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REVIEW ARTICLE

Insights into the role of reactive sulfhydryl groups of Carbonic Anhydrase III and VII during oxidative damage

Daria M. Monti[a], Giuseppina De Simone[b], Emma Langella[b], Claudiu T. Supuran[c], Anna Di Fiore[b] and Simona M. Monti[b]

[a]Department of Chemical Sciences, University of Naples Federico II, Naples, Italy; [b]Institute of Biostructures and Bioimaging, CNR, Naples, Italy; [c]Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Università degli Studi di Firenze, Florence, Italy

ABSTRACT

Carbonic anhydrases (CAs) III and VII are two cytosolic isoforms of the α-CA family which catalyze the physiological reaction of carbon dioxide hydration to bicarbonate and proton. Despite these two enzymes share a 49% sequence identity and present a very similar three-dimensional structure, they show profound differences when comparing the specific activity for CO_2 hydration reaction, with CA VII being much more active than CA III. Recently, CA III and CA VII have been proposed to play a new role as scavenger enzymes in cells where oxidative damage occurs. Here, we will examine functional and structural features of these two isoforms giving insights into their newly proposed protective role against oxidative stress.

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Introduction

Carbonic anhydrases (CAs) are ubiquitous metalloenzymes, present throughout most living organisms and encoded by six unrelated gene families: the α-, β-, γ-, δ-, η-, and ζ-CAs. These enzymes contain a Zn(II) ion in the active site that can be replaced by Fe(II) or Co(II) in the gamma family and by Cd(II) in the zeta one[1–6]. Human CAs (hCAs) belong to the α class which consists of 15 isoforms, among which 12 are enzymatically active (CAs I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, and XIV) and 3, called CA-related proteins (CARPs VIII, X, and XI), catalytically inactive. Catalytically active hCAs have different cellular localization; in particular, 5 are cytosolic (CAs I–III, VII, and XIII), 4 are membrane-associated (CAs IV, IX, XII, and XIV), 2 are mitochondrial (CAs VA and VB), and 1 is a secretory protein present in milk and saliva (CA VI)[1,2,7].

CAs catalyze a very simple reaction: the hydration of carbon dioxide to bicarbonate and proton. These enzymes are expressed in many tissues where they participate in numerous physiological processes such as acid–base balance, respiration, ureagenesis, dioxide and ion transport, bone resorption, gluconeogenesis, body fluid generation, and lipogenesis[1,7]. Interestingly, apart from these biological activities, CAs are also associated to several pathological processes when abnormal levels and/or activities are registered. For instance, glaucoma and epilepsy are CA-related diseases where CA II and CA VII are involved[8,9]. Other CAs, such as CA IX and CA XII, have been associated to tumors where they cause extracellular pH lowering, thus helping the progression of malignant cells[10,11].

CA III and CA VII are two of the least understood CA isoforms. These two cytosolic enzymes are both localized in tissues that have a high oxygen consumption rate, such as skeletal muscle, liver, and brain; moreover, they share 49% sequence identity and present a very similar three-dimensional structure[12,13]. Despite these similarities, these isoforms present a very different catalytic efficiency for CO_2 hydration reaction, being CA VII one of the more efficient isoforms and CA III the least efficient (CA III activity is 0.35% compared to CA VII activity)[14]. Recently it has been suggested that these enzymes could participate in defense processes in cells where oxidative damage occurs upon the generation of reactive oxygen species (ROS)[15–17]. ROS are generated by normal metabolic activity, as well as lifestyle factors such as smoking, exercise, and diet. Their overproduction can be induced by different factors and perturb the normal cell redox balance, shifting cells into a state of oxidative stress. Since in conditions of severe stress, survival of the cells depends on their ability to adjust or resist to stress[18–20], cells have developed an antioxidant defense system, involving gluthathione, antioxidant vitamins, sulfhydryl groups, and antioxidant enzymes[21]. Recently, it has been suggested that CA III and CA VII could be part of this antioxidant defense system. Indeed, the comparative analysis of the amino acid sequence of CA III and CA VII with that of the other cytosolic hCAs clearly showed the presence of a higher number of cysteine residues (Figure 1). Biochemical and structural studies have indicated for some of these cysteines a particularly high reactivity, thus suggesting that both enzymes could have a role in scavenging reactive species through their reactive sulfhydryl groups[14,17,22,23]. This is a new interesting finding considering that CA VII has been principally studied as an antiepileptic target being involved into the GABAergic transmission[24].

