

# Crystal structure of the catalytic domain of the tumor-associated human carbonic anhydrase IX

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**Carbonic anhydrase (CA) IX is a plasma membrane-associated member of the  $\alpha$ -CA enzyme family, which is involved in solid tumor acidification. It is a marker of tumor hypoxia and a prognostic factor in several human cancers. An aberrant increase in CA IX expression in chronic hypoxia and during development of various carcinomas contributes to tumorigenesis through at least two mechanisms: pH regulation and cell adhesion control. Here we report the X-ray structure of the catalytic domain of CA IX in complex with a classical, clinically used sulfonamide inhibitor, acetazolamide. The structure reveals a typical  $\alpha$ -CA fold, which significantly differs from the other CA isozymes when the protein quaternary structure is considered. Thus, two catalytic domains of CA IX associate to form a dimer, which is stabilized by the formation of an intermolecular disulfide bond. The active site clefts and the PG domains are located on one face of the dimer, while the C-termini are located on the opposite face to facilitate protein anchoring to the cell membrane. A correlation between the three-dimensional structure and the physiological role of the enzyme is here suggested, based on the measurement of the pH profile of the catalytic activity for the physiological reaction, CO<sub>2</sub> hydration to bicarbonate and protons. On the basis of the structural differences observed between CA IX and the other membrane-associated  $\alpha$ -CAs, further prospects for the rational drug design of isozyme-specific CA inhibitors are proposed, given that inhibition of this enzyme shows antitumor activity both in vitro and in vivo.**

Carbonic anhydrases (CAs) are ubiquitous metallo-enzymes, acting as catalysts in the reversible hydration of CO<sub>2</sub> to HCO<sub>3</sub><sup>-</sup> and H<sup>+</sup>. All human CAs (hCAs) belong to the so-called  $\alpha$ -class and differ widely in their cellular localization: CA I, II, III, VII, and XIII reside in the cytosol, CA IV, IX, XII, and XIV are associated with the cell membrane, CA VA, and VB occur in mitochondria, whereas CA VI is secreted into saliva and milk (1). Structural studies on isozymes I, II, III, IV, V, XII, XIII, and XIV (2–10) have revealed that all these isoforms present a considerable degree of three-dimensional similarity and a typical fold characterized by a central antiparallel  $\beta$ -sheet surrounding a Zn<sup>2+</sup> critical for catalysis. The active site is located in a large, cone-shaped cavity that reaches the center of the protein molecule. The zinc ion is close to the bottom of the cavity and is coordinated by three conserved His residues in a tetrahedral geometry with H<sub>2</sub>O or OH<sup>-</sup> as the fourth ligand.

CA IX is a very peculiar member of the CA family since it is generally expressed in a limited number of normal tissues (mainly in the gastrointestinal tract), whereas its overexpression is seen on the cell surface of a large number of solid tumors, where it is invariably linked with the hypoxic phenotype, mediated by the transcription factor HIF-1 (11). Moreover, CA IX overexpression is often associated with a poor responsiveness to classical radio- and chemotherapy (12). Since CA IX has also been shown to contribute to pH regulation of tumor cells, cell proliferation, cell adhesion, and malignant cell invasion (13, 14),

this protein has been recently recognized as a valuable target for cancer diagnostics and treatment (1).

A cDNA coding for the hCA IX protein was first cloned and investigated by Pastorek et al. (15), and the *CA9* gene was further characterized by the same group (16). On the basis of the sequence similarity, these authors proposed that hCA IX is a multidomain protein consisting of an N-terminal proteoglycan-like (PG) domain, a CA catalytic domain, a transmembrane segment (TM), and an intracytoplasmic (IC) portion (16). The first domain is absent in all other CA isozymes and resembles the keratan sulfate attachment domain of a large aggregating proteoglycan, namely aggrecan; in contrast, the CA catalytic domain presents a significant sequence identity (from 30% to 40%) to other catalytic hCA isozymes. Both CA and PG domains are glycosylated (17). According to recent biochemical reports, the CA IX protein is dimeric, and the dimerization is mediated by the formation of an intermolecular disulfide bond between the same Cys residue located on two CA catalytic domains (17). Despite all of the data available on CA IX, no information is currently available on the three-dimensional structure of its isolated domains or their relative orientation within the whole CA IX three-dimensional structure. This information is fundamental for the rational design of selective CA IX-directed anti-tumor drugs and diagnostic tools.

