Regulation of assimilatory nitrate reduction at the level of nitrite in *Chlorella fusca*

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**Abstract.** Batch cultures of *Chlorella fusca* excreted nitrite into the medium if gassed with air (0.03% CO₂), but they did not if supplied with air containing 5% CO₂. After a change from high to low CO₂ concentration in the gas stream, nitrite excretion started immediately. After an increase in CO₂ concentration to 5%, nitrite uptake started within only 30 min. Changes of in-vitro activities of nitrate reductase, nitrite reductase and glutamine synthetase did not correspond to changes of nitrite concentration in the medium and therefore could not explain these observations. A nitrite-binding site, whose activity corresponded with both nitrite excretion and uptake, was detected at the chloroplast envelope. From these data an additional regulatory step in the assimilatory nitrate-reduction sequence is suggested. This includes an envelope-protein fraction probably regulating the availability of nitrite within the chloroplast.

**Key words:** *Chlorella* – Chloroplast envelope – Nitrate reduction – Nitrite

**Introduction**

The utilization of nitrate by plant cells needs a reduction to ammonium before the incorporation of the nitrogen into amino acids. This reduction is catalyzed by two enzymes, nitrate reductase (NR) and nitrite reductase (NIR). The former transfers two, the latter six electrons. Both reduction steps are separated in the cell by compartmentation, the first step occurs in the cytosol, the second is located inside the chloroplast (Guerrero et al. 1981).

While intensive work has been done on the characterization of the two enzymes involved and on the regulation of nitrate reduction (Losada et al. 1981), less attention has been paid on the regulation of nitrite reduction. However, it is known that reduced ferredoxin supplies the electrons for nitrite reduction (Miflin 1974) and that the inhibition of phosphorylation causes an excretion of nitrite into the medium (Kessler 1955; Kessler and Bücker 1960). It has also been reported that a sufficient CO₂ supply is necessary for nitrite reduction (Strotmann 1967). The latter result was considered to be an example of feedback inhibition since CO₂ was a growth limiting factor (Canvin and Atkins 1974). However, only few data drew attention to the regulation of NIR by the availability of nitrite inside the chloroplast. It was suggested that nitrite should enter the chloroplast in its protonated form by diffusion (Kaiser and Heber 1983). Therefore the entrance from the cytosol into the chloroplast was hardly taken into account as a regulatory step (Kessler et al. 1970; Hofmann 1972). In this report we present data indicating the existence of such a regulatory site attached to the envelope of the chloroplast.

**Material and methods**

*Organism.* *Chlorella fusca* 211-8b (Sammlung von Algenkulturen der Universität Göttingen, FRG) was cultured according to Schmidt (1972), but the sulfate concentration was changed to 0.3 mM. Cultures were gassed either with air or air enriched with 5% CO₂. *Chlorella sorokiniana* 211-8k (high-temperature strain) was grown as reported in detail by Peuke et al. (1986). The pea plants (*Pisum sativum* L.) used were germinated and cultivated on vermiculite. They were rinsed with water and grown for 11 d in a light-dark change of 16:8 h at 22°C and 200 μmol·m⁻²·s⁻¹.

*Algal cell disruption for enzyme assays.* Cells were broken in a French press at 12000 PSI (1 PSI≈7 kPa) using a buffer sys-
Preparation of the membranous material

Chlorella sorokiniana cells were broken using glass beads (0.11 mm diameter) in a Bühler homogenisator; the procedure for separation of the membranes was according to Peuke et al. (1986). Pea plants were extracted with buffer containing 0.25 M sucrose, 50 mM Tris-2-(N-morpholino)ethane sulfonic acid (Mes) pH 7.5, 1 mM dithioerythritol (DTE), 10 mM KCl and 0.1 mM MgCl₂ using a Waring blender.

Preparation of chloroplasts, envelopes and thylakoids. The preparation of intact pea chloroplasts includes a Percoll-gradient-centrifugation step according to Douce et al. (1973). These intact chloroplasts (producing oxygen at a rate of 110 μmol. (mg chlorophyll) 1 h⁻¹) were used for the preparation of envelope and thylakoid fractions according to Douce et al. (1973).

Enzyme activities

(i) Nitrate reductase (NR). This enzyme was determined in a total volume of 0.5 ml containing: 100 μmol potassium phosphate pH 8.0; 1 μmol potassium nitrate; 75 nmol NADH and crude extract. Incubation was for 15 min at 30°C. The assay was terminated by adding 2 ml of the diazotation reagent for nitrite determination (see below).

(ii) Nitrite reductase (NIR). Nitrite-reductase activity was measured in a total volume of 1 ml: 300 μmol Tris-HCl pH 7.5; 3 μmol sodium nitrite; 700 nmol methylviologen; 100 nmol ethylenediaminetetraacetic acid (EDTA); 0.15 ml of dithionite solution (250 mg sodium dithionite and 250 mg sodium hydrogen-carbonate in 10 ml of water) and crude extract. After incubation for 15 min at 30°C, 0.1 ml of the assay was diluted to 1 ml with water and oxidized using a whirlmix. An aliquot of 0.2 ml was used for nitrite determination (see below).

(iii) Glutamine synthetase (GS). Glutamine-synthetase activity was determined using the transcrase assay according to Rhodes and Stewart (1974).

Determination of nitrite-binding capacity of membrane fractions. Aliquots of the membrane fractions were incubated in a total volume of 2 ml containing: 150 μmol Tris-HCl pH 7.0; 6 μmol sodium nitrite; 160 nmol riboflavin 5'-phosphate (FMN); 3 μmol t-cysteine; and 0.3 ml of dithionite solution (as above). After incubation for 10 min at 30°C, 0.1 ml of the assay mix was diluted tenfold with 0.8 M methylviologen solution needed to oxidize dithionite using a whirlmix. An aliquot of 0.2 ml of this suspension was used for nitrite determination (see below). The same assay without membrane addition was incubated as reference. The amount of nitrite reduced was calculated from the difference between the nitrite in the membrane assay and the nitrite in the reference. It was expressed as specific activity and taken as a measure for binding capacity.

Nitrite determination. Nitrite was determined according to Nicholas and Nason (1957), but the procedure was modified using 1 ml of 1% sulfanilic acid in 3 M HCl and 1 ml of 0.02% N-(1-naphthyl)-ethylenediamine hydrochloride. After 45 min the absorbance at 538 nm was determined and nitrite was calculated from a standard curve.

Ammonium determination. Ammonium was determined using the phenol-nitroprusside assay mixture of Sigma (München, FRG). When ammonium excretion from algal cultures was determined, the gas stream leaving the culture was passed through 0.01 N HCl, trapping ammonium released to the gas stream. Total ammonium was calculated from ammonium in the medium and ammonium in the trap.

Protein determination. (i) For crude extracts the Coomassie-blue method of Bradford (1976) was used with the dye reagent preparation from Biorad (München, FRG) and bovine serum albumin as reference.

(ii) For membrane fractions the method of Lowry et al. (1951) was employed with bovine serum albumin as reference.

Results

Nitrite excretion during growth with 0.03% CO₂ (air). Batch cultures of Chlorella fusca grown with air started to excrete nitrite into the medium about 3 d after inoculation (Fig. 1). Concentration values up to 5 mM at the fifth day were measured. The onset of nitrite excretion is correlated with an increase of the pH of the culture medium; however, culture density (F₆₈₀) still increased. Nitrite excretion was not accompanied by ammonium accumulation in the growth medium, thus nitrate is reduced to nitrite and excreted as the result of a