Pyrroloquinoline Quinone as Cofactor in Galactose Oxidase (EC 1.1.3.9)*

(Received for publication, November 29, 1988)

Robert A. van der Meer, Jacob A. Jongejan, and Johannis A. Duine

From the Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Galactose oxidase from Dactylium dendroides was shown to contain one molecule of covalently bound pyrroloquinoline quinone (PQQ)/enzyme molecule. From the spectroscopic characteristics reported for the enzyme forms, a mechanistic role for PQQ could be deduced. In analogy with other quinoproteins, the initial formation of a PQQ-substrate adduct is proposed. Following internal hydrogen transfer, leading to aldehyde product and reduced pyrroloquinoline quinone, reoxidation of the organic cofactor with molecular oxygen could be mediated by the PQQ-ligated copper ion with concomitant formation of hydrogen peroxide. With PQQ as an additional (two-electron) redox center and the occurrence of a "superoxidized" enzyme form must be considered. Possible consequences of this view, in relation to a physiological function of the enzyme and interpretation of ESR data, are discussed.

Galactose oxidase (GAO, EC 1.1.3.9) is a fungal enzyme catalyzing the oxidation of the primary alcohol function of several carbohydrates and aliphatic alcohols to the corresponding aldehyde. The enzyme form that is excreted into the medium shows a high specificity for galactose. Until now, the enzyme was considered to be a type 2 mono-copper protein containing no additional cofactor. Obviously, as a monomeric enzyme, activation was shown to occur to a form (GAOa) of GAO that was separated and washed with 200-ml portions of water until neutral.

Recently, Whittaker and Whittaker (1) reported the characterization of three spectroscopically distinct redox forms of GAO. In addition, they were able to show that enzyme preparations that are obtained by routine procedures contain variable amounts of an inactive form of the enzyme (GAOin). By raising the redox potential of solutions containing inactive enzyme, activation was shown to occur to a form (GAOa) with distinct properties. Substrate addition to GAOin produced a third enzyme form, GAOme. From these findings, the authors (1) concluded that an additional redox center might be involved. Since no other cofactor was thought to be present, investigation of an amino acid residue was proposed.

Until recently, a similar situation existed for soybean lipoygenase 1 (EC 1.13.11.12). The enzyme is also isolated in an inactive form, activation requiring treatment with the peroxidase product, and the proposals for the catalytic mechanism assuming the involvement of only a single iron ion. It appears now that this view is incorrect, the iron ion being coordinated to certain sites of the cofactor PQQ, the latter covalently bound to the protein chain. This complex forms an electron relay system allowing electron removal from the substrate followed by stereoselective O2 insertion (2). Since the presence of PQQ as cofactor has apparently been overlooked in this well studied iron-protein as well as in several copper-proteins (bovine serum amine oxidase (3), porcine kidney diamine oxidase (4), human placental l-lysyl oxidase (5), dopamine β-hydroxylase from bovine adrenal medulla (6)), the possibility was considered whether the second redox center in GAO could be PQQ instead of an amino acid residue.

MATERIALS AND METHODS

Purification of GAO

In view of the reported (7) presence of proteolytic enzymes and other impurities, a commercial enzyme preparation from Dactylium dendroides (Sigma) was purified according to a published procedure (8), using affinity chromatography on Sepharose 6B (Pharmacia LKB Biotechnology Inc.). Enzyme activities were determined (after redox activation) at 25 °C by measuring the rate of oxygen consumption with a Clark electrode cell and galactose as substrate (9). Redox-activated GAO was prepared by adding K2Fe(CN)6 (to 50 mM) in the enzyme solution (0.1 M sodium phosphate buffer, pH 7.0) and removing the redox agent by gel filtration.

The amount of purified GAO was calculated using the specific absorption coefficient determined by Kosman et al. (10) (A280nm = 15.4).

Determination of PQQ

Determination of PQQ in homogeneous preparations of GAO was performed by a two-step procedure (a and b). The intermediate, PQQ-5,5-dihexyl ketal, was also characterized by comparison with an authentic sample (c).

a. Extraction of Cofactor as PQQ-5,5-dihexyl Ketal—A mixture of GAO (4.8 mg) in 0.1 M sodium phosphate, pH 7.0 (7.5 ml), 8 M hydrochloric acid (7.5 ml), and 1-hexanol (5 ml) was boiled under reflux for 2 h. After cooling to room temperature the organic layer was separated and washed with 200-ml portions of water until neutral. The hexanol was evaporated under reduced pressure at elevated temperature. Traces of hexanol were removed by keeping the residue overnight under vacuum over phosphorous pentoxide. The dried preparation was dissolved in 10 mM sodium phosphate buffer, pH 7.0 (4 ml).

