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MONOCLONAL ANTIBODIES IDENTIFY MULTIPLE EPITOPES ON MAIZE LEAF NITRATE REDUCTASE

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SUMMARY Nine hybridoma cell lines secreting antibodies against the maize leaf nitrate reductase have been distinguished by reciprocal competition for binding to the antigenic site. Inhibition of enzymatic activities, and western blots of native enzyme and denatured subunits revealed different behaviors of individual antibodies towards the antigen. Two classes of monoclonal antibodies are inhibitory of NADH and methyl viologen nitrate reductase activities, but only one affects also NADH cytochrome c reductase activity. The associated epitopes are sensitive to antigen conformation. Among the 4 other classes, one is specific for the native conformation of the molecule, another binds more strongly to the denatured antigen, and two recognize equally well the two forms.

Higher plant nitrate reductase, which catalyses the first step of nitrate assimilation, has been the subject of many investigations, largely because of its key role in nitrogen metabolism. Purified enzyme contains a heme component, FAD, and a molybdenum cofactor (1). The classical model proposes a dimeric structure with two basic subunits of about 100 kD (2,3), and one molybdenum cofactor (MoCo). The electron flow is thought to be :

$$NADH \longrightarrow FAD \longrightarrow Heme\ (cyt.b\ 557) \longrightarrow MoCo \longrightarrow NO_3^-$$

This chain can be partly shunted by introduction of artificial electron donors or acceptors. Cytochrome c can be reduced in the presence of NADH and the FAD component of nitrate reductase. Methyl viologen can transfer electrons to the heme and to the molybdenum cofactor to reduce nitrate.

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid, Na salt; SDS, sodium dodecyl sulfate; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline, pH 7.2; PBS-T, PBS containing 0.05% Tween 20; PAGE, polyacrylamide gel electrophoresis.

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The first interest of hybridoma technology in the case of nitrate reductase lies in the difficulty to obtain strictly specific polyclonal antibodies. This is largely due to contaminations remaining in purified extracts of this enzyme which is present in very small amounts in crude extracts of higher plants. Monoclonal antibodies would also have specific applications in the study of the different domains of the molecule, the analysis of their three dimensional arrangement and search for the contribution of each one to the maintenance of enzymatic activity. Furthermore they would allow the detection and isolation of different forms of nitrate reductase in the plant, potential precursors, degradation products, and altered enzyme present in some nitrate-reductase-deficient mutants. We report here the production and characterization of monoclonal antibodies against the maize leaf enzyme.

MATERIALS AND METHODS

- **Nitrate reductase extraction and purification:** Maize leaves (cv INRA 508) of 8 days old plantlets were frozen in liquid nitrogen and ground as in (4). The crude extract was centrifuged 1h at 30,000 x g and nitrate reductase was partially purified on a Blue Sepharose CL-6B column, as described by Commère et al (submitted for publication). The nitrate reductase activity peak fractions were pooled, then concentrated by ultrafiltration. The concentrated eluate (called hereafter NRC) was an enriched fraction in which nitrate reductase represented about 20% of the total protein content, with a specific activity of 8 units/mg protein, determined with NADH as electron donor. One unit of enzymatic activity was defined by the production of 1 µmole NO_2^- per minute.

- **Immunization procedure:** Nine Balb/c mice were injected with NRC. The highest number and proportion of specific clones was achieved with a mouse receiving intraperitoneally the equivalent of 1 µg of nitrate reductase, mixed with Freund's complete adjuvant, and boosted twice 17 and 129 days later with 7 and 3 µg of enzyme, without adjuvant. The spleen was removed for fusion 3 days after the last injection.

- **Fusion:** Splenocytes were fused with SP2/0-Ag14 cells according to the general procedure of Köhler and Milstein (5), as modified by Nowinsky et al (6). Selection of hybrids was performed in hypoxanthine - azaserine medium as described by Zagury et al (7), complemented with 3 weeks old mouse thymocytes.

- **Screening:** Ten to fifteen days after fusion, cell cultures were selected by an ELISA assay of the supernatants, on plates coated with NRC diluted to 0.8 µg/ml in sodium carbonate buffer, 0.1M pH 9.6. The specificity for nitrate reductase was established after "Western blotting" with the native enzyme (see below).

