

An Investigation on the Quinoprotein Nature of Some Fungal and Plant Oxidoreductases*

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The presence of pyrroloquinoline quinone (PQQ) as the organic cofactor of *Dactylium dendroides* galactose oxidase and lentil (*Lens culinaris*) seedling amine oxidase, purported PQQ-containing oxidoreductases (Van der Meer, R. A., Jongejan, J. A., and Duine, J. A. (1989) *J. Biol. Chem.* 264, 7792-7794; Citro, G., Verdina, A., Galati, R., Floris, G., Sabatini, S., and Finazzi-Argo', A. (1989) *FEBS Lett.* 247, 201-204), was reinvestigated using the nitro blue tetrazolium redox-cycling method (Paz, M. A., Gallop, P. M., Torrelío, B. M., and Flückiger, R. (1988) *Biochem. Biophys. Res. Commun.* 154, 1330-1337; Paz, M. A., Flückiger, R., Boak, A., Kagan, H. M., and Gallop, P. M. (1991) *J. Biol. Chem.* 266, 689-692) and the enzyme-linked immunosorbent assay with polyclonal antibodies against PQQ. The possible quinoprotein nature of the laccases from *Polyporus versicolor* and *Rhus vernicifera* was also investigated because of the similarities in spectroscopic and kinetic features of these enzymes and the laccase from *Phlebia radiata*, reported to be a PQQ protein (Karhunen, E., Niku-Paavola, M.-L., Viikari, L., Haltia, T., Van der Meer, R. A., and Duine, J. A. (1990) *FEBS Lett.* 267, 6-8). The presence of a quinonoid cofactor in lentil seedling amine oxidase is confirmed, whereas galactose oxidase and both laccases do not display any quinoprotein nature.

Since the discovery of quinoproteins as a novel class of dehydrogenases containing the organic cofactor pyrroloquinoline quinone (6), many prokaryotic and eukaryotic oxidoreductases were reported to contain PQQ¹ (7). This cofactor was reported to occur in dehydrogenases, oxidases, oxygenases, hydratases, and decarboxylases (8). Methylophilic bacteria are able to synthesize free PQQ from L-glutamic acid and L-tyrosine as precursors (9, 10). The synthesis of this cofactor in eukaryotic enzymes is supposed to occur *in situ* (10, 11). Whether eukaryotic cells synthesize PQQ has yet to be established, and indeed, the nutritional value of pyrroloquinoline quinone as a vitamin or growth factor has been already pointed out (12).

Eukaryotic quinoproteins were reported to contain covalently bound PQQ (13). Subsequently, a different structure, termed pro-PQQ, was proposed as the quinonoid cofactor of bacterial methylamine dehydrogenase (14, 15) and was suggested to be the genuine cofactor of all enzymes previously reported to contain covalently bound PQQ (16). Recent experiments have demonstrated that the redox prosthetic group of methylamine dehydrogenase is tryptophan tryptophylquinone instead of PQQ or pro-PQQ (17). Furthermore, other proteins originally proposed to contain covalently bound PQQ have been shown to contain 3,4,6-trihydroxyphenylalanine (6-hydroxydopa) (TOPA) instead of PQQ (18). In addition, some putative quinoproteins have recently been found to contain neither PQQ nor any other quinonoid cofactor (19-22).

In light of the apparent controversies regarding the quinonoid cofactors and the methodology to detect their presence, we studied the quinoprotein nature of some fungal and plant oxidoreductases. Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9), secreted by the fungus *Dactylium dendroides*, is a monocopper enzyme that catalyzes the oxidation of the primary alcohol to aldehyde by reducing O₂ to H₂O₂ (23). Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) from the fungus *Polyporus versicolor* and the Japanese lacquer tree *Rhus vernicifera* are multicopper oxidases containing a blue (or type 1) copper site; they oxidize a wide range of substrates with a four-electron reduction of oxygen to water (24). Lentil (*Lens culinaris*) seedling amine oxidase (amine:oxygen oxidoreductase, EC 1.4.3.6) belongs to the class of copper-containing amine oxidases that oxidize only primary amines producing the corresponding aldehyde, NH₃, and H₂O₂ (25).

Galactose oxidase and lentil seedling amine oxidase, previously reported to contain PQQ as the organic cofactor (1, 2), were reinvestigated by the redox-cycling assays (3, 4) and the use of anti-PQQ polyclonal antibodies (2). The study of the possible quinoprotein nature of *P. versicolor* and *R. vernicifera* laccases was attempted because of their similarities to the laccase from the white-rot fungus *Phlebia radiata*, claimed to be a PQQ enzyme (5).

