MECHANISM OF NITRATE REDUCTION IN CHLORELLA

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SUMMARY

In Chlorella, the reduction of nitrate to ammonia takes place in two independent enzymatic steps: 1) the reduction of nitrate to nitrite, involving 2 electrons, catalyzed by NADH-nitrate reductase and 2) the reduction of nitrite to ammonia, involving 6 electrons, catalyzed by ferredoxin-nitrite reductase. Both enzymes have been purified and characterized, and some of their properties have been investigated.

Although the pioneer work of Warburg and Negelein (1) on the reduction of nitrate to ammonia was carried out with living Chlorella cells, little has yet been revealed about the mechanism of the process in this alga at the enzymatic level.

The presence of nitrate reductase in cell-free extracts of Chlorella vulgaris was demonstrated by Syrett and Morris (2), who showed that the enzymatic reduction of nitrate to nitrite specifically required NADH as electron donor and did not proceed with NADPH. Trebst and Burba (3) partially purified the enzyme by ammonium sulfate fractionation of cell-free extracts from Chlorella pyrenoidosa, using for the assay methyl viologen and dithionite as reductant, in order to study its inhibition by disalicylidenediamines.

Knutsen (4) investigated the induction of nitrite reductase in C. pyrenoidosa and demonstrated enzymatic activity in

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a homogenate from cells that had been incubated with nitrite, using reduced methylene blue as electron donor, but Ahmed and Morris (5) failed in preliminary attempts to detect enzymatic reduction of nitrite by cell-free extracts from this organism, using various combinations of benzyl viologen, dithionite and NADPH.

The present report is concerned with the elucidation of the mechanism of the reduction of nitrate to nitrite and of nitrite to ammonia by two independent enzymes purified from Chlorella cells. The results indicate that in this alga the enzyme involved in the first step of nitrate reduction is essentially similar to that occuring in higher plants (6) and apparently differs from that found in the blue-green alga Anabaena cylindrica by Hattori and Myers (7).

MATERIALS AND METHODS

The alga used in this work was Chlorella fusca Shihira et Krauss (= pyrenoidosa) 211-15 from Pringsheim's culture collection at Göttingen. The alga was grown at 30º under the conditions described by Kessler (8). Three days old cells were broken with alumina in a cold mortar, using 0.1 M Tris pH 8.0 for extraction of enzymes. The crude extract was centrifuged for 1 h. at 100,000 x g and the supernatant passed through a DEAE-cellulose bed equilibrated with the same buffer in order to adsorb the ferredoxin. The enzymes which went through the column were fractionated with $(NH_4)_2SO_4$. The precipitate up to 40% saturation which contained nitrate reductase was further purified following the method for spinach nitrate reductase of Paneque et al. (9). The precipitate between 40% and 70% $(NH_4)_2SO_4$ saturation, which contained nitrite reductase and NADP reductase was redisolved and desalted by passing it through a Sephadex G-25 column, and the enzymes purified by chromatography on DEAE-cellulose and on Sephadex G-100. Chlorella ferredoxin was recovered from the original DEAE-cellulose bed and rechromatographied on DEAE-cellulose. Where not speci-

fied, the experimental conditions were as in previous works (9-11).

RESULTS AND DISCUSSION

Reduction of nitrate to nitrite

As was previously shown for nitrate reductase from spinach and other higher plants (9,10,12), it has now been found that highly purified preparations from Chlorella can not directly accept electrons from ferredoxin kept in the reduced state either chemically with dithionite or enzymatically with NADPH and NADP reductase (cf. 7), but can use both NADH (but not NADPH) and FNH_2 (or reduced viologens) as electrons donor for the reduction of nitrate to nitrite. In this case also, two enzymatic activities have been found to participate one after another in the transfer of electrons from NADH to nitrate: first, a NADH-specific diaphorase, and second a nitrate reductase proper. The reduction of nitrate with FNH_2 (or reduced viologens) involves exclusively the second activity. Since the diaphorase activity is inhibited not only by 0.1 mM pCMB but also by heating at 45º for 5 minutes, this mild treatment completely blocks the reduction of nitrate with NADH, but does not affect the reaction with FNH_2 or reduced viologens. This latter reaction is however totally poisoned by 1 mM cyanide.

It can be seen in Figure 1 that after gel filtration with agarose of a highly purified nitrate reductase preparation from Chlorella, which before this treatment was able to use both NADH and reduced methyl viologen as reductant, the protein fraction eluted from the column which exhibited nitrate reductase activity was much more effective with the viologen than with the nucleotide, due to the lability of the diaphorase activity. By this procedure, and using ovalbumin, globulin and apoferritin as markers, it could besides be estimated a molecular weight of about 500,000 for Chlorella nitrate reductase.The spectrum of the purified enzyme did not show any indication of absorption bands for flavin nucleotides.

