Immunochemical studies on *Rhodotorula gracilis* D-amino acid oxidase

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Summary. Polyclonal antibodies were prepared from rabbit sera after immunization with holo- and apo-D-amino acid oxidase purified from *R. gracilis*. Both anti-holo- and anti-apoenzyme IgG fractions (as well as affinity-purified IgG) were highly specific: in blot-transfer analyses after SDS-PAGE only a 39 kDa band, correspondig to enzyme monomer, was recognized even in the partially purified yeast extract. No cross-reaction was detected with pig kidney D-amino acid oxidase. As a difference from the mammalian enzyme, yeast D-amino acid oxidase anti-holo- and anti-apoenzyme IgGs had different properties in inactivation and precipitation experiments, indicating the existence of different antigenicity sites related to the FAD-binding domain in the enzyme.
Key words. D-amino acid oxidase; polyclonal antibody; FAD binding.

D-amino acid oxidase (DAAO, EC 1.4.3.3) is a flavoprotein which catalyzes the oxidative deamination of D-amino acids. Its activity has been detected in a wide variety of organisms [1], although only in two instances has a homogeneous protein been isolated and spectrally characterized: from pig kidney [2] and more recently from the yeast *R. gracilis* [3, 4] where it is inducible [3, 4].

The *R. gracilis* enzyme contains one mol of non-covalently but tightly bound FAD per 39 kDa protein monomer; the native enzyme is a homodimer (M_r = 79 kDa)[4], while in the native condition the apoenzyme exists as a monomer [5]. Most of the properties of yeast DAAO involving the flavin moiety are typical of those of flavoprotein dehydrogenases/oxidases [6]. Catalytic specificity and tight binding of FAD to the protein moiety sharply distinguish the yeast enzyme from the well-known pig kidney DAAO, which has been extensively studied and is regarded as a model flavo-oxidase enzyme [7].

The present work concerns the immunochemical characterization of yeast DAAO. We used polyclonal antibodies raised against purified holo- and apo- forms of this oxidase to examine their immunoreactivity; cross-reactivity with pig kidney DAAO and the effect of antibody on FAD binding to the apoprotein were also investigated, the latter to gain some insight into the active site area where the coenzyme binds.

Materials and methods

Protein A from *Staphylococcus aureus* Cowan I strain was purchased from Miles (Kankakee, USA); standard molecular weight proteins were from Pharmacia (Uppsala, Sweden). The streptavidin-biotinylated peroxidase complex and the biotinylated donkey anti-rabbit $F(ab')_2$ fragments were from Amersham (Amersham, UK); Accell-QMA was from Waters (Milford, USA) and Affigel 10 from Bio-Rad (Richmond, USA). Pure pig kidney DAAO was a generous gift of Prof. Bruno Curti.

DAAO activity was assayed polarographically at 30 °C and pH 8.5 with D-alanine as substrate [3]. One unit (U) of activity corresponds to the uptake of 1 μmole of oxygen per min under the assay conditions. *R. gracilis* (ATCC 26217) cells were grown in a fermenter on synthetic medium [8] containing 30 mM D-alanine. DAAO was purified as reported previously and the final preparation had a specific activity of 185 U/mg protein, and an E_{274}/E_{455} of 8.2, indicating the absence of contaminating apoprotein. The fully reconstitutable apoprotein was prepared from the holoenzyme as described by Casalin et al. [5]. Concentrations of IgG fractions and purified holo- and apoenzyme were determined spectrophotometrically using A_{280} = 1.35 [9], A_{274} = 2.78 [4] and A_{278} = 2.14 [6] for 1 mg/ml solutions respectively.

Antibodies to the holo- and apoenzyme were prepared by inoculating male New Zealand rabbits each with a total of 1.5 mg of pure protein (in three injections)[9]. IgG fractions from the anti-holo- and anti-apoenzyme antisera (IgG_H and IgG_A respectively) were precipitated using ammonium sulfate [10] and then chromatographed on an Accell-QMA (Waters) column equilibrated in 20 mM potassium phosphate (pH 7.2) and eluted with a linear gradient of NaCl (0–0.4 M).

Ligand-affinity purified antibodies were prepared using pure D-amino acid oxidase coupled to Affigel 10. Rabbit serum was loaded (9.2 mg of total protein) onto the gel-immobilized D-amino acid oxidase column in 20 mM potassium phosphate (pH 7.5). Unbound protein was eluted with the same buffer, followed by 0.1 M borate buffer (pH 8.5) containing 0.5 M NaCl and 0.1% Nonidet P-40. Bound antibody was eluted with 200 mM glycine-HCl solution containing 10% ethylene glycol and buffered to about pH 8.0 with solid Tris.

