Plant Physiol. (1984) 75, 285–289
0032-0889/84/75/0285/05/$01.00/0

Characterization of Nitrate Reductase from Corn Leaves (*Zea mays* cv W64A × W182E)[1]

TWO MOLECULAR FORMS OF THE ENZYME

Received for publication August 8, 1983 and in revised form January 26, 1984

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ABSTRACT

The primary leaves from corn seedlings grown for 6 days were harvested, frozen with liquid N_2 and extracted in a Tris buffer (pH 8.5, 250 millimolar) containing 1 millimolar dithiothreitol, 10 millimolar cysteine, 1 millimolar EDTA, 20 micromolar flavin adenine dinucleotide and 10% (v/v) glycerol. Nitrate reductase (NR) in the crude extract was stable for several days at 0°C and for several months at −80°C. The enzyme was purified using $(NH_4)_2SO_4$ fractionation, brushite-hydroxyl-apatite chromatography and blue-sepharose affinity chromatography. The enzyme was eluted from the blue-sepharose column with a linear gradient of NADH (0–100 micromolar) or with 0.3 molar KNO_3. About 10% of the original activity was recovered with NADH (NADH-NR). It had a specific activity of about 60 to 70 units (micromoles NO_2^- per minute per milligram protein). A sequential elution with NADH followed by KNO_3 (0.3 molar) or KCl (0.3 molar) yielded 2 peaks. Rechromatography of each peak gave two peaks again. These results indicate that we are dealing with two forms of the same enzyme rather than two different NR proteins. The two NRs had different molecular weights as judged by chromatography on Toyopearl. The NADH-NR was more sensitive than the NO_3^--NR to antibody prepared against barley leaf NR. In Ouchterlony assays a single precipitin line, with completely fused boundaries, was observed.

Nitrate reductase (EC 1.6.6.1) in *Zea mays* catalyzes an NADH dependent reduction of nitrate to nitrite. Solomonson (26) successfully purified the enzyme from *Chlorella vulgaris* using blue dextran sepharose affinity chromatography (the chromophore of blue dextran being Cibacron Blue F3G-A) and this technique has also proven to be a simple and rapid technique for the isolation of NR[3] from higher plants (3, 8, 9, 10, 12, 16, 21, 24). However, attempts to purify NR to homogeneity have been relatively unsuccessful because of the instability of the enzyme and the relatively low levels of NR in higher plant tissues. Recently, Kuo *et al.* (13) have developed a buffer system which stabilizes the NR obtained from barley leaves. Using a similar buffer in conjunction with affinity chromatography they have been able to purify the barley leaf NR and to obtain an enzyme with a single subunit with a mol wt of about 110,000 D (12).

The main objective of the present research was to develop a procedure for the purification of corn leaf NR using BS affinity chromatography and to characterize the enzyme. We have been able to show that two molecular forms of NR (NADH-NR and NO_3^--NR) can be eluted from a BS column and that these two forms are interconvertible.

MATERIALS AND METHODS

Plant Material. Corn (*Zea mays* cv W64A × W182E), purchased from the Wisconsin Seed Foundation, Madison, WI, was planted in vermiculite and sand, and grown for 6 d. The plants were watered daily with a one-tenth Hoagland solution which contained 10 mM KNO_3. The growth chamber was maintained on 16 h days with a light intensity of about 150 $\mu E\ m^{-2}\ s^{-1}$. The day and night temperatures were 28 and 26°C, respectively.

Preparation of Crude Extract. Primary leaves from 6-d seedlings were frozen with liquid N_2, ground to a powder with a mortar and pestle, and then stored at −80°C. The powder was thawed for 10 min at 4°C and homogenized in a blender in 250 mM Tris-HCl buffer, pH 8.5, containing 10 mM cysteine, 1 mM EDTA, 20 μM FAD, 1 mM DTT, and 10% (v/v) glycerol (13). The homogenate was centrifuged at 10,000*g* for 30 min.

