Screening of monoclonal antibodies to scarce and labile enzymes: A functional immunoassay for isolating monoclonal antibodies to NADPH:nitrate reductase

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A functional immunoassay, that has proved very useful, is described for screening and identifying monoclonal antibodies (McAbs) against scarce and labile enzymes. This method does not require purified enzyme or antigen and it has been successfully applied to isolate three hybridomas secreting McAbs to NADPH:nitrate reductase from the chloronema cells of the moss *Funaria hygrometrica*. Briefly, the protocol involves: adsorption of murine antibodies from hybridoma supernatants by rabbit antimouse IgG antibody pre-adsorbed to *Staphylococcus aureus* cells (SAC), reaction with crude extract for 15 min, sedimentation of the SAC complex by centrifugation and measurement of residual enzymatic activity in the supernatant. A depletion indicates the presence of antibodies that bind to the active enzyme. The method is rapid, sensitive and versatile enough to be used to isolate McAbs with exquisite specificities. The three isolated McAbs recognized nitrate reductase protein in a conformation-independent and/or a conformation-dependent manner.

Introduction

Monoclonal antibodies (McAbs) are invaluable for studying the enzymes or antigens which occur in low amounts or are also labile and difficult to purify. In plants, the enzyme nitrate reductase (NR) catalyzing the reduction of nitrate into nitrite is one such protein. In the past few years, polyclonal as well as monoclonal antibodies to a few NRs have been isolated. The NADPH:NR is unique to fungi and a few bryophytes (Rudolph 1990). Among higher plants, corn roots have also been reported to contain a NADPH:NR besides the NADH:NR and both are nitrate inducible (Li and Oaks 1993). As so little is known about NADPH:NR in plants, we have been studying its properties and regulation using the chloronema cell cultures of the moss *Funaria hygrometrica* Hedw.

For our studies McAbs to *Funaria* NR were needed. The McAbs against other plant NRs have been produced using either the purified enzyme (Notton *et al* 1985; Hyde *et al* 1989) or the extensively enriched preparations (Cherel *et al* 1985) in the screening protocols. These methods could not be applied to *Funaria* NR as it was difficult to obtain sufficient amounts of purified, or even extensively enriched NR. In most organisms NR is labile and present in low amounts estimated to be about 0·005 to 0·03% of the total soluble protein (Campbell 1989).

In the absence of pure enzyme or antigen, McAbs can be identified on the basis of some unique characteristic of the antigen such as its intracellular localization, biochemical property or the size of its subunit (Harlow and Lane 1988). A screening method based on dot-immunobinding assays combined with a chemiluminescence detection system has also been described for semi-purified antigen (Bakkali *et al* 1994). With impure preparations of soluble enzymes, functional screens such as assay for the depletion of antigenic activity from supernatant (ADAAS) (Crawford *et al* 1982; Nakane and Deguchi

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1982; Krishnaswamy and Bryan 1986; Lenman *et al* 1990) or assay of immunoadsorbed enzyme (Frackelton and Rotman 1980; Jemmerson and Fishman 1982; Mierendorf and Dimond 1983; Lenman *et al* 1990) have been used to identify McAbs. These assays require prolonged incubation periods lasting a few hours and are unsuitable for labile antigens or enzymes. A rapid method based on the enzymatic activity right at the unfractionated cell-free, crude extract stage would be ideal to screen supernatants. We wish to describe here a modified functional immunoassay based on the depletion of NR activity from the crude extracts and it was successfully employed to isolate three hybridomas against the NADPH:NR.

2. Materials and methods

2. *Culture of chloronema cells*

The chloronemal cells of the moss *Funaria hygrometrica* Hedw. were grown in axenic, liquid cultures in minimal medium with 1% glucose (Johri 1974). The cell line was maintained routinely in low-calcium medium buffered at pH 7·0 with 10 mM Hepes. The NR enzyme was induced by inoculating stationary phase cells into 250 ml of the above medium at an inoculum density of 0·1 mg/ml. Four or five days after inoculation, the cells were harvested by filtration at a cell density of 0·9 ± 0·2 mg/ml.

