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The Structure of Carbonic Anhydrase IX Is Adapted for Low-pH Catalysis

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Abstract

Human carbonic anhydrase IX (hCA IX) expression in many cancers is associated with hypoxic tumors and poor patient outcome. Inhibitors of hCA IX have been used as anticancer agents with some entering Phase I clinical trials. hCA IX is transmembrane protein whose catalytic domain faces the extracellular tumor milieu, which is typically associated with an acidic microenvironment. Here, we show that the catalytic domain of hCA IX (hCA IX-c) exhibits the necessary biochemical and biophysical properties that allow for low pH stability and activity. Furthermore, the unfolding process of hCA IX-c appears to be reversible, and its catalytic efficiency is thought to be correlated directly with its stability between pH 3.0 and 8.0 but not above pH 8.0. To rationalize this, we determined the X-ray crystal structure of hCA IX-c to 1.6 Å resolution. Insights from this study suggest an understanding of hCA IX-c stability and activity in low-pH tumor microenvironments and may be applicable to determining pH-related effects on enzymes.

Graphical abstract

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Author Contributions

B.P.M. performed and analyzed the experiments, designed the hCA IX-c, and wrote the paper. L.S., J.M.D., C.O., A.B., C.L.L., C.T., M.Y.M., and J.J.K. performed and analyzed the experiments. S.C.F. and R.M. designed the study and reviewed the paper. All authors discussed the results and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

Supporting Information

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Figures pertaining to sequence alignments used for hCA IX-c construct design, SDS-PAGE gel and hCA IX-c crystals, hCA IX-c protonogram and pH profile of CO₂ hydration, surface charge comparisons among hCA IX-c, hCA II, and hCA XII, DSC thermograms, extended structural observations, such as a glycerol binding site, from the hCA IX-c crystal structure, a table of calculated T_M values and associated errors from DSF experiments, and a table comparing active site residues of hCA II, hCA IX, and hCA XII (PDF)

Hypoxia is a condition commonly seen in primary and metastatic tumors where a deprivation of oxygen is caused by rapid proliferation and/or abnormal surrounding vasculature.¹⁻³ This environment results in a shift in cancer cell metabolism that is commonly described as the Warburg effect, where mitochondrial oxidative phosphorylation is superseded by anaerobic glycolysis.^{1,4,5} This switch to the glycolytic phenotype results in the production and export of lactic acid, causing the reduction of the extracellular pH (pHe) from ~7.4 to as low as 6.0, which is unfavorable for cell viability.⁶ To circumvent this continuous decrease in pH, cancer cells upregulate the expression of human carbonic anhydrase IX (hCA IX) that contributes to the pH equilibrium by the rapid hydration/dehydration of $\text{CO}_2/\text{HCO}_3^-$. However, for hCA IX to be active, it must be functionally stable and active at low pH. Here it is postulated that hCA IX is ideally suited to fulfill this need for cancer cell survival. As such, we provide insights into its biophysical, structural, and biochemical characteristics that permit the enzyme to maintain stability and activity under acidic conditions for pH regulation. Results from this study may be directly applicable to understanding pH-related effects on enzyme stability in acidic microenvironments.

Like all active hCA isoforms, hCA IX is a zinc-metalloenzyme. hCA IX is homodimeric, with each protomeric unit comprised of four distinct domains: an N-terminal proteoglycan-like domain (PG), an extracellular catalytic domain (hCA IX-c), a single-transmembrane anchor, and a C-terminal intracellular tail.^{7,8} To date, only the hCA IX-c has been structurally characterized, and limited information regarding the function of the PG domain exists. However, it has been proposed that the PG domain is important for the enzyme's stability and activity in low-pH environments.^{7,9}

Structural alignments between hCA IX-c and the other hCA isoforms display a high degree of structural and sequence conservation between the catalytic sites with amino acid variations occurring primarily on the surface.^{10,11} Accordingly, hCA IX follows the same general mechanism of catalysis, the reversible interconversion of HCO_3^- and H^+ to CO_2 .^{12,13} The reaction is a simple zinc hydroxide “ping-pong” mechanism composed of two steps (eqs 1 and 2, where E is the enzyme and BH^+ a proton donor in the bulk solvent). In tumor microenvironments, CA-mediated dehydration of HCO_3^- is considerably more important for pH regulation; therefore, the general CA mechanism is discussed as such.¹³

The first step in the dehydration direction (eq 1) is the formation of the metal-bound water ($\text{EZn}^{2+} - \text{H}_2\text{O}$) from proton donors in bulk solvent (BH^+) via sequestration of a proton through an ordered water network and a residue acting as a weak base, which is typically a His at the entrance of the active site. In the second step, the zinc-bound water molecule is displaced by a molecule of HCO_3^- to form $\text{EZn}^{2+} - \text{HCO}_3^-$ (eq 2).^{14,15} The $\text{EZn}^{2+} - \text{HCO}_3^-$ can then be further decarboxylated to form $\text{EZn}^{2+} - \text{OH}^-$ and is then poised for another cycle of catalysis.^{12,14}

The expression profile of hCA IX has prompted its utilization as a prognostic marker for several aggressive cancers, including breast, liver, lung, brain, and prostate.⁸ More importantly, hCA IX activity has been directly correlated with tumor cell proliferation, migration, growth, survival, and resistance to chemo- and radiotherapies.^{16,17} Therefore, hCA IX has been termed a “cancer-associated” CA and has been identified as a drug target for a wide range of cancers.¹⁸ In tumor microenvironments, disruption of hCA IX activity via inhibitors has shown favorable therapeutic responses in aggressive cancers.^{16,17,19,20} These observations have prompted the recent advancement of compound SLC-0111 to Phase 1 clinical trials (see clinicaltrials.gov; NCT0221585).^{21,22}

Most hCAs are not stable below pH 5.0 and show large reductions in their catalytic efficiency when approaching acidic conditions.¹³ Interestingly, this is not the case for hCA IX, which remains catalytically active and stable in low-pH environments. In this study, we examine the catalytic activity and stability of the catalytic domain of hCA IX and show that it maintains its fold as low as pH 2.0 and remains active at pH 3.0 in the absence of its PG domain. Further, we provide evidence to suggest that the unfolding of the catalytic domain by pH is reversible. To rationalize these observations, we utilize a high-resolution crystal structure of an engineered form of the hCA IX-c. Insights from this work will contribute to our understanding of CA stability and activity at low pH and may be further applied to a broader scope of understanding pH-related effects on enzymes in acidic microenvironments.