In this review, by examining CA III and CA VII functional and structural features, we will provide insights into their newly proposed protective role against oxidative stress.

CONTACT Anna Di Fiore anna.difiore@cnr.it; Simona Maria Monti simonamaria.monti@cnr.it Institute of Biostructures and Bioimaging, CNR, via Mezzocannone, 16, I-80134, Naples, Italy

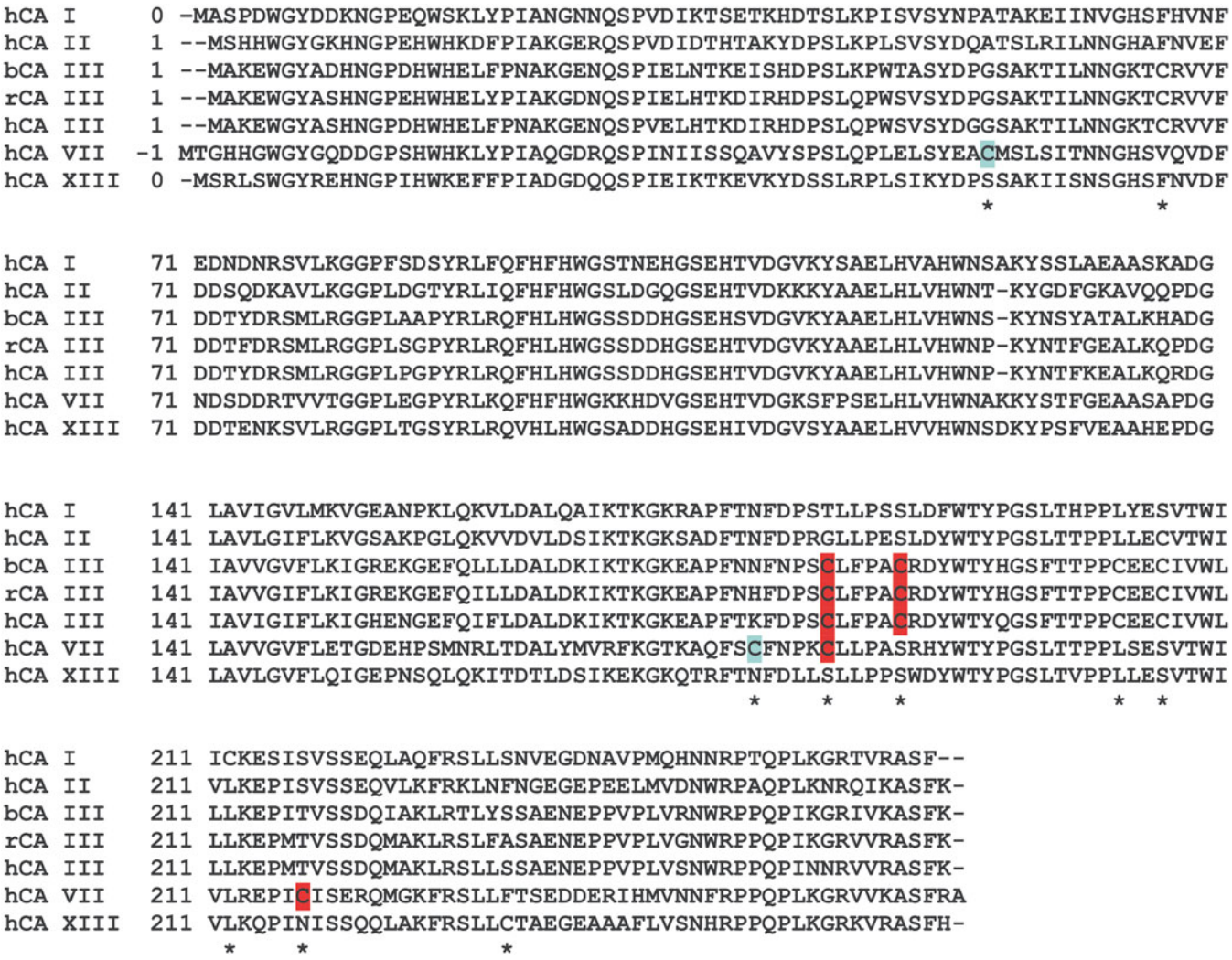


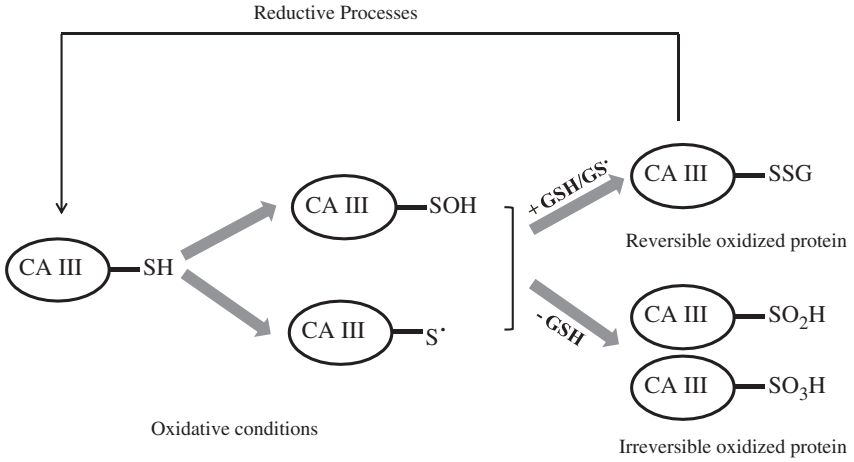
Figure 1. Multiple sequence alignment of cytosolic CAs. All cysteine residues are indicated with an asterisk; the reactive cysteines of isoforms III and VII are highlighted in dark gray, whereas those involved into hCA VII intramolecular disulfide bridge are highlighted in light gray. The sequence of bovine (b), rat (r) and human (h) CA III are reported showing that all cysteine residues are conserved. The alignment has been performed using Clustal Omega server[84].

CA III biochemical and functional features

The distribution pattern of CA III in the different tissues has been largely investigated by means of both Western blotting and immunohistochemistry experiments[25], showing that CA III is abundantly expressed in liver and skeletal muscle. Several other tissues contain detectable quantities of CA III although in a smaller quantity[25–33]. The great amount of CA III in tissues with a great oxidation potential suggested that this protein could function *in vivo* as an oxyradical scavenger, protecting cells from oxidative damage. In agreement with this hypothesis, it was demonstrated that in NIH/3T3 cells, transfected with rat CA III cDNA, the intracellular steady-state level of ROS was lower with respect to the parental cells, and addition of exogenous hydrogen peroxide (H_2O_2) did not induce an increase of ROS contrary to what observed for not transfected cells. Moreover, in proliferation assays CA III overexpressing cells grew faster and were more resistant to cytotoxic concentration of H_2O_2 with respect to not-transfected cells[34].

