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Nitrate Reductase from *Monoraphidium braunii*

IMMUNOCYTOCHEMICAL LOCALIZATION AND IMMUNOLOGICAL CHARACTERIZATION[1]

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ABSTRACT

Homogeneous nitrate reductase (EC 1.6.6.2) from *Monoraphidium braunii* was obtained by means of affinity chromatography in blue-Sepharose and gel filtration. After electrophoresis in polyacrylamide, gel slices containing pure nitrate reductase were disrupted and injected into previously unimmunized rabbits. The antiserum produced after several weeks was found to inhibit the different activities of nitrate reductase to a similar degree. Monospecificity of the antiserum was demonstrated by Ouchterlony double diffusion and crossed immunoelectrophoresis. The antibodies were purified by immunoabsorption to Sepharose-bound nitrate reductase.

The intracellular location of nitrate reductase in green algae was examined by applying an immunocytochemical method to *M. braunii* cells. Ultrathin frozen sections were first treated with immunopurified anti-nitrate reductase monospecific antibodies, followed by incubation with colloidal gold-labeled goat antirabbit immunoglobulin G as a marker. The enzyme was specifically located in the pyrenoid region of the chloroplast.

The reduction of NO_3^- to NH_4^+ is catalyzed by two enzymes working sequentially, NR[2] and NiR. Plant-type NR catalyzes the initial step of nitrate assimilation: the reduction of nitrate to nitrite using pyridine nucleotides as electron donors (16). Besides this physiological activity, the enzyme can act as an NAD(P)H diaphorase, catalyzing the reduction of ferricyanide, Cyt *c*, and other acceptors, and can also catalyze the reduction of nitrate with reduced viologens or flavins as electron donors (16, 27).

Whereas a close relationship between CO_2 assimilation and the light reactions of photosynthesis has been traditionally accepted, the connection between these reactions and nitrate assimilation has been a matter of controversy (2, 16). However, it has been shown that the reducing power used in nitrate and nitrite reduction is provided by the photosynthetic light reactions (16). In order to substantiate these functional relationships, it would be of primary interest to establish definitively the intracellular location of both enzymes. So far, no attempts to localize NR in green algae have been reported. However, NiR is widely accepted as a chloroplastic enzyme in both green algae and higher plants (4, 12, 16, 30). On the other hand, working with Chl deficient mutants of barley, Sawhney (19) has suggested that NR is localized in the chloroplast, although other reports indicate very different locations for the enzyme in higher plants (4, 15, 30). Recent experiments with soybean cotyledons suggest that NR is located in the cytoplasm (25, 26).

By using immunocytochemical methods, NR from *Neurospora crassa* has been located in the cell wall-plasmalemma region and the tonoplast (18). Similar immunochemical approaches have been successfully applied to the localization of other proteins (6, 28). In the present work, we have used a similar direct approach to demonstrate the precise localization of NR in the green alga *Monoraphidium braunii*.

MATERIALS AND METHODS

Growth of Algae. *Monoraphidium braunii* (formerly *Ankistrodesmus braunii*) strain 202-7d from Göttingen University's culture collection was grown at 25°C in the light on 5 mM KNO_3 as previously described (14), but AP 31 (Merck) was added as an antifoaming agent. The cultures were bubbled with a stream of 5% (v/v) CO_2 in air. After 3 to 4 days of growth, the cells were harvested by centrifugation and stored frozen at −20°C until used.

Enzyme Assays. NAD(P)H-NR, MVH-NR, FMNH-NR, and NAD(P)H-Cyt *c* reductase assays were performed as previously described (7). A unit of activity is the amount of enzyme which catalyzes the formation of 1 μmole of product per min.

Preparation of Crude Extracts and Purification of Nitrate Reductase. Crude extracts were prepared and NR was purified according to the method of de la Rosa *et al.* (5) with the following modifications: (a) the enzyme was eluted from the blue-Sepharose column with 1 M KNO_3; (b) the fractions containing NR activity were pooled and subjected to $(NH_4)_2SO_4$ fractionation between 25 to 50% saturation; and (c) the enzyme preparation was applied to a Sephacryl S-300 column (90 × 1.6 cm).

