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The proteoglycan region of the tumor-associated carbonic anhydrase isoform IX acts as anintrinsic buffer optimizing CO_2 hydration at acidic pH values characteristic of solid tumors

Alessio Innocenti [a], Silvia Pastorekova [b], Jaromir Pastorek [b], Andrea Scozzafava [a], Giuseppina De Simone [c], Claudiu T. Supuran [a,*]

[a] Universita degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della, Lastruccia 3, I-50019, Sesto Fiorentino (Firenze), Italy
[b] Centre of Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Dubravska, Cesta 9, 84505 Bratislava, Slovak Republic
[c] Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, 80134 Napoli, Italy

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ABSTRACT

The enzymatic activities of carbonic anhydrase (CA, EC 4.2.1.1) isozymes CA I, II, IX (catalytic domain (cdCA IX) and catalytic domain plus proteoglycan, flCA IX), XII and XIV were investigated as a function of pH for the CO_2 hydration to bicarbonate and a proton. The cytosolic isoforms CA I and II as well as the catalytic domain of CA IX, together with the transmembrane isoforms CA XII and XIV showed sigmoid pH dependencies of k_{cat}/K_M, with a pK_a of 6.90–7.10, showing thus optimal catalytic efficiency around pH 7. The full length CA IX had a similar shape of the pH dependency curve but with a pK_a of 6.49, having thus maximal catalytic activity at pH values around 6.5, typical of hypoxic solid tumors in which CA IX is overexpressed. The proteoglycan domain of CA IX (present only in this transmembrane isoform) may thus act as an intrinsic buffer promoting efficient CO_2 hydration at acidic pH values found in hypoxic tumors.

Carbonic anhydrases (CAs, EC 4.2.1.1) are among the most efficient catalysts know innature.[1] Some of the 16 isozymes characterized so far in mammals (for example the human isozymes hCA II (cytosolic) and hCA IX (transmembrane with an extracellular active site),[1–3] catalyze CO_2 hydration to bicarbonate and a proton (the physiological reaction in which CAs participate) with turnover numbers close to the limits of the diffusion controlled processes, that is, of around 10^8 $M^{-1} \times s^{-1}$ (see Table 1).[1–7] Many of the CA isozymes found in mammals play crucial roles in various physiological processes of such organisms, such as CO_2/HCO_3^- transport between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion, biosynthetic reactions (gluconeogenesis, lipogenesis and ureagenesis), bone resorption and tumorigenicity.[1,4–10] As a consequence, many CA isoforms are drug targets of interest for the design of various pharmacological agents, such as diuretics, antiglaucoma, antiobesity, antiepileptic or antitumor drugs/diagnostic agents.[1]

Among all these applications, the potential use of CA inhibitors (CAIs) for the management of solid hypoxic tumors which overexpress two isoforms, CA IX and XII, recently became a hot topic, with many investigations aiming to develop potent and isoform-selective compounds targeting these two isozymes.[1,6–11] Indeed, hypoxia, through the hypoxia inducible factor (HIF) cascade, leads to a strong overexpression of CA IX/XII in many tumors.[8] The overall consequence of these phenomena is a pH imbalance, with most hypoxic tumors having acidic extracellular pH values as low as 6.2–6.5, in contrast to normal tissue which have characteristic pH values around 7.4.[8–10] Constitutive expression of human CA IX (hCA IX) was recently shown to decrease extracellular pH (pHe) in hypoxic Madin-Darby canine kidney (MDCK) epithelial cells (which normally do not express CA IX) by our group.[8] Potent sulfonamide inhibitors of CA IX were then shown to bind specifically only to hypoxic cells expressing CA IX and to reduce the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions.[8] This finding supported the view that tumor cells decrease their pHe both by production of lactic acid (due to the high glycolysis rates), and by CO_2 hydration catalyzed by the tumor-associated CA IX/XII, possessing extracellular catalytic domains.[8–10] A low pHe has been associated with malignant transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of the

* Corresponding author. Tel.: +39 055 4573005; fax: +39 055 4573385.
E-mail address: claudiu.supuran@unifi.it (C.T. Supuran).