Activity Measurements. During enzyme purification, activity was measured as the rate of NO_2^- production at 28°C. The assay mixture contained, KNO_3 (10 mM), Hepes (0.065 M; pH 7.0), NADH (0.5 mM) in phosphate buffer (0.04 M; pH 7.2) and enzyme in a final volume of 1.5 ml. The reaction was started by adding the NADH. After 15 min it was terminated by adding 1 ml of 1 N HCl solution containing 1% sulfanilamide followed by 1 ml of 0.02% aqueous *N*-1-naphthylethylene-diamine-dihydrochloride (NED). The *A* was read at 540 nm after 10 min. Nitrite concentrations were calculated from a standard curve which contained NO_3^-, NADH, and phosphate in addition to NO_2^-. One unit of NR activity was defined as the amount of enzyme catalyzing the formation of one μmol of NO_2^- per min.

Electrophoresis. Analytical disc gel electrophoresis of NR was performed at 4°C using the Tris-glycine buffer system of Davis (6). Samples were applied to 7.5% acrylamide gels, and electrophoresis was carried out first at 1 mamp/gel for 20 min and then at 2 mamp/gel until the tracking dye front was close to the gel bottom (about 2 h). Proteins were located by staining with 0.25% Coomassie Brilliant Blue R-250 in 7% acetic acid for 1 h followed by diffusion-destaining with several changes of 7% acetic acid. NR was detected after electrophoresis either in the gel by the method of Solomonson *et al.* (27) or in sliced gel sections (2.5 mm) with the standard NADH-NR assay.

Determination of Stokes Radius. The Stokes radius was deter-

[1] Supported by the Natural Sciences and Engineering Research Council of Canada (Grant No. A2818). H. N. supported by the Natural Sciences and Engineering Research Council (Canada) visiting scientist program, and by a travel grant from the Ministry of Education of Japan.

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[3] Abbreviations: NR, nitrate reductase; BS, blue-sepharose; 2-ME, 2-mercaptoethanol; FAD, flavin adenine dinucleotide.

mined as described by Siegel and Monty (25). We used a Toyopearl WH-55 column (2.5 × 50 cm) equilibrated with K-phosphate (250 mM; pH 7.5), containing EDTA (1 mM), 2-ME (1 mM) either in the presence or absence of KCl (0.1 M). Two ml samples were layered on the top of the column and were eluted at a flow rate of 22 ml/h·cm². Elution volume (Ve), void volume (Vo), and total volume (Vt), were determined in the usual manner (25). Standards of known Stokes radius were: ovalbumin, 2.7 nm; BSA, 3.5 nm; yeast alcohol dehydrogenase, 4.6 nm; catalase, 5.2 nm; ferritin, 7.8 nm; fibrinogen, 10.7 nm.

Sensitivity of Maize NRs to Antibodies Prepared against Barley Leaf NR. The behavior of NADH- and NO_3^--NRs was studied using an immunoprecipitation procedure. Constant amounts of enzyme were incubated for 3 h on ice with increasing concentrations of antibody in a total volume of 0.5 ml. The preparation was then centrifuged in an Eppendorf Microfuge for 5 min and the activity remaining in the supernatant solution was assayed. The antibody was a gift of D. A. Somers, Tsung-Min Kuo, and R. L. Warner, Washington State University, Pullman WA.

Gel Immunodiffusion. NADH- and NO_3^--NRs (eluted from the BS column) were assayed in the presence of antibodies by immunodiffusion (5) in agar plates (1% agarose, in 50 mM Tris-citrate buffer, pH 8.5). After 48 h, the plates were stained for 15 min with 0.5% amido black in methanol:acetic acid:water (50:10:40)(v/v/v). The plates were subsequently destained with the methanol:acetic acid:water (50:10:40)(v/v/v).

Protein Determination. Protein concentrations were determined by the Bio-Rad dye-binding assay (standard assay procedure) for crude preparations, $(NH_4)_2SO_4$, and hydroxylapatite fractions. Protein obtained at later stages of purification, was determined by the Bio-Rad dye-binding assay (microassay procedure).

Chemicals and Supplies. FAD, NADH, catalase and alcohol dehydrogenase (yeast) were purchased from Sigma. Blue-sepharose CL-6B (BS), purchased from Pharmacia Fine Chemicals, was regenerated according to the method of Noland *et al.* (15). Toyopearl WH-55 was supplied by Toyo Soda Mfg. Co. Ltd., Tokyo, Japan. Hydroxylapatite and brushite were prepared by the methods of Levin (14) and Rice *et al.* (22). All other chemicals used were the purest available from commercial sources.

