MECHANISM OF NITRATE AND NITRITE REDUCTION IN Chlorella CELLS GROWN IN THE DARK

M.G. Guerrero, J. Rivas, A. Paneque, and M. Losada

Departamento de Bioquímica, Facultad de Ciencias y CSIC, Universidad de Sevilla, Spain.

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Summary: The assimilatory nitrate-reducing system operating in dark-grown Chlorella cells seems to be identical to that previously found in cells autotrophycally-grown in the light, and involves the same two reactions catalyzed by NADH-nitrate reductase and ferredoxin-nitrite reductase. Ferredoxin isolated from dark-grown Chlorella cells was reduced by NADPH with the aid of the NADP reductase present in the same cells, and, when assayed for other prominent and specific activities, it was also effective in mediating the photoreduction of NADP and nitrite by spinach chloroplasts.

The mechanism for the overall reduction of nitrate to ammonia in the photosynthetic tissues of higher plants and in Chlorella cells grown in the light have been thoroughly investigated during the last years and shown to be similar (1-4). In Chlorella, the complete reaction proceeds in a stepwise fashion as follows: a) nitrate is reduced to nitrite by the FAD-dependent molybdoprotein NADH-nitrate reductase (4-8), and b) nitrite is reduced to ammonia by the ironprotein ferredoxin-nitrite reductase (4,5,7).

In previous work (1-4), it has been firmly established that ferredoxin can act as electron carrier in mediating the transfer of electrons from illuminated grana to nitrite in the presence of nitrite reductase. It was moreover shown (1-4) that the enzymatic reduction of nitrite to ammonia can also be achieved in the dark with ferredoxin reduced either chemically by sodium dithio-

nite or enzymatically by a variety of electron donor systems: 1) molecular hydrogen-hydrogenase; 2) NADPH-NADP reductase. Since to date (2,3) the presence of ferredoxin in plants has been reported only in photosynthetic tissues, and nongreen tissues can also metabolize nitrite, the nature of the cofactor for nitrite reduction in nonphotosynthetic tissues remains to be establish. Of great interest is also the identification of the physiological reducing system for this cofactor.

The present report is concerned with the elucidation of the mechanism for the assimilatory reduction of nitrate and nitrite in Chlorella cells grown in the dark. The results indicate that the nitrate-reducing system operating in the dark is the same previously found to operate in the light (4). Of particular relevance has been the identification of ferredoxin as the electron carrier for nitrite reduction, and that of NADPH-NADP reductase as its electron donor system.

Materials and Methods: Chlorella 1.1 10.14.1 -a colorless mutant from Prof. E. Kessler at Erlangen-Nüremberg- was aerobically grown with vigorous shaking at 27º on the medium used for the wild type strain (5), but supplemented with 0.5% glucose and buffered with 20 mM phosphate, pH 7.0. 50 g of three days old cells were collected by low speed centrifugation, washed with distilled water, and broken in a vibration homogenator (Bühler) with glass beads of 0.3 mm. The broken material was resuspended in 25 mM Tris-HCl buffer, pH 7.5, passed through cheesecloth and centrifuged at 25,000 g for 45 minutes. After discarding the sediment, the resulting crude extract was applied, in order to adsorb the ferredoxin, to a DEAE-cellulose bed (60 mm height,

30 mm diameter), beforehand equilibrated with 25 mM Tris-HCl buffer, pH 7.5. Nitrite reductase and NADP reductase were eluted together from the column by washing with the same buffer supplemented with 150 mM NaCl. Ferredoxin was prepared from the DEAE-cellulose bed used in the previous treatment by elution with 25 mM Tris-HCl buffer, pH 7.5, 800 mM NaCl. The crude ferredoxin preparation thus obtained was diluted 5-fold with water and again adsorbed on a DEAE-cellulose bed (30 mm height, 15 mm diameter) equilibrated with 25 mM Tris-HCl buffer, pH 7.5. Ferredoxin was finally eluted with 25 mM Tris-HCl buffer, pH 7.5, 600 mM NaCl.

Results and Discussion: Reduction of nitrate to nitrite. Table I

Table I. EFFECT OF DIFFERENT ELECTRON DONORS ON THE REDUCTION OF NITRATE BY A CRUDE EXTRACT FROM DARK-GROWN Chlorella

Electron donor	Nitrite formed (nmoles per min)
NADH	20.7
NADPH	0.9
$FADH_2$	7.6
$FMNH_2$	7.6
MVH	3.1
None	0.6

Except in the experiments with reduced pyridine nucleotides, where no sodium dithionite was used, the conditions were as previously described for the standard assay of nitrate reductase (9). Cell-free extract, 0.1 ml, containing 4.4 mg protein; NADH or NADPH, 0.3 µmoles; FAD or FMN, 0.2 µmoles; methyl viologen (MV), 1.5 µmoles.

shows that nitrate reductase from dark-grown Chlorella cells could use NADH -but not NADPH- as electron donor for the reduction of nitrate to nitrite. As previously shown for the enzyme from light-grown cells (5), FAD or FMN (and methyl viologen) chemically kept in the reduced state by dithionite were also relatively effective as electron donors. With dithionite only, i.e. in the absence od added cofactors, the reaction did not practically occur.