2.2 *Preparation of crude extract*

The harvested cells were washed in sterile water, frozen in liquid nitrogen and ground to a fine powder. For every gram of cell powder, 1 ml of double-strength buffer A was added (buffer A: 50 mM Na-phosphate, 1 mM EDTA and 5 μM FAD; pH 7·5). The homogenate was spun at 40,000 *g* for 15 min at 4°C and the volume of the supernatant was adjusted with water to achieve the composition of single-strength buffer A. For the screening protocol, the crude extracts were diluted with buffer A such that 20 μl contained 1 mU of NR activity (1 mUnit is equivalent to 1 nmol of nitrite formed per min).

2.3 *Enrichment and assay of NR*

The enzyme from the crude extract was fractionated using polyethylene glycol 6000 precipitation (8–18% fraction), dye-ligand chromatography with Blue Sepharose (eluted with 0·3 M KNO_3), concentrated by ultrafiltration and further purified by gel-filtration chromatography (Sephadex G-200). This enriched preparation contained at least four other proteins besides NR, and was used as the immunogen. NR activity was estimated in 500 μl buffer A, containing 20 mM KNO_3, 1–4 mU of NR and 125 μM NADPH, by incubating it at 35°C for 7·5 min (Padidam and Johri 1991).

2.4 *Generation of hybridomas*

Balb/c mice were immunized intraperitoneally with the enriched NR emulsified in Freund's adjuvant (2·5 U NR/mouse for the primary and 1·25 U for the booster injections). After administering four booster injections over a period of four months, the serum was tested for the presence of antibodies which could inhibit the activity of NR. The mice were then injected with 1 U NR without adjuvant on the three successive days prior to fusion. One mouse was sacrificed and the spleen cells were fused with Sp2/0-Ag 14 myeloma cells using polyethylene glycol as described by Galfre and Milstein (1981). The fused cells were dispensed into a total of 456 wells using 96-well plates. The culture medium contained 40% conditioned medium instead of a feeder layer of cells (Lovborg 1982). The myeloma cells and the hybridomas generated were normally cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Four days after fusion, a microscopic examination showed that all wells contained hybridomas. This suggested that there were multiple clones in each well. Hence, on account of this high efficiency of fusion, cells from 72 wells were frozen and those from 24 wells were grown in larger wells (four 24-well plates). After three days, 1 ml of the supernatant was tested for the presence of anti-NR antibody as described below. Positive wells were cloned by limiting dilution. The mice were handled and cared for in accordance with the institutional guidelines.

2.5 *Modified ADAAS for screening for anti-NR antibodies*

The conventional functional assay was modified to identify the hybridomas secreting the anti-NR antibodies. The major modification made was to first adsorb from the hybridoma supernatants the murine antibodies using a suitable matrix. In order to ensure that all classes of murine immunoglobulins were adsorbed, the rabbit anti-mouse IgG (RAMI) antibodies were first pre-adsorbed on to the SAC (or protein A-Sepharose). Ten ml of 10% suspension of formalin-fixed SAC were mixed with saturating amounts of RAMI (heavy and light chain specific, 18·4 mg protein as specified by supplier) in buffer B (50 mM Na-phosphate, 1 mM EDTA, pH 7·5). Twenty μl of this suspension was added to 1 ml of hybridoma or myeloma supernatant (the latter served as negative control), and allowed to mix for at least 3 h on a rotator at room temperature. The SAC-RAMI-murine antibody complexes were pelleted by centrifugation, washed with buffer A containing 20 mM KNO_3 and the

pellet was resuspended in 3 µl of the same buffer. Crude extract containing 1 mU of NR/20 µl was added to SAC complex, mixed and incubated at 25°C for 15 min. The incubation was stopped by diluting it with 530 µl of ice-cold buffer. The SAC complex was spun down and 500 µl of the supernatant was assayed for NR activity. The pellet was washed and assayed for NR activity. A 50% reduction in NR activity in the supernatant (as compared to the control) or the appearance of NR activity in the pellet, was taken to indicate the presence of anti-NR antibody.

