*Biochem. J.* (1968) **106**, 575
*Printed in Great Britain*



# Short Communication

## The Nature of Copper in Pig Plasma Benzylamine Oxidase

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(*Received* 2 *November* 1967)

Pig plasma benzylamine oxidase is a copper pyridoxal enzyme (Buffoni & Blaschko, 1964; Blaschko & Buffoni, 1965). Copper is known to be required for enzymic activity since (i) treatment of the enzyme with sodium diethyldithiocarbamate results in a copper-free preparation that is devoid of activity (Blaschko & Buffoni, 1965), (ii) the enzyme is inhibited by cupric chelating agents such as cuprizon (Blaschko & Buffoni, 1965) and (iii) the enzymic activity is lacking in copper-deficient pigs (Blaschko, Buffoni, Weissman, Carnes & Coulson, 1965).

In the present work, the valence state of copper in the native, reduced and perchloric acid-denatured enzyme was examined by ESR* spectroscopy and correlated with data from chemical copper assays.

*Methods.* The ESR spectra were studied at $-165°$ on a Varian model V 4502X band spectrometer. The instrument was fitted with a dual-sample cavity. Hyperfine splittings and G (gauss) values were determined by reference to a sample of 0·1% MnO in MgO that had been calibrated against a proton-resonance probe. Quantitative determination of the copper content was carried out as described by Wyard (1965) and by Beinert & Kok (1964), with 2mM-$Cu^{2+}$ in 25mM-disodium EDTA as standard.

Crystalline benzylamine oxidase, specific activity 0·18 international unit at 25° in the manometric assay with benzylamine as substrate (Buffoni & Blaschko, 1964), which had been stored as a suspension in $(NH_4)_2SO_4$ solution, was centrifuged and dialysed at 4° against 0·01M-sodium–potassium phosphate buffer, pH 7. The protein concentration, determined by the biuret method against albumin standards (Lowry, Rosebrough, Farr & Randall, 1951), was 16·6mg./ml.

Samples of the enzyme were treated at pH 7 in anaerobic ESR tubes with either a two-fold excess of $Na_2S_2O_4$ or a 60-fold excess of benzylamine hydrochloride. A reaction time of 15min. at 25° was allowed before freezing in liquid $N_2$ and study of the ESR spectra. Further addition of a fourfold excess of $Na_2S_2O_4$ was made to the $Na_2S_2O_4$-reduced sample and the ESR spectra were again measured.

In other experiments, duplicate samples (0·2ml.) of the enzyme were transferred to ESR tubes and their spectra recorded. Then 72% (w/v) $HClO_4$ (0·2ml.) was added to each according to the procedure described by Broman, Malmström, Aasa & Vänngård (1962) and the ESR spectra were again studied. Portions (0·1ml.) of these $HClO_4$-treated samples were further digested with $HClO_4$–$H_2SO_4$ mixture and analysed for copper by the neocuproin method of Smith & McCurdy (1952).

*Results and discussion.* The reaction catalysed by pig plasma benzylamine oxidase can be separated into two steps: an anaerobic step in which the addition of substrate bleaches the visible absorption spectrum of the enzyme (Buffoni & Blaschko, 1964) and an aerobic phase that occurs when $O_2$ is introduced. During the anaerobic phase, evidence has been obtained that the substrate is linked to the enzyme in the form of an imine bond (Buffoni, 1966), The results from the present ESR study clearly show that, irrespective of whether low (17·5mw) or high (175mw) microwave power is used to observe the resonance condition (Beinert & Palmer, 1965), the copper in the enzyme remains bivalent after this anaerobic phase with benzylamine as substrate. Reduction with $Na_2S_2O_4$, however, does result in a greatly decreased cupric ESR signal.

The observation that under our experimental conditions copper does not change its valency during the enzyme reaction is in agreement with the findings on similar oxidases (Yamada, Yasunobu, Yamano & Mason, 1963; Mondovì *et al.* 1967). It could indicate either that copper is only important for the chemical structure of or substrate binding in the enzyme, or that it participates in the catalytic process by a mechanism similar to that proposed by Hamilton (1966), where a valency change would not have been detected.

Spectral constants for the oxidized and reduced enzyme compared with those of copper EDTA are given in Table 1. The hyperfine splitting constant ($A$) found for benzylamine oxidase is closely similar

* Abbreviation: ESR, electron spin resonance.

Table 1. *ESR-spectral constants of pig plasma benzylamine oxidase compared with those of cupric EDTA*

The spectra were recorded at a microwave power of 17·5 mW and a modulation amplitude of 4·3 G (gauss).

| | $g_{\parallel}$ | $g_{\perp}$ | Hyperfine splitting constant ($A$) (cm.$^{-1}$) |
|---|---|---|---|
| Cupric EDTA | — | 2·070 | 0·015 |
| Oxidized enzyme | 2·266 | 2·060 | 0·015 |
| Reduced enzyme (benzylamine) | 2·271 | 2·059 | 0·015 |
| Reduced enzyme ($Na_2S_2O_4$) | 2·268 | 2·058 | 0·015 |

to those reported for other copper- and pyridoxal-containing oxidases (Yamada *et al.* 1963; Mondovì *et al.* 1967) as well as for simple copper complexes (Malmström & Vänngård, 1960), and is quite different from those found for laccase and caeruloplasmin (Malmström & Vänngård, 1960). In these latter two enzymes, the copper has been shown (Broman, Malmström, Aasa & Vänngård, 1963) to undergo a valency change during the catalytic cycle, and a high degree of delocalization of the unpaired hole on the cupric atom (as reflected in the decreased value of $A$) would facilitate such a process. The ESR data can be interpreted as indicating that the cupric atoms in benzylamine oxidase are equivalent and that the copper is present as a symmetrical tetragonal complex with oxygen, sulphur or more probably nitrogen as ligands in which the unpaired electron is largely localized on the copper. Mondovì *et al.* (1967) have provided ESR evidence that, in the related enzyme pig kidney diamine oxidase, the substrate amine nitrogen is not a ligand atom, suggesting that either the pyridoxal or amino acids in the protein, possibly the Schiff-base amino acid nitrogen atom bonded to the pyridoxal (Buffoni, 1966), provide the ligand atoms.

Integration of the ESR signal gave 1·9 atoms of $Cu^{2+}$/mol. of enzyme before and 2·2 atoms of $Cu^{2+}$/mol. of enzyme after $HClO_4$ treatment. This latter value was in good agreement with the copper content determined chemically (2·3 atoms of $Cu^{2+}$/mol. of enzyme). In our studies and those of Yamada *et al.* (1963) on ox plasma amine oxidase, the copper concentration given by integration of the ESR signal of the native enzyme is less than that given by chemical assay. This would indicate either that these enzymes contain cuprous copper or that the cupric atoms are interacting with one another or with some other paramagnetic species. Yamada & Yusonobu (1962) by chemical assay found no indication of cuprous copper. Equally, there is no ESR evidence for cupric interactions; according to Broman *et al.* (1962) such interactions would result in broadening of the signal relative to that from cupric EDTA. The reason for the small discrepancy between the copper content determined by ESR integration and chemical analysis thus remains unexplained.

We thank Dr H. Blaschko, F.R.S., and Dr R. C. Bray for helpful discussions and Miss F. M. Pick and Miss A. Toccafondi for valuable technical assistance. The work was supported by grants from the Medical Research Council, the British Empire Cancer Campaign for Research, U.S. Public Health Service Grant no. CA-03188-10, the Instituto Farmochimico Falorni and Consiglio Nazionale delle Ricerche, Rome.

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