- **Electrophoresis:** Electrophoresis under non-denaturing conditions was performed according to Davis (8),using 7.5% polyacrylamide gels, with a

4% stacking gel. Nitrate reductase was located in the gel by its enzymatic activity in 2 mm slices. Denaturing electrophoresis was carried out in 10% separating gels, 5% stacking gels, after heating to 100°C during 2.5 min in the presence of 2-mercaptoethanol and SDS (9).

- **Western blot:** After electrophoresis of NRC, the proteins were electrophoretically transferred onto a nitrocellulose sheet (Schleicher and Schuell BA 85) (10). The sheet was then soaked overnight in blocking solution (1% BSA in PBS) after which it was cut into strips for incubation with supernatants. Immune complexes identification was done using anti-mouse immunoglobulin-peroxidase conjugates (Institut Pasteur Production) (11). A supernatant of a hybridoma cell line secreting an antibody against a glycosylated envelop protein of a coronavirus was used as negative control.

- **Inhibition of enzymatic activity:** Ten µl of ascitic fluid were incubated with 1.5×10^{-3} units of NADH nitrate reductase activity, in 0.5 ml of potassium phosphate buffer 0.1M pH 7.5, 1 mM EDTA (buffer A), during 10 min. The enzymatic assay was started by addition of the substrates. NADH (12) and methyl viologen (13) nitrate reductase activities were measured by NO_2^- accumulation. Cytochrome c reductase activity was assayed as described by Wray and Filner (12). The anti-coronavirus ascite fluid was used as negative control.

- **Antibody labeling:** Immunoglobulins from ascitic fluids were purified by gel filtration on sephacryl S-300, in tris/HCl buffer 50 mM pH 8, 0.5 M NaCl. The anti-nitrate reductase immunoglobulin fractions were concentrated and equilibrated in borate buffer 0.2 M pH 8.9. Tritiation was carried out using the method of Tack (14), as follows: 0.1 ml of solution containing 0.5 to 1 mg of purified immunoglobulin was allowed to react with 5 µl of 0.4M formaldehyde and 5mCi of tritiated potassium borohydride during 15 min, after which unreacted borohydride was eliminated by centrifugation desalting (15).

- **Competitive radioimmunoassay:** The microtitration plates were coated with 50 µl of NRC diluted in buffer A to 1.6 µg of nitrate reductase equivalent per ml. After 3 washes in PBS-T, 50 µl of a mixture of labeled (0.16 ug) and unlabeled antibodies in 1% BSA/PBS-T were added. After 3 hours at 37°C, the plates were washed 4 times with PBS-T. Bound radioactivity was eluted from each well with 2% SDS and measured by scintillation counting.

RESULTS AND DISCUSSION

Two fusions resulted in positive clones. From the 120 ELISA positive populations 36 secreted anti-nitrate reductase antibodies. Nine of them were selected and subcloned; all except 28(2) came from the same fusion.

- **Western blot analysis:** Revelation of antigen-antibody complexes after blotting of electrophorised non-denatured NRC resulted in one major band, which was associated with the main peak of nitrate reductase activity (fig.1). The antibodies which reacted with a high intensity (8(23), 28(2), 30(6), 30(17), 42(22) and 96(9)25) could also detect a slower migrating band, which was coincident to the smaller peak of nitrate reductase activity. A faint band of higher migration was observed with antibody 30(17). Since

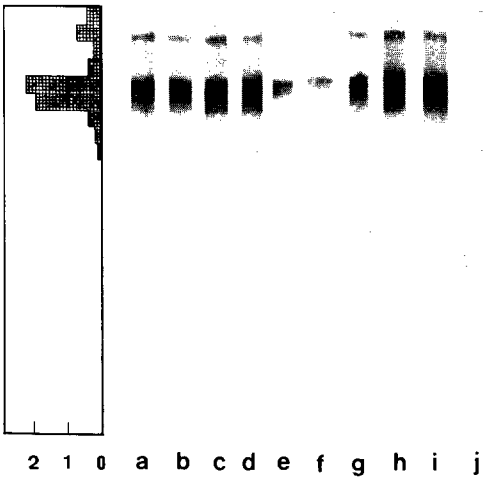

Fig. 1. **PAGE of NRC in non-denaturing conditions.**
Left: NADH-nitrate reductase activity in 2-mm gel slices (OD 540 nm x 10, 45 min reaction). Right: Western blots with antibodies a: 28(2), b: 7(10)12, c: 8(23), d: 30(17), e: 15(21), f: 25(15), g: 30(6), h: 42(22), i: 96(9)25, j: control.

this band is also visible on the control strip, it is probably a non specific reaction.