The involvement of CA III in the response to the oxidative stress was also supported by the observation that among the five cysteines present in its sequence (Figure 1), two of them, i.e. Cys183 and Cys188, were shown to form *in vivo* a disulfide link with glutathione (GSH)[35] in a process referred as S-glutathiolation[36]. Reversible protein S-thiolation is an early cellular response towards oxidative stress due to the partial oxidation of cysteines to sulfenic acid or thiol radicals, which subsequently react with cellular GSH or GS radicals to form disulfide adducts[36] (see Scheme 1). The analysis of the 3D structure of S-glutathiolated CA III suggested that the forming bonds between CA III reactive cysteines and GSH moiety were not to be ascribed to a specific recognition of GSH, but rather to the high reactivity of Cys183 and Cys188 and the great abundance of GSH in cell[37], which reaches *in vivo* millimolar concentration[36,38,39].

The behavior of the two reactive sulfhydryl groups of CA III, upon exposure to H_2O_2, peroxy radicals, or hypochlorous acid (HOCl) in presence or absence of GSH, was investigated in detail by Mallis and coworkers[40]. These authors found that irreversible oxidation was prevented only when GSH was approximately equimolar to protein thiols, thus allowing the S-gluthathiolation process[40]. Accordingly, at low GSH concentration, sulfenic acids (cysteine SOH) were irreversibly oxidized to sulfinic or sulfonic acid (cysteine SO_2H and SO_3H, respectively) which were not reducible by S-disulfide exchange (see Scheme 1). Similar results were obtained in cultured rat hepatocytes when treated with diethyl maleate for GSH depletion and menadione as oxidative agent[40]. In agreement with these data, protein extracts from liver of aged rats, which presented reduced levels of GSH, contained increased amounts of irreversibly oxidized CA III[40,41].