EXPERIMENTAL PROCEDURES

hCA IX-c Design and Molecular Cloning

Previously, expressing high levels of hCA IX that can readily produce well-ordered crystals has been challenging. Therefore, to overcome these challenges, we have designed a recombinant form of the catalytic domain of hCA IX (hCA IX-c) that is easily expressed in *Escherichia coli* and crystallized, without the use of an inhibitor to stabilize the enzyme. To design the hCA IX-c, we utilized the following strategies: (1) eliminating the intermolecular disulfide bridge (dimerization site) at Cys174 (full-length sequence containing signal peptide and transmembrane domains) to limit aggregation, (2) reducing hydrophobicity of the hCA IX surface, and (3) exploiting properties of the easily expressed, purified, and crystallized

hCA II at structurally conserved locations. The following substitutions were implemented: C174S (removal of the intermolecular disulfide bridge), L180S and M360S (reduction of surface hydrophobicity), and A210K, A258K, and F259Y (to mimic the hCA II surface) (Figure S1). These positions correspond to hCA II residues 28, 47, 227, 77, 126, and 127, respectively. For the purpose of comparison, hCA II numbering will be used unless otherwise stated, and parentheses proceeding residue numbers will signify full-length hCA IX numbering.

The gene containing the hCA IX-c was synthesized in a pUC57-cloning vector and purchased from GenScript. The hCA IX-c coding region was cloned into a pET15b vector suitable for *E. coli* expression. Site-directed mutagenesis was used to introduce an NcoI cut site into the pUC57 vector using Quick Change Mutagenesis (Stratagene) with the following primers: 5'-CAGTCCATGGGCGACCCGCAAGAA-3' and 5'-TTCTTGCGGGTCGCCCATCCATCCATGGACTG-3'. An NcoI cut site was chosen because it is positioned downstream from the poly-His tag of the pET15b vector, allowing expression of an "untagged" hCA IX-c. The polymerase chain reaction (PCR) products were transformed into *E. coli* DH5 α competent cells (New England BioLabs) for DNA amplification. Plasmid purification was performed using a QIAprep Spin Miniprep kit (Qiagen). Concentrations of purified plasmid were determined using a BioTek Epoch Multi-Volume Spectrophotometer System. Single- and double-restriction enzyme digests were performed to screen for incorporation of NcoI and BamHI sites. After confirmation of PCR products, a second restriction digest was performed (1) to isolate the hCA IX-c gene and (2) to linearize the pET15b expression vector for use in ligation reactions. The restriction digests for the pET15b plasmid followed a phosphatase treatment with recombinant shrimp alkaline phosphatase (rSAP) to reduce the chance of vector religation. The ligation reaction was performed with a 1:3 vector:insert concentration ratio according to the New England BioLabs protocol for a 20 μ L reaction mixture. Reaction mixtures were incubated at room temperature for ~16 h. T4 DNA ligase (New England BioLabs) used in the reaction was inactivated through heat shock at 65 °C for 10 min following the reaction. Transformation and subsequent plating of ligation products were then performed as described above. Colonies containing the pET15b-hCA IX-c gene were purified and screened by restriction digestion. Successful ligation of the hCA IX-c coding region into the pET15b vector was confirmed using Sanger sequencing performed at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR).

Protein Expression and Purification

The hCA IX-c was expressed in recombinant BL21(DE3) competent cells as previously described.²³ Briefly, *E. coli* cells that contain the plasmid encoding hCA IX-c were grown in 2 L of Luria broth, supplemented with 100 μ g/mL ampicillin, to an OD₆₀₀ between 0.6 and 1.0, at which point hCA IX-c expression was induced by the addition of isopropyl β -D-1-thiogalactopyrano-side for ~4 h at 37 °C in the presence of 1 μ M zinc sulfate. Cells containing hCA IX-c were harvested, resuspended using a glass homogenizer, and enzymatically lysed overnight at 4 °C. Expressed hCA IX-c was purified by a simple two-step process that involves (1) affinity chromatography followed by (2) size-exclusion chromatography. Affinity separation of hCA IX-c followed the same protocol that has been

utilized for hCA II.²³ A gravity-fed column containing agarose resin coupled to the inhibitor *p*-(aminomethyl)benzenesulfonamide [*p*-AMBS (Sigma)] was used for affinity purification followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of eluents to estimate hCA IX-c purity. SDS–PAGE from affinity chromatography displayed two distinct bands that migrated at ~100 and 33 kDa (Figure S2A). The higher-MW band was not identified; the band that migrated at ~33 kDa was determined to be the monomeric hCA IX-c through mass spectroscopic analysis (data not shown). Separation of the confirmed monomer of hCA IX-c was performed using size-exclusion chromatography on an Äkta Pure 150 Fast Protein Liquid Chromatography (FPLC) purification system (GE healthcare), equipped with a prepacked Superdex 75 10/300 GL gel filtration column (exclusion range of 3–70 kDa; GE Healthcare Biosciences AB, Uppsala, Sweden). The column was equilibrated with 50 mM Tris-HCl (pH 7.8), 100 mM NaCl buffer. The protein was eluted at a flow rate of 0.5 mL/min, yielding 1 mL fractions. Protein fractions were detected by absorbance at 280 nm, and data acquisition and processing were performed using the *FPLC UNICORN* software. The final protein concentration was estimated to be 12 mg/mL, as determined by UV/vis spectroscopy at 280 nm using an extinction coefficient of 36565 M⁻¹ cm⁻¹ (calculated from the amino acid sequence using *ExPASy* software). The hCA II used for this study was expressed and purified as previously described by Mahon et al.²⁴ to a final concentration of 25 mg/mL.

Protonography

Protonography was used to screen for hCA IX-c activity. Protonography is a newly developed technique used for qualitatively screening CA activity via monitoring proton production in an SDS gel.²⁵ In brief, the SDS gel, or protonogram, is stained with bromothymol blue (a widely used pH indicator) following electrophoresis. The dye appears blue in its deprotonated form, while its color changes to yellow as it is protonated. This is directly proportional to the number of protons produced in a solution. Therefore, in the case of CA activity, the production of H⁺ following CO₂ hydration causes a drop in pH (in areas of the gel containing CA) until the color transition point of the dye is reached (pH 6.8), resulting in gel bands containing active CA to change to a yellow color.²⁶ Here, wells of a 12% SDS gel were loaded with purified hCA II (positive control), BSA (negative control), and the hCA IX-c, mixed with Laemmli loading buffer without 2-mercaptoethanol (BME) and without boiling. The gel was run at 90 V for 30 min and then continued at 150 V until completion was reached. Following electrophoresis, the gel was soaked in 2.5% Triton X-100 for 1 h on a shaker at ~70 rpm. The gel was then soaked in 100 mM Tris (pH 8.2) containing 10% isopropanol for 10 min followed by incubation in 0.04% bromothymol blue in 100 mM Tris (pH 8.2) for 45 min. The gel was then rinsed and stored in ddH₂O. To initiate CA-mediated CO₂ hydration, dry ice was added to ddH₂O surrounding the gel, and color changes of CA active bands were monitored. Bands pertaining to active hCA II and hCA IX-c were visible after 45 s. To test the reannealing potential of the hCA IX-c, we repeated these experiments using Laemmli buffer containing 5 mM BME and 8 M urea and heated the samples at 100 °C for 15 min prior to running SDS–PAGE.

