Human carbonic anhydrase II–cyanate inhibitor complex: putting the debate to rest

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The binding of anions to carbonic anhydrase II (CA II) has been attributed to high affinity for the active-site zinc. An anion of interest is cyanate, for which contrasting binding modes have been reported in the literature. Previous spectroscopic data have shown cyanate behaving as an inhibitor, directly binding to the zinc, in contrast to previous crystallographic data that implied that cyanate acts as a substrate mimic that is not directly bound to the zinc but overlaps with the binding site of the substrate CO₂. Wild-type and the V207I variant of CA II have been expressed and X-ray crystal structures of their cyanate complexes have been determined to 1.7 and 1.5 Å resolution, respectively. The rationale for the V207I CA II variant was its close proximity to the CO₂-binding site. Both structures clearly show that the cyanate binds directly to the zinc. In addition, inhibition constants (~40 µM) were measured using ¹⁸O-exchange mass spectrometry for wild-type and V207I CA II and were similar to those determined previously (Supuran *et al.*, 1997). Hence, it is concluded that under the conditions of these experiments the binding of cyanate to CA II is directly to the zinc, displacing the zinc-bound solvent molecule, and not in a site that overlaps with the CO₂ substrate-binding site.

1. Introduction

The α -class carbonic anhydrases (α -CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton. Much is known about the inhibition of α -CAs and the binding of anions in the active site; in particular, CA II has been well studied (Lindskog, 1966; Bertini *et al.*, 1982; Maren & Sanyal, 1983; Håkansson *et al.*, 1992; Jönsson *et al.*, 1993).

In CA II, simple anions such as bromide and azide have been shown to bind *via* a tetrahedral coordination (Fig. 1*a*) involving the three zinc-coordinating histidines (His94, His96 and His119) and the displacement of the zinc-bound solvent molecule (Jönsson *et al.*, 1993). This binding site is located near the backbone amide of Thr199 between the zinc and the hydrophobic pocket that includes Val121, Val143, Leu198 and Val207 (Håkansson *et al.*, 1992; Jönsson *et al.*, 1993). This hydrophobic region has been shown to be essential for the binding of the CO₂ substrate (Fierke *et al.*, 1991; Nair *et al.*, 1991; Krebs *et al.*, 1993; Domsic *et al.*, 2008; Sjöblom *et al.*, 2009; West *et al.*, 2012) and is highly conserved between α -CA isozymes.

Pentacoordinate binding has also occasionally been observed with zinc(II)-containing CAs and more frequently with cobalt(II)-containing CAs. In this binding mode the anion typically contains a carboxyl group that can accept a hydrogen bond from the backbone amide of Thr199, while the carboxyl O atom of Thr199 points towards the metal-bound solvent molecule. The position of the zinc-bound solvent is displaced almost 1 Å from its position in the apoenzyme, resulting in both the zinc-bound solvent and ligand coordination to the zinc. An example of this binding is CA II in complex with thiocyanate (Fig. 1*b*).

The coordination geometry for CA II in complex with cyanate has remained controversial. Previous crystallographic studies of cyanate bound to CA II proposed that cyanate forms a hydrogen bond to the backbone amide of Thr199 and displaces a solvent molecule in the hydrophobic pocket, termed the 'deep water' (Lindahl *et al.*, 1993). This suggested that cyanate binding was mimicking that of the natural

substrate CO₂. Since this study, the CO₂ binding in the active site of CA II has been identified (Fig. 1c; Domsic *et al.*, 2008).

In addition, studies with CA II variants in which Val143 (a residue in close proximity to the CO₂-binding site) was replaced by Ile, Leu or Ala showed a decrease in the catalytic rate and displacement of CO₂ binding compared with wild-type CA II (West *et al.*, 2012). Hence, a ligand mimicking CO₂ binding would behave differently when comparing the wild type and the V207I CA II variant.

We have determined the X-ray crystal structures of wild-type and V207I CA II in complex with cyanate to 1.7 and 1.5 Å resolution, respectively. In addition, inhibition constants (~40 µM) were measured using ¹⁸O-exchange mass spectrometry for both wild-type and V207I CA II and were similar to those determined previously. Hence, we conclude, as shown by both the structures and the inhibition constants, that cyanate binds directly to the zinc, forming a tetrahedral complex. These data indicate that cyanate does not mimic CO₂ binding.