Scheme 1. Schematic representation of reversible and irreversible oxidation of CA III reactive cysteines.

Subsequent investigations on the putative antioxidant role of CA III were performed in 2004 by Zimmerman and coworkers; these authors analyzed the S-glutathiolation and the irreversible oxidation of CA III in skeletal muscle subjected to ischemia or exhaustive exercise. These studies showed that the two reactive sulfhydryl groups of CA III were differentially and progressively oxidized in skeletal muscle when it was exposed to oxidative insult. Under a mild or brief stress, reversible S-glutathiolation of one of CA III reactive cysteines was observed, involving only 20% of skeletal muscle protein, whereas under prolonged or harsh stress both sulfhydryl groups were irreversible oxidized. These results suggested that the high content of CA III in skeletal muscle might serve as a reservoir of reactive sulfhydryl groups able to repair acute and chronic insults. It is worth noticing that less than 10% of S-glutathiolated enzyme was detected in resting skeletal muscle indicative of a regulative physiological function[22]. The same authors analyzed, by microarray, *ca3* knockout and wild-type mice and identified a transcriptional alteration of about 500 genes (out of 12 000), all associated to the GSH mediated anti-oxidative system. However, the *ca3* knockout mice exhibited a normal development, fertility, and life span, at least under the standard laboratory conditions used[42].

Finally, interesting data were presented on the repression of transcription of CA III gene driven by EVI1[23]. EVI1, a multi-domain protein belonging to *cys2hys2* zinc finger family[43,44], is involved into cancer progression through several mechanisms, including enhanced cell proliferation, impaired differentiation, and evasion of apoptosis[45]. It has been shown that high levels of EVI1 expression in Rat1 cells induce an enhanced sensitivity to H_2O_2-induced apoptosis due to the down-regulation of CA III gene expression. However, the molecular mechanism by which EVI1 acts as CA III repressor has not yet been understood. Authors hypothesized a direct binding of EVI1 to CA III promoter sequence, which was shown to contain *cis*-regulatory elements necessary for EVI1-mediated transcriptional repression[46]. Since EVI1 is overexpressed in some human cancers[47,48], it would be interesting to assess whether these types of tumors present reduced levels of CA III. If this is the case, a novel strategy could be developed for the treatment of tumors overexpressing EVI1 as they might be vulnerable to therapeutic agents that induce oxidative stress[23].

CA VII biochemical and functional features

CA VII is known to be predominantly expressed in the cytosol of rat[49] and mice neurons starting around postnatal day 10[50]. In humans, CA VII is expressed in several normal tissues, including liver, brain, colon, and skeletal muscle[51–53], and only few years ago Parkkila's group found high CA VII expression levels in brain tumor cells, allowing them to suggest CA VII as a tumor marker[54]. On the other side, Yang and coworkers, by using qPCR and Western blot analysis, recently found that CA VII mRNA and protein levels were down-regulated in colon tumors. Immunohistochemical staining also showed a weak signal in colon tumors with respect to normal colon tissues. Moreover, a correlation between reduced CA VII protein levels and shorter disease-specific survival was also found[55]. These results confirmed those already obtained by others who, analyzing different samples from colon (normal and tumor tissues) by a gene expression profiling study and a bioinformatics-based analysis, noted that CA VII mRNA was down-regulated in colorectal carcinoma clinical specimens[56,57]. Altogether, these data provided evidence for the potential utility of CA VII as a prognostic marker for patients with colorectal carcinoma[55].

A recent study from our group unveiled for this enzyme another potential role as an oxygen radical scavenger for protecting cells from oxidative damage[17]. Indeed, we found that among the 4 cysteine residues present in the amino acid sequence of the enzyme (Figure 1), those in position 183 and 217 were particularly reactive, being quantitatively S-glutathiolated during the *in vitro* purification[14]. The glutathiolated protein, as well as a variant in which the two reactive cysteine residues were substituted by serines (C183S/C217S), showed an enzymatic activity similar to that reported for the wild-type protein (K_{cat}/K_M of 6.5×10^7 $M^{-1}s^{-1}$ for the variant, 8.0×10^7 $M^{-1}s^{-1}$ for the glutathiolated protein and 7.2×10^7 $M^{-1}s^{-1}$ for the wild-type enzyme). The same results were obtained for the inhibition constants using sulfonamide inhibitor acetazolamide (K_I of 2.8, 3.0, and 2.7 nM for wild-type, C183S/C217S and the glutathiolated protein, respectively). Altogether these results were indicative of two sulfhydryl groups very reactive and not involved in the enzyme catalytic mechanism, thus suggesting, in analogy with what observed for CA III, that CA VII could function *in vivo* as an oxygen radical scavenger through its reactive cysteines, protecting cells from oxidative damage. To verify this hypothesis, the CA VII ability to protect human cells from oxidative stress was investigated[17]. In particular, HeLa cells, that do not express endogenous CA VII, were transiently transfected with a plasmid encoding the wild-type protein and then stressed by using sodium arsenite (SA). It was observed that CA VII was able to protect cells from death, as the induction of apoptosis was lower in cells expressing CA VII with respect to