The same cell culture supernatants were also tested for their ability to recognize the nitrate reductase subunits, after SDS-PAGE and western blot. A single band of 105 kD was detected by antibodies 7(10)12, 8(23), 15(21) and 42(22) (fig.2). Antibody 30(17) gave an additional band of 63 kD. This band,

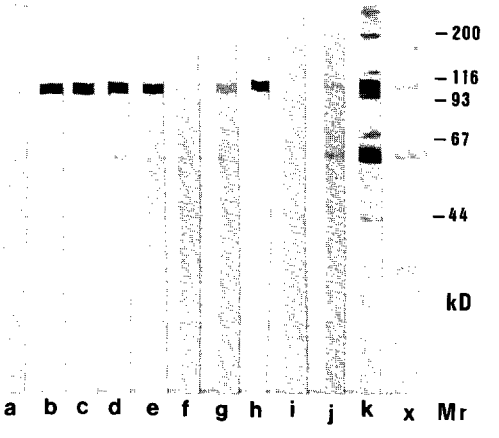

Fig. 2. **SDS-PAGE of NRC after protein denaturation.**
a to j: see fig.1, k: western blot with rabbit anti-nitrate reductase polyclonal serum, x: amido-schwartz staining of transferred proteins

which was also detectable on the control, although with a weaker intensity, is likely due to a non specific binding. Antigen denaturation greatly enhanced the affinity of 15(21), which bound poorly to nitrate reductase under its "native" form. Antibodies 30(6) and 28(2) gave a weak or very weak response restricted to the 105 kD band, which only appeared after a long time of revelation, leading to the colored background. No band at all appeared with 96(9)25 and 25(15).

- **Inhibition of enzymatic activity:** Antibodies 28(2), 30(6) and 96(9)25 were found to be inhibitory of NADH and methyl viologen nitrate reductase activities, confirming that they very likely recognize conformation-dependant epitopes. For these 3 clones 10 µl of a 1/1000 dilution of ascitic fluid is sufficient to completely inhibit the nitrate reductase activities under our test conditions.

NADH cytochrome c reductase activity was inhibited only by 30(6) and 96(9)25. Clone 28(2) supernatant had no effect. This suggests that 28(2) recognizes a different epitope than 30(6) and 96(9)25, and that this epitope is apparently linked to a site involved in the last steps of the electron transfer. The other antibodies neither inhibited nor stimulated any of the enzymatic activities.

- **Epitope mapping:** Two monoclonal antibodies are supposed to recognize the same epitopic site when there is a reciprocal competition between them for binding to the antigen. This condition was fulfilled with two groups of antibodies: 30(6) / 96(9)25 and 7(10)12 / 8(23) (fig.3). Immunoglobulins 96(9)25 and 30(6) could not bind in the presence of 28(2), but 28(2) could bind while in the presence of 96(9)25 or 30(6) and therefore forms a third group. In the same manner, antibody 25(15) prevented the binding of all the other monoclonals except 42(22), while no one was able to inhibit its own binding. These non reciprocal reactions can be explained by an indirect effect of antibody on antigen conformation rather than by epitope overlap. Monoclonals 15(21) and 42(22) were not related to any of the other antibodies, neither inhibiting nor being inhibited by one of them. Thus six

