Regulation of Nitrate Reductase Activity in Cultured Spinach Cells as Studied by an Enzyme-Linked Immunosorbent Assay

Received for publication March 14, 1986 and in revised form June 14, 1986

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ABSTRACT

An enzyme-linked immunosorbent assay permitting the determination of nanogram quantities of nitrate reductase (NR) in cultured spinach cells has been developed and used for studies of the mechanism by which NR activity is regulated as a function of culture age. When 8-day old spinach cells were transferred to fresh medium, NR activity increased markedly in 2 days and thereafter decreased gradually until it became undetectable on the 10th day after the transfer. Determination of the amounts of NR by the immunosorbent assay indicated that the unique alteration of NR activity could be accounted for by the concomitant change in the amount of NR protein. Immunoblotting analysis of the subunit of NR also supported this result. It is concluded that the regulation of NR in spinach cells as a function of culture age is mediated by changes in the amount of the enzyme protein rather than by activation and inactivation of the preexisting proteins.

Nitrate reductase (NR, NADH-nitrate oxidoreductase, EC 1.6.6.1) occurs ubiquitously in higher plants and catalyzes the first step of nitrate assimilation (2). NR activity in higher plants is finely regulated in response to changes in physiological, environmental and other conditions (6). However, its regulatory mechanism is not yet fully understood, partly because of the lack of suitable methods for quantitation of NR protein. ELISA is a method capable of determining a specific protein in crude mixtures (3, 19). We report here an ELISA that permits accurate determination of ng quantities of NR in crude spinach extracts. Application of this assay to spinach cells in suspension culture has provided evidence that alterations in NR activity as a function of culture age are due to changes in the amount of NR protein. Immunoblotting experiments have also supported this conclusion.

MATERIALS AND METHODS

Chemicals and Biochemicals. The following chemicals and biochemicals were purchased from sources indicated in parentheses: NADH, Triton X-100, Tween 20, and complete Freund's adjuvant (Wako Chemical Co., Tokyo); polystyrene beads (Sekisui Chemical Industries, Ltd., Tokyo); horseradish peroxidase (type IV, Sigma Chemical Co.); Toyopearl HW 55F (Toyo Soda Co., Tokyo); Sephadex G-25 (Pharmacia Fine Chemicals); and peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Amersham International). All other chemicals used were of highest quality available commercially.

Preparation of Anti-NR Antibodies. NR was purified from fresh spinach leaves and subjected to PAGE under nonnaturating conditions as described previously (11). The gel section containing NR activity, as located by the method of Solomonson et al. (14), was excised and finely dispersed in 2 ml (for about 120 μg of NR protein) of phosphate buffered saline (PBS, 0.1 M K-phosphate [pH 7.2] containing 0.15 M NaCl) with a syringe. This suspension was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. The injection was made every 2 weeks and about 120 μg of NR was injected at each time. Eight weeks after the first injection, whole blood was obtained by cardiac puncture and the antiserum was collected by centrifugation. The IgG fraction was prepared from the antiserum by repeated precipitation with 33% saturated (NH₄)₂SO₄, followed by chromatography on a Toyopearl HW 55F column. Anti-NR antibodies were purified from the IgG fraction as follows. Ten polystyrene beads (ϕ, 6 mm) were added to 2 ml of PBS containing 50 μg of purified spinach NR and the mixture was incubated overnight at 4°C. The beads were then washed three times with 5 ml of PBS containing 0.05% (v/v) Triton X-100. The beads thus coated with NR were added to the IgG fraction (20 μg protein/bead) and the mixture was incubated overnight at 4°C. After washing the beads three times as above, the anti-NR antibodies adsorbed were eluted from the beads with 8 M urea containing 0.5% (w/v) BSA. The eluted antibodies were subjected to gel filtration through a Sephadex G-25 column (1.5 × 30 cm) equilibrated with PBS and protein-containing fractions were combined. The monospecificity of the antibodies was confirmed by immunoelectrophoresis (5).