2.6 *Kinetics of immunoadsorption of NR using the modified ADAAS*

The SAC with pre-adsorbed RAMI antibody was mixed with an excess of 5F1 McAb as described in § 2.5. In this experiment the IgG fraction from the hybridoma supernatant was concentrated by ammonium sulphate. The SAC-RAMI-murine antibody complex was suspended in buffer B and stored at 4°C in the presence of 0·01% sodium azide. This complex was stable for at least six months. For each treatment, 20 µl of a 10% immunoadsorbent suspension was spun, washed thrice with buffer B and the pellet was suspended in 3 µl of buffer A containing 20 mM KNO_3. Crude extract (ca. 1·3 mU of NR activity in 20 µl) were added to each tube and kept in ice. At various intervals, 850 µl of ice-cold buffer A with KNO_3 was added and tubes were spun rapidly for 2 min at 4°C in a microcentrifuge. Aliquots of supernatant in duplicate were assayed for NR activity. When the adsorbed enzymic activity had to be measured, the pellet was washed once with the above buffer, resuspended in 850 µl of the same buffer and aliquots in duplicate were assayed.

2.7 *Electrophoresis and Western blotting*

Crude extracts equivalent to 200–250 µg protein were separated by 8% non-denaturing PAGE. The gels were stained with reduced methyl viologen (Lund and DeMoss 1976). A colourless band representing NR activity appeared within 20 min and was recorded by photography. The M_r markers used were apoferritin (443 kDa), sweet potato β-amylase (200 kDa), and bovine serum albumin (monomer 66 kDa; dimer 132 kDa; trimer 198 kDa). The proteins were transferred on to nitrocellulose sheets (Otter *et al* 1987) and probed with McAbs as described below.

To study the NR subunits, the samples of crude extract were denatured with SDS and β-mercaptoethanol, separated by SDS-PAGE and transferred to nitrocellulose paper (Towbin *et al* 1979). After transfer the nitrocellulose sheet was incubated for 2 h in BLOTTO [5% non-fat dry milk in TBS (Tris 10 mM, NaCl 13 mM; pH 8·0)]. Nitrocellulose was then incubated (room temperature, 2 h) with the hybridoma supernatant diluted ten-fold in BLOTTO or BLOTTO with 0·2% Tween-20. The sheet was next washed thrice with TBS containing 0·1% Tween-20 and reacted with peroxidase conjugated anti-mouse Ig antibody. After another washing cycle the bound peroxidase was visualized by incubating the nitrocellulose sheet in 1·3 mM diaminobenzidine, 0·02% v/v H_2O_2, in TBS pH 7·4. The pre-stained M_r markers used were fumarase (48·5 kDa), pyruvate kinase (58 kDa), fructose-6-phosphate kinase (84 kDa), β-galactosidase (116 kDa) and α-macroglobulin (180 kDa).

2.8 *Chemicals*

Staphylococcus aureus cells, tissue culture media and peroxidase conjugated anti-mouse Ig antibodies were obtained from Sigma Chemical Co.; polyethylene glycol 1540 from Union Carbide; nitrocellulose filters from Sartorius GmbH; class- and subclass-specific biotinylated antibodies and peroxidase conjugated streptavidin from Amersham and RAMI antibody from Bio-Rad Laboratories and Sigma Chemical Co.

