Bovine serum amine oxidase: a mammalian enzyme having covalently bound PQQ as prosthetic group

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In addition to the metal ion, copper-containing amine oxidases possess an organic prosthetic group, the nature of which has long been controversial. We show here that in the case of bovine plasma amine oxidase, this second prosthetic group is covalently bound pyrroloquinoline quinone (PQQ). Until now the coenzyme PQQ has been found in several bacterial dehydrogenases. Thus the finding reported here is the first example of a quinoprotein oxidoreductase discovered in a eukaryotic organism.

Bovine serum amine oxidase *Pyrroloquinoline quinone (PQQ)* *Quinoprotein* *Copper-containing amine oxidase* *Prosthetic group*

1. INTRODUCTION

Oxidative deamination of amines is catalyzed by two groups of enzymes. One is the class of copper-containing amine oxidases (e.g., EC 1.4.3.6), in which a covalently bound prosthetic group can also be detected after removal of copper [1]. It is generally accepted that this prosthetic group has a carbonyl function since the absorption spectrum of the enzyme changes on addition of carbonyl-group reagents like hydrazine [2,3]. As the maximum in the spectrum of the modified enzyme is similar to that of pyridoxal phosphate (PLP)-containing enzymes treated with a hydrazine [4], this observation has played a suggestive role in the formation of a concept about the nature of the prosthetic group. It should be noted, however, that a PLP-adduct has never been isolated as such from pure enzyme. Furthermore, based on mechanistic studies [5,6], it seems unlikely that PLP is involved in the catalytic cycle, although the difficulties can be circumvented by assuming [7] that copper participates in the redox steps. A quite different proposition, avoiding these difficulties, has been put forward [8], namely that a ring-opened flavin derivative could be the prosthetic group. However, in this respect pyrroloquinoline quinone (PQQ) is also a good candidate, showing carbonyl-group functionality as well as redox behaviour [9]. As no information is yet available on the presence of this coenzyme in eukaryotes, we decided to investigate the possible occurrence of PQQ in bovine serum amine oxidase, a mammalian copper-containing amine oxidase.

2. METHODS

2.1. *Enzyme isolation*

Fresh bovine serum was treated with solid $(NH_4)_2SO_4$ and the fraction precipitating between 30 and 60% saturation was dissolved in and dialyzed against 10 mM potassium phosphate (pH 7.0). This solution was applied to a DEAE–Sepharose column equilibrated with 10 mM potassium phosphate (pH 7.0). After washing with this buffer, the enzyme was eluted with a linear gradient to 0.5 M potassium phosphate (pH 7.0). Active fractions were pooled and dialyzed against 10 mM potassium phosphate (pH 7.0), containing 0.15 M NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The solution was applied to a ConA–Sepharose column, equilibrated with the latter buffer. After

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washing with this buffer, the enzyme was eluted with this buffer, containing 0.5 M α-D-methylglucoside. Active fractions were dialyzed against 10 mM potassium phosphate (pH 7.0) and applied to a Procion red–Sepharose column, prepared as in [10]. After washing with 20 mM Tris–HCl (pH 7.0) containing 5 mM KCl, the enzyme was eluted with 20 mM Tris–HCl (pH 7.0) containing 0.5 M KCl. Active fractions were pooled and dialyzed against 70 mM potassium phosphate (pH 7.0).

2.2. *Modification of the enzyme*

Adduct of the prosthetic group in the enzyme was obtained by incubating 4.5 ml enzyme (14 mg protein) in 70 mM potassium phosphate (pH 7.0) with 80 μl of 2 mM 2,4-dinitrophenylhydrazine (DNP) in 0.1 M HCl at 37°C for 16 h. Excess reagent was removed by gel filtration on a Sephadex G-25 column in 70 mM potassium phosphate, pH 7.0.

