Contribution from the Department of Chemistry, University of Florence, 50121 Florence, Italy, and Institute of Agricultural Chemistry, University of Bologna, 40126 Bologna, Italy

Characterization of Cu_2Co_2- and Co_2Co_2-Alkaline Phosphatase Complexes at Acidic pH

L. Banci,† I. Bertini,*† C. Luchinat,‡ M. S. Viezzoli,† and Y. Wang†

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Alkaline phosphatase (AP) has been shown to bind selectively copper in the A site and cobalt in the B site, at pH 6. Magnesium can be present in the C site. On account of the short distance between A and B sites the electronic relaxation rates of copper decrease, although no magnetic coupling interactions can be detected via magnetic susceptibility measurements at room temperature. The water ^1H nuclear magnetic relaxation dispersion measurements show a dramatic decrease in relaxation rates when cobalt is added to Cu_2E_2AP (E = empty) as a result of the decrease of the correlation time of protons feeling the copper electron. The ^1H NMR spectra of the $Cu_2Co_2Mg_2AP$ derivative show well-shaped signals of protons of histidines bound to both copper and cobalt. The assignment is performed through a comparison between the spectra of $Cu_2Co_2Mg_2AP$ and $Co_2Co_2Mg_2AP$; the histidine NH's are assigned by recording the spectra in H_2O and D_2O. In the absence of magnesium the latter protons exchange rapidly on the NMR time scale.

Introduction

Alkaline phosphatase (AP) is a dimeric enzyme of molecular weight 94 000, each subunit containing two zinc ions and possibly one magnesium ion.[1-5] The enzyme is an excellent system for investigating the interaction among metal ions in each subunit as well as between subunits. It also provides a frame in which, in principle, different metal ions can be placed close to each other and their interactions can be studied. We are particularly interested in the investigation of magnetic coupling within pairs of different metal ions. Since the molecular tumbling is slow due to the large molecular weight, any time-dependent phenomena occurring in a time domain shorter than the rotational correlation time can be fruitfully studied.[6] In particular the effect of magnetic coupling on the electronic relaxation times of the two metal ions can in principle be investigated.

The metal sites in AP are called A, B, and C. A and B are those occupied by zinc.[7,8] The X-ray structure of the Cd_6 derivative has been solved up to 2.8 Å of resolution.[9,10] It appears that sites A and B are 3.9 Å apart and that the former has three histidine nitrogens as donor atoms,[9,10] one of which was shown to be Nδ1 and the others Nϵ2.[11] The B site has been shown to have one histidine and one aspartic residue coordinated to the metal; furthermore, an aspartic residue is bridging metal B and metal C. The distance between sites B and C is 4.9 Å whereas that between sites A and C is 7.2 Å. The four- or five-coordination of the A site and the six-coordination for the B site, as shown from spectroscopic measurements,[2,12] are reached through water molecules or unidentified protein ligands.

We had already reported the site occupation sequence of cobalt(II) with respect to apoAP at low pH.[12] We have now prepared the derivative in which Cu^{2+} is in the A site and Co^{2+} is in the B site, with and without Mg^{2+} in the C site. If magnetic coupling between copper and cobalt is going to occur, the electronic relaxation times of copper will be reduced and it may be conceivable to measure the ^1H NMR spectra and to detect the signals of protons of residues bound to both copper and cobalt.[6,13-15]

Materials and Methods

Alkaline phosphatase from *Escherichia coli* was isolated and purified according to the procedures followed by Applebury et al.[16] The apoenzyme was prepared by dialysis against 2 M $(NH_4)_2SO_4$ in 10 mM Tris-10 mM CH_3COONa at pH 9.[17] The apoenzyme had an activity <5% of that of the native protein. Protein concentration was determined spectrophotometrically at 280 nm, with an $\epsilon_{1\ cm}^{0.1\%} = 0.72$,[5] and a molecular weight of 94 000. Unbuffered apoenzyme solution, at pH 6, was concentrated in a metal-free Amicon ultrafiltrating cell to final concentration of approximately 1 mM. Solutions at different concentrations were prepared by dilutions of the stock solution.

Metal derivatives were prepared by addition of aqueous solutions of $CuSO_4$, $CoSO_4$, and $MgSO_4$ to the unbuffered apoenzyme solution, at a pH of around 6. Deuteriated samples were prepared by repeated dilution with D_2O and concentration under nitrogen flow to a final D_2O content larger than 90%.

