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Monoclonal Antibody Recognizes Different Quinone Moieties in Enzymes*

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We produced monoclonal antibodies against the coenzyme pyrrolequinoline quinone (PQQ). These antibodies were obtained by immunizing mice with PQQ conjugated to a chemically modified polypeptide in order to induce a strong immune response. Among the various antibodies obtained, one was found to bind (besides PQQ and 6-hydroxydopamine conjugated to carrier proteins) several different quinoenzymes, namely lentil seedling and bovine serum diamine oxidases and methylamine dehydrogenase. This antibody was able to inhibit the catalytic activity of these enzymes. Moreover, the monoclonal antibody recognized different proteins of lentil seeds on Western blots. Even the variable fragment of immunoglobulin heavy chains of this monoclonal antibody expressed in *Escherichia coli* is able to recognize the active site of different quinoenzymes.

Antibodies directed toward cofactors at the active site of enzymes may be used to study structure-function relationships and, alternatively, to build up new catalytic activities (1-3). We have elicited polyclonal antibodies against the coenzyme pyrrolequinoline quinone (PQQ)[1] (methoxatin) that were able to react with lentil seedling amine oxidase (4). PQQ, first found in some dehydrogenases from prokaryotes (5), has been claimed to be widespread in nature, being present also in eukaryotes, where it seems to be involved in many redox and non-redox reactions (6). Its possible role as a vitaminic factor has been also proposed (7).

However, the presence of PQQ in some purported quinoenzymes has recently been challenged (8, 9). In fact, methylamine dehydrogenase from *Thiobacillus versutus*, one of the first quinoproteins described, was found to contain tryptophan tryptophylquinone instead of PQQ (10). Furthermore, amine oxidases were reported to contain 6-hydroxydopamine (2,4,5-trihydroxyphenylalanine (Topa)) as their organic redox center (11). Since we had obtained circumstantial evidence that antibodies obtained by sensitizing rabbits with pure PQQ reacted strongly with lentil amine oxidase, we extended our investigation to monoclonal antibodies elicited against poly-L-lysine-PQQ adducts. One of these, which reacted with the redox moiety of quinoenzymes, was expanded and characterized. The results showing that this antibody recognizes at least three different quinone prosthetic groups are reported here.

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[1] The abbreviations used are: PQQ, pyrrolequinoline quinone (2,7,9-tricarboxy(1*H*)-pyrrolo(2,3-*f*)quinoline-4,5-dione; Topa, 2,4,5-trihydroxyphenylalanine; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Chemicals and Enzymes—PQQ was obtained from Fluka (Buchs, Switzerland), poly-L-lysine and *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide were from Sigma, and sodium borohydride was from Merck (Schuchardt, Germany). All other chemicals were of the highest purity commercially available.

Lentil seedling amine oxidase (EC 1.4.3.6) and bovine serum amine oxidase (EC 1.4.3.6) were purified according to Refs. 12 and 13, respectively. Methylamine dehydrogenase (amine dehydrogenase, EC 1.4.99.3) was purified according to Ref. 14. Ascorbate oxidase (EC 1.10.3.3) was purified according to Ref. 15. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was purchased from Boehringer Biochemia Robin (Mannheim, Germany).

Preparation of Immunogen—To minimize the interactions between the coenzyme and the carrier, PQQ was conjugated to a carrier polypeptide obtained in our laboratory. Poly-L-lysine was modified to induce stronger T and B cell determinants by conjugating a small amount of hydrophilic amino acids, *i.e.* lysine, arginine, and glutamic acid (0.3 mol/mol of ϵ-amino group), and PQQ (PQQ/ϵ-amino group molar ratios ranging from 0.2 to 1) to poly-L-lysine. Conjugation was performed by incubating the mixture in 0.1 M phosphate buffer, pH 4.5, with excess *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide essentially as previously described (16). The complex was used after dialysis against phosphate-buffered saline. Binding of PQQ to poly-L-lysine was checked spectrophotometrically: about one PQQ molecule was bound per every eight poly-L-lysine ϵ-amino groups. For antibody screening and determination of affinity, the antigens used were (*a*) PQQ-gelatin, obtained by conjugating porcine gelatin with different amounts of PQQ as reported above, and (*b*) BSA-Topa, obtained as described below.