2.3. *Proteolysis of modified enzyme*

Modified enzyme (4.5 ml) was denatured anaerobically by adding 1 ml of 8 M urea, 0.3 ml of 3 M Tris–HCl (pH 8.6), and 10 μl β-mercaptoethanol. Free SH-groups were blocked by adding 200 mg β-bromoethylamine. After desalting on a Sephadex G-25 column in 10 mM potassium phosphate (pH 7.5), proteolysis was started by adding 0.3 mg α-chymotrypsin and incubating at 37°C for 48 h. The solution was acidified to pH 3.0 with acetic acid and proteolysis continued by adding 0.3 mg pepsin and incubating at 37°C for 48 h. Thereafter the solution was brought to pH 7.5, 0.3 mg pronase was added and the incubation continued at 37°C for 48 h.

2.4. *Isolation of the adduct*

After proteolysis of the modified enzyme, the solution was brought to pH 5.5, and passed through a Seppak C_{18} cartridge. After washing with 1 M HCl, followed by H_2O, the retained adduct was eluted with methanol/0.01 M potassium phosphate (pH 7.0) (22.5:77.5, v/v).

2.5. *Preparation of the model compound*

PQQ (15 mg) and 37 mg DNP in 4 M HCl were incubated for several hours at 20°C. After centrifugation, the precipitate was dissolved in 0.1 M potassium phosphate (pH 7.0) and the solution passed through a Seppak C_{18} cartridge. The cartridge was washed with 1 M HCl, followed by H_2O, and the model compound eluted with methanol.

2.6. *Analytical procedures*

^{1}H-NMR spectroscopy of the model compound was performed in $(C^2H_3)_2SO$ with a Varian SC 300 spectrometer, operating at 300 MHz, using the pulse Fourier-transform mode (max 32 K data table of 32 bits) and tetramethylsilane as an internal reference.

The HPLC system consisted of a Waters 6000 A pump, equipped with a U6K injection block. Gel filtration was performed on a Serva SI 200 Polyol (5 μm) column and as eluant (1 ml/min) 0.1 M potassium phosphate (pH 7.0). Reversed phase chromatography of the model compound and the adduct was performed with an RCM 100 module containing a 10 μm C_{18} RCM cartridge while the eluant (1.5 ml/min) consisted of methanol/0.01 M potassium phosphate (pH 7.0) (22.5:77.5, v/v). The eluates were monitored with a Hewlett-Packard 1040 A photodiode-array detector, taking absorption spectra of the eluting peaks, upslope, at the top, and downslope, to check homogeneity and identity.

Conversion of PQQ, adduct or model compound into fluorescing product was achieved by adding 25-μl samples to 1 ml of 50% (w/w) NaOH solution in an ampoule. After evacuation and sealing, it was kept at 100°C for 45 min. The ampoule was opened and the contents diluted 10-fold with H_2O. The solution was acidified to pH 2.0 with a concentrated HCl solution and passed through a Seppak C_{18} cartridge. The cartridge was washed with H_2O and the fluorescing compound eluted with methanol. Reversed phase chromatography of the fluorescing compound was performed as above, using methanol/0.4% H_3PO_4 (50:50, v/v) as the eluant. The effluent was monitored with fluorescence detection (Waters model 420 AC, λ_{exc} = 360 nm, and $\lambda_{em} > 418$ nm).

3. RESULTS

3.1. *The model compound*

The product obtained by reacting PQQ with DNP was homogeneous as revealed by HPLC

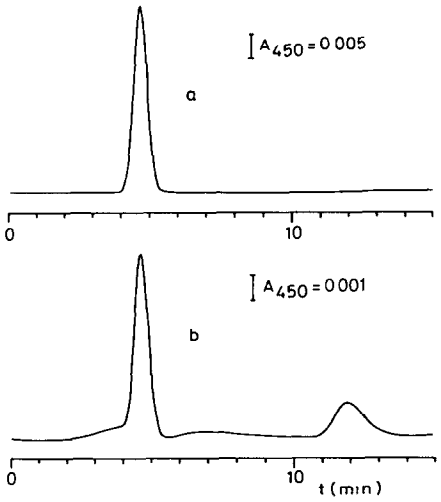
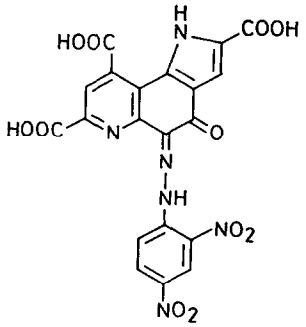


