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Phenylhydrazine as probe for cofactor identification in amine oxidoreductases

Evidence for PQQ as the cofactor in methylamine dehydrogenase

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Homogeneous methylamine dehydrogenase (primary-amine:(acceptor) oxidoreductase (deaminating), EC 1.4.99.3, MADH) from the bacterium *Thiobacillus versutus* was treated with the inhibitor phenylhydrazine (PH). Derivatization of the cofactor in MADH took place in a fast reaction to give compound I. A different product, compound II, was formed in a slow reaction at high O_2 concentrations. The compounds I and II could be removed from the protein by proteolysis with pronase and purified to homogeneity. Products showing identical absorption spectra and chromatographic behaviour were isolated from the reaction mixture after incubatng pyrroloquinoline quinone (PQQ) with PH. Upon dissolving in dimethyl sulphoxide, both the enzyme-derived as well as the model-system-derived compounds I and II were nearly quantitatively transformed into PQQ. The conclusion is, therefore, that MADH from *T. versutus* contains covalently bound PQQ, removable from the protein with pronase, and not a structural analogue of this cofactor without the carboxylic acid groups, as was recently proposed for MADH from *Bacterium* W3A1 [(1986) Biochem. Biophys. Res. Commun. 141, 562–568]. The properties of compounds I and II suggest that they are the 'azo adduct' and the 'hydrazone adduct' of PH and PQQ at the C(5)-position, respectively. The finding that the reaction of a hydrazine with PQQ can lead to two different products, in enzymes as well as in a model system, has important implications for the interpretation of recent comparative studies aimed at detection of PQQ in amine oxidoreductases with Raman spectroscopy.

Methylamine dehydrogenase; Pyrroloquinoline quinone; Prosthetic group; Phenylhydrazine; Hydrazone; *(Thiobacillus versutus)*

1. INTRODUCTION

Several methylotrophic bacteria use methylamine as the sole source of carbon and energy and degrade this compound via methylamine dehydrogenase (MADH) (EC 1.4.99.3) to formaldehyde and ammonia according to the equation:

$$CH_3NH_3^+ + H_2O \rightarrow HCHO + NH_4^+ + 2H^+ + 2e^-$$

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In the first description of this enzyme [1], it was mentioned as having an organic prosthetic group, most probably covalently bound at two sites in the protein chain of the small subunit, as was later reported [2]. ESR spectroscopy [3] showed that the prosthetic group was a free radical, having properties in common with pyrroloquinoline quinone (PQQ) in its free radical form (PQQH$^\cdot$). However, in the same report it was mentioned that electron nuclear double resonance (ENDOR) spectroscopy revealed three protons, considered to originate from PQQH$^\cdot$. Since ENDOR spectra of other PQQH$^\cdot$-containing enzymes revealed two protons, we concluded that the prosthetic group in MADH

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was PQQ or a derivative thereof [3]. Continuing work [4] showed that ENDOR spectroscopy is unable to detect the protons of PQQH$^{\cdot}$, the signals detected in quinoproteins most probably belonging to amino acid residues in the vicinity of PQQH$^{\cdot}$. Although arguments derived from ENDOR spectroscopy against PQQ as such were absent, uncertainty remained as further data about the structure were lacking.

Detection of covalently bound PQQ in enzymes by direct hydrolysis was considered inappropriate by us, since free amino acids react with PQQ to a complex mixture of products (although it has been claimed [5] that PQQ as such can be detected in these hydrolysates by fluorescence spectroscopy and biological assays, as was recently confirmed, the biological assay fails to detect the amino acid adducts and fluorescence is not specific enough [6]). The approach developed by us consists of derivatization of the cofactor still bound to the enzyme with a hydrazine compound, degrading the protein with pronase, isolating the cofactor adduct, and comparing it with model compounds prepared from PQQ and the hydrazine. In this way, the presence of PQQ could be established in several mammalian and microbial copper-containing amine oxidases [7–10], showing that the cofactor has a wide distribution [11,12].

Recently, McIntire and Stults [13] described the isolation of the cofactor-semicarbazide adduct from MADH isolated from *Bacterium* W3A1. Mass spectroscopy revealed a PQQ-like compound, missing the three carboxylic acid groups and being bound to the protein via a cysteine thioether bridge and a serine ether link. In order to obtain more structural details of this cofactor, the hydrazine derivatization method was applied to the MADH from *T. versutus*, an enzyme previously characterized by us [14].

