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The Cobalt(II)-Alkaline Phosphatase System at Alkaline pH*

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The uptake of cobalt(II) ions by apoalkaline phosphatase at pH 8 (the pH optimum for activity) has been investigated by the combined use of electronic and ^{1}H NMR spectroscopies. The presence of fast-relaxing high spin cobalt(II) ions in the active site cavity of the protein induces sizable isotropic shifts of the ^{1}H NMR signals of metal-coordinated protein residues, allowing us to propose a metal uptake pattern by the various metal binding sites both in the presence and in the absence of magnesium ions.

In the absence of magnesium the active site is not organized in specific metal binding sites. The first equivalent of cobalt(II) ions per dimer binds in an essentially unspecific and possibly fluxional fashion, giving rise to a six-coordinated chromophore. The second and third equivalents induce the formation of increasing amounts of metal ions pairs, cooperatively arranged into the A and B sites of the same subunit with a five- and six-coordinated geometry, respectively. The fourth and fifth equivalents induce the formation of fully blocked A-B pairs in both subunits.

Magnesium shows the property of organizing the metal binding sites, probably through coordination to the C sites. Electronic and ^{1}H NMR titration with Co^{2+} ions show that the initial amount of fluxional cobalt is smaller than in the absence of magnesium and that A-B pairs are more readily formed.

Titration of fully metalated Co_4Mg_2alkaline phosphatase samples with phosphate confirms binding of only one phosphate per dimer.

The enzyme alkaline phosphatase (AP)[1] is a quite complex enzyme that gives rise to a variety of metal derivatives and of interactions between metal sites which cannot be framed within a unique picture.

The enzyme is a dimer; in AP from *Escherichia coli*, the most studied among the many isolated from different sources, each monomer has three metal sites, which in nature are occupied by two zinc ions (sites named A and B) and one magnesium ion (site C) (1–5). The native enzyme will be termed $Zn_2Zn_2Mg_2$AP hereafter. The x-ray structure of the cadmium-substituted derivative (Cd_6AP) has shown that three histidines (His-372, His-412, and His-331) are coordinated to the A site, whereas one histidine (His-370) and two aspartates (Asp-51 and Asp-369) are coordinated to the metal in the B site. In the C site the residues Asp-51, Asp-153, Glu-322, and Thr-155 have been identified as coordinated residues. The metal ions A and B are approximately 3.9 A apart, whereas C is located 4.9 A from B and 7.2 A from A (6, 7).

The Zn_4AP and Zn_6AP bind 1 phosphate ion per dimer whereas Cd_4AP and Cd_6AP bind 2 phosphate ions per dimer (8). The anion binds to a serine residue (Ser-102) at acidic pH and to the metal of the A site at higher pH (9–11). We should notice that the pK_a of the equilibrium between the metal-bound and the serine-bound species is 5.0 for the Zn_4 derivative whereas the pK_a of enzymatic activity is 7.5. The former value is an apparent pK_a that reflects the pH dependence of the ratio between the kinetic constants of dephosphorylation and phosphorylation of Ser-102; the latter value is the real pK_a of the enzymatic reaction, probably related to the pK_a of a solvent molecule coordinated to the metal ion the A site (2, 12, 13). The two pK_a values for the Cd_4 derivative are 2–3 units higher than those of the Zn_4 derivative (2). Zn_2AP, Co_2AP, and Cd_2AP bind one phosphate per dimer at pH 8 (2, 8, 14). The same happens for Cd_2AP at pH 6.5 where the serine-bound species is still predominant. Furthermore, phosphorylation of this derivative at pH 6.5 induces a slow migration of Cd(II) from the A site of one subunit to the B site of the other subunit. Cadmium migration is also observed in absence of phosphate by increasing the pH from 6.5 to 9.0 (15, 16).

