Hydrazone formation of 2,4-dinitrophenylhydrazine with pyrroloquinoline quinone in porcine kidney diamine oxidase

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Homogeneous diamine oxidase (EC 1.4.3.6) from porcine kidney was treated with the inhibitor 2,4-dinitrophenylhydrazine (DNPH). The coloured compounds formed were detached with pronase and purified to homogeneity. When the reaction with DNPH was conducted under an O_2 atmosphere, the product (obtained in a yield of 55%) was the C(5)-hydrazone of pyrroloquinoline quinone (PQQ) and DNPH, as revealed by its chromatographic behaviour, absorption spectrum and ^{1}H-NMR spectrum. Only 6% of this hydrazone was formed under air, the main product isolated being an unidentified reaction product of DNPH with the enzyme. Porcine kidney diamine oxidase is the second mammalian enzyme shown to have PQQ as its prosthetic group. In view of the requirements for hydrazone formation with DNPH, it is incorrect to assume that inhibition of this type of enzymes with common hydrazines is simply due to blocking of the carbonyl group of its cofactor.

(Porcine kidney) *Diamine oxidase* *Pyrroloquinoline quinone* *Copper-containing amine oxidase* *Prosthetic group* *2,4-Dinitrophenylhydrazine* *Hydrazone*

1. INTRODUCTION

Recently we established that the organic prosthetic group in bovine serum amine oxidase is pyrroloquinoline quinone (PQQ) [1]. This could be demonstrated by derivatizing the covalently bound prosthetic group with 2,4-dinitrophenylhydrazine (DNPH) before hydrolyzing the protein. Although the coloured product isolated from the hydrolysate appeared to be identical to the hydrazone prepared from PQQ and DNPH, the yield was only 6%. Despite this imperfectness, at that time we considered derivatization with DNPH as the only feasible procedure since PQQ reacts with amino acids to give rise to many unidentifiable products, so that direct hydrolysis seemed out of the question [1]. Although others [2] claimed that they could identify PQQ as the fluorescing compound in such hydrolysates, we are unable to detect any PQQ under such circumstances (Van Kleef et al., unpublished), confirming that our original statement and strategy were correct.

In the meantime we have found an explanation for the low yield of hydrazone; reaction of DNPH with the enzyme also leads to a coloured product having quite different properties. In this respect, although it is generally assumed that hydrazines react with the carbonyl group of the prosthetic group in this type of amine oxidases, it should be realized that other oxidoreductases were inhibited by reactive species formed from hydrazine oxidation (e.g. the reaction of flavoprotein amine oxidases with phenylhydrazine results in covalent attachment of the phenyl group to the flavin [3]). Since such a reaction could give reduced cofactor, unable to form a hydrazone with DNPH, it was investigated whether the O_2 concentration during derivatization influenced the extent of hydrazone

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formation. The results obtained were similar for bovine serum amine oxidase and porcine kidney diamine oxidase. However, the data presented here are those of the latter enzyme in order to show that at the time the finding of PQQ in the first enzyme [1] was not fortuitous.

2. MATERIALS AND METHODS

2.1. *Enzyme purification*

Since commercial diamine oxidase preparations were not homogeneous, the following purification scheme was developed. 1 g lyophilized enzyme (Sigma) was dissolved in 100 ml of 10 mM sodium phosphate (pH 7.0). To the solution, powdered $(NH_4)_2SO_4$ was added and the fraction precipitating between 30 and 65% saturation was dissolved in 25 ml of 10 mM sodium phosphate (pH 7.0), followed by dialysis against the same buffer. The retentate was applied to a DEAE-Sepharose (fast flow, Pharmacia) column equilibrated with 10 mM sodium phosphate (pH 7.0). After washing with this buffer, a linear gradient was applied from 0.01 to 0.5 M sodium phosphate (pH 7.0). Active fractions were pooled and dialyzed against 10 mM sodium phosphate (pH 7.0), containing 0.15 M NaCl, 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$. The retentate was applied to a Con A-Sepharose column equilibrated with the same buffer. After washing with this buffer, the enzyme was eluted with this buffer, containing 0.5 M α-D-methylglucoside. Active fractions were pooled and dialyzed against 0.2 M sodium phosphate (pH 7.0).

