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Crystal structure of the C183S/C217S mutant of human CA VII in complex with acetazolamide

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ABSTRACT

Human carbonic anhydrase VII (hCA VII) is a cytosolic member of the α-CA family. This enzyme is mainly localized in a number of brain tissues such as the cortex, hippocampus and thalamus and has been noted for its contribution in generating neuronal excitation and seizures. Recently, it has been also proposed that hCA VII may be involved in the control of neuropathic pain, thus its inhibition may offer a new approach in designing pain killers useful for combating neuropathic pain. We report here the X-ray crystallographic structure of a mutated form of human CA VII in complex with acetazolamide, a classical sulfonamide inhibitor. These crystallographic studies provide important implications for the rational drug design of selective CA inhibitors with clinical applications.

Fifteen α-carbonic anhydrase (CA) isoforms have so far been identified in humans (hCA I–hCA XIV), 12 of which are catalytically active (hCAs I–IV, hCAs VA–VB, hCAs VI–VII, hCA IX and hCAs XII–XIV).[1] These isozymes differ widely in their cellular localization: in particular, hCAs I–III, VII and XIII reside in the cytosol, hCAs IV, IX, XII and XIV are associated with membranes, hCAs VA and VB occur in mitochondria, whilst hCA VI is secreted.[1] Since these enzymes participate in various physiological and pathological processes linked to the catalytic hydration of carbon dioxide to bicarbonate, in recent years they have become an interesting target for the design of inhibitors or activators with biomedical applications.[1–5] To date, none of the clinically used hCA inhibitors show selectivity for a specific isozyme;[1,6] however, a large number of recently reported structural studies have provided a scientific basis for the rational drug design of isoform selective enzyme inhibitors.[6–16]

CA VII is one of the least investigated and understood cytosolic CA isoforms. This enzyme presents a limited distribution, being mainly localized in a number of brain tissues such as the cortex, hippocampus and thalamus.[17,18] CA VII has been noted for its contribution in generating neuronal excitation,[19] establishing a functionally excitatory GABAergic transmission by supplying bicarbonate anions, which can mediate current through channels coupled to $GABA_A$ receptors.[20] The observation that this activity is suppressed by membrane-permeating CA inhibitors corroborates the involvement of CA VII in neuronal excitation and seizures.[18,21]

Recently, it has been proposed that CA VII may be also involved in the control of neuropathic pain, and its inhibition may constitute a new pharmacologic mechanism in designing pain killers useful for combating neuropathic pain,[22] a condition which currently offers few therapeutic options.

As part of a general research project based on the structure-based drug design of isoform-selective hCA inhibitors we have undertaken a structural study on all hCAs with unknown 3D structure. Here we report the X-ray crystallographic characterization of a mutated form of hCA VII as a complex with the inhibitor acetazolamide (AZM).[23]

Two recombinant hCA VII enzyme forms were produced using the *Escherichia coli* expression system: the first form corresponded to the native enzyme while the second, called MhCA VII, contained two amino acid substitutions.[24] In particular, the cysteine residues in position 183 and 217 were mutated to serines. This mutant form was used for the structural studies since it avoided potential crystallization problems caused by the mixture of structurally different reduced and oxidized enzyme forms. Both recombinant products were purified to homogeneity by affinity and size exclusion chromatography.[15]

Crystallization experiments were performed both on native hCA VII and MhCA VII in their unbound and inhibitor-bound form. AZM, a strong inhibitor with a nanomolar affinity for hCA VII,[1] was used

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Table 1
Crystal parameters, data collection and refinement statistics