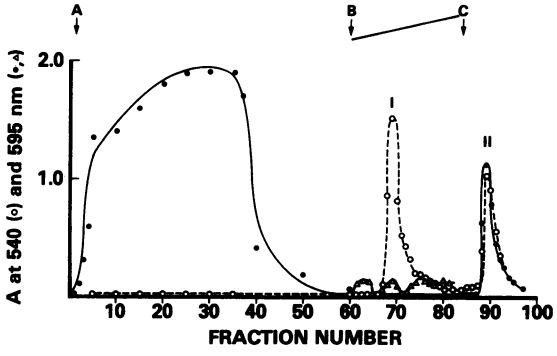

FIG. 1. BS affinity chromatography of corn NR. NR was absorbed on a BS column (2.5 × 6 cm) and washed overnight with 50 mM Tris-HCl buffer (pH 8.3) containing EDTA, 2-ME, FAD, and glycerol. Arrows indicate where the different elution buffers were applied: A, 50 mM Tris-HCl, 1 mM EDTA, 1 mM 2-ME, and 20 μM FAD (pH 8.3); B, a 0 to 100 μM NADH linear gradient in the original buffer (pH 8.3); C, 300 mM KNO_3 in the original buffer (pH 8.3). Flow rate was adjusted to about 50 ml/h·cm². NADH-NR activity was expressed as *A* at 540 mm (○) and the protein concentrations were measured at 595 nm by the standard (●) or microbiorad (△) assays.

RESULTS

The Effect of Age on the Stability of NR Activity. In the early experiments, leaves were harvested at daily intervals from 5 to 10 d after planting. The NR activity in the $(NH_4)_2SO_4$ fraction (27.5–50% saturation) was measured either immediately or after the preparation had been stored on ice for 24 h. Preparations, from leaves harvested 6 d after planting, had the highest initial activities and were the most stable. Hence, in subsequent extractions, primary leaves from 6-d-old plants were used. Crude extracts were also prepared with Hepes (100 mM, pH 7.4), K-phosphate (50 mM; pH 7.5 or 8.5), or Tris-HCl (250 mM; pH 8.5) buffers and the activity was measured either immediately or at various times after storage at 4°C. Tris-buffer yielded both the most active and the most stable preparations. It was, therefore, used in subsequent extractions.

Purification of Nitrate Reductase. Solid $(NH_4)_2SO_4$ was added to the crude extract to 27.5% saturation. After removing the precipitate by centrifugation, solid $(NH_4)_2SO_4$ was added again to give 50% saturation. The precipitate thus formed was collected by centrifugation and dissolved in a minimal amount of 10 mM K-phosphate (pH 7.5), containing 1 mM EDTA, 1 mM 2-ME, and 10% (v/v) glycerol. The solution was dialyzed for a total of 4 h against 2 L of the 10 mM K-phosphate which was changed 2 times. After centrifugation, the dialyzed supernatant solution was applied to a hydroxylapatite brushite (1:3 v/v) column (5 × 5 cm) equilibrated with 10 mM K-phosphate (pH 7.5) containing 1 mM EDTA, 1 mM 2-ME, and 10% glycerol. After washing the column with 750 ml of the same buffer, elution was performed with 100 mM K-phosphate (pH 7.5) containing 1 mM EDTA, 1 mM 2-ME, and 10% glycerol. The fractions having high NR activities were combined and applied to a BS column (2.5 × 6 cm) equilibrated with 10 mM K-phosphate (pH 7.5) containing 1 mM EDTA, 1 mM 2-ME, 20 μM FAD, and 10% (v/v) glycerol. The column was then washed overnight with 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA, 1 mM 2-ME, 20 μM FAD, and 10% glycerol. The flow rate was 50 ml/h·cm² and 7 ml fractions were collected. Adding 0.3 M Tris-HCl (pH 8.3) after this treatment failed to elute NR activity. When a linear concentration gradient of NADH (0–100 μM) in 50 mM Tris-HCl buffer (pH 8.3) containing the usual additives, was used, a major peak of NR activity was eluted with 10 μM NADH (Fig. 1). To prevent inactivation of this NR, fractions were collected into tubes which contained 0.1 ml of 1 M KNO_3. Subsequently, the BS column was washed with 50 mM Tris-HCl buffer (pH 8.3) plus the usual additives, and then with the same buffer containing 0.3 M KNO_3. A second NR peak (NO_3^--NR) was eluted with 0.3 M KNO_3 (Fig. 1). Adding 0.3 M KCl instead of KNO_3 also resulted in a peak of NR activity. However, adding 1 M KCl after KNO_3 resulted in no further elution of NR. The two or three fractions possessing high NR activities were combined and concentrated by means of an immersible-membrane obtained from Millipore.