2. MATERIALS AND METHODS

2.1. *Isolation of MADH*

T. versutus (formerly *Thiobacillus* A2) ATCC 25364^{T} [15] was cultured and MADH purified as described [14]. Protein concentrations of the pure enzyme were calculated from the specific absorption coefficient $A_{280}^{1\,mg/ml} = 1.14$, determined with UV spectroscopy [16]. Enzyme was assayed in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM methylammonium chloride, and 100 μM Wurster's blue as the electron acceptor, by following the decrease in absorbance at 600 nm (molar absorption coefficient $9 \times 10^3\ M^{-1} \cdot cm^{-1}$) using freshly prepared test mixtures (final volume 1.1 ml).

2.2. *Oxidation of the enzyme*

MADH as isolated has its prosthetic group in the free radical form [3]. Since hydrazone formation requires PQQ in its oxidized form, oxidation of MADH was considered necessary. Oxidation was performed with an excess of Wurster's blue while MADH was bound to a Q-Sepharose (fast flow, Pharmacia) column, equilibrated with 10 mM sodium phosphate (pH 7.0), enabling removal of excess Wurster's blue by washing with this buffer. Enzyme was eluted with 0.5 M sodium phosphate, pH 7.0.

2.3. *Cofactor adducts in the enzyme*

Derivatization of the cofactor in the enzyme occurred by incubating 200 ml enzyme solution (420 mg oxidized MADH in 0.2 M sodium phosphate, pH 7.0) with PH (1 mg PH · HCl) at 40°C for 5 min in air (for preparing compound I) or for 16 h while blowing a stream of O_2 over the surface of the mixture (for preparing compound II). After the indicated period, the solution was brought to pH 7.5 and proteolysis performed by incubation with 20 mg pronase E (Boehringer) at 40°C for 6 h. Subsequently, the solution was brought to pH 2.0 with 1 M HCl and passed through a Sep-Pak C_{18} cartridge equilibrated with 10 mM HCl. After washing with successively 10 ml of 10 mM HCl, 10 ml of 10% methanol, and 100 ml H_2O, the cofactor adduct was eluted with methanol and the coloured fractions pooled. Methanol was removed in a rotary evaporator and the residue was dried over P_2O_5 in vacuo.

2.4. *Preparation of the model compounds*

To a saturated solution of PQQ in methanol at 40–50°C, a slight molar excess of PH (in the form of PH · HCl) was added. The suspension was stirred for 10 min at 50°C, whereafter the copious precipitate was collected and washed with cold methanol. The orange-red solid was dissolved in large amounts of boiling methanol, yielding a microcrystalline red solid upon cooling. The solid

mainly consisted of two compounds which were further purified by HPLC on a reversed-phase column, using the conditions described in section 2.7.

2.5. *Transformation into PQQ*

The model compounds and cofactor adducts were dissolved in Me_2SO (Merck, Darmstadt, pro analyse, art. 2931) and incubated at room temperature for 4 h. The incubate was mixed with 10 ml of 10 mM HCl and the solution was applied to a Sep-Pak C_{18} cartridge equilibrated with 10 mM HCl. After washing with successively 10 ml of 10 mM HCl, 10 ml of 10% methanol, and 100 ml H_2O, PQQ was eluted with methanol and the coloured fractions pooled. PQQ concentrations were determined with a biological assay as in [17].

2.6. *Analytical procedures*

Reversed-phase HPLC was performed on a Waters HPLC system with an RCM 100 module containing a 10 μm C_{18} RCM cartridge. The eluent (flow rate 1.5 ml/min) consisted of a linear gradient (20 min) of 7–63% methanol in 10 mM sodium phosphate, pH 7.0, containing 10 mM NH_4Cl. The eluate was monitored with a Hewlett-Packard 1040A photodiode-array detector, taking absorption spectra of the eluted peaks, upslope, at the top, and downslope, to check homogeneity and to establish identity.

2.7. *NMR measurements*

^{1}H-NMR spectroscopy was performed in $(C^2H_3)_2SO$ (Janssen Chimica Belgium, 99.9% ^{2}H) or in $^2HCON(C^2H_3)_2$ (Janssen Chimica Belgium, 99% ^{2}H) on a Nicolet NT-200 WB spectrometer, operating at 200 MHz, using tetramethylsilane as an internal reference.

