NAD from the adsorbent loaded by $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$ (purification factor subsequently – 15, 20, 17, yield – 56–68%) but not $Ni^{2+}$, $Mn^{2+}$ (yield 2.2 and 15.8%) or $Co^{2+}$, $Fe^{3+}$ (not eluted). This study supports the idea that the interaction of such metal ions as $Cu^{2+}$ and $Zn^{2+}$ ($Cd^{2+}$) with LRY chromophore (in contrast to the metal ions $Mn^{2+}$ and $Ni^{2+}$) stabilizes a particular conformation of dye that is sterically acceptable to the NAD-binding site in ADH.

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## P11

## EPR Studies of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Activated With $Cu^{2+}$

STENBJÖRN STYRING, ROLF BRÄNDÉN and THOMAS NILSSON

*Department of Biochemistry and Biophysics, University of Göteborg and Chalmers Institute of Technology, S-412 96 Gothenburg, Sweden*

Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase catalyzes the primary reaction in two metabolic pathways in plants.

A: in $CO_2$fixation:

RuBP + $CO_2$ → 2,3-phosphoglyceric acid (PGA)

B: In photorespiration:

RuBP + $O_2$ → PGA + phosphoglycolate

In the carboxylase reaction a 6-carbon intermediate has been identified. In the oxygenase reaction a 5-carbon peroxointermediate has been suggested. However, there is little experimental evidence for its existence [1].

RuBP carboxylase/oxygenase requires a divalent metal ion for activity. The metal ion stabilizes the carbamate formed between the enzyme and a $CO_2$ molecule in the activation process [2]. Whether the metal also has a function during catalysis is not clear. $Mg^{2+}$ is the normal activator *in vivo* but the enzyme is also activated by $Fe^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$.

$Cu^{2+}$ gives little activity and has not been much studied in carboxylase research. Despite this, $Cu^{2+}$ may be a useful paramagnetic probe.

Our results show that $Cu^{2+}$ in the presence of $HCO_3^-$ (50 m*M*) is specifically bound to the enzyme. The high field part of the EPR spectrum shows nitrogen hyperfine splitting indicating that the metal in the Enz-$CO_2$–$Cu^{2+}$ complex has at least one nitrogen

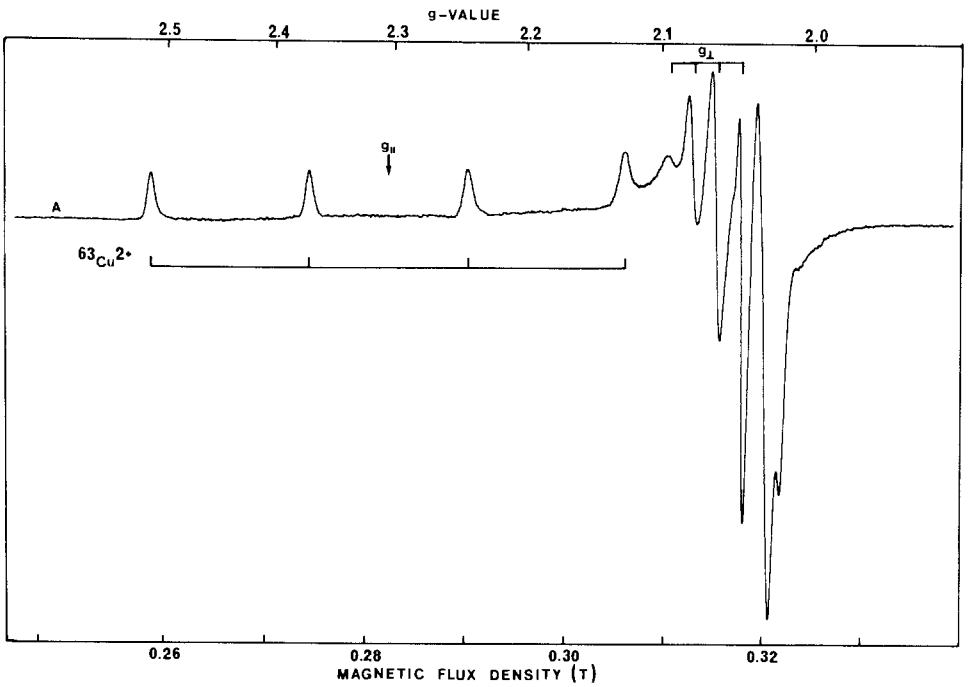


Fig. 1. Effect of addition of RuBP in the presence of $HCO_3^-$ and $Cu^{2+}$. To 0.25 ml enzyme (41 mg/ml in 50 m*M* HEPPS buffer at pH 8.3) was added 0.015 ml $NaHCO_3$ (0.5 *M*) followed by 0.025 ml RuBP (0.1 *M*) and 0.010 ml $^{63}CuCl_2$ (12.5 m*M*). The spectrum was recorded at 77 K. The spectrometer gain was $1 \times 10^5$ and the modulation amplitude was 0.5 mT. From the spectrum the following parameters are obtained: $g_\parallel = 2.301$, $g_\perp = 2.055$, $A_\parallel = 15.75$ mT and $A_\perp = 2.39$ mT.

ligand [3]. The yield of the specific EPR signal is lowered by $Mg^{2+}$ indicating that $Cu^{2+}$ binds in or very near the normal metal activator site [3].

