Carbon dioxide hydration activity and metal–substrate distances of manganese (II) human carbonic anhydrase B determined by ^{13}C magnetization–transfer NMR

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A CO_2 hydration activity for Mn(II) human carbonic anhydrase B (MnHCAB) of 7% of the activity of the native Zn^{2+} enzyme has been determined using a ^{13}C magnetization–transfer NMR approach, that involves two complementary experiments. As this approach also allows a determination of the individual relaxation rates of the enzyme-bound CO_2 and HCO_3^-, an evaluation could be made of the distances between these substrates and the paramagnetic Mn^{2+} in the active site. Thus HCO_3^- is found to bind directly to Mn^{2+}, whereas CO_2 is attached relatively weakly to the enzyme without a direct bond to the metal ion.

Mn(II) human carbonic anhydrase B *CO_2 hydration activity* *Substrate binding* *^{13}C Magnetization–transfer NMR*

1. INTRODUCTION

Inconsistent reports on the CO_2 hydration activity of Mn(II) carbonic anhydrase giving rise to the catalysis of the reversible reaction

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+ \quad (1)$$

have been given in the literature. Thus, Lindskog and Nyman [1] using the colometric procedure in a stopped-flow apparatus [2], found that the residual activity of the Mn(II) derivatives at 0.5°C was $8 \pm 2\%$ of the activity of the native Zn(II) enzyme in case of bovine carbonic anhydrase B (BCAB), and 12 ± 2% in case of the human B (HCAB) enzyme, whereas Coleman [3], by employing the colometric method of Wilbur and Anderson [4,5], measured a residual activity, at 4°C, of 4% for both the inactive HCAB apo-enzyme and its Mn(II) derivative (MnHCAB), indicating that also the latter is inactive.

Undoubtedly, these inconsistencies are due to the inherent experimental difficulties of the applied methods [6–8] in controlling the CO_2 concentration, and determining the precise end-point of the observed reaction, as well as the pH-value to which the activity corresponds. Also the instability of the enzyme at the very low concentration required by the method, contributes to the difficulties [8].

Here, we have measured a significant CO_2 hydration activity of MnHCAB at pH 8.5, using the modified magnetization-transfer (MT) NMR technique detailed in [9]. Unlike the steady state kinetic methods [1–7], this technique allows the activity to be measured at chemical equilibrium and, therefore, at fixed and well-defined values of pH, CO_2- and active enzyme concentrations. As the MT method also yields the individual ^{13}C relaxation rates of the enzyme-bound substrate and product, valuable information about the binding of these compounds relative to the enzyme Mn(II) is obtained, in addition to the measurement of activity.

2. MATERIALS AND METHODS

HCAB was purified from human erythrocytes obtained from Københavns Amts Sygehus, using

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affinity chromatography as in [10]. The resulting enzyme solution was thoroughly desalted and lyophilized.

Apo-enzyme was prepared by dialyzing 20 ml of a 500 μM solution of HCAB in 0.1 M phosphate buffer (pH 7.0) and 0.040 M in dipicolinic acid, against a 20-fold excess of the enzyme-free buffer for 24 h followed by desalting and dialysis against a 10-fold excess of 50 mM Tris–sulfate buffer at pH 8.5. The buffer was demetalized by dithizone before use. All equipment in contact with the enzyme was washed with 4 M sulfuric acid followed by quartz-distilled water, to remove traces of Zn(II) and other complexing metals. Similarly, all contact with the inhibitory halide ions [11], and in particular Cl^-, was avoided. The final apo-enzyme contained <2% ZnHCAB as monitored by esterase activity.

The samples were prepared from Zn(II)- and apo-enzymes using demetalized 50 mM Tris–sulfate buffer, a 12 mM solution in quartz-distilled water of ultrapure $MnSO_4 \cdot 5\ H_2O$ from Johnson and Matthey Chemical Ltd., and $NaHCO_3$, (90% ^{13}C) from Stohler Isotope Chemicals. The Zn(II) content of the bicarbonate was found to be <0.2 ppm, using a Perkin Elmer 5000 atomic absorption spectrophotometer. The 2 ml samples were sealed-off in 8 mm (OD) NMR tubes 9–10 cm long, to prevent loss of CO_2 and reduce the gas volume above the sample to a minimum. The 8 mm tubes were mounted co-axially in 10 mm NMR sample tubes, allowing the interstitial annulus to accommodate D_2O for lock purpose.