In order to understand the system better, we have undertaken an investigation of the cobalt(II)-AP system which is expected to be similar to the zinc(II)-AP system on chemical grounds and therefore to be a good model of the native system. Its activity (for the Co_4 derivative) is 30% that of the native protein (17). The advantage of studying the cobalt derivatives is that the electronic spectra may provide information on the nature and number of donor atoms (18, 19) and that it is possible to record the ^{1}H NMR spectra of the resulting paramagnetic species to obtain information on the groups coordinating the metal ions (19–23).

In a previous report we have shown that at pH 6, which we call acidic pH in relation to the pK_a of 7.5 for the catalytic activity, the Co^{2+} ions selectively occupy the A sites, giving rise to a $Co_2E_2E_2$ derivative (E for empty). The ^{1}H NMR spectra have been recorded and the NH signals of coordinated histidines assigned (24). Later the ^{1}H NMR spectra were assigned also for the $Co_2Co_2E_2$AP and $Co_2Co_2Mg_2$AP species (25). In this study we have tackled the problem of the cobalt(II)-AP system at pH 8, which we refer to as alkaline pH. We will also report on the interaction of phosphate with the $Co_2Co_2Mg_2$ derivative at the same pH.

At the moment there are reports in the literature about the uptake of cobalt(II) by AP at alkaline pH (pH = 8) but the agreement is limited to a narrow range of conditions (26, 27).

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[1] The abbreviation used is: AP, alkaline phosphatase.

EXPERIMENTAL PROCEDURES

Alkaline phosphatase from *E. coli* was isolated and purified according to the method reported by Applebury *et al.* (28). The native metals were removed by extensive dialysis against 2 M $(NH_4)_2SO_4$ in 10 mM Tris, 10 mM CH_3COONa (pH 9). Ammonium sulfate was removed by dialysis against a 0.1 M NaCl solution in the same buffer and then against a 10 mM Tris solution at pH 8 (8). The apoenzyme solution at pH 8 was concentrated in a metal-free Amicon ultrafiltration cell to a final concentration of about 1 mM. The activity of the resulting apoprotein was less than 5% compared with the native protein. The concentration of the protein was determined spectrophotometrically at 278 nm and calculated on the basis of a molecular weight of 94,000 and using an $\epsilon_{1\,cm}^{0.1\%} = 0.72$ (16, 29). Cobalt and magnesium were added to the apoprotein using solutions of the metals in the same buffer (10 mM Tris) at pH 7. The phosphate adduct of the cobalt derivative was prepared by adding a solution of Na_2HPO_4 at pH 8, in stoichiometric amounts, to the cobalt-alkaline phosphatase sample. Deuterated samples were prepared by repeated dilution with D_2O and concentration under nitrogen flow to a final D_2O content higher than 95%.

The ^1H NMR spectra were recorded on a Bruker CXP 90 spectrometer using the modified driven equilibrium Fourier transform pulse sequence (30, 31). The spectra were obtained through block-averaging of 10–20 spectra of 16,000 scans each. The T_1 values were determined by measuring the signals' intensity as a function of the time between subsequent pulses of the modified driven equilibrium Fourier transform sequence.

RESULTS

Titration of apoAP with Co^{2+} in Presence of Mg^{2+}—Titration of apoAP with Co^{2+} was followed in the presence of 10 mM Mg^{2+} in solution, at pH 8.0. Magnesium binds to the C and B sites (32), probably organizing the active site structure in a conformation close to that achieved by the fully metallized protein. Indeed, through x-ray studies it was seen that there are many conformational changes at the active site of apoAP compared with the metal protein (33). Stepwise addition of Co^{2+} produces an electronic spectrum with a maximum at 550 nm and bands at 510, 605, and 640 nm. The spectrum, more than 50% developed when 2 cobalt ions per dimer are added, is almost completely developed with 3 cobalt ions per dimer, with $\epsilon_{550} = 441\ M^{-1}\ cm^{-1}$. The final spectrum, corresponding to the Co_4 derivative, is in agreement with the one reported by Anderson *et al.* (27). From Fig. 1 we can see that the addition of the first pair of Co^{2+} ions produces an increase of molar absorbance higher than the second pair, suggesting that the first two Co^{2+} ions fill sites with a lower coordination number than that of the second sites. The overall behavior is similar to that reported by Anderson *et al.* (27) (Fig. 1, *inset*), although the latter titration curve is somewhat shifted to higher Co/AP ratios.