3. Results

3. *Screening and identification of anti-NR hybridomas*

By employing the modified ADAAS during screening, we identified and then successfully isolated three hybridomas producing anti-NR McAbs (5B2, 5F1 and 5F9). During screening, whereas hybridoma supernatants from 5B2 and 5F1 depleted more than 50% of NR from crude extract, that from 5F9 depleted it only slightly. The hybridoma 5F9 was identified on the basis of NR activity associated with the pellet. After recloning, the lack of NR activity in the supernatant (5B2, 5F1 and 5F9 hybridomas) was not due to an inhibition of enzymic activity by immunoadsorbent as most of the enzyme activity was recovered in the pellet following immunoadsorption and centrifugation. Also, there was no inhibition of NR when heterologous antibody was added to SAC-RAMI complex (table 1). The question of inhibition of NR by components of hybridoma supernatant does not arise because the constituents of culture medium get eliminated during the preparation of the immunoadsorbent.

3.2 *Characteristics of the anti-NR monoclonal antibodies*

Following immunoadsorption of NR by SAC-RAMI-5F1 McAb complex, the polypeptides were separated by SDS-PAGE and gels were stained with silver. As compared to the control, a major polypeptide of about

116 kDa and traces of 110 and 64 kDa were found (figure 1). The two smaller polypeptides seem to represent the degradation products (results presented later). The size of 116 kDa is consistent with the subunit of NR found in higher plants (Campbell 1989). In order to further establish the specificity of McAbs towards NR, the crude extracts were separated by electrophoresis on non-denaturing gels. A single spot of NR activity corresponding to about 450 kDa was detected (figure 2, lane 1). The proteins from such gels were transferred to nitrocellulose sheets and the blots were probed with the hybridoma supernatants. The McAb 5F1 reacted strongly with a single band of about 450 kDa which matched with the position of NR activity (figure 2, lane 2). Though the McAbs 5B2 and 5F9 depleted NR activity from crude extracts (table 1), they did not react with 450 kDa NR protein on the blots of non-denaturing gels (table 2). This result can be explained if the conformation of 450 kDa NR protein in solution is different from that after transfer to the nitrocellulose sheet. Under these conditions, an epitope recognized by an antibody may not be exposed and be accessible after transfer. Thus the epitope on native NR detected by McAb 5F1 is different from that recognized by McAbs 5B2 and 5F9.

The three McAbs differed in their ability to bind to NR subunit also. The crude extracts were denatured, separated by SDS-PAGE and blotted on to nitrocellulose. The McAb 5F1 reacted with the 116 kDa polypeptide both in the presence and absence of Tween-20 (figure 3). A polypeptide of 64 kDa was also recognized by McAb 5F1. Tween-20 enhanced the binding of McAb 5F1 to the 64 kDa polypeptide and was essential for the recognition of the 116 kDa polypeptide by McAb 5B2 on the blots (figure 3). Tween-20 seems to denature the polypeptides completely so that the epitopes are fully exposed for recognition by the antibodies. The McAb 5F9 did not recognize any polypeptide on the blots. The 64 kDa polypeptide seems to be associated with native NR, as all the three McAbs depleted it from solution (figure 4). The 110 and 64 kDa polypeptides were present in traces and were not detected by McAb 5F1 in carefully prepared crude extracts (figure 4, lane 6). The occurrence of traces of 110 and 64 kDa polypeptides and their absence in carefully prepared extracts suggests that they are degradation products of the labile NR.

The above results establish the specificity for NR of the three McAbs, which were identified on the basis of modified ADAAS. The three anti-NR McAbs antibodies differed in their ability to recognize the 116 kDa NR subunit and also the 450 kDa NR native protein in solution or on the blots. As argued above the conformation of polypeptide and the consequent exposure of epitope may be involved. Thus, the three antibodies are distinct and recognize different epitopes in a conformation-dependent or -independent manner.

Table 1. Depletion of NR activity by different McAbs.

Treatment	NR activity in the supernatant	
	mUnits	% of initial
Initial activity (control)	3·48	100
After 15 min in ice	3·23	93
After 15 min at 25°C	2·57	74
SAC-RAMI-control McAb*	2·46	71
SAC-RAMI-5B2 McAb	0·16	4·6
SAC-RAMI-5F1 McAb	0·18	5·2
SAC-RAMI-5F9 McAb	0·15	4·3

Immunoadsorption for 15 min at 25°C. The supernatants were obtained after cloning the hybridomas and were used as the source of different McAbs.
*Specific for *Drosophila* protein.