In this study, we report the X-ray structure of the catalytic domain of CA IX. Significant differences were identified with respect to the other  $\alpha$ -CA isozymes, whose three-dimensional structure has been determined. The molecular bases for dimerization of CA IX in the membrane are provided together with a correlation between the three-dimensional structure and the physiological role of the enzyme. Because of the structural differences observed between CA IX and the other membrane-associated  $\alpha$ -CA isoforms, further prospects for the rational drug design of isozyme-specific CA inhibitors are also proposed.

## Results

**Protein Cloning, Expression, and Purification.** Four recombinant hCA IX enzyme forms were produced using the baculovirus-insect cell expression system (17) (Fig. S1). A recombinant product (P1) had both the PG and CA domains of hCA IX; the

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID code 3IAI).

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**Table 1. Crystal parameters, data collection, and refinement statistics for P1 and P4 constructs**

Protein	P1	P4
Crystal parameters		
Space group	P6 <sub>3</sub>	P6 <sub>1</sub>
Unit-cell parameters		
a = b (Å)	142.4	144.18
c (Å)	104.9	208.89
Data collection statistics		
Temperature, K	100	100
Resolution range, Å	20.0–3.2	20.00–2.20
Total reflections	93,041	827,770
Unique reflections	19,866	122,974
Completeness, %	99.4 (99.6)	99.3 (95.8)
$R_{\text{sym}}^*$	0.097 (0.433)	0.054 (0.231)
Mean $I/\sigma(I)$	14.2 (2.9)	28.5 (5.2)
Refinement statistics		
Resolution range, Å	20.0–3.2	20.00–2.20
Number of reflections used	19,009	119,944
R factor <sup>†</sup> , %	22.9	15.7
R free <sup>†</sup> , %	27.3	18.1
rmsd from ideal geometry		
Bond lengths, Å	0.011	0.007
Bond angles, °	1.8	1.3
Protein atoms	7,588	8,116
Water molecules	39	1,662
Inhibitor atoms	—	52
Average B factor, Å <sup>2</sup>	45.9	24.6
Ramachandran plot		
Most favored, %	84.1	91.8
Additionally allowed, %	15.6	8.2

\* $R_{\text{sym}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ , where  $I_i(hkl)$  is the  $i$ th measurement and  $\langle I(hkl) \rangle$  is the weighted mean of all measurements of  $I(hkl)$ .

<sup>†</sup>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. Values in parentheses refer to the highest resolution shell (3.3–3.2 Å and 2.28–2.20 Å for P1 and P4, respectively).

remaining ones (P2–P4) contained only the CA catalytic domain. In P4, Cys-41 (hCA I numbering system), a residue involved in an intermolecular disulfide bond responsible for CA IX dimerization (17), was mutated into Ser. Proteins were purified to homogeneity by Ni<sup>2+</sup>-based chromatography; the His-tag was removed using a protease site that was included in the sequences. Proteins were further purified by size exclusion chromatography. Protein purity and homogeneity were assessed by SDS/PAGE in reducing and non-reducing conditions, and by LC-ESI-IT MS analysis. In the latter case, MS spectra confirmed the occurrence of high mannose-type glycans N-linked to all recombinant products (17).

**Crystallization, Structure Resolution, and Refinement.** Initial crystallization experiments were performed on the P1 recombinant product. Large well-formed crystals were obtained with the hanging-drop vapor diffusion method. Upon diffraction with synchrotron radiation, data were collected to 3.2-Å resolution. The crystals belonged to the space group P6<sub>3</sub> with four molecules per asymmetric unit (Table 1). The structure was solved by molecular replacement using the crystallographic structure of mCA XIV as starting model (9). Despite low-resolution data, most of the residue main chains of the catalytic domain were defined in the electron density maps. In contrast, no electron density was observed for the entire PG domain, strongly suggesting the absence of this portion within the crystals. To check this hypothesis, crystals were dissolved and analyzed by SDS/

PAGE, revealing a band migrating at the molecular mass of the sole CA domain. This result was confirmed by MALDI-TOF PMF analysis. The absence of the entire PG domain was then ascribed to P1 degradation as a consequence of contaminants present within the purification/crystallization solutions. Additional crystallization experiments performed on the P1 construct were not successful.