Crystal parameters	
Space group	$P2_1$
Unit-cell parameters	
a (Å)	43.78
b (Å)	66.28
c (Å)	46.46
β (°)	113.84
Data collection statistics	
Temperature (K)	100
Resolution range (Å)	20.00–2.05
Total reflections	55,467
Unique reflections	15,321
Completeness (%)	99.9 (99.1)
R-sym[a]	0.046 (0.094)
Mean $I/\sigma(I)$	26.5 (10.4)
Refinement statistics	
Resolution range (Å)	20.00–2.05
Number of reflections	15,099
R-factor[b] (%)	16.6
R-free[b] (%)	20.7
RMSD from ideal geometry	
Bond lengths (Å)	0.008
Bond angles (°)	1.5
Number of protein atoms	2099
Number of water molecules	261
Number of inhibitor atoms	13
Average B-factor (A^2)	13.9
Residues in most favoured regions (%)	88.2
Residues in additional allowed regions (%)	11.8

[a] *R*-sym = $\sum_{hkl}\sum_i|I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(h\,k\,l)$ is the *i*th measurement and $\langle I(hkl)\rangle$ is the weighted mean of all measurements of $I(h\,k\,l)$.
[b] *R*-factor = $\sum_{hkl}||F_o(hkl)| - |F_c(hkl)||/\sum_{hkl}|F_o(hkl)|$, *R*-free calculated with 5% of data withheld from refinement. Values in parentheses refer to the highest resolution shell (2.12–2.05 Å).

to this purpose.[25] Crystals were obtained only in the case of the MhCA VII/AZM complex, using the hanging drop vapour diffusion method and PEG 4000 as precipitant. They belong to the space group $P2_1$ with one molecule per asymmetric unit, according to a solvent content of 41% (Table 1). Upon diffraction with synchrotron radiation, data were collected to 2.05 Å resolution. All residues were well defined in the electron density maps except the first five N-terminal residues, whose large conformational flexibility was also confirmed by high *B*-factor values.

MhCA VII is a monomeric compact globular protein, whose roughly ovoidal shape is approximately $40 \times 44 \times 40$ Å^3 in size. As already observed for other α-CAs,[6] its structure consists of a central 10-stranded β-sheet surrounded by four α- and four 3_{10}-helices and five additional β-strands (Fig. 1A). An intramolecular disulfide bond is present in the structure between Cys54 and Cys178. However, the observation that these two cysteines are not conserved within the α-CA family[6] and that disulfide bonds are extremely rare in cytosolic proteins[26] suggests that this disulfide bond is not present in hCA VII under physiological conditions, but is an artefact generated by the oxidizing conditions that arise during protein handling. Further studies are currently under way to clarify this point.

As observed for other α-CA isozymes, the active site is located in a conical cavity about 15 Å wide and 15 Å deep, which spans from the surface of the protein to the centre of the molecule. The catalytic zinc ion is located at the bottom of this cavity, coordinated by three histidine residues. The fourth coordination position is occupied by the deprotonated sulfonamide NH^- group of the AZM inhibitor which co-crystallized with the enzyme. Figure 1B shows the main protein–inhibitor interactions.

The active site cavity is divided in two very different regions bordered by hydrophobic or hydrophilic amino acids. In particular, Val121, Leu198, Ala135, Leu141, Val143, Val207 and Phe131 delimit the hydrophobic region, while Asn62, His64, Gln67, Lys91 and Gln92 identify the hydrophilic one. Very well defined electron density maps were observed for all these residues with the exception of His64, which on the contrary was disordered. The high