For the inactivation and immunoprecipitation experiments, increasing aliquots of antibody were incubated in separate tubes, with between 0.012 and 0.017 units of DAAO for 10 min at room temperature (longer incubation times did not modify the results). A constant final volume was obtained by adding 10 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1% bovine serum albumin and 10% glycerol. Enzyme activity was measured against a control in which the antibody solution had been replaced by buffer. 370 μl of 10%

protein A suspension per mg of antibody was added to each tube, and the mixtures incubated for 30 min in the cold. After centrifugation at 12000 × g for 10 min, residual enzyme activity was measured in the supernatant.

CNBr digestion was performed for 48 h, according to the procedure of Gross[11], on pure holo- and apoenzyme previously carboxymethylated with iodoacetic acid[12]. Digestion kinetics and fragment composition were analyzed by SDS-PAGE electrophoresis[13].

Slab PAGE electrophoresis under native conditions was performed as described by Davis[14] and SDS-PAGE according to Laemli[13]. Upon completion of the gel electrophoretic run, the gel was used for electrotransfer to a nitrocellulose sheet[15]. The sheets were stained for protein with Amido Black and antigen was detected at room temperature with streptavidin-biotinylated peroxidase complex and 3,3'-diamino-benzidine-tetrahydrochloride as substrate. The blotted nitrocellulose sheets were washed five times for 5 min in 0.05 M Tris-HCl (pH 7.4) containing 0.2 M NaCl (buffer A) and 0.05% Triton X-100. They were then incubated in blocking solution (10% dried milk in buffer A) for 1 h at 40 °C and for 0.5 h at room temperature. They were further incubated for 1 h in blocking solution supplemented with 500 µg of antibody (100-fold dilution). The sheets were then washed as above and incubated for 1 h in blocking solution supplemented with biotinylated donkey anti-rabbit $F(ab')_2$ fragments at 800-fold dilution. After washing again as above, the sheets were incubated for 1 h in blocking solution supplemented with the streptavidin-biotinylated peroxidase complex (2000-fold dilution). Staining of the sheet-bound peroxidase was by adding a freshly prepared solution containing 0.05% 3,3'-diamino-benzidine-tetrahydrochloride and 0.02% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.4).

The presence of anti-DAAO antibodies in each preparation and the activity of the immunoprecipitate was assayed by the Ouchterlony technique[16]. Staining to show enzyme activity was carried out by incubating agar gel slides in 35 mM sodium pyrophosphate buffer pH 8.5 containing 23 µM FAD, 93 µg/ml iodonitrotetrazolium chloride and 65 mM D,L-alanine at 37 °C. Gels were stained for protein with Coomassie Brilliant Blue R 250.

Results and discussion

The homogeneous holo-DAAO from the yeast *R. gracilis* was used to obtain the corresponding apoprotein as described by Casalin et al.[5]. Flavin fluorescence at 530 nm was not detected, nor was there any enzyme activity (in the absence of exogenous FAD) in the final apoprotein preparation. Both holo- and apoenzyme forms were used to raise polyclonal antibodies in rabbit serum. Both proteins were highly antigenic, with antibody titres against the enzyme (as determined by an ELISA method[17]) of about 1:8000. IgG fractions were obtained from the antisera, after ammonium sulfate precipitation, by ion-exchange chromatography on an Accell-QMA column. The purity of both Accell-chromatographed and affinity-purified IgG fractions was checked by SDS-electrophoresis; no contaminant protein was detected (not shown).

