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The Cu_A Center of a Soluble Domain from *Thermus* Cytochrome ba_3. An NMR Investigation of the Paramagnetic Protein: A Proton* NMR Study of the Paramagnetic Active Site of the Cu_A Variant of Amicyanin†

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Condensation of the Research

Purpose of the Studies:

To show that ^{1}H NMR spectroscopy can be used to probe the structure and physical properties of the delocalized mixed-valent CuA center and to provide insight into the ability of CuA to function in the electron transfer pathways of both cytochrome c oxidase and nitrous oxide reductase

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Background:

Cytochrome *c* oxidase (CcO) and nitrous oxide reductase ($N_2$0R) both contain an unusual dicopper electron transfer center, namely Cu_A, that appears to function in a similar fashion to blue copper centers.[1,2] CcO participates in cellular respiration by coupling the reduction of O_2 to H_2O with transmembrane proton pumping, while N_2OR is involved in the alternative respiration system of denitrifying bacteria by reducing N_2O to N_2. Cu_A was initially thought to be a mononuclear type I copper center; however, Kroneck et al.[3] suggested that it was actually a dinuclear mixed-valence [Cu(1.5)Cu(l.5)] center on the basis of an unusual seven-line ^{63}Cu hyperfine pattern observed in the X-band electron paramagnetic resonance (EPR) spectrum of oxidized N_2OR. A similar seven-line hyperfine pattern was also observed for intact CcO at frequencies less than X-band.[4] A mixed-valent dinuclear center was confirmed by EXAFS data that indicated a Cu—Cu distance of ~2.5 Å.5 These data suggested that a Cu-Cu bonding interaction existed for Cu_A, a notion recently substantiated in an elegant analysis of the electronic absorption, MCD, and S K-edge EXAFS spectra of both CcO and several Cu_A model complexes.[6]

Structural details of the Cu_A center were recently revealed by X-ray crystallographic studies on mammalian and bacterial CcO as well as the soluble domain of subunit II of the cytochrome *bo*$_3$ quinol oxidase.[7-10] Each of these structures indicates that Cu_A is in fact a dicopper center in which each copper ion resides in a distorted tetrahedral coordination environment. Both copper ions are bridged by two thiolate sulfur cysteine ligands (Fig. 1), and each copper ion is in turn coordinated by terminal N$^{\delta}$-bound histidine ligands that are *anti* to each other with respect to the Cu—Cu axis. One copper ion is further coordinated by a methionine sulfur ligand, whereas the second copper ion is bound by a backbone carbonyl oxygen atom of a glutamic acid residue. The symmetric nature of the dicopper center, the short metal-metal distance, and the unusual spectroscopic properties indicate that Cu_A is in fact a paramagnetic (S = 1/2), fully delocalized Cu(I)Cu(II) center. Moreover, these X-ray crystallographic studies demonstrated a structural relationship between the Cu_A center in the soluble domain of subunit II of the cytochrome *bo*$_3$ quinol oxidase and type I blue

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copper proteins (cupredoxins). Using protein engineering techniques, researchers replaced the blue copper sites of amicyanin and azurin with Cu_A binding loops. These blue copper mutants provide authentic Cu_A centers as shown by spectroscopic techniques.[11-13]

What Researchers Accomplished

In these two articles, the proton NMR spectra for the soluble Cu_A domain from *Thermus thermophilus* cytochrome ba_3 and the Cu_A variant of amicyanin were reported.[14,15] Several remarkably sharp, well resolved hyperfine shifted ^1H NMR signals were observed in the 300 to - 10 ppm chemical shift range for *T. thermophilus* cytochrome ba_3 (Fig. 2), whereas the Cu_A variant of amicyanin exhibited hyperfine shifted signals between 110 and - 10 ppm (Fig. 2). The T_1 values of aJI of the signals observed for both Cu_A centers were in the 1- to 17-ms range (Table 1). Six solvent exchangeable signals were observed upon substitution of H_2O for D_2O in the soluble Cu_A domain from *T. thermophilus* cytochrome ba_3, whereas only three exchangeable signals were observed for the Cu_A variant of amicyanin. The two most downfield shifted signals were assigned to the N- H protons of the two N^{δ}-coordinated histidine residues; the remaining exchangeable signals in each sample were assigned to backbone N-H protons in close proximity to the dinuclear copper cluster. The X-ray crystallographic information for Cu_A[7-10] shows that the N-H proton of one coordinated histidine ligand is hydrogen-bonded to a peptide carbonyl, and the second histidine ligand has a solvent-exposed N-H proton. Therefore, the observation that one of the downfield shifted solvent exchangeable signals in both samples exchanges slowly over the course of several days suggested that it belonged to His145 of the soluble Cu_A domain from *T. thermophilus* cytochrome ba_3 and His54 of the Cu_A variant of amicyanin. These data allowed the authors to further assign all of the observed hyperfine shifted signals in a sequence-specific manner.