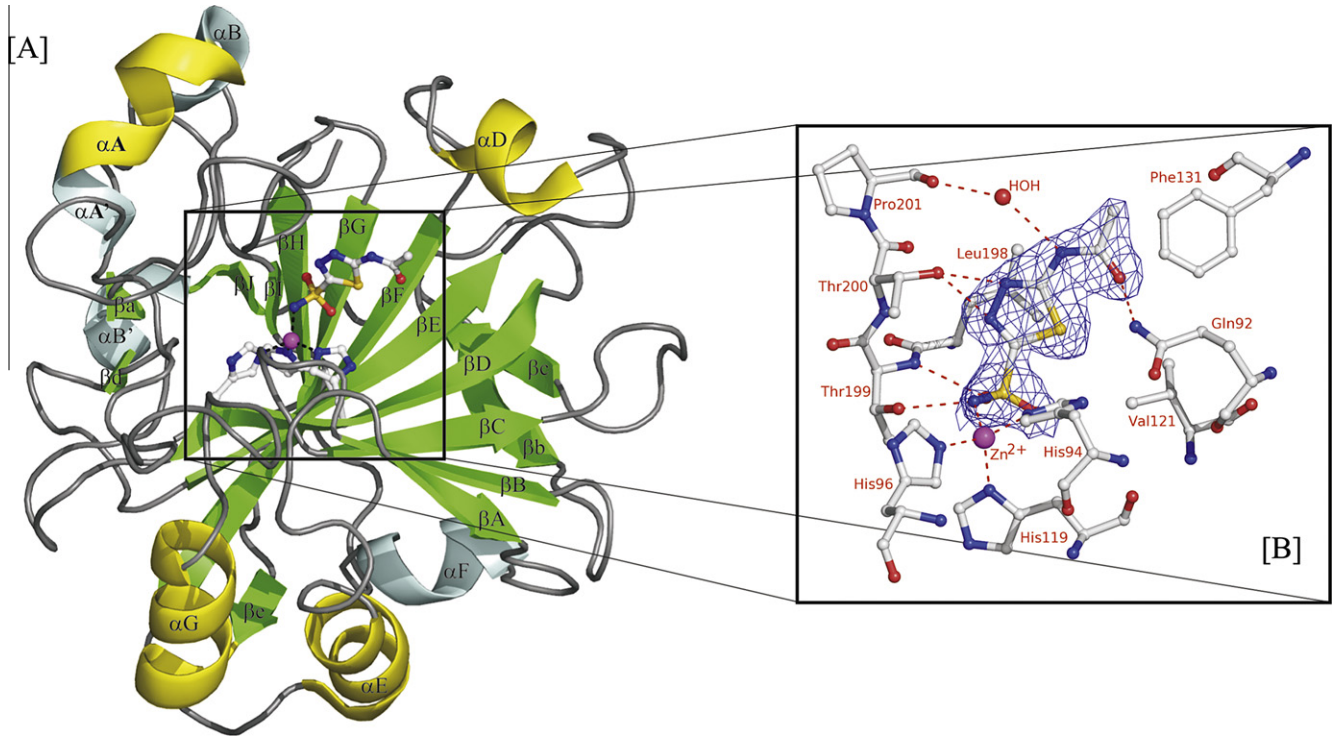

Figure 1. (A) MhCA VII overall fold. β-Strands are reported in green, α-helices in yellow and 3_{10}-helices in cyan; (B) enlarged view of the active site of the MhCA VII/AZM complex. Hydrogen bonds, Zn^{2+} coordination and residues establishing strong van der Waals interactions (distance <4.5 Å) with the inhibitor are shown. The simulated annealing omit $|2F_o - F_c|$ electron density map, relative to the inhibitor molecule is reported.