The electronic spectra were recorded on a Cary 17D spectrophotometer by using the apoenzyme in the same concentration as reference.

X-Band EPR spectra were run on a Bruker ER 200 spectrometer at room temperature, calibrated with diphenylpicrylhydrazyl (g = 2.0037).

Water proton longitudinal relaxation measurements were performed on a field cycling relaxometer described elsewhere,[6] in the proton Larmor frequency range 0.01-40 MHz.

Room-temperature susceptibility measurements were performed with the Evans method on a Bruker MSL 200 spectrometer at 200 MHz using DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) as inert standard. The accuracy of the measurements is ±0.1 Hz, over total bulk susceptibility shifts of 5-10 Hz.[6,18,19]

The ^1H NMR spectra were recorded on a Bruker CXP 90 spectrometer with the modified DEFT (MODEFT)[20,21] pulse sequence. 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 DEFT sequence. Estimated errors are of the order of 10%.

Results and Discussion

Electronic Spectra. The addition of a 10^{-2} M solution of $CuSO_4$ to a 10^{-4} M solution of apoAP in the ratio 2 mol of Cu^{2+}/dimeric protein gives the Cu_2E_2AP derivative, where copper occupies the A site and E stands for the empty B site.[22-25] The electronic

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† University of Florence.
‡ University of Bologna.

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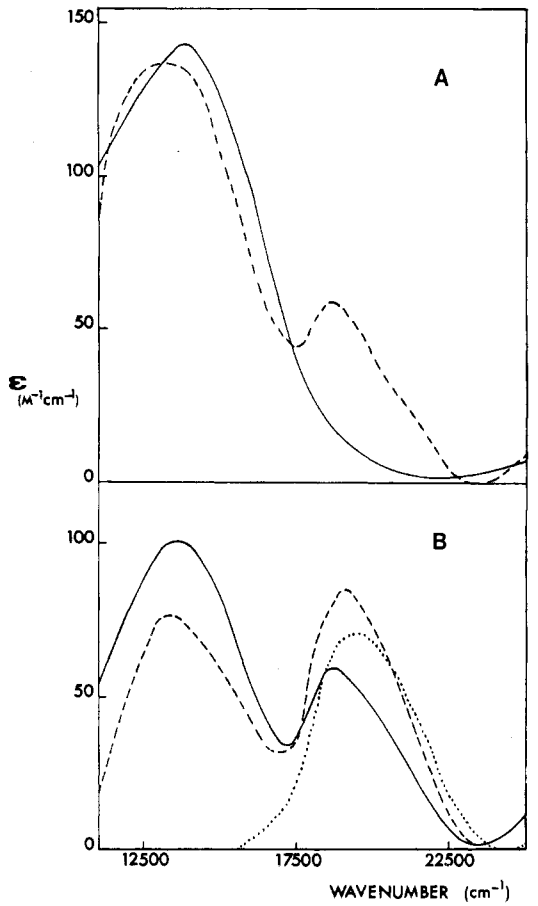

Figure 1. (A) Electronic absorption spectra of Cu_2E_2AP (—) and Cu_2Co_2AP (- - -). (B) Electronic absorption spectra of Co_2E_2AP (···), Co_2Cu_2AP (- - -) (spectrum run immediately after the addition of copper), and Co_2Cu_2AP (—) (final spectrum recorded after 8 h). The concentrations of the samples were 0.2–0.3 mM and the pH was 6.

spectra at pH 6 are typical of the derivative as previously reported[25,26] (Figure 1A). When a solution of 10^{-2} M $CoSO_4$ is added to the solution of Cu_2E_2AP (in the ratio 2 mol of Co^{2+}/dimeric protein), the low-intensity spectrum of the cobalt in the B site develops.[12] The spectrum does not change with time. It seems we succeeded in preparing Cu_2Co_2AP.

When a solution of $CoSO_4$ is first added to the solution of apoAP the spectrum of the already described Co_2E_2AP derivative develops.[12] The spectrum of cobalt in the A site is well shaped and relatively intense (Figure 1B). After addition of copper a new absorption appears, typical of copper, indicating that a Co_2Cu_2AP derivative may be formed. However, the spectrum of cobalt fades with time (of the order of hours) tending to the spectrum previously obtained and that of copper increases. This behavior suggests that copper migrates to the A site and cobalt to the B site. The spectrum of the Cu_2Co_2AP derivative as obtained from the previous procedure is never reached, even after days. The same holds in presence of 2×10^{-2} M $MgSO_4$. We have further investigated only samples obtained with the former procedure.