3. RESULTS

3.1. *Enzyme purification*

The purification procedure yielded an enzyme

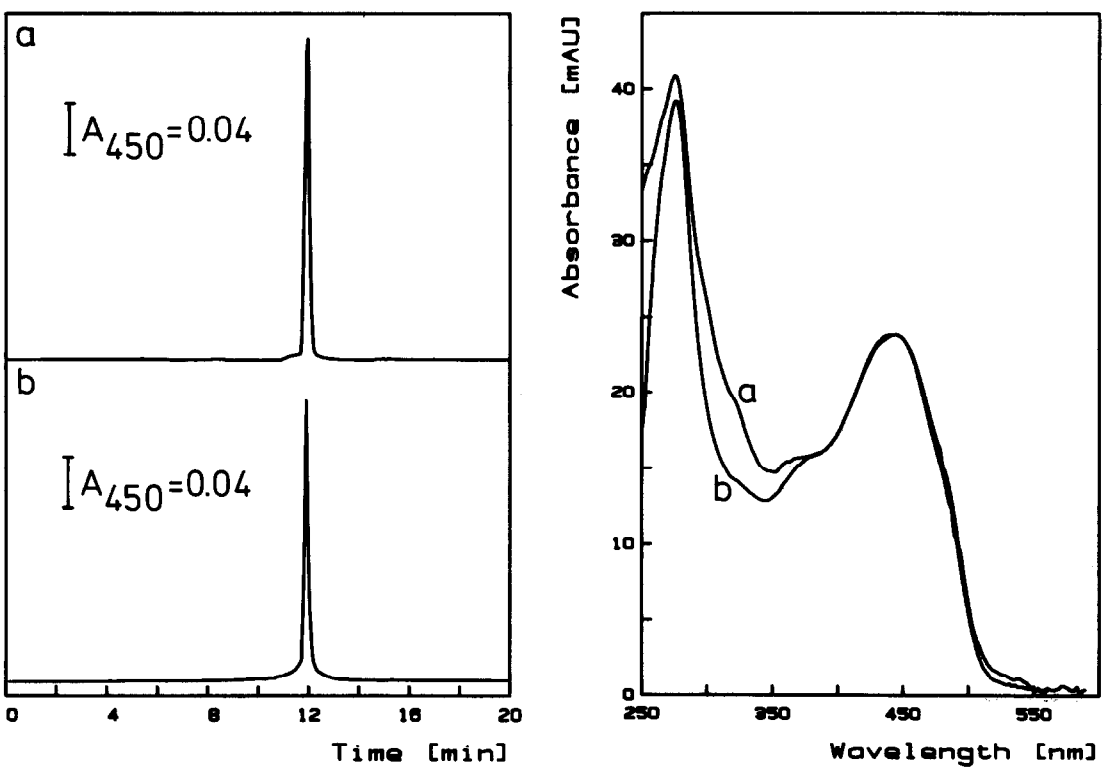

Fig.1. Chromatograms (left) and absorption spectra (right) of compound I: (a) cofactor adduct isolated from enzyme treated with PH for 5 min under air; (b) corresponding model compound. HPLC and photodiode array detection were performed as described in the text. Spectra were taken at the top of the peak.

preparation with a specific activity of 3.9 μmol Wurster's blue reduced $\cdot min^{-1} \cdot mg$ protein^{-1}, comparable to that found previously [14]. The preparation appeared to be homogeneous, as revealed by electrophoresis and by a photodiode array detection method applied to the peak eluting from an HPLC gel-filtration column [18].

3.2. *Cofactor adducts*

The procedure used to derivatize the enzyme is essentially the same as that developed [8,10] for copper-containing amine oxidases, except that PH was used instead of 2,4-dinitrophenylhydrazine (DNPH) since PH was an inhibitor for MADH and DNPH was not. In accordance with that observed for the derivatization of amine oxidases with DNPH, an immediate reaction between PH and the prosthetic group in MADH took place, giving the product (designated as compound I) with chromatographic and spectral properties shown in fig.1. When the incubation was performed for a much longer time under an O_2 atmosphere, a different product (designated as compound II) was found with chromatographic and spectral properties shown in fig.2. Both derivatization methods produced only a single product in an almost quantitative way, as will be discussed below. The behaviour of MADH is not unique since derivatization of diamine oxidase from porcine kidney with DNPH under similar conditions also leads to two different products [8] and its derivatization with PH to compounds I and II (not shown).