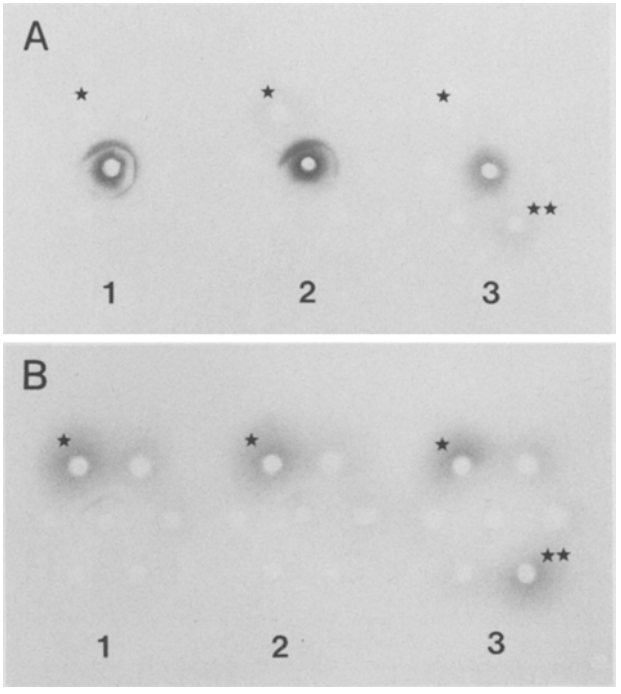

Figure 1. Double diffusion of holo- and apo-DAAO with anti-holoenzyme IgG on agarose gel. (1) center well: 0.125 mg of anti-holoenzyme IgG; external wells (clockwise from the asterisk): *R. gracilis* DAAO: 7.8, 1.6, 0.78, 0.31, 0.16 and 0.08 µg. (2) center well: 0.125 mg of anti-holoenzyme IgG; external wells (clockwise from the asterisk): *R. gracilis* apo-DAAO: 7.8, 1.6, 0.78, 0.31, 0.16 and 0.08 µg. (3) center well: 20 µl of preimmune serum; external wells (clockwise from the asterisk): *holo-DAAO: 7.8, 1.6, 0.78 µg and **apo-DAAO: 7.8, 1.6, 0.78 µg.
A Staining for total protein with Coomassie Blue. *B* Staining for DAAO activity as described in Materials and Methods.

The specificity of the anti-holo-DAAO serum and of the corresponding purified IgG fractions was examined by the Ouchterlony method in a double-diffusion gel: both preparations gave a single immunoprecipitate against holo- and apoprotein, and this retained its catalytic activity (fig. 1). Identical results were obtained in experiments performed with anti-apo-DAAO IgG under the same conditions. No immunoprecipitate was detectable when anti-holo- and anti-apoenzyme sera were replaced with preimmune sera.

The specificity of the IgG fractions and of the affinity-purified antibody was confirmed in blot transfer experiments after SDS-PAGE electrophoresis using both pure enzyme preparations and a partially-purified yeast extract: no additional forms of the enzyme were detected, since only the 39 kDa band corresponding to the molecular weight of purified protein monomer reacted specifically; faint additional bands can be ascribed to degradation products (fig. 2). No aspecific bands were detected when preimmune serum was used; in the same experiments, the two enzyme forms cross-reacted with IgG_A or