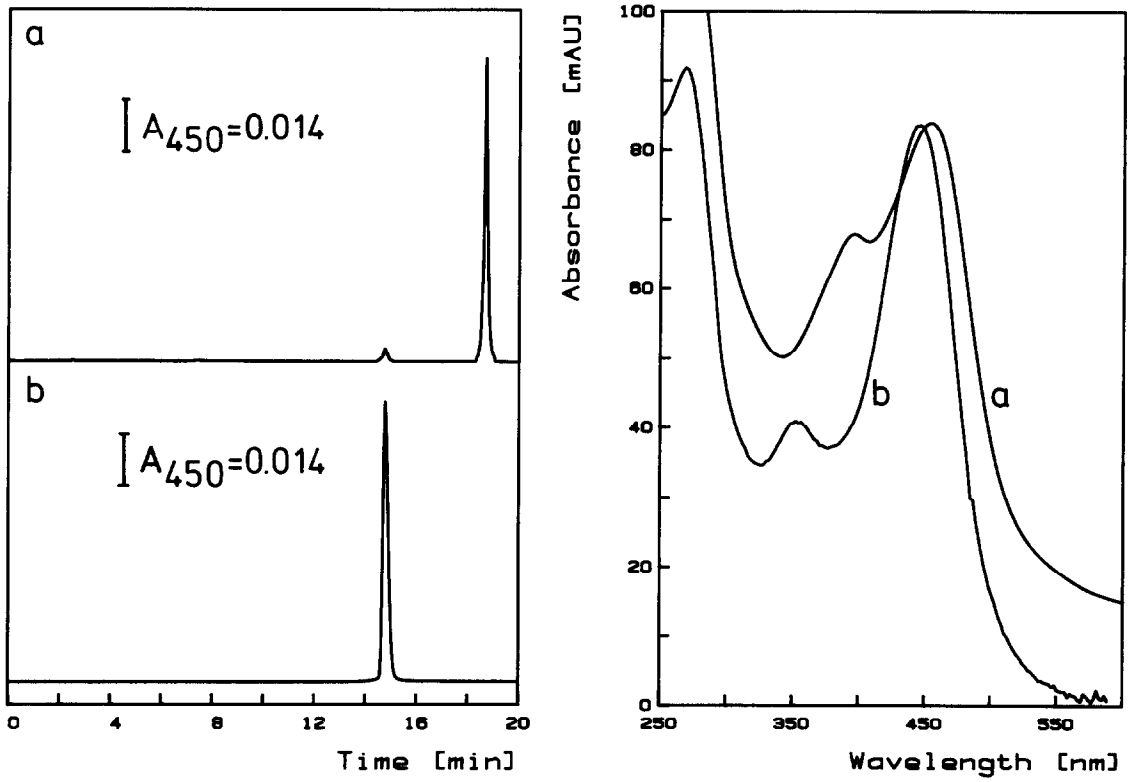

Fig.1. Chromatograms (left) and absorption spectra (right) of the products isolated from enzyme treated with DNPH under air (a) and under oxygen (b). The absorption spectra were taken from the peak at 18.7 min (a), and from the peak at 14.6 min (b). The Sep-Pak eluates were injected on a 10 μm C_{18} RCM cartridge in a Waters RCM 100 module. The eluant (1.5 ml/min) consisted of a linear gradient (20 min) of methanol from 7 to 63% in 10 mM sodium phosphate (pH 7.0) plus 10 mM NH_4Cl. The eluates were monitored at 450 nm with a Hewlett-Packard 1040 A photodiode-array detector taking absorption spectra throughout the peaks.

2.2. *Adduct isolation*

Derivatization of the prosthetic group in the enzyme was achieved by incubating 200 ml enzyme solution (125 mg enzyme in 0.2 M sodium phosphate, pH 7.0) with 70 μl of 0.1 M DNPH in H_3PO_4/ethanol (1:1, v/v) [4] at 40°C for 16 h. The mixture was either exposed to air or a stream of oxygen blown over the solution. Excess reagent was removed by gel filtration on a Sephadex G-25 column in 10 mM sodium phosphate (pH 7.0) giving the inhibited enzyme solution. The pH of this solution (200 ml) was brought to 7.5 and proteolysis performed by incubation with 6 mg pronase E (Boehringer) at 40°C for 6 h. After proteolysis, the solution was brought to pH 2.0 with HCl and passed through a Sep-Pak C_{18} cartridge equilibrated with H_2O (pH 2.0 with HCl). After washing with subsequently 10 ml H_2O (pH 2.0), 10 ml 10% methanol, and 100 ml H_2O, the red-coloured compound was eluted with methanol.

2.3. *Preparation of the model compound*

The C(5)-hydrazone of PQQ and DNPH was prepared by adding a slight excess of DNPH reagent (0.1 M DNPH in H_3PO_4/ethanol (1:1, v/v) [4]) to a saturated solution of PQQ in methanol at 40–50°C. The suspension was stirred for 10 min at 50°C, whereafter the copious precipitate was collected and washed with methanol. The orange-red solid was dissolved in large amounts of boiling methanol, affording the hydrazone as a microcrystalline orange solid upon cooling.

3. RESULTS

3.1. *Product formation*

The purification procedure yielded an enzyme preparation with a specific activity of 1.4 U/mg protein, comparable to that found by others [5]. The preparation appeared to be homogeneous, as revealed by electrophoresis and HPLC gel-filtration chromatography so that it could be safely used for the identification of its prosthetic group.

