Dynamics of Functional Water in the Active Site of Native Carbonic Anhydrase from ^{17}O Magnetic Relaxation Dispersion

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The reversible hydration of CO_2 catalyzed by carbonic anhydrase is among the most efficient enzymatic reactions known.[1−4] Water is not only a substrate in this reaction but also participates in a network of H-bonds that mediates the rate-limiting proton-transfer step.[1,3,5] A full understanding of the catalytic events in carbonic anhydrase will therefore require quantitative information about water dynamics in the active site. We report here ^{17}O magnetic relaxation dispersion (MRD) data from human carbonic anhydrase II (HCA II), yielding bounds on the residence times of the zinc-bound hydroxide ion and nearby water molecules.

The catalytic zinc ion is firmly bound to three rigid histidine side-chains near the apex of a 15 Å deep conical cavity, with a strongly polarized water molecule ($pK_a \approx 7$) completing the tetrahedral coordination.[6] Under the conditions of the present study (pH* 9.0), the solvent-donated zinc ligand (referred to as W263) is essentially in the OH^- form. The crystal structure of HCA II reveals a network of extensively H-bonded water molecules in the catalytic pocket (Figure 1).[6] Of particular importance for the catalytic events are the "deep water" W338, which is displaced by the incoming substrate (CO_2 or HCO_3^-), and the two "shuttle waters" W318 and W292, which are thought to relay protons between W263 and His64.[5,7]

Residence times of structural water molecules in proteins can be determined by the ^{17}O MRD technique, where the longitudinal relaxation rate, R_1, of the water ^{17}O resonance is measured as a function of resonance frequency.[8] A water molecule contributes to the relaxation dispersion only if its residence time, τ_W, is longer than the rotational correlation time, τ_R, of the protein (ca. 10 ns) but shorter than the intrinsic ^{17}O relaxation time (ca. 2 μs). Long-lived water molecules ($\tau_W > 10$ ns) can usually be identified from high-resolution crystal structures as those that (simultaneously) have small solvent-accessible surface area (<10 Å^2) and small Debye−Waller factor ($B < 20$ Å^2) and are extensively H-bonded (≥2 H-bonds with $R_{OX} < 3.2$ Å to protein or to other long-lived waters).[9] On these criteria, HCA II contains 17 potentially long-lived water molecules outside of the catalytic pocket. Any contribution to the relaxation dispersion from water in the catalytic pocket must therefore be observed against a large background, requiring highly accurate data. Within the catalytic pocket, five water molecules satisfy the criteria for a long residence time: W263, W264, W292, W318, and W369.

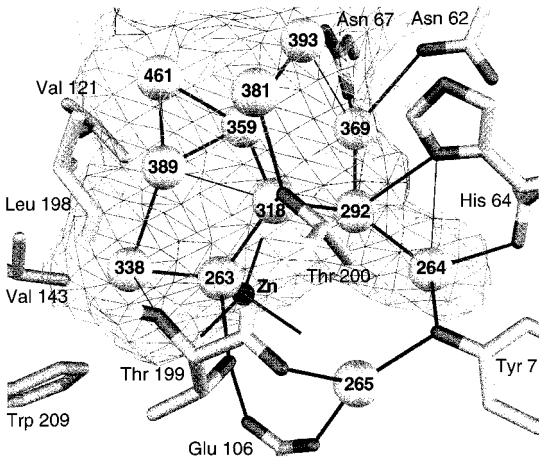

Figure 1. Crystal structure of the catalytic pocket of native HCA II (PDB file 2CBA[6]), highlighting the H-bond network of water molecules and side-chains. The cavity surface was constructed with a probe radius of 1.2 Å. Potential H-bonds are represented by thick ($R_{OX} < 3.2$ Å) or thin (3.2−3.4 Å) lines. The coordination of the zinc ion to three histidines is also indicated. For clarity, the water oxygens and the zinc ion are represented at half their van der Waals radii.