Production of Antisera. Purified NR was subjected to disc gel electrophoresis in 5% polyacrylamide and localized on the gels (22). Gel sections containing NR were pooled, disrupted and emulsified in 4 ml of 1:1 mixture of sodium-buffered physiological saline solution and Freund's complete adjuvant and injected subcutaneously into unimmunized rabbits. The process was repeated three times, at 15-d intervals, but using incomplete adjuvant. Whole blood was obtained from the ear 1 week after the last injection. New boosting injections were given at 1-month intervals, and the rabbits were bled 1 week after each injection,

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[2] Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; anti-NR, antibodies against nitrate reductase; MV, methyl viologen; FMN, flavin adenine mononucleotide.

which contained approximately 50 μg of homogeneous NR.

Anti-NR Purification. After centrifugation and inactivation of complement, the serum was precipitated with 45% $(NH_4)_2SO_4$ and the pellet resuspended in, and dialyzed against, 17.5 mM phosphate (pH 6.3). The antiserum was then transferred to a column of DEAE-Sephacel equilibrated with the same buffer. The IgG fraction eluted with the washing buffer.

Immunoaffinity Purification of Anti-NR. Two ml of DEAE-purified anti-NR serum were loaded on a NR-Sepharose column (7 × 1.6 cm). A flow rate of 25 ml/h was used throughout the procedure. After washing with sodium-buffered physiological saline solution, the same buffer was supplemented with either 1 M NaCl or 3 M $MgCl_2$ to elute nonspecifically absorbed proteins. Specific anti-NR was eluted with 0.2 M glycine (pH 2.5). These antibodies formed a single precipitate against NR crude extract when subjected to Ouchterlony double diffusion test, and inhibited the different activities of the enzymic complex.

Gel Immunodiffusion and Immunoelectrophoresis. Double immunodiffusion experiments were performed according to the method of Ouchterlony and Nilson (17). In order to estimate the antibody titer, the Sewell method was followed (20). Crossed immunoelectrophoresis (29) was carried out in 1% (w/v) agarose plates using Tris barbital buffer (pH 8.6) for both the gel and electrophoresis buffer. The process was conducted for 6 h at 10 v/cm and 4°C. NR activity was detected in gels by following the nitrate-dependent oxidation of chemically reduced MV (5). In all cases, immunoprecipitates were stained with Coomassie brilliant blue R 250.

Electron Microscopy. The procedure described by Tokuyasu (24) was essentially followed. Cells from liquid cultures were washed twice and fixed with 0.5% (v/v) glutaraldehyde, 3% (v/v) paraformaldehyde on 0.1 M sodium phosphate buffer (pH 7.3) for 2 h. After washing, they were infused with 0.8 M sucrose for 10 to 12 h. The cells were centrifuged and the pellet mounted on a copper rod and frozen in liquid nitrogen. Ultrathin cryosections were made at −90°C in a Sorwall MT 2B ultramicrotome equipped with a FTS attachment. The sections were collected and transferred to Formvar carbon-coated grids, using the sucrose droplet method (24).

Immunostaining consisted of sequential incubation of the sections in solutions of anti-NR antibodies, buffer, gold-labeled goat anti-rabbit antibodies (GAR 20-Janssens, Pharmaceutica), buffer, uranyl acetate, and finally methylcellulose, with which they were covered. For the nonimmune controls, the primary antibody was replaced by a solution of nonimmune rabbit IgGs. Sections were studied in a Jeol 100 B electron microscope.

RESULTS

NR, purified as described in "Materials and Methods" (47 units/mg), showed a single band when subjected to disc gel electrophoresis. The purity of the antiserum was tested by using Ouchterlony double diffusion test (Fig. 1). Figure 1A shows the reaction of increasing concentrations of partially purified NR (Blue-Sepharose eluate; outer wells) with partially purified antiserum (center well). Figure 1B shows the reaction of increasing concentrations of antiserum (outer wells) with the partially purified NR preparation (center well). In both cases, a single immunoprecipitate was found. No contaminants were detected in any of the dilutions employed.