¹⁸O Mass Spectrometry

The kinetic rates of the hCA IX-c were obtained by measurement of the exhaustion of ¹⁸O from species of CO₂ at chemical equilibrium by way of membrane inlet mass spectrometry as described by Tu et al.^{27,28} In brief, the reaction proceeds via the continuous measure of various isotopic species of CO₂ diffusing across a dissolved gas-permeable membrane. The membrane is submerged in the reaction solution and connected by glass tubing to a mass spectrometer (Extrel EXM-200).²⁸ The catalyzed exchange and uncatalyzed exchange of ¹⁸O between CO₂ and water at chemical equilibrium were measured in an unbuffered solution at a total substrate concentration of 25 mM bicarbonate. The reaction solution was maintained at 25 °C, and the ionic strength of the solution was normalized at 0.2 M by adding Na₂SO₄. The catalytic mechanism of isotope-labeled substrates is described as follows: (1) the dehydration of ¹⁸O-labeled HCO₃⁻ (eq 3) and (2) the protonation of the zinc-bound ¹⁸O-labeled hydroxide, forming H₂¹⁸O, which is then released into solution (eq 4).

The catalytic rate for the interconversion of CO₂ and HCO₃⁻ at chemical equilibrium is defined as R_1 (eq 5). The rate constant for the maximal interconversion of CO₂ to HCO₃⁻ is defined as $k_{\text{cat}}^{\text{ex}}$. $K_{\text{eff}}^{\text{CO}_2}$ is the effective binding constant for binding of either CO₂ or HCO₃⁻ to CA. Therefore, the ratio $k_{\text{cat}}^{\text{ex}}/K_{\text{eff}}^{\text{CO}_2}$ is equivalent to the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of hydration under steady-state conditions.²⁸

$$\frac{R_1}{[\text{E}]} = \frac{k_{\text{cat}}^{\text{ex}} [\text{CO}_2]}{k_{\text{eff}}^{\text{CO}_2} [\text{CO}_2]} \quad (5)$$

The rate of proton transfer is obtained by measuring the diffusion of ¹⁸O-labeled water from the enzyme to the solvent ($R_{\text{H}_2\text{O}}$). $R_{\text{H}_2\text{O}}$ is dependent on the donation of protons to the ¹⁸O-labeled zinc-bound hydroxide by the proton-shuttling residue (His64) as the second independent step of CA catalysis (eq 2) and reiterated in terms of labeled species in eq 4.²⁷ The rate constant for proton transfer to the zinc-bound hydroxide is defined in eq 6 by k_{B} , where $(K_{\text{a}})_{\text{His64}}$ and $(K_{\text{a}})_{\text{ZnH}_2\text{O}}$ are the ionization constants of the proton donor (represented by His64 from hCA II) and the zinc-bound water molecule, respectively.

$$\frac{R_{\text{H}_2\text{O}}}{[\text{E}]} = \frac{k_{\text{B}}}{\left[1 + \frac{(K_{\text{a}})_{\text{His64}}}{[\text{H}^+]}\right] \left[1 + \frac{[\text{H}^+]}{(K_{\text{a}})_{\text{ZnH}_2\text{O}}}\right]} \quad (6)$$

Equation 5 and 6 were fitted to the data using a nonlinear least-squares methods in Enzfitter (Biosoft) and also in a log scale and fitted using nonlinear least-squares methods in GraphPad. Results are listed in Figure S3B and Table 1.

Differential Scanning Fluorimetry

Differential scanning fluorimetry (DSF) was used to assay hCA IX-c conformational stability in terms of melting temperature (T_{M}) against a pH range of 3.0–9.5. Samples of purified hCA II and hCA IX-c at a concentration of 0.25 mg/mL were buffer exchanged into a citrate-phosphate buffer adjusted to the desired pH. A citrate-phosphate buffer system was used for varying the pH while keeping the ionic strength constant.²⁹ The pH values selected for these experiments were 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.5. Prior to data collection, samples were incubated with 0.01% Sypro-Orange dye (no. S6651, Invitrogen Inc.) for ~30 min on ice. Melting curve assays were conducted in a quantitative PCR (qPCR) instrument (RG-3000, Corbett Research) with the temperature increasing from 30 to 99 °C, increasing at a rate of 0.1 °C/6 s. Solutions containing only buffer were also assayed in the same manner to use for background subtraction during data processing. The T_{M} was defined as the maximal value of the first derivative ($\text{dRFU}/\text{d}T$; change in fluorescence/change in temperature) of the signal that is produced in terms of relative fluorescent units (RFU). Each experiment was performed in triplicate. Results are summarized in Figure 1A and Table S2.

Differential Scanning Calorimetry

DSC experiments were performed to confirm the thermal stability and calculate the thermodynamic parameters of hCA IX-c and hCA II at various pH values. Experiments were performed using a MicroCal VP-DSC instrument (Malvern Instruments, Worcestershire, U.K.) with a cell volume of 250 μL for each of the sample and buffer cells. Samples of each enzyme (10–20 μM) were dialyzed against a citrate-phosphate buffer corresponding to pH values of 7.0, 4.0, 3.0, and 2.0 prior to data collection. The DSC thermograms were collected in a passive feedback mode from 25 to 90 °C with a 3 min prescan thermostat, a scan rate of 60 °C/h, and a 10 s filtering period between data points. The final dialysis buffer at each pH was added to both the sample and buffer cells for an initial run to be used for buffer subtraction during data processing. Analysis of thermographs was performed using the MicroCal VP-DSC Analysis software provided by Malvern Instruments. Curves were fitted using a non-two-state model to obtain calorimetric values with one transition point defined to obtain a single peak fit. T_{M} values for both enzymes were obtained from the midpoints of the DSC peaks, indicating a two-state transition. The calorimetric enthalpies of unfolding were calculated by integrating the area under the peaks in the thermograms after buffer subtraction.

The denaturation enthalpies (H_m^D) were calculated at a given temperature using the Kirchoff's law equations (eq 7).³⁰

$$\Delta H^\circ(T) = \Delta H_m^\circ + \Delta C_p(T - T_M) \quad (7)$$

The van't Hoff enthalpy (H^H) of unfolding was calculated assuming a two-state reversible model, such that the ideal narrow peak of unfolding would result in $H_m^D \approx H^H$.

Thermograms displaying buffer-subtracted data and a non-two-state model fit are given in Figure S3, and T_M and enthalpy values are summarized in Table 2. No baseline corrections were performed to preserve raw data quality in thermograms.

Circular Dichroism

Circular dichroism (CD) was utilized to identify any major shifts (unfolding) in secondary structure in hCA IX-c and hCA II as a function of pH. We utilized a citrate-phosphate buffer (similar to that used in the experiments described above) to generate the desired pH for CD experiments. Experiments were performed using an Aviv model 430 circular dichroism spectrometer using a cuvette with a cell path length of 0.1 cm and an incubation temperature of 25 °C. Optics were continuously purged with nitrogen gas during data collection such that oxygen concentrations were less than 7 ppm. Ten scans were performed for each hCA sample in the far-UV wavelength range of 260–190 nm at intervals of 1 nm. The resulting plots were averaged and smoothed, and the CD signal from buffer at each pH was subtracted prior to further processing and analysis. The mean residue molar ellipticity was calculated using a concentration of 0.5 mg/mL for all samples.

