

(Co(II)-E,NAD', alcoxide). Decreasing the pH below the pK_a of the presumed enzyme-bound alcohol again prevents the formation of this ternary complex.

We have tentatively assigned the absorption bands centered at 650 nm and 680 nm to the active site Co(II)-ion in the open and closed conformation, respectively, of the protein. Irrespective of the conformation state, binding of an anionic ligand to the Co(II)-ion creates the transition at 575 nm. Scheme I summarizes the transients observed hitherto and their spectral characteristics.

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¹H NMR INVESTIGATION OF INHIBITOR BINDING TO COBALT(II) SUBSTITUTED LIVER ALCOHOL DEHYDROGENASE (LADH)

It is known from X-ray studies that imidazole and pyrazole bind at the catalytic zinc ion in LADH [1]. They substitute the coordinated water molecule whereas the remaining residues are essentially in unchanged positions. We have substituted cobalt(II) for zinc(II) at the catalytic site and allowed the two ligands to interact with the new derivative [2]. The electronic spectra in the 12,000-25,000 cm^{-1} region show some changes indicating that binding occurs at the metal ion. The ¹H NMR spectra in H₂O [3] show as sharp signals in the downfield region up to 50 ppm from water the NH signal of the coordinated histidine, the NH of imidazole and the corresponding *meta*-like proton, or the *meta*-like protons of pyrazole. The signals of the cysteine β -CH₂'s and of the *ortho*-like protons of the two ligands are quite broad and often shifted very far downfield. Such spectra provide definitive evidence that the metal-coordinated residues system does not change upon water substitution by either ligand. Furthermore, the ligand exchange is slow on the NMR time scale. The very same considerations apply to the ternary systems with imidazole and NADH on one side and pyrazole and NAD⁺ on the other.

^{13}C NMR studies have been performed on $\text{CH}_3\text{-}^{13}\text{CO}_2^-$ 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 $5 \pm 1 \text{ M}^{-1}$ through electronic spectroscopy.

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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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