MATERIALS AND METHODS

Chemicals were of the purest analytical grade. Nitro blue tetrazolium (NBT), PQQ, TOPA and bovine serum albumin were purchased from Sigma. Nitrocellulose filters (0.45 µm) were from Bio-Rad. All solutions were prepared by using MILLI-Q water filtered through a five-cartridge system (Millipore).

Enzymes—Galactose oxidase from *D. dendroides* was purchased from Sigma. Laccases from *P. versicolor* and *R. vernicifera* were a gift from Dr. B. G. Malmström (University of Göteborg). Lentil seedling amine oxidase, purified to homogeneity from *L. culinaris* as described (26), was a gift from Dr. A. Finazzi-Agro' (University of Rome "Tor Vergata").

Electrophoresis and Electroblothing—SDS-polyacrylamide gel electrophoresis (12%) was performed under reducing conditions (27)

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¹ The abbreviations used are: PQQ, pyrroloquinoline quinone; TOPA, 3,4,6-trihydroxyphenylalanine; BSAr, NaBH₄-reduced bovine serum albumin; NBT, nitro blue tetrazolium; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

using a Mini Protean II apparatus (Bio-Rad) with 0.75-mm spacer arms. All samples (40 μ g each) were heated at 95 °C for 15 min before being loaded. The electrophoretic runs were stopped as soon as the bromophenol blue dye reached the bottom of the gel, and then proteins were transferred to the nitrocellulose filter for 2 h at 100 V. The transfer was performed with a Mini Trans Blot apparatus (Bio-Rad), cooled to 4 °C, in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3, essentially following a published procedure (28). Gel proteins were stained using 0.2% Coomassie Brilliant Blue R-250.

NBT Staining—Soon after the transfer, the nitrocellulose filter was subjected to quinoprotein-specific staining based on the NBT/glycinate redox-cycling procedure in the dark (3, 4). The membrane was incubated for 25 min at room temperature in 0.24 mM NBT, 2 M potassium glycinate solution, pH 10.0, and was then dipped in 100 mM sodium borate, pH 10.0. Quinoproteins were specifically stained as purple-blue bands due to NBT reduction to formazan. When the nitrocellulose filter was counterstained based on the negative staining procedure (29), proteins other than quinoproteins appeared as yellow bands on a blue background.

Spectrophotometric Assays—The quinoprotein nature of galactose oxidase, *P. versicolor* and *R. vernicifera* laccases, and lentil seedling amine oxidase was further investigated by using the direct and amplified assay methods previously described (3, 4). Direct assay was performed in the dark by incubating overnight at 37 °C different amounts of each protein (ranging from 0.25 to 1.0 μ M) with its specific substrate at saturating concentrations, namely 1 M galactose (galactose oxidase), 20 mM coniferyl alcohol (*P. versicolor* and *R. vernicifera* laccases), and 1.2 mM putrescine (lentil seedling amine oxidase). Reaction mixtures always contained 0.25 mM NBT in 0.1 M NaCO₃, pH 9.5 (1.0-ml final volume), and 0.5 mg of BSA (bovine serum albumin reduced under N₂ with 100-fold molar excess of NaBH₄ and dialyzed overnight at 4 °C against deionized water, previously degassed and flushed with N₂). Controls were made by incubating reaction mixtures containing substrates alone or enzymes alone. After overnight incubation, the A₅₃₀ values were recorded with a Hewlett-Packard 8450A double-beam diode-array spectrophotometer.

Amplified detection was performed by either denaturing the samples with overnight incubation in 2% SDS at room temperature or reducing the samples with NaBH₄ (same conditions as described for BSA). Different amounts of each denatured (or reduced) enzyme, ranging from 25 to 200 nM, were then incubated for 1 h at 37 °C in 1.0 ml of 2 M sodium glycinate, pH 10.0, 0.24 mM NBT in the dark; and eventually, the absorbances at 530 nm were recorded. Control experiments included bovine serum albumin (in the range of 25–200 mM) and reaction mixtures without enzymes.

Antibodies—Polyclonal antibodies against PQQ were raised in rabbits and prepared according to the procedure of Citro *et al.* (2). Rabbit nonimmune serum, anti equine chorionic gonadotropin polyclonal antibodies (a gift from Dr. G. W. K. van Dedem (Diosynth B. V., Oss, The Netherlands), and the anti-lipoxygenase antiserum (prepared according to Ref. 30) were used as control antibodies.