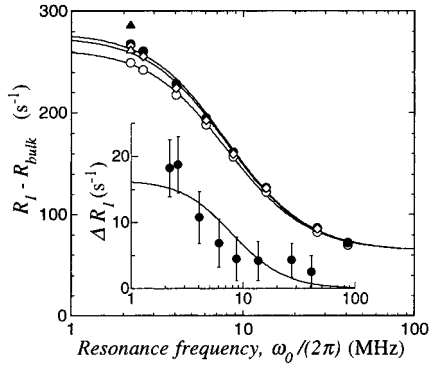

Figure 2. Frequency dependence of the water ^{17}O excess relaxation rate, $R_1 - R_{bulk}$, for 2.5 mM D_2O solutions of native HCA II, pH* 9.0 (●), apo-HCA II, pH* 9.4 (○), and azide-inhibited HCA II, pH* 9.0 (◇). The curves represent fits according to eq 1 with common α and τ_R for the three data sets. At the lowest frequency, excess transverse rates, $R_2 - R_{bulk}$ (▲,△) are also shown, demonstrating that there is no further dispersion step at lower frequencies. The insert shows the native − apo difference dispersion and the one-parameter fit with $\Delta\alpha = 0$ and τ_R as for the individual dispersions. Recombinant HCA II in apo form was expressed and purified as described.[25,26] Pure (unbuffered) D_2O enriched in ^{17}O was used for the solutions, which were filtered three times (0.22 μm) with several hours delay. The native enzyme was reconstituted by adding (under an argon atmosphere) 3 ± 1 equiv of dry $ZnSO_4 \cdot H_2O$ to the apo enzyme. Nearly complete (ca. 90%) inhibition by azide at pH* 9 was achieved by a large excess (210 mM) of NaN_3.[27]

Figure 2 shows the ^{17}O MRD profiles from solutions of HCA II in the native and apo (without zinc) forms and in the presence of azide inhibitor. The data are well represented by the theoretical expression[8]

$$R_1 - R_{bulk} = \alpha + \beta\tau_R\,[0.2/(1 + x^2) + 0.8/(1 + 4x^2)] \quad (1)$$

where $x = \omega_0\tau_R$ and ω_0 is the resonance frequency. When the dispersion magnitude β is scaled with the rigid-lattice water ^{17}O

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quadrupole frequency, $\omega_Q = 7.61 \times 10^6$ rad s^{-1}, and the water/protein mole ratio, $N_T = 20\,600 \pm 500$, according to $\beta N_T/\omega_Q^2$, it yields the product $N_\beta S^2$ of the number of long-lived water molecules (N_β) and their mean-square orientational order parameter (S^2).[8] The high-frequency relaxation enhancement α is essentially due to the kinetically labile surface hydration.[8]

For native HCA II we find $N_\beta S^2 = 6.9 \pm 0.2$, consistent with 7 fully ordered ($S = 1$) water molecules with residence times in the range 10 ns $-$ 2 μs or with a larger number of less ordered water molecules (e.g., $N_\beta = 20$ for $S = 0.6$). The correlation time, $\tau_R = 11.3 \pm 0.3$ ns, is slightly shorter than previously reported values for the tumbling time of carbonic anhydrase (14$-$16 ns, scaled to 27 °C and a solvent viscosity of 1.09 cP).[10,11] The present ^{17}O MRD data for native HCA II are consistent with the previously reported ^{17}O line broadening (at 8 MHz) for the bovine enzyme.[12] Published ^{1}H MRD data[13] on native HCA I imply that $N_\beta S^2$ increases from ca. 15 at pH 5.5 to ca. 30 at pH 9.9, indicating a substantial contribution from labile HCA protons.[14]