b. Conversion into PQQ—Conversion of the PQQ-5,5-dihexyl ketal into PQQ was effected by hydrolysis at high pH (approximately 2 M sodium carbonate) at 90 °C for 2 h. The resulting free PQQ was quantified by a biological assay (11) after neutralization with 6 M HCl.

c. Characterization of the Intermediate PQQ-5,5-dihexyl Ketal—Characterization of the PQQ-5,5-dihexyl ketal was accomplished by comparing its visible and 1H NMR spectral properties with authentic material, prepared by heating synthetic PQQ in excess hexanol with trace amounts of p-toluenesulfonic acid using a water trap. High pressure liquid chromatography of the preparation obtained after work-up as described for the product extracted from GAO revealed the presence of small amounts of hexyl esters in addition to the 5,5-dihexyl ketal.

d. Determination of Noncovalently Bound PQQ—The presence of noncovalently bound PQQ was checked by denaturing GAO with...
RESULTS AND DISCUSSION

Evidence for PQQ—The purification step yielded an enzyme preparation with a specific activity of 135 μmol of oxygen consumed per min " per mg protein", comparable with the value (140) ascribed to a homogeneous GAO preparation (1).

Extraction of GAO with the sodium dodecyl sulfate procedure (d) did not reveal any PQQ so that a method suited to detect covalently bound cofactor seemed necessary to detect the presumed presence of PQQ. In view of the reported (1, 9) heterogeneity in redox state of GAO preparations, the commonly used hydrazine method (13) in our laboratory was considered inappropriate because it requires derivatization of the cofactor in its oxidized state. A recently developed method (14), the so-called hexanol extraction procedure, seemed more attractive since it is based on removal of PQQ under denaturing conditions, enabling oxidation to occur of any reduced cofactor. Briefly, the rationale behind the method is the following. The higher aliphatic alcohol, hexanol, adds to PQQ at the C-5 position, forming a ketal, and provides a high temperature during refluxing so that HCl hydrolysis proceeds in an efficient way; the protonated PQQ-5,5-dihexyl ketal is soluble in the organic layer, escaping unwanted attack by other nucleophilic compounds (amino acids liberated from the protein).

Application of the hexanol extraction procedure to GAO yielded a compound which was identical to PQQ-5,5-dihexyl ketal, as judged from the spectral and chromatographic data (Fig. 1). To obtain further proof for the identity of the extracted compound, hydrolysis (b) was attempted. Using a biological assay for PQQ (11), the procedure was optimized, and in a typical experiment (with conditions described under "Materials and Methods"), a quantity of 66 nmol of PQQ per 4.8 mg of protein was found. Based on a M, of 70,000 (1), it appears that 0.96 PQQ is present per enzyme molecule. Thus GAO contains at least 1 PQQ/enzyme molecule. Hydrolysis is judged to be quantitative since a similar value was found on calculating the amount of extracted ketal compound, using a molar absorption coefficient of 8,700 M⁻¹·cm⁻¹ at 357 nm (determined for the PQQ-5,5-dihexyl ketal model compound).

Spectroscopic Features—Given the finding of PQQ in GAO, its presence should be traceable in the reported spectroscopic data of the enzyme. Since this is indeed the case and the data could give information on a possible interaction and role of the two cofactors, each enzyme form is discussed separately.

GAO∞ is an ESR-silent enzyme form with respect to Cu²⁺ but not to organic free radicals since it shows three very sharp signals in the g = 2 region (1). Assuming rhombic splitting, these could originate from PQQH⁻ (gₘ = 2.0043 (15)) but not from a plausible candidate like the free radical of a tyrosine residue. Since only 1% of the theoretically possible amount is observed and the free radical content does not change on substrate addition, the significance is not obvious at first sight. Assuming that it is not an artifact, the observation could be indicative for the presence of a complex of the copper ion and PQQ. For instance, the tiny amount of PQQH⁻ could result from dissociation of a complex consisting of Cu²⁺ and PPQH⁻, coupled anti-ferromagnetically. However, in view of the spectral properties, the formally overall equivalent redox couple, Cu²⁺/PQQ, seems a better candidate.

So on inspection of the absorption spectrum of this enzyme form, showing an intense peak around 445 nm, it appears that the maximum and the overall shape of the spectrum are strikingly similar to those of the quinoprotein (PQQ-containing) methylamine dehydrogenase (EC 1.4.99.3) in its fully oxidized form (16) (except at very high wavelengths around 800 nm, possibly related to the fact that methylamine dehydrogenase contains covalently bound PQQ (17) but no copper ions). The redox potential estimated for this enzyme form