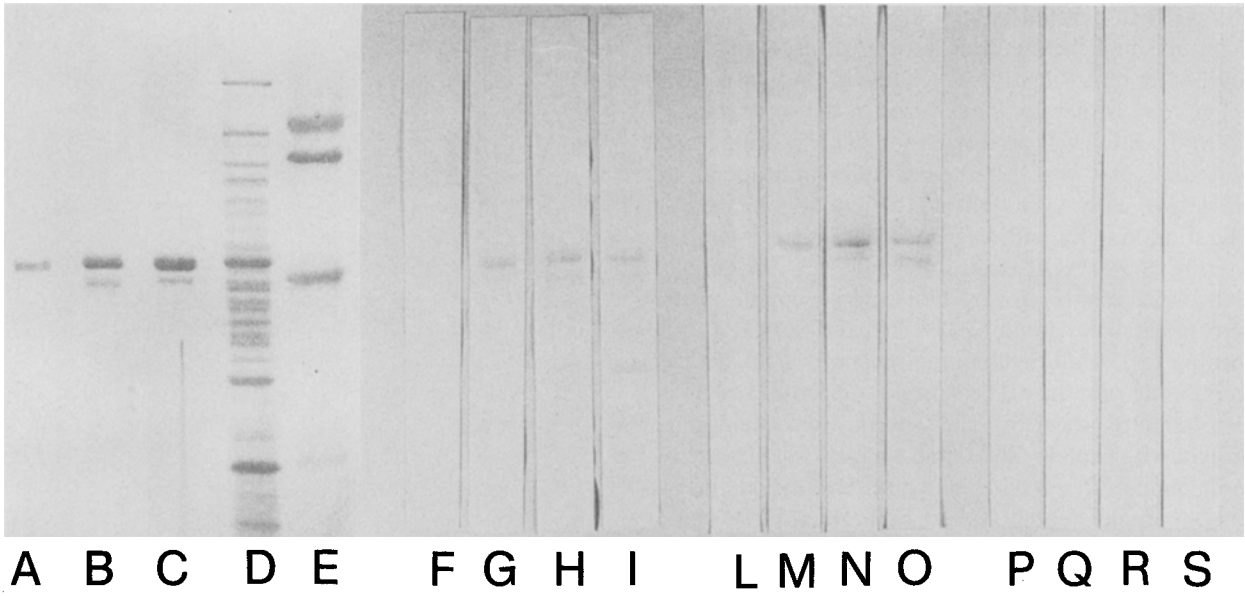

Figure 2. Immunoblot of yeast crude extract and purified DAAO. Total proteins were stained with Amido Black (lanes A–E) and DAAO was detected as described in Materials and Methods. Lanes F, G, H and I were treated with anti-holo DAAO IgG diluted 1:100 in blocking solution; lanes L, M, N and O were treated with anti-apo DAAO diluted as above; lanes P, Q, R and S were treated with corresponding preimmune IgG. (A, F, L and P) 2.5 µg/each of pig kidney DAAO; (B, G, M and Q) 2.5 µg/each of holo-D-amino acid oxidase; (C, H, N and R) 2.5 µg/each of apo-D-amino acid oxidase; (D, I, O and S) 100 µg/each of *R. gracilis* crude extract; (E) 3 µg/each of Pharmacia standard weight proteins: albumin (67 k), catalase (60 k), lactate dehydrogenase (36 k) and ferritin (18.5 k).

IgG_H, respectively. DAAO purified from pig kidney was not recognized by antibodies against yeast DAAO, indicating limited homology between the two enzymes.

The holo-DAAO was incubated in the presence of increasing quantities of IgG_H and IgG_A respectively, and the residual activity was measured after 10 min against a control to which preimmune serum had been added (fig. 3A). Quantitatively different inhibition patterns were observed: IgG_H caused 50% inhibition at a higher IgG/DAAO ratio, and IgG_A caused 75% inhibition (fig. 3B, C); this contrasts with the behavior of pig kidney DAAO where the anti-holo and anti-apo antibodies showed no significant differences in their ability to inhibit the enzyme.

In all instances, addition of protein A caused a complete precipitation of the enzyme after a 30-min incubation with either antibody (fig. 3B, C) indicating that the enzyme was present entirely as antibody-enzyme complex. The affinity-purified antibodies showed similar reactivities to their corresponding IgG_H and IgG_A fractions, though at much lower IgG/enzyme ratios (10-fold less). Similar experiments with anti yeast DAAO antibodies on the pig kidney enzyme showed 25–30% activity inhibition and immunoprecipitation (data not shown).

Reactivation of the yeast apoprotein in the presence of excess FAD was investigated in the presence of both apo- and holo-antibodies. The apoprotein is almost completely reactivated by excess FAD, reaching a specific activity of about 90% of that of the original holoenzyme. As shown in the table, when either IgG_H or IgG_A was added to apoprotein, either at the same time or after FAD addition, both exerted the same minimal effect on enzyme reconstitution. When IgG_A was added to the incubation mix before FAD, however, a much more pronounced inhibition of reconstitution was observed, and this was also considerably greater than the inhibitory effect exerted by IgG_H under the same conditions.

The differences in holoenzyme inhibition properties and apoenzyme reconstitution-inhibition properties between the two antibodies prompted us to investigate their reactions with the four peptide fragments produced by CNBr digestion of the pure enzyme.

Blot transfer analysis of these fragments with IgG_H and IgG_A did not reveal sites with a particular antigenicity relating to linear sequence epitopes; all four fragments were recognized by both the types of antibody (data not shown).

That a catalytically active precipitate is obtained in the Ouchterlony double-diffusion test seems to be confirmed by the experiments shown in figure 3, where a clear effect of antibodies on the enzyme activity is detectable; the

Effect of anti-holo- and anti-apoenzyme antibodies on reactivation of DAAO apoprotein by excess FAD

Time of IgG addition to FAD-apoprotein incubation	Preimmune IgG	IgG_H	IgG_A
Before FAD addition	100.0	73.7	56.5
Together with FAD addition	100.0	76.2	81.5
After FAD addition	100.0	79.1	82.0

Enzymatic activity was measured in the presence of 5 µM FAD after 10 min incubation of 0.01 units of apoenzyme at room temperature with IgG_H and IgG_A (mg IgG/U DAAO = 20).