Conjugation of 2,4,5-Trihydroxyphenylalanine to Carrier Protein—The whole procedure was performed under anaerobic conditions in order to prevent the autoxidation of Topa. It was conjugated at different ratios to BSA. BSA dissolved in water (4 mg/ml) was incubated with a 1000 molar excess of sodium borohydride for 20 min. Thereafter, the solution was dialyzed against water, and different amounts of Topa were added (BSA/Topa molar ratios ranging from 0.1 to 1). Twelve hours later, the solution was dialyzed against water and stored frozen. The amount of covalently bound Topa was checked spectrophotometrically.

Immunization of Mice and Immunoassays—Five-week-old mice (BALB/c) were injected intraperitoneally with 0.1 mg of antigen (poly-L-lysine-PQQ) in phosphate buffer emulsified in complete Freund's adjuvant (total volume of 0.5 ml). The route of immunization and the following fusion were performed essentially as previously described (17).

The production of ascitic fluid was obtained by intraperitoneally injecting 10^6 hybridoma cells into pristane-primed (0.5 ml intraperitoneally on days −10 and −3) BALB/c mice. The ascitic fluid was harvested within 2 weeks. Antibody (characterized as IgM) purification from ascitic fluid was performed essentially as described (18) using polyethylene glycol precipitations. Based on SDS-PAGE analysis and immunoassay of pellets and supernatants, the concentration of polyethylene glycol precipitating most of the IgM was found to be 6.5%, followed by a second precipitation at 5.5% polyethylene glycol. Immunodetection of quinoproteins was performed via dot-blot analy-

sis by spotting different amounts of native proteins on nitrocellulose (0.22 μm; Schleicher & Schuell) or Problott (Applied Biosystems, Inc.) sheets using a Bio-Rad immunodot-blot apparatus. Filters were incubated for 2 h with blocking solution (phosphate-buffered saline containing 3% BSA, 0.25% gelatin, 0.05% Tween 20, and 1% polyvinylpyrrolidone). After three washings in phosphate-buffered saline, 0.1% Tween 20, the filters were incubated for 12 h in a cold room with the purified monoclonal anti-PQQ antibody. The filters were then fixed with glutaraldehyde to block the primary monoclonal antibody-antigen complex on nitrocellulose or polyvinylidene difluoride sheets as described (19). To increase the sensitivity of the test, immunodot blots were then developed using the ECL Western blotting detection system (Amersham Corp.). ELISA and Western blotting were performed essentially as previously described (20). ELISA was performed by coating polyvinyl chloride plates (Falcon Labware) with different amounts of antigens. Isotypes of monoclonal antibodies were determined in cell culture supernatants as previously described (20). To calculate the dissociation constant of the antibody-antigen complex, indirect ELISA was carried out as described (21). Lentil seedling proteins were analyzed for the presence of antigens as follows. Lentil seeds were soaked for 24 h in H_2O and germinated for 8 days in moist sawdust layered on plastic trays in the dark at room temperature. The seedlings were then homogenized in a Waring Blendor with deionized water for 4 min. The homogenate was centrifuged, and an aliquot was run under reducing conditions on 10% SDS-polyacrylamide gel. Western blotting was then performed as previously described (20). Blot scanning was performed on a Bio-Rad Ultrascan using the software supplied with the instrument.

Inhibition of Enzyme Activity—Oxygen consumption of lentil seedling amine oxidase was measured as follows. Five microliters of enzyme (2 mg/ml) were incubated overnight at +4 °C with the same amount (200 μl) of the purified nonspecific IgM or specific anti-PQQ antibody. The molar ratios of lentil seedling amine oxidase to purified antibodies were 1:0.5, 1:1, and 1:1.25. Due to the high molecular weight and low solubility of the whole IgM molecule, no higher lentil seedling amine oxidase/purified antibody molecular ratio could be achieved. The reaction was started by adding 1–100 μM putrescine in the polarographic chamber to the mixtures in 3 ml (final volume) of 0.1 M phosphate buffer, pH 7.2, at 21 °C. The oxygen uptake was followed using a Yellow Springs Instruments oxygen-sensitive electrode.