Derivatization of the enzyme with DNPH under air leads to 6% of the C(5)-hydrazone of PQQ (as will be argued below) and a product with a retention time of 18.7 min and absorption spectrum depicted in fig.1a (the amount of hydrazone was calculated using an M_r of 172000 [5], the assumption that one enzyme molecule contains one prosthetic group [6,7], and a molar absorption coefficient for the hydrazone of 31400, the latter being determined with pure model compound). On the other hand, under an oxygen atmosphere, a 10-times higher amount of hydrazone was formed (fig.1b). Derivatization under an N_2 atmosphere gave only a product identical to the main compound formed in air (retention time 18.7 min), not the hydrazone (not shown). The products were homogeneous, as revealed by the overlay of normalized spectra taken throughout the eluting peaks.

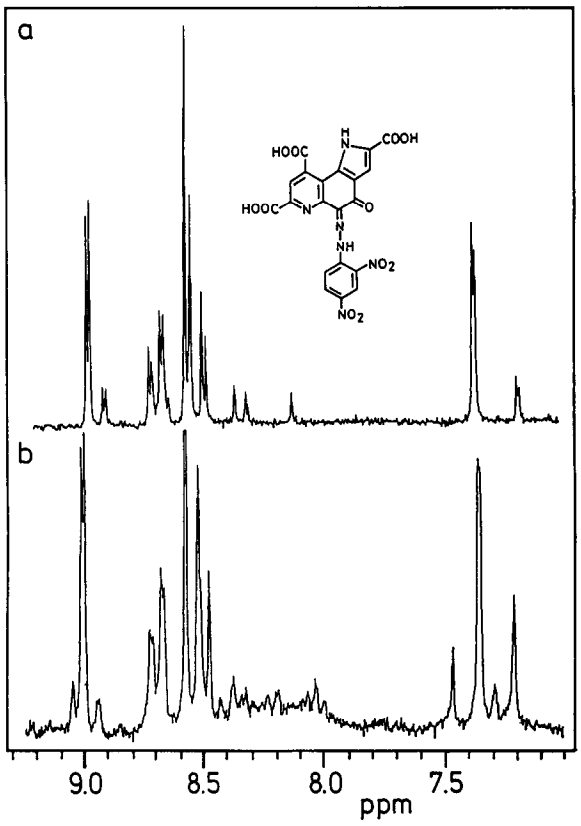

Fig.2. ^{1}H-NMR spectra of the model compound (a) and the adduct (retention time 14.6 min) isolated from the enzyme derivatized under O_2 (b). ^{1}H-NMR spectroscopy was performed in $(C_2H_3)_2SO$ on a Varian SC300 spectrometer, operating at 300 MHz, using the pulse Fourier transform mode (max 32 K data table of 32 bits) and tetramethylsilane as an internal reference.

3.2. *Identification of the hydrazone*

The procedure used to prepare the model compound was more convenient than that described in [1] and resulted in a homogeneous product with the same characteristics as found before. Its ^{1}H-NMR spectrum is depicted in fig.2, showing signals in agreement with the structure of the C(5)-hydrazone of PQQ and DNPH [8,9]: signals at 7.38 (s, 3-H), 8.72 (s, 8-H), 8.97 (doublet 1.5 Hz, 1 proton), 8.57 (multiplet 1.5 Hz and 9.5 Hz, 1 proton) and 8.32 ppm (doublet 9.5 Hz, 1 proton).

The adduct isolated from enzyme derivatized under an O_2 atmosphere (retention time 14.6 min, fig.1b) had the same chromatographic properties, absorption spectrum and ^{1}H-NMR spectrum (fig.2) as the model compound.

4. DISCUSSION

Hydrazines have long been known as inhibitors of copper-containing amine oxidases; in particular, the stoichiometry of the reaction has been studied intensively. Although it has been generally assumed that they react with the carbonyl moiety of the prosthetic group, the structure of the adduct remained unknown. Recently we established that reaction of DNPH with bovine serum amine oxidase resulted in the formation of the C(5)-hydrazone of DNPH and PQQ, obtained, however, only in a 6% yield. The present report shows that the same hydrazone and yield were obtained with diamine oxidase, that another coloured compound is formed (being the main product under this condition), and that a 10-times higher yield of hydrazone could be obtained by derivatizing under an O_2 atmosphere.

The results imply that under the usual conditions of inhibition with common hydrazines, hydrazone formation in amine oxidases probably does not take place. An amount of 6% is found with DNPH, but it should be noted in this respect that hydrazones were not formed from PQQ and other hydrazines, PQQ being reduced by these compounds [1,9]. The structure of the other coloured product (fig.1a, retention time 18.7 min) is still unknown, but first results (unpublished) indicate that the aromatic ring structure of DNPH is conserved. An interesting possibility could be the enzyme-catalysed oxidation of DNPH, leading to a different product from PQQ and DNPH, or to a product with an amino acid in the active site. Nevertheless, it is clear that the results have important consequences for inhibition studies with these enzymes. The possibility of directing the reaction towards hydrazone formation and the establishment of the presence of PQQ in a second amine oxidase now give confidence to the undertaking of the structure elucidation of prosthetic groups in related enzymes.

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