Interaction of CO_2 and Copper(II) Carbonic Anhydrase

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Abstract: ^{13}C NMR studies on the $CO_2 \rightleftharpoons HCO_3^-$ interconversion in the presence of copper(II) carbonic anhydrase (CuCA) permit detection of two signals, both of them containing information on the copper–CO_2 or –HCO_3^- interactions. Such measurements are now performed as a function of CO_2 concentration up to 1 M. It is shown that the upper limit of the apparent affinity constant of CO_2 for the CuCA–HCO_3^- system is 0.1 M^{-1}. On the other hand, a pure diffusion model does not account for the $^{13}CO_2$ nuclear relaxation properties. It is proposed that the K_M of the hydration reaction in the native enzyme does not represent the thermodynamic dissociation constant. The kind of interaction between CO_2 and metalloproteins is discussed.

Carbonic anhydrase (CA) catalyzes the reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. A major point, yet unsettled, concerning the catalytic cycle is the binding mode of CO_2 to the enzyme and, in particular, whether there is a species CA–CO_2 as a real intermediate.[1]

Carbonic anhydrase obeys Michaelis–Menten kinetics, with K_M values of 12 mM for the high-activity bovine CA II and 4–8 mM for the low-activity human CA I.[2] It is likely, however, that even if the species CA–CO_2 exists, K_M does not reflect its thermodynamic dissociation constant.[2b,3]

In order to obtain information on the CA–CO_2 system, ^{13}C NMR T_1 data have been recorded on ^{13}C-enriched substrate in the presence of the inactive, paramagnetic derivatives CuCA[4] and MnCA.[5,6] In these experiments HCO_3^- acts as a ligand of the metal ion, so that what is monitored is the interaction of CO_2 with the CA–HCO_3^- adduct. Such interaction may still mimic a productive binding of CO_2, especially if the binding site is not the metal ion itself. From these experiments, an upper limit for the distance between the metal and the CO_2 carbon has been estimated to be 7 Å by assuming that the dissociation constant of CO_2 equals K_M.[4,5]

We here report on a series of ^{13}C NMR T_1 experiments on the system CuCA–HCO_3^-–CO_2 (CA being the high-activity bovine isoenzyme BCA II and the low-activity human isoenzyme HCA I) as a function of CO_2 pressure in the range 1.6–35 atm, corresponding to CO_2 concentrations of 4.5×10^{-2}–9.1×10^{-1} M. The aim of the research is to estimate the affinity of CO_2 for CuCA–HCO_3^- and to better understand the type of interaction.

Experimental Section

Human CA I was a gift from S. Lindskog, whereas bovine CA II was purchased from Sigma Chemical Co., St. Louis. MO. CuCA derivatives were prepared as previously described.[4] Aliquots of unbuffered, concentrated (1–3 mM) solutions of the above derivatives were placed in a thick-wall, 10-mm NMR tube; weighted amounts of $NaH^{13}CO_3$ (≥90% ^{13}C) were dissolved in the samples, which were then frozen in liquid nitrogen. Solid CO_2 was added on top of the frozen solutions in roughly equimolar amount—estimated by weight—with respect to the hydrogen carbonate present. The tubes were capped and allowed to slowly warm to room temperature. ^{13}C NMR spectra (20 °C, 20 MHz) were recorded on a Varian FT80-A spectrometer. T_1 measurements were performed with the inversion–recovery method. Concentrations of CO_2 in the samples were determined from the integrated intensity of the CO_2 signal with respect to the known concentration of HCO_3^-. pH values were estimated from the CO_2/HCO_3^- ratios by using $pK_a(CO_2) = 6.08$. The resulting values ranged between 5.33 and 6.27. Blank experiments were similarly performed on solutions containing the diamagnetic native carbonic anhydrase and the paramagnetic copper–zinc superoxide dismutase (purchased from Diagnostic Data Inc., Mountain View, CA).