Inhibition of bovine serum amine oxidase by antibodies was determined essentially as described above for lentil seedling amine oxidase using benzylamine as the substrate. Inhibition of lentil seedling amine oxidase activity was also tested spectrophotometrically. The chromogenic substrate *p*-dimethylaminomethylbenzylamine, used as substrate, was diluted (0.2 mM) in 0.1 M potassium phosphate, pH 7, just before use. Five microliters of 2 mg/ml purified lentil seedling amine oxidase alone or the mixture of lentil seedling amine oxidase/specific IgM or lentil seedling amine oxidase/nonspecific IgM (lentil seedling amine oxidase/antibody ratios of 1:0.75, 1:1, and 1:1.25) were added to 1 ml of substrate, and the absorbance increase at 250 nm was measured for at least 5 min. Methylamine dehydrogenase activity was tested as follows. A solution of 0.1 M potassium phosphate containing 0.01 M methylamine HCl, 0.5 mM phenazine methosulfate, and 60 μM 2,6-dichloroindophenol was prepared just before use. Five microliters of purified methylamine dehydrogenase (1 mg/ml) were added to this solution, and the absorbance change at 600 nm was recorded for 5 min. In inhibition experiments, different amounts of methylamine dehydrogenase/specific or nonspecific purified monoclonal antibodies (methylamine dehydrogenase/antibody ratios of 1:0.75, 1:1, and 1:1.25) were added to the incubation mixtures. To control the specificity of inhibition, the monoclonal antibody against PQQ was tested under similar conditions with ascorbate oxidase or with glucose 6-phosphate dehydrogenase and their appropriate substrates.

RESULTS

Production of Monoclonal Antibodies—Four fusions were performed using different immunization protocols. All of them gave only a few positive wells. Different carrier proteins were tried (keyhole limpet hemocyanin, BSA, gelatin, etc.) All monoclonal antibodies obtained reacted strongly with the immunogens, but only weakly with quinoproteins. The fusion showing the highest efficiency in terms of poly-L-lysine-PQQ-positive clones and quinoprotein-specific clones was obtained using a poly-L-lysine-PQQ conjugate. Among the positive clones obtained, all secreting IgM, one was found to be stable and to secrete specific antibodies. It was further cloned and injected into mice to produce large amounts of the monoclonal antibody. The ascitic fluid was then purified as previously described. Electrophoretic analysis under reducing conditions showed two main bands representing the μ chain and the light chain of IgM. A small contaminating band due to albumin was also present. Densitometric scanning of the gel allowed us to evaluate the purity of the IgM preparation to ~95% (data not shown).

Affinity of Monoclonal Antibody for Different Antigens—Preliminary ELISA experiments showed that the purified monoclonal antibody obtained against PQQ was able to bind lentil seedling amine oxidase-, methylamine dehydrogenase-, BSA-Topa-, bovine serum amine oxidase-, and PQQ-gelatin-coated plates. The antibody (diluted at the concentration giving 50% binding onto antigen-coated plates) was incubated overnight with different concentrations of antigens at +4 °C. The mixtures were then plated onto plates coated with the same antigen, which was incubated overnight with the monoclonal antibody, except for poly-L-lysine-PQQ, for free PQQ K_d measurement. The amount of antibody trapped was detected as described above. Dissociation constants for the antibody-antigen complexes were measured after 12 h since it was found that inhibition of antibody binding to the plates was time-dependent and reached a plateau after 12 h. The dissociation constants (K_d) for the antibody-protein complex were 7.2×10^{-7} M (correlation coefficient (r) = 0.98), 8.4×10^{-6} M (r = 0.98), and 2.4×10^{-4} M (r = 0.99) for lentil seedling amine oxidase, methylamine dehydrogenase, and free PQQ, respectively. The dissociation constants for BSA-Topa (molar ratio of 10:1) and bovine serum amine oxidase were 1.3×10^{-5} M (r = 0.98) and 3.5×10^{-7} M (r = 0.99), respectively. A strong correlation between monoclonal antibody binding and the amount of PQQ or Topa in gelatin or BSA conjugates was found (Table I). Using regression analysis by plotting the residual peroxidase activity *versus* the log of the amount of PQQ-gelatin or BSA-Topa, it was possible to measure the amount of antigen recognized in lentil seedling amine oxidase, confirming the data obtained from Ref. 4. The specificity of the purified monoclonal antibody was demonstrated using ELISA plates coated with different non-quinone enzymes such as ascorbate oxidase, cytochrome oxidase, etc., in which no binding activity was observed. The affinity of the antibody for lentil seedling amine oxidase reduced in the absence of air decreased by ~30%.