EPR Spectra. When copper(II) is added to a solution of apoAP the EPR spectrum of the metal ion in the A site develops (Figure 2). The intensity increases up to a 2:1 metal:dimeric protein molar ratio. The measurements are performed at room temperature where the slow rotation of the molecule provides a powderlike spectrum. When cobalt(II) is added titrationwise, the intensity

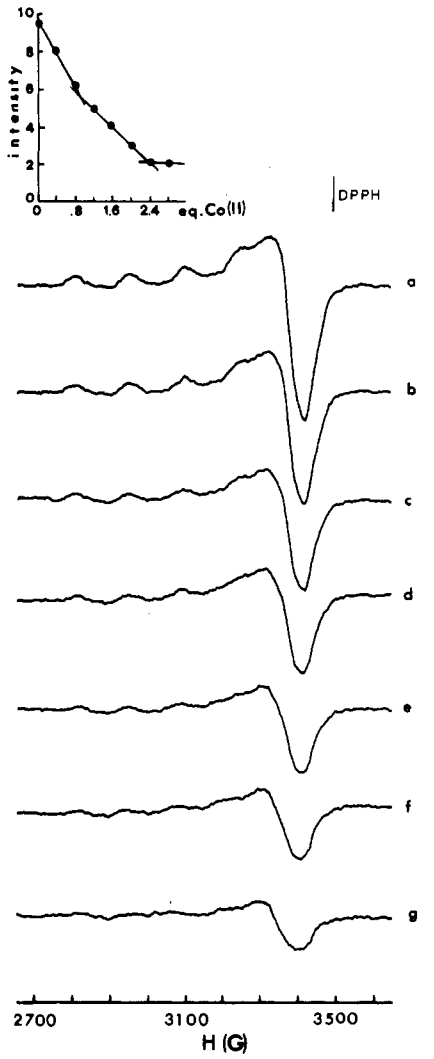

Figure 2. EPR titration of alkaline phosphatase with an increasing amount of cobalt: (a) $Cu_{1.7}E_2AP$; (b) $Cu_{1.7}Co_{0.4}AP$; (c) $Cu_{1.7}Co_{0.8}AP$; (d) $Cu_{1.7}Co_{1.2}AP$; (e) $Cu_{1.7}Co_{1.6}AP$; (f) $Cu_{1.7}Co_{2.0}AP$; (g) $Cu_{1.7}Co_{2.4}AP$. Concentration of the starting sample was 1 mM, the $CoSO_4$ solution was 0.1 M, and the pH was 6. The spectra were recorded at 9.95 GHz. In the inset at the top the intensity (arbitrary units of the signals in the $g_\perp$ region) is reported, as a function of the equivalents of cobalt added. The values are corrected for the dilution factor.

of the copper signal decreases to almost nothing, at least in the $g_\parallel$ region, for the Cu_2Co_2AP derivative (inset of Figure 2). The EPR spectrum of high-spin cobalt(II) is not detectable at room temperature because the electronic relaxation times are very short, of the order of 10^{-11}–10^{-12} s.[13] If copper(II) feels the unpaired electrons of cobalt(II), a further mechanism for electronic relaxation for copper is provided and its EPR line width increases until it eventually is no longer detected. Other contributions to the line width may come from the proximity of the metal ions and the powderlike spectra.[27] A similar phenomenon happens with Cu_2Co_2SOD (SOD = superoxide dismutase) where the magnetic coupling[28] between the two metal ions makes their electronic relaxation times rather similar in magnitude.[14] The EPR spectra behavior is an evidence of some kind of magnetic interaction between copper and cobalt in AP.

Magnetic Susceptibility Measurements. The magnetic susceptibility has been measured with the Evans method at room temperature, at different metal:protein ratios. For this experiment two coaxial tubes are used, one of which contains the protein and

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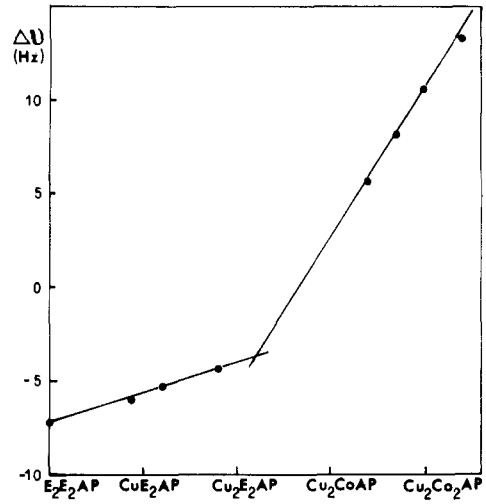

Figure 3. Differences in chemical shift between the two ^1H NMR signals of DSS in water and in an aqueous solution of alkaline phosphatase at increasing concentrations of copper and cobalt. The starting concentration of the protein was 1 mM, the pH was 6, and the concentration of DSS was 10 mM. The values are corrected for the dilution factor.