Once the purification procedure was established, the specific activities recovered routinely had about 70 units of NADH-NR activity per mg protein. The two NR peaks were also obtained when the dialyzed crude enzyme or the $(NH_4)_2SO_4$ precipitated NR was transferred directly to the BS column. Redinbaugh and Campbell (20) and Oji *et al.* (18) have also reported two forms of NR obtained from root tissue after affinity chromatography. Usually, however, only one form of the enzyme has been reported (12, 16, 24).

Characterization of Nitrate Reductase Proteins. *(a) Polyacrylamide Gel Electrophoresis.* NADH-NR on 7.5% acrylamide gels at pH 8.3 yielded one major and two minor protein bands. The major protein band was also associated with methyl viologen-NR activity (Fig. 2). The NO_3^--NR had several protein bands and two bands associated with methyl viologen-NR activity. The R_m value for NADH-NR was 0.21 and for NO_3^--NR, 0.20, and

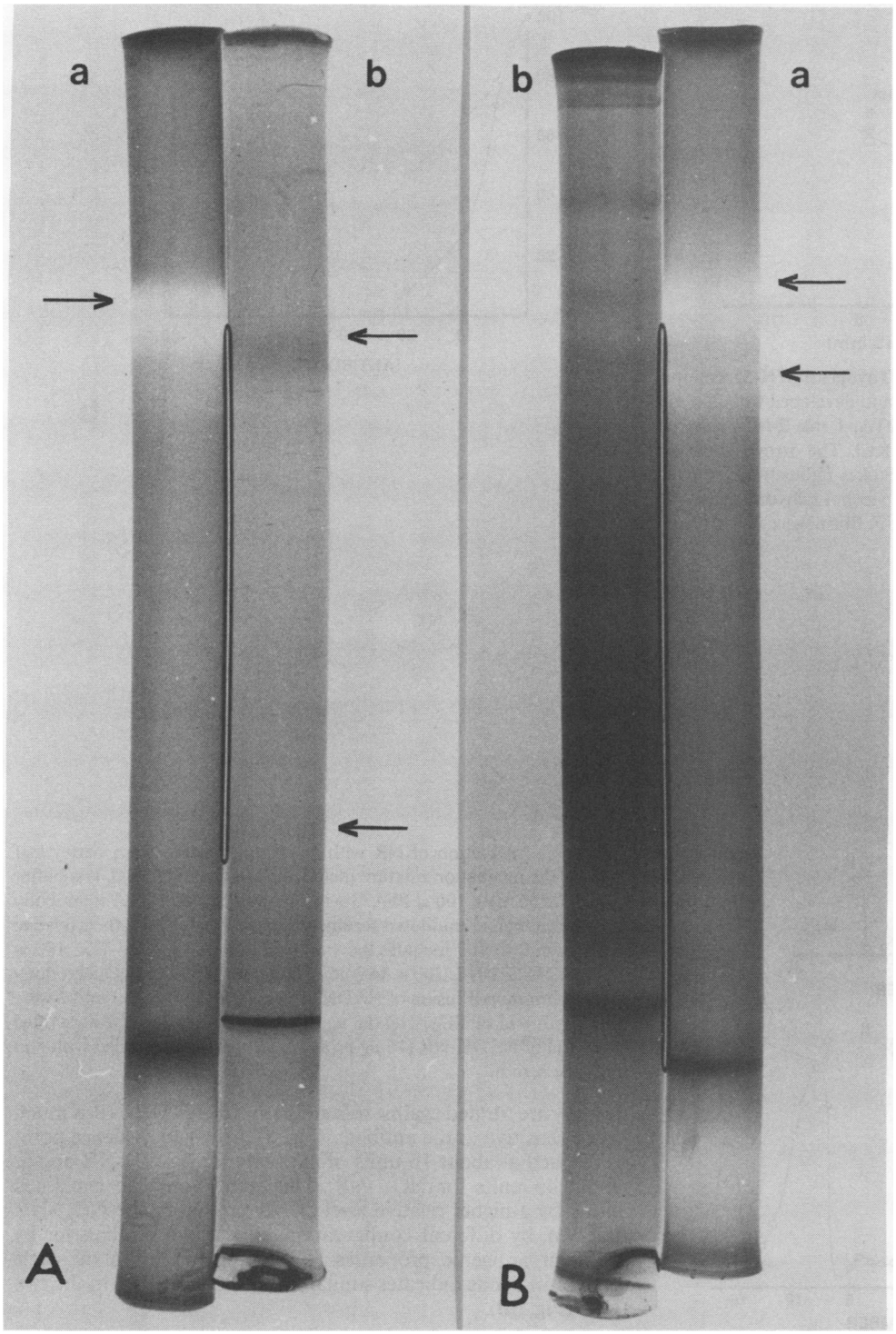

FIG. 2. Separation of NADH- (A) and NO_3^- (B) NRs on polyacrylamide gels. Activity bands determined with methyl viologen (a) and protein bands (b) of the affinity-column purified NRs are compared. The arrows indicate the position of the protein or NR activity bands. Difference in RF values are related to amounts of extract required to give protein or activity bands.

0.24.