Crystallization, Data Collection, and Processing

Crystals of hCA IX-c were grown using the sitting-drop vapor diffusion method in a 96-well IntelliPlate (Hampton Research) as described by Díaz-Torres et al.³¹ Premade crystal screens were used to screen 96 different conditions (Crystal Screen HT, Hampton Research) following incubation at 17 °C. Several conditions produced crystals of hCA IX-c; however, the largest crystals (0.1 mm × 0.2 mm × 0.3 mm), which were used for data collection, formed in 0.1 M Tris-HCl (pH 8.5) and 8% (w/v) PEG 8000 (Figure S2B). Crystals were observed after 2 weeks. Prior to data collection, crystals were cryoprotected with 20% glycerol and stored in liquid nitrogen.

X-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS) on beamline F1 using a wavelength of 0.9177 Å. The data sets were collected using an ADSC Quantum 270 CCD detector at a crystal-to-detector distance of 150 mm with a 0.5° oscillation angle and an exposure time of 1 s per image. A total of 360 images were collected. The data were indexed, integrated, and scaled using *HKL2000*.³² Data were scaled to the orthorhombic $P2_12_12_1$ space group (unit cell parameters $a = 57.9$ Å, $b = 102.7$ Å, $c = 109.0$ Å, and $\alpha = \gamma = \beta = 90^\circ$) and to a high resolution of 1.60 Å with a completeness of 100.0%, and an R_{sym} of 10.0%. A summary of other statistics is provided in Table 3.

Structure Determination and Refinements

The structure of hCA IX-c was determined by molecular replacement (MR) using the previously determined hCA IX-AZM structure (PDB entry 3IAI) as a search model, with solvent, zinc, and AZM removed.⁷ MR solutions were calculated using *PHENIX*.^{33,34} The starting phases of the hCA IX-c model yielded a unique solution comprised of two molecules in the asymmetric unit. Refinement of structural solutions was also completed using *PHENIX*.³³ Each refinement was performed with 5% of the unique reflections selected at random and excluded to calculate R_{free} .³⁵ Manual refitting of the model between each refinement was done using *Coot*.³⁶ Superimpositions of the C $_{\alpha}$ atoms of chain A onto chains A and B (rmsd = 0.14 Å) indicated there were no major structural perturbations between the main chain of each monomer. Therefore, noncrystallographic symmetry (NCS) operators were employed for the remainder of the refinement. The final model of hCA IX-c was refined to an R_{cryst} of 16.8% and an R_{free} of 19.1% (Table 3). Model geometries and statistics were assessed by *PROCHECK*.³⁷ All figures were made using *PyMOL*.³⁸

RESULTS AND DISCUSSION

Expression, Purification, and Structure of hCA IX-c

Expression of the hCA IX-c *E. coli* cell culture produced ~10–20 mg of soluble, active enzyme per liter. Affinity purification schemes, similar to those used previously for hCA II,²⁴ provided specific separation of hCA IX-c from the cell lysate. Through size-exclusion chromatography, it was determined that hCA IX-c exists as a monomer in solution (data not shown). Results from protonography experiments indicated that hCA IX-c retained its catalytic activity after purification (Figure S3A). This was compared to hCA II and bovine serum albumin (BSA) as positive and negative controls, respectively. Treatment with denaturing conditions prior to protonography experiments showed that hCA IX-c still maintained activity most likely due to reannealing within the gel. This indicates that hCA IX, similar to hCA II, is able to refold readily in solution.²⁵ Thus, these data indicate that our new recombinant design and purification scheme provides a simple method for producing high yields of a stable and active form of the catalytic domain of hCA IX.

The overall structure of the hCA IX-c exhibited the typical α -CA fold, consisting of a 10-antiparallel β -strand core interlaced with surface loops and helical structures. An overlay of the hCA IX-c structure with the previously determined hCA IX-AZM complex structure (PDB entry 3IAI)⁷ shows minimal structural change with an rmsd of 0.28 Å (Figure 2A). In addition, overlays of the hCA IX-c structure with recently published structures of hCA IX (produced from a yeast expression system) show minimal differences (determined by an rmsd of 0.35 Å).³⁹ An overlay of hCA IX-c with hCA IX-AZM shows that no major structural perturbations were induced by the surface substitutions (Figure 2B). In addition, the presence of surface substitutions had apparent minimal effects on the overall charge of the enzyme as depicted by estimations of the theoretical isoelectric point (pI) values in comparison to that of the wild type. The theoretical pI values for hCA IX-c and the wild type were calculated on the basis of the amino acid sequence to be 5.4 and 5.1, respectively (*ExPASy*) (Table 4). These observations indicate that the catalytic domain of hCA IX has a

conserved fold regardless of the expression system and further suggests that the *E. coli*-produced hCA IX-c is suitable for “high-throughput” structural and biochemical studies.

Similar to those of other hCAs, the hCA IX-c active site cleft is divided into distinct hydrophobic and hydrophilic sides (Figure 3A). It has been proposed that the hydrophobic side allows entry of CO₂ into and exit of CO₂ from the active site while the hydrophilic pocket assists in HCO₃⁻ exit and entry and the stabilization of an ordered water network required for proton shuttling to and from the zinc-bound solvent.^{23,40} The residues that line the hydrophilic and hydrophobic sides contain a high degree of variability between the CA isoforms.^{10,24} As such, attempts have been made to exploit these differences for isoform selective inhibitor design.^{11,24} Inspection of the hCA IX-c active site architecture shows the expected distorted tetrahedral coordination of His94 (226), His96 (227), and His119 (251) with Zn²⁺ (Figure 3B). In the uninhibited hCA IX-c, the electron density is indicative of the presence of the catalytic Zn–OH/H₂O. Furthermore, the ordered water network, which is essential to the proton transfer step in the enzyme catalysis (eq 2), is well-defined (Figure 3B).^{41,42}

Catalytic Activity of hCA IX-c

Kinetic parameters of hCA IX-c were assayed using ¹⁸O mass spectroscopic analysis. The activity of hCA IX-c was compared to previously published kinetic data for hCA II,¹⁵ the catalytic domain of wild-type hCA IX (hCA IX_W),⁴³ hCA IX containing the PG domain (hCA IX_{PG}),⁹ and hCA XII,⁴⁴ a CA that is also expressed in tumors. The maximal $k_{\text{cat}}/K_{\text{M}}$ of CO₂ hydration for hCA IX-c was determined by measuring a pH profile, with data appearing as a bell-shaped curve fit to eq 5, and was determined to be $13 \pm 1 \mu\text{M}^{-1} \text{s}^{-1}$ (Table 1). This is ~10-fold lower than those of both hCA II ($120 \mu\text{M}^{-1} \text{s}^{-1}$) and hCA IX_{PG} ($150 \mu\text{M}^{-1} \text{s}^{-1}$), ~5-fold lower than that of hCA IX_W ($55 \mu\text{M}^{-1} \text{s}^{-1}$), and ~3-fold lower than that of hCA XII ($34 \mu\text{M}^{-1} \text{s}^{-1}$) (Table 1). It is unclear why there is a difference in catalytic efficiency between hCA IX-c and hCA IX_W, although it is possible that the oligomeric state of hCA IX_W (which is dimeric, because of the presence of the intermolecular disulfide bridge at Cys174) may in part cause an increase in catalytic efficiency via a pseudocooperativity mechanism, a phenomenon observed in other types of multimeric enzymes.⁴⁵ This is further supported by measurements of the catalytic efficiency of the hCA IX catalytic domain expressed in Sf9 cells (hCA IX_{SF9}), which also exists as a dimer and displays a catalytic efficiency similar to that of hCA IX_W.⁹ These data rule out a contribution to the catalytic efficiency by the N-linked glycosylation at Asn346 (full-length hCA IX numbering) in hCA IX_{SF9}. Similar conclusions were reached by Li et al. when studying full-length hCA IX in MDA-MB-231 human breast cancer cells.⁴⁶

