2.17: What Do We Learn?

From Copper Substitution

The coordination chemistry of CuCA is not yet fully understood, since the electronic spectra are not very pH-sensitive. Nevertheless, the affinity of anions is pH-dependent, as it is for CoCA.[82] As could be anticipated from Section III.B, the affinity of anions, including HCO_3^-, is higher than that of CoCA. Water is usually present in the coordination sphere, along with the anion, as checked by water ^1H NMRD.[83,84] The steric requirements of the three histidines and of the cavity allow the anion and the water molecule to arrange in an essentially square pyramidal geometry (Figure 2.17).

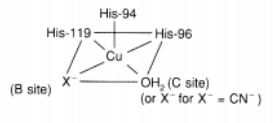

Figure 2.17 - Schematic representation of the suggested coordination geometry for the anion adducts of CuCA.

This is consistent with the electronic and EPR spectra. In particular, the EPR spectra are all axial, with g-values decreasing from 2.31 in the nonligated enzyme to 2.24 in the various anion adducts.[84] The water molecule would be in the C site or hydrophilic binding site, and the anion would be in the B site or hydrophobic pocket. His-94 would be in the apical position of the square pyramid. It has been shown by EPR spectroscopy that at low temperature two cyanide anions bind to copper. The donor atoms are two cyanide carbon and two histidine nitrogen atoms in the basal plane, and the third histidine nitrogen in the axial position.[85] The hyperfine splitting is observed only with nuclei in the basal plane. It is observed both with ^{13}C nuclei of ^{13}C-enriched CN^- and with the two ^{14}N of two histidines. The second cyanide may thus displace the coordinated water (Figure 2.17). Oxalate and sulfonamides displace water from the coordination sphere.[85,86] For the oxalate ion this may occur through bidentate behavior. Coordination to an oxygen of the sulfonamide cannot be ruled out, although the electronic and EPR spectra of the sulfonamide complex are more consistent with a pseudotetrahedral chromophore. The SO_2 moiety would in any case point toward the B binding site. It is likely that sulfonamides bind as in ZnCA. Bicarbonate also shows less water relaxivity than other monodentate anions.[83,84.86]

^{13}C NMR spectroscopy has been used to investigate the location of the two substrates, CO_2 and HCO_3 ,with respect to the metal ion in CuCA.[86-88] As was pointed out in Section IV.B, the interconversion between the two species is slow on the NMR timescale in the absence of catalysts. Therefore, two signals are observed (Figures 2.6 and 2.18). In the presence of the catalytically active CoCA, only one signal is observed at suitable enzyme concentrations, and individual information on CO2 binding cannot be obtained.[89,90] In the presence of inactive CuCA, two signals are again observed, which are broadened to different extents.

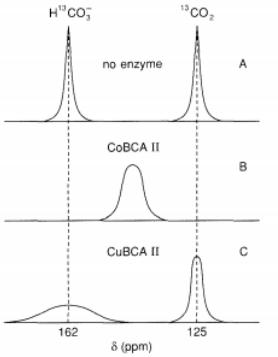

Figure 2.18 - Schematic representation of the ^{13}C NMR spectra of the CO_2/HCO_3^- system (A) in pure water, (B) in the presence of CoCA, and (C) in the presence of CuCA.[56]

For the HCO_3^- signal the T_2^{-1} values as estimated from the linewidth are much larger than T_1^{-1}. Since the equation for T_2^{-1}, analogous to Equation (2.14), would predict similar T_1 and T_2 values,[69,72] a sizeable broadening due to chemical exchange must be present. Indeed, unlike T_{1p}^{-1} (Equation 2.1l), T_{2p}^{-1} may be a complicated function of the exchange time τ_M and of the isotropic shift, $\Delta\omega_M$,

$$T_{2p}^{-1} = \frac{f_M}{\tau_M} \frac{T_{2M}^{-2} + T_{2M}^{-1}\tau_M^{-1} + (\Delta\omega_M)^2}{(T_{2M}^{-1} + \tau_M^{-1})^2 + (\Delta\omega_M)^2} \qquad (2.15)$$

In the slow-exchange region, i.e., when two separate signals are observed and the broadening is due to exchange, $T_{2p}^{-1} = f_M \tau_M^{-1}$. This region is characterized by a marked increase in linewidth with increasing temperature, as confirmed by measurements at 4 and 25 °C. Therefore, T_{2p} gives a direct measure of τ_M.[56] The ^{13}C T_1^{-1} values of HCO_3^- are consistent with bicarbonate bound to the metal. The Cu—C distance would be 2.5 Å if the unpaired electron were completely on the copper ion, as estimated by using Equation (2.1) and a value of $\tau_c = 2.1 \times 10^{-9}$ s independently obtained from water ^1H NMRD.[83] This distance is much too short for a coordinated bicarbonate; however, electron delocalization on the bicarbonate ligand may account for such a short calculated distance; the possibility of a bidentate type of ligation cannot be discarded. The dissociation rate, which is very low, by itself accounts for the lack of activity of the derivative.