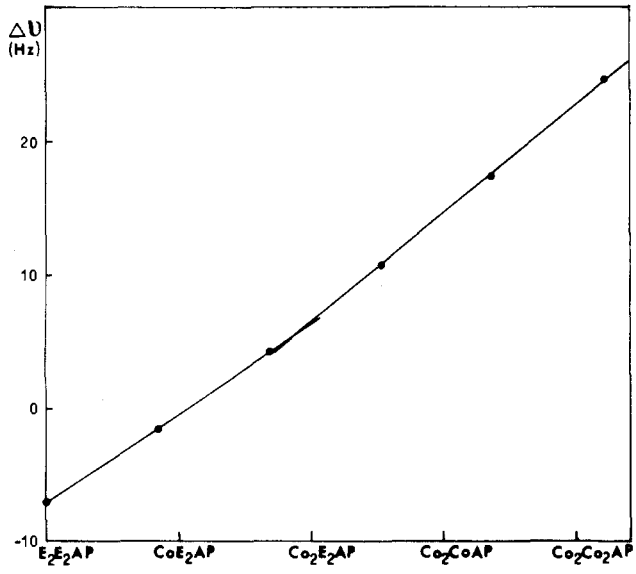

Figure 4. Differences in chemical shift between the two ^1H NMR signals of DSS in water and in a aqueous solution of alkaline phosphatase at increasing concentrations of cobalt. The starting concentration of the protein was 1 mM, the pH was 6, and the concentration of DSS was 10 mM. The values are corrected for the dilution factor.

the other contains water, and both contain the same reference compound for the NMR signal. The separation in shift between the two signals, one for each tube, is proportional to the overall magnetic susceptibility. In Figure 3 the separation in hertz at 200 MHz is reported as a function of metal concentration (corrected for dilution). It appears that the shift separation is linear with the metal content for each metal. The absolute values for the effective magnetic moment are 2.0 ± 0.1 and 4.7 ± 0.1 μ_B for copper and cobalt, respectively. The same procedure provided a value of 4.9 ± 0.1 μ_B for the hexaaqua complex. The small reduction compared to the hexaaqua complex cannot be taken as evidence of magnetic coupling at room temperature.[29]

The measurements have been performed also for the titration of apoAP with cobalt only up to a metal:protein ratio of 4:1 (Figure 4). Again no magnetic coupling was detected. If it is assumed that cobalt occupies first the A site and then the B site, the

(29) If a reduction from 4.9 to 4.7 μ_B is taken as indicative of magnetic coupling, a value of $J = 30$ cm^{-1} ($2J = 60$ cm^{-1}) is estimated. This could be taken as an upper limit.

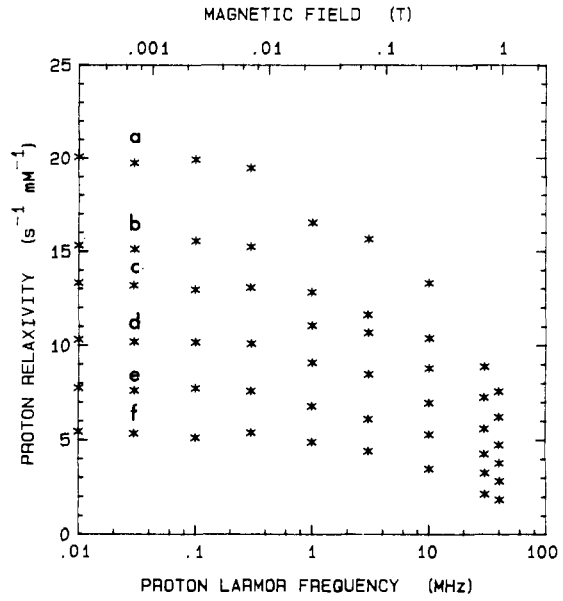

Figure 5. Water proton relaxivity of (a) Cu_2E_2AP, (b) $Cu_2Co_{0.4}AP$, (c) $Cu_2Co_{0.8}AP$, (d) $Cu_2Co_{1.2}AP$, (e) $Cu_2Co_{1.6}AP$, and (f) Cu_2Co_2AP. The concentration of the protein was 0.32 mM and the pH was 6, at 298 K.