Enzyme-linked Immunosorbent Assay—ELISA was performed as previously described (31). PQQ/BSAr or TOPA/BSAr was prepared as reported (3, 20) by mixing different volumes of 45 μ M solutions of PQQ or TOPA (freshly prepared with deionized water, degassed and flushed with N₂) with 50 μ l of BSAr (2 mg/ml). These model compounds were immediately used to coat each well of polystyrene 96-well Nunc Immuno plate (GIBCO) and covered the range of 450–1450 pmol of quinonoid cofactor. The anti-PQQ polyclonal antibodies (100 μ l, diluted 1:100) were added to each well, and then goat anti-rabbit IgGs conjugated with alkaline phosphatase (Bio-Rad) were used as second antibody (diluted 2000-fold) with *p*-nitrophenyl phosphate as substrate. Plates were read at 405 nm by means of an EAR 400 ELISA plate reader (SLT-Lab Instruments).

Native galactose oxidase, *P. versicolor* and *R. vernicifera* laccases, and lentil seedling amine oxidase were subjected to the same test. The amounts of each enzyme used to coat the wells were in the same range of cofactor concentration as the model compounds based on a PQQ:enzyme molar ratio of 1:1 (1, 2, 5). Controls were made by performing the same ELISA tests with the aspecific polyclonal antibodies instead of the anti-PQQ ones. Controls also included different amounts of BSAr alone.

RESULTS

NBT Staining of Quinoproteins Electroblotted onto Nitrocellulose Filters—The oxidoreductases under investigation (galactose oxidase, *P. versicolor* and *R. vernicifera* laccases,

and lentil seedling amine oxidase) were subjected to electrophoresis followed by Coomassie staining before (Fig. 1, upper) and after (lower) transfer to the nitrocellulose filters. All proteins were transferred almost completely to the nitrocellulose, indicating the adequacy of the electroblotting.

When the nitrocellulose filter was subjected to quinoprotein-specific NBT/glycinate staining, only lentil seedling amine oxidase reacted, whereas none of the other samples did (Fig. 2, upper). The counterstaining of the same filter showed proteins other than quinoproteins as yellow bands on a blue background, whereas lentil seedling amine oxidase remained blue-purple (Fig. 2, lower).

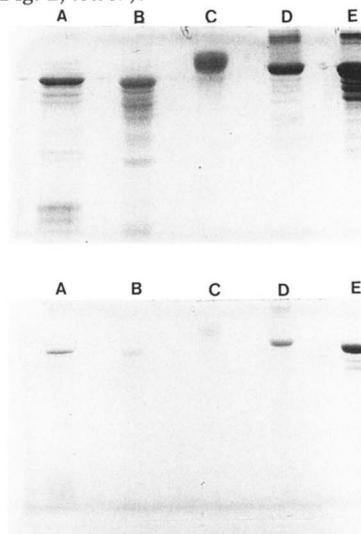


FIG. 1. Coomassie staining of polyacrylamide gel. Proteins were stained before (upper) and after (lower) transfer to the nitrocellulose filter. 40 μ g of the following proteins/lane were loaded: lane A, galactose oxidase; lane B, *R. vernicifera* laccase; lane C, *P. versicolor* laccase; lane D, lentil seedling amine oxidase; lane E, bovine serum albumin (used as control).

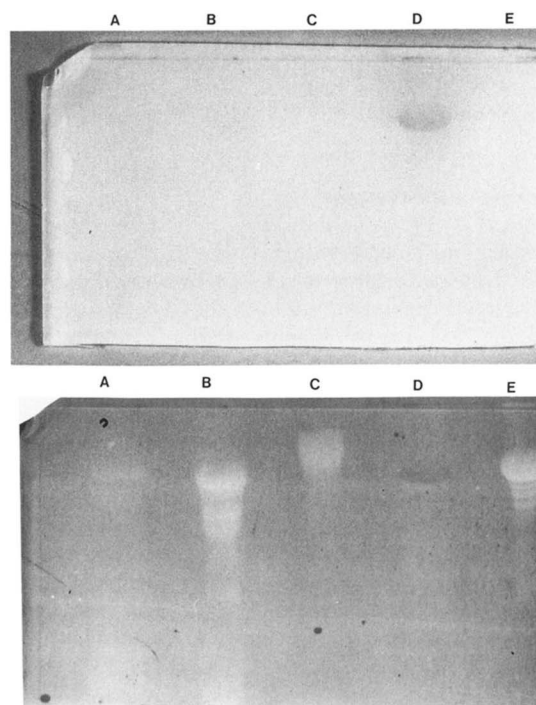


FIG. 2. Quinoprotein-specific staining of polyacrylamide gel. Upper, NBT/glycinate staining of the nitrocellulose membrane after electrotransfer of the same samples as described for Fig. 1; lower, counterstaining of the nitrocellulose filter (cf. upper).

