the resuspended pellet and soluble fractions.

**Inactivation of PM NR.** The U3 pellet was solubilized with Triton X-100 (TX-100; octylphenoxyethoxyethanol) in buffer 2, incubated in the presence of anti-NR serum or preimmune serum for 2 h, after which NR activity was determined.

**Enzyme analysis.** Marker enzymes were determined as described by Quail (1979). Nitrate-reductase activity was determined as described by Tischner (1976) except that 0.1% TX-100 (Triton/protein ratio = 10) was included in the assay mix for the membrane fractions.

**Electrophoresis and blotting.** Polyacrylamide gel electrophoresis in the presence of 10% sodium dodecyl sulfate (SDS) was performed according to Laemmli (1970). Electrophoretic transfer to nitrocellulose was performed as described by Clausen (1974). Peptide molecular weights were estimated with biotinylated molecular-weight markers (Biorad Laboratories, Richmond, Cal., USA). After electrophoretic transfer, the nitrocellulose sheets were air-dried and then incubated with blocking solution consisting of 20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 1% BSA (buffer 3) for at least 1 h and then incubated in the same solution lacking BSA (buffer 4) containing anti-NR serum (1:1000) for 2 h. The blot was then washed twice for 30 min in buffer 3 and once in buffer 4 containing 0.05% (v/v) Tween 20, polyoxyethylene sorbitan monolaurate. After rinsing in buffer 3, the blot was incubated with horseradish-peroxidase (HRP)-conjugated, goat anti-rabbit IgG, and HRP-avidin-conjugate, diluted 1:1000 in buffer 3 for 1 h. Anti-NR cross-reactive bands and molecular-weight markers were detected by development of the blot with 30 mg of 4-chloro-l-naphthol (in 10 ml of methanol) and 40 μl of 30% H2O2 in 100 ml of buffer 3. Autoclaving the blots to destroy endogenous peroxidase activity did not affect the results (Rohringer and Holden 1985).

**Results**

Immunoglobulin-G fragments purified from *Chlorella NR* antiserum inhibited nitrate uptake by 95% but had no affect on nitrite uptake by *Chlorella cells* (Table 1). The inhibition of nitrate uptake by IgG fragments was only partially reversible; uptake recovered to about 50% of the control after two rinses in fresh uptake solution. Intact anti-NR serum and preimmune-serum IgG fragments did not affect nitrate uptake.

Since the plasma membrane (PM) is the primary barrier of ions (and IgG fragments) to the cell cytosol, the inhibition of nitrate uptake by anti-NR IgG fragments indicated that NR or an antigenically related protein involved in nitrate transport

**Table 1.** Effect of anti-NR IgG fragments on nitrate and nitrite uptake into intact *Chlorella cells* (± SD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptake rate (nmol·(mg chlorophyll)⁻¹·min⁻¹)</th>
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<tbody>
<tr>
<td>Nitrate</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>64.3 ± 0.5</td>
</tr>
<tr>
<td>Preimmune fragments</td>
<td>63.1 ± 0.7</td>
</tr>
<tr>
<td>Intact anti-NR serum</td>
<td>64.0 ± 0.7</td>
</tr>
<tr>
<td>Anti-NR IgG fragments</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>Anti-NR IgG fragments wash*</td>
<td>29.3 ± 0.3</td>
</tr>
</tbody>
</table>

* Cells were resuspended in fresh phosphate buffer twice after treatment with anti-NR IgG fragments