magnetic moments for the two sites are 4.2 ± 0.1 and 4.9 ± 0.1 μ_B, respectively. This difference, which is outside the experimental error, indicates that the coordination number is different for the two sites. The absolute values indicate that cobalt in the A site is four-coordinated whereas it is six-coordinated in the B site.[30,31] The high value of the magnetic moment of copper in the A site is also indicative of a pseudotetrahedral coordination.[32]

^1H NMRD Measurements. Water ^1H nuclear magnetic relaxation dispersion (NMRD) has been measured in the magnetic field range 0.01–40 MHz for Cu_2E_2AP in the presence of increasing amounts of cobalt. The water ^1H NMRD values for Cu_2E_2AP are equal to those already observed,[25] which are typical of a copper(II) protein with a water molecule coordinated to the metal ion and in fast exchange with the bulk solvent.[33-35] Since the correlation time of copper is about 3×10^{-9} s, the water ^1H T_1^{-1} values are rather large. High-spin cobalt(II) coordinated to proteins have correlation times of the order of 10^{-11}–10^{-12} s, and the effect on water ^1H NMR relaxation is very small. Indeed, both Co_2E_2AP and Co_2Co_2AP gave ^1H NMRD profiles indistinguishable from those of the demetalized protein. It follows from the above reasoning that in absence of any interaction between copper and cobalt, addition of cobalt(II) to Cu_2E_2AP would not change the water proton relaxation values. On the contrary, addition of cobalt induces a decrease in nuclear relaxation rates (Figure 5). The final profile relative to Cu_2Co_2AP has low relaxation rates and is reminiscent of that of cobalt(II) carbonic anhydrase with a quite short correlation time.[36] This is a neat evidence of the occurrence of magnetic coupling between the two metal ions. From our previous studies it was proposed that when the extent of magnetic coupling, as expressed in terms of the isotropic coupling constant J, is such that $J > \hbar\tau_c^{-1}(Cu)$, where $\tau_c(Cu)$ is the correlation time for copper, a decrease in the correlation time of copper is expected.[15,37] From Figure 5 it appears

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Table I. Longitudinal Relaxation Times, T_1 (ms), of ^1H NMR Signals (ppm) of Co_2E_2AP, Co_2Co_2AP, Cu_2Co_2AP, and $Cu_2Co_2Mg_2AP$

					Co_2E_2AP						
signals	98.4	68.0	53.1	32.6							
T_1	9	18	11	12							
					Co_2Co_2AP						
signals[a]	110.6 (c)	83.7 (d)	57.0 (i)	44.8 (k)	34.9 (l)	24.6 (n)	−15.5 (t)	−30.0 (w)	−38.6 (x)	−45.2 (y)	−56.6 (z)
T_1	5.6	5.7	4.6	8.9	16.4	13.9	16.3	7.0	11.3	16.6	5.8
					Cu_2Co_2AP						
signals[b]	110.8 (b)	83.9 (d)		45.8 (i)	34.9 (l)	24.2 (m)		−30.3 (u)	−38.1 (v)	−45.6 (x)	−56.8 (y)
T_1	5.8	8.1		8.1	9.9	8.8		7.0	8.9	7.9	7.3
					$Cu_2Co_2Mg_2AP$						
signals[c]	116.0 (b)	75.0 (d)	54.7 (f)	44.3 (g)	39.0 (h)	35.2 (i)	29.7 (j)	26.5 (k)			
T_1	3.1	5.9	13.6	9.0	8.1	7.4	14.6	14.9			

[a] Labeled as in figure 6A. [b] Labeled as in Figure 8A. [c] Labeled as in Figure 7A.