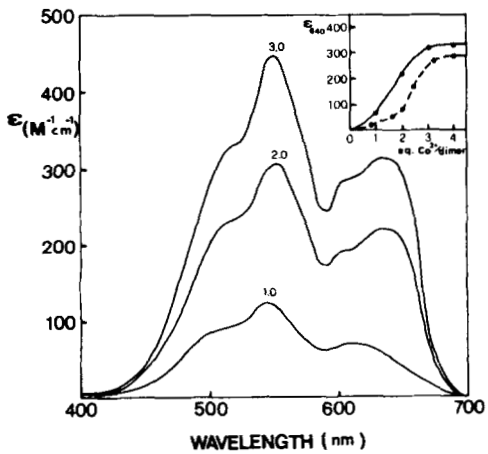

FIG. 1. **Electronic absorption spectra of apoAP plus increasing amounts of Co^{2+}: the numbers show the Co^{2+}/dimeric protein ratio.** Conditions: 10 mM Tris, 10 mM Mg^{2+} (pH 8), room temperature; the concentration of the protein was 0.1–0.2 mM. The *inset* at the *top* shows the values of ϵ_{640} as a function of the Co^{2+}/dimeric protein ratio obtained by us (——) and by Anderson *et al.* (– – –) (from Ref. 27).

The addition of up to 1 Co^{2+} ion per dimer to the apoprotein in the same conditions (presence of magnesium, pH 8) gives rise a ^1H NMR spectrum that shows isotropically shifted signals in the range 130 to −45 ppm (Fig. 2*A*). With the second Co^{2+} ion the spectrum still shows the typical signals of this first species (Fig. 2*B*). Two (perhaps three) of these signals (signals *d* and *h* (and possibly *f*)) disappear in D_2O and can be attributed to NH protons of two (perhaps three) coordinated histidine residues. The downfield signals, above 90 ppm, *a*, *b*, and *c*, for their broad lines and their position, seem to belong to ortho-like protons of the histidine rings. Signal *g* with a sharp line and relatively long T_1 value (10.3 ms, Table I) might arise from a meta-like proton of a coordinated histidine ring which could be the Hδ2 proton of His-331 which is coordinated through Nδ1 (34). We can note that in spectrum 2B new signals (*a'*, *b'*, *c'*, *d'*) begin to appear; with further additions of cobalt these four signals develop further (Fig. 2*C*). One of them (*d'*) disappears in D_2O and can be attributed to an NH proton of a coordinated histidine. With the fourth Co^{2+} ion per dimer the spectrum does not change much, except for the relative intensity of the two sets of signals.

The interaction with inorganic phosphate (P_i) has been also studied on the above $Co_2Co_2Mg_2AP$ sample. Increasing amounts of P_i were added and the titration followed through ^1H NMR. Fig. 3 shows that addition of P_i causes the appearance of new signals at 51.2 and 39.2 ppm. The binding of P_i at $Co_2Co_2Mg_2AP$ seems to cause changes in the relative positions of some coordinated residues, without detatchment of any residue. The ^1H NMR spectrum does not change any further for addition of phosphate above 1:1 (Fig. 3*B*). This is in agreement with the electronic spectra of the $Co_2Co_2Mg_2AP$ with increasing amount of phosphate (Fig. 4). The addition of P_i causes a change in the position of the bands at 550 and 510 nm toward lower wavelengths and a sizable decrease of absorbance at 605 and 640 nm. With 1 eq of P_i per dimer, 80% of the whole decrease is obtained (*inset* of Fig. 4).