Monospecificity of the antiserum was further demonstrated by the formation of a single immunoprecipitate when partially purified NR was subjected to crossed immunoelectrophoresis and the gels were stained for protein (Fig. 2). The immunoprecipitate also stained positively for MVH-NR activity (not shown) indicating that immunoprecipitates were formed by the specific reaction of antibodies with NR. Similar results were obtained when using homogeneous NR.

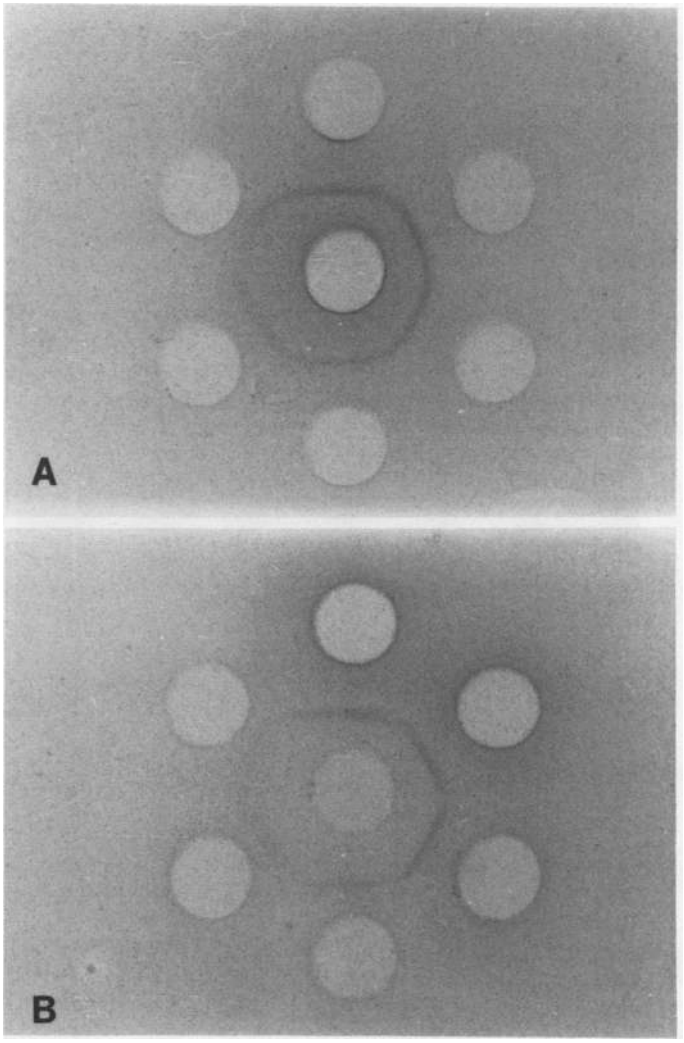

FIG. 1. Ouchterlony double immunodiffusion. A, The center well contained 16 μl of DEAE-purified anti-NR, and the outer wells contained 16 μl of serial dilutions of partially purified NR; B, NR in center well, antiserum in surrounding wells. NR activity was 17 units/ml.

Anti-NR from different sources such as *Chlorella vulgaris* (9), *Neurospora crassa* (1), squash (21), and barley (23) inhibit their respective NR activities. Figure 3 shows the inactivation of *Monoraphidium* NR when increasing amounts of antiserum were incubated with a constant amount of blue-Sepharose purified enzyme. The inhibition was very strong, since small amounts of antiserum inactivated completely the nitrate reductase. All the activities of the enzymic complex were inhibited. However, MVH-NR and FMNH-NR activities were more sensitive to the antiserum than NADH-NR and NADH-Cyt *c* reductase. Preimmune control serum did not inhibit any enzymic activity of NR.