Fig.1. HPLC of the model compound (a) and the adduct isolated from the enzyme (b). The system consisted of a Waters model 6000 A pump, equipped with U6K injection block and an RCM module, containing a 10 μm C18 cartridge. The eluant (1.5 ml/min) was methanol/0.01 M potassium phosphate (pH 7.0) (22.5:77.5, v/v) and was monitored by a Hewlett-Packard 1040 A photodiode-array detector. Peaks were investigated for homogeneity by taking absorption spectra, upslope, at the top, and downslope.

(fig.1) and had an absorption spectrum as shown in fig.2. Product formation easily occurred under the strong acid conditions but very slowly at pH 7.0. ^{1}H-NMR spectroscopy showed signals at 7.04 (s, 3-H), 8.37 (s, 8-H), 8.84 (doublet 1.5 Hz, 1 proton), 8.44 (multiplet 1.5 Hz and 9.5 Hz, 1 proton) and 8.26 ppm (doublet 9.5 Hz, 1 proton). These and earlier results [12] indicate that the model compound is the monohydrazone of PQQ (most probably as the isomer depicted).



3.2. *Modified enzyme*

The purified enzyme had a specific activity of 480 units/mg protein, measured at 25°C as reported [11], and using $A^{1\%}_{280nm,1cm} = 17.4$ for the protein estimation. Specific activity as well as the absorption spectrum (fig.3) show that the purity of the preparation is comparable to that reported. HPLC gel filtration revealed only minor contaminants, having no absorbance above 300 nm.

Attempts to isolate the prosthetic group as such failed as many compounds were found after degradation of the enzyme. Since PQQ reacts with certain amino acids to yield several products [12], we therefore tried to derivatize the prosthetic group in the enzyme into a stable adduct.

Bovine serum amine oxidase is inhibited immediately on addition of phenylhydrazine. This has been ascribed to reaction with the organic prosthetic group as the absorption spectrum in the visible region changes dramatically [2,3]. However, as we could not prepare a model compound from PQQ and phenylhydrazine, other reagents were tested. Since DNP gave a model compound which appeared to be reasonably stable, this reagent was tried on the enzyme. Although the reaction of enzyme with DNP is slow, after incubation under the indicated conditions the enzyme is completely inhibited. The absorption spectrum of the modified enzyme (fig.3)

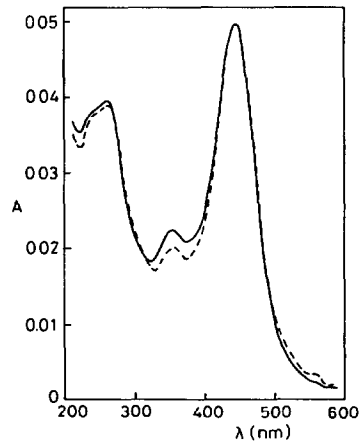


Fig.2. Absorption spectra of the model compound (——) and the adduct isolated from the enzyme (– – –). Spectra were measured with the photodiode-array detector (see fig.1 legend). For comparison, the spectrum of the adduct was multiplied by a factor 5.

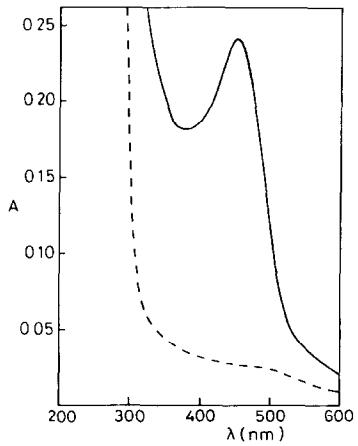


Fig.3. Absorption spectra of bovine serum amine oxidase (– – –) and after modification with DNP (——). Enzyme was incubated with DNP and excess reagent removed by gel filtration on a Sephadex G-25 column.