To isolate any contribution to the relaxation dispersion from long-lived waters in the catalytic pocket, we recorded MRD profiles from native HCA II complexed with two different inhibitors. Azide, which binds to the zinc ion, displacing W263 and W338,[2,15,16] has no significant effect on the dispersion (Figure 2). The displacement of a single, fully ordered water molecule with 10 ns $\ll \tau_W \ll 2$ μs would have reduced the dispersion step by $\omega_Q^2 \tau_R / N_T = 32$ s^{-1}.[17] This is an order of magnitude larger than the experimental uncertainty in R_1 and would therefore easily have been detected.[18] Consequently, the zinc-bound hydroxide ion (W263) and the deep water (W338) must either have residence times outside the range 10 ns to 2 μs or must undergo large-amplitude local reorientational motion on time scales $\ll$10 ns. Since the strongly interacting W263 is not likely to be disordered or short-lived, we conclude that it has a residence time much longer than 2 μs at 27 °C. On the other hand, since W338 does not interact strongly with the protein and has a relatively large B factor (27 Å^2), it is probably short-lived or weakly ordered. The larger inhibitor acetazolamide, which displaces four additional waters in the upper part of the catalytic pocket (Figure 1),[2,19,20] like azide, has no significant effect on the relaxation dispersion (data not shown), indicating that the additional displaced waters are short-lived.

Removal of the zinc ion produces a small but significant reduction of the ^{17}O relaxation dispersion step (Figure 2), corresponding to $N_\beta S^2 = 0.52 \pm 0.07$. This reduction cannot be caused by the loss of the zinc-bound W263, since the inhibitor experiments demonstrate that W263 does not contribute to the native dispersion.[21] The observed decrease therefore indicates a reduced order parameter or a reduced residence time (<10 ns) for one or more other waters. We cannot exclude that this change involves waters outside the catalytic pocket, but the virtual identity of the apo and native protein structures and the insignificant change (<3 Å^2) of the B factors of these structural waters argue against this possibility.[6] We therefore attribute the reduction of the dispersion step upon zinc removal to one or more waters in the catalytic pocket, with the shuttle waters W318 and W292 being the most likely candidates since they are near the zinc site and have substantially increased B factors (by 22 and 7 Å^2, respectively) in the apo form.[6] If this assignment is correct, either or both of the shuttle waters must have residence times in the range 10 ns to 2 μs in the native enzyme.

Measurements of the rate of exchange of ^{18}O from the zinc-bound hydroxide ion into the bulk solvent have been used to probe the rate and mechanism of the proton-transfer step in the catalytic reaction[3,7,22,23] on the assumption that ^{18}O in OH^- produced by decomposition of HCO_3^- dissociates from the zinc as H_2O (i.e., protonation precedes dissociation and limits its rate). It has been suggested, however, that the oxygen atom of the zinc-bound OH^- can exchange by an associative mechanism involving a five-coordinated intermediate with fast proton transfer from a transient H_2O ligand (presumably W338) to the OH^- ligand.[13] When the zinc reverts to the normal tetrahedral coordination, it still ligates a OH^- ion, but the original oxygen atom now belongs to an adjacent water molecule which quickly diffuses out of the catalytic pocket. The observed leveling out of the buffer-free ^{18}O-exchange rate at high pH[7,22] (when the rate of proton transfer into the active site should decrease) and the merely 2-fold reduction of this rate at pH 8.5 on elimination of buffer[22] or mutation of His64,[7] suggest that the associate mechanism dominates at pH > 8.

The present ^{17}O MRD result for the residence time of the zinc-bound OD^- in native HCA II, $\tau_W \gg 2$ μs at 27 °C and pH* 9, is consistent with the residence times ca. 50 μs (25 °C)[22] and ca. 130 μs (10 °C)[7] implied by ^{18}O-exchange rates for HCA II at pH > 8 and in the absence of external buffer. (A factor 6.5 correction for the solvent H/D isotope effect[23] has been applied here.) For the cobalt-substituted bovine isozyme B, the part of the paramagnetic ^{1}H relaxation enhancement that is eliminated by azide and sulfonamide inhibitors yields an upper bound for the residence time of the cobalt-coordinated hydroxide ion: $\tau_W \ll 17$ μs at 25 °C and pH 8.9.[24] This implies that the associative exchange mechanism is faster on cobalt than on zinc, consistent with the stronger affinity of cobalt for oxygen ligands and the consequent greater propensity for five-coordination.[2]

Our results on active-site water dynamics in carbonic anhydrase support the current picture of the catalytic mechanism: the inferred kinetic lability of W338 is consistent with a diffusion-controlled substrate binding rate, and the longer residence time for W318 or W292 may reflect the rate-limiting H-bond rearrangement along the proton conduction pathway.

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