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Table 1
Kinetics parameters for the CO_2 hydration reaction catalyzed by the cytosolic mammalian CA isozymes I, II, and the transmembrane ones CA IX (catalytic domain, cd, and full length, fl), CA XII and CA XIV at 25 °C and pH 7.4, and their inhibition with acetazolamide (5-acetazmido-1,3,4-thiadiazole-2-sulfonamide)

Isozyme[a]	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1} \times s^{-1}$)	K_I (acetazolamide) (nM)	Ref.	pK_a
hCA I	2.0×10^5	4.0	5.0×10^7	250	1	6.90
hCA II	1.4×10^6	9.3	1.5×10^8	12	1	7.10
hCA IX (cd)	3.8×10^5	6.9	5.5×10^7	9	7	7.01
hCA IX (fl)	1.1×10^6	7.5	1.5×10^8	16	7	6.49
hCA XII	4.2×10^5	12.0	3.5×10^7	5.7	7	6.90
hCA XIV	3.1×10^5	14.2	3.5×10^7	41	7	6.92

The pK_a for the titration curve describing the pH dependency of k_{cat}/K_M, determined in this work for all these CA isoforms, is also shown in the last column of the table.
[a] Recombinant proteins obtained in *E. coli* as described in Refs. 4,6,8.

expression of cell growth factors and protease activation.[10] Thus, the proof-of-concept study mentioned above[8] showing the role of CA IX in tumor acidification, also established that sulfonamide inhibitors bind only to hypoxic cells overexpressing CA IX (and not to their normal counterparts) and that CA IX inhibition with such compounds reverts the tumor acidification processes, restaurating a more physiologically normal pHe. Dubois et al.[8c] also proved recently the possibility to use sulfonamide CA IX-specific inhibitors for imaging purposes in a xenograft tumor model in which CA IX is overexpressed. Recently, Pouyssegur's group[9] showed that in hypoxic LS174Tr tumor cells expressing either CA IX or both CA IX and XII, in response to a CO_2 load, both enzymes contribute to extracellular acidification and to maintaining a more alkaline resting intracellular pH (pHi), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0–6.8) values, and low bicarbonate medium. Thus, hypoxia-induced CA IX and CA XII are major tumor prosurvival pH regulating enzymes, and their combined inhibition targeting has great potential for the design of anticancer drugs with a novel mechanism of action.[9] However, the in vivo proof-of-concept that sulfonamide CA IX inhibitors may indeed show antitumor effects, has been only very recently published by Neri's group,[11] by using membrane-impermeant sulfonamides. This group demonstrated the strong tumor growth retardation in mice with xenografts of a renal clear cell carcinoma line (SK-RC-52) when the animals were treated for one month with some of these CAIs. Such preliminary data of our,[8,10] Pouyssegur's[9] and Neri's[11] groups show indeed the great promise of tumor CA IX/XII inhibition with sulfonamides or related agents for the development of alternative anticancer drugs. Furthermore such compounds may be also used for the imaging of hypoxic tumors.[8c]

A distinctive feature of the tumor-associated isoform CA IX as the only among all known CAs, is the presence of a proteoglycan (PG)—like region of around 60 amino acid, situated extracellularly at the very amino-terminal region of the protein, in front of the CA catalytic domain of the protein, as shown schematically in Figure 1.

The PG region of CA IX was shown to be involved in cell–cell adhesion and intercellular communication, thus suggesting that it may be the CA IX moiety most probably involved in tumor invasion processes.[12] It has been shown that hypoxia induces tumor invasion via decreased E-cadherin mediated cell–cell adhesion, and CA IX participates in this process, by reducing this adhesion through interactions with β-catenin.[12] However, the precise role of the PG domain for the biochemical function of CA IX is largely unknown at this moment, although it is clear that this region has an important role both for the catalytic activity (as the flCA IX has a much higher catalytic efficiency compared to cdCA IX, see also Table 1)[7] and thus for the tumor acidification processes[8] as well as for the adhesion and tumor invasion processes.[12]

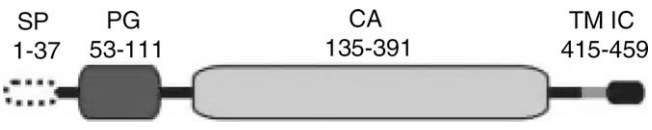

Figure 1. Domain organization of the CA IX protein: SP = signal peptide; PG = proteoglycan-like domain, CA = catalytic domain; TM = transmembrane segment; IC = intracellular, cytoslic tail.