Enzyme-Linked Immunosorbent Assay. To a small test tube (ϕ, 8 mm) containing one polystyrene bead (ϕ, 6 mm) was added 400 μl of an anti-NR IgG solution in PBS (10 μg IgG/ml) and the tube was incubated overnight at 4°C. The fluid was then removed from the tube with the aid of a Pasteur pipette. One ml of PBS containing 0.05% (v/v) Tween 20 (PBST) was added to the tube and after stirring for 30 s the buffer was removed. The bead was washed two more times with PBS in the same way. Four hundred μl of PBST containing 0.5 to 4 ng of purified spinach NR (for preparation of a standard curve) or an appropriately diluted spinach cell extract was added to the IgG-coated bead and the tube was incubated for 20 h at 30°C. The bead was then washed once with PBST and twice with PBS as described above. Four hundred μl of PBS containing 0.55 μl of peroxidase-labeled anti-NR IgG, prepared as described below, was added to the tube, which was then incubated for 20 h at 30°C, followed by washing with PBST and PBS as above. To the tube was then added 2 ml of 0.1 M citrate-0.2 M Na₂HPO₄ (pH 5.5) containing α-phenylenediamine (3 mg/ml), 0.02% H₂O₂, and 0.01% sodium

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1 Supported by Grant-in-Aid for Scientific Research (No. 60166001) from the Ministry of Education, Science and Culture.
2 Abbreviations: NR, nitrate reductase; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay.
ethylmercurithiosalicylate. This mixture had to be heated to 30°C before the addition. The peroxidase reaction was run at 30°C for 10 min, and the peroxidase activity was determined by measuring the A at 492 nm by the method of Joyce et al. (8). The amount of NR present in the sample was estimated from the peroxidase activity measured with the aid of the standard curve. The peroxidase-labeled anti-NR IgG was prepared by covalently conjugating horseradish peroxidase to the antibodies as described by Nakane and Kawai (12).

Immunoblotting. Cultured spinach cells were harvested and a cell-free extract was prepared therefrom as described previously (10). The extract (7.5 μl) was subjected to SDS-PAGE as described previously (11) and the protein bands thus separated on the gel were transferred to a nitrocellulose filter (Schleicher and Schull, GmbH) in a gel destainer (Marsoru Corp.) as described by Towbin et al. (18). Additional protein binding sites on the filter were blocked by incubating the filter overnight at 20°C in 10 mM K-phosphate (pH 7.5) containing 0.15 M NaCl and 2% (w/v) BSA. The filter was incubated with anti-NR IgG (8 μg/ml) dissolved in PBS for 2 h at room temperature, and rinsed three times in 10 mM K-phosphate (pH 7.5)-0.15 M NaCl (10 mM PBS) containing 0.05% (v/v) Tween 20. This was further incubated for 2 h at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies, which had been diluted 2000-fold with carrier solution. The filter was then rinsed three times in 10 mM PBS containing 0.05% Tween 20 and then three times in 10 mM PBS. Peroxidase activity staining of the filter was performed for 2 to 10 min by the method of Voller et al. (19).

Other Methods. Suspension culture of spinach cells and preparation of cell-free extracts were carried out as described earlier (9, 10). NR activity was determined as described previously (11). Protein determination was performed as reported earlier (11) by using BSA as a standard.

RESULTS AND DISCUSSION

Enzyme-Linked Immunosorbent Assay of NR. The standard curve obtained in this study for determination of spinach NR by the ELISA is shown in Figure 1. The peroxidase activity observed was linearly dependent on the amount of purified NR in the range from 0.5 to 4 ng. The sensitivity of the assay could not be improved by increasing the amount of antibodies used for coating and by prolongation of incubation times of the coating and steps in the assay procedure. The amounts of NR protein determined by the ELISA in spinach cell extracts were somewhat (10-15%) higher than those expected from NR activities (data not shown). This discrepancy might be due to the presence in the extracts of small amounts of inactive NR molecules that can be recognized by the antibodies. To examine the effects of coexisting proteins, 2.28 ng of purified spinach NR was added to 10 μl of a crude extract prepared from 10-d-old spinach cells, which contained no detectable amount of NR protein (see below). When this mixture was subjected to the assay, 2.28 ng of NR was detected. This indicated that NR determination by the ELISA was not affected by the coexistence of other spinach proteins. The reproducibility of the assay was tested by estimating intra-assay variance by using a crude extract from 8-d-old spinach cells (containing 330 ng of NR per ml). On intra-assay, the coefficient of variation was 3.1% in three determinations.