![FIG. 1. Chromatograms (left) and absorption spectra (right) of: a, adduct isolated from GAO; b, the PQQ-5,5-dihexyl ketal model compound. The samples were injected on a 5-μm C₇ RCM cartridge in a Waters RCM 100 module. The eluent (flow rate, 1.5 ml/min) consisted of a linear gradient (20 min) of methanol from 7 to 63% in 0.4% H₃PO₄ (v/v). The eluate was monitored at 357 nm with a Hewlett-Packard 1040 A photodiode-array detector taking absorption spectra throughout the peaks.](image1)

![FIG. 2. Structure of PQQ, its reduced forms (PQQH⁻, PQQH₂), its adducts (R = –OH, hydrated PQQ (PQQ·H₂O); R = –CN, cyanide adduct; R = –O–C₆H₅, ethanol adduct), and ketals (R = –O–C₆H₁₃).](image2)

![SCHEME 1. Catalytic cycle proposed for the oxidation mechanism of GAO.](image3)
(between 0 and +100 mV (1)), is also in accordance with the proposed redox system (the PQQ/PQQH$_2$ couple has a redox potential of +90 mV at pH 7.0 (18)).

GAO$_{av}$ is produced from GAO$_{av}$ under reducing conditions (1). Since the copper ion is fully visible with ESR spectroscopy as Cu$^{2+}$, but no organic free radical was observed with this technique, the redox system should consist of Cu$^{2+}$/PQQH$_2$. The significant shoulder observed in the absorption spectrum around 340 nm is indeed indicative for PQQH$_2$ (the fully reduced forms of methylamine dehydrogenase and methanol dehydrogenase have maxima at 326 and 340 nm, respectively (16, 19)). From model studies it is known, however, that PQQH$_2$ becomes immediately oxidized by Cu$^{2+}$ (20). Thus, this suggests that the two cofactors in the enzyme are separated from each other (perhaps resulting from a conformational change occurring during the transition of GAO$_{av}$ into GAO$_{av}$). Another possibility, however, is that the Cu$^{2+}$ ion is complexed in such a way that the redox potential becomes lower than that of free PQQ, as has been found for the model system Cu$^{2+}$/bipyridine-PQQ (21).

GAO$_{red}$ is obtained on reaction of GAO$_{av}$ with substrate under anaerobic conditions (1). Based on the redox systems proposed for the other two enzyme forms, GAO$_{red}$ should contain Cu$^{2+}$/PQQH$_2$. The absorption spectrum of this enzyme form is very curious since it is structureless. This could either result from the overlapping of very broad absorption bands due to the interaction of the two cofactors or to a blue shift of its PQQH$_2$ absorption maximum (the maximum of free PQQH$_2$ is at 302 nm (18)), being nondetectable in the protein absorption spectrum of the enzyme form.

Finally, it should be noticed that a fourth enzyme form, containing a Cu$^{2+}$/PQQ system, is theoretically possible. Unfortunately, qualitative and quantitative data on heterogeneity are not available so that clear evidence for its occurrence in reported preparations cannot be given. Perhaps some indications for the existence of such a form can be derived from the fact that cyanide displaces a ligand of Cu$^{2+}$ in GAO which is not an imidazole but which has been proposed to be a phenolate ion (22, 23). H$_2$O, cyanide, alcohols, and many other nucleophilic compounds easily form adducts with the carbonyl group of PQQ at the C-5 position (24) (Fig. 2). The PQQ-H$_2$O adduct has a $pK_a$ value between 7 and 8.$^2$ Therefore, the effects of cyanide and the pH regime on the kinetics and ESR spectra of GAO can be ascribed to the presence of this enzyme form, a close interaction between the two cofactors, perhaps in a configuration as has been proposed for the iron/PQQ couple in lipoxigenase-1 (2).

Mechanism of Action—PQQ is involved as the only cofactor in several types of quinoprotein alcohol dehydrogenases (25), converting the alcohol into an aldehyde under PQQH$_2$ formation. Therefore, it could be easily imagined that it is also the primary cofactor in GAO, abstracting the reduction equivalents from the substrate in a similar way, followed by reoxidation of PQQH$_2$ to PQQ via the Cu$^{2+}$ ion with O$_2$ under H$_2$O formation (see Scheme 1). As mentioned already, spectroscopic characteristics suggest that an interaction of Cu$^{2+}$ with the o-quinone moiety of PQQ occurs in GAO. Additional evidence is provided by the fact that cyanide and galactose compete for the same binding site (23), most probably the C-5 carbonyl group of PQQ, while cyanide has a pronounced effect on the ESR spectrum.

As mentioned already, the existence of superoxidized GAO has not been reported so far, although some evidence can be derived from ESR studies. If this enzyme form reacts with substrate, GAO$_{av}$ should be the resulting enzyme form (Scheme 1). The point of varying amounts of GAO$_{av}$ in purified preparations raises, therefore, the question as to which enzyme form occurs in vivo and whether GAO is a genuine oxidase or in fact a dehydrogenase, fortuitously detached from the membranes on cell lysis.

REFERENCES