In this Letter we report the pH dependency of the catalytic activity for the physiologic reaction for two cytosolic CA isozymes (CA I and II) and three transmembrane mammalian isoforms, CA IX (cdCA IX and flCA IX), XII and XIV, showing that important differences are observed between all these isozymes and flCA IX (which has the PG domain). Our data thus suggest that the PG domain is an evolutionarily evolved feature of CA IX enabling this protein to act as an excellent catalyst for CO_2 hydration to bicarbonate and protons in acidic media, characteristic of hypoxic tumors. Indeed, catalysis of CO_2 hydration to bicarbonate occurs via deprotonation of a water molecule bound to the Zn(II) ion of the enzyme active site, with generation of a zinc hydroxide species.[1] This process is highly disadvantaged at acidic pH values, and it is thus difficult to explain the very high catalytic activity of flCA IX, which is similar to that of the cytosolic CA II, with a k_{cat}/K_M of 1.5×10^8 M^{-1} s^{-1} (Table 1).[7]

We measured the pH dependency of hCA I, II, IX (cd and fl enzymes), XII and XIV for the physiologic raction, CO_2 hydration to bicarbonate and a proton, by a stopped flow assay, in the pH range of 5.8–7.8.[13] The pH profiles of these enzymes are shown in Figures 2–4. It may be observed that for all isoforms, a sigmoidal titration curve describes the dependency of k_{cat}/K_M on pH, with the pK_a of this curve showing the optimal catalytic activity as well as the value at which the zinc-bound water molecule from the enzyme active site is deprotonated (Figs. 2–4 and Table 1). Interestingly, all CAs except flCA IX have a pK_a of the titration curve in the range

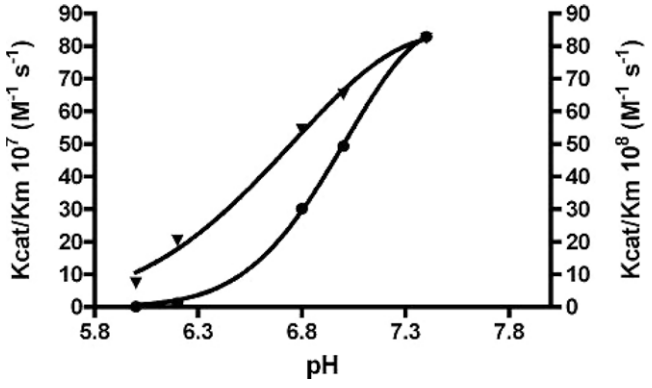

Figure 2. Variation of k_{cat}/K_M for hCA I (▼) and hCA II (●) versus pH, for the CO_2 hydration reaction at 25 °C. A pK_a of 6.90 ± 0.12 (n = 3) was obtained for the hCA I curve and of 7.10 ± 0.07 (n = 3) for hCA II. Left-hand y-axis referes to hCA I, and right-hand one to hCA II (the two enzymes show one order of magnitude catalytic activity differences).

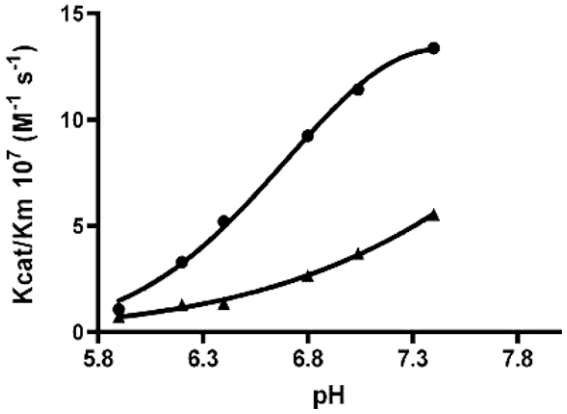

Figure 3. Variation of k_{cat}/K_M for hCA IX recombinant forms (flCA IX ·, and cdCA IX ▲) versus pH, for the CO_2 hydration reaction at 25 °C. The titration curve of the flCA IX has a pK_a of 6.49 ± 0.06 (n = 3); that of cdCA IX a pK_a of 7.01 ± 0.08 (n = 3).