3.3. *Model compounds*

Two main compounds were found in comparable yields after reacting PH with PQQ. Their chromatographic and spectral properties are shown in figs 1 and 2. This result is somewhat different compared to that for PQQ and DNPH

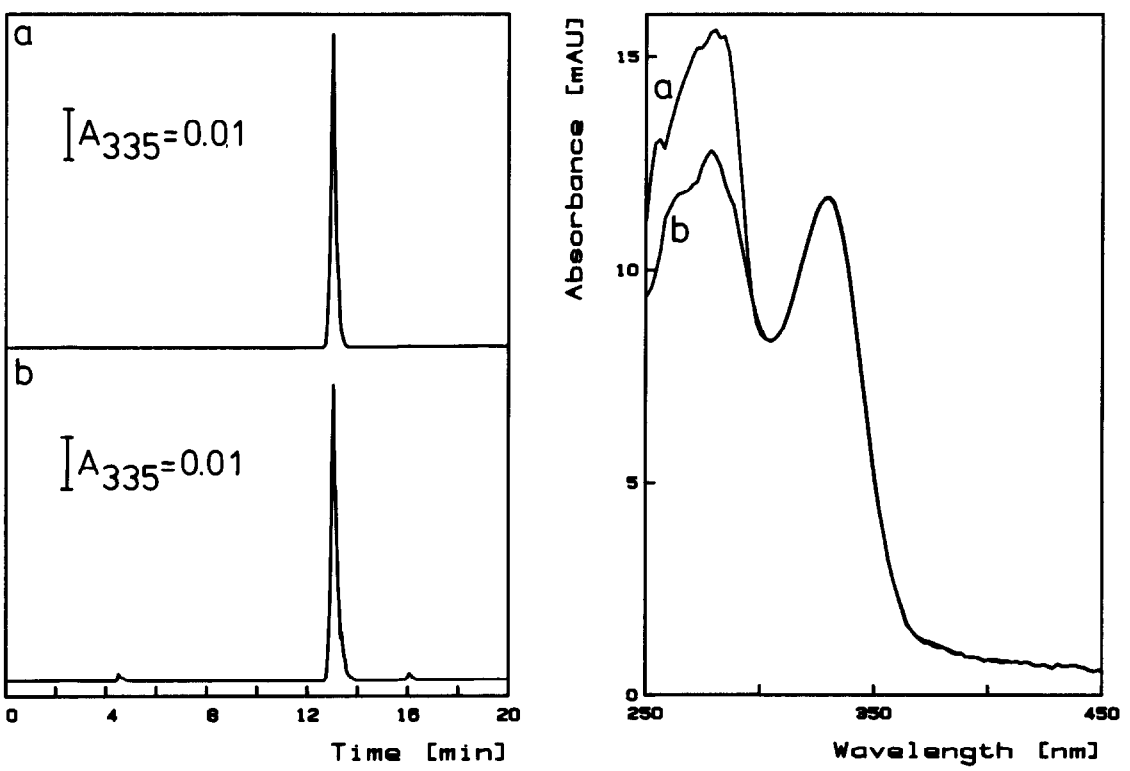

Fig.2. Chromatograms (left) and absorption spectra (right) of compound II: (a) cofactor adduct isolated from enzyme treated with PH for 16 h under O_2; (b) corresponding model compound. HPLC and photodiode array detection were performed as described in the text. Spectra were taken at the top of the peak.

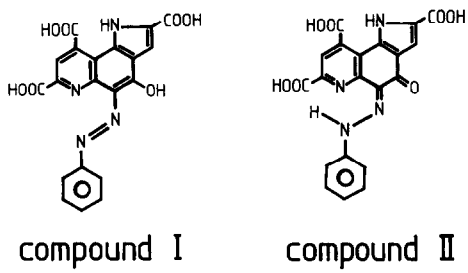

Fig.3. Structures assigned to compound I (azo adduct) and compound II (hydrazone adduct).

where only one model compound was obtained, namely the C(5)-hydrazone of PQQ and DNPH [8].

3.4. *Characterization*

Compounds I and II were stable in water-containing solvents and did not convert into each other. However, upon dissolving them (adducts as well as model compounds) in deuterated Me_2SO, ^{1}H-NMR spectroscopy revealed weak signals of the phenyl ring of PH, only at the very first moment. The signals disappeared rapidly and the well-known signals of PQQ arose. Although this behaviour was repeatedly seen, no attempts were made to elucidate the reaction mechanism.