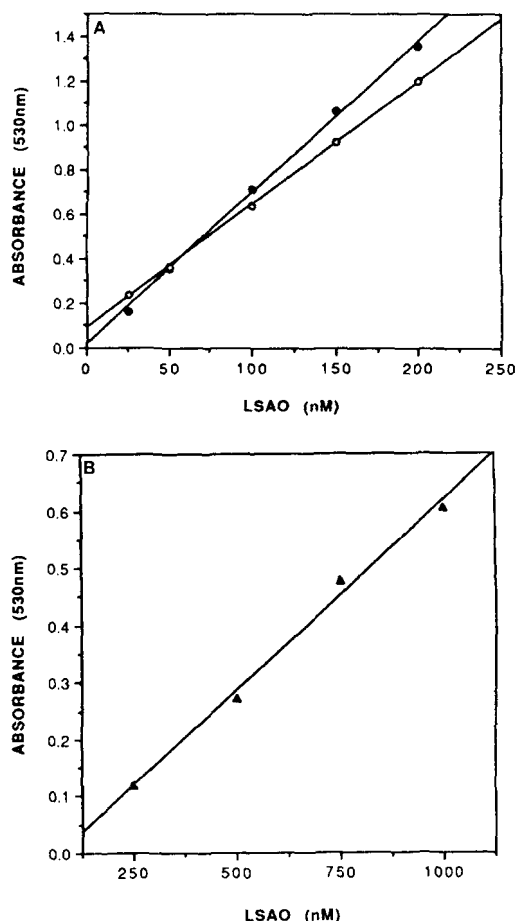


FIG. 3. Spectrophotometric assays of quinoproteins. *Upper*, Amplified detection of the quinonoid cofactor of lentil seedling amine oxidase. ●, SDS-denatured lentil seedling amine oxidase; ○, NaBH_4 -reduced lentil seedling amine oxidase. Galactose oxidase and *P. versicolor* and *R. vernicifera* laccases did not show any increase in the A_{530} values when subjected to the same treatments as lentil seedling amine oxidase. *Lower*, direct assay of native lentil seedling amine oxidase. Analysis of the native forms of galactose oxidase and *P. versicolor* and *R. vernicifera* laccases was not feasible with this procedure (*cf.* "Results"). The experimental points represent the mean values of three different determinations (S.D. < 4%).

Spectrophotometric Redox-cycling Assays—The quinoprotein nature of galactose oxidase, *P. versicolor* and *R. vernicifera* laccases, and lentil seedling amine oxidase was further investigated by performing spectrophotometric assays. The amplified detection showed that both the SDS-denatured and the NaBH_4 -reduced lentil seedling amine oxidases reacted positively by yielding an increase in the A_{530} values proportional to the amounts of protein tested (Fig. 3, *upper*). However, none of the other oxidases reacted under the conditions outlined in Fig. 3 (*upper*).

When direct assay was attempted, native lentil seedling amine oxidase displayed a linear relationship between the A_{530} values and the protein concentration (Fig. 3, *lower*). Direct assays were not feasible for galactose oxidase and *P. versicolor* and *R. vernicifera* laccases because of the high formazan production due to the substrates alone (galactose and coniferyl alcohol) at the concentrations required for enzyme activity.

Immunochemical Detection—The possible presence of a quinonoid cofactor was investigated by testing the native oxidoreductases in ELISA. A proportional increase in color development was observed with increasing amounts of PQQ/BSAr or TOPA/BSAr when the anti-PQQ polyclonal antibodies were used (Fig. 4, *upper*). A linear relationship was

