^{13}C NMR studies have been performed on CH_3-$-^{13}CO_2^-$ in presence of cobalt(II) substituted LADH. Evidence is provided that acetate binds at the metal ion, presumably substituting the water molecule, with an affinity constant that has been estimated to be 5 ± 1 M^{-1} through electronic spectroscopy.

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PS3.3 — TH

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^1H NMR SPECTRA OF ACTIVE SITE RESIDUES IN COBALT(II) ALKALINE PHOSPHATASE

Dimeric alkaline phosphatase (AP) contains a total of three pairs of metal binding sites [1], which are occupied by four zinc(II) and two magnesium(II) ions in the native enzyme. Extensive work mostly based on metal substitution has shown a very complicated pattern of metal binding to such sites both from the thermodynamic and kinetic points of view [2].

On the ground of the experience gained by some of us in the use of cobalt(II) as an NMR shift probe for zinc metalloenzymes we have reacted cobalt(II) with apo AP obtained from the *E. coli* enzyme. Extensive checks were performed through electronic spectroscopy on the binding sequence of cobalt(II) ions as a function of pH. It was established that in the pH region 5-7 the first two cobalt(II) ions selectively bind to one set of sites which, by means of comparison with mixed copper-cobalt derivatives and by obvious extension of literature data [3,4] have been assigned as A sites. Titration of cobalt(II) into AP solutions at pH 5--6.5 results in the development of ^1H NMR signals sizeably isotropically shifted outside the region of the bulk protein signals and relatively well resolved. The titration was first limited to one cobalt(II) ion per protein, *i.e.* half occupancy of A sites, to minimize possible anticooperative effects between A sites. From these initial sets of data, which include measurement of longitudinal relaxation times of the isotropic shifted signals, it can be already established that the number and shape of signals in the downfield region of the spectrum confirms the presence of three histidines in the coordination sphere of cobalt(II) in the A site. The ^1H NMR spectra in D_2O solution indeed show the disappearance of three signals from the exchangeable NH protons of the coordinated histidines. Such spectra also indicate that at least one, and possibly two, of the three histidines are coordinated through N1.

Work is in progress to investigate the effect of higher cobalt(II)-protein ratios on the NMR spectra and the pH dependence of the latter.

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CD AND ^{1}H NMR STUDIES ON COBALT(II) SUBSTITUTED OVOTRANSFERRIN--OXALATE COMPLEXES

Although the X-ray structure of the transferrins is not as yet available, other spectroscopic techniques allowed to identify the nature of the metal ligands that probably are two tyrosines, two histidines, the synergistic anion and a water or hydroxo moiety [1].

The ^{1}H NMR spectra of mono and dicobalt ovotransferrin at pH 8.3 in presence of bicarbonate as synergistic anion show rather well resolved signals. The pattern of the signals is in good agreement with the above proposed cromophore; no difference between the sites is observed [2].

By substituting oxalate for bicarbonate as synergistic anion the two sites become spectroscopically non equivalent. The CD spectra relative to each site are deeply different as shown in Fig. 1.

^{1}H NMR spectra of ovotransferrin oxalate derivatives are able to differentiate between the two sites; the existence of different conformational states of the cromophore in equilibrium among them can also be assessed.

The pH dependent properties of the cromophore were also studied. In the low pH region the *C*-terminal site appears to be more stable than the *N*-terminal one. In the high pH region there is evidence for a pH dependent equilibrium with a pK_a value of about 9.5. Deprotonation of a third tyrosine group in the proximity of the cromophore is suggested to be responsible for such equilibrium.

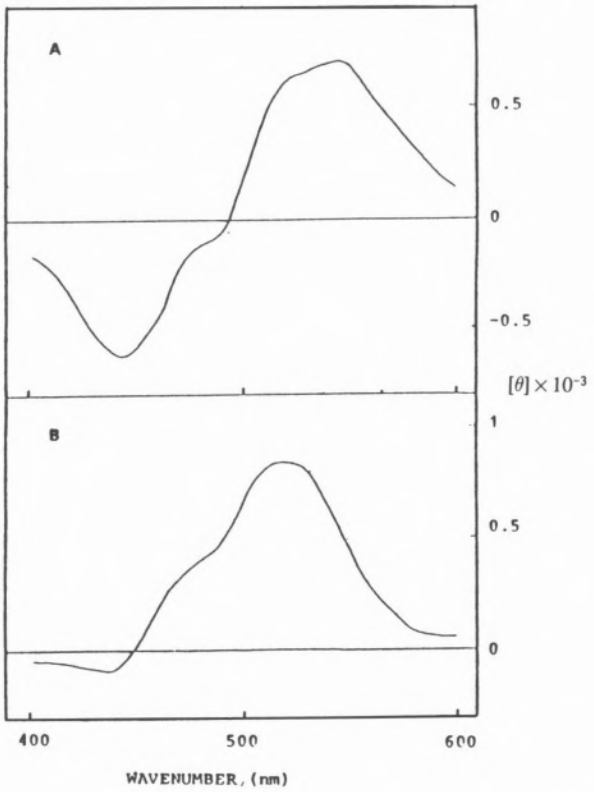

Fig. 1
Visible CD spectra of the C-terminal (A) and N-terminal site (B) of cobalt(II)$_2$-ovotransferrin-oxalate

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