Titration of apoAP with Co^{2+} in Absence of Mg^{2+}—Addition of 1 Co^{2+} ion per dimer to apoalkaline phosphatase solutions at pH 8 generates an electronic spectrum of low intensity with maximum around 500 nm (Fig. 5). The following additions of cobalt up to three ions per dimer produce little increase in molar absorbance, while the intensity of the spectrum increases sharply from 3 to 5 Co^{2+} per dimer (see *inset* of Fig. 5). The final spectrum, obtained with 6 cobalt ions per dimer, shows bands at 640, 605, 550, 510 nm, with maximum at 550 nm. This behavior is again similar to that reported by Anderson *et al.* (27).

The ^1H NMR spectrum obtained upon addition of up to 1 Co^{2+} ion per dimer shows isotropically shifted signals in the region from 100 to −25 ppm (Fig. 6, *A* and *B*). Two of these signals, at 92.1 and 70.2 ppm, disappear in D_2O, suggesting that they might belong to two NH protons of coordinated histidines. Addition of the second Co^{2+} ion changes the previous spectrum completely: some new signals appear and the signals at 92.1, 70.2, 46.4 ppm disappear (Fig. 6*B*). The spectrum remains almost unchanged with 3 Co^{2+} ions per dimer (Fig. 6*C*), while the fourth equivalent of cobalt again induces drastic changes; the spectrum of the previous species disappears almost completely (Fig. 6*D*). After 4 Co^{2+} ions per dimer the spectrum does not change any further. The analysis of these data is complicated by the fact that the system is very sensitive to the experimental conditions: repeated exper-