Fig. 1 shows a dot-blot analysis performed with the same amount of different quinoproteins in order to find the respective binding affinity. The correlation between the amount of Topa and antibody binding is clear-cut. The lowest binding capacity was observed for methylamine dehydrogenase, and the strongest for lentil seedling and bovine serum amine oxidases. No binding was detected with the carrier proteins gelatin, BSA, and cytochrome *c*.

Inhibition of Quinoenzymes by Antibodies—Since the monoclonal antibody obtained was capable of recognizing the redox

TABLE I

Monoclonal antibody binding in different conjugates

BSA/Topa ratio	Absorbance	Gelatin/PQQ ratio	Absorbance
BSA alone	0.104 ± 0.015	Gelatin alone	0.075 ± 0.021
1:0.1	0.238 ± 0.067	1:0.1	0.327 ± 0.043
1:0.5	0.358 ± 0.078	1:0.5	0.453 ± 0.079
1:1	0.685 ± 0.103	1:1	0.768 ± 0.146
1:5	0.884 ± 0.175	1:5	1.013 ± 1.383
1:10	0.964 ± 1.428	1:10	1.541 ± 1.641

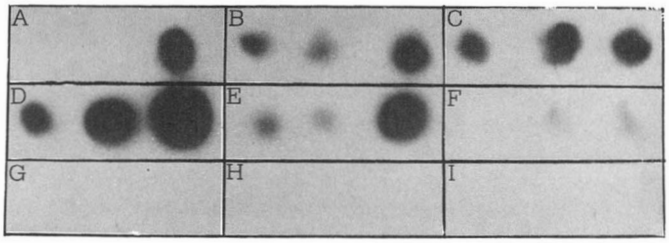

FIG. 1. **Dot-blot analysis of different quinoproteins.** BSA-PQQ conjugated at different molar ratios (1:1 (*A*), 1:5 (*B*), and 1:10 (*C*)), lentil seedling amine oxidase (*D*), bovine serum amine oxidase (*E*), methylamine dehydrogenase (*F*), aprotinin (*G*) BSA (*H*), and gelatin (*I*) were spotted on Problott polyvinylidene difluoride paper. Sheets were then treated as described under "Materials and Methods" and developed using the ECL detection system (Amersham Corp.) following the manufacturer's instructions. Serial dilutions of each sample at a total of 5 μl were spotted (final amounts of protein of 1, 0.25, and 0.1 mg/ml starting from the left).

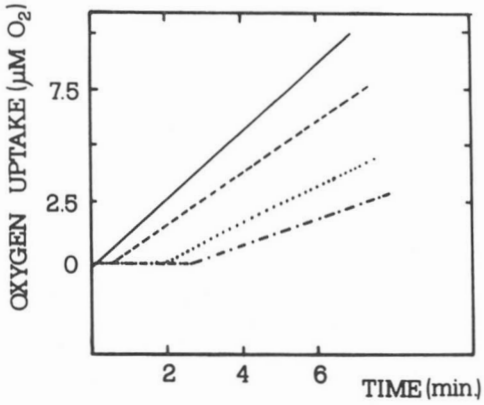

FIG. 2. **Inhibition of amine oxidase by antibody.** Five microliters of lentil seedling amine oxidase (2 mg/ml) were incubated at room temperature with 200 μl of purified nonspecific IgM or specific anti-PQQ antibody (molar ratios of lentil seedling amine oxidase to antibody of 1:0.5, 1:1, and 1:1.25). Purified antibodies were diluted in the polarographic chamber to 3 ml (final volume) with 0.1 M phosphate buffer, pH 7.2, containing putrescine as the substrate. The reaction was started by adding the nonspecific (——) or specific (1:0.5 (– – –), 1:1 (· · · ·), and 1.25 (– · – ·) enzyme/antibody mixtures and was performed at 21 °C.

moiety of quinoenzymes, experiments on the possible inhibitory activity of this antibody were performed. Either specific or nonspecific purified monoclonal IgM was used. Increasing amounts of antibodies were incubated with lentil seedling or bovine serum amine oxidase or methylamine dehydrogenase.

