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Sequestration of Carbon Dioxide by the Hydrophobic Pocket of the Carbonic Anhydrases

John F. Domsic and Robert McKenna*

Department of Biochemistry and Molecular Biology, University of Florida, College of Medicine, Gainesville, FL, USA 32610-0245

Abstract

The interaction between carbon dioxide (CO₂) and the α -class carbonic anhydrase, human CA 2 (HCA2) exists for only a short period due to the rapid catalytic turnover by this enzyme. The fleeting nature of this interaction has led to difficulties in its direct analysis, with previous studies placing the CO₂ in the hydrophobic pocket of HCA2's active site. A more precise location was determined via the crystal structure of CO₂ trapped in both wild-type (holo) and zinc-free (apo) HCA2. This provided a detailed description of the means by which CO₂ is held and orientated for optimal catalysis. This information can be extended to the β and γ class enzymes to help elucidate the binding mode of CO₂ in these enzymes.

Keywords

carbonic anhydrase; carbon dioxide; sequestration of carbon dioxide

In order to truly understand the means by which an enzyme acts it is necessary to know how the substrate(s), transition state(s), and product(s) are coordinated within the enzyme's active site. This analysis can be confounded by several factors, including a high turnover rate and multiple binding modes. An exceptional example of the former is the α -class carbonic anhydrase (CA), human CA2 (HCA2), which is among the fastest enzymes known, with a turnover rate of approximately 10^6 s^{-1} [1]. The reaction catalyzed by the CAs is a two-step cycle: the conversion of carbon dioxide to bicarbonate and the regeneration of an active enzyme. Initially a hydroxide (pK_a ~7.0, for the conjugate acid of the zinc-bound hydroxide), which is coordinated by the zinc ion, nucleophilically attacks the carbon of the CO₂ group, leading to the formation of product bicarbonate. A water molecule then displaces the bicarbonate by coordinating directly to the zinc ion. To regenerate the hydroxide a proton must be transferred off of the zinc-bound water. This is accomplished by a network of amino acid-coordinated water molecules, termed the "proton wire" [2,3]. The penultimate step of the proton transfer is the protonation of His64, which proceeds to release the excess proton to bulk solvent.

This two-stage reaction is vital for the efficiency of the CAs and is made possible by the amphiphilic active site (Fig. 1). That is, on one side is a hydrophobic patch that, as will be discussed in further detail, is responsible for the binding of carbon dioxide. This patch consists of the following residues: Val121, Val143, Leu198, Val207, and Trp209. On the other side is

*Corresponding author: Tel: 352 392-5696; Fax: 352 392-3422; rmckenna@ufl.edu.

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a patch of hydrophilic residues that act to orient the CO₂ (specifically Thr199) and also form the aforementioned proton wire [2,3].

A large number of studies, spanning several decades, have led to the same conclusion; that CO₂ binding is accomplished via an interaction with the hydrophobic half of the HCA2 active site. Early studies of substrate binding, due to the rapid rate of catalysis by HCA2, relied on the use of substrate analogs and inhibitors to probe the CO₂ binding site [4]. This led to the conclusion that CO₂ would most likely interact with the hydrophobic pocket that was occupied, for example, by the hydrophobic benzene ring of the inhibitor 3-acetoxymercuri-4-aminobenzenesulfonamide. Interestingly, it was seen that the binding of inhibitors displaced a water molecule, termed the “deep water”, which is coordinated by the amide nitrogen of Thr199, thereby suggesting a possible place holder that is commonly seen in HCA2 crystal structures [5]. Additionally, previous spectroscopic studies also indicated that the binding of CO₂ does not require any inner-sphere coordination by the zinc ion [6,7,8]. Combined, these results suggest that CO₂ would bind in a way as to interact ephemerally with the hydrophobic half of the active site. The hydrophilic half also was suggested to play a role, specifically in the orienting of CO₂. This would be due to an interaction with Thr199, via one of the CO₂ oxygens interacting with the amide nitrogen.

Later, the first simulations of CO₂ interacting with HCA2 were accomplished using molecular dynamics [9,10]. Despite not identifying identical binding sites, the results indicated that there was one catalytic binding site located on the hydrophobic face of the enzyme, in support of the previous inhibitor studies. In addition, second and third, lower affinity sites were discovered that were suggested to allow for the rapid diffusion of CO₂ into the active site for the next round of catalysis, with the caveat that these sites, in order to be productive, could not interfere with product release [10].