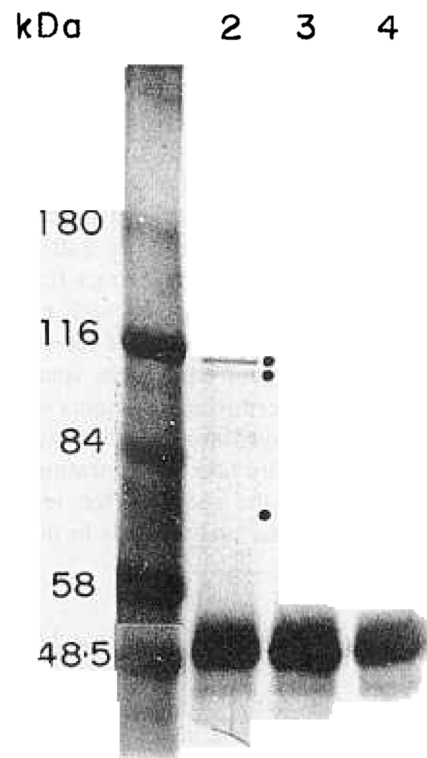

Figure 1. Characterization of anti-NR McAb. Imunoadsorbents consisting of 5F1 McAb or a heterologous antibody (specific for *Drosophila* nervous system protein) bound to protein A-Sepharose-RAMI were prepared. These were mixed individually with crude extract samples (20 μl, 1 mU NR) and the NR was adsorbed. The protein A-Sepharose antigen-antibody complex was pelleted, washed twice with 1 ml of buffer A, suspended in SDS gel-loading buffer and denatured by heating to 100°C for 3 min. The protein A-Sepharose was removed by centrifugation and the polypeptides were separated by 8% SDS-PAGE and detected by staining with silver (Harlow and Lane 1988). Lane 1, M_r markers. Lanes 2–4, crude extracts treated respectively with complexed 5F1 McAb (lane 2), anti-*Drosophila* protein McAb (lane 3) and protein A-Sepharose-RAMI (lane 4). The 64 kDa polypeptide barely detectable on the gel is not visible in the photograph.

3.3 *Kinetics of immunoadsorption of NR activity*

The modified ADAAS can also be used to immunopurify the NR for studying its properties. In order to adsorb the maximum amount of NR in the shortest time, the SAC-RAMI complex was allowed to bind saturating levels of McAb 5F1. To study the kinetics of NR depletion, the crude extracts were incubated in ice-bath (to ensure minimal inactivation of NR) with SAC-RAMI-5F1 McAb complex. After various intervals, assay buffer was added and the SAC-complex pelleted by centrifugation. The NR activities in the supernatants and in the crude extract left in ice for 40 min were measured. The enzyme activity was adsorbed rapidly by the immobilized anti-NR antibody, and about 90% of it was bound in 30 min (figure 5). The NR enzyme activity was not affected by binding to immobilized 5F1 McAb, as most of the activity depleted from the supernatant was recovered in the pellet.

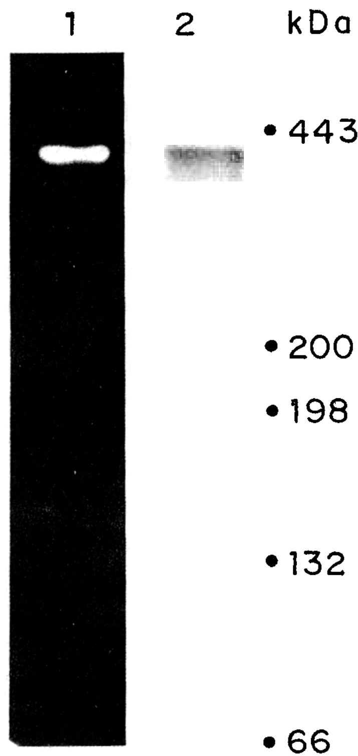

Figure 2. Characterization of anti-NR McAb. Crude extract (5·2 mU NR and 200 μg protein) was separated by 8% non-denaturing PAGE. Lane 1, gel stained for NR activity. Lane 2, Western blot of the gel track shown in lane 1, probed with 5F1 McAb.