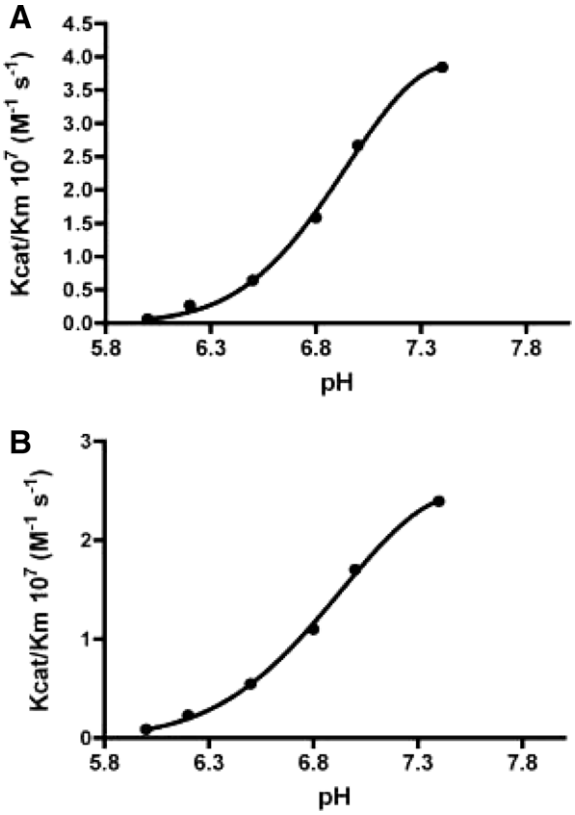

Figure 4. (A) Variation of k_{cat}/K_M for hCA XII recombinant isoform versus pH, for the CO_2 hydration reaction at 25 °C. The titration curve has a pK_a of 6.90 ± 0.10 (n = 3). (B) Variation of k_{cat}/K_M for hCA XIV recombinant isoform versus pH, for the CO_2 hydration reaction at 25 °C. The titration curve has a pK_a of 6.92 ± 0.06 (n = 3).

of 6.90–7.10, proving that the zinc-bound water is deprotonated at a pH value of around 7, and that the optimal catalytic activity of these enzymes (for the physiologic reaction) is around the neutral pH. Indeed, the amino acid residues and the 3-dimensional structure of the active site in all α-CAs are very similar,[1,2] which may explain these results. However, the CA IX protein possessing the unique PG domain (flCA IX), showed a pK_a for the titration curve of 6.49, that is, 0.5 pH units lower than the catalytic domain-only enzyme (cdCA IX, Fig. 3). This is a very interesting result which clearly shows that the PG domain enables flCA IX to act as a better catalyst for CO_2 hydration at more acidic pH values, allowing for a microenvironment buffering effect, with an optimal activity at pH 6.49. Interestingly this slightly acidic pH value is within a typical pH range of solid and hypoxic tumors, where CA IX is generally expressed.[7–10]

Another feature of the PG domain which could not be explained up to now is that this domain is rich in acidic amino acid residues (8 Asp and 18 Glu residues in a total of 58 residues are present in its sequence).[14] The presence of 26 COOH side chains from the aspartic acid and glutamic acid residues within the PG domain enables this region to act in a buffer-like manner, as it is well known that the carboxylate/carboxylic acid conjugate base are excellent biological buffers. Thus, we propose here that the PG domain of the CA IX molecule may act as an intrinsic buffer, facilitating the high catalytic turnover of this enzyme at acidic pH values (around 6.5) typical of hypoxic solid tumors where CA IX is overexpressed. The fact that the other two extracellular CA isozymes, CA XII and XIV, do not have this PG domain, is also reflected in their pH dependency similar to that of the cytosolic isoforms CA I and II. The same is true for the CA IX construct possessing only the catalytic domain, which had a pH dependency for its CO_2 hydration activity similar to those of CA I, II, XII and XIV. It should be noted that the overall buffering effect at the tumor site, where salts and many other proteins may contribute to buffering, may be independent of the PG domain of CA IX. However, as intramolecular proton transfer reactions are much more effective than intermolecular ones,[1,5] it cannot be excluded that the microenvironment buffering effect mentioned above is the main player in regulating the CA IX catalytic activity. Surely, this topic deserves further investigations. As the PG domain of CA IX is glycolsylated in mammalian cells,[7] it must be mentioned that its in vivo functioning may be affected by the glycosylation, another topic which warrants further studies.

In conclusion, we report here that the flCA IX, unlike the cytosolic (CA I, II) or transmembrane (CA XII and XIV) isoforms, or the catalytic domain CA IX, has an optimal catalytic activity for CO_2 hydration to bicarbonate and a proton in acidic pH ranges (pK_a of 6.5). All other isoforms investigated so far (together with cdCA IX) have this parameter in the pH range of 6.90–7.10. flCA IX has thus evolved to act as an excellent catalyst for CO_2 hydration at acidic pH values, typical of hypoxic solid tumors, through its unique PG domain which acts as an intrinsic buffer.

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