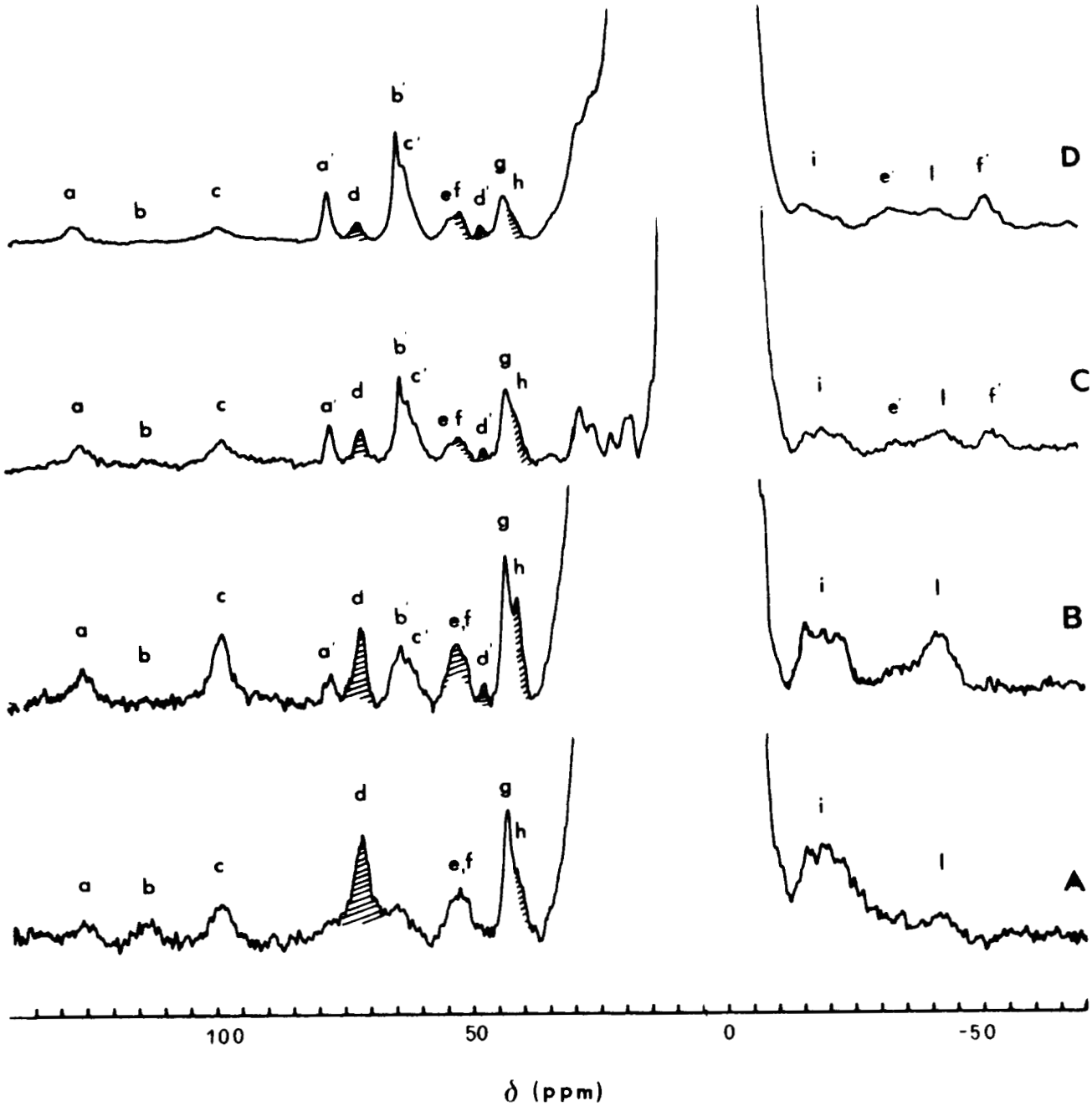

FIG. 2. **90 MHz ^{1}H NMR spectra of apoAP plus 1 Co^{2+}/dimer (*A*); 2 Co^{2+}/dimer (*B*); 3 Co^{2+}/dimer (*C*); 4 Co^{2+}/dimer (*D*).** The *shaded signals* disappear in D_2O. Conditions: 10 mM Tris, 10 mM Mg^{2+} (pH 8), 301 K; the concentration of the protein was 1 mM.

TABLE I

Longitudinal relaxation times, T_1 (ms), of some ^{1}H NMR signals of $Co_2Co_2Mg_2AP$ at pH 8

Signals[a]	T_1
ppm	*ms*
127.4 (*a*)	4.1
98.8 (*c*)	7.7
77.6 (*a'*)	5.4
71.5 (*d*)	8.1
64.1 (*b'*)	7.9
62.4 (*c'*)	4.7
51.8 (*e*)	6.4
51.6 (*f*)	12.4
47.8 (*d'*)	6.5
43.4 (*g*)	10.3

[a] Labeled as in Fig. 2*D*.

iments produced similar but not identical results in terms of relative signal intensities, reflecting fluctuations in the distribution of various species along the titration. Even deuteration of the Co_4AP sample yielded a spectrum that is correlated with the spectrum in H_2O but which is not exactly the same.

DISCUSSION

The uptake of cobalt and the order of population of the various metal sites at alkaline pH has up to now been quite puzzling. The combined analysis of the electronic spectra and of the ^{1}H NMR spectra may shed some light on the complicated problem. When 1 eq of cobalt(II) is added to dimeric apoAP in the absence of magnesium a weak absorption spectrum develops. The ^{1}H NMR spectra of this derivative show very weak signals, *i.e.* with a signal-to-noise ratio much lower