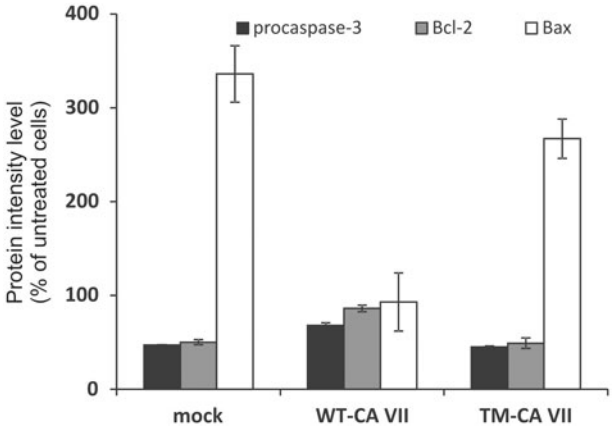


Figure 2. Quantitative analysis of apoptotic marker levels in transfected HeLa cells after treatment with SA for 16 h. In each histogram, the protein intensity level was normalized to endogenous actin and expressed as percentage with respect to untreated cells. Black histograms refer to procaspase-3 levels, gray histograms to Bcl-2 and white histograms to Bax levels.

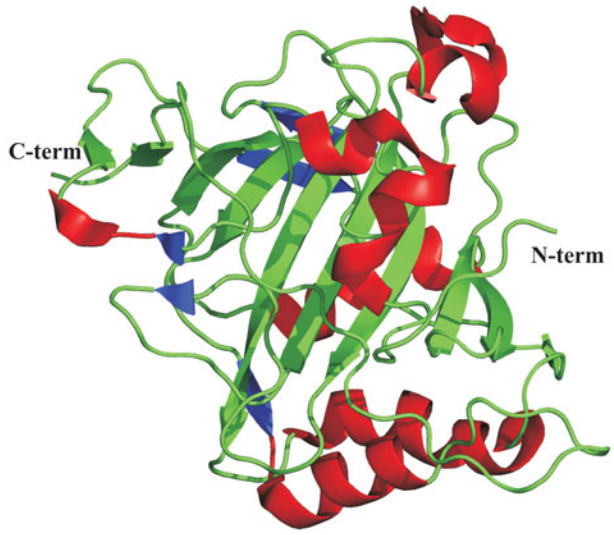


Figure 3. Ribbon representation of the overall fold of CA III.

mock-transfected cells[17]. In particular, in mock-transfected cells the percentage of apoptotic cells increased from 15% to 49% after SA treatment, whereas cells expressing CA VII showed a lower percentage of apoptotic cells after induction of oxidative stress (from 18% to 39%). The activation of apoptosis mediated by mitochondria was then analyzed by Western blotting, showing that CA VII was able to protect HeLa cells from oxidative stress (Figure 2). In fact, levels of both Bcl-2 (Figure 2, gray bars) and Bax (Figure 2, white bars), which are, respectively, anti- and pro-apoptotic markers involved in mitochondria-mediated apoptosis, were almost unchanged in SA-treated cells expressing WT-CA VII with respect to untreated cells (86% and 93%, respectively, bars in the middle of Figure 2). By contrast, mock-transfected cells showed a significant alteration in Bcl-2 and Bax levels after induction of oxidative stress (50% and 336%, respectively, left side of Figure 2), as well as in the decrease of procaspase-3 levels, one of the final effectors of apoptosis (47% and 68%, for mock-transfected and CA VII-expressing cells, black bars in Figure 2). It is worth to note that, after treatment with SA, a stronger CA VII signal was observed probably due to a protective attempt of the cell to contrast oxidative stress injury[17].