Table 2. Binding characteristics of different McAbs.

	Monoclonal Antibody		
	5F	5B2	5F9
Native NR in solution	++++	+++	++++
Native NR protein (blotted)[c]	+++		–
Denatured (blotted)[b]	++++	++	–

++++, Strong recognition; ++, weak recognition; –, no recognition.

[a]NR fractionated by non-denaturing PAGE and blotted on to nitrocellulose.

[b]Denatured in SDS and β-mercaptoethanol at 100°C for 3 min, separated by SDS-PAGE and blotted.

3.4 *Mapping the location of the epitope recognized by McAb 5F1*

As mentioned earlier in § 3.2, the McAb 5F1 recognized a polypeptide of 64 kDa also besides the intact NR subunit polypeptide of 116 kDa. These two polypeptides must share the same epitope and furthermore since the moss NR is a homotetramer, the 64 kDa fragment must arise due to proteolysis of the 116 kDa polypeptide. In order to ascertain the presence of proteases and specific proteolytic pattern, unfractionated cell free extracts were stored in ice for 2 h, later separated by SDS-PAGE and the blot was probed with McAb 5F1. In these extracts, polypeptides of 64 and 84 kDa were found to be the predominant products of proteolysis (figure 4, lane 7). The formation of discrete bands indicates that certain regions of the polypeptides are more susceptible to proteolysis than others. These regions are likely to be the hinge regions of the NR polypeptide and as argued in the discussion, the epitope recognized by 5F1 McAb must be located on the N-terminal domain.

4. Discussion

In general, functional assays have not found wide application as screening protocols because they are often

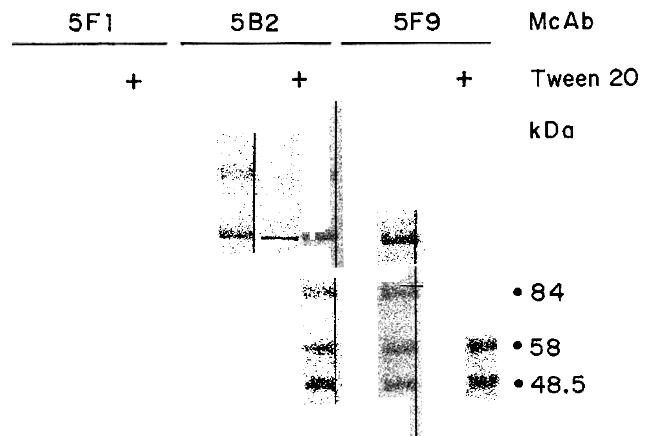

Figure 3. Recognition of NR subunit polypeptide by the three anti-NR McAbs. Crude extracts (2 mU NR and 100 μg protein) were denatured and separated by 8% SDS-PAGE. Strips of Western blots were probed individually with the three McAbs in the presence or absence of 0·2% Tween-20. Each strip contained paired tracks of crude extract and pre-stained markers.