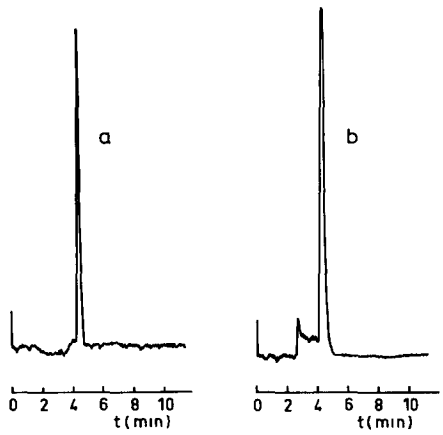


Fig.4. HPLC on a reversed phase column of fluorescing product obtained by degradation in NaOH solution of PQQ (a) and isolated adduct (b). Degradation and chromatography conditions are described in section 2.

is very similar to that of the model compound (fig.2) but quite different from that obtained by incubating PLP with DNP (not shown).

3.3. *Properties of the adduct*

Following the above isolation procedure, adduct was obtained showing exactly the same retention time (fig.1) and absorption spectrum (fig.2) as the model compound.

To obtain further evidence for the presence of PQQ, we tried to dissociate the adduct into its constituents. However, as conventional methods to dissociate the hydrazone failed and a very sensitive method was available for the conversion of PQQ into a fluorescing product (unpublished), the latter method was tried on the model compound and adduct. The structure of the fluorescing compound is not exactly known at the moment, but the results indicate that it is comparable to the products obtained from phenanthroline–diones under similar conditions [13]. Although the fluorescing compound is a minor product in the reaction, experiments under standardized conditions showed that the amount of fluorescing compound, as measured by HPLC, was proportional to the amount of PQQ in the sample. On degradation of model compound or adduct, a fluorescing product with the same retention time as in the case of PQQ was obtained (fig.4).

Based on an M_r of 172000 (determined by gradient polyacrylamide gel electrophoresis) and assuming one prosthetic group (or reactive carbonyl group) per enzyme molecule [2,3], a yield of 2% adduct was calculated. However, as it was found that the model compound is relatively unstable at pH 3.0, we omitted the pepsin digestion step. This resulted in a yield of 6.2% adduct. Further experiments showed that the model compound is stable in solutin with α-chymotrypsin but, unfortunately, not with pronase. However, as the pronase proteolysis step appeared to be essential for obtaining the adduct, this step could not be omitted. On incubation of DNP with the proteases, no adduct was found.

4. DISCUSSION

The model compound and the adduct have identical absorption spectra and chromatographic properties. Alkaline degradation of these compounds produces a fluorescing compound which has identical chromatographic properties to that obtained from PQQ. This indicates that the enzyme preparation contains covalently bound PQQ which can be detached by proteolysis.

A yield of only 6% adduct was obtained. It should be stressed, however, that the absorption spectra of modified enzyme and model compound

are very similar. Based on absorbance measurements, it appeared that all the adduct is still present before the step with pronase. However, during this step a large decrease in the absorbance values occurs. Since large losses of the model compound were found on incubation with pronase (as revealed by HPLC), the low yield of isolated adduct can be explained from the destructive action of this step. As no compound with a different visible absorption spectrum was detectable in the modified enzyme, it is concluded that the organic prosthetic group in bovine serum amine oxidase is covalently bound PQQ.

PQQ has only been found in bacterial oxidoreductases [9]. The fact that it has been detected now in this amine oxidase provides a stimulus to look for its presence in other mammalian enzymes and to investigate the eventual role of PQQ as a vitamin.

Plasma amine oxidase converts spermine and spermidine into their corresponding aldehydes. These compounds function in the regulation of cell division [14,15]. As it is clear now that PQQ is involved in these conversions, PQQ-directed inhibitors, important for chemotherapy [16], might be designed. Furthermore, if it appears that other copper-containing amine oxidases also contain PQQ, these specific inhibitors could clarify the physiological role of these enzymes.

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