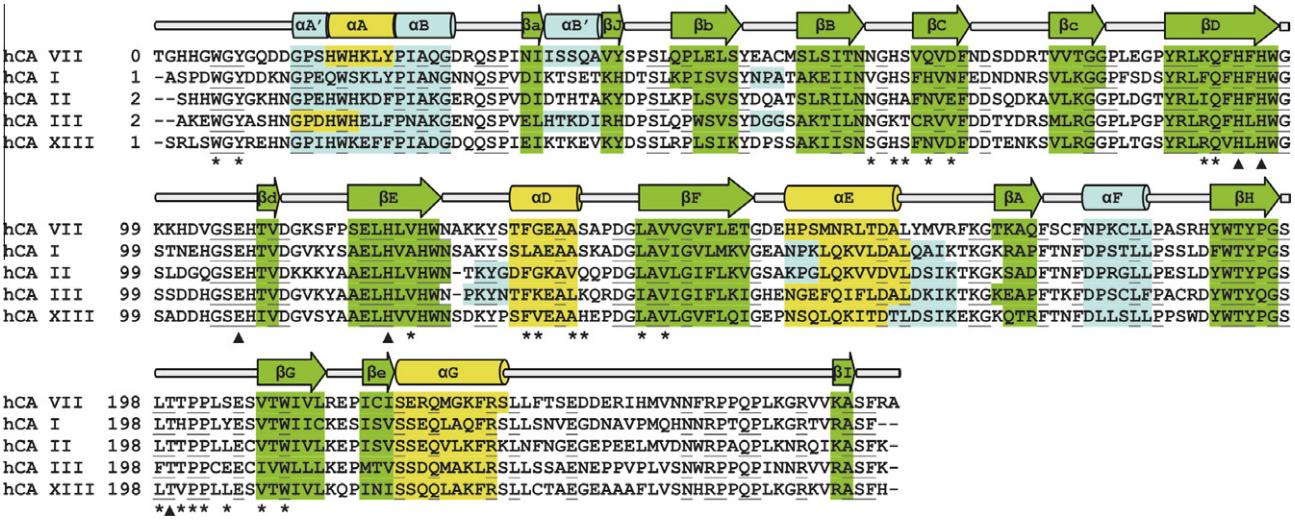

Figure 2. Sequence alignment of cytosolic α-CAs. hCA VII secondary structure elements are shown schematically: helices are represented by solid cylinders and β-strands as arrows. These regions are named as reported by Eriksson et al.[8] for hCA II. Secondary structure elements not present in the hCA II structure are primed. α-Helices, 3_{10}-helices and β-strands for hCA I (PDB code 2CAB),[7] hCA II (PDB code 1CA2),[8] hCA III (PDB code 1Z93)[14] and hCA XIII (PDB code 3D0N)[15] are highlighted in yellow, cyan and green, respectively. Conserved residues are underlined, catalytic triad, Thr199 and Glu106 are indicated with black triangles, while residues delimiting the active site cavity are marked with asterisks.

conformational mobility of this residue is likely associated with its role as a proton shuttle in catalysis, as already observed for the same residue in other α-CA isozymes.[27] Moreover, similarly to that observed for hCA II, it is reasonable to suppose that the hydrophobic region is involved in the capture of the CO_2 substrate, opportunely orienting it for nucleophilic attack by the zinc-bound hydroxide,[28–30] while the hydrophilic region is involved in a well ordered solvent network which assists His64 in the proton transfer reaction.[28,31–34]

hCA VII shows very high sequence identity with all other cytosolic hCAs (hCA VII/hCA I = 50%, hCA VII/hCA II = 55%, hCA VII/hCA III 49% and hCA VII/hCA XIII 52%) and consequently a substantial degree of 3D similarity. Indeed, the RMSD for the superposition of the Cα atoms of MhCA VII with the corresponding atoms in hCA I,[7] hCA II,[8] hCA III[14] and hCA XIII[15] is calculated as 0.91, 0.55, 0.72 and 0.69 Å, respectively. The main differences among these proteins are observed in the loops connecting βb and βB, βC and βc, βE and αD, αD and βF, and in the C-terminal region. Since these regions are located distant to the active site, the observed differences should not influence either catalytic activity or substrate binding. Limiting the comparison to the active site region, it is possible to observe that most of the residues which delimit this cavity are generally conserved either by nature or conformation (Fig. 2). This finding is particularly true in the comparison between hCA VII and hCA II. Indeed, in this case only seven substitutions (S65A, Q67N, D69E, K91I, A135V, S136Q and S204L) are observed among the 23 residues which delimit the cavity. These findings are particularly interesting considering that, despite their high sequence and structural similarity, all cytosolic hCAs are characterized by very different kinetic features.[1] Indeed, hCA II and hCA VII are very efficient as catalysts (for hCA II $K_{cat}/K_M = 1.5 \times 10^8\ M^{-1}\ s^{-1}$ and for hCA VII $K_{cat}/K_M = 8.3 \times 10^7\ M^{-1}\ s^{-1}$), hCA I and hCA XIII are 10–50 times less efficient (for hCA I $K_{cat}/K_M = 5.0 \times 10^7\ M^{-1}\ s^{-1}$ and for hCA XIII $K_{cat}/K_M = 1.1 \times 10^7\ M^{-1}\ s^{-1}$), while hCA III is a very poor catalyst ($K_{cat}/K_M = 3.0 \times 10^5\ M^{-1}\ s^{-1}$).[1,15] Mutagenesis experiments are currently under way to clarify the role of the different amino acid substitutions within the active site cavity on the enzyme's catalytic efficiency.