Definitive assignment of the remaining observed hyperfine shifted signals was obtained by a combination of one-dimensional nuclear Overhauser effect (NOE) difference and two-dimensional NOESY spectroscopy. In both studies, all of the signals in the 40- to 10-ppm chemical shift range were assigned to ligand histidine ring protons except one signal at 27.4 ppm observed in the soluble Cu_A

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domain from *T. thermophilus* cytochrome ba_3 that was assigned to a bridging Cys $C^{\alpha}H$ proton. The corresponding resonance observed for the Cu_A variant of amicyanin was alternatively assigned to the $C^{\epsilon 1}H$ proton of a coordinated histidine ligand, although the assignment of this resonance to a Cys $C^{\alpha}H$ proton could not be ruled out. The two resonances observed upfield (-1 to - 10 ppm) in both samples were assigned to either His $C^{\beta}H$ ligand protons or backbone amine N- H protons. A third upfield shifted resonance at -0.2 ppm observed for the Cu_A variant of amicyanin was assigned to an Ile 26 $C^{\gamma}H_3$ proton. Full assignment of the Cu_A variant of amicyanin allowed the authors to calculate the hyperfine coupling constants for these protons from which the spin-density distribution on the Cu_A ligands was calculated. The authors suggested that 50 to 60% of unpaired spin-density was distributed on the coordinated cysteine and histidine ligands; the two weak axial methionine and backbone carbonyl ligands contained less than l% spin density.

The proton NMR spectrum of the soluble Cu_A domain from *T thermophiles* cytochrome ba_3 revealed four additional very broad hyperfine shifted signals in the 300 to 105 ppm region that were not observed for the Cu_A variant of amicyanin. NOEs from three of these signals to other observed resonances were not observed; however, the signal at 116 ppm shows a clear NOE connection to the signal at 27.4 ppm that had previously been assigned to a Cys $C^{\alpha}H$ proton, suggesting that the 116-ppm feature can be assigned to a Cys $C^{\beta}H$ proton. The similar NMR properties of the four broad peaks in the 300- to 105-ppm region and their disappearance upon selective deuteration of the Cys $C^{\beta}H$ protons[16] thus assign these four protons to Cys $C^{\beta}H$ protons of bridging Cu_A Cys ligands.

The researchers also recorded the temperature dependence of the observed hyperfine shifted ^1H NMR signals for both Cu_A samples. All of the observed signals in the Cu_A variant of amicyanin exhibited Curie-type behavior (contact shift decreases with increasing temperature); however, extrapolation of the fits of the temperature data to infinite temperature indicated that two of the signals fell well outside the expected diamagnetic range. The authors postulated that the unusual temperature behavior of these two resonances was due to a temperature induced conformational change in the Cu_A site. For the

soluble Cu_A domain from *T thermophilus* cytochrome ba_3, all but three signals observed exhibited Curie-type behavior. One of these signals exhibited anti-Curie behavior (contact shift increases with increasing temperature) while two had little or no temperature dependence. The authors ascribed the anomalous temperature behavior of these three resonances, all of which belonged to bridging cysteine ligand protons, to distinct orbital interactions of the bridging sulfur atom with each copper ion. Similar effects have been observed for low-spin cytochromes.[17]