The possible protective effect of different enzyme ligands against inactivation of NR by anti-NR antiserum was tested. No protection was found when the inactivation was carried out in the presence of $NAD(P)^+$, NAD(P)H, FAD, FMN, ADP, NO^-_3 KCN, or azide under a wide range of conditions. These results suggest that there are not antigenic determinants, at least to a significant extent, at the ligand binding sites of NR.

The main interest of the work reported here was to determine the intracellular location of NR in *M. braunii* cells. To perform the EM immunolocalization, the monospecific antiserum was further purified by immunoaffinity chromatography in a purified NR-Sepharose CL 4B column as indicated in "Materials and

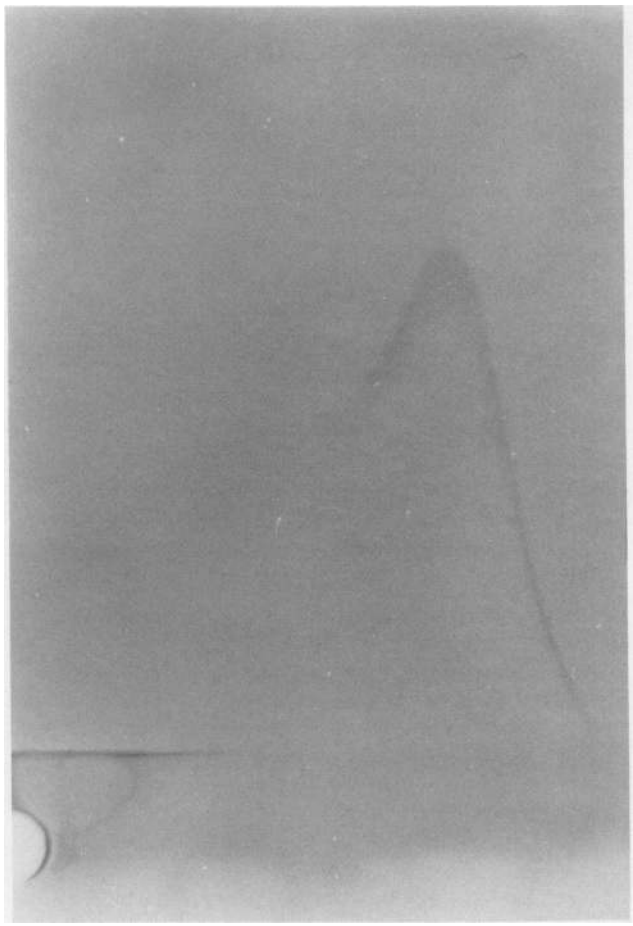

FIG. 2. Crossed immunoelectrophoresis of blue-Sepharose purified *M. braunii* NR (0.59 units). The gel contained 0.13% (v/v) of DEAE-purified antiserum. Protein was stained as described in "Materials and Methods."

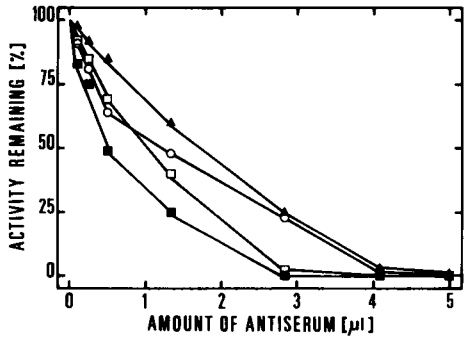

FIG. 3. Inhibition of NR activity by anti-NR antiserum. The designated amount of serum was mixed with blue-Sepharose purified NR (0.1 unit), and sodium phosphate-buffered physiological saline solution in a final volume of 0.1 ml. After 15-min incubation at 30°C, aliquots were taken to assay the different activities. (○), NADH-NR; (▲) NADH-Cyt *c* reductase; (□), MVH-NR; (■), FMNH-NR.

Methods."