Interestingly, the presence of the PG domain seems to have little effect on the $\text{p}K_{\text{a}}$ of the Zn-bound solvent when comparing hCA IX_{PG} to both hCA IX_W and hCA IX-c. This result contradicts previous data that suggest the PG domain acts as an “internal buffer” and affects the pH-dependent catalytic efficiency.⁷ It should be noted, however, that activity assays previously reported on hCA IX_{PG} utilized an esterase activity assay to determine catalytic parameters, which often shows differences in kinetic parameters compared to values determined via ¹⁸O mass spectrometry.⁴⁴ In the case of hCA XII, upon comparison of results

from both types of activity assays, there was an increase of ~3-fold in values for k_{cat}/K_M determined by esterase activity assays over ^{18}O mass spectrometry while measuring CO_2 hydration.⁴⁴ This suggests that the value previously reported for hCA IX_{PG} may be directly comparable to those for hCA IX_W if the experiments were to be repeated with ^{18}O mass spectrometry.

The kinetic profile of hCA IX-c shows that enzymatic activity, in terms of CO_2 hydration, appears to be retained under low-pH conditions (as low as pH 5.5) (Figure S3B). This phenomenon is not observed in hCA II or hCA XII, each of which shows a rapid decrease in activity at pH <6.0.^{44,47} At high pH, there is a large decrease in hCA IX-c activity (Figure S3B). The reason for this is unknown as there were no observable structural changes between hCA IX-c (crystals grown at pH 8.5 and presumably inactive) and the previously published 3IAI (crystals grown at pH 4.0). An overlay of active site residues of both hCA IX-c and 3IAI displays no major structural changes; however, there are noticeable yet subtle differences observed in Tyr7. It is unclear why this difference exists; however, it is possible this may be a result of the structure of hCA IX-c being determined at a higher pH, where it shows reduced activity, and thus a slight rearrangement of residues. This observation also differs from that seen in hCA II and XII where an increase in overall activity is observed as the pH is increased.^{44,47} As previously mentioned, the residues that contribute to the active site architecture in hCA IX, relative to II and XII, are highly conserved with only a few variations occurring in residues located toward the opening of the catalytic site (Table S1). It is possible that these few variations in active site residues in hCA IX-c contribute to the difference in pH-dependent activity under more basic conditions, perhaps through altering the pK_a of the active site microenvironment to favor catalysis at a lower pH. However, more work will be required to provide definitive conclusions.

Previously published data suggest that the presence of the PG domain is necessary for the enzyme to retain its activity at low pH.⁹ This conclusion is supported by the large number of acidic residues contained in the PG domain that cause an apparent overall reduction in the theoretical pI of hCA IX from 5.4 (5.1 for the wild type) to 4.5 (Table 4). In addition, previous activity assays show that hCA IX_{PG} retains its catalytic activity at pH <6.0.⁷ However, the overall fold and amino acid composition of the catalytic domain alone appear to be sufficient for the enzyme to retain activity at acidic pH values. This further correlates with previous experiments that assayed full-length hCA IX activity in MDA-MB-231 cells that determined the catalytic efficiency of hCA IX was comparable to that of hCA IX_W.⁴⁶

Rates of Proton Transfer

The $R_{\text{H}_2\text{O}}$ (eq 6), corresponding to the rate of release of H_2^{18}O from the active site, correlates directly to the rate of proton transfer. We determined this using a pH profile with data fit to eq 6 that gave a rate constant for intramolecular proton transfer (k_B) of $1.8 \pm 0.1 \mu\text{s}^{-1}$ for hCA IX-c (Table 1). This was only slightly higher compared to the k_B of hCA II and hCA IX_W, suggesting that hCA IX-c uses a similar proton transfer mechanism and shuttle at residue His64 (200). The proton transfer rates of hCA XII were also comparable but produced the lowest k_B value ($0.4 \mu\text{s}^{-1}$) (Table 1). As mentioned above, hCA IX_{PG}

activity was determined using an esterase activity assay, so rates of proton transfer have currently not been reported.

Structural comparison of the positions of the ordered waters and surrounding residues between hCA IX-c and hCA II (PDB entry 3KS3) shows they are in very similar positions. It should be noted that the previous structure of hCA IX, 3IAI, was crystallized with AZM, and therefore, the ordered water network could not be compared. The stabilization of these waters occurs via interactions formed between O γ and N α of Thr199 (332) with the Zn–OH/H₂O and deep water (DW; important for the escape of HCO₃[−] from the active site⁴⁷), respectively, O γ of Thr200 (333) interacting with W1, O η of Tyr7 (143) interacting with W3a, and N δ 2 of Asn62 (198) and O ϵ 1 of Gln67 (203) interacting with W3b (Figure 3B). Small deviations (~0.2 Å) in bonding distance between W2 and W3b and between Zn–OH/H₂O and “deep-water” (DW) in hCA IX-c were observed compared to hCA II (Figure 3C). This is most likely a result of the substitution of N67Q (N203Q; hCA II to hCA IX) (Figure 3C). The replacement of an Asn with a Gln presents a small perturbation of O ϵ 1 toward W3b, which may induce a slight shift in its position in hCA IX relative to hCA II and further influence the positioning of DW. This observation may explain the slight difference in the rate of proton transfer ($k_B = 1.0 \mu s^{-1}$) between hCA IX-c and hCA II, as both waters are predicted to influence the Zn–OH pK_a (Table 1).⁴⁷ However, both hCA IX-c and hCA II utilize the same proton shuttling residue in His64 (200), indicated by the similar Zn–H₂O pK_a (Table 1 and Figure 3C). Replacement of a Lys with a Glu at position 170 (from hCA II to hCA IX, respectively) provides another explanation for the small change in proton transfer rate. Previous studies have shown that residues at position 170 influence the pK_a of His64 by causing a significant reduction from 7.2 to 6.3.⁴⁸ The reduction in the His pK_a enhances its ability as a proton donor in the dehydration direction of CA-mediated catalysis and therefore influences rates of proton transfer (eq 2).⁴⁸ The pK_a of the Zn–H₂O during proton transfer in hCA XII is much higher than those of the other isoforms (pK_a = 8.0). This alone would suggest the use of an alternative proton shuttling residue other than His64 (200). However, previous experiments using site-directed mutagenesis confirmed that this is not the case.⁴⁴ Most likely, the higher Zn–H₂O pK_a is attributed to more basic residues in the active site (compared to hCA IX and II), causing a shift in pK_a.⁴⁴ Specifically, along with Lys170 and His64, hCA XII has a Lys at position 67 [Asn in hCA II and Gln in hCA IX (Table S2)]. The influence of these residues may cause an increase in the pK_a of His64 and, because of their proximity to the ordered water network, may also act as poor proton transfer residues; a similar mechanism is utilized by hCA III, whereby position 64 is occupied by a Lys.⁴⁹ This also explains the increase in the net positive charge of the hCA XII active site (theoretical pI of 8.6) compared to those of the other hCAs (Table 4).