Results and Discussion

The effect of the paramagnetic copper center in CA on the longitudinal relaxation rates of CO_2, T_{1p}^{-1}, obtained by subtracting the rates measured on the diamagnetic blank, is shown in Figure 1 as a function of CO_2 concentration. All the data are normalized to 1 mM copper(II). If binding of CO_2 to a specific site is assumed, under fast-exchange conditions, T_{1p}^{-1} is equal to the full paramagnetic effect, T_{1M}^{-1}, times the molar fraction of bound CO_2, f_M. Two limit cases are possible: (i) CO_2 is competitive with HCO_3^-; (ii) CO_2 can bind simultaneously with HCO_3^- in a noncompetitive way.[7] Since under the present experimental conditions the metal site is saturated with HCO_3^- (see later text), in case (i) f_M and hence T_{1p}^{-1} should decrease linearly with $C_{HCO_3^-}$ ($\simeq C_{CO_2}$). The relationship

$$f_M \simeq \frac{[E\text{-}CO_2]}{[CO_2]} = \frac{C_E K_{CO_2}}{C_{HCO_3^-} K_{HCO_3^-} + 1 + C_{CO_2} K_{CO_2}}$$

holds where C_E is the total enzyme concentration, C_{CO_2} and $C_{HCO_3^-}$ are the concentrations of the substrates, and K_{CO_2} and $K_{HCO_3^-}$ are their thermodynamic binding constants. In case (ii) f_M and T_{1p}^{-1} should be constant with C_{CO_2} below saturation and decrease linearly above saturation. In this case f_M is given by

$$f_M = C_E \frac{K_{CO_2}}{1 + K_{CO_2} C_{CO_2}}$$

There is no apparent decrease of T_{1p}^{-1} up to $C_{CO_2} \simeq 1$ M (actually, a moderate increase is observed, probably due to the large concentration of the various species). This definitely rules out case (i). In case (ii), which apparently holds, it is possible to extract information about the affinity for CO_2 alone. Since we are going to show that under the present experimental conditions bicarbonate is saturating, the experimental data can be related to the affinity of CO_2 for the E–HCO_3^- system. The experimental pattern is consistent with a binding constant $\ll 1$ M^{-1}. Comparison with the T_{1p}^{-1} data obtained on bovine copper–zinc superoxide dismutase solutions (Figure 1) indicates that

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(7) Product inhibition by HCO_3^- is known to be mixed noncompetitive at low pH: Tibell, L.; Forsman, C.; Simonsson, I.; Lindskog, S. *Biochim. Biophys. Acta* **1984**, *789*, 302. Pocker, Y.; Deits, T. L. *J. Am. Chem. Soc.* **1982**, *104*, 2424. However, since K_M may not reflect the thermodynamic constant, *binding* competition may still occur.

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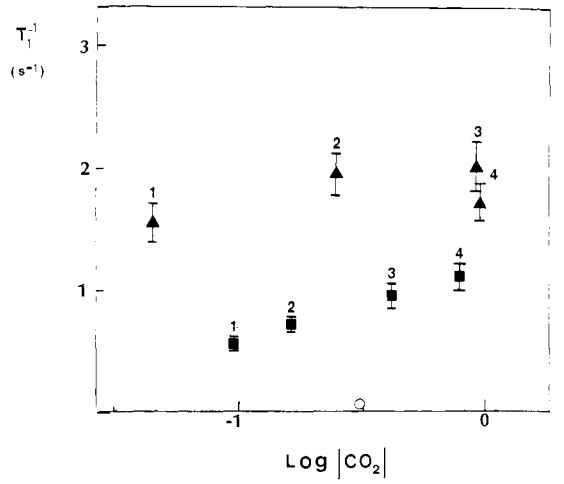