Ultrathin sections of *Monoraphidium* cells were incubated first with the immunopurified anti-NR IgG, and subsequently treated with goat anti-rabbit IgG marked with colloidal gold. The immunoelectron microscopy of such sections is shown in Figure 4. In this immunocytochemical procedure, positive staining is seen as electron-dense and very defined spots. In the sections, most of the cellular structures could be clearly recognized. Although resolution in frozen sections is generally lower than in plastic sections, membrane resolution was found in nearly all cell sections. Plasmalemma could be clearly discerned, but membrane resolution was most obvious in the chloroplast, where the pyrenoid matrix is traversed by thylakoid membranes. No shell of starch inclusions is present, surrounding the pyrenoid matrix of *M. braunii*. In control sections, only negligible gold staining was found (Fig. 4A). Specific nitrate reductase label was found only in the chloroplast (Fig. 4B). The pyrenoid was clearly the site of preferential NR location. In the sections, it was totally covered with the colloidal gold particles. A few label particles occurred, also on the Chl-containing regions. This latter signal, however, was very weak when compared to the nonimmune controls. No specific label at all could be found at the nucleus, cytoplasm, and plasmalemma.

Sewell titration of anti-NR (Fig. 5) showed the equivalence point for 3.50 µg of anti-NR IgG and 2.23 µg of pure NR. Assuming a mol wt of 160 kD for IgG and 460 kD for the enzyme of *M. braunii* (5), 4.5 mol of IgG are required to precipitate one mol of enzyme under the described conditions.

DISCUSSION

Ouchterlony double diffusion analysis showed that the antiserum against *M. braunii* NR was apparently monospecific. This was confirmed by crossed immunoelectrophoresis, a much more sensitive method. Monospecific antisera have previously been prepared against NR from different sources, such as *N. crassa* (1), *C. vulgaris* (9), squash cotyledons (21), barley (23), and spinach (8).

The antibodies raised against NR from *M. braunii* inhibited all the activities of the NAD(P)H-NR complex from this organism, the inhibition being stronger for MVH-NR and FMNH-NR than for the pyridine nucleotide depending activities. These results differ from those reported for *C. vulgaris* (9) and barley (23), where a similar inhibition pattern of the different NR activities have been shown. However, the activities of the NR complex from *N. crassa* (1) and squash (21) were affected in a different way by incubation with their corresponding antisera.

The results obtained in Sewell titration indicate that immunoprecipitates contain 4.5 molecules of anti-NR IgG per molecule of NR. On the other hand, the protection by pyridine nucleotides against antiserum inhibition of several dehydrogenases has been reported (3, 13) and used as an indication of the existence of antigenic determinants in the nucleotide binding domain. However, no such protection was found with *M. braunii* NR, suggesting that none of the antigenic determinants of NR is in the nucleotide domain of the enzyme.

On the basis of different experimental approaches, NiR is widely accepted as a chloroplastic enzyme (12, 27, 30). In higher plants, NR location has been generally studied by differential isolation of subcellular fractions followed by detection of the enzyme activity. The results obtained have led some authors to claim that NR is located in the chloroplast (10, 16), whereas others have reported NR is a cytoplasmic enzyme (2, 4, 30). Recently, by using a histochemical approach, Vaughn and Duke (25) have proposed that NR of soybean cotyledons is located in small cytoplasmic vesicles.

In the last years, localization studies have been approached by more precise methods involving specific antibodies labeled for use in light and EM. This approach has demonstrated the location of assimilatory NR in cytoplasm of soybean cotyledons (26) and its association with the cell-wall plasmalemma and the tonoplast in *N. crassa* (18). No evidence of NR localization in green algae has been reported, to date. Our results clearly demonstrate the association of NR with the chloroplast in *M. braunii*. The pyrenoid is proposed as the specific site of NR location. Therefore, the cellular location of NR differs in green algae and