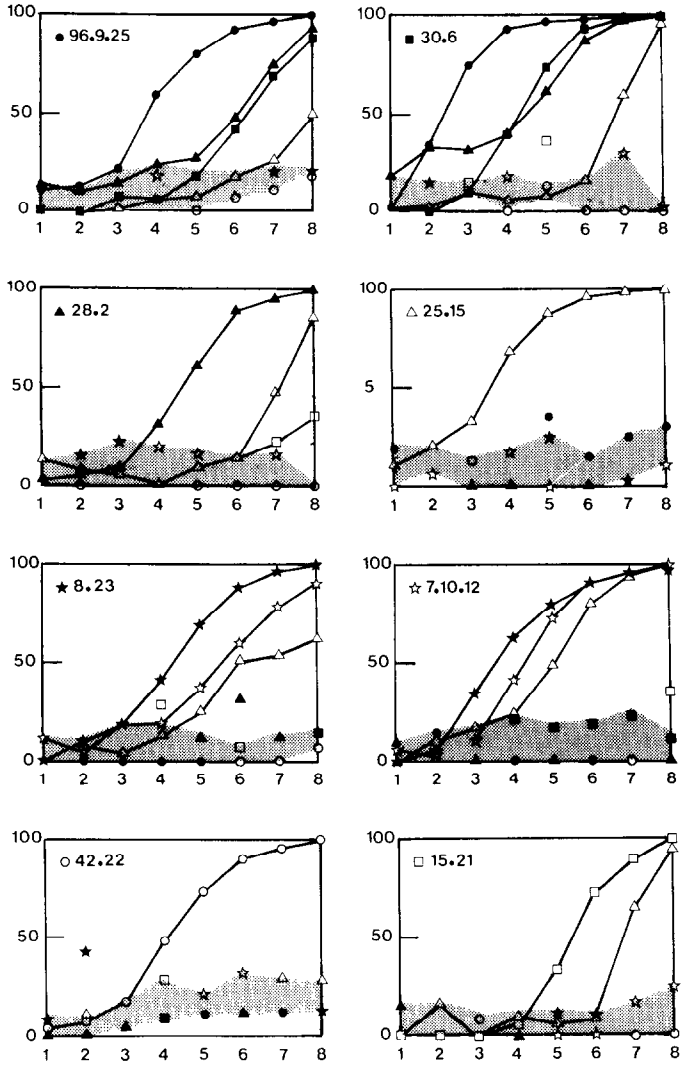

Fig. 3. **Competitive binding assays between monoclonal antibodies.** The radiolabeled antibody (indicated inside the figure near its symbol) was mixed with the indicated amounts of various unlabeled antibodies: 1: 0.6ng, 2: 3ng, 3: 16ng, 4: 80ng, 5: 400ng, 6: 2µg, 7: 10µg, 8: 50µg. The results were expressed as percentage of competition: P=100(1-(X-B)/(T-B)), where X= cpm bound, B= background and T= cpm bound in the absence of competitor antibody. ● : 96(9)25, ■ : 30(6), ▲ : 28(2), △ : 25(15), ★ : 8(23), ☆ : 7(10)12, ○ : 42(22), □ : 15(21). The grey area includes all the curves obtained with the non competing antibodies.

different classes have been defined : 1: 30(6) + 96(9)25, 2: 28(2), 3: 25(15), 4: 7(10)12 + 8(23), 5: 42(22), 6: 15(21).

As shown in table 1, this classification fits with the binding properties of the antibodies. Three classes (1, 2 and 3), two of which are inhibitory (1 and 2), poorly or do not at all recognize the antigen after its

Table 1. Summary of the properties of the different monoclonal antibodies

EPITOPIC GROUP	1	2	3	4	5	6
CLONES	30(6) 96(9)25	28(2)	25(15)	8(23) 7(10)12	42(22)	15(21)
WESTERN BLOTTING						
NATIVE FORM	+++	+++	+	+++	+++	+
DENATURED	+/-,-	-	-	+++	+++	+++
ENZYMATIC ACTIVITY INHIBITION						
NADH / NO_3^-	+	+	-	-	-	-
M.V. / NO_3^-	+	+	-	-	-	-
NADH cyt.c	+	-	-	-	-	-

complete denaturation. Antibodies of class 4, 5 and 6, which react with the subunits after protein denaturation, might correspond to sequential epitopes.

Immunoglobulins of class 1 have been found to be the only ones that cross react with nitrate reductase extracted from fairly distant plant species (to be published). The 96(9)25 monoclonal antibody seems to be a universal reagent able to bind with high affinity to most higher plant nitrate reductases. This panel of antibodies could therefore be used as a tool for the characterization and specific detection of the nitrate reductase molecule. We are now investigating different aspects of regulation, molecular architecture and enzymatic mechanisms of nitrate reductase under different physiological and genetic conditions.

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