2. Materials and methods

The V207I CA II variant was synthesized *via* site-directed mutagenesis using an expression vector with a wild-type CA II coding region. The single point mutation was made by polymerase chain reaction (Pulst, 2003) using the QuikChange II and QuikChange Lightning Kits from Agilent (Håkansson *et al.*, 1994; Fisher, Tu *et al.*, 2007). Verification of this mutation was accomplished by purification of the DNA using the Eppendorf plasmid miniprep kit (Schowen & Schowen, 1982) followed by DNA sequencing of the entire CA II coding region and simulated translation using ExPASy Translate. Both the wild-type and V207I variant CA II were then transformed into *Escherichia coli* BL21(DE3)pLysS cells, a cell line specific for protein expression and one that does not express CA under experimental conditions.

Following transformation, the cells were subjected to expression (Baneyx, 1999) at 37°C in LB and enriched 2× YT medium containing ampicillin. Wild-type and V207I CA II production was induced at an OD₆₀₀ between 0.6 and 1.2 by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. Zinc sulfate was also added to a final concentration of 1 mM to provide a source of zinc for proper folding and functioning of the enzyme. Cells were harvested 4 h after induction, spun down and stored at -80°C overnight. The cell pellets were lysed and each protein was purified by affinity chromatography using a 2 ml *p*-(aminomethyl)-benzene-

sulfonamide resin column. The wild-type and V207I CA II cell extracts were loaded onto separate columns pre-washed with 20 ml pH 9 buffer (0.2 M Na₂SO₄, 0.1 M Tris) and allowed to flow through the columns followed by 60 ml of pH 9 buffer. Each flowthrough was collected and measured for absorbance. Once the absorbance reached 0.2 or lower, a maximum of 40 ml pH 7 buffer (containing the same reagents as pH 9 buffer) was added to remove stronger binding cell debris. Protein was eluted off the columns with 0.5 M sodium azide. The sodium azide was removed and proteins were purified by buffer exchange into 0.1 M Tris pH 8 in a 15 ml Millipore centrifugal filter unit with a 10 kDa molecular-weight cutoff.

Crystals of wild-type and V207I CA II were obtained using the hanging-drop vapour-diffusion method. The drops were prepared by mixing 5 µl protein at a concentration of 10 mg ml⁻¹ in 50 mM Tris-HCl pH 8.0 with 5 µl precipitant solution consisting of 50 mM Tris-HCl pH 8, 1.4 M sodium citrate and were equilibrated against 1 ml precipitant solution at room temperature. Crystals were stored in polystyrene containers and allowed to sit undisturbed. Crystals were observed 3 d after setup (McPherson, 1982; Fisher, Maupin *et al.*, 2007). The crystals were soaked in a drop of 0.01 M sodium cyanate for 5–10 s, allowing diffusion of cyanate into the crystal. This was immediately followed by immersion into a cryoprotectant solution consisting of glycerol (30%) and 1 M Tris-HCl (70%) for 5–10 s; the crystals were then flash-cooled and mounted for data collection.

X-ray diffraction data sets for the wild-type and V207I CA II crystals were collected on an in-house R-Axis IV⁺⁺ image-plate detector using an RU-H3R rotating Cu anode (Cu Kα = 1.5418 Å) operating at 50 kV and 22 mA. The crystal-to-detector distance was set to 80 mm and the oscillation steps were 1° with a 5 min exposure time. The crystal data were integrated, merged and scaled using HKL-2000 (Otwinowski & Minor, 1997).

The wild-type and V207I CA II structures were solved using *phenix.refine* in PHENIX (Adams *et al.*, 2010). Preceding refinement, random test sets of ~10% of the reflections were flagged for *R*_{free} calculations. The method of structure determination was molecular replacement (*Phaser*) with wild-type CA II (PDB entry 2ili; Fisher, Maupin *et al.*, 2007) stripped of the zinc and solvent and with amino acid Val207 replaced by alanine. The structural solution from *Phaser* was used for the initial phase calculations in *phenix.refine*. The cyanate ligand was built using PRODRG (Schüttelkopf & van Aalten, 2004) and the cif files needed for refinement were generated using PHENIX (Adams *et al.*, 2010). Following three cycles of refinement in PHENIX (Adams *et al.*, 2010), the refined structure was visually inspected using the Coot molecular-imaging system (Emsley

Figure 1

Coordination states of the zinc ion in the active site of CA II. (a) Tetrahedral coordination with azide. (b) Pentacoordination with thiocyanate. (c) CO₂ binding in the active site prior to nucleophilic attack by ZnOH⁻ (tetrahedral coordination maintained).

Table 1

Crystallographic data and refinement statistics.

Values in parentheses are for the highest resolution bin.