On the basis of the observation that the PG domain was not present in the obtained crystals, subsequent crystallization experiments were performed on the other CA IX recombinant products (P2–P4) containing only the catalytic domain either in their unbound or inhibitor-bound form. Acetazolamide (AZM), a strong inhibitor with a nanomolar affinity for hCA IX (17), was used to this purpose. Crystals were obtained only in the case of the P4/AZM complex and they belonged to the space group P6<sub>1</sub> with four molecules per asymmetric unit. Upon diffraction with synchrotron radiation, data were collected to 2.20-Å resolution (Table 1). The structure was solved by molecular replacement using the P1 low resolution structure as starting model. In this case, all residues were well defined in the electron density maps, except for the N-terminal region of chains B, C, and D (residues 2 and 3). The structure of the P4 recombinant product was identical to that of P1; hence, we shall describe only its structure in the following sections, referring to P4 as CA IX catalytic domain, unless otherwise stated.

**Overall Structure and Active Site.** The four molecules in the asymmetric unit of the CA IX catalytic domain crystals, called A, B, C, and D, revealed only minor differences: selecting A as the reference, B, C, and D showed an rmsd for the superimposition of the C $\alpha$  atoms of 0.27 Å, 0.28 Å, and 0.30 Å, respectively. For this reason, the subsequent discussion is based on one arbitrarily chosen monomer, unless otherwise stated.

The CA IX catalytic domain appeared as a compact globular domain, whose roughly ovoid shape is approximately 47 × 35 × 42 Å<sup>3</sup> in size. Its structure revealed a fold characteristic of other  $\alpha$ -CAs, whose three-dimensional structure has been solved (2–10), in which a 10-stranded antiparallel  $\beta$ -sheet forms the core of the molecule. Two glycans of the pentasaccharide core N-linked to Asn-213 (17) were clearly visible in the electron density maps of all molecules in the asymmetric unit.

As observed in the structure of other  $\alpha$ -CA isozymes, the CA IX active site is located in a large conical cavity, which spans from the surface to the center of the protein. The zinc ion is located at the bottom of this cavity. Two distinct regions made of hydrophobic or hydrophilic amino acids delimit the active site. In particular, Leu-91, Val-121, Val-131, Leu-135, Leu-141, Val-143, Leu-198, and Pro-202 define the hydrophobic region, while Asn-62, His-64, Ser-65, Gln-67, Thr-69, and Gln-92 identify the hydrophilic one. A clear electron density for AZM molecule was evident within the active site of each molecule in the asymmetric unit (Fig. 1). The main protein-inhibitor interactions are depicted in Fig. 1.

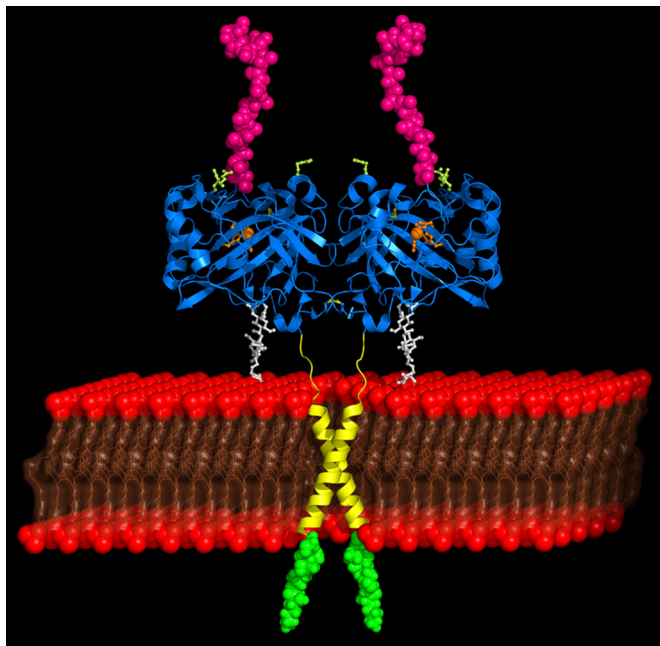
**P1 and P4 Dimeric Arrangement.** We recently reported that purified hCA IX is a dimeric protein, and its dimerization is mediated by an intermolecular disulfide bond involving Cys-41 (17). In agreement with these findings, two identical dimers (consisting of the pairs AB and CD), resulting from a Cys-41-mediated intermolecular disulfide bond between two adjacent monomers, were observed in the asymmetric unit of the P1 crystals (Fig. 2A). Within each dimer, the two monomers were related by a non-crystallographic 2-fold symmetry axis. The two dimers interacted with each other generating a tetramer that was recognized by the protein interfaces, surfaces and assemblies service PISA ([http://www.ebi.ac.uk/msd-srv/prot\\_int/pistart.html](http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html)) (18) as a potential stable oligomeric arrangement in solution. However, further size-exclusion chromatography and dynamic light-scattering experiments, performed at different pH