Based on these data it became evident that the hydrophobic pocket was vital to CO₂ binding and that any perturbation of this site would hinder the catalytic efficiency of the enzyme. Utilizing this knowledge, a series of mutations were made at Val143, one of the residues constituting the hydrophobic pocket [11]. It was discovered that larger substitutions at this site resulted in an enzyme that was essentially catalytically dead, with V143F having 0.2% activity and V143Y having <0.02% activity of wild-type. Structural analysis of V143Y revealed that its phenol ring protruded into the hydrophobic pocket, blocking access to that part of the active site, and thereby creating a dead enzyme [12].

A recent study by our group provided for the first time structural proof that CO₂ does in fact bind in the hydrophobic pocket, suggested by the previous studies, and provided detailed analysis of the orientation of CO₂ in this pocket [13]. It should be noted, a very similar study which reports almost identical results, has also been reported by Sjöblom *et al.* 2009 [14].

To overcome the issues associated with obtaining such a structure, namely trapping the binding of the substrate, an apparatus originally developed for the cryo-cooling of protein crystals with helium at high pressures was utilized [15]. Crystals of HCA2 were placed in a room temperature CO₂-pressurized environment above a liquid nitrogen bath. Almost assuredly, the CO₂ environment lowered the pH of the crystal, thereby ensuring that the zinc-bound solvent molecule was a water. This would thereby prevent any catalytic activity towards the CO₂. The crystals were then dipped slowly, over a 2 minute period, into the liquid nitrogen to trap the CO₂ in its place. Due the nature of the experimental set up an accurate value of the pH could not be ascertained.

As would be expected of an enzyme with such a high turnover rate, the binding of substrate causes virtually no structural changes in the enzyme with respect to the unbound form. In fact, the CO₂-bound structure at 1.1 Å resolution and the unbound structure at 1.05 Å resolution have

an overall C α RMSD of only 0.21Å over the entire structure [13]. This lack of structural perturbation is explained by the passive mode of binding of CO₂ inside of the hydrophobic pocket, with interaction distances of about 4Å with regard to the hydrophobic residues (Fig. 2). More literally this interaction can be viewed as a sequestration and is analogous to CO₂'s preferential solvation in organic solvents.

However, this is only part of the story as the crystal structure, in agreement with previous molecular dynamics work, suggests that there is also a role of some of the hydrophilic residues in orienting the molecule. Specifically, the amide nitrogen of Thr199 interacts with one of the oxygens of the CO₂ molecule with a distance of 3.5Å (Fig. 2). Although a seemingly weak interaction, coupled with the linear geometry of the CO₂ molecule, this places the oxygens of CO₂ roughly equidistant from the zinc-bound solvent molecule (3.0 and 3.1Å). This results in a side-on orientation of CO₂ with respect to the zinc-bound solvent, placing the CO₂ carbon 2.8Å away. Together, these interactions place the CO₂ in a way as to optimize it for nucleophilic attack by the lone-pair on the zinc-bound hydroxide.

Interestingly, it was seen that the zinc plays only a minor, secondary role in interacting with the CO₂. The structure of CO₂-bound, metal-free (apo) HCA2 revealed a strikingly similar orientation of the CO₂ when compared to the CO₂-bound holo HCA2 structure (Fig. 2). In the apo structure, a water molecule exists roughly where the zinc-bound water sits in the holo structure, albeit ~0.6Å closer to the histidine ligands (His94, His96, and His119). The result of this slight shift in the position of the water is that the CO₂ molecule rotates about the oxygen nearest the zinc and thereby has a slightly shorter interaction distance with the amide nitrogen of Thr199 (3.1Å for apo, 3.5Å for holo).

It could be argued that the CO₂-bound crystal structures may not fully represent productive binding sites. However, this placement of CO₂ in the hydrophobic pocket fully supports the results of previous studies in this field. Firstly, the results of the mutagenesis at Val143 are fully explained by this binding orientation [11,12]. A superposition of the V143Y mutant and the CO₂-bound structures distinctly shows that this mutation would completely prohibit CO₂ from binding in this location, thus blocking the catalytic activity of the enzyme (Fig. 3).