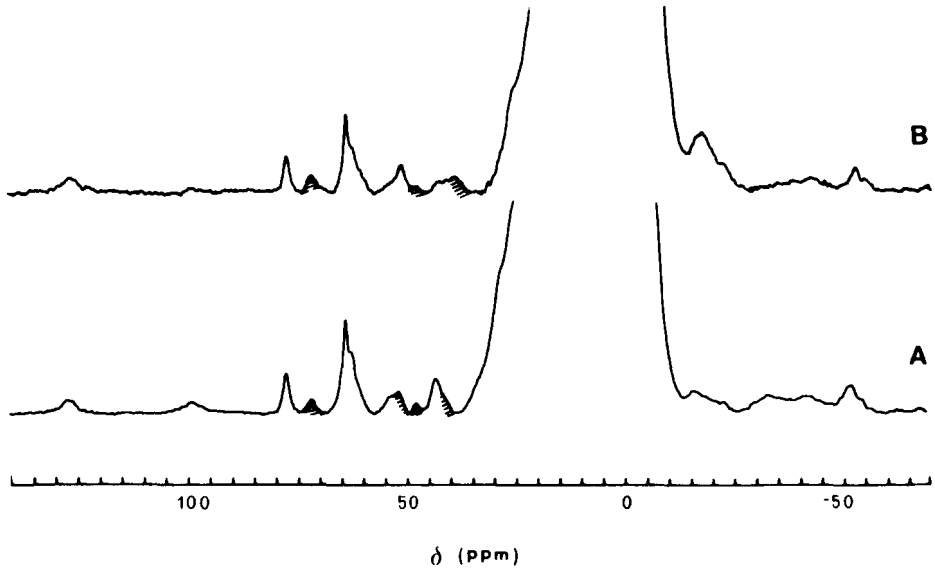

FIG. 3. **90 MHz ^{1}H NMR spectra of $Co_2Co_2Mg_2AP$ (*A*) and $Co_2Co_2Mg_2AP$ + 1.0 eq of P_i per dimer (*B*).** The *shaded signals* disappear in D_2O. Conditions as in Fig. 2.

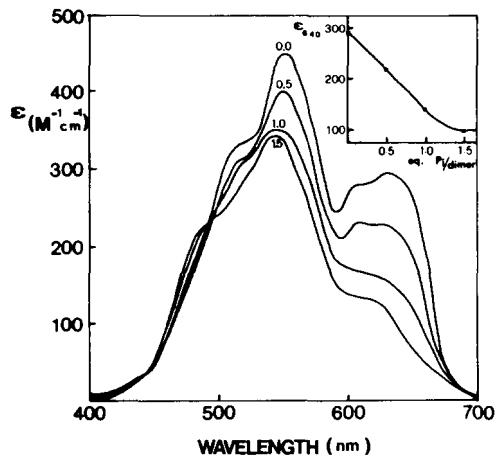

FIG. 4. **Electronic absorption spectra of $Co_2Co_2Mg_2AP$ plus phosphate.** The numbers show the P_i/dimeric protein ratio. The *inset* shows the values of ϵ_{640} as a function of the amount of P_i. Conditions as in Fig. 1. The concentration of the protein was about 0.2 mM.

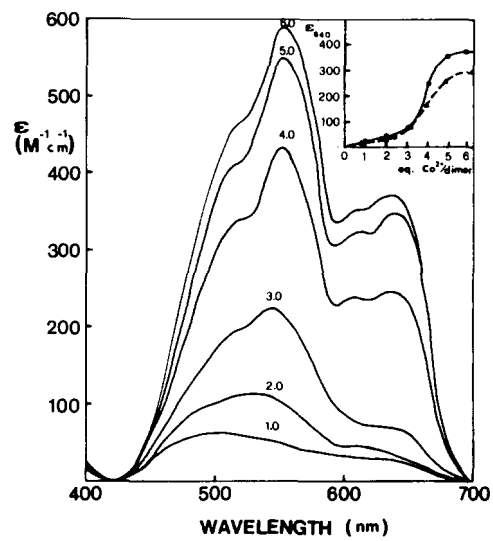

FIG. 5. **Electronic absorption spectra of apoAP plus increasing amount of Co^{2+}.** The numbers show the Co^{2+}/dimeric protein ratio. Conditions: 10 mM Tris (pH 8), room temperature; the concentration of the protein was 0.1–0.2 mM. The *inset* shows the values of ϵ_{640} as a function of the Co^{2+}/dimeric protein ratio obtained by us (——) and by Anderson *et al.* (– – –) (from Ref. 27).

than that expected on the ground of the cobalt-protein concentration. This observation can be accounted for by suggesting that most of the cobalt(II) ions are coordinated in a fluxional fashion, not giving rise to observable signals. The fluxional behavior is suggested by the observation that the active site structure of the apoprotein is quite disordered (33) and may not have the binding sites organized to bind the metal ions. The metal may interact with donor groups from both the A and B sites and solvent molecules may complete the coordination, up to a total of six coordination. The weak signals observed, on the other hand, may be relative to a small fraction of cobalt ions bound to one or more binding sites which are kinetically inert on the NMR time scale.