The general McConnell equations [14] for exchange between two sites, A and B, applied in a slightly modified form [9], are the basis for the data analysis. As shown in [9], a simultaneous analysis of the data of two complementary MT experiments, using these modified equations, allows the exchange rate and the relaxation rates of the individual signals to be determined independently even in an extreme and non-ideal case as the one presented here; that is, where the intensities as well as the widths of the signals corresponding to the two exchanging sites are significantly different, and one or both of the relaxation rates are considerably smaller than the exchange rate. Also, it should be noted that unlike the NMR linewidth approach [15,16], the MT method, as applied here, yields exchange rates, that are unaffected by paramagnetic contributions to the relaxation rates of the enzyme-bound substrate and product.

3. RESULTS AND DISCUSSION

3.1. *CO_2 hydration activity*

The result of a least squares fitting of the model parameters [9] to the peak heights of the spectra in fig.1 is illustrated in fig.2, while the obtained exchange and relaxation rates are given in table 1. Here k^{CO_2} is the $CO_2 \rightarrow HCO_3^-$ flux rate per unit CO_2, and is related to the Michaelis–Menten parameters, $k_{cat}^{CO_2}$ and $K_M^{CO_2}$, and the maximal exchange rate constant, k_{cat}^{exch}, as described in [15,16]:

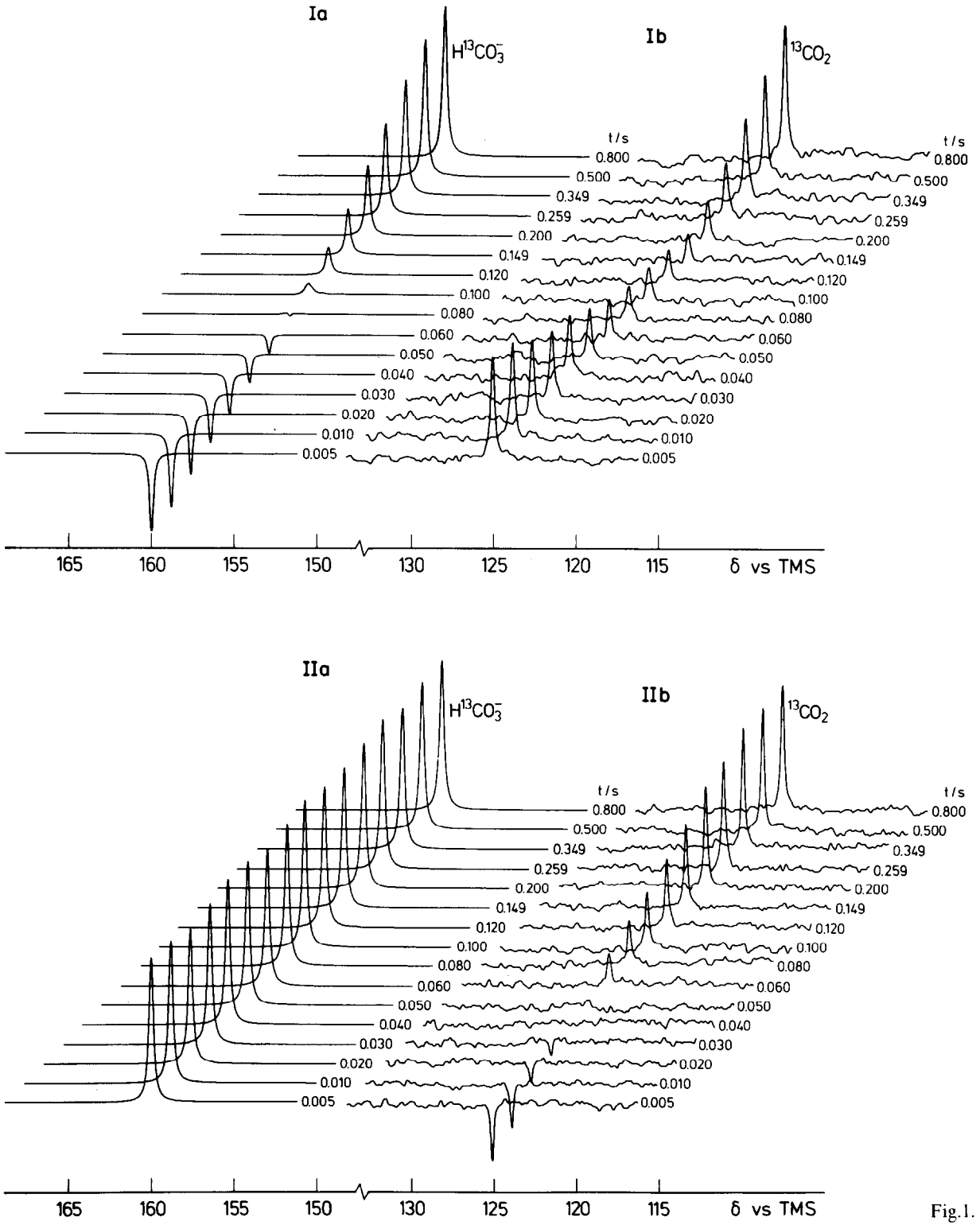
$$k^{CO_2} = \frac{k_{cat}^{CO_2}[E_o]}{K_M^{CO_2}\left(1 + \frac{[S^{CO_2}]}{K_{eff}^{CO_2}}\right)} = \frac{k_{cat}^{exch}[E_o]}{K_{eff}^{CO_2} + [S^{CO_2}]} \quad (2)$$