The importance of cysteine residues was validated by performing the same set of experiments in cells transfected with a CA VII mutant (TM-CA VII), in which all the cysteines present in the amino acid sequence (Cys54, Cys178, Cys183, and Cys217) were replaced by serine residues. Noteworthy, it was observed that cells expressing the TM-CA VII were susceptible to oxidative stress, as the increase in Bax levels as well the decrease in Bcl-2 and procaspase-3 levels were similar to those reported for mock-transfected cells (267%, 49%, and 45%, respectively, right side of Figure 2). These findings indicated lack of protection from oxidative stress in the presence of the mutated protein. Since this mutated enzyme possessed the same catalytic activity as the native protein (see above), it was possible to conclude that the protective role of CA VII against oxidative stress was not related to the enzyme catalytic activity, but rather to the presence of very reactive cysteine residues, which could act as oxygen radical scavenger.

A great amount of data support the idea that *in vivo* oxidative stress and the accompanying ROS are genotoxic and contribute to the development of several human cancers including colorectal carcinoma[58–61]. Since the latter presents a reduced CA VII expression[55], it is tempting to speculate that the absence of the antioxidant action of CA VII could be responsible of a higher sensibility to the oxidative stress and consequently of the disease progression. Thus, the reported protective role of CA VII might suggest a novel tumor-suppressing function for this enzyme.

X-ray structural studies on CA III and CA VII

Several crystallographic structural studies have been reported on CA III[12,37,62,63], the first one being on the bovine isoform which was solved in 1993[12], showing that the enzyme three-dimensional structure presents the typical α-CA fold[13,64–72], characterized by a central 10-stranded β-sheet surrounded by several helices and additional β-strands (Figure 3). Structural data on the human isoform have been reported later, together with different site-specific variants aimed at identifying which residues were responsible for the low catalytic efficiency of CA III[62,63].

In particular, McKenna's group reported a kinetic and structural characterization of three hCA III mutants where an active site residue was substituted by a histidine one, namely mutants K64H, R67H, and K64H-R67N[63]. It is well known that the rate-limiting step of CO_2 hydration reaction, catalyzed by α-CAs, is the proton transfer reaction from the zinc-bound water molecule to the external medium, to regenerate the zinc-bound hydroxide which is the reactive species[1]. This reaction is assisted by the proton shuttle His64 in hCA II[73], the most catalytically active member of α-CA class[2]. Interestingly, the same position is occupied by a lysine in hCA III enzyme, thus suggesting that restoring a histidine in such position could greatly improve the CA III catalytic efficiency. Surprisingly, the kinetic data of K64H variant revealed only a small increase in the rate constant for proton transfer from proton donors to the zinc-bound hydroxide (k_B values were 3.0 and 20 ms^{-1} for native hCA III and K64H variant, respectively)[63]. Moreover His64 in K64H variant had a capacity for proton transfer that was only 2.5% that of His64 in hCA II. Comparison of the crystal structures of K64H hCA III mutant and hCA II suggested that the different ability in proton transfer could be related, at least in part, to the limited conformational mobility of His64 observed in the mutant compared to hCA II, where His64 adopts two different conformations (referred to as inward and outward)[73]. In agreement with this hypothesis, it was previously reported that the high

