Immunological Comparisons of Nitrate Reductases of Different Plant Species Using Monoclonal Antibodies

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ABSTRACT
Six monoclonal antibodies against different epitopes of maize leaf nitrate reductase were used to compare plant nitrate reductases in enzyme linked immunosorbent assay and enzyme activity inhibition tests. The number of cross-reacting antibodies was shown to vary with species according to phylogenetic classification, ranging from five (sugarcane) to one (dicotyledonous species). Cross-reactions were restricted to higher plant nitrate reductases.

Plant nitrate reductase (EC 1.6.6.1) has been shown to have a dimeric structure with two identical subunits (6, 11) and to contain a heme component, FAD1 and a molybdenum cofactor (4). NR has two partial activities in the presence of electron donors or acceptors which shunt the electron flow: NADH-Cyt c reductase and methyl viologen-NR (4).

Polyclonal antisera have been obtained against NR of spinach (Spinacia oleracea) (3), barley (Hordeum vulgare) (7), and squash (Cucurbita maxima) (14). Using a rabbit polyclonal antiserum prepared against squash NR in an Ouchterlony test, NRs of squash and spinach appeared to be similar, but somewhat different from corn and soybean NRs and very different from Chlorella vulgaris and Neurospora crassa NRs (14). Results obtained by rocket immunoelectrophoresis with a polyclonal antiserum raised against barley NR showed differences between NRs of monocotyledonous and dicotyledonous species (15).

Recently, MAbs against plant NR have been obtained (1, 12). MAbs prepared against spinach NR were shown to inhibit NR activity of different monocotyledonous and dicotyledonous species (12).

Rabbit polyclonal antiserum and mouse MAbs have been obtained against maize leaf NR. The properties of these MAbs have been previously described (1). The aim of this work was to study the reactivity pattern of the MAbs, prepared against maize NR, with NRs of different plant species.

MATERIALS AND METHODS
Plant Materials and Culture Conditions. Seedlings of maize (Zea mays L. cv INRA 508), soybean (Glycine max L. cv L 28), tobacco (Nicotiana tabacum L. cv Xanthi), Nicotiana plumbaginifolia L. cv Viviani, barley (Hordeum vulgare L. cv Sonia), and pearl millet (Pennisetum americanum L.), and shoots of sugarcane (Saccharum officinarum L.) were grown in the greenhouse, watered with a nutrient solution as previously described (2), and harvested as young green plantlets 2 or 3 weeks after sowing. Sunflower (Helianthus annuus L. cv HA 89) was available as adult plants grown as the other species. Asparagus (Asparagus officinalis L.) was grown in the field and harvested as young green shoots. Chlorella pyrenoides was grown according to Lefebvre-Drouet and Calvet (9) and Neurospora crassa as previously described (10).

Enzyme Preparation. Leaves (or shoots for asparagus) were frozen and ground in liquid N2 and then placed into extraction buffer (15 g of leaves for 100 ml of buffer), containing 50 mM Hepes (pH 8.2), 0.1 mM NaCl, 1 mM Na2EDTA, 1 mM sodium molybdate, 5 mM FAD, 1 mM leupeptin, 5 mM cysteine, and 3 g Polyclar AT (water-insoluble PVP), according to T. Moureaux (personal communication). After centrifugation at 16,000 g for 45 min, the supernatant was precipitated with (NH4)2SO4 (45% of saturation) for 2 h at 4 to 6°C and centrifuged for 45 min at 16,000 g. The pellet was dissolved in a 0.05 M Tris-HCl buffer (pH 7.5) containing 1 mM Na2EDTA, 1 mM sodium molybdate, 1 mM leupeptin, 5 mM FAD, and 5 mM cysteine and desalted on a GF55 column (Pharmacia). The resulting final volume of the extract was 1 ml of Tris-HCl buffer for 15 g of leaves. C. pyrenoides cells were sedimented, resuspended in distilled H2O, and broken with the microtip of a Branson B15 sonicator (30% of maximal output). N. crassa nitrate reductase was extracted as previously described (10).

Rabbit Polyclonal Antiserum. The rabbit polyclonal antiserum was prepared against maize leaf NR, purified by affinity chromatography (2a).

Monoclonal Antibodies. The MAbs were used as ascite fluid (after (NH4)2SO4 precipitation) prepared as previously described (1).