$[E_o]$ = the total concentration of active enzyme;
$[S^{CO_2}]$ = the equilibrium CO_2 concentration;
$K_{eff}^{CO_2}$ = an effective dissociation constant, which depends on the binding of both CO_2 and HCO_3^- [15]

Qualitatively, table 1 shows that the applied MnHCAB (sample I) gives rise to a significant catalysis of reaction (1), since the obtained $k^{CO_2} \cdot [S^{CO_2}]$ rate is at least 2 orders of magnitude faster than the non-enzymatic hydration rate [7], even when the OH^--catalyzed hydration, which approaches the uncatalyzed one at pH 8.5 [17], is taken into account.

Table 1 also shows, that the activity observed in sample I is almost entirely caused by MnHCAB, whereas the residual amount of Zn-enzyme, which is present in the large surplus of apo-enzyme that was applied, contributes only to a small extent. Thus a k^{CO_2} (19°C) value corresponding to 1.8 ± 0.2 s^{-1} at pH 8.5 [7] was found for a sample (II, table 1), similar to sample I in apo-enzyme but without Mn(II).

Quantitatively, the residual activity of MnHCAB was determined by comparison with the rate obtained for a 10 mM sample of native ZnHCAB at pH 7.5 (III, table 1), which corresponds to k^{CO_2} (19°C) = 125 ± 20 s^{-1} when adjusted to pH 8.5 [7]. Hence, the rate obtained for sample I, which contains 9.8 μM Mn-enzyme


Ia
$H^{13}CO_3^-$
Ib
$^{13}CO_2$
t/s
0.800
0.500
0.349
0.259
0.200
0.149
0.120
0.100
0.080
0.060
0.050
0.040
0.030
0.020
0.010
0.005
165
160
155
150
130
125
120
115
δ vs TMS
IIa
$H^{13}CO_3^-$
IIb
$^{13}CO_2$
t/s
0.800
0.500
0.349
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0.200
0.149
0.120
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0.050
0.040
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δ vs TMS

Fig.1.