When excess substrate (10 m*M* RuBP) is added to the $Cu^{2+}/Co_2$ activated enzyme another EPR spectrum is obtained (Fig. 1). This signal has very narrow hyperfine lines which is only compatible with oxygen atoms being liganded to the $Cu^{2+}$ ion. The nitrogen ligand in the Enz–$CO_2$–$Cu^{2+}$-complex must therefore have been displaced by an oxygen atom probably derived from RuBP. This experiment has two important implications. First it proves that $Cu^{2+}$ binds in the active site of the enzyme and secondly it implies that the metal is involved in substrate binding to the active site [3].

When stoichiometric amounts of RuBP (0.5 m*M*) are added to the $Cu^{2+}/CO_2$ activated enzyme a transient EPR signal is obtained. This signal is converted to a third signal with time. Both these signal possess the same narrow hyperfine lines as the Enz–$CO_2$–$Cu^{2+}$–RuBP complex (Fig. 1) but have different parameters. The third signal can also be obtained by addition of 3-PGA to the Enz–$CO_2$–$Cu^{2+}$ complex [4].

The transient signal is dependent on the concentration of oxygen. In the presence of $^{17}O_2$ the signal is broadened. This proves that at least one oxygen ligand to $Cu^{2+}$ is derived from $O_2$. Preliminary experiments have shown that this effect is not derived from $^{17}O$ incorporated in any of the expected products of the oxygenase reaction. $H_2O_2$ and $O_2$ are also excluded as ligands. We therefore suggest that the transient EPR signal is derived from a peroxointermediate formed in the oxygenase reaction. If so the $Cu^{2+}$-activated enzyme is very attractive for further research directed towards an understanding of the oxygenase reaction.

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## P12

### Cobalt(II) as an NMR Probe for the Investigation of the Coordination sites of Conabulmin

I. BERTINI, C. LUCHINAT, A. MESSORI and A SCOZZAFAVA

*Department of Chemistry, University of Florence, Florence, Italy*

Conalbumin and transferrin in the presence of bicarbonate bind metal(III) and also metal(II) ions, although the former oxidation number is preferred.

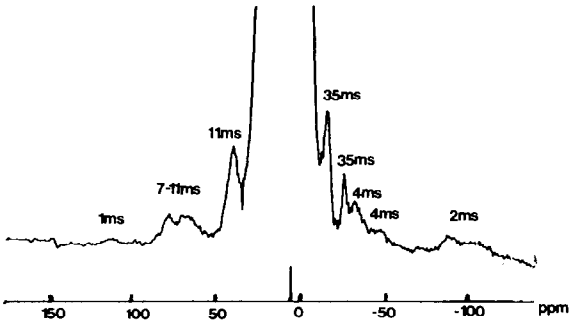


Fig. 1. 60-MHz $^1H$ NMR spectra of cobalt(II) conalbumin at pH = 8.2.

In the absence of oxidizing agents, the cobalt(II) derivatives can be prepared; the conalbumin derivative is particularly inert and both 1:1 and 1:2 cobalt to protein derivatives have been characterized during this research.

The 60 MHz proton spectra of cobalt(II) conalbumin recorded in water is shown in Fig. 1. It was obtained using an appropriate pulse sequence (modified DEFT [1]) to suppress the slowly relaxing signals of water and of the diamagnetic protons of the protein. The same sequence was used to evaluate the longitudinal relaxation times of the isotropically shifted signals through a saturation recovery type of experiment.

The spectrum shows several well shaped resonances. Within the resolution determined by the linewidth, no difference was observed between the 1:1 and 1:2 derivatives.

Assignment of the signals can be attempted through the analysis of the $T_1^{-1}$ values which are mainly determined by the distance from the paramagnetic center. There is a very broad signal downfield at 100 ppm with a $T_1$ of 1 ms. Position [2, 3] and $T_1$ indicate that the signal is due to a histidine H$\alpha$. Another quite similar signal is detected when measuring $T_1$ under the peaks at +67 and +58 ppm. This is a second histidine H$\alpha$. The other peaks at 67 and 58 ppm are in the same position as peaks found for other tetrahedral and five coordinate cobalt(II) proteins which have been assigned as histidine H$\beta$ protons [2, 3]; even the $T_1$ values (7–11 ms) are consistent with the assignment. There are then four peaks upfield at –37, –51, –90 and –105 ppm with $T_1$ values ranging from 2 to 4 ms. They could be the four ortho signals of the two proposed tyrosinate ligands. The signals at –30 and –20 ppm may be assigned to two of the four meta protons of the tyrosinates since the $T_1$ values of 35 ms indicate a larger proton metal distance. The other two meta signals could be under the intense absorption in the diamagnetic region. Two resonances are still to be assigned, *i.e.* that at 31 ppm of intensity 2 and at 19 ppm of intensity 1. Both of them have a $T_1$