(b) Toyopearl Chromatography. The NADH-NR and NO_3^--NR were eluted from a calibrated Toyopearl column at a position corresponding to a Stokes radius of 5.8 and 7.1 nm, respectively (Fig. 3) when calculated according to the method of Siegel and Monty (25); of 4.9 and 6.0 nm according to the protocol currently recommended by Pharmacia (Gel filtration calibration kit instruction manual for protein mol wt determinations by gel filtration). Thus, the apparent mol wt for the two forms of NR are different.

(c) Interconvertability of NADH-NR (NR-I) and NO_3^--NR (NR-II). A sequential elution from the BS column with NADH followed by KNO_3 yielded 2 peaks (Figs. 1 and 4), designated NR-I and NR-II, respectively (Fig. 4). When fractions from each peak were pooled, dialyzed, and rechromatographed on BS, we again recovered two peaks (Fig. 4, B and C). Thus, NR recovered from the primary leaf of corn exists in two forms which are interconvertible.

(d) Reaction with Antibody Prepared against Barley Leaf NR. Preliminary tests showed that corn NR in crude preparations was inactivated by antibody prepared against barley leaf NR and that the antibody-NR complex could be removed by centrifugation (A. Oaks, T.-M. Kuo and R. L. Warner, unpublished data). When equal amounts of NR protein as determined by NR

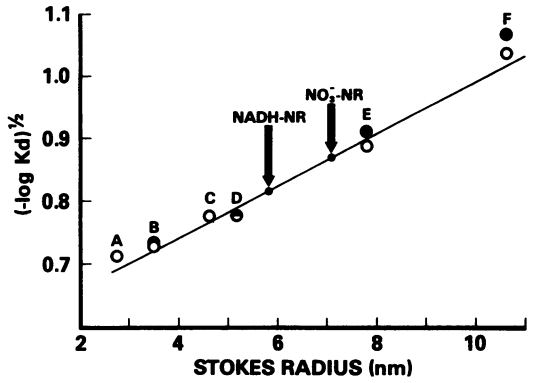

FIG. 3. Gel filtration of corn NR on a Toyopearl WH-55 column. The column (2.5 × 50 cm) was equilibrated and developed with 250 mM K-phosphate, pH 7.5, containing 1 mM EDTA, 1 mM 2-ME, either in the presence (○) or absence (●) of 0.1 M KCl. The arrows show the positions of the NRs. Standards of known Stokes radius were: A, ovalbumin, 2.75 nm; B, BSA, 3.5 nm; C, yeast alcohol dehydrogenase, 4.6 nm; D, catalase, 5.2 nm; E, ferritin, 7.8 nm; F, fibrinogen, 107 nm.