The concentrations of NR specific immunoglobulins in the different ascites were 0.2 mg/ml for 25(15), 0.3 for 96(925), 0.3 for 28(2), 0.3 for 8(23), 0.7 for 15(21), and 0.9 for 42(22). We used also, as control ascite, a MAb against a glycosylated protein of a coronavirus TGE (8). This control antibody did not react with NR.

NR Assay and Inhibition of NR Activity. The NR activity determinations were made according to Robin (13), except soybean NR activities which were measured according to Jolly et al. (5). For inhibition experiments 0.2 ml of plant extract were incubated with 0.1 ml asicte fluid (dilution 1/50, 1/150, 1/450, etc.), during 20 min in 0.5 ml K-phosphat, 0.1 mM, pH 7.5, before the NR assay. The most active extracts (maize, barley) were diluted in order to have the same activities as the less active ones (asparagus, sunflower) to compare NR inhibition of the different plant species.
Two Sites ELISA Test. Ascite fluids (dilution 1/200 in 0.1 M sodium carbonate buffer, pH 9.6) were distributed into the wells of ELISA plates (Linbro ISFB 96) (100 μl per well) and incubated 90 min at 37°C. The plates were then washed 3 times with ELISA buffer (0.01 M K-phosphate (pH 7.5), 0.15 M NaCl, 0.05% (v/v) Tween 20, and 0.02% (v/v) NaN₃) and carefully emptied. The plant concentrated extract (100 μl) was added at different concentrations after dilution in the ELISA buffer with 10% (v/v) sheep serum, then incubated 90 min at 37°C and washed as above. The third step was the addition of 100 μl of the rabbit anti-NR polyclonal antiserum (dilution 1/200 in ELISA buffer with sheep serum). After incubation 90 min at 37°C and washing, an anti-rabbit IgG alkaline phosphatase conjugate (Sigma) was added (dilution 1/400 in ELISA buffer with sheep serum). After incubation 90 min at 37°C and washing, the phosphatase assay was performed (with PNPP 2.7 mM in 0.1 M diethanolamine buffer, pH 10.2, 1 mM MgCl₂). The OD at 405 nm was measured 15 min later using a Dynatech ELISA autoreader.

RESULTS

NR Activity. Extracts of the different species had variable NR activities and the exact quantity of NR in each extract is unknown, so the affinities of a MAb for NR of different species could only be qualitatively compared. The NADH-NR activity of the extracts was about 100 to 120 nmol nitrite/min·g of fresh weight for maize, pearl millet, barley, and sugarcane; 50 for tobacco and N. plumbaginifolia; 20 for soybean; and 10 for sunflower and asparagus.

Immunological Comparison of NRs. We verified that the six classes of MAbs gave a positive response with maize NR and that the control MAb TGE gave a negative response in a two sites ELISA test (Fig. 1). We also verified that a maize extract reacted only very slightly with the six antibodies after passage of the extract on a blue Sepharose column (due to some escape from the affinity column) (result not shown); this type of column retained NR and allowed a purification of NR after elution with NADH (6).

The two sites ELISA technique (Fig. 1) showed that the 96(9)25, 28(2), 25(15), and 15(21) MAbs cross-reacted with barley NR, that the 96(9)25, 28(2), and 25(15) MAbs cross-reacted with pearl millet NR, and that only the 96(9)25 and the 28(2) MAbs gave a positive response with asparagus NR. Three MAbs, 96(9)25, 28(2), and 25(15), cross-reacted strongly with sugarcane, but the 15(21) and 8(23) antibodies gave a low yet reproducible response.

Only the 96(9)25 antibody could recognize NR of all the dicotyledonous species (Fig. 1; result shown only for tobacco).

NR Activity Inhibition. The MAbs 96(9)25 and 28(2) inhibited NR activity of all monocotyledons. The 96(9)25 antibody inhibited also dicotyledon NR activity, but not N. crassa and C. pyrenoides NR activity (Table I). The inhibition level depended on plant species. The MAb 96(9)25 inhibited very similarly NR activity of all monocotyledonous species and of two dicotyledonous species (tobacco and N. plumbaginifolia), but that of sunflower and particularly that of soybean were inhibited to a lesser extent. Soybean extracts exhibited two NR activities, NADH- and NADPH-NR (5). The NADPH-NR activity of soybean was inhibited at the same level than the NADH-NR activity (result not shown). The MAb 28(2) does not inhibit NR activity of dicotyledonous species and, among the monocotyledons, asparagus NR was inhibited less than NRs of other species.