In conclusion, in this Letter we have reported the X-ray structure of a mutated form of hCA VII, completing the structural characterization of human cytosolic CAs and providing the basis either for mutagenesis experiments devoted to identify specific residues responsible for the different catalytic properties of these enzymes or for the rational drug design of selective hCA inhibitors with clinical applications.

Acknowledgments

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23. While this Letter was under revision, the crystal structure of the native hCA VII in complex with ethoxzolamide was released by the Protein Data Bank (Accession code 3MDZ), even if no Letter was associated with this deposition. A structural comparison of this structure with the one reported here did not reveal significant differences.
24. Bacterial expression of hCA VII was performed using pGex-6P-1 vector (GE Healthcare) which encodes a GST-fused protein containing a PreScission protease cleavage site upstream of hCA VII. Site-directed mutagenesis of MhCA VII was prepared according to the manufacturer's instructions (QuikChange Site-Directed Mutagenesis Kit, Stratagene). Purification of recombinant products was performed as previously described.[15]
25. The MhCA VII/AZM complex was prepared by adding a 5 M excess of the inhibitor to a 7 mg/mL protein solution in 20 mM Tris–HCl, pH 9.0, 100 mM sodium chloride. This mixture, equilibrated for 1 h at room temperature, was used for the crystallization experiments. In particular, MhCA VII/AZM co-crystals were obtained at 20 °C by the hanging drop vapour diffusion method. Drops were prepared by mixing 1 μl of enzyme/inhibitor solution with 1 μl of precipitant solution containing 30% (w/v) PEG 4000, 0.2 M magnesium chloride, 0.1 M Tris–HCl, pH 8.5, and further equilibrated over a well containing 500 μl of precipitant buffer. Crystals appeared in the drops within 2–3 days and grew to a maximum dimension of $0.2 \times 0.15 \times 0.2$ mm^3 in about 2 weeks.
X-ray data collection was performed at the ELETTRA synchrotron source (Trieste, Italy) using a MAR CCD detector. Prior to data collection, crystals were transferred into a cryo-protectant solution containing 20% (v/v) glycerol as cryogenic agent, and frozen at 100 K under a nitrogen stream. Data were indexed, processed, and scaled with HKL-2000 package.[35] Crystal parameters and data collection statistics are reported in Table 1.
The MhCA VII/AZM crystal structure was solved by molecular replacement using the AMORE[36] program and the crystallographic structure of hCA II (PDB code 1CA2)[8] as starting model. The rotation and translation functions were calculated using data between 15.0 and 3.5 Å resolution.
Few cycles of rigid body refinement were followed by a simulated annealing and group *B*-factor refinement performed with CNS 1.1.[37] Clear electron density for the inhibitor was observed in the difference map after this single round of refinement (*R*-free = 0.360; *R*-factor = 0.321). Introduction of the AZM molecule was followed by several cycles of addition of water molecules, manual rebuilding, and energy minimization and thermal *B*-factor refinement, reducing the *R*-free and *R*-factor values to 0.207 and 0.166, respectively. Refinement statistics are summarized in Table 1. Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (Accession code 3ML5).
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