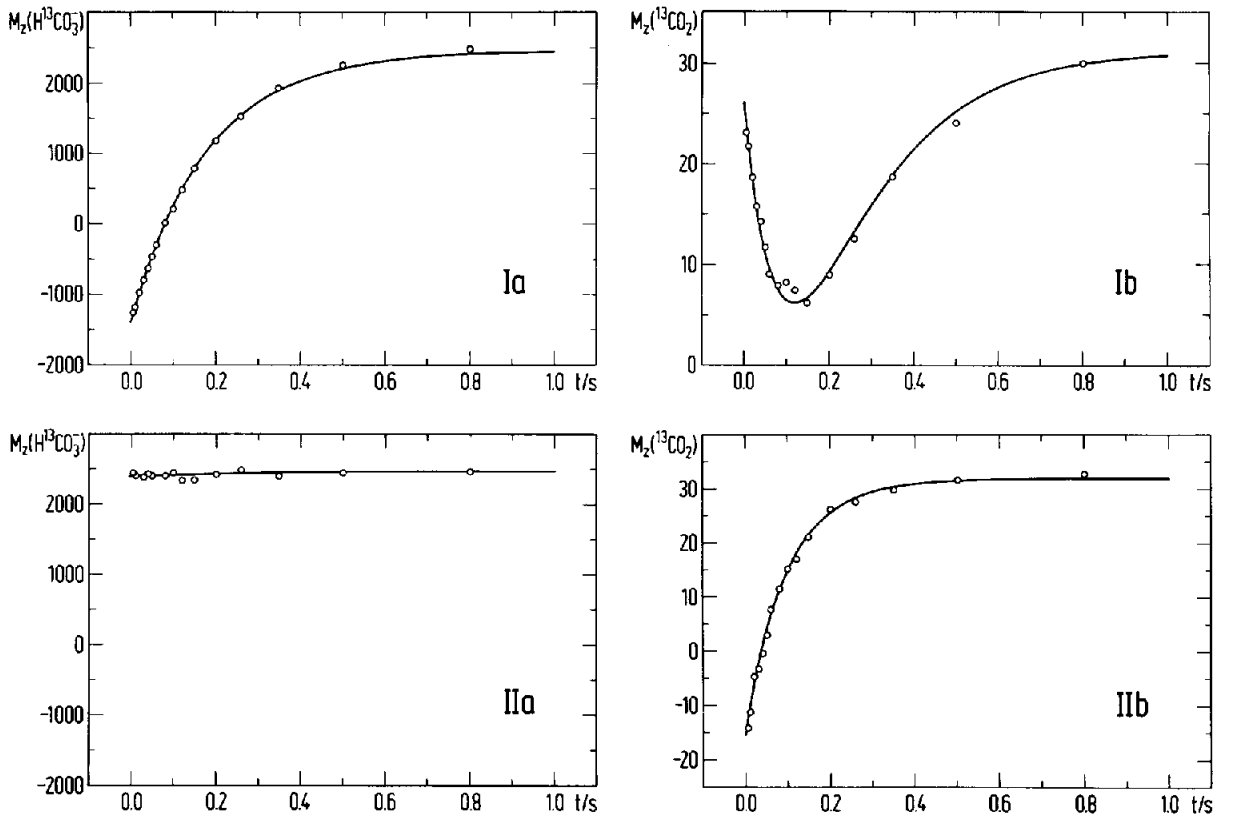

Fig.2. Plots of the time-dependent peak heights of the set of complementary spectra in fig.1. The curves represent the best fit obtained by the least squares analysis.

assuming a metal–enzyme stability constant as found for MnBCAB at the same pH [18], corresponds to k^{CO_2} (19°C) = (10.2 −1.8)/0.98 ≅ 8.6 ± 0.6 s^{-1} for a 10 μM solution of MnHCAB, which results in a residual CO_2 hydration activity of the Mn-enzyme of 6.9 ± 1.2% of the activity of the native enzyme.

The confidence in the activities thus obtained is further strengthened by the close agreement between the k^{CO_2}-value measured for sample III, and the results obtained for the same enzyme by means of NMR linewidth measurements. Thus, according to the equation for $k^{HCO_3^-}$ corresponding to eq. (2), the values of $k_{cat}^{HCO_3^-}/K_M^{HCO_3^-}$ and $K_{eff}^{HCO_3}$ given in [15] predict $k^{HCO_3^-}$ (25°C) = 2.0 s^{-1} for the concentrations and pH of sample III, while the value k^{CO_2} (19°C) = 56 ± 9 s^{-1}, obtained here, corresponds to $k^{HCO_3^-}$ (19°C) = 2.0 ± 0.4 s^{-1} when applying the equilibrium relation $[HCO_3^-] \cdot k^{HCO_3^-} = [CO_2] \cdot k^{CO_2}$ and a ratio $[HCO_3^-]/[CO_2]$ = 0.036 ± 0.004 s^{-1}. This ratio was obtained by integrating the CO_2 and HCO_3^- signals of a completely relaxed spectrum of sample III, while a calculation, using pK 6.08 [19] for the acid dissociation of

←

Fig.1. Sets of complementary ^{13}C magnetization–transfer NMR spectra of $H^{13}CO_3$ and $^{13}CO_2$ in the presence of Mn(II) human carbonic anhydrase B (sample I of table 1) obtained at 19°C and 67.89 MHz using a Bruker HX 270 spectrometer). Each spectrum consists of 8500 scans accumulated with a delay time of 0.6 s between the individual scans. The $H^{13}CO_3^-$ and $^{13}CO_2$ signals are selectively inverted in I and II, respectively, using a DANTE pulse sequence [12] consisting of 16 consecutive pulses, each 2.6 μs long and separated by 0.9 ms, while placing the carrier frequency on the signal to be inverted. The 90° non-selective, analyzing pulse was 16.5 μs. No proton decoupling was performed. The temperature was measured using a ^{13}C thermometer [13].