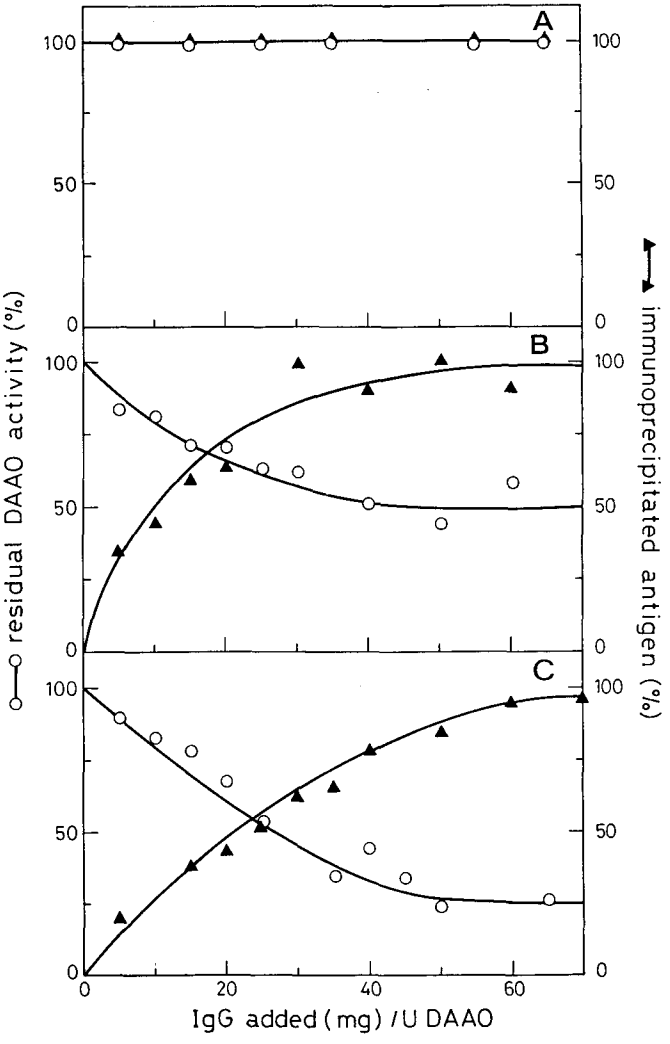

Figure 3. Inactivation and protein A-aided immunoprecipitation of holo-DAAO by multispecific IgG: *A* normal serum; *B* anti-holoenzyme IgG; *C* anti-apoenzyme IgG. 0.017 units of enzyme were incubated with increasing amounts of antibody in 10 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1% bovine serum albumin and 10% glycerol added to give final volumes equal to that of a control incubation mixture without antibody. After 10 min incubation at room temperature, an aliquot of sample was removed and assayed for enzyme activity (○); protein A immunoprecipitation was performed on the remaining samples as described in Materials and Methods; enzyme activity was then assayed on the supernatants after centrifugation (▲). 100% activity was defined as the enzyme activity of a control sample where the antibody solution had been replaced by buffer.

observed 50% residual activity persisted even at higher antibody-enzyme ratios, though it was shown that all the enzyme was present as antibody-enzyme complex. Under very similar conditions the pig kidney enzyme was also incompletely inactivated[19,20]. However, while the anti-holo- and the anti-apoprotein antibodies showed identical reactivity for pig kidney DAAO, our results indicate that, in addition to there being common epitopes in the two forms of the enzyme, distinct epitopes connected with the FAD-binding domain (presumably involving the N-terminal region of the protein[21]) are probably present on the two proteins, or an altered exposure of the same epitopes might occur. The differences between the yeast and pig kidney DAAO include, as our data show, differences in the FAD binding domain. The low cross-activity between the mammalian and yeast enzymes with respect to their corresponding antibodies, as revealed by the inactivation experiments and blot transfer analyses, is a further demonstration of their lack of close similarity. It may be supposed that this lack of similarity is a reflection of different biochemical roles played by these enzymes. We note finally that the very high specificity of the antibodies raised against *Rhodotorula gracilis* DAAO suggest that they could be used as markers to investigate the sub-cellular compartmentalization of this enzyme, and thus contribute to further elucidation of its metabolic role.

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