For CO_2, a carbon-copper distance could be calculated if the affinity constants of the substrate for the protein were known. When the binding site, if any, starts being saturated, fast exchange with excess ligand (in this case, CO_2) decreases the observed paramagnetic effect. From this behavior, the affinity constant may be estimated. For CO_2 the paramagnetic effect remained constant up to 1 M CO_2; i.e., the affinity constant is smaller than 1 M^{-1}. This means that practically there is no affinity for copper; yet the paramagnetic effect is paradoxically high.[88]

Another picture comes by analyzing the NMR data in terms of a pure diffusive model.[88] Here Hubbard's equation[91] has been used:

$$T_{2p}^{-1} = N_M \left(\frac{\mu_0}{4\pi}\right)^2 \frac{8\pi}{225} \frac{\gamma_I^2 n_e^2 \mu_B^2 S(S+1)}{d(D_N + D_M)} \left(13 f(\omega_S, \tau_D) + 3 f(\omega_S, \tau_D)\right) \tag{2.16}$$

where

$$f(\omega, \tau_D) = \frac{15}{2} I(u) \tag{2.17.1}$$

$$u = [\omega \tau_D]^{1/2} \tag{2.17.2}$$

$$I(u) = u^{-5}\{u^2 - 2 + e^{-u}[(u^2 - 2)\sin u + (u^2 + 4u + 2)\cos u]\} \tag{2.17.3}$$

d is the distance of closest approach, D_N and D_M are the diffusion coefficients of the molecules containing the nucleus under investigation, and $\tau_D = 2d^2/(D_N + D_M)$. The experimental paramagnetic effect can be reproduced with a CO_2 concentration inside the cavity much larger than the one in the bulk solution. This result indicates that substrate does not bind to a specific site, but probably binds in the hydrophobic region. Note that CO_2 is more soluble in organic solvents than in water.

The effect of the cavity is to attract CO_2 by interaction either with the metal ion or with a hydrophobic part of the cavity itself. But the affinity constant is in any case lower than expected from the Michaelis constant (see Section IV.B) measured under steady-state conditions, indicating that the latter does not represent the dissociation constant of the enzyme-CO_2 system.

In summary, the main information concerning the catalytic cycle obtained from the copper derivative is the structural and kinetic characterization of both CO_2 and HCO_3^- species when they are not interconverting but present within the cavity. In this way we have further proof that HCO_3^- is bound to the metal and that CO_2 is attracted inside the cavity either by hydrophobic interactions or by the metal ion or both. The data obtained on the geometry around copper are consistent with those obtained on cobalt.

From Manganese and Cadmium Substitutions

Several studies have been performed on MnCA. Although CA is not the protein for which Mn(II) has been most extensively used as a paramagnetic probe to map substrates and inhibitors within the metal cavity, by measuring the T_{2M}^{-1} values of protons of the inhibitor N-acetyl-sulfanilamide, and by assuming that dipolar contributions are dominant, researchers have mapped the orientation of the inhibitor inside the active cavity (Figure 2.19).[92] This orientation is consistent with x-ray data on stronger binding sulfonamides.[64-66] MnCA is not completely inactive. ^{13}C NMR studies of the $CO_2 \rightleftharpoons HCO_3^-$ interconversion at pH 8.5 showed that the interconversion rate is about 4 percent that of the native enzyme.[93] The T_1^{-1} and T_2^{-1} values of $H^{13}CO_3^-$ suggest that bicarbonate might be bidentate in the central step of the catalytic cycle.[93]

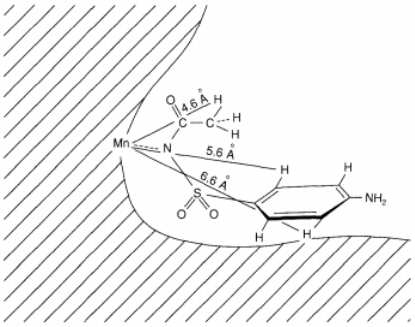

Figure 2.19 - Schematic drawing of the geometric arrangement of the inhibitor N-acetyl-sulfanilamide in the active cavity of manganese(II)-substituted CA, as revealed by ^{1}H NMR spectroscopy.[92]

Data from ^{113}Cd studies that have been performed on CdBCA II and CdHCA I are consistent with the general picture presented here.[94] The ^{113}Cd chemical shifts are indeed consistent with a donor set of three nitrogens and two oxygens. The cadmium(II) derivative could thus be five-coordinate with two water molecules, in agreement with the expectation based on its ionic radius being larger than that of zinc(II). The ^{113}Cd signal of CdBCA II in the presence of benzene-sulfonamide enriched in ^{15}N is split into a doublet because of the nitrogen-cadmium coupling (Figure 2.20).[95] This result provides direct evidence for metal-nitrogen bonding in sulfonamides, which has been confirmed by x-ray data.[65]

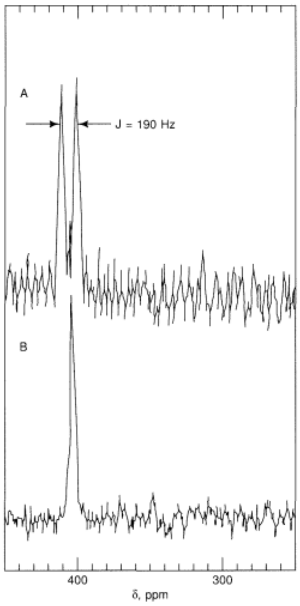

Figure 2.20 - ^{113}Cd NMR spectra of Cd-substituted bovine carbonic anhydrase II in the presence of ^{15}N-enriched (A) or ^{14}N-enriched (B) benzenesulfonamide inhibitor.[95]