Table 1

Exchange and relaxation rates[a] obtained fromt the least squares analysis of the experimental data

Sample	Enzyme	pH	k^{CO_2} (s^{-1})	$R_{1,obs}^{HCO_3^-}$ (s^{-1})	$R_{1,obs}^{CO_2}$ (s^{-1})
I[b,c]	MnHCAB	8.5	10.2 ±0.5	5.50 ±0.07	0.2 ±0.3
II[c]	apoHCAB	7.4[e]	0.67±0.05	0.0088±0.003	0.04±0.03
III[d]	ZnHCAB	7.5[e]	56.0 ±9	0.175 ±0.004	<0.2

[a] At 19 ± 1°C in 0.05 M Tris–sulfate buffer, ionic strength 0.2 M
[b] [Mn(II)] = 10 μM; [c] [apoHCAB] = 100 μM; [d] [ZnHCAB] = 10 μM
[e] This pH-value was chosen since the slow ^{13}C relaxation rates in sample II and III make it impractical to measure k^{CO_2} at the very low [CO_2] at pH 8.5

eq. (1), corresponding to the ionic strength of 0.2 M in the sample, gave the ratio 0.038 at pH 7.5. Also, a comparison between sample II and III showed a Zn-enzyme induced increase of the CO_2 linewidth of 50 ± 10 s^{-1} at 19°C. Thus, this study shows that MnHCAB is active at pH 8.5. This result is in agreement with those achieved by the experimentally handier esterase method [20], which gave residual MnBCAB activities of 3.9% [21] and 4.5% [22] of the activity of the Zn enzyme, at 25°C in Tris–sulphate buffer (pH 7.5) with an ionic strength of 0.1 M. For MnHCAB a somewhat larger residual esterase activity (14%) at pH 7.5 and 23°C has been reported [3]. In [18] it was found not only that the stability of MnBCAB, like the activity, increase with pH, but also that the inflection point of the pH dependence of the esterase activity is at pH 8.2, that is 0.7 pH unit higher than for the native Zn enzyme, resulting in a residual esterase activity of 8% in the alkaline pH region. A similar shift in the pH-dependence of the CO_2 hydration activity of MnHCAB could explain the inconsistent results obtained in [1,3], since in [1] phenol-red (pH 8.4–6.8) was used as indicator, whereas in [3] bromothymol blue (pH 7.6–6.0) was applied.

3.2. *Mn(II)–substrate distances*

The observation (table 1), that Mn(II) increase $R_{1,obs}^{HCO_3^-}$ dramatically, whereas $R_{1,obs}^{CO_2}$ is unaffected by the presence of these ions, as well as lack of a similar effect in a sample without the apo-enzyme, but otherwise identical to I, indicates that HCO_3^- binds considerably closer than CO_2 to the Mn(II) ion in the active site of the enzyme. By using the expression in [23]:

$$R_{1P} = R_{1,obs}(\text{MnHCAB}) - R_{1,obs}(\text{apo}) = \frac{P_M}{T_{1M} + \tau_M} \quad (3)$$

for the paramagnetic contribution, R_{1P}, to the relaxation rate of an observed nucleus, the Mn(II)–^{13}C distances for the two substrates, HCO_3^- and CO_2, can be evaluated from the obtained ^{13}C relaxation rates. For each of the two substrates, T_{1M} in eq. (3) is the ^{13}C relaxation time of the enzyme-bound substrate molecules, τ_M^{-1} is the rate constant for the dissociation of the enzyme–substrate complex, and P_M is the fraction of the substrate bound to the enzyme.

In the case of HCO_3^-, where $R_{1P}^{HCO_3^-} = 5.5 - 0.1 = 5.4\ s^{-1}$, eq. (3) gives $T_{1M}^{HCO_3^-} = 1.82 \cdot 10^{-5}$ s, if $T_{1M}^{HCO_3^-} \gg \tau_M^{HCO_3^-}$, and $P_M^{HCO_3^-} = [E_o]/[HCO_3^-]$ corresponding to a 1:1 saturation of the enzyme with HCO_3^-. For a dipole–dipole correlation time, τ_R, of $3 \cdot 10^{-9}$ s [18] this $T_{1M}^{HCO_3^-}$ value results in a Mn(II)–^{13}C distance of $r^{HCO_3^-} = 3.2$ Å, when applying the Solomon equation [24]. If $T_{1M}^{HCO_3^-} \leq \tau_M^{HCO_3^-}$ or $P_M^{HCO_3^-} < [E_o]/[HCO_3^-]$, a somewhat smaller distance would be found. In any case, the result leads to the conclusion, that HCO_3^- is bound directly to the Mn(II) in the active site of the enzyme.