Figure 1. 20-MHz, 20 °C T_1^{-1} values of the ^{13}C nucleus of CO_2 in the presence of CuBCA II (▲) and CuHCA I (■) as a function of CO_2 concentration. CuBCA II: (1) $[HCO_3^-] = 7.00 \times 10^{-2}$ M, pH 6.27; (2) $[HCO_3^-] = 8.45 \times 10^{-2}$ M, pH 5.61; (3) $[HCO_3^-] = 1.69 \times 10^{-1}$ M, pH 5.33; (4) $[HCO_3^-] = 6.40 \times 10^{-1}$ M, pH 5.93. CuHCA I: (1) $[HCO_3^-] = 4.2 \times 10^{-2}$ M, pH 5.92; (2) $[HCO_3^-] = 8.43 \times 10^{-2}$ M, pH 5.80; (3) $[HCO_3^-] = 1.68 \times 10^{-1}$ M, pH 5.68; (4) $[HCO_3^-] = 3.41 \times 10^{-1}$ M, pH 5.72. The open circle refers to native SOD. All data are normalized to a 1 mM concentration of copper(II).

the measured effects are much larger than any nonspecific, long-range interaction with a paramagnetic protein (see also later text). Contributions to T_{1p}^{-1} of CO_2 from a noncompletely quenched $CO_2 \rightleftharpoons HCO_3^-$ interconversion were already suggested to be negligible[4] and can be definitely ruled out from the fact that T_{1p}^{-1} for HCO_3^- decreases linearly with concentration—as expected from the saturating behavior of the ion—and so should be the reflected effect on CO_2.

As a result of the fact that a strong paramagnetic effect is detected up to $C_{CO_2} \simeq 1$ M, the upper limit for K_{CO_2} is 0.1 M^{-1}. From the Solomon equation[8] the upper limit of the metal–^{13}C distance r can now be evaluated:

$$T_{1M}^{-1} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{r^6}\left(\frac{7\tau_s}{1+\omega_S^2\tau_s^2} + \frac{3\tau_s}{1+\omega_I^2\tau_s^2}\right)$$

Here γ_N is the nuclear magnetogyric ratio, $g_e = 2.0023$, μ_B is the Bohr magneton, S is the electron spin multiplicity, r is the metal–nucleus distance, ω_I and ω_S are the nuclear and electronic Larmor frequencies at the magnetic field of the experiment, and τ_s is the electronic relaxation time. This equation is shown to hold for copper(II) in the case of magnetic fields larger than 0.2 T.[9] By using the electronic relaxation time of the CuHCAI–HCO_3^- adduct previously estimated (τ_s 2.4×10^{-9} s),[4b] the results are $r \leq 2.5$ Å for CuBCA II and $r \leq 2.8$ Å for CuHCA I. Such values are indeed very short and would imply direct coordination of CO_2 to the metal ion. Let us assume that the interaction between CO_2 and the enzyme is outer-sphere diffusion-controlled. The Hubbard[10] equation should describe the interaction as follows

$$T_{1p}^{-1} = N_M\left(\frac{\mu_0}{4\pi}\right)^2 \frac{16\pi}{225}\frac{\gamma_N^2 g_e^2 \mu_B^2 S(S+1)}{d(D_N + D_M)}[7f(\omega_s\tau_D) + 3f(\omega_I\tau_D)]$$