Thermostability of hCA IX-c

DSF, DSC, and CD were used to assay the stability of hCA IX-c at a range of pH values, and the results were compared to the stability of hCA II and previously published results for hCA XII. Stability in the case of DSF and DSC was determined by the two-state transition that can be correlated to the melting temperature (T_M) of the enzyme. DSF experiments were performed to assay a wide range of pH values from 3 to 11 (Figure 1A and Table S2). DSF allows for screening of a wide range of conditions while using minimal protein. The pH

stability profiles for hCA IX-c indicate that the enzyme maintains a high level of stability throughout the pH values tested with a significant decrease in stability at pH <5.0. Despite this decrease in stability, hCA IX-c still shows some stability near physiological temperatures (~37 °C) even at a pH of 3.0 (Figure 2A and Table S2). In contrast, hCA II was shown to be stable only at pH 5.0 (Figure 1A). Values lower than pH 5.0 resulted in complete denaturation of hCA II, as shown by a loss of signal in the DSF experiments.

To confirm the DSF results, DSC was performed on selected pH values as it provides a higher level of accuracy in determining T_M values for enzymes. In addition, DSC was used to calculate thermodynamic parameters to assess the nature of unfolding of both hCA IX-c and hCA II. The major transition peak for each enzyme displayed a single unfolding dynamic (Figure S5). The transitions were calculated to be endothermic from eq 3 and were centered at the melting temperature (T_M), with a maximal heat capacity (C_p) occurring at the midpoint of the peak. A summary of the calculated T_M and enthalpies values is presented in Table 2. The van't Hoff enthalpy (H^H) of unfolding was calculated assuming a two-state reversible model (based on previous data from the observation of unfolding transitions of hCA II),³⁰ such that an ideal narrow peak of unfolding would result in $H^H_m \approx H^H$ and larger transition peaks in lower H^H values (Table 2). For both hCA II and hCA IX-c at pH 7.0, the H^H is within the same order of magnitude as H^H_m . This relative equivalence confirms that the unfolding process is most likely a reversible two-state transition for both hCA IX-c and hCA II at neutral pH. These data correlate with protonography experiments that suggest that hCA IX-c (and hCA II) can refold once under stable conditions and can regain activity. At pH 4.0 and 3.0, hCA II did not exhibit unfolding, indicating that the protein did not undergo a transition at these pH values and, most likely, the enzyme was denatured prior to the experiment. However, hCA IX-c displayed a T_M transition at 51.9 °C for pH 4.0, a T_M of 38.4 °C for pH 3.0, and a T_M of 30.1 °C for pH 2.0. The thermogram at pH 3.0 had an unusual spike in signal prior to the unfolding peak, which likely indicates that a population of protein molecules were undergoing an unfolding transition at or below the starting recorded temperature of 28 °C (because of the 3 °C thermostat from 25 to 28 °C), and this initial transition could not be fitted using the buffer subtraction. Similarly, the peak obtained for hCA IX-c at pH 2.0 indicated that while 50% of the enzyme population was unfolded by 30.1 °C, unfolding began at or before data collection at 28 °C (Figure S5). Interestingly, the enthalpy values for hCA IX-c at pH 4.0 were equivalent (Table 2), indicating that the enzyme was able to refold at this pH. This is not mirrored in hCA II, which appeared to be unfolded prior to DSC measurements at pH 4.0. In addition, on the basis of previously published data, it is assumed that the same trend would be true for hCA XII, which shows denaturation at pH ~4.5 (Table 2).⁵⁰ Unlike the enthalpy values obtained for hCA IX-c at pH 7.0 and pH 4.0, the H^H_m and H^H values were not equivalent at pH 3.0 and 2.0 (Table 2). This indicates that in these lower-pH ranges, while the enzyme is able demonstrate some stability and can unfold at temperatures above ambient, this process is likely irreversible, and reannealing may not occur.

The T_M values determined from DSC show that hCA IX-c is the most stable isoform with a T_M of 59.3 °C at pH 7.0, compared to values of 56.3 and 53.0 °C for hCA II and XII, respectively (Table 2). As predicted, hCA II is no longer stable at pH <5.0, resulting in no determinable T_M for low pH values. Interestingly, hCA XII, which also exists in acidic

tumor microenvironments, had reduced acid stability compared to that of hCA IX-c via a T_M of 45 °C at pH ~4.5 and was completely denatured at pH 3.0.⁵⁰ The presence of the PG domain does not seem to influence the stability of hCA IX. This is displayed by a T_M comparable to that of hCA IX-c at neutral pH (Table 2), implying that the presence of the PG domain in hCA IX does not contribute significantly to the overall stability of the enzyme (Table 2). However, it should be noted that hCA IX_{PG} has not been assayed over a broad pH range. It is currently unknown how this domain might influence the stability of the enzyme in an acid environment, although it is predicted that the majority of the acid stability of hCA IX, based on our current data, is a direct result of the conformation and chemical structure of its catalytic domain.

To confirm that hCA IX-c maintains its conformation at low pH, we utilized CD to assay hCA IX-c and hCA II at neutral and acidic pH values of 7.0 and 3.0, respectively (Figure 1B). As shown in Figure 1B, the ellipticity trends for the hCA IX-c samples at pH 7.0 and 3.0 are fairly similar to both possessing minima at 208 nm, indicating that the structural dynamics and folding of hCA IX-c remain largely unchanged at low pH. Conversely, the spectra for hCA II show a large shift in ellipticity from pH 7.0 to 3.0, especially around 200 nm where the hCA II signal becomes negative with low pH. As a reference standard, we note that the spectra observed for the hCA II pH 7.0 sample are consistent with previously collected CD data for the enzyme.⁵¹ The difference in the spectra for hCA II at pH 3.0 indicates possible changes in β -sheet content after incubation in an acidic environment as the negative peak changes from 210 nm at pH 7.0 to 202 nm at pH 3.0. This further correlates to DSF and DSC data that suggest a complete denaturation of hCA II at pH <5.0.