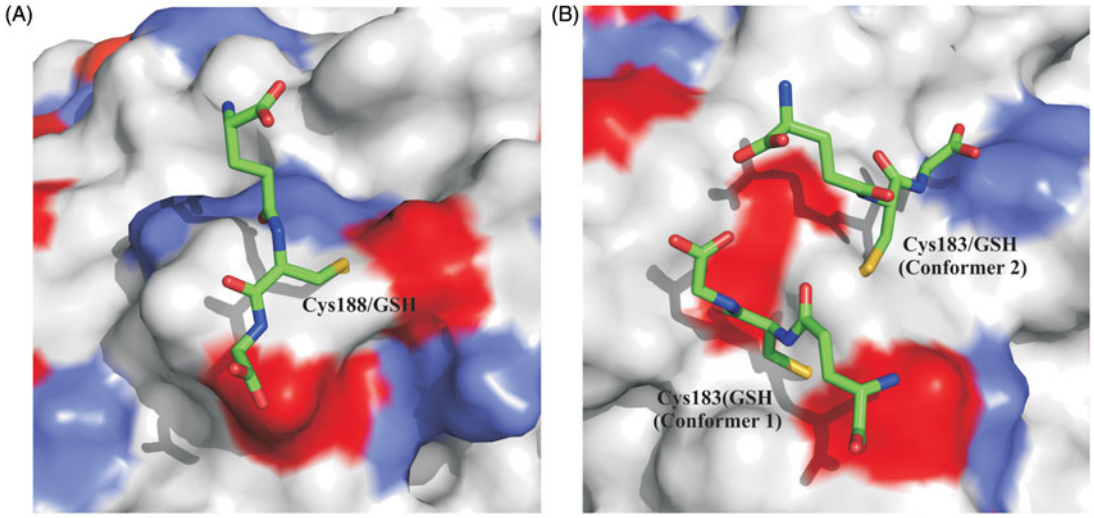


Figure 4. Schematic representation of (A) Cys188-GSH adducts and (B) Cys183-GSH. The two Cys183 conformers are indicated as 1 and 2. Surface representation of CA III is also reported showing the positive charged residues (Lys and Arg) colored light gray, while negative ones (Asp and Glu) colored black.

conformational mobility of His64 in hCA II would be an important feature for its ability in proton transfer[74].

A higher reaction rate for proton transfer was achieved with R67H and K64H-R67N mutants (k_B value of 81 and 100 ms^{-1} for R67H and K64H-R67N, respectively), indicating that other residues of the active site cavity can be involved into catalytic mechanism. Additional site-specific mutagenesis studies also showed the influence of amino acid replacement at position 198, suggesting that the hydrophobicity of Phe198 can affect the pK_a of the zinc-bound water lowering CA III catalytic efficiency[75].

The X-ray structure of CA III extracted from rat liver was also solved providing very interesting data for understanding the molecular determinants responsible for the above described high reactivity of Cys183 and Cys188[37]. These two residues are located on the molecular surface of the protein and were found S-glutathiolated in the crystal structure. The analysis of the structure revealed that the presence of disulfide linkages between cysteine residues and GSH molecule did not alter the overall structure of the protein, nor the conformation of residues located near to Cys183 and Cys188. This observation was in agreement with the experimental data that S-glutathiolation has no effect on the catalytic activity of the enzyme, as the GSH moieties are distant from enzyme active site[37]. Moreover, the analysis of electron density in correspondence of Cys-GSH adducts indicated conformational flexibility of the glutathionyl moieties, with the disulfide bridge involving Cys183 adopting two different conformations (conformation 1 and 2), and that involving Cys188 with only one orientation observed.

Previously reported data indicated Cys188 as the most reactive cysteine[76]; this was explained by the examination of S-glutathiolated CA III structure. In fact, Cys188 was located in an environment characterized by a lower negative charge that could justify its greater propensity to react with uncharged or charged species (Figure 4)[35,76,77]. On the contrary, Cys183 was located in a depression of the surface showing a greater negative charge making this residue less reactive. Further clarifications on the Cys188 high reactivity were obtained from site-specific mutagenesis studies[78]. Oxidation of a thiol group involves its ionization to the thiolate. As the pK_a of free cysteine is approximately 8.5, it was expected that, at physiological pH, cysteine was almost completely in its protonated form. However, within a protein 3D structure, the cysteine pK_a can be altered and, in particular, lowered by the

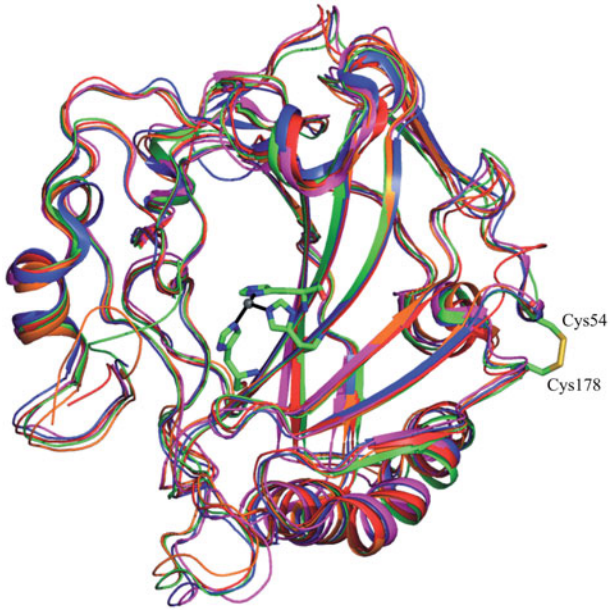


Figure 5. Structural superposition of all cytosolic α-CAs. The zinc ion coordination and intramolecular disulfide bridge of hCA VII are also depicted.