where d is the distance of closest approach, D_N and D_M are the diffusion coefficients of CO_2 and the protein, respectively, N_M is the number of metal ions per cubic meter of solution, and τ_D is the diffusional correlation time. $f(\omega,\tau_D)$ is given by $f(\omega,\tau_D) = (^{15}/_2)I(u)$, where $u = [\omega\tau_D]^{1/2}$ and $I(u) = u^{-5}\{u^2 - 2 + e^{-u}[(u^2 - 2)\sin u + (u^2 + 4u + 2)\cos u]\}$. Diffusion coefficients of 10^{10} $Å^2\ s^{-1}$ for the protein and 2.4×10^{11} $Å^2\ s^{-1}$ for CO_2 are estimated from the relationship $D = kT/6\pi a\eta$ where a is the radius of the molecule. τ_D is evaluated from $\tau_D = 2d^2/(D_N + D_M)$. The experimental data can be reproduced through a computer program by a distance of closest approach <1 Å, i.e., too short for a nonbonding situation. The Hubbard equation, on the other hand, accounts for the T_{1p}^{-1} values observed for CO_2 in superoxide dismutase solutions with $d = 4.0$ Å and $\tau_D = 1.5 \times 10^{-10}$ s. These values are absolutely reasonable. Similar to CuCA, copper(II) in SOD binds to histidine nitrogens and a water molecule. It seems therefore that a description of the CuCA system should arise within the dipolar coupling model described by the Solomon equation. The results shown above are consistent either with a single CO_2 directly coordinated to the metal ion or with several molecules, all within a few angstroms of the metal ion. For example, for the bovine enzyme, the effect can be reproduced by one molecule at 3 Å and four more within 4 Å of the metal (the average affinity constant being taken $=0.1\ M^{-1}$).

It is important now to discuss which information can be transferred to the zinc enzyme. Probably copper has a different coordination than zinc: tetragonal five-coordinated for the former and tetrahedral for the latter. However, it is possible that the bicarbonate adducts are five-coordinated in both cases. Independently of that, zinc(II) is expected to have less affinity for any molecule, CO_2 included, than copper(II), whereas the cavity possibly has the same affinity for CO_2 independent of the metal ion. Therefore the observed K_M for the native enzyme probably simulates a dissociation constant much smaller than the equilibrium value and then reflects the accumulation of one intermediate further down the catalytic pathway. In the case where CO_2 binds the cavity, or binds zinc and copper with similar affinity, CA–CO_2 is a true intermediate and not a transition-state complex between an incoming CO_2 and the metal-coordinated hydroxide, since pure diffusion does not account for the present data.

Coming back to CuCA, the present experiments show that the active site of CA markedly favors binding of CO_2 in the close proximity of the metal ion even in the presence of bicarbonate, since the $^{13}CO_2$ T_1^{-1} values are much larger than in presence of superoxide dismutase. An apparent affinity constant of 0.1 M^{-1}, despite the fact it is very low, still indicates a real interaction. There is no Cu(II)–CO_2 complex in the inorganic literature, but the possibility of its occurrence with the above affinity constant cannot be ruled out. On the other hand, if CO_2 does not bind the metal, the cavity has to be able to accommodate a number of CO_2 molecules either by H bonding through the oxygen atoms or by hydrophobic interactions with nonpolar residues, or both. The overall effect could be described as an increased "solubility" of CO_2 inside the cavity, leading to a higher concentration of partially desolvated and—possibly—activated molecules available for nucleophilic attack by the metal-coordinated hydroxide.

As far as HCO_3^- is concerned, the T_{1p}^{-1} (70 s^{-1} at $p(CO_2)$ = 1.6 atm and 2 s^{-1} at $p(CO_2)$ = 35 atm) are dependent on the molar fraction of bound anion according to the relationship[11]

$$T_1^{-1}(\text{measd}) = T_{1dia}^{-1} + T_{1p}^{-1} = T_{1dia}^{-1} + f_M(T_{1M} + \tau_M)^{-1}$$

This indicates that the coordination site of HCO_3^- is fully saturated in the concentration range of these experiments. On the other hand, HCO_3^- exchanges slowly on the NMR time scale, and T_{1p}^{-1} is essentially a measure of the exchange time τ_M, which is 2.0×10^{-4} s for bovine and 2.3×10^{-3} s for human enzyme.[4] The data are consistent with HCO_3^- bound to the metal ion.

Registry No. CO_2, 124-38-9; HCO_3^-, 71-52-3; CuCA, 9001-03-0; Cu, 7440-50-8.

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