Changes in NR Activity and NR Protein in Cultured Spinach Cells. When spinach cells that had been cultured for 8 d were transferred to fresh medium, NR activity per unit cell mass increased markedly for 2 d and then declined gradually (Fig. 2), as has been reported for tobacco cells in culture (20) and for barley (15). NR activity became practically undetectable 10 d after the transfer. In the case of tobacco cells (20) and barley (15), evidence has been reported to indicate that the marked induction of NR activity accompanying the transfer to fresh medium is due to de novo synthesis of NR protein. In the case of nitrate-induced increase in NR activity in Chlorella cells, on the other hand, activation of an inactive form of NR protein has been observed (4, 13, 17). The gradual decline of NR activity observed in spinach cells after the rapid increase was probably caused by the decrease in nitrate concentration in the medium. In fact, it has been observed in a number of higher plants that removal of nitrate from the medium results in a decrease in NR activity (2, 7, 15, 20). Such decrease in NR activity in Neurospora crassa (1, 16) and barley (15) have been reported to be caused by repression of NR synthesis and enhancement of its degradation.

It was, therefore, of interest to examine whether the alteration of NR activity observed in spinach cells after the transfer to fresh

![Fig. 1. ELISA of spinach NR. The assay was carried out as described in "Materials and Methods," using the amount of homogeneous protein shown.](https://www.plantphysiol.org/)

![Fig. 2. Changes in NR activity and NR protein in cultured spinach cells during their growth age. Extracts from cultured spinach cell were prepared and used for the determination of NR protein by the ELISA (O) or enzymic activity (●).](https://www.plantphysiol.org/)
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either too small to be recognized by the antibodies or present at concentrations too low to be detected by this technique.

CONCLUSION

The ELISA developed in this study is sufficiently sensitive and useful for studies of regulation of NR activity in higher plants. Application of this technique to cultured spinach cells has provided evidence that the unique alteration of NR activity observed when cells were transferred to fresh medium was caused by the synthesis and degradation of NR protein and not by activation of an inactive protein and inactivation of the active enzyme. It is, however, to be noted that the operation of activation-inactivation regulation of NR under other conditions cannot be excluded.

Acknowledgments—The authors express their deep appreciation to Professor R. Sato and A. Oaks for stimulating discussion and for critically reading the manuscript.

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FIG. 3. Immunoblotting detection of NR subunit in spinach extracts. Crude extracts from cultured cells or leaves were subjected to SDS-PAGE followed by immunoblotting using antiserum raised against NADH-NR from spinach leaves. Lanes 2, 4, 6, 8, and 10 are 2, 4, 6, 8, and 10-d-old cells, respectively. Lane L is spinach leaves.

medium was due to an activation-inactivation mechanism. The amounts of NR protein that can be recognized by anti-NR IgG were determined in spinach cells after the transfer by the ELISA developed in this study. As shown in Figure 2, the level of NR protein changed in parallel to that of NR activity. Assuming that the inactive form of NR, if existing, can be recognized by anti-NR IgG, it can be concluded from Figure 2 that the alteration in NR activity was effected by the synthesis-degradation mechanism rather than by the activation-inactivation mechanism. This conclusion is consistent with observations that cycloheximide blocked the induction of NR activity in spinach cells (data not shown). The alteration of NR protein in spinach cells probably reflects the balance between its synthesis and degradation rather than mere synthesis or degradation. Zielke and Filner (20) have reported that tobacco cells synthesize NR even when NR activity is declining.

Immunoblotting Detection of NR Subunit in Spinach Extracts. At various intervals after the transfer to fresh medium, crude extracts were prepared from cultured spinach cells and subjected to SDS-PAGE. The polypeptide bands thus separated on the gel were transferred to nitrocellulose filters and the immunochromically reactive NR subunit was visualized as described under "Materials and Methods." As shown in Figure 3, a single immunologically labeled polypeptide was detected in crude extracts from 2-d-old cells (line 2). This band showed the same electrophoretic mobility as the subunit band of NR from spinach leaves (lane L) and its mol wt 110,000–120,000 agreed with that reported for the spinach NR subunit 114,000 (11), indicating that the immunoblotting technique could detect the NR subunit specifically. The intensity of immunochromical staining of the subunit band decreased gradually as the culture age was increased (lanes 4, 6, and 8) and became undetectable on the 9th and 10th d (lanes 9 and 10). This provided support for the conclusion that the decay of NR activity on aging is due to degradation of NR protein. No immunochromically reactive fragments of the NR subunit could be detected by this method during the decay of NR activity (Fig. 3). It seemed that breakdown products were