time-consuming and tedious (Harlow and Lane 1988). In the conventional ADAAS, for instance, the enzyme (or antigen) is added to the hybridoma supernatant and incubated for an hour or more (Crawford *et al* 1982; Nakane and Deguchi 1982; Krishnaswamy and Bryan 1986; Lenman *et al* 1990). After the addition of RAMI antibody and SAC, a further incubation is necessary to allow the binding of soluble antigen-antibody complexes to SAC. The SAC along with the bound complexes are removed by centrifugation and the supernatant is assayed for enzyme activity. We reasoned that it should be possible to shorten the incubation time by concentrating the reactants. The concentrated NR enzyme activity was obtained by extracting the enzyme in a small volume of buffer while the murine antibodies were concentrated by pre-adsorbing them with SAC-RAMI complex. The enzyme extract was added to the immobilized antibody and by employing this strategy, the time of incubation of enzyme in solution was further reduced. The foregoing reasoning turned out to be right and ultimately led to the development of the modified functional assay. In order to ensure that murine IgGs of all classes get adsorbed and bovine antibodies (from fetal bovine serum) do not inhibit binding, rabbit anti-mouse IgG antibodies (directed against the whole molecule) were pre-adsorbed on to SAC.

Of the different functional assays, only ADAAS can identify both inhibitory as well as non-inhibitory antibodies that bind to the native enzyme. In the modified protocol described here, the SAC-RAMI complex is first added to the hybridoma supernatant and incubated for at least 3 h, so that the hysteresis effect may ensure a tighter binding of the adsorbed antibody (Absolom and van Oss 1986). The SAC-RAMI-McAb complex is then centrifuged and washed. It is worth mentioning that unlike the conventional ADAAS where SAC are used only to help sediment antigen-antibody complexes, in the present studies the SAC-RAMI complex was used to concentrate, purify and immobilize murine antibodies.

During screening, after the immunoadsorption step, both supernatant and pellet were assayed for the enzyme activity. The usefulness of this strategy makes it possible to identify even those cases where the ratio of non-NR antibody secretors to anti-NR secretors in the original well was very high (for 5F9 the observed ratio was

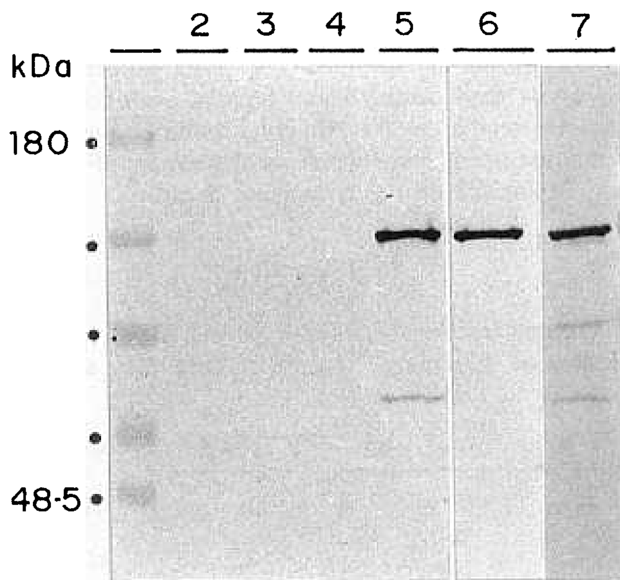

Figure 4. Specificity of the anti-NR McAbs. Lane 1, pre-stained M_r markers. Lanes 2–5 and 7, crude extracts (2 mU NR and 100 μg protein) were treated individually with immobilized (on to SAC) McAb 5F1 (lane 2), McAb 5B2 (lane 3), McAb 5F9 (lane 4) and anti-*Drosophila* protein McAb as negative control (lane 5 and 7). After adsorption the contents were spun, the proteins in the supernatants were denatured and separated by 8% SDS-PAGE. The Western blot was probed with McAb 5F1. The 116 and 64 kDa polypeptides present in the crude extract were not adsorbed by the control McAb. They were adsorbed and removed from the extract by each of the three anti-NR McAbs. Lane 6, crude extract prepared rapidly in the presence of protease inhibitors and separated and probed as described above. Only a single 116 kDa polypeptide was detected in such carefully made extracts. Lane 7, crude extract was pre-incubated in ice for 4 h and then it was treated as described for the sample in lane 5.