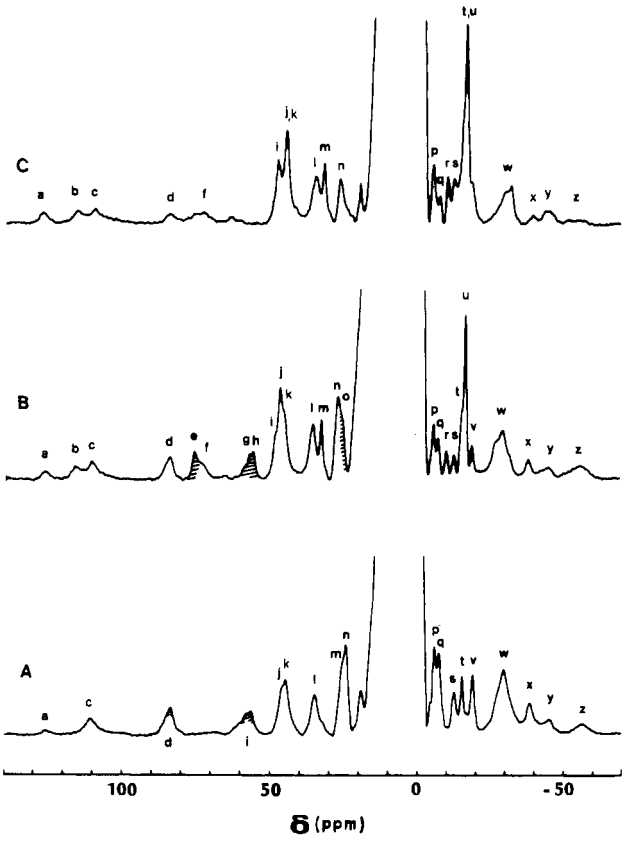

Figure 6. 90-MHz ^1H NMR spectra of (A) Co_2Co_2AP in H_2O, (B) $Co_2Co_2Mg_2AP$ in H_2O, and (C) $Co_2Co_2Mg_2AP$ in D_2O, at 301 K. Co^{2+} was added in stoichiometric amount; the concentration of Mg^{2+} was 10 mM. The pH was 6.0–6.3. Shaded signals disappear in D_2O. The labels indicate correspondence of signals in the three derivatives. Some of them are only tentative (see text).

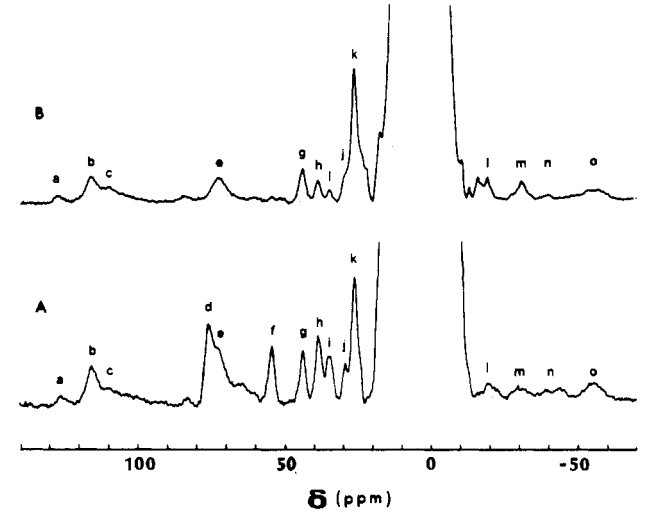

Figure 7. 90-MHz ^1H NMR spectra of (A) $Cu_2Co_2Mg_2AP$ in H_2O and (B) $Cu_2Co_2Mg_2AP$ in D_2O. The experimental conditions were the same as in Figure 6.

that τ_c for the whole system (and probably for copper) is (2–3) $\times 10^{-11}$ s. Therefore J may be sizable, although not large enough to allow its precise evaluation from susceptibility measurements at room temperature. A lower limit for J can be estimated[37] to be 0.2 cm^{-1}.

^1H NMR Spectra and Their Assignments. If we compare the ^1H NMR spectra of Co_2Co_2AP and $Co_2Co_2Mg_2AP$ (parts A and B of Figure 6), it is apparent that several more signals are present in the latter spectrum. They are b, e, f, r, and u, at 116.6, 75.0, 73.3, −10.7, and −16.9 ppm, respectively. Furthermore signals, g, h, and o, at 56.3, 55.0, and 24.7 ppm, respectively, are new because they disappear in D_2O, whereas no signal disappears in D_2O in spectrum A, (although signals d and i decrease in intensity). The signals that disappear in D_2O for the $Co_2Co_2Mg_2AP$ sample are, therefore, e, g, h, and o (Figure 6C). It appears that binding of Mg^{2+} causes a slowing down of the exchange rate of labile protons, which makes them observable. It is possible that these protons belong to the four histidines binding both at sites A and B. Signals e, g, and h are all above 50 ppm downfield and are likely to correspond to coordinated histidine NH protons. Signal o could also belong to a His NH proton, thereby completing the assignment of the four active-site histidines; however, its smaller isotropic shift might leave the possibility that it belongs to another active site exchangeable proton experiencing essentially dipolar shift. At this stage we cannot assign the His NH of each site.