It has been predicted that several structural elements contribute to the pH stable conformation of hCA IX-c. Similar to other hCAs, hCA IX-c contains an intramolecular disulfide bond (Cys156–Cys336; full-length hCA IX numbering). This intramolecular disulfide bond is also present in hCA XII, which may explain its higher stability at pH <5.0 compared to that of hCA II (Table 2). This is further supported by data from Boone et al., in which the same disulfide bond was engineered into hCA II and increased its stability under acidic conditions.³⁰ The more compact structure of hCA IX-c relative to that of hCA II may also contribute to stability. In that regard, Diaz-Torres et al. have proposed that CAs from extremophilic organisms have more compact structures that may underlie their enhanced stability.³¹ However, the most striking or unusual characteristic of hCA IX-c is that it contains a surface far more net negatively charged than that of hCA II or XII (Table 4). This is revealed by observing the predicted theoretical pI of hCA IX-c [pI = 5.4 (Table 4)]. The electrostatic potential of the hCA IX-c surface supports this by showing large regions of negatively charged residues compared to hCA II and XII (Figure S4). In addition, the ratio of negatively charged residues (Asp and Glu) to those most likely to have a positive charge at low pH (Arg, Lys, Asn, Gln, and His) is increased in hCA IX-c (0.73) relative to that in hCA II and XII where the ratio is reduced (0.53 and 0.44, respectively) (Table 4). The increase in net negatively charged residues on the surface of hCA IX-c, upon protonation as pH levels of the solution drop, delays the point at which excess positive charge induces a destabilizing effect.^{52–54}

Alternatively, the presence of net positive charges (mostly from Arg and Lys) on protein surfaces has been correlated with protein instability, because, as the pH becomes more acidic, these residues will become protonated and create charge–charge repulsion forces that will act to destabilize the protein.⁵⁵ Interestingly, hCA IX-c has two added positively charged residues (Lys210 and Lys258) compared to the wild type. This may imply that hCA IX-c may actually be less acid stable than wild-type hCA IX, further highlighting the acid resistant nature of the enzyme's overall fold. The same is seen in hCA IX_{PG} because of the large number of Glu residues in the PG domain, which increases the ratio of net negatively charged residues from 0.73 to 1.12 (Table 4). However, the limited effect of the PG domain on hCA IX stability raises questions about this domain's contribution to the conformational rigidity of the enzyme at low pH. It should be noted that the presence of counterions in a buffered solution could possibly contribute to the observed thermostability of hCA IX-c as seen in experiments by Goto et al.;⁵⁶ however, this has not been tested in the study presented here. Nonetheless, it is apparent that hCA IX-c exhibits an acid stability higher than those of hCA II and XII.

Correlations between pH Stability and Activity of hCA IX-c

On the basis of kinetic and stability data, it is evident that hCA IX-c remains stable and appears to retain CO₂ hydration capabilities at acidic pH. Interestingly, hCA II remains catalytically functional at pH values as low as 5.0 but thereafter quickly loses its activity. Because hCA II loses its native conformation at pH <5.0, it is likely that the loss of activity is a function of conformational stability. The same is probably true for hCA XII, which shows a rapid decrease in catalytic efficiency at lower pH values. Denaturation is likely to occur in hCA IX-c, but at lower pH. Our CD and kinetic data imply that the enzyme most likely retains its conformation and therefore its activity at pH values as low as 3.0 (Figure 2C and Table 2). A different story is revealed at the opposite end of the spectrum, pH >8.0, where hCA IX-c appears to become inactive despite having conformational stability, implying a different mechanism affecting catalytic efficiency at high pH (Figure 2A and Table S1). In addition, and as mentioned previously, there were no major structural differences between hCA IX-s (crystals grown at pH 8.5) and 3IAI (crystals grown at pH 4.0), thus complicating our rationale about why hCA IX-c reveals a decrease in activity at pH >8.0. Together, DSC and protonography data suggest that the denaturation of hCA IX-c and hCA II is a reversible process (Figures S3 and S4 and Table 2) at pH >3.0 and >5.0, respectively. This suggests a two-part equilibrium associated with hCA IX-c that (1) correlates to the enzyme activity in its native conformation in areas of pH >3.0 and (2) a loss of activity that is independent of stability at pH <8.0 (Figure 4A). In contrast, a single-step equilibrium is proposed whereby the activity of each enzyme (hCA II and hCA XII) is correlated with the native structure at pH >5.0 (Figure 4B). At this point, the physiological significance of this is unknown; however, this model may imply that hCA IX activity occurs more readily in highly acidic tumor micro-environments (pH ~5.0) but may be functionally replaced by the activities of hCA II and hCA XII once the pH becomes more stabilized (pH >6.5). This would further suggest that, to target CA activity in tumors, administration of a combination of hCA IX and XII specific inhibitors would be needed to produce the greatest therapeutic advantage. In addition, this model implies the importance of pH-regulated

enzymatic reactions in tumor microenvironments that should be considered and exploited therapeutically.

CONCLUSIONS

In this study, we have presented a high-resolution X-ray structure of the catalytic domain of hCA IX-c and have shown that this domain appears to be stable and active at low pH in the absence of the PG domain. The acid stability and activity of hCA IX confer an advantage, relative to other CAs, in effective pH control in acidic tumor microenvironments. Under acidic conditions, the activity of hCA IX appears to be directly correlated with the ability for the enzyme to retain its native conformation at pH values as low as 3.0 (Figure 1). This contrasts with the much higher pH required for hCA II and XII (pH >5.0). It appears that the major factors contributing to acid stability are the compact nature of hCA IX-c, the presence of an intramolecular disulfide bridge, and the increase in the net negative charge on the enzyme surface. The pH-dependent denaturation for hCA IX is reversible at pH >3.0, suggesting it can reanneal under more stabilizing conditions (Figure 1 and Table 2). hCA II and most likely hCA XII also show this feature, but at higher pH (>5.0). Interestingly, hCA IX loses its catalytic efficiency at pH >8.0 despite retaining its native conformation (Figure S3B and Table 1). The reversible nature of the (1) correlation between acid stability and activity at pH >3.0 and (2) the stability-independent inactivation of hCA IX-c at pH <8.0 presents a possible two-stage equilibrium that differs from that observed in hCA II and XII (Figure 4) and can have implications for preferential hCA IX activity in highly acidic tumor microenvironments. The results presented here provide insights into hCA stability and activity at low pH that may be applied to a broader scope of understanding pH-related effects on enzymes in acidic microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

hCA	human carbonic anhydrase
PG	proteoglycan-like
-c	soluble and crystallizable form of the hCA IX catalytic domain
DW	deep water

rmsd root-mean-square deviation

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Figure 1.

Stability of hCA IX-c and hCA II at a range of pH values. (A) T_M of hCA IX-c (red) and hCA II (blue) over a pH range from 3.0 to 11.0 as determined by DSF. (B) CD spectra of hCA IX-c and hCA II at pH 7.0 and 3.0.

Figure 2.