Secondly, the structure of the substrate analog and potent inhibitor, cyanate (NCO⁻), indicates a very similar orientation in the hydrophobic pocket [16]. Cyanate was seen to displace the “deep water” which forms a hydrogen bond with the amide nitrogen of Thr199. Moreover, the carbon of cyanate interacts with the zinc-bound solvent at a distance of ~2.4Å, a distance that is similar to that seen for the CO₂-bound holo enzyme. Thus, binding of the catalytically inactive cyanate to HCA2 would prevent the binding of the physiological substrate.

One last piece of evidence for this being the productive CO₂ binding site is provided by the crystal structure of bicarbonate-bound HCA2 [17]. The authors accomplished this by making a mutation, T200H, which has a higher affinity for bicarbonate than wild-type. A superposition with the CO₂-bound holo enzyme reveals that the CO₂ molecule lies nearly in plane with the bicarbonate (Fig. 4). From a structural perspective, the hydration of the CO₂ molecule via the addition of the zinc-bound hydroxide would place the bicarbonate in nearly the same position seen in the bicarbonate-bound structure.

Taken together these results agree with a mechanism proposed by Lindskog [18], based on theoretical free energy calculations (Fig. 5). In this mechanism, the zinc-bound hydroxide nucleophilically attacks the carbon of the CO₂ molecule. The addition of the hydroxide forms bicarbonate, which in turn interacts with the zinc in a bidentate fashion. That is, one of the CO₂ oxygen atoms becomes a direct ligand of the zinc ion, resulting in a penta-coordinated metal ion, with trigonal bipyramidal geometry. At this point, a water molecule displaces the

zinc-bound bicarbonate and the proton transfer event occurs, cycling to the next round of catalysis.

It is of note that a candidate water molecule was identified in the CO₂-bound crystal structures, which may play the role of displacing the bicarbonate [13]. Remember that the binding of CO₂ releases the “deep water” from its position in the hydrophobic pocket. In both the CO₂-bound apo and holo enzyme structures, a new water molecule, termed W_I, was found in a position that had not been previously seen in crystal structures of HCA2: between the side chain of Thr200 and the CO₂ molecule (Fig. 2). This location would allow for easy access to the bicarbonate molecule and could aid in increasing the rate of catalysis (as opposed to having a water diffuse from the bulk solvent into the active site).

Hydrophobic patches in β and γ -class CAs

The reaction catalyzed by the carbonic anhydrases is virtually the same throughout the three structurally-distinct CA classes. Because of this, the CAs represent one of the prime examples of convergent evolution, the development of nearly identical traits in completely unrelated lineages. Therefore, it is highly probable that hydrophobic pockets exist in the β - and γ -class enzymes that would allow for optimal orientation of CO₂. In fact, the crystal structures of members of both of these classes would suggest such an occurrence.

The crystal structure of the β -class carbonic anhydrases from the red alga *Porphyridium purpureum* and the dicotyledonous plant *Pisum sativum* reveal similar designs in their hydrophobic pockets [19,20]. The active site of these enzymes sits in a cleft that is at the interface between two monomers (*P. sativum*) (the *P. purpureum* CA is monomeric, with each monomer having a near-identical domain repeat) (Fig. 6A). The hydrophobic pocket, as in HCA2, forms one face of the active site, and is composed of several residues including Val184 from one monomer and Phe179, Tyr205, and Leu210 from the other monomer (*P. sativum* numbering). The only difference is that Val184 is replaced by an isoleucine in *P. purpureum* CA. This binding would place the CO₂ directly adjacent to the active site, and in particular, the catalytic aspartate that is present in these enzymes. Interestingly, the hydrophobic pocket of another β -class CA, from the archaeon *Methanobacterium thermoautotrophicum* has a different composition. The tyrosine and phenylalanine that exist in the previously mentioned β -class CAs are replaced by alanine and lysine, respectively [21]. This would suggest that the hydrophobic pocket of these CAs may play a similar, though lesser role in capturing and orienting the CO₂ molecule.

Yet another structurally distinct class of CAs is suggested to have a hydrophobic binding pocket as suggested from the crystal structure of the γ -class CA from the archaeon *Methanosarcina thermophila* [22]. This enzyme exists as a homotrimer with three active sites, each existing at the interface between two monomers. The active site is accessible at the surface of the enzyme at these interfaces, thereby being open to substrate diffusion (Fig. 6B). This would suggest that a hydrophobic pocket is necessary to trap the CO₂ to allow for catalysis. Such a hydrophobic pocket does exist on one side of the active site and is made up of residues from both monomers: Leu83, Phe138, Phe140, Ile157, and Val172 from monomer A, and Phe132 and Met135 from monomer B. This provides an adequate area for substrate binding while placing the CO₂ in a position for hydration by the enzyme, most likely by nucleophilic attack from a zinc- or iron-bound hydroxide [23].