These findings allow us to make an attempt to assign the signals of Co_2Co_2AP (without magnesium), which has almost identical spectra in water and D_2O[12] (Figure 6A). The signals downfield, a, c, and d, above 75 ppm, must be ortholike protons of the histidine rings. Their T_1 values (Table I) are long enough (5–6 ms) to indicate that they belong to octahedral or five-coordinated Co^{2+}.[6,13] A metalike proton that is present in one histidine belonging to the A site could be assigned to either the signal at 45.4 ppm (signal j) or at 44.8 ppm (signal k) or at 34.9 ppm downfield (signal l). The many other signals are assigned either to CH_2 protons of carboxylic residues or to protons of protein close to the metal ion but not belonging to residues coordinated to it. They have in general long T_1s and sharp lines. The isotropic shift of the latter kind of protons is dipolar in origin. Octahedral Co(II) complexes are known to give rise to sizable dipolar shifts.[6]

When the spectrum of $Cu_2Co_2Mg_2AP$ (Figure 7) is compared to that of $Co_2Co_2Mg_2AP$ (Figure 6B,C), we observe that, in the former, four exchangeable signals are also apparent. Signals d and f are in the same position of signals e and h in Figure 6B. One of them is likely to correspond to B site His NH. Tentatively, we may assign signal d (e in figure 6B) to such a proton, due to its larger isotropic shift. Then signals f, h, and i would belong to the A site. A possible candidate for the metalike proton of the A site is the signal at 29.7 ppm (signal j). The signals assigned to Co^{2+} in the B site above 70 ppm are the same in both Cu_2-

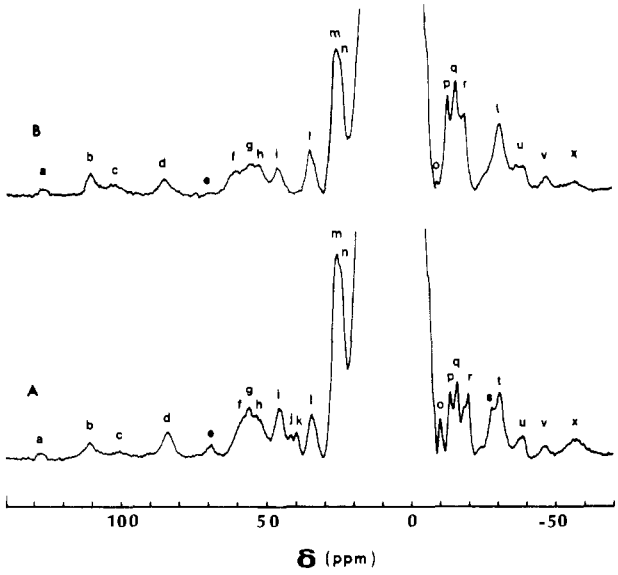

Figure 8. 90-MHz ^{1}H NMR spectra of (A) Cu_2Co_2AP in H_2O and (B) Cu_2Co_2AP in D_2O. The experimental conditions were the same as in Figure 6.

Co_2Mg_2AP and $Co_2Co_2Mg_2AP$. Some differences can be noted in the upfield part of the spectrum and near the diamagnetic region, indicating that some conformational changes occur, as detected from changes in dipolar shifts on passing from cobalt to copper in the A site.

A final comment regards the spectra of Cu_2Co_2AP (Figure 8) where, similar to the spectrum of Co_2Co_2AP (Figure 6A), very small intensity changes are observed when passing from H_2O to D_2O. This indicates that the histidine NH protons are in fast exchange with the solvent. However, two signals of fractional intensity at 41.0 and 39.0 ppm (signals j and k) disappear; they are not present in Co_2Co_2AP and may be assigned to residual NH signals of the A site. The slight decrease of signals d and i in the spectrum shown in Figure 6A upon deuteriation might suggest a correlation with signals j and k of Figure 8. Their positions give the shift values of exchangeable protons in the A site when occupied by cobalt. They experience larger isotropic shifts than signals j and k in Figure 8, consistent with the expected larger contact contributions for cobalt with respect to copper.