Oxygen uptake data (Fig. 2) indicated that the specific monoclonal antibody reduces by 15–40% lentil seedling amine oxidase activity for putrescine depending on the antibody concentration added. Spectrophotometric measurements confirmed that the monoclonal antibody inhibits by 15–40% the enzymatic activity of lentil seedling amine oxidase as well as that of methylamine dehydrogenase (Fig. 3) as a function of the amount added. In the case of bovine serum amine oxidase, the inhibition ranged from 35% at an enzyme/antibody ratio of 2 to 80% at a ratio of 0.25. No inhibition of two different redox enzymes such as ascorbate oxidase and glucose-6-phosphate dehydrogenase was observed (data not shown), thus confirming the specificity of the monoclonal antibody for the quinone moieties. The inhibition of lentil seedling and bovine serum amine oxidases by monoclonal antibodies was noncompetitive (data not shown). The low solubility of the purified monoclonal antibody did not allow higher enzyme/antibody ratios and thus higher inhibition values to be reached. Western blotting of the lentil seedling homogenate was performed. The data reported as a blot scan (Fig. 4) suggest that the monoclonal antibody recognizes several proteins present in the homogenate.

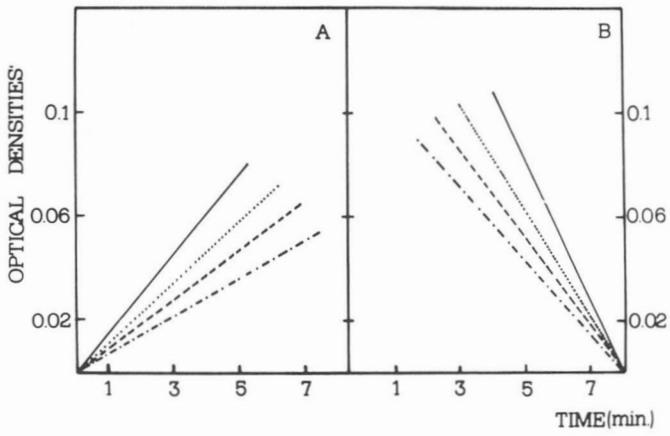

FIG. 3. **Enzymatic activity of lentil seedling amine oxidase (*A*) and methylamine dehydrogenase (*B*).** *A*, lentil seedling amine oxidase activity was tested as described under "Materials and Methods." Ten (· · · ·), 15 (– – –), or 20 (– · – ·) μl of purified monoclonal antibody (lentil seedling amine oxidase/antibody ratios of 1:0.75, 1:1, and 1:1.25) or 15 μl of purified nonspecific IgM (——) were incubated with 5 μl of lentil seedling amine oxidase for 12 h. *B*, methylamine dehydrogenase activity was measured as described under "Materials and Methods." Five microliters of purified methylamine dehydrogenase (1 mg/ml) were added to the incubation mixtures, and the absorbance at 600 nm was recorded for 5 min. For inhibition experiments, 10 (· · · ·), 15 (– – –), or 20 (– · – ·) μl of purified monoclonal antibody (methylamine dehydrogenase/antibody ratios of 1:0.75, 1:1, and 1:1.25) or 15 μl of purified nonspecific IgM (——) were added to 5 μl of methylamine dehydrogenase 12 h before testing the activity.

DISCUSSION

Using the hybridoma technology introduced by Kohler and Milstein (22), we obtained the first described monoclonal antibody directed against the redox moieties of an array of quinoenzymes. A monoclonal antibody was raised by immunizing mice with PQQ coupled to the newly designed carrier poly-L-lysine. This low immunogenic polypeptide was modified in order to stimulate a stronger T and B cell response and to express efficiently the immune system-stimulating redox moiety. Nevertheless, the antigenicity of this complex was still low, as demonstrated by the production of an IgM response (average antibody titer measured with an anti-IgM peroxidase-labeled secondary antibody, 2500 ± 500; average antibody titer measured with an anti-IgG peroxidase-labeled secondary antibody, 200 ± 100). Also, the low affinity of the monoclonal antibody obtained, like that of most monoclonal antibodies so far described, is probably due to the low antigenicity of the immunizing complex. ELISA and Western blot experiments confirmed the specificity of the monoclonal antibody, which could react with the redox moieties of all the quinoproteins so far analyzed. The antibody-antigen reaction was further tested by inhibition of lentil seedling and bovine serum amine oxidases and methylamine dehydrogenase. Monoclonal antibody binding to lentil seedling amine oxidase inhibited its enzymatic activity by 15–40% in a noncompetitive way. A similar extent of inhibition was obtained for methylamine dehydrogenase. The high molecular weight of the whole IgM molecule and its low solubility did not allow us to achieve greater inhibition values. However, the correlation between the amount of added monoclonal antibody and inhibition confirms the specificity of inhibition. The produc-

