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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P14

Evidence for Inner-sphere Coordinated Alkoxide Ion Intermediates in the Catalytic Mechanism of Co(II)-Substituted Liver Alcohol Dehydrogenase

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Substitution of certain divalent transition metal ions, *i.e.* Co(II), Ni(II), Cu(II) and Fe(II), for the active site zinc ion of horse liver alcohol dehydrogenase yields catalytically active enzyme derivatives [1–4]. Since catalysis occurs *via* inner-sphere coordination of substrate [3, 5, 6], the ligand-to-metal charge-transfer (LMCT) and d–d electronic transitions of these metal ions are useful spectroscopic probes of the chemical events occurring during catalysis [1, 2, 7]. As will be shown in this report, when reaction is monitored *via* rapid-scanning, stopped-flow (RSSF) UV-visible spectroscopy, the resulting time-resolved spectra (300 nm to 700 nm) provide detailed temporal information about transient intermediates formed during catalysis. We report here evidence derived from RSSF spectroscopy for the existence of ternary enzyme–NAD^+–alkoxide ion intermediates both in the Co(II)E-catalyzed reduction of aldehydes and in the Co(II)E-catalyzed oxidation of alcohols.

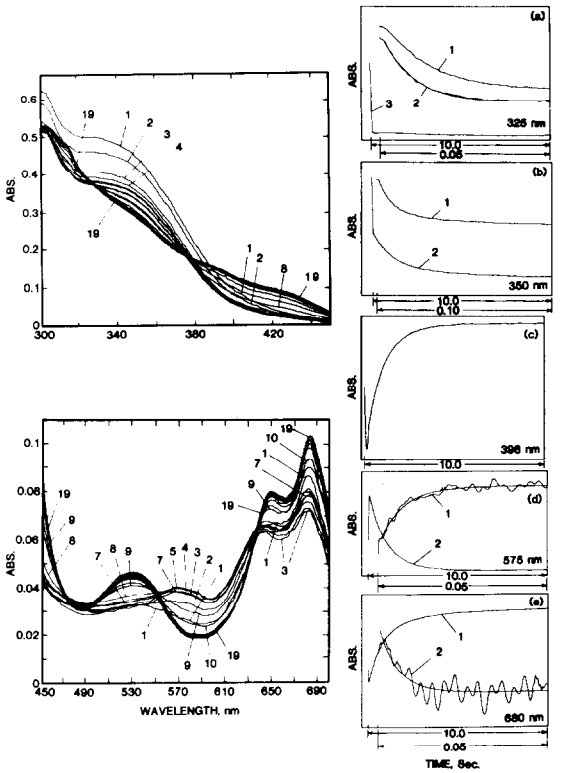

Fig. 1. Rapid-scanning, stopped-flow spectra for the Co(II)E-catalyzed reduction of benzaldehyde by NADH at 25 °C. Spectral acquisition times (measured in msec from the moment flow stopped): 1 (8.6), 2 (17.6), 3 (25.8), 4 (34.4), 5 (43.0), 6 (51.6), 7 (103.3), 8 (129.1), 9 (516.3), 10 (929.3), 11 (1979), 12 (4130), 13 (6282), 14 (9293), 15 (11875), 16 (14456), 17 (17038), 18 (19619), 19 (24782). (A) Conditions after mixing: (Syringe 1) [Co(II)E] = 34.4 μN; [EDTA] = 11.3 μM; [KCl] = 13.6 mM; (Syringe 2) [Benzaldehyde] = 0.955 mM; [NADH] = 47.4 μM; [pyr] = 24.3 mM; 0.1 M potassium glycinate buffer pH 9.04. (B) (Syringe 1) [Co(II)E] = 68.4 μN; [EDTA] = 10.4 μM; [KCl] = 23.9 mM; 25 mM potassium-TES buffer (pH 7 before mixing); (Syringe 2) [benzaldehyde] = 0.942 mM; [NADH] = 0.186 mM; [pyr] = 24.0 mM; 0.05 M potassium glycinate buffer, final pH 8.94. Insets (a)–(e) show single-wavelength time-courses. Conditions after mixing: (Syringe 1) [Co(II)E] = 53.6 μN; [EDTA] = 12.5 μM; [KCl] = 44.9 mM; (syringe 2) [benzaldehyde] = 0.951 mM; [NADH] = 0.107 mM; [pyr] = 24.2 mM; 0.1 M potassium glycinate buffer pH 9.04. Trace 2 in inset (a) was measured with 0.105 mM NADD in place of NADH.

Experimental

Site-specifically-substituted Co(II)E was prepared by the method of Maret *et al.* [1]. The preparation used in this study was found to have 76 percent active site occupancy relative to total protein. The remaining active sites contained no metal ion. Substrates were purified prior to use by vacuum distillation. The highest purity grades of NADH and NAD^+ were purchased from either Sigma Chemical Company or Boehringer Mannheim Corporation and used without further purification. For kinetic isotope studies, both (4R)-4-deuterio-nicotinamide adenine dinucleotide (NADD) and isotopically normal NADH were prepared and purified as previously described [5, 8, 9]. Pyrazole (Aldrich) was purified by vacuum sublimation, isobutyramide (Aldrich) was purified by recrystallization from hot water. The rapid kinetics systems for rapid-scanning, rapid-mixing and for single-wavelength studies have been previously described [10, 11].