DISCUSSION

Using MAbs raised against maize NR and belonging to six epitopic classes, we observed similarities between NRs of different plant species. The two sites ELISA test showed that NR of

Fig. 1. Two sites ELISA test of NR of maize, barley, tobacco, asparagus, sugarcane, and pearl millet, using ascite fluids of MAbs 96(9)25, 28(2), 15(21), 25(15), 8(23), 42(22) (prepared against leaf maize NR and belonging to six epitopic classes) and TGE (control MAb), and a rabbit polyclonal antiserum also prepared against leaf maize NR. Results obtained with soybean, sunflower, and N. plumbaginifolia are similar to those described for tobacco and are not presented. Only the first number of the MAbs is mentioned in the figure. Note the scale change for ordinate plate (O.D., e.g. reduction of PNPP) of sugarcane and pearl millet.
Table 1. Inhibition of NADH-NR Activity by MAbs 96(9)25 and 28(2)

Concentrations of antibodies necessary to inhibit 50% of NR activity of different plant species have been compared to the concentration of antibody inhibiting 50% of NR activity of maize. MAbs 96(9)25 and 28(2) inhibited maize NR activity at about the same concentration.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Relative Concentration of Antibody for 50% Inhibition of NR Activity</th>
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<tbody>
<tr>
<td>Maize</td>
<td>1.0*</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>1.3</td>
</tr>
<tr>
<td>Pearl millet</td>
<td>1.8</td>
</tr>
<tr>
<td>Asparagus</td>
<td>1.4</td>
</tr>
<tr>
<td>Barley</td>
<td>1.3</td>
</tr>
<tr>
<td>N. tabacum</td>
<td>1.8</td>
</tr>
<tr>
<td>N. plumbaginifolia</td>
<td>1.8</td>
</tr>
<tr>
<td>Sunflower</td>
<td>5.4</td>
</tr>
<tr>
<td>Soybean</td>
<td>18.2</td>
</tr>
<tr>
<td>C. pyrenoides</td>
<td>NI</td>
</tr>
<tr>
<td>N. crassa</td>
<td>NI</td>
</tr>
</tbody>
</table>

* Value 1.0 = 0.1 ml ascite fluid 1/9000 incubated with 0.2 ml of plant extract producing 1 nmol nitrite per min in our experiment conditions.

No inhibition even with ascite fluid dilution 1/10.

Monocotyledonous plants presented more common epitopes with maize NR than dicotyledon NRs. The absence of cross-reactivity with C. pyrenoides or N. crassa NR confirmed results obtained with squash polyclonal antibody (14). The immunological data obtained are in agreement with the botanical classification based mostly on morphological criteria. Nevertheless, pearl millet NR was expected to have more common epitopes with maize NR than barley NR according to this classification.

Generally, antigenic sites involved in enzyme activity (epitopes of antibodies 28(2) and 96(9)25) are more conserved than other sites. The conservation of active sites has also been observed with an antisera against barley NR (15) and with MAbs against spinach NR (12). The epitope of antibody 28(2), which is correlated with methyl viologen-NR activity (1), is similar to the monocotyledon NRs, but different in dicotyledon NRs. On the contrary, the 96(9)25 epitope is very interesting, because of its central position in NR. Indeed, antibody binding to that site inhibits altogether the two NR partial activities (1), usually thought to be performed by separate domains of the molecule. This epitope is the most conserved one, being present in monocotyledonous and dicotyledonous species, but absent in gymnosperms (data not shown), C. pyrenoides and N. crassa.

We observed differences, however, in the NR activity inhibition among the different species tested. The recognition site of the 96(9)25 antibody is probably modified in soybean NR compared to maize NR, in agreement with results obtained with polyclonal antiserum against barley NR (15). Our observations show that, among the two soybean NRs, at least the NAD(P)H-NR is recognized. Similarly the 28(2) epitope in asparagus is also probably slightly different from maize NR site.

The three MAbs, 8(23), 42(22), and 15(21), which react with denatured enzyme in western blotting (1), have a more restricted cross-reactivity with other plant species NRs.

For the present time, NR immunological comparison had been carried out only with polyclonal antibodies (7, 14) and with MAbs inhibiting NR activity (12). MAbs have the ability to react with restricted sites of the molecule, each of which could be compared separately. Additional MAbs will, however, allow a more detailed immunological classification of plant NRs.

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LITERATURE CITED

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