**Fig. 5.** Proposed model showing the structural arrangement of the full-length CA IX dimer on the cellular membrane. The X-ray structure of the dimeric catalytic domain is reported in cyan, with Arg-58, Arg-60, and Arg-130 in green and the glycan moieties in white. The hypothetical arrangements of the PG domains, the transmembrane helices and the cytoplasmic portions are schematically reported in magenta, yellow, and green, respectively.

speculate that the PG domain could interact with positively charged residues that delimit the active site, sterically controlling substrate accession or participating in the proton-transfer reaction (1). Further experiments are currently under way to confirm these hypotheses.

The crystal structure resolution of the CA IX catalytic domain also provides important results for the structure-based drug design of selective CA IX inhibitors with potential as anticancer drugs. Owing to the extracellular location of isozymes CA IV, IX, XII, and XIV, an initial step in the design of selective inhibitors can be obtained with the development of membrane-impermeant inhibitors, which inhibit membrane-associated CAs without interacting with the cytosolic or mitochondrial isoforms. This selectivity has been recently obtained through the design of positively charged sulfonamides that, being permanently charged, are restricted to the extracellular space (1, 27, 28). Structural comparison with the other membrane-associated CA isozymes reported herein, offers some additional insights for the design of more selective CA IX inhibitors. In particular, since the region 125–137 differs both in structure and sequence in all these isozymes, it represents a “hot spot” to be considered in structure-based drug design.

The results presented in this work provide structural background for the elucidation of the complex enzymatic machinery associated to CA IX, a multidomain membrane protein consisting of extra-, trans-, and intra-cellular portions, which act together to generate different biochemical functions (21). Interestingly, a similar PG-CA domain combination has also been reported in extracellular parts of some human receptor protein Tyr phosphatases (RPTP $\beta/\gamma$ ) and a related rat phosphacan, which contain inactive CA domains and are expressed in the form of proteoglycans (29). These proteins also contain a cytoplasmic phosphorylation-based signaling module, function as receptor molecules and, through the binding of neuronal adhesion molecule contactin/tenascin, mediate adhesive communication between astroglial cells and gonadotrophin-releasing hormone neurons (30, 31). In some cases, RPTPs modulate their

activity through a disulfide-based dimerization process involving the same Cys residue present in two monomers (32–34). We believe that the occurrence of a number of structural/functional analogies with some RPTPs as well as the recognition of CA IX as a valuable target for cancer diagnostics and treatment will promote further crystallographic studies to completely elucidate the whole CA IX structure. These investigations will definitively allow the rational drug design of isozyme-specific inhibitors for the different CA IX functions.

## Materials and Methods

**Production and Purification of Recombinant CA IX Enzymes.** The recombinant CA IX enzymes used for the crystallization studies were produced using the Bac-To-Bac Baculovirus Expression System (Invitrogen) (17). Four CA IX proteins, whose sequences are reported in Fig. S1, were constructed. All of the enzymes had a CA IX signal sequence, whose codon usage was optimized for *Spodoptera frugiperda*, eight His residues for protein purification and a protease cleavage site (thrombin or PreScission protease, GE Healthcare) to remove the His-tag. The cDNA sequences were constructed by stepwise elongation sequence-PCR similarly to what recently described (17).

The production and purification of the recombinant enzymes as well as the thrombin treatment for P1 were performed as previously described (17). Before protease treatment of P2–P4 products, they were changed to a buffer of 50 mM Tris-HCl, pH 7.5. To remove the His-tag from P2, the protein was treated with 170  $\mu$ L resin-coupled thrombin (Thrombin CleanCleave KIT, Sigma-Aldrich) per 1 mg protein, with gentle mixing at 25 °C for 1 h. The thrombin and the His-tag were removed from the solution as previously described (17). For P3 and P4, the His-tag was removed by incubating proteins with 2 U GST-tagged PreScission protease per 1 mg protein at 4 °C for 20 h. PreScission protease was removed using Bulk GST Purification module (GE Healthcare), and the His-tag was removed from the solution as previously described (17). As final purification step the proteins were loaded on a Superdex 200 10/30 (P1) or Superdex 75 10/30 (GE Healthcare) (P2–P4) column equilibrated with 50 mM phosphate, 100 mM NaCl, pH 8.0 (P1), or 30 mM Tris-HCl, 100 mM NaCl, pH 7.5 (P2–P4). Highly purified proteins were pooled, concentrated to 5 mg/mL and buffer exchanged to water before crystallization experiments. Purity was assessed by SDS/PAGE and LC-ESI-IT MS as reported previously (10).