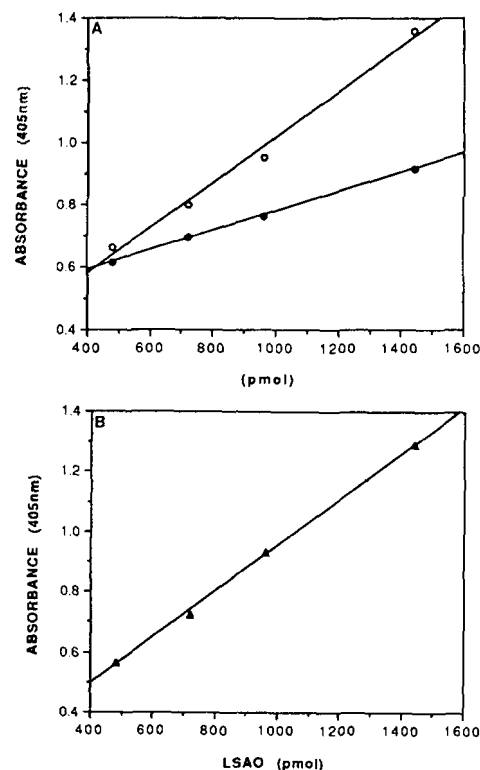


FIG. 4. Immunochemical detection of quinonoid cofactors. *Upper*, reaction of the model compounds PQQ/BSAr (○) and TOPA/BSAr (●) with the anti-PQQ polyclonal antibodies; *lower*, same ELISA test as described (*upper*) performed on native lentil seedling amine oxidase. Galactose oxidase and *P. versicolor* and *R. vernicifera* laccases in their native forms were not recognized by the antibodies against PQQ. These experiments were carried out in triplicate (S.D. < 4%).

also found when lentil seedling amine oxidase was subjected to the same ELISA test (Fig. 4, *lower*); none of the other oxidoreductases showed any reactivity to the antibodies against PQQ. Control experiments with different aspecific antibodies indicated no reactivity at any dilution used. BSAr alone did not react.

DISCUSSION

The quinoprotein nature of galactose oxidase, *P. versicolor* and *R. vernicifera* laccases, and lentil seedling amine oxidase was investigated by different methods, namely (a) NBT/glycinate staining of SDS-polyacrylamide gel electrophoresis electroblots, (b) direct assay, (c) amplified detection based on the redox-cycling method (3, 4), and (d) enzyme-linked immunosorbent assay (2). Despite controversies on the ability of the redox-cycling method to quantitate PQQ and quinonoid compounds (32–35), a general agreement exists on its reliability for the qualitative detection of quinoproteins (36). We used this procedure for the detection of PQQ in soybean lipoxygenase 1, previously reported to be a PQQ enzyme (13); and our results, indicating the absence of any quinonoid cofactor in the protein (20), have been recently confirmed using different experimental approaches (37).

Electrophoretic NBT staining showed the absence of PQQ or related cofactors in galactose oxidase from *D. dendroides*. Because the conditions during electrophoresis/electroblotting may be unfavorable for the detection of all PQQ loci, spectrophotometric assays were performed; but once again, the denatured and reduced galactose oxidase did not behave as a quinoprotein. Additionally, recognition of any quinonoid co-

factor in the native enzyme by the specific antibodies failed. These data are in contrast with the recently reported presence of PQQ in galactose oxidase (1). New data derived from the x-ray absorption near-edge structure spectra of galactose oxidase (38) do not seem to favor the mechanistic model proposed in Ref. 1 for the catalytic cycle of the enzyme. Moreover, results from the x-ray crystallographic study of galactose oxidase at 1.7-Å resolution fail to support the presence of PQQ (22).

The laccases from *P. versicolor* and *R. vernicifera* yielded the same results as the galactose oxidase, indicating the absence of a quinonoid cofactor in these proteins as well. This finding is interesting since there are close similarities in the EPR spectroscopic features (39, 40) and reaction mechanisms (41, 42) of these enzymes and the laccase from *P. radiata*, reported to be a quinoprotein containing covalently bound PQQ (5).

At variance with galactose oxidase and *P. versicolor* and *R. vernicifera* laccases, lentil seedling amine oxidase reacted positively to the redox-cycling assays both in the native and the denatured or reduced forms. This evidence, in addition to confirming the quinoprotein nature of lentil seedling amine oxidase (2), indicates that the cofactor must be easily accessible to the reactants and to the natural substrate (putrescine), a feature this protein shares with the porcine kidney diamine oxidase (3). The immunochemical detection of native lentil seedling amine oxidase corroborated the redox-cycling assays.

It is not possible to identify the cofactor as PQQ because the redox-cycling method cannot discriminate between PQQ- and TOPA-containing quinoproteins (4) and neither can the anti-PQQ polyclonal antibodies. The ability of these antibodies to recognize TOPA, albeit to a lesser extent, is not surprising in light of the structural similarities between the two cofactors (18). New data based on the EPR analysis of lentil seedling amine oxidase clearly indicate the presence of TOPA, instead of PQQ (2), in the enzyme.² Similar evidence has been presented for other plant amine oxidases (43). In conclusion, we confirmed the quinoprotein nature of lentil seedling amine oxidase and showed that galactose oxidase and two laccases do not contain any quinonoid cofactor.

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² A. Finazzi-Agró, personal communication.