Reduction of nitrite to ammonia. Nitrite reductase and ferredoxin from dark-grown Chlorella cells were found to be similar to the respective enzyme and electron carrier-protein previously isolated from Chlorella cells grown in the light (5). As can be seen in Table II, the reduction of nitrite by nitrite reductase depended on ferredoxin. Among several electron carriers tested, and using also dithionite as the reductant, only methyl viologen

Table II. REDUCTION OF NITRITE WITH CHEMICALLY-REDUCED FERREDOXIN BY NITRITE REDUCTASE FROM DARK-GROWN Chlorella

System	Nitrite reduced (nmoles per min)
Complete	120
Ferredoxin omitted	0
Nitrite reductase omitted	0
Complete, nitrite reductase heated	0

Experimental conditions as previously described for the standard assay of the enzyme (10,11). Nitrite reductase preparation, 1.0 mg; ferredoxin, 1.8 mg.

-but not even benzyl viologen- was capable of replacing ferredoxin in mediating the transfer of electrons from dithionite to the nitrite-nitrite reductase system; FAD or FMN were not effective. The product of the reduction was stoichiometrically identified as ammonia.

Since the crude extracts from Chlorella cells grown in the dark contained, besides nitrite reductase and ferredoxin, a NADP reductase which specifically catalyzed the oxidation of NADPH -but not of NADH- by ferredoxin, such extracts could also carry into effect the reduction of nitrite with NADPH as electron donor (Table III). It can be seen that the reaction catalyzed by a concentrated preparation from a crude extract was absolutely dependent on ferredoxin: When the preparation was freed from ferredoxin by

Table III. FERREDOXIN-MEDIATED REDUCTION OF NITRITE WITH NADPH BY A PREPARATION FROM DARK-GROWN Chlorella

System	Nitrite reduced (nmoles per min)
Enzyme preparation	34
Fd-free enzyme preparation	0
Fd-free enzyme preparation plus Fd	56

The reaction mixture included in a final volume of 2.5 ml: 100 µmoles Tris-HCl buffer, pH 7.5; NADPH-generating system (glucose-6-phosphate, 15 µmoles; glucose-6-phosphate dehydrogenase, 0.05 mg; $MgCl_2$, 2 µmoles, NADP, 1 µmole); sodium nitrite, 4 µmoles; and where indicated, enzyme preparation (a crude extract concentrated by adsorcion on a DEAE-cellulose bed, elution with 25 mM Tris-HCl buffer, pH 7.5, 800 mM NaCl, and finally filtration with Sephadex G-25), 11 mg; ferredoxin (Fd)-free enzyme preparation, 6.8 mg; ferredoxin (Fd), 1.8 mg. Nitrite disappearance was estimated as previously described (10,11).

passing through a DEAE-cellulose bed, no reaction occurred; if ferredoxin purified from the same cells was then added back, the reaction did proceed again.

By gel filtration with Sephadex G-100, NADP reductase and nitrite reductase could be separated, and their molecular weights so estimated (41,000 and 62,000 respectively) were in accord with those reported by Zumft et al. (5) for the same enzymes isolated from light-grown Chlorella.

Ferredoxin-mediated photoreduction of NADP and nitrite by spinach chloroplast fragments. Prominent among the reactions mediated by ferredoxins are the transfer of strongly reducing electrons -as those supplied in the light by chloroplasts- to NADP and to nitrite, catalyzed by NADP reductase and nitrite reductase, respectively (12).

Ferredoxin from photosynthesizing Chlorella cells was isolated under the name of "red enzyme" by Gewitz and Völker in Warburg's laboratory in 1962 (13). It was characterized, among other properties, by an absorption spectrum which showed bands at 330, 420 and 460 nm, and by catalyzing the photoreduction of NADP by spinach chloroplasts. Fig. 1 shows the absorption spectrum of the new isolated dark-grown Chlorella ferredoxin. It could also replace spinach ferredoxin in mediating the reduction of NADP by illuminated spinach grana.

Table IV shows that, in the presence of spinach chloroplast fragments, nitrite could be reduced in the light with the aid of nitrite reductase and ferredoxin isolated from Chlorella cells grown in the dark.

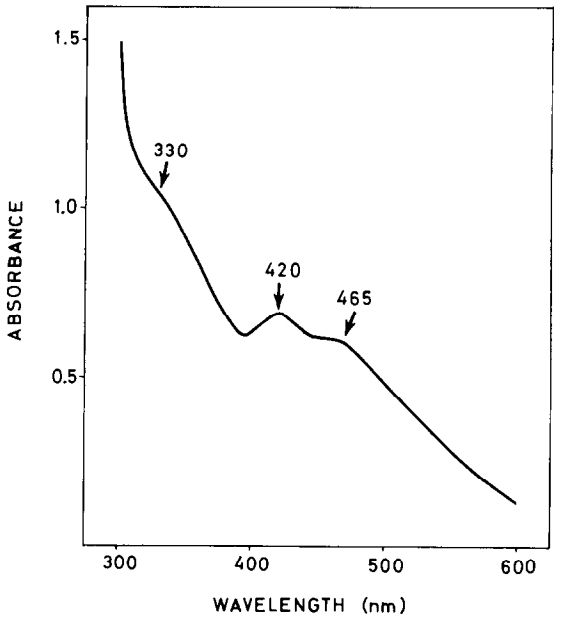

Fig. 1. Absorption spectrum of dark-grown Chlorella ferredoxin.

Table IV. PHOTOREDUCTION OF NITRITE BY SPINACH CHLOROPLAST FRAGMENTS FORTIFIED WITH FERREDOXIN AND NITRITE REDUCTASE FROM DARK-GROWN Chlorella

System	Nitrite reduced (nmoles per min)
Complete	190
Ferredoxin omitted	20
Nitrite reductase omitted	25
Complete, dark	0

Experimental conditions as previously described (10,14). Nitrite reductase, 1 mg; ferredoxin, 1.8 mg.

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