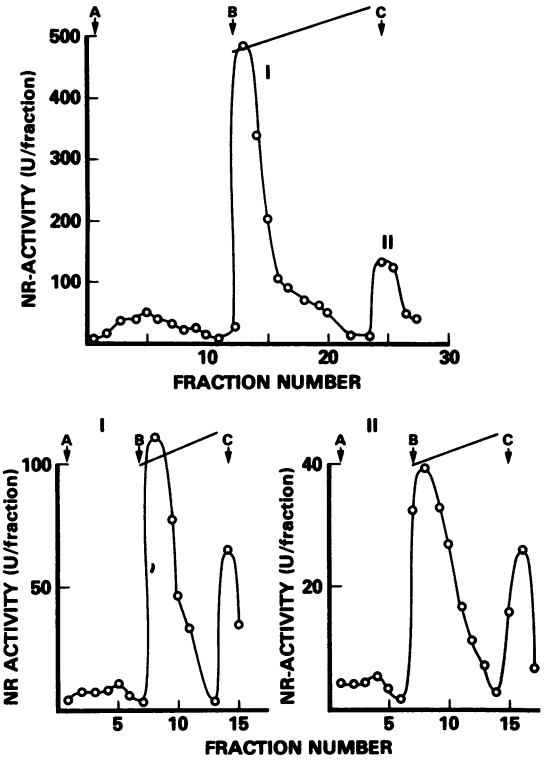

FIG. 4. Interconvertibility of NADH- and NO_3^--NR. NR obtained from the hydroxylapatite brushite column was applied to a BS column (0.7 × 3 cm) equilibrated with the 100 mM K-phosphate, pH 7.5 containing 1 mM EDTA, 1 mM 2-ME, and 10 μM FAD (buffer A). The BS was washed successively with 25 ml buffer A, with a linear NADH concentration gradient from 0 to 100 μM (20 ml) in buffer A and finally with 0.3 M KNO_3 in buffer A. Tubes containing each NR were pooled and concentrated to about 2 ml, were dialyzed for 3 h against buffer A, and the dialyzed solutions were reapplied to a BS column (0.7 × 2 cm) equilibrated with buffer A. Arrows indicated where the different elution buffers were applied: A, buffer A; B, 0 to 100 μM NADH in buffer A; C, 0.3 M KNO_3 in buffer A. NR activity was expressed as nmol NO_2^- produced/min·fraction.