As far as T_1 (Table I) values are concerned, an overall shortening is observed on passing from Co_2E_2AP to all the other derivatives. This is somewhat surprising since magnetic coupling should give the opposite effect, if any.[14,27,37] Possibly, the A site chromophore experiences a change in electronic relaxation times when the B site is filled. A factor of 2, which is in principle a small change on the scale of the electron relaxation rates, could well account for the observed behavior.[38]

Conclusions

The present results have shown that at acidic pH apoAP is capable of binding first Cu^{2+} in the A site and then Co^{2+} in the B site. Mg^{2+} can be finally added and its major effect is that of freezing down histidine proton exchange on the NMR time scale. Minor conformational changes have been detected from the position of the ^{1}H NMR proton signals in the presence or the absence of magnesium.

The interest in these kinds of derivatives resides in the investigation of metal–metal interactions that can be monitored through the occurrence of magnetic coupling. Magnetic susceptibility measurements at room temperature do not reveal any measurable magnetic coupling. The magnetic moment of Cu^{2+} is 2.0 μ_B, typical of pseudotetrahedral copper. Magnetic moments of Co^{2+} in the B site range from 4.7 to 4.9 μ_B, which are typical of Co^{2+} with a coordination number larger than four.[39-41] Despite the fact that the magnetic susceptibility of the metal cluster is the sum of that of the separated ions, the measurements of the electronic relaxation times have revealed a much more sensitive tool in detecting weak magnetic interaction. Indeed, the EPR signal of copper in Cu_2Co_2AP broadens beyond detection, and the correlation time of water proton interaction with the copper electron is reduced by the presence of cobalt.

The ^{1}H NMRD profile of Cu_2Co_2AP qualitatively shows that (a) the relaxivity is low and (b) τ_s may be dramatically shortened down to $\simeq 10^{-11}$ s. It is difficult to determine τ_s because the dispersion is smoother than expected on the basis of a simple Solomon approach and the end of the dispersion is not observed. The τ_s value of copper in Cu_2E_2AP, i.e. without any magnetic interaction, is 4×10^{-9} s. We have then to consider that magnetic coupling causes a decrease in the squared hyperfine coupling between unpaired electrons and nuclei and therefore causes a reduction in the nuclear relaxation rate enhancements. Such reduction is three-eights in the case of protons feeling copper in a copper–cobalt pair.[14,38] We may therefore expect an overall decrease in the ^{1}H NMR line width of about 2 orders of magnitude. Indeed the ^{1}H NMR spectra of histidines bound to copper are observed.

This is the second case in which the ^{1}H NMR spectra of groups coordinated to copper in proteins have been detected. The other example is Cu_2Co_2SOD in which a J of 17 cm^{-1} has been found to reduce the electronic relaxation times of copper to 1.7×10^{-11} s from 1.8×10^{-9} s in Cu_2Zn_2SOD.[14] The shifts of the histidine protons (the NH of the AP derivatives) are in the same range for the two systems. The T_1 values of protons feeling copper are rather close to those of protons feeling cobalt for Cu_2Co_2AP whereas the T_1 values of the former protons are relatively longer than those of the latter protons in the case of Cu_2Co_2SOD. This may mean that the magnetic coupling in the former system is smaller and that the electronic relaxation times of copper are somewhat longer. Since the nuclear T_1^{-1} value is given by the product of $S(S + 1)$ by a function of the electronic relaxation times, cobalt and copper can give rise to the same nuclear T_1^{-1} enhancement only if their τ_s values are different.

As far as the enzyme is concerned, we are learning that metal ions in the A and B sites feel each other, consistent with the short distance (3.9 Å) found in the Cd_6AP derivative. The X-ray structure however has not revealed any bridging ligand. In the case of Cu_2Cu_2AP a relatively large magnetic coupling was observed (J = 120 cm^{-1}) and it was proposed that a hydroxo or alkoxo group could bridge the two ions.[25] A reasonable candidate could be Ser-102, which has been proposed to have an important role in catalysis.[2] In the present Cu_2Co_2 and Co_2Co_2 systems, the magnetic coupling is smaller and it may be that there is no bridging ligand. If the magnetic coupling were entirely dipolar, it would be estimated to be 0.2 cm^{-1} for a 3.9 Å distance. This value is the same as the upper limit for J estimated from the correlation time of copper in the Cu_2Co_2AP. Therefore, such dipolar coupling may well be effective by itself in reducing the electronic relaxation times of copper.

Finally, from the ^{1}H NMR data it should be inferred that when the B site is occupied, some changes occur in the coordination sphere of the A site, and something similar happens, although to a minor extent, on the A and B sites when C is occupied by magnesium.

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