With the second and third cobalt ion the electronic spectra begin to shape up, although they are still of low intensity. The ^{1}H NMR spectra, with a better signal-to-noise ratio, may be relative to cobalt ions organized in an A-B couple of the same subunit. From the intensity of the electronic spectra and the signal-to-noise ratio, the amount of organized cobalt can be estimated on the order of 20% in spectrum C and 35% in spectrum D of Fig. 6. Such figures are calculated by assuming that only the A site cobalt in an A-B pair contributes to the electronic spectra. The assumption is justified by the low pH titration (24), where the B site cobalt shows a molar absorption of 10 M^{-1} cm^{-1} which is consistent with hexacoordination. The fluxional cobalt is also expected to be hexacoordinated and of low intensity. Between three and four cobalt ions the electronic spectra largely increase in intensity, maintaining the overall features. This may mean that the cobalt ions organize themselves cooperatively in the A and B sites of both subunits. Occupancy of both subunits may give rise to different ^{1}H NMR spectra relative to the occupancy of a single subunit. At four cobalt ions we have a large share of ions bound to A-B pairs of the two subunits, plus some residual fluxional cobalt which is also forced to bind specifically by further increasing the Co/AP ratio. This scheme accounts for all the experimental observations and is consistent with the data relative to the titration in presence of magnesium. The latter ion binds to the C sites and, at least partially, also to the B sites (32). Such binding helps in organizing the A site; indeed, the electronic spectra develop at lower Co/AP ratios with respect to the sample without