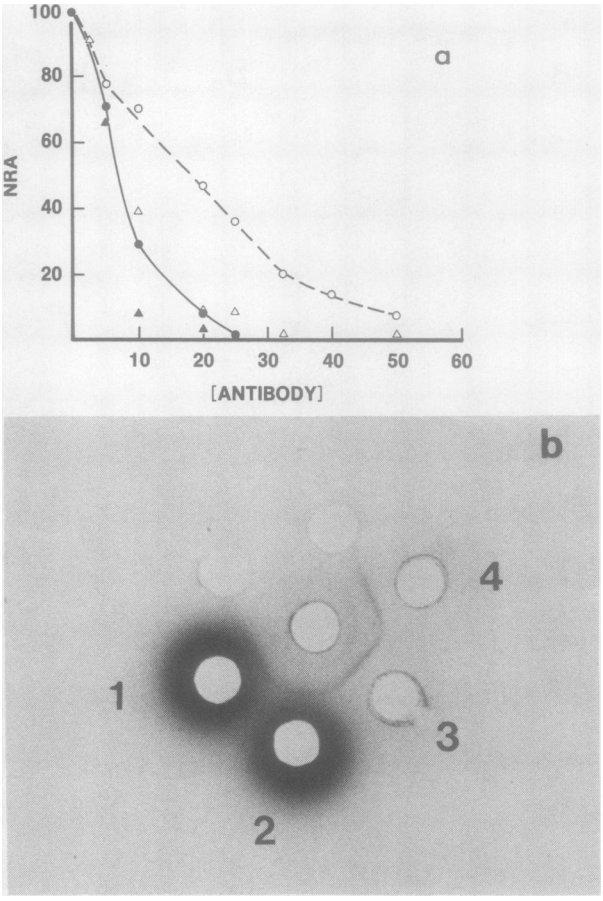

FIG. 5. Interaction of NR with antibody prepared from barley leaf NR: A, the incubation mixture included 50 μl enzyme, 50 μl Tris-buffer (pH 8.5, 250 mM), 100 μl BSA (3% in Tris-buffer), and 100 μl of antibody or water. Original undiluted serum was given a value of 100. A relative scale from 0 to 100 includes the various dilutions. ○, NO_3^--NR: 100 = 92.1; ●, NADH-NR: 100 = 44.3 μmol NO_2^- per reaction. B, Ouchterlony double-immunodiffusion of NADH- and NO_3^--NRs. Wells 1 and 2 were filled with 8 μl of NO_3^--NR (65 μg of protein); wells 3 and 4 were filled with 8 μl of NADH-NR (16 μg protein). Center well was filled with 8 μl of crude serum.

activity are titrated against this antibody, the NADH-NR is much more sensitive to the antibody (Fig. 5A). The equivalence point is reached at about 16 units of antibody for NADH-NR and at about 43 units for NO_3^--NR. This result could be explained either by a higher relative level of NR protein in the NO_3^--NR fraction, by different configurations of the two proteins, or by different antigenic properties. The complete fusion of single precipitin bands indicates similar antigenic properties in the two NRs (Fig. 5B).

DISCUSSION

The NR examined in several plant species has a relatively high turnover rate *in vivo* (1, 17, 23, 28). The enzyme from corn leaves, for example, has a half-life of 6 h (1, 23). This instability is also seen after extraction and has made purification of the enzyme difficult. Recently, buffers introduced by Kuo *et al.* (12, 13) have ameliorated this problem. The use of affinity chromatography with BS has also enhanced the ease with which NR can be purified (4, 20). Other affinity gels have also been used successfully in purifying NR from various sources (3, 7, 10, 12, 16, 18, 21, 24, 26).

There have been previous reports of NR being eluted from BS

by high salt (1.0 M KCl) or high NO_3^- (0.3 M KNO_3). Campbell and Smarrelli (4) found, for example, that NR from squash and corn could be eluted from BS specifically with NADH and either 0.3 M KNO_3 or some other salt. In our experiments, both the difference in the Stokes radius between the NADH- and NO_3^--NR and the difference in distance moved by the NR proteins in the acrylamide gels suggest that there are two different configurations of NR in corn leaf extracts. In *Chlorella vulgaris* there are oxidized and reduced forms of NR (26, 27) which appear to be related to the metabolic status of the cells (19). However, it is difficult to demonstrate reversible oxidized and reduced forms of NR prepared from corn leaves and, hence, we suspect that the two forms of NR that we see are not related to this phenomenon.

Recently Howard and Solomonson (11) have been able to stabilize *Chlorella* NR in various states of polymerization from a monomer to a tetramer. The degree of polymerization depended on the dilution of the protein. Since the two forms of NR obtained from corn leaves have different apparent mol wt, we could also be dealing with different levels of polymerization. For example, the approximate mol wt of the NADH-NR (using the Pharmacia method of quantitation) is about 160,000 D, of the NO_3^--NR is about 433,000 D. Results from barley (12) or *Chlorella* (11) suggest a monomer of about 100,000 D as the basic subunit of the NR protein. Our preliminary results also indicate a similar subunit size at least in the NO_3^--NR (M. Poulle and A. Oaks, unpublished data). Thus, the NADH-NR could be a polymer of two basic subunits and the NO_3^--NR a polymer of four basic subunits. This interpretation is supported both by the different mol wt and by the different sensitivities of the two forms of NR to antibody prepared against barley leaf NR (Fig. 5A). It remains to be seen whether the two forms of NR obtained from corn leaves reflect a particular physiological status of the cells.

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