**Crystallization and Data Collection.** The crystals of the P1 recombinant product were obtained at 20 °C using the hanging-drop vapor diffusion method. Drops of 2  $\mu$ L, prepared mixing 1  $\mu$ L protein solution with an equal volume of precipitant solution (0.4 M  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.1 M sodium citrate, pH 5.0), were suspended over wells containing 1.0 mL precipitant. In about two weeks crystals of  $0.2 \times 0.1 \times 0.1$  mm<sup>3</sup> were grown. P1 diffraction data were collected at 3.20-Å resolution from a single crystal at the temperature of 100 K, using a MAR CCD detector, at the synchrotron source Elettra in Trieste. The crystals were transferred to a cryoprotectant solution containing 35% (vol/vol) glycerol, in addition to the precipitant buffer, before X-ray data diffraction collection. Data were processed using the HKL crystallographic data resolution package (Denzo/Scalepack) (35) (Table 1). The crystals belonged to space group P6<sub>3</sub> with four molecules in the asymmetric unit according to a solvent content of 53.4%.

P4/AZM complex was prepared by adding a 10-M excess of inhibitor to a 5 mg/mL protein solution in water. The crystals were grown using the hanging drop vapor diffusion technique at 20 °C by using a precipitant solution whose formulation was similar to that of P1 enzyme (1.0 M  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.1 M sodium acetate, pH 4.0). Crystals appeared in the drops within 3 days and grew in about 2 weeks to maximum dimensions of  $0.4 \times 0.2 \times 0.2$  mm<sup>3</sup>.

P4/AZM X-ray diffraction data were collected and processed as for P1, with the exception that the cryoprotectant solution contained 25% glycerol. The crystals belonged to the hexagonal space group P6<sub>1</sub> with four complex molecules in the asymmetric unit and showed an unusual solvent content of 77.2%, which corresponded to a Matthews coefficient of 5.4 Å<sup>3</sup> Da<sup>-1</sup>. Data reduction statistics for both P1 and P4 are recorded in Table 1.

**Structure Resolution.** The P1 crystal structure was solved by molecular replacement using the program AMoRe (36) and the crystallographic structure of mCA XIV (PDB code 1RJ5) (9) as starting model. P1 structure refinement was performed with CNS (37) and model building was performed with O (38). The four molecules in the asymmetric unit were refined by using NCS-restraints and thermal B-factors were initially fixed at 20.0 Å<sup>2</sup>. Many cycles of positional and temperature-factor refinement alternated with manual rebuilding were necessary to reduce the crystallographic Rfactor and Rfree values to 0.229 and 0.273, respectively.

The structure of P4/AZM complex was solved by molecular replacement em-

playing the refined P1 structure as search model. After initial refinement limited to the enzyme structure, a model for the inhibitor, clearly visible in the electron density maps, was built, and introduced into the atomic coordinate set for further refinement. The first cycles of the refinement were carried out with 4-fold NCS-restraints. After Rfactor and Rfree reached 0.287 and 0.297, respectively, the NCS-restraints were removed and further cycles of manual rebuilding and positional and temperature-factor refinement were necessary to reduce the crystallographic Rfactor and Rfree values to 0.157 and 0.181, respectively. Refinement statistics for both P1 and P4 are summarized in Table 1.

**CA Activity Assay.** The CA-activity was measured as previously described (17). Assays were performed on P1 and P4 recombinant CA IX forms. For

obtaining the pH dependency, the following buffer/indicator pairs were used: pH 6.0, Mes, methyl red, working at 433 nM; pH 6.2 and 6.5, Mes, chlorophenol red, working at 574 nM; pH 6.8, HEPES, 4-nitrophenol, 400 nM; pH 7.0, HEPES, neutral red, working at 540 nM; pH 7.4, HEPES, phenol red, 557 nM (this is the buffer-indicator pair in which most measurements of CA catalytic and inhibition are usually performed, due to the physiological pH value of 7.4) (39).

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