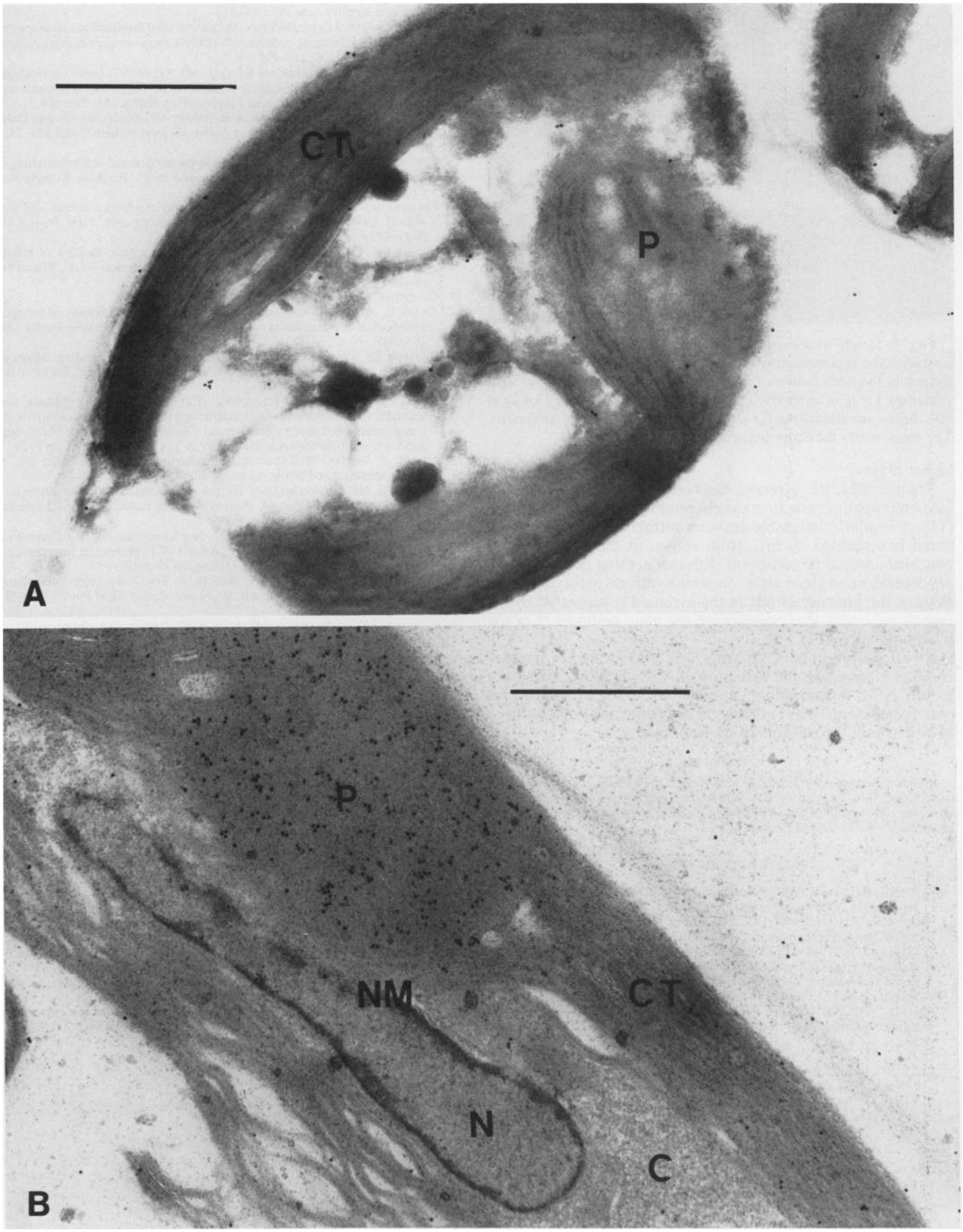

FIG. 4. Immunoelectron-microscopic localization of NR in *M. braunii* cells. Bar is 1 μm. A, Control section incubated with preimmune serum; B, cell section treated with specific anti-NR antibodies. C, cytoplasm; CT, chloroplast thylakoids; N, nucleus; NM, nuclear membrane; P, pyrenoid.