After the conversion of cofactor adducts or model compounds in Me_2SO, samples were analyzed by HPLC, this showing peaks with retention times and absorption spectra identical to those of PQQ. The presence of PQQ was also confirmed with the biological assay. Quantitative determinations of the PQQ isolated from cofactor adducts from the enzyme (by HPCL using a molar absorption coefficient for PQQ of 25 400 $M^{-1} \cdot cm^{-1}$ at 249 nm) showed that compound I was obtained in 70% yield and compound II in 86% yield (assuming that the transformation into PQQ occurred quantitatively and that MADH contains 2 PQQs per enzyme molecule).

^{1}H-NMR spectroscopy of compound II in deuterated $HCONMe_2$ showed signals in agreement with the structure of the C(5)-hydrazone of PQQ and PH: 7.26 (s, 3-H), 8.83 (s, 8-H) and 8.02 ppm (multiplet 1.5 and 9.5 Hz, 5 protons). Upon dissolving compound I in deuterated $HCONMe_2$, the same phenomena were observed as described above when using Me_2SO.

4. DISCUSSION

4.1. *Identity of the cofactor in MADH*

The cofactor in MADH of *T. versutus* could be derivatized in high yield with PH to either compound I or compound II. Compounds I and II were also obtained from the reaction of PQQ with PH. This indicates that the cofactor in MADH is PQQ, a conclusion that is substantiated by the fact that the adducts could be converted into PQQ as such.

In accordance with the almost quantitative yield of PQQ obtained from each cofactor adduct, no other coloured compounds were found in the hydrolysates besides compounds I or II. The two PQQs in MADH from *T. versutus* are presumably bound to the protein chains of the two small subunits via one or more peptide or ester bonds since pronase treatment was able to remove the derivatized PQQs. These findings are in contradiction with those of McIntire and Stults [13] for the cofactor of MADH from *Bacterium* W3A1. Derivatization of their enzyme with semicarbazide, followed by proteolysis and purification, gave a PQQ-like compound missing 3 carboxylic acid groups but containing amino acids attached to the cofactor via ether bonds. Although it could be reasoned that the MADHs may have different cofactors, this seems highly improbable since the absorption spectra of the enzymes are very similar [14,19]. Unfortunately, the conclusions were only based on the mass spectrum of a product isolated from the derivatized enzyme (criteria to follow this product during isolation steps were not mentioned so that it is unclear how to prepare it). Since data about yield or absorption spectrum were not given, it is difficult to explain the discrepancy.

4.2. *Structure of compounds I and II*

In analogy with derivatization of diamine oxidase from porcine kidney with DNPH under similar conditions, the structure of compound II could be the hydrazone of PH and PQQ at the C(5)-position (fig.3). ^{1}H-NMR spectroscopy of compound II in deuterated $HCONMe_2$ indeed showed signals which are in accordance with such an assignment. The much higher absorption maximum of compound I suggests a more conjugated structure. Since adducts of PQQ at the C(4)-position have not been observed so far, the most

likely candidate seems the 'azo structure' depicted in fig.3.

Although MADH is a dye-linked dehydrogenase and (as far as known) contains no copper ions, with respect to the hydrazines it behaves similarly to the amine oxidases (EC 1.4.3.6), the covalently bound PQQ reacting to either compound I or compound II.

4.3. *Critical notes on the detection of PQQ by Raman spectroscopy*

The fact that the reaction of PQQ with hydrazines can lead to different products has important consequences for the evaluation of the results reported in several recent papers [20–22] where Raman spectroscopy was used as a tool to provide evidence for the existence of PQQ in amine oxidases. To that end, Raman spectra of model compounds prepared from PQQ and hydrazine were compared with those of enzyme derivatized with hydrazine. Although it was assumed by the authors that they were comparing 'hydrazones', the (usual) conditions used for derivatization suggest that the azo compound was formed in the enzymes (the high absorption maxima mentioned in some of the publications confirm this view). Since details about comparisons of absorption spectra of the cofactor adducts in the enzymes and model compounds were not presented, the discrepancy between the Raman spectra of model compounds and derivatized enzymes (explained by the authors as originating from interaction of the hydrazone with local protein structural elements) could be due to comparison of the adduct in the enzyme with the wrong model compound. In view of these uncertainties and the easy isolation procedure developed by us for derivatized cofactor from amine oxidoreductases, it is questionable whether Raman spectroscopy can be advocated as the tool to reveal the identity of the cofactor in these enzymes. On the other hand, the nature of the cofactor adducts being established by isolation, Raman spectroscopy could be very useful to probe the environment of the (derivatized) cofactor.

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