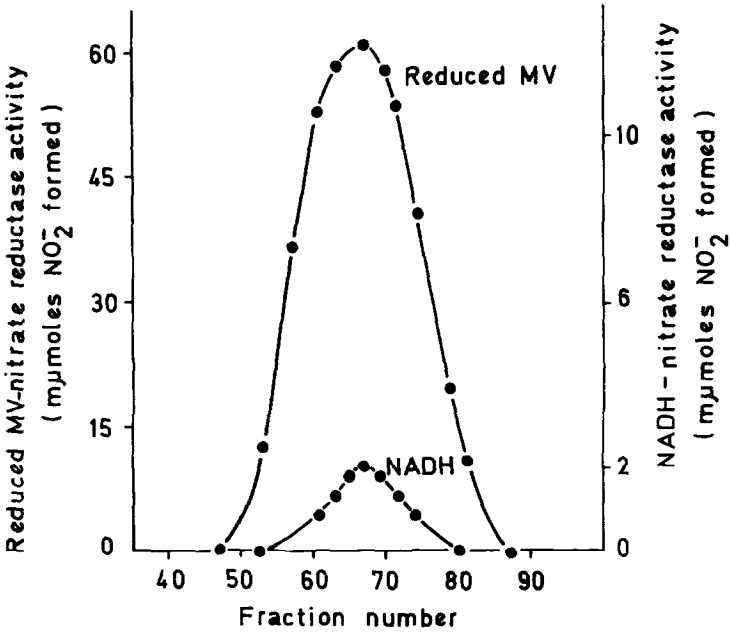

Figure 1. Elution of nitrate reductase from agarose. 2 mg of nitrate reductase were applied to a column of agarose (Bio-Gel A-1.5 m, Calbiochem, 100-200 mesh), 2.5 x 75 cm, and eluted with 0.05 M Tris buffer, pH 7.5, containing 0.2 M NaCl and 0.002 M KNO_3. 2 ml fractions were collected and 0.2 ml aliquots were assayed as previously described for nitrate reductase activity using NADH or chemically reduced methyl viologen as electron donor, and running the reaction for 10 min (9,10).

Reduction of nitrite to ammonia.

Nitrite reductase purified from Chlorella has been found to be quite similar to the enzyme previously isolated from higher plants (11,13,14) and Anabaena (15).It does not seem to be, according to its absorption spectrum, a flavoprotein, and catalyzes the reduction of nitrite to ammonia, specifically requiring reduced ferredoxin as electron donor.

Table I shows that Chlorella nitrite reductase can not use by itself NADH or NADPH as electron donor.Among the natural electron carriers tested, using dithionite as reductant, only ferredoxin was effective. Neither FMN or FAD were capable of replacing ferredoxin in mediating the transfer of electrons from dithionite to the nitrite-nitrite reductase system. Among

TABLE I

EFFECT OF DIFFERENT ELECTRON DONORS ON THE REDUCTION OF NITRITE BY *Chlorella* NITRITE REDUCTASE

Electron donor	Concentration (mM)	Nitrite reduced (micromoles)
NADH	1.5	0
NADPH	1.5	0
Fd	0.1	1.2
MV	0.5	2.6
BV	0.5	0.3
FAD	0.1	0
FMN	0.1	0

Except in the experiments with reduced pyridine nucleotides, where no sodium dithionite was used, the conditions were as in the standard assay of Ramirez *et al*. (11). Nitrite reductase, 0.25 mg.

the artificial electron carriers examined, methyl viologen was very active, but benzyl viologen was rather sluggish. This may explain the failure of Ahmed and Morris (5) in their attempts to find the enzyme.

Table II shows that, under well characterized conditions and using methyl viologen and dithionite as reductant, the enzymatic reduction of nitrite was accompanied by the formation of stoichiometric amounts of ammonia.

NADPH could serve as electron donor for the reduction of nitrite in a *Chlorella* enzyme system containing, besides nitrite reductase, NADP reductase and ferredoxin. In this case, the reaction proceeded in two independent steps: 1) the reduction of oxidized ferredoxin by NADPH, catalyzed by NADP reductase and 2) the oxidation of reduced ferredoxin by nitrite catalyzed by nitrite reductase. By gel filtration with a Sephadex G-100

TABLE II

STOICHIOMETRY OF NITRITE REDUCTION AND AMMONIA FORMATION IN THE REACTION CATALYZED BY *Chlorella* NITRITE REDUCTASE

System	NO_2^- reduced (micromoles)	NH_3 formed (micromoles)
Complete	2.4	2.4
Methyl viologen omitted	0	0.1
Nitrite reductase omitted	0	0
Sodium dithionite omitted	0	0.1
Complete, nitrite reductase heated	0	0.1

The experimental conditions were as in the standard assay of Ramirez *et al.* (11). Nitrite reductase, 0.25 mg.

column, *Chlorella* NADP-reductase and nitrite reductase could be separated and their molecular weights estimated by calibrating the column with serum albumin, ovalbumin and myoglobin. The value of 63,000 calculated for *Chlorella* nitrite reductase is in accord with those reported, using the same technique, by Hewitt and Hucklesby (14) and by Hattori and Uesugi (15) for squash and spinach nitrite reductase, and for *Anabaena* nitrite reductase, respectively. The value of 40,000 calculated for *Chlorella* NADP reductase differs apreciably from that of 65,000 estimated also for *Chlorella* NADP reductase by Gewitz and Völker (16) on the basis of its FAD content.

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