Structural comparisons between of hCA IX-c and the wild-type catalytic domain of hCA IX (salmon; PDB entry 3IAI⁷) with surface substitutions highlighted. (A) Overlay of hCA IX-c (pale cyan) with 3IAI and labeled N- and C-termini (rmsd = 0.28 Å). (B) Overlay of surface substitutions shown as sticks in hCA IX-c with corresponding residues in 3IAI. Labeled are specific residue substitutions. All residues are normalized to hCA II numbering for the sake of clarity.

Figure 3.

Active site of hCA IX-c. (A) Surface rendition of hCA IX-c (pale cyan) with highlighted hydrophilic (purple) and hydrophobic (orange) pockets. (B) Detailed view of the ordered water network in the hCA IX-c active site (shown as red spheres) and associated residues (shown as sticks) both with corresponding labels. (C) Overlay of the ordered water network and associated residues in hCA II (PDB entry 3KS3) with hCA IX-c with corresponding labels. Highlighted (and expressed in terms of Δd , where d is the distance in angstroms) are variations in water positions in hCA IX-c vs hCA II. In addition, variable residues at position 67 are shown. All residues are normalized to hCA II numbering for the sake of clarity.

Figure 4.

Model representation of the reversible pH-dependent denaturation (D) and activation (N^A) processes of hCA IX and II. (A) In hCA IX, catalytic activity under acidic (pH < 3.0) conditions is predicted to be directly correlated to the rate of stability (shown as the native conformation of hCA IX-c in pale cyan; N^A). In addition, under more basic conditions (pH > 8.0), hCA IX-c remains stable but displays a loss of catalytic efficiency (shown as the native conformation of hCA IX-c in black; N^X). This produces a two-sided equilibrium. (B) In hCA II (blue), the correlation between pH-dependent catalytic efficiency is directly correlated to enzyme stability, which produces a single-transition equilibrium.

Table 1

Catalytic Parameters of the hCA IX-c Compared to Those of Other hCAs

parameter	hCA IX-c	hCA II ^a	hCA XII ^b	hCA IX _w ^c	hCA IX _{pc} ^d
k_{cat}/K_M ($\mu M^{-1} s^{-1}$)	13.0 ± 0.1	120	34	55	150
pK_{a,ZnH_2O} ^e	6.6 ± 0.2	6.9	7.1	6.3	6.5
k_B (μs^{-1})	1.82 ± 0.06	0.8	0.4	1.4	–
pK_{a,ZnH_2O} ^f	6.3 ± 0.2	6.8	8.0	6.4	–

^aData from ref 31.

^bData from ref 43.

^cData from ref 7. Note that ¹⁸O MS was not used to assay CA activity.

^dData from ref 44.

^eDetermined by ¹⁸O exchange from calculation of $k_{cat}^{ex}/k_{eff}^{CO_2}$ in the hydration of CO₂.

^fDetermined from calculation of $R_{H_2O}/[E]$.

Table 2

Thermostability of hCA IX-c, II, and XII

pH	$T_M (^{\circ}\text{C})^a$			
	hCA IX-c	hCA II	hCA XII ^b	hCA IX _{PG} ^c
7.0	59.3 ± 0.03	56.3 ± 0.01	53	55.5
4.0	51.9 ± 0.06	no peak	45 ^d	–
3.0	38.4 ± 0.07	no peak	–	–
2.0	30.1 ± 0.04	–	–	–

pH	hCA IX-c		hCA II	
	H_m (kcal mol ⁻¹) ^d	H^{*H} (kcal mol ⁻¹) ^e	H_m (kcal mol ⁻¹) ^d	H^{*H} (kcal mol ⁻¹) ^e
7.0	72 ± 0.57	97 ± 0.96	49 ± 0.29	20 ± 0.10
4.0	47 ± 0.50	70 ± 0.93	–	–
3.0	9 ± 0.20	110 ± 3.1	–	–
2.0	12 ± 0.22	140 ± 3.5	–	–

^aCalorimetric parameters determined by DSC.^bResults estimated from ref 50.^cResults from ref 57. Note that the value was recorded at pH 7.4 using DSF.^dData point recorded at pH 4.5.^eThe van't Hoff enthalpy (H^{*H}) was determined from a two-state reversible unfolding model.

Table 3

X-ray Crystallography Statistics for Data Processing and Refinement of hCA IX-c

PDB entry	5DVX
space group	$P2_12_12_1$
cell dimensions	$a = 57.9 \text{ \AA}, b = 102.7 \text{ \AA}, c = 109.0 \text{ \AA}, \alpha = \beta = \gamma = 90.0^\circ$
resolution ($\text{\AA}$)	19.8–1.60 (1.62–1.60) ^e
total no. of reflections	155687
$R_{\text{sym}},^a R_{\text{pim}}^b$ (%)	10.0 (56.5), ^e 4.0 (23.3) ^e
I/σ	19.7 (2.94) ^e
completeness (%)	100.0 (99.9) ^e
redundancy	7.2 (6.7) ^e
R_{cryst}^c (%)	16.8 (20.7) ^e
R_{free}^d (%)	19.1 (23.5) ^e
CC _{1/2}	0.971
V_M ($\text{\AA}^3 \text{ Da}^{-1}$)	2.49
residues ^f	140–395
no. of protein atoms	4166 (all chains)
no. of water molecules	642 (all chains)
Ramachandran stats (%) (favored, allowed, outliers)	97.8, 1.8, 0.4
average B factor ($\text{\AA}^2$) (main chain, side chain, solvent, Zn, ^g glycerol ^g)	17.0, 21.7, 31.1, 9.37, 39.2

$$^a R_{\text{sym}} = (\Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle) \times 100.$$

$$^b R_{\text{pim}} = [(\Sigma 1/N - 1) \Sigma |I - \langle I \rangle| / \Sigma \langle I \rangle] \times 100.$$

$$^c R_{\text{cryst}} = (\Sigma |F_O - F_C| / \Sigma |F_O|) \times 100.$$

^d R_{free} is calculated in the same way as R_{cryst} except it is for data omitted from refinement (5% of reflections for all data sets).

^e Values in parentheses correspond to those of the highest-resolution shell.

^f Chains numbered relative to full-length hCA IX.

^g Indicates molecules in the active site.

Table 4

Comparison of Surface and Active Site Charges between hCA Isoforms

isoform	pI ^a		Cys-Cys (intra)	neg. res. (%) ^b	pos. res. (%) ^c	±res. ratio ^d
	surface	active site				
hCA IX-c	5.4	7.1	Y, 1	12.3	16.5	0.73
II	6.9	7.1	N	12.2	21.5	0.57
IX _{PG}	4.5	7.1	Y, 1	17.2	15.4	1.12
IX _{cut}	5.1	7.1	Y, 1	–	–	–
XII	5.8	8.6	Y, 2	9.7	22.0	0.44

^a Calculation of theoretical pI estimated on the basis of sequence using *Expasy*.^b Negatively charged residue percentage calculated by the summation of Asp and Glu relative to chain length.^c Positively charged residue percentage calculated by the summation of Arg, Asn, His, and Gln relative to chain length.^d Calculated as $\pm\text{res. ratio} = (\text{neg. res.})/(\text{pos. res.})$.