Commentary on the Research

These two studies are extremely important contributions because ^{1}H NMR spectroscopy has been largely overlooked as a probe of dicopper centers in biological systems. This lack of attention stems from the fact that mononuclear copper(II) ions exhibit long electronic relaxation times making them poor paramagnetic ^{1}H NMR probes[18] Therefore, the fact that relatively sharp hyperfine shifted signals are observed from mixed-valent Cu_A centers suggests an alternative electronic relaxation pathway. The ^{1}H NMR relaxation data reported by Bertini et al.[14] suggests a room temperature electron relaxation rate of $\sim 10^{11}$ s^{-1} for Cu_A. This rate can be compared to mononuclear Cu(II) centers that have electronic relaxation rates of $\sim 10^8$ to 10^9 s^{-1} .[18] Increased electronic relaxation rates have also been observed for spin-coupled dicopper(II) centers and, consequently, relatively sharp, hyperfine shifted ^{1}H NMR signals are observed.[19-27] For dicopper(ll) systems, the nuclear relaxation rate enhancements are all decreased by a factor of 2 because of spin-coupling between the two metal ions.[27] Antiferromagnetic coupling, for example, creates a dicopper(II) system in which the ground (S = 0) state is separated from the first excited (S = 1) state by the exchange constant, 2J.[24] Therefore, two low-lying energy levels are present that provide a facile electronic relaxation pathway.[25,27]

The descriptions of enhanced electronic relaxation rates for a spin-doublet dicopper system in these two papers are the first such examples reported. The electronic relaxation rate enhancement for Cu_A centers was postulated by Bertini et al.[14] to be the result of two low-lying doublets that are spaced by ≤6 kT for an Orbach process or

slightly more for a Raman process. This suggestion was recently substantiated in an elegant analysis of the electronic absorption, MCD, and S K-edge EXAFS spectra for both CcO and two Cu_A model complexes.[6] From these data, a molecular orbital diagram was constructed for Cu_A that described the individual contributions from Cu-S and Cu-Cu bonding interactions to inter-ionic coupling. For Cu_A from CcO, it was shown that a significant contribution from a Cu-Cu interaction occurs resulting in two low-lying energy levels that stabilize the electron delocalization in Cu_A centers. Therefore, the unique mixed-valent dinuclear Cu_A center has a distinct advantage over a mononuclear blue copper center in accepting an electron from cytochrome *c* and transferring it to the proton-pumping unit of cytochrome oxidase.

References

[1] Babcock, G.T., Wilkstrom, M. *Nature* 1992, *356,* 301.

[2] Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Korner, H., Jakob, W. *Eur. J. Biochem.* 1985, *153,* 459.

[3] Kroneck, P.M.H., Antholine, W.A., Riester, J., Zumft, W.G. *FEBS Lett.* 1988, *242,*70.

[4] Antholine, W.E., Kastrau, D.H.W., Steffens, G.C.M., Buse, G., Zumft, W.G., Kroneck, P.M.H. *Eur. J. Biochem.* 1992, *209,* 875.

[5] Blackburn, N.J., Barr, M.E., Woodruff, W.H., van der Oost, J., de Vries, S. *Biochemistry* 1994, *33,* 10401.

[6] Williams, K.R., Gamelin, D.R., LaCroix, L.B., Houser, R.P., Tolman, W.B., Mulder, T.C., de Vries, S., Hedman, B., Hodgson, K.O., Solomon, E.I. *J. Am. Chem. Soc.* 1997, *119,* 613.

[7] Iwata, S., Ostermerier, C., Ludwig, B., Michel, H. *Nature* 1995, *375,* 660.

[8] Willmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., Saraste, M. *Proc. Natl. Acad. Sci. USA* 1995, *92,* 11955.

[9] Tsukihara, T., Aoyana, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawaltoh, K., Nakashima. R., Yaono, R., Yoshikawa, S. *Science* 1996, 272, 1136.

[10] Tsukibara, T., Aoyana, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawaltoh, K., Nakashima. R., Yaono, R., Yoshikawa, S. *Science* 1995, *269,* 1069.

[11] Andrew, C.R., Lappalainen, P., Saraste, M., Hay, M.T., Lu, Y., Dennison, C., Canters, G.W., Fee, J.A., Slutter, C.E., Nakamura, N., Sanders-Loehr, J. *J. Am. Chem.Soc.* 1995, *117,* 10759.

[12] Dennison, C., Vtjgenboom, E., de Vries, S., van der Oost, J., Canters, G.W. *FEBS Lett.* 1995, *365,* 92.

Chemtracts: Inorganic Chemistry, Vol. 11 (1998): pg. 120-126. Publisher Link.