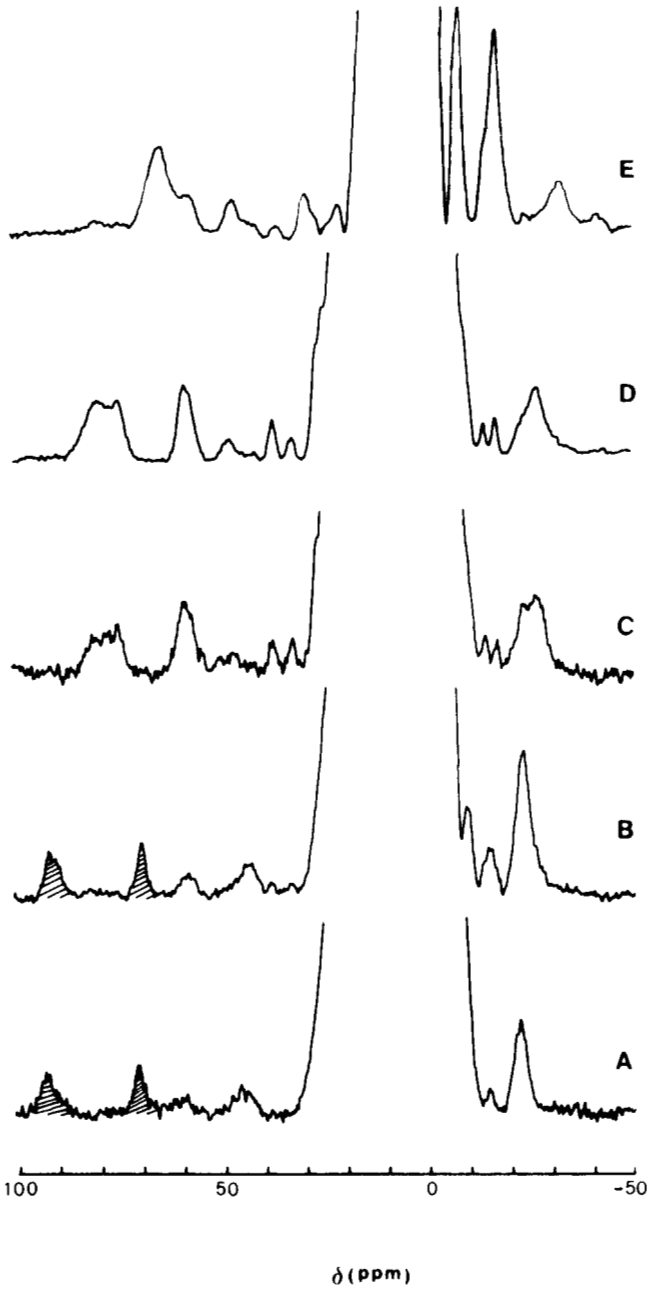

FIG. 6. **90 MHz ^{1}H NMR spectra of apoAP plus Co^{2+}: *A*, 0.5 Co^{2+}/dimer; *B*, 1.0 Co^{2+}/dimer; *C*, 2.0 Co^{2+}/dimer; *D*, 3.0 Co^{2+}/dimer; *E*, 4.0 Co^{2+}/dimer.** The *shaded signals* disappear in D_2O. Conditions: 10 mM Tris (pH 8), 301 K; the concentration of the protein was 1 mM.

magnesium, although the increase in molar absorbance is not linear with cobalt but increases less at low Co/AP ratios. This may mean that we still have some fluxional cobalt which is hexacoordinated. The ^{1}H NMR spectra reveal signals which we may refer to the signals of an A or pseudo-A site. Indeed, in spectrum A of Fig. 2 there is more than one signal which disappears in D_2O, consistent with the fact that the A site has three histidines as donor groups. The electronic spectra show relatively low intensity. At a Co/AP ratio of about 2 a new species arises which develops with increasing amount of cobalt. This species may be relative to A site cobalt in the presence of metals in both B and C sites. Binding of cobalt in the B site may occur in partial competition with magnesium. The presence of magnesium in the C site facilitates the occupancy of the A and B sites of both subunits. The final electronic spectra are quite similar for the two titrations, because they are both dominated by the cobalt ion in the A site, which presumably is 5-coordinated (24). The ^{1}H NMR spectra are quite different because they are sensitive to the orientation of the coordinated groups which may depend on the mechanism through which the metal binding has taken place. One could think that the difference in the ^{1}H NMR spectra is due to the presence of magnesium in the C site. However, at acidic pH the occupancy of the C sites by magnesium produces only small variations in the shifts. It appears to us more reasonable that the ligands may arrange around the metal ions in different protein conformations (even involving the backbone) and that the final arrangement is due to the history of the sample. Indeed, the titrations reported in Figs. 1 and 5 by us and others (27) and other titrations performed by us and not reported here are similar to each other but not identical. In the light of these findings, a report by Applebury and Coleman (26) on stoichiometric binding of 2.2 cobalt(II) ions per dimeric protein, even in the apparent absence of magnesium, can be rationalized on the ground of a different starting conformation of the apoprotein, already suitable for A site binding.

Phosphate binds at the metal in the A site as shown before (11). The electronic spectrum changes considerably. Consistent with the above picture, the ^{1}H NMR spectrum (Fig. 3*B*) shows the appearance of two new signals (51.2 and 39.2 ppm), probably originating from the A site of the subunit which binds P_i. The other subunit shows no appreciable changes. We confirm that essentially one phosphate ion can bind the two subunits.

This study confirms the complexity of behavior of apoAP toward metal uptake. At alkaline pH copper has large affinity only for the A site (35), manganese binds the A and the B site consecutively only if there is magnesium in excess,[2] whereas cobalt binds in a random fashion until the A and B sites are cooperatively occupied.

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