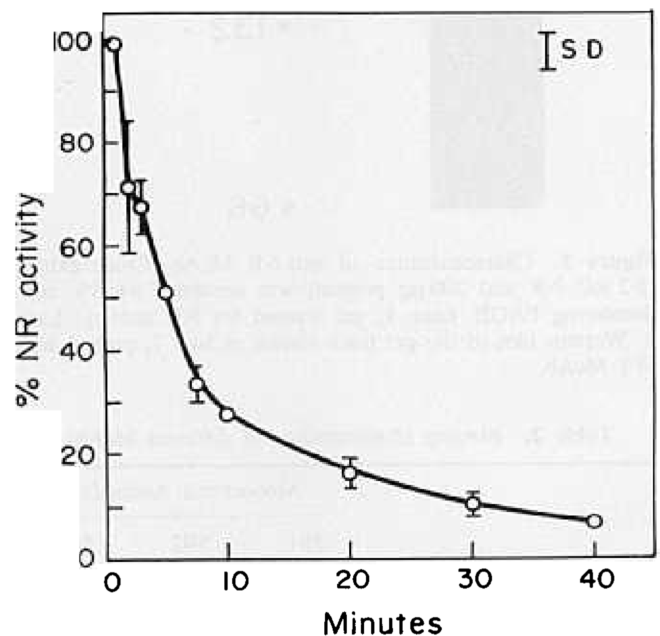

Figure 5 Kinetics of immunoadsorption by the modified ADAAS. Samples of crude extract were incubated in ice with immunoadsorbant consisting of SAC-RAMI-5F1 complex for various durations and then centrifuged. NR activity was assayed in the supernatants and pellets; the data constitute the mean of two separate experiments. Where no vertical bars have been shown the SD is contained within the symbol.

36 : 1) and the concentration of anti-NR antibody in the supernatant was low. In such cases the depletion of enzymatic activity from the supernatant is not sensitive enough to detect the anti-NR antibody. Such cases, e.g. hybridoma 5F9 can be identified by the association of enzyme activity in the pellet. The only instance where such a strategy will fail is with McAbs which block enzyme activity.

The results showing the specific degradation pattern of the 116 NR polypeptide and the known consensus structure of the functional domains of NR subunit (Campbell and Kinghorn 1990) can be used to map the domain containing the epitope. In plants, the 112 kDa (and also 116 kDa) NR subunit polypeptide consists of three adjacent functional domains – the N-terminal molybdenum-binding domain (the **N domain**), the haem-binding domain (the **H domain**) and the C-terminal flavin-binding domain (the **F domain**). The N, H and F domains consist typically of 482, 78 and 262 amino acid residues respectively. The N and H domains and H and F domains are joined by a hinge region of 60 and 35 amino acids respectively. The hinge regions are highly susceptible to proteolysis during handling. A partial proteolysis of NR will yield 5 different polypeptides corresponding respectively to N + H, H + F, N, H, and F domains (assuming cleavage in the middle of the hinge, the number of amino acid residues in these 5 polypeptides is calculated to be 637, 405, 512, 125 and 280). The fact that 5F1 McAb recognized polypeptides of 64 and 84 kDa, the epitope must be located on the N-terminal domain. The sizes of N and N + H domains are estimated to be respectively 64 and 84 kDa and these estimates are in excellent agreement with the results obtained (figure 4, lane 7).

The modified method described in the current studies, besides being rapid, is also versatile enough to be used to isolate McAbs with exquisite specificities. An important feature of this protocol is that the composition of the binding environment (buffer containing the crude extract) can be manipulated. Hence, hybridomas secreting McAbs against different conformational states of the enzyme can be isolated. These may be induced by pH, ionic strength, by adding substrate, or allosteric modulators to the crude extract. Since the McAb reacts with the antigen in the absence of hybridoma supernatant, the McAbs to enzymes which are affected by the hybridoma supernatant (e.g., proteases inhibited by serum α_2-macroglobulin), can also be detected.

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