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

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

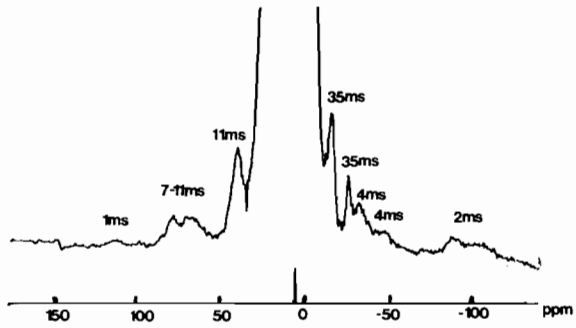

Fig. 1. 60-MHz ^1H NMR spectra of cobalt(II) conalbumin at pH = 8.2.

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 Hα. Another quite similar signal is detected when measuring T_1 under the peaks at +67 and +58 ppm. This is a second histidine Hα. 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 Hβ 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

of 11 ms, *i.e.* they are due to protons relatively close to the metal. The one at 19 ppm could be assigned to a meta signal of a tyrosinate bent in such a way as to give rise to a short proton metal distance. However, a proton of a CH_2 attached to a histidine in a α position seems to fit better with the characteristics of the signal. The assignment of the peak at 31 ppm of intensity two is important even for the chemical implications. It is reasonable to assign the signal to two additional histidine Hβ protons. The spectrum recorded in D_2O after allowing the apoprotein to exchange with D_2O for a week at 35 °C shows that the intensity of the 31 ppm signal is halved, while the signal at +67 ppm disappears. If we assign the 67 ppm signal and one of the 31 ppm signals to histidine NH protons, then signal at +58 and the residual signal at +31 ppm can be assigned as histidine 4H protons.

Based on the present assignment, the NMR spectrum confirms the nature of the ligands in iron transfer proteins and suggests that: i) the histidines bind with the nitrogen close to CH_2; ii) the tyrosinates are in the apical positions of a pseudooctahedral chromophore since only such positions have been found to provide large upfield dipolar shifts.

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P13

The Binding of Cobalt(II) to Apo Alkaline Phosphatase

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Alkaline phosphatase from *Escherichia Coli* is a dimeric zinc enzyme which catalyzes the hydrolysis of monophosphoric esters; it contains four zinc(II) and two magnesium(II) ions per molecule. Each subunit contains a 'catalytic' zinc ion, a 'structural' zinc ion and a 'regulatory' magnesium ion. The low resolution crystal structure shows that the catalytic and structural zinc ions are about 5 Å apart, while the magnesium ion is about 30 Å away from the above-mentioned ions [1]. The enzyme can be totally depleted of metal ions and reconstituted by the addition of two, four or six like or unlike metal ions.

Since a high resolution X-ray structure is not yet available, the geometry of the metal sites and the donor group should be argued only from different spectroscopic data. Otvos *et al.* proposed, on the basis of ^{113}Cd and ^{13}C NMR data, that the catalytic metal ion could be coordinated by four histidine nitrogens [2]. Previous water proton NMR data indicated that a water molecule is present in the first coordination sphere of Mn^{2+}, Cu^{2+} and Co^{2+} alkaline phosphatase, substituted at the catalytic sites [3]. Combining these data, the catalytic metal ion should be five-coordinated; this hypothesis is in agreement with our recent proposal of five coordination of cobalt(II) ion in the catalytic site, based on the relatively low molar absorbance of the electronic spectra [4]. Structural and regulatory sites were assigned as pseudo-octahedral on the basis of the low molar absorbance of the cobalt(II) derivative.

We have titrated apoalkaline phosphatase solutions at different pH values with increasing amounts of cobalt(II) ions in order to shed light on the distribution of metal ions among the various metal sites, which is still a matter of discussion. We worked with unbuffered and unsalted solutions either in the presence or absence of magnesium(II) ions. When excess Mg^{2+} is present, only two cobalt(II) ions are required to develop fully the electronic spectrum typical of the catalytic site, while in its absence four cobalt(II) ions are required. We thus propose that when magnesium is present, cobalt(II) has a higher affinity for the catalytic site than for the structural sites, while in the absence of magnesium the affinity of cobalt(II) ions for the two sites is comparable; this holds both for the low and high pH limits. We also recorded the spectrum of cobalt(II) in the structural site at low pH in the presence of a 2:1 ratio of copper(II) ions to apoenzyme molecule, where copper is selectively bound only to the structural sites [5]; the difference spectrum gives a molar absorbance of around 10 per cobalt, which we assign to the cobalt(II) ions bound in the pseudo-octahedral environment of the structural site. It was previously reported that for the system M_2AP only one mol of inorganic phosphate is required to obtain the limit spectrum for the phosphate adduct [6], while for the M_4AP system, two equivalents of phosphate are required. We have titrated both Co_2Mg_4AP and Co_4Mg_2AP with inorganic phosphate and we found that in both cases only one mol of phosphate is required to develop fully the spectrum of the phosphate adduct. This result is in contrast with the model worked out for the enzymatic activity of AP [7].

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