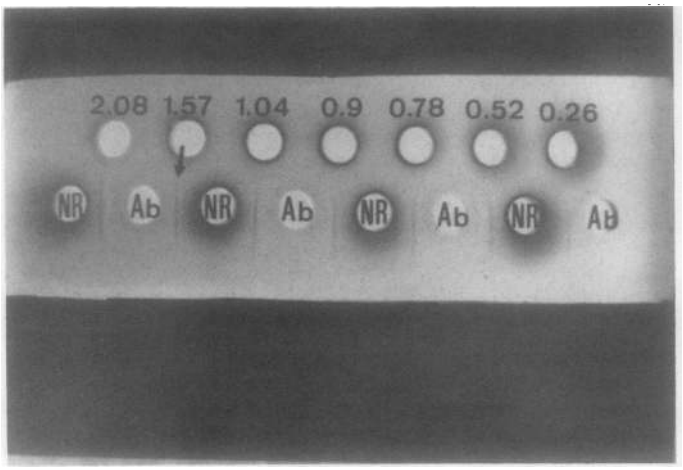

FIG. 5. Sewell titration of immunopurified anti-NR. Upper wells contained the supernatants of purified NR and anti-NR mixtures. The figures on the wells indicate the antibody:antigen ratio. All these wells contained 3.5 μg of antibody. The outer right well contained 13.4 μg of NR. Below are alternating 6.7 μg of antigen and 3.5 μg of antibodies. The arrow marks the equivalence point.

higher plants.

Traditionally, the pyrenoid has been considered as a specialized chloroplastic structure closely related with starch synthesis (11). A similar relationship between nitrate reduction and pyrenoid is supported by the results shown in this work. Such a conclusion would be consistent with the fact that both processes are dependent, in green algae, on photosynthetic reducing power. Besides, the presence of NR in the pyrenoid is supported by the reports indicating that proteins are the main component of the pyrenoid matrix (11).

If NiR, a chloroplastic enzyme, were also located in the pyrenoid of green algae, the reduction of NO^-_3 to NH^+_4 would not require NO^-_2 translocation, increasing the efficiency of the process. Experiments are in progress to determine the exact location of NiR within the chloroplast of *M. braunii*.