[13] Hay, M.T., Richards, J.H., Lu, Y. *Proc. Natl. Acad. Sci. USA* 1996, *93,* 461.
[14] Bertini, 1., Bren, K.L., Clemente, A., Fee, J.A., Gray, H.B., Luchinat, C., Malmstrom, B.G., Richards, J.H., Sanders, D., Slutter, C. *J. Am. Chem. Soc.* 1996, *118,* 11658.
[15] Dennison, C., Berg, A., Canters, G.W. *Biochemistry* 1997, *36,* 3262.
[16] Stutter, C.E., Sanders, D., Wittung, P., Malmstrom, B.G., Asas, R., Richards, J.H., Gray, H.G., Fee, J.A., *Biochemistry* 1996, *35,* 3387.
[17] Shokhirev, N.V., Walker, F.A. *J. Chem. Phys.* 1996, /00, 17795.
[18] Bertini, I., Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems.* Benjamin & Cummings: Menlo Park, CA, 1986.
[19] Byers, W., Williams, R.J.P. *J. Chem. Soc., Dalton Trans.* 1972, *555.*
[20] Ze1onka, R.A., Baird, M.C. *Inorg. Chem.* 1972, *11,* 134.
[21] Maekawa, M., Kitagawa. S., Munakata, M., Masuda, H. *Inorg. Chem.* 1989, *28,* 1904.
[22] Holz, R.C., Brink, J.M., Gobena, F.T., O'Connor, C.J. *Inorg. Chem.* 1994, *33,* 6086.
[23] Holz, R.C., Brink, J.M., *Inorg. Chem.* 1994, *33,* 4609.
[24] Holz, R.C., Brink, J.M., Rose, R.R. *J. Mag. Res, A* 1996, *119,* 125.
[25] Brink, J.M., Rose, R.R., Holz, R.C. *Inorg. Chem.* 1996, *35,* 2878.
[26] Satcher, J.H., Balch, A.L. *Inorg. Chem.* 1995, *34,* 3371.
[27] Murthy, N.N., Karlin, K.D., Bertini, I., Luchinat, C. *J. Am. Chem. Soc.* 1997, *119,* 2156.

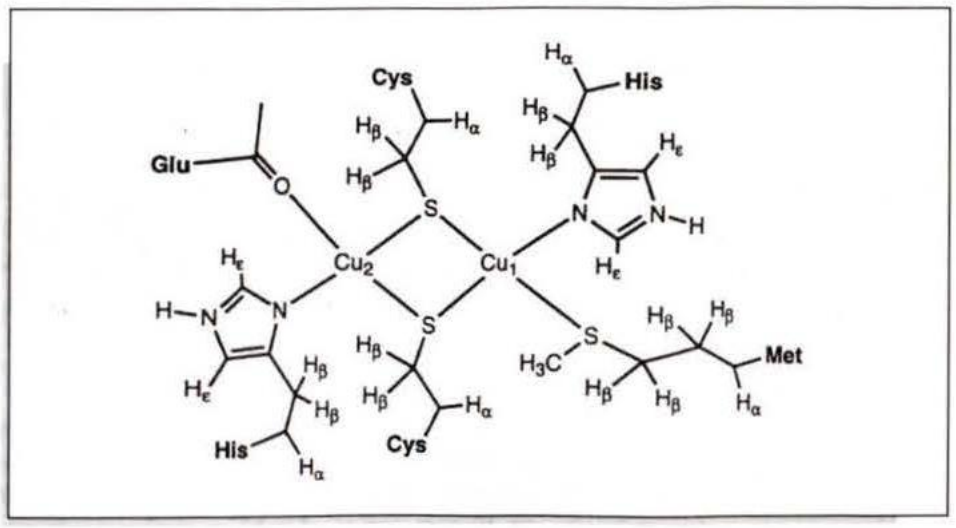


Figure I. Representation of the Cu_A site as found in the purple CyoA mutant and mammalian and bacterial CcO.