CONCLUSION

The binding of CO₂ to HCA2 is not simply a matter of capture and catalyze. Rather, for the rapid α -class CA, HCA2, it is an issue of optimal orientation of the substrate for nucleophilic attack by the zinc-bound hydroxide. It is evident from analysis of the structures of all classes

of CAs that these hydrophobic pockets are a ubiquitous feature and play an integral role in the catalytic mechanism. Furthermore, slight variations in the structure of the hydrophobic pocket could in part explain the high variation in catalytic efficiencies seen throughout the various human isozymes as well as the other classes of CAs. To truly understand how the catalytic mechanism is carried out in the other structurally-distinct classes, it will be necessary to repeat the CO₂ trapping experiments for the other enzymes.

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Figure 1.

Active site of HCA2. The active site of HCA2 is found in a pocket near the center of the protein. It is divided into two regions surrounding the zinc, which is coordinated by three histidines (cyan) and a water (Zn-HOH) (PDB 2ili, [3]). On one side is a hydrophobic patch (magenta) that has been shown to be responsible for the binding of CO₂ and is typically occupied a water molecule (W_{deep}) in most HCA2 structures. On the opposite side is the hydrophilic patch (blue) that is responsible for the orientation of CO₂ and the formation of the solvent-mediated proton wire (red spheres).

Figure 2.

CO₂ binding to apo and holo HCA2. Stereo view of the binding of CO₂ to both holo (orange) and apo (cyan) HCA2 (PDB 3d92 (holo) and 3d93 (apo), [13]). The CO₂ molecule appears to be mostly unaffected by the absence of the zinc, with only a slight rotation about the proximal (to the zinc) oxygen. In the apo structure, a water molecule has moved into a position closer to where the zinc ion would normally exist. Interestingly, a new water molecule, W₁, is found in a position that is not seen in any other HCA2 structures, and may represent the new location of the “deep water.”

Figure 3. V143Y closes the hydrophobic pocket. From previous mutational studies of HCA2, it was discovered that a V143Y (pink) variant had virtually no activity (PDB 9ca2, [11,12]). This can be explained as a blocking of the hydrophobic patch (magenta mesh) that directly prohibits CO₂ binding in its optimal orientation (PDB 3d92, [13]).

Figure 4. Structural basis of catalysis. The location of CO₂ in the crystal structure of holo HCA2 lies nearly in the plane of the bicarbonate molecule in the T200H HCA2 variant (PDB 3d92, [13]; PDB 1bic, [17]). These two structures help visualize how the binding of CO₂ in the hydrophobic pocket (magenta mesh) leads to a nucleophilic attack of the zinc-bound hydroxide that results in a bidentate zinc-bicarbonate complex.

Figure 5.

The catalytic mechanism of HCA2. The mechanism of CO₂ hydration by HCA2 begins with the capture of CO₂ by the hydrophobic pocket of HCA2 (top middle) [18]. Nucleophilic attack by the zinc-bound hydroxide (top right) leads to the formation of a bidentate zinc-bicarbonate complex (bottom right). The bicarbonate is displaced by a water molecule, possibly WI (bottom left). The final stage of catalysis is a transfer of a proton from the water molecule to the bulk solvent, via the water-mediate proton wire and the proton shuttling residue, His64.

Figure 6.

Hydrophobic pockets in β and γ CA. The structures of representatives of the β and γ CAs show that the hydrophobic patch is a common feature throughout the three structurally unrelated classes. (A) β CA from *P. Purpureum* (PDB 1ddz, [19]) exists as a dimeric structure with the active site located at a cleft. A closer look at the active site reveals a hydrophobic patch (green) that leads from the surface of the molecule into the active site (catalytic aspartate shown in yellow). (B) γ CA from *M. Thermophila* (PDB 1qre, [22]) is a homotrimeric enzyme with an active site located at the interface between each monomer. Here we see the active site is again slightly buried with a hydrophobic patch (green) leading into the active site (zinc shown in magenta).