	Wild-type CA II	V207I CA II
PDB code	4e5q	4qef
Space group	$P2_1$	$P2_1$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 42.2$, $b = 41.0$, $c = 71.5$, $\beta = 104.5$	$a = 42.4$, $b = 41.5$, $c = 72.5$, $\beta = 104.4$
Resolution ($\text{\AA}$)	20–1.7 (1.76–1.70)	35–1.5 (1.55–1.50)
$R_{\text{merge}}^\dagger$ (%)	8.8 (5.5)	4.4 (2.5)
$\langle I/\sigma(I) \rangle$	16.5 (4.0)	28.1 (5.2)
Completeness (%)	91.1 (89.2)	92.2 (87.9)
Average multiplicity	6.8 (7.0)	3.8 (3.7)
No. of unique reflections	23839	38496
$R_{\text{cryst}}^\ddagger/R_{\text{free}}^\S$ (%)	17.2/20.0	13.9/17.1
No. of atoms		
Protein	2090	2073
Water	287	320
B factors ($\text{\AA}^2$)		
Main chain	13.2	13.8
Side chain	17.4	17.4
Solvent	24.8	25.4
R.m.s.d.		
Bonds ($\text{\AA}$)	0.006	0.010
Angles ($^\circ$)	1.092	1.300
Ramachandran plot (%)		
Most favored	88.4	88.0
Allowed	11.6	12.0
Outliers	0.0	0.0

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, $^\ddagger R_{\text{cryst}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|} \times 100$, $^\S R_{\text{free}}$ is calculated in same manner as R_{cryst} , except that it uses 10% of the reflection data omitted from refinement.

& Cowtan, 2004) to display the model and electron density. The zinc and the amino acid at position 207, as well as improperly positioned side chains, were manually placed in their respective densities. The refined models were then submitted for subsequent rounds of refinement and the cyanate was built into residual $F_o - F_c$ electron density, followed by solvent placement. During the final stages of refinement in conjunction with *Coot* (Emsley & Cowtan, 2004), the models were viewed and solvent with little or no $2F_o - F_c$ density was deleted until the R_{work} and R_{free} values had converged. Data statistics are given in Table 1.

Catalysis by both wild-type and V207I CA II was measured by the exchange of ^{18}O between CO_2 and water (Silverman, 1982). The ^{18}O content of CO_2 was determined using a membrane inlet attached to an Extrel EXM-200 mass spectrometer. Solutions consisted of 25 mM total substrate ($\text{HCO}_3^-/\text{CO}_2$), 100 mM HEPES pH 7.6 at 25°C . The

uncatalyzed exchange reaction was measured for 2 min, following which enzyme was added to measure the catalyzed rate. Cyanate was added every minute while the ^{18}O -exchange rate was continuously measured to obtain a series of rates at accumulated concentrations of cyanate. Owing to the very high value of the dissociation constant (K_{diss}) for CO_2 and bicarbonate, the mode of inhibition (competitive, noncompetitive or uncompetitive) cannot be determined using this method and the IC_{50} equals the inhibition constant K_i . Calculations of K_i from experimental data were performed using *Enzfitter* (Biosoft).

3. Results and discussion

The wild-type and V207I CA II cyanate complex structures were refined to 1.7 and 1.5 $\text{\AA}$ resolution with final R_{cryst} values of 17.2 and 13.9%, respectively (Table 1). Both structures clearly showed cyanate bound directly to the zinc. The cyanate anion extends into the hydrophobic pocket, with the cyanate N atom positioned 2.0 $\text{\AA}$ from the zinc and its O atom 2.9 $\text{\AA}$ from the amide N atom of the Thr199 main chain (Figs. 2*a* and 2*b*). The zinc-bound solvent was not observed, indicating that the cyanate had displaced it. There also appears to be no significant changes in side-chain conformations caused by the cyanate binding or the valine-to-isoleucine substitution at residue 207. The r.m.s.d. value of 0.25 $\text{\AA}$ for C^α atoms between wild-type and V207I CA II with cyanate bound compared with unbound wild-type CA II indicates that there are negligible perturbations in the structure of the variant or on cyanate binding.

Both structures show that the cyanate is linear and the overlay of both cyanate-bound structures shows that this anion is bound in the same proximity to the Zn atom (Fig. 2*c*).

The K_i of cyanate for wild-type and V207I CA II was determined from ^{18}O exchange between CO_2 and water measured by mass spectrometry. The K_i measured for the wild type agrees with previously published data (Supuran *et al.*, 1997) and there was no difference in the K_i values for wild-type and V207I CA II (Table 2).