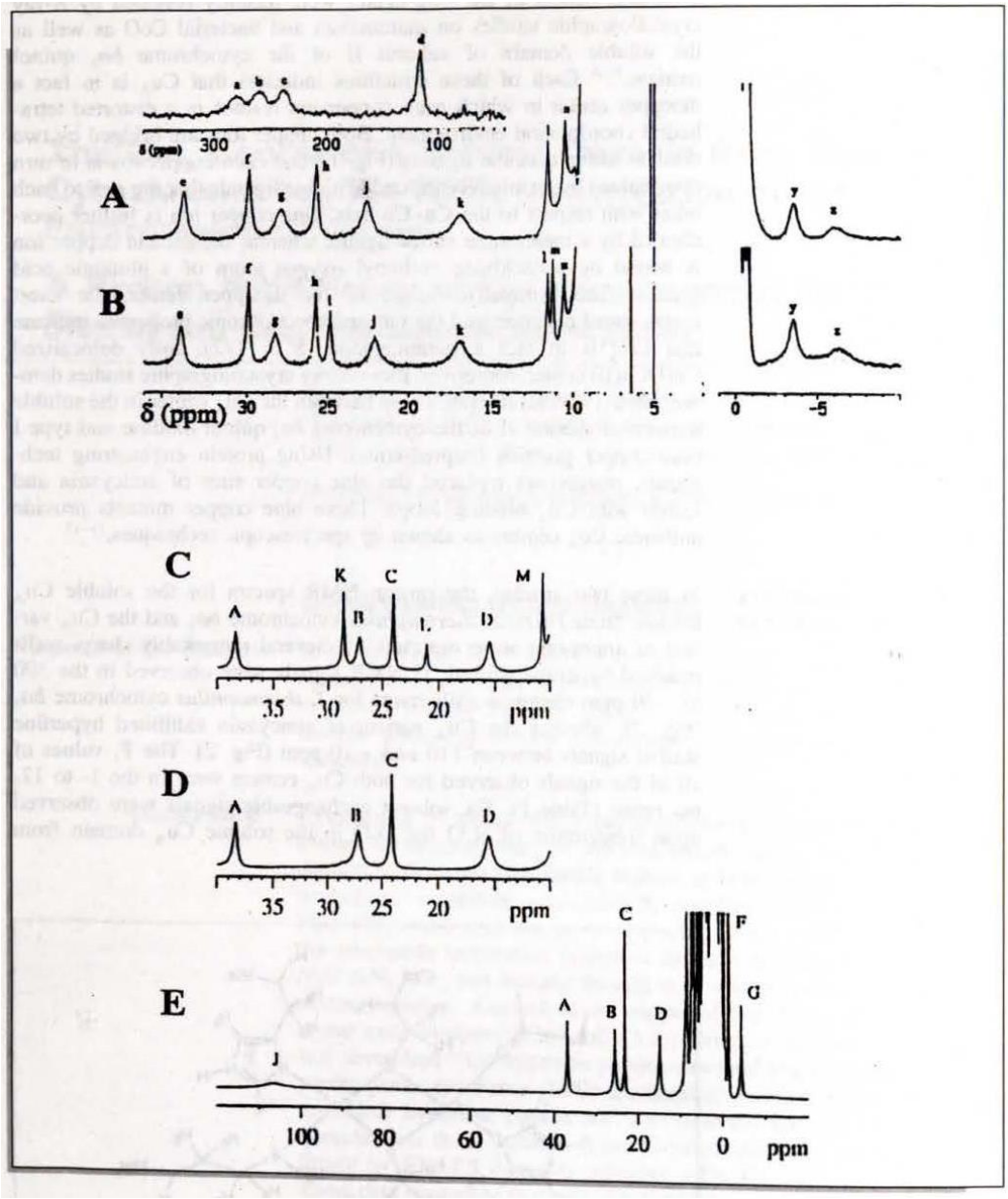
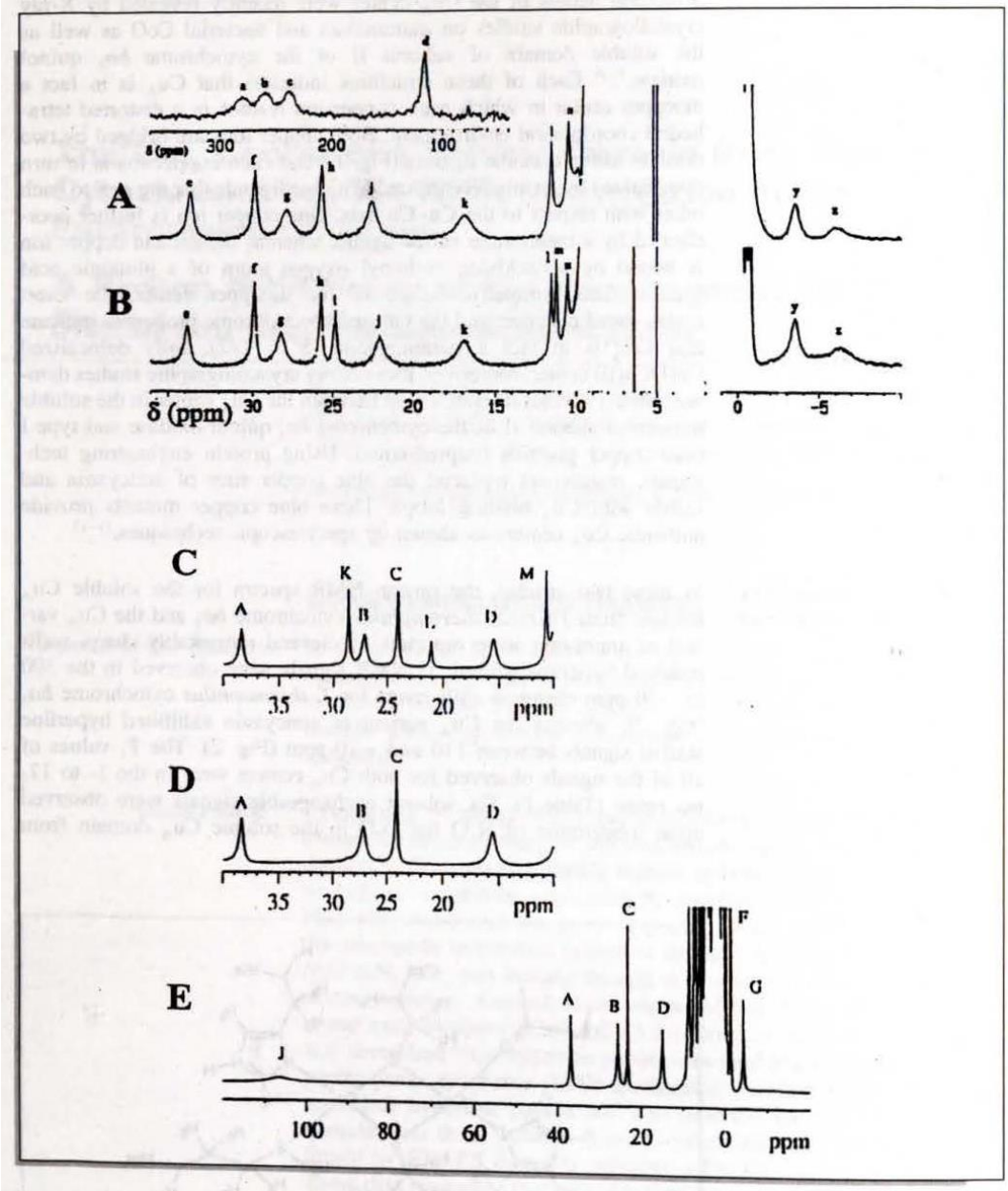


Figure 2. ^{1}H NMR spectra of *Thermus* Cu_A domain in H_2O at 278 K, and 600 Mhz at (A) pH 8 and (B) pH 4.5. The inset shows the far-downfield region of the pH 8 spectrum recorded in D_2O at 298 K. (C) 600 Mhz ^{1}H NMR spectrum of the 40- to 10-ppm region of the Cu_A amicyanin mutant in H_2O at 280 K and pH 6. (D) 600 Mhz ^{1}H NMR spectrum of the Cu_A amicyanin mutant in D_2O at 280 K. (E) Full 600 Mhz ^{1}H NMR spectrum of the Cu_A amicyanin mutant in D_2O at 298 K and pH 6.



[a] Bertini, I., Bren, K.L., Clemente, A., Fee, J.A., Gray, H.B., Luchinat, C., Malmstrom, B.G., Richards, J.H., Sanders, D., Slutter, C.J. *Am. Chem. Soc.* 1996, *118,* I 1658.
[b] Dennison, C., Berg, A., Canters, G.W. Biochemistry 1997, *36.* 3262.
[c] Not determined.

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