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LITERATURE CITED

1. AMY NK, RH GARRET 1979 Immunoelectrophoretic determination of nitrate reductase in *Neurospora crassa*. Anal Biochem 95: 97–107
2. BEEVERS L, RH HAGEMAN 1972 The role of light in nitrate metabolism in higher plants. Photophysiology 7: 85–113
3. CARLBERG I, B ALTMEJD, B MANNERVIK 1981 Purification and immunological studies of glutathione reductase from rat liver. Evidence for an antigenic determinant at the nucleotide binding domain of the enzyme. Biochim Biophys Acta 677:146–152
4. DALLING MJ, NE TOLBERT, RH HAGEMAN 1972 Intracellular location of nitrate reductase and nitrite reductase. I. Spinach and tobacco leaves. Biochim Biophys Acta 283: 505–512
5. DE LA ROSA MA, J DIEZ, JM VEGA, M LOSADA 1980 Purification and properties of assimilatory nitrate reductase NAD(P)H from *Ankistrodesmus braunii*. Eur J Biochem 106: 249–256
6. DEL RIO LA, DS LYON, I OLAH, B GLICK, ML SALIN 1983 Immunocytochemical evidence for a peroxisomal localization of manganese superoxide dismutase in leaf protoplasts from a higher plant. Planta 158: 216–224
7. DIEZ J, A CHAPARRO, JM VEGA, A RELIMPIO 1977 Studies on the regulation of assimilatory nitrate reductase in *Ankistrodesmus braunii*. Planta 137: 231–234
8. FERRARIO S, B HIREL, P GADAL 1983 Immunochemical characterization of nitrate reductase from spinach leaves and roots. Biochem Biophys Res Commun 113: 733–737
9. FUNKHOUSER EA, CS RAMADOSS 1980 Synthesis of nitrate reductase in *Chlorella* II. Evidence for synthesis in ammonia-grown cells. Plant Physiol 65: 944–948
10. GRANT BR, CA ATKINS DT CANVIN 1970 Intracellular location of nitrate reductase and nitrite reductase in spinach and sunflower leaves. Planta 94: 60–72
11. GRIFFITHS DJ 1970 The pyrenoid. Bot Rev 36(1): 29–58
12. HAREL E, PJ LEA, BJ MIFLIN 1977 The localization of enzymes of nitrogen assimilation in maize leaves and their activities during greening. Planta 134: 195–200
13. KATIYAR SS, JW PORTER 1983 Antibodies for NADPH-binding region of enzymes possessing dehydrogenase activities. Proc Natl Acad Sci USA 80: 1221–1223
14. KESSLER E, W LANGNER, I LUDWIG, H. WIECHMANN 1963 Bildung von sekundar Carotinoiden ber Stickstoffmangel und Hydrogenase-Aktivität als taxonomishe Merkmale in der Gattung *Chlorella*. *In* Studies on Microalgae and Photosynthetic Bacteria. University of Tokyo Press, Tokyo, pp 7–20
15. LIPS H, Y AVISSAR 1972 Plant-leaf microbodies as the intracellular site of nitrate reductase and nitrite reductase. Eur J Biochem 29: 20–24
16. LOSADA M, MG GUERRERO 1979 The photosynthetic reduction of nitrate and its regulation. *In* J Barber, ed, Photosynthesis in Relation to Model Systems Elsevier, Amsterdam, pp 365–408
17. OUCHTERLONY O, NILSSON 1973 Immunodiffusion and immunoelectrophoresis. *In* DM Weir, ed, Handbook of Experimental Immunology. Blackwell Scientific Publications, Oxford, pp 19.1–19.34
18. ROLDAN JM, J-P VERBELEN, WL BUTLER, K TOKUYASU 1982 Intracellular localization of nitrate reductase in *Neurospora crassa*. Plant Physiol 70: 872–874
19. SAWHNEY SK, V PRAKASH, MS NAIK 1972 Nitrate and nitrite reductase activities in induced chlorophyll mutants of barley. FEBS Lett 22: 200–202
20. SEWELL MMH 1967 A semi-quantitative technique using LKB immunodiffusion apparatus. Science Tools 14: 11–12
21. SMARELLI J, WH CAMPBELL 1981 Immunological approach to structural comparisons of assimilatory nitrate reductases. Plant Physiol 68: 1226–1230
22. SOLOMONSON LP, GH LORIMER, RL HALL, R BORCHERS, JL BAILEY 1975 Reduced nicotinamide adenine dinucleotide nitrate reductase of *Chlorella vulgaris*. J Biol Chem 250: 4120–4127
23. SOMERS DA, T-M KUO, A KLEINHOFS, RL WARNER 1983 Nitrate reductase-deficient mutants in barley. Immunoelectrophoretic characterization. Plant Physiol 71: 145–149
24. TOKUYASU KT 1980 Immunochemistry of ultrathin frozen sections. Histochemistry 12: 181–203
25. VAUGHN KC, SO DUKE 1981 Histochemical localization of nitrate reductase. Histochemistry 72: 191–198
26. VAUGHN KC, SO DUKE, EA FUNKHOUSER 1984 Immunochemical characterization and localization of nitrate reductase in norflurazon-treated soybean cotyledons. Physiol Plant 62: 481–484
27. VENNESLAND B, MG GUERRERO 1979 Reduction of nitrate and nitrite. *In* M Gibbs, E Latzko, eds, Encyclopedia of Plant Physiology, New Series, Vol 6. Springer Verlag, Berlin 425–444
28. VERBELEN J-P, LH PRATT, WL BUTLER, K TOKUYASU 1982 Localization of phytochrome in oats by electron microscopy. Plant Physiol 70: 867–871
29. WALLENBERG B, V-B ANDERSON 1978 Immunoelectrophoretic techniques with the LKB multiphor. LKB Application Note 249
30. WALLSGROVE RM, PJ LEA, BJ MIFLIN 1979 Distribution of the enzymes of nitrogen assimilation within the pea leaf cell. Plant Physiol 63: 232–236