In the case of CO_2 it can be verified, that eq. (3) reduces to:

$$R_{1P}^{CO_2} = \frac{P_M^{CO_2}}{T_{1M}^{CO_2}} \quad (4)$$

since $(\tau_M^{CO_2})^{-1}$, as the rate constant for the dissociation $E \cdot CO_2 \rightarrow E + CO_2$, is given by $(\tau_M^{CO_2})^{-1} = k^{CO_2} \cdot [CO_2]/[E \cdot CO_2] = k^{CO_2}/P_M^{CO_2}$, according to a detailed balance. Thus $\tau_M^{CO_2} = P_M^{CO_2}/k^{CO_2} \simeq 0.1\ P_M^{CO_2}$ s, when introducing the k^{CO_2} value of 10.2 s^{-1}, obtained for sample I (table 1). When comparing this result with the relation $T_{1M}^{CO_2} + \tau_M^{CO_2} \simeq 5.0\ P_M^{CO_2}$ s, obtained by introducing $R_{1P}^{CO_2} = 0.2\ s^{-1}$ into eq. (3), it is immediately apparent that $\tau_M^{CO_2} \ll T_{1M}^{CO_2}$, which reduces eq. (3) to eq. (4).

According to eq. (4) the observation of a substantial smaller R_{1P} value for CO_2 than for HCO_3^- could be due to a larger value of T_{1M} caused by a longer Mn(II)–^{13}C distance ($r^{CO_2} > r^{HCO_3^-}$). However, it could also be a result of a smaller $P_M^{CO_2}$ value ($P_M^{CO_2} < P_M^{HCO_3^-}$), which could be the case if CO_2 binds more loosely than HCO_3^- to the enzyme. In fact, this latter possibility has been suggested on basis of X-ray [25] and perturbed angular spectroscopy [26] studies. A precise evaluation of the two possibilities would necessitate a knowledge of the still unknown formation constants for the enzyme–substrate complexes. However, from an examination of the two extremes:

(i) $P_M^{CO_2} = [E_o]/[CO_2]$ corresponding to a 1:1 saturation of the enzyme with CO_2;

(ii) $r^{CO_2} = r^{HCO_3^-}$ corresponding to a direct bonding between CO_2 and Mn(II);

a qualitative evaluation of the $E \cdot CO_2$ interaction can be made. Thus for the largest possible value of 0.2 s^{-1} for R_{1P} (table 1), eq. (4) gives $r^{CO_2} = 9.7$ Å in (i), and $P_M^{CO_2} = P_M^{HCO_3^-}/27$ in (ii), respectively, when applying the Solomon equation and a correlation time, $\tau_R = 3 \cdot 10^{-9}$ s, as with HCO_3^-. While the large Mn(II)–^{13}C distance obtained in (i) is incompatible with any realistic model for the mechanism of the CO_2 hydration [25,27], the small formation constant, which corresponds to the $P_M^{CO_2}$ value obtained in (ii), seems in itself to exclude the assumed direct Mn(II)·CO_2 complexation. Hence, it is clearly suggested that the correct solution lies between the two extremes; i.e., CO_2 binds looser than HCO_3^- to the enzyme, and is not attached directly to Mn(II).

The latter suggestion, which is in agreement with a previous NMR investigation of the inactive CuHCAB [28], is further supported by the observation here of a significant Mn(II)-induced paramagnetic broadening of the HCO_3^- signal in I ($R_{2P}^{HCO_3^-} = 36 \pm 2\ s^{-1}$), and the complete absence of a similar effect on the CO_2 signal, which merely is broadened by an amount ($11 \pm 2\ s^{-1}$) corresponding to the obtained k^{CO_2} value. Again, since R_{2P}, undoubtedly, is caused exclusively by a scalar Mn(II)–^{13}C coupling [29,30] which depends on a covalent interaction between Mn(II) and ^{13}C [31], these observations suggest that only HCO_3^- binds covalently to Mn(II) in the active site, whereas CO_2 does not.

Thus, although a precise NMR determination of the position of CO_2 relative to the metal ion in the active site must await an evaluation of the formation constant of the $E \cdot CO_2$ complex, the relaxation rates of the present study clearly suggest that CO_2 associates relatively weakly with the enzyme, without a direct bond to the metal ion in the active site. The data also show, that, unlike this, the HCO_3^- substrate is attached directly to the metal ion during the catalysis.

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