The crystallographic study reported here shows cyanate bound as an inner-sphere ligand forming a tetrahedral complex with the zinc in wild-type and V207I CA II (Figs. 2*a* and 2*b*). A common factor in the tetrahedral coordination of anionic ligands appears to be the formation of hydrogen bonds to Thr199, either to the backbone amide or the side-chain hydroxyl group (Liljas *et al.*, 1995). With cyanate bound directly to the zinc, it is positioned 2.9 $\text{\AA}$ from the backbone amide of Thr199 in both the wild type and the V207I variant (Figs. 2*a* and 2*b*). Previous NMR data showed that cyanate, as

Figure 2

Stick diagrams of (a) wild-type and (b) V207I CA II in complex with cyanate. $F_o - F_c$ electron-density map contoured at 3σ . The hydrogen-bonding distance (2.9 $\text{\AA}$) between the main-chain amide of Thr199 and the O atom of cyanate is indicated as a dashed line. (c) Overlay of wild-type (dark grey) and V207I (light grey) CA II in complex with cyanate. Active-site residues are as labelled and the zinc is represented as a gray sphere. This figure was produced using *PyMOL* (v1.5.0.4; Schrödinger).

well as sulfonamides, bind to the zinc of CA II through the N atom (Kanamori & Roberts, 1983), which allows the O atom to accept a hydrogen bond from Thr199. Our structures are similar to those of other anions that form tetrahedral coordination complexes with the Zn atom and coordinating histidines in CA II. The cyanate was built assuming that it was orientated with the N atom in direct contact with the zinc, as at the resolution of our electron-density maps we could not distinguish between an N and an O atom.

While these data indicate direct coordination of cyanate to zinc in CA II, it is contrary to an early study in which cyanate was observed to bind in a position similar to the natural substrate CO₂, with a water or hydroxide ion bound to the zinc (Lindahl *et al.*, 1993; Liljas *et al.*, 1995). While the central C atom of CO₂ has been shown to be positioned 2.8 Å from the zinc, cyanate appears closer, with its N atom only 2.0 Å from the zinc. The overlay of cyanate- and CO₂-bound structures in Fig. 3 illustrates that there are significant differences in their position in the active site. Many small anions show metal–ligand interaction in CA II and it appears that cyanate follows the same pattern (Alterio *et al.*, 2012). Work has shown that azide and thiocyanate, which are triatomic anions with the same linear structure as cyanate, bind directly to the zinc (Supuran *et al.*, 1997) and their position in the active site matches our findings for cyanate in wild-type and V207I CA II.

However, owing to structural similarities, cyanate could mimic the natural substrate CO₂ and bind in a similar position. Both cyanate and CO₂ have a linear conformation and the same number of electrons (they are isoelectronic), and these multiple structural similarities could explain why cyanate was previously found in a position mimicking CO₂ binding. It is possible that cyanate can have different binding positions depending on the enzyme variant, the environment and the experimental conditions. Some structures of CA II with bicarbonate indicate that it can form a pentacoordinated complex with solvent still also bound to the zinc (Håkansson *et al.*, 1992; Liljas *et al.*, 1995). However, it has also been shown that bicarbonate can form a tetrahedral complex, displacing water (Sjöblom *et al.*, 2009).

The X-ray crystal structure of the cyanate–CA II complex described previously (Lindahl *et al.*, 1993) showed the ligand in a

Table 2
Inhibition constants for cyanate.

CA II	Inhibition constant (K_i) (μ M)
Wild type†	20
Wild type	37 ± 3
V207I	42 ± 3

† Data from Supuran *et al.* (1997).

similar position to the natural substrate CO₂ bound in the hydrophobic pocket (Håkansson & Wehnert, 1992; Domsic *et al.*, 2008). However, based on the data presented here, it appears that the binding of cyanate is that of a metal-binding ligand rather than a weak substrate. The K_i for cyanate is similar for wild-type and V207I CA II, the latter of which perturbs the binding site of CO₂ (Table 2). This is consistent with our conclusion that under our conditions cyanate, while similar in electron configuration and conformation to CO₂, does not bind to the CO₂ site in CA II but binds directly to the zinc, displacing the zinc-bound solvent (Fig. 3).

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Figure 3
Overlay of wild-type CA II in complex with cyanate (dark gray) and CA II in complex with CO₂ (white; Domsic *et al.*, 2008; PDB entry 3d92). The root-mean-squared deviation between all C^α atoms is 0.25 Å. This figure was produced using PyMOL (v.1.5.0.4; Schrödinger).