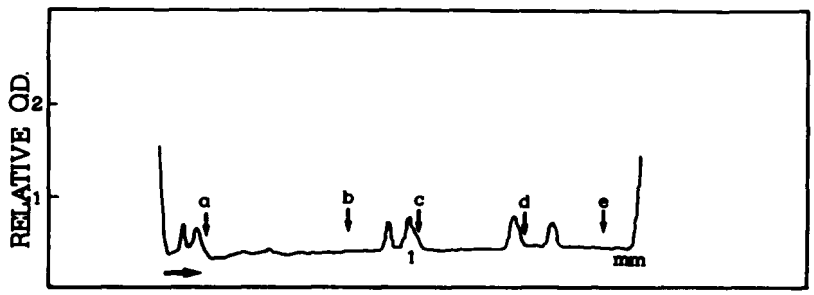

FIG. 4. **Immunoelectrophoretic detection of amine oxidase.** A lentil seedling homogenate was run under reducing conditions on 8% SDS-polyacrylamide gel. Western blotting was performed and analyzed as described under "Materials and Methods." Molecular weight markers were myosin (205,000; *arrow a*), phosphorylase *b* (97,400; *arrow b*), bovine plasma albumin (66,000; *arrow c*) egg albumin (45,000; *arrow d*), and carbonic anhydrase (29,000; *arrow e*). *Peak 1* corresponds to lentil seedling amine oxidase.

tion via recombinant techniques of the Fab fragment still in progress will allow higher enzyme/antibody ratios.

Dot-blot analysis showed that this monoclonal antibody raised against PQQ recognizes, although at a different extent, different quinoproteins. This finding is particularly interesting since these proteins do contain different quinone compounds. In fact, bovine serum and lentil seedling amine oxidases have been shown to contain Topa (11, 23), whereas methylamine dehydrogenase contains a ditryptophanyl derivative (10). Therefore, the antibody should recognize a chemical feature common to all these molecules. The most likely possibility is that it reacts with just the *o*-quinone group. Thus, this monoclonal antibody allows the detection of a whole class of proteins, the quinoproteins. As an example, Western blot analysis of a lentil seedling homogenate detected different molecules reacting with the monoclonal antibody, thus indicating the possibility that a number of proteins in these seedlings could have a quinone moiety. Since the immunizing antigen is PQQ, it can be excluded that the monoclonal antibody is directed against a tertiary structure motif common to all the analyzed quinoproteins. The different affinity of the antibody for the various quinoproteins may therefore reflect the different structure of the quinones. In this context, it is interesting to note that the affinity of the antibody for lentil seedling amine oxidase decreases significantly when the coenzyme Topa is reduced. A further cause of the different reactivity may arise from the steric hindrance of the large IgM molecule in reaching the coenzyme, possibly embedded inside the protein. Thus, we tried to obtain a variable fragment of immunoglobulin heavy chains (VH fragment) in order to determine whether it could more easily reach the coenzyme. The VH domains from monoclonal antibodies could express higher affinity for a ligand than the intact molecule essentially for two reasons. (i) The smaller size of the molecule allows a better interaction with the active site; (ii) the hydrophobicity of the VH fragment could increase the affinity of the single domain antibody for an hydrophobic active site.

Preliminary experiments were performed using the methods described in Ref. 24 and the vectors kindly provided by Dr. G. Winter (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom). The sequenced VH gene was found to belong to the subgroup I (B) family (25). This purified recombinant VH gene product is able to bind specifically lentil seedling amine oxidase and methylamine dehydrogenase with a higher affinity (1.03×10^{-8} and 9.3×10^{-8} M, respectively) under the same conditions described for the entire IgM molecule. The purified VH gene product is also able to inhibit efficiently the catalytic activity of lentil seedling amine oxidase and methylamine dehydrogenase. In fact, the purified recombinant VH gene product was found to inhibit by 20–40% the activity of these enzymes when used at enzyme/VH gene molar ratios ranging from 0.1 to 0.2. Production of larger amounts of both VH and Fab fragments by recombinant technologies and further sequence analysis will shed more light on the relationships between the complementarity of antibody and antigen structures.

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