interaction with basic amino acids. Several charged residues were identified from CA III crystal structure that could affect the pK_a of Cys188 (i.e. Lys213, Arg189, Asp190, and Glu214, numbering referring to that used for the crystal structure of CA III from rat liver). In order to elucidate their role, site-specific mutants were produced and characterized showing that Lys213 was the major responsible for the lowering of the pK_a , whereas Arg189 seemed not to affect it. Notably, the acidic Asp190 and Glu214 reduced the reactivity of Cys188, thus suggesting that their conformational rearrangement could serve to modulate the CA III propensity to glutathiolation[78].

The crystal structure of hCA VII, solved in 2010 by our group[13], showed structural features similar to those previously reported for other cytosolic CAs (Figure 5)[12,64,65,71]. Indeed, the protein was a monomer characterized by a central 10-stranded anti-parallel β-sheet surrounded by additional β-strands and three α- and four

3_{10} helices. The hCA VII active site was located in a conical cavity about 15 Å wide and 15 Å deep, which extended from the surface of the protein to the center of the molecule. The catalytic zinc ion was located at the bottom of this cavity, coordinated by three histidine residues and the deprotonated nitrogen atom of sulfonamide moiety of acetazolamide molecule, which co-crystallized with the enzyme. The active site cavity was divided in two very different portions delimited by hydrophobic or hydrophilic amino acids. In particular, Val121, Leu198, Ala135, Leu141, Val143, Val207, and Phe131 delineated the hydrophobic region, while Asn62, His64, Gln67, Lys91, and Gln92 identified the hydrophilic one. This peculiar active site arrangement, already observed for other members of the α-CA family, was directly correlated to the catalytic mechanism of the enzyme. In particular, the hydrophobic region was supposed to be involved in the sequestration of the CO_2 substrate and its opportune orientation for nucleophilic attack by the zinc-bound hydroxide[79], whereas the hydrophilic region was supposed to be involved in the formation of a well ordered hydrogen-bonded solvent network, which assists the proton transfer reaction[74,80,81].

An intramolecular disulfide bond was observed in hCA VII structure between Cys54 and Cys178 (Figure 5). However, the observation that these two cysteines are not conserved within the α-CA family[82] and that disulfide bonds are extremely rare in cytosolic proteins[83], suggested that this disulfide bond could be a result of the oxidizing conditions that arise during protein handling.

No structural data were available on the two cysteines (Cys183 and Cys217) involved into S-glutathiolation *in vitro*[14], as a variant form, containing the substitution of these cysteines with serines, was used for the structural studies. However, the analysis of the structure of this variant showed that these residues were located on the protein surface and were completely accessible to the solvent. Molecular modeling studies, corroborated by site-specific mutagenesis, should be performed in order to clarify the structural determinants of the high chemical reactivity of these two sulfhydryl groups.

Conclusion

To respond to oxidative stress cells have developed antioxidant defense systems, including gluthathione, antioxidant vitamins, sulfhydryl groups, and antioxidant enzymes. CA III and CA VII have been proposed to be involved in these antioxidant defense systems, acting, through their reactive cysteines, as scavenger enzymes of reactive species in cells where oxidative damage occurs. Biochemical and structural studies of CA III allowed identification of the molecular determinants responsible for the high reactivity of cysteine residues involved in the proposed scavenger function and confirmed the critical role of the charged residues to modulate the reactivity of such residues.

On the other hand, fewer data are available for CA VII, which presents many similarities with CA III, starting from its propensity to undergo S-glutathiolation *in vitro*, ending with its ability to protect cells from oxidative insults. Interestingly, available data clearly indicate that the protective role of CA VII is not related to the enzyme catalytic activity, since a CA VII variant, in which the cysteine residues were replaced by serines, showed the same catalytic activity of the wild-type protein, but was not able to protect cells from oxidative stress injury. Further studies are needed to investigate the influence of the residues surrounding the reactive cysteines in order to highlight the molecular mechanisms regulating this phenomenon.

Disclosure statement

The authors report no declarations of interest.

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