# Structure of carbonic anhydrase IX is adapted for low pH catalysis

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**Supplemental Material**

Supplemental Figure S1


**Figure S1.** Sequence alignment of the catalytic domain of hCA IX (denoted with "ca") and II with surface residues that have been substituted labelled. The orange box indicates Cys 174 (full-length sequence containing signal peptide and transmembrane regions) that is important for hCA IX dimerization. Blue circles highlight residues that align structural in hCA IX and II that have been changed. Green boxes indicate residues that align through sequence alignment and spatially. Underlined in red are conserved residues of the active site. Sequence alignment was done using *ExPasy.*

Supplemental Figure S2

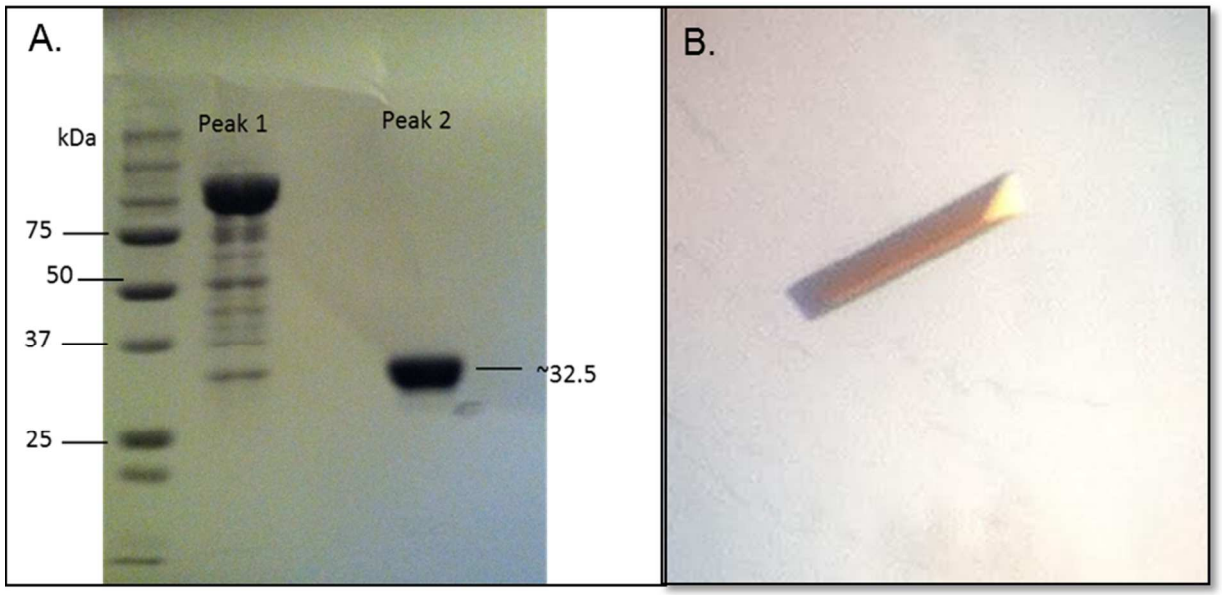



**Figure S2.** Results from hCA IX-c purification and crystallization. A) SDS-PAGE gel following size-exclusion chromatography (SEC). Shown are two bands that correspond to the first peak of SEC containing non-specific material and peak 2 which corresponds to monomeric hCA $IX_{xtal}$. Labelled is the relative MW of hCA IX-c B) Crystal used for data collection of hCA IX-c that formed in the following condition: 0.1 M Tris HCl, pH 8.5, 8% (w/v) PEG 8000.

**Supplemental Figure S3**

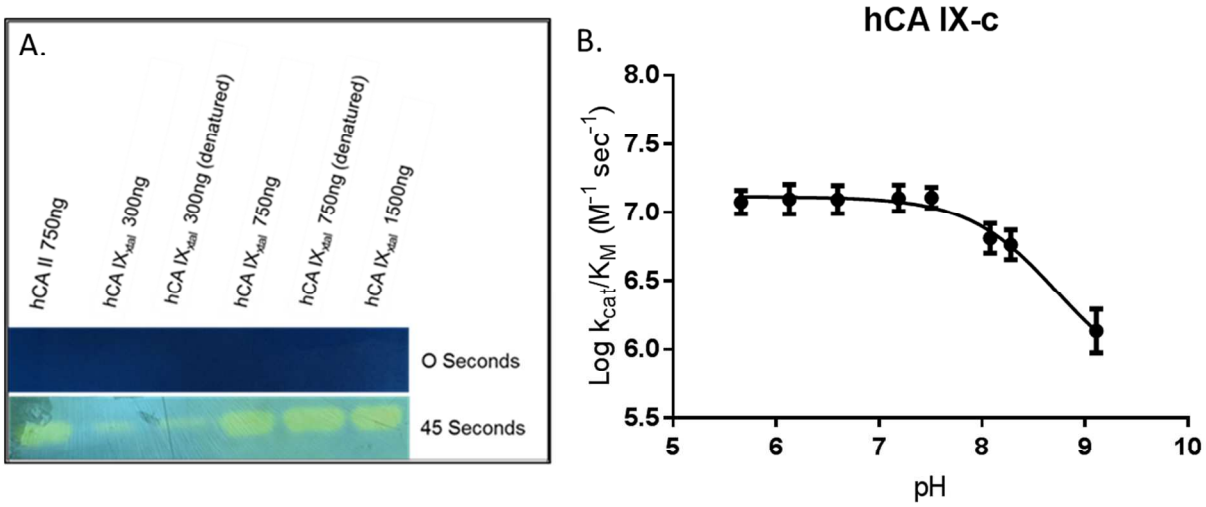



**Figure S3.** Results from activity assays of hCA IX-c. A) Protonogram of hCA IX-c activity. Activity is assessed by the presence of a yellow band displayed in the SDS-PAGE gel. Labelled are lanes containing hCA IX-c at different concentrations and with/without denaturation prior to the experiment (defined by incubation of samples with Laemelli dye containing 5 mM BME, 8 M Urea, and heated at 100$^{o}$C for ~15 mins). Also shown is a lane containing hCA II as a positive control. BSA was used as a negative control but is not shown in the protonogram since it did not produce a band. B) Activity of hCA IX-c ($k_{cat}/K_M$; corresponding to $CO_2/HCO_3^-$ hydration/dehydration, see Experimental Sections of main text for details) determined at a wide range of pH values by $O^{18}$-mass spectroscopy from Eq. 5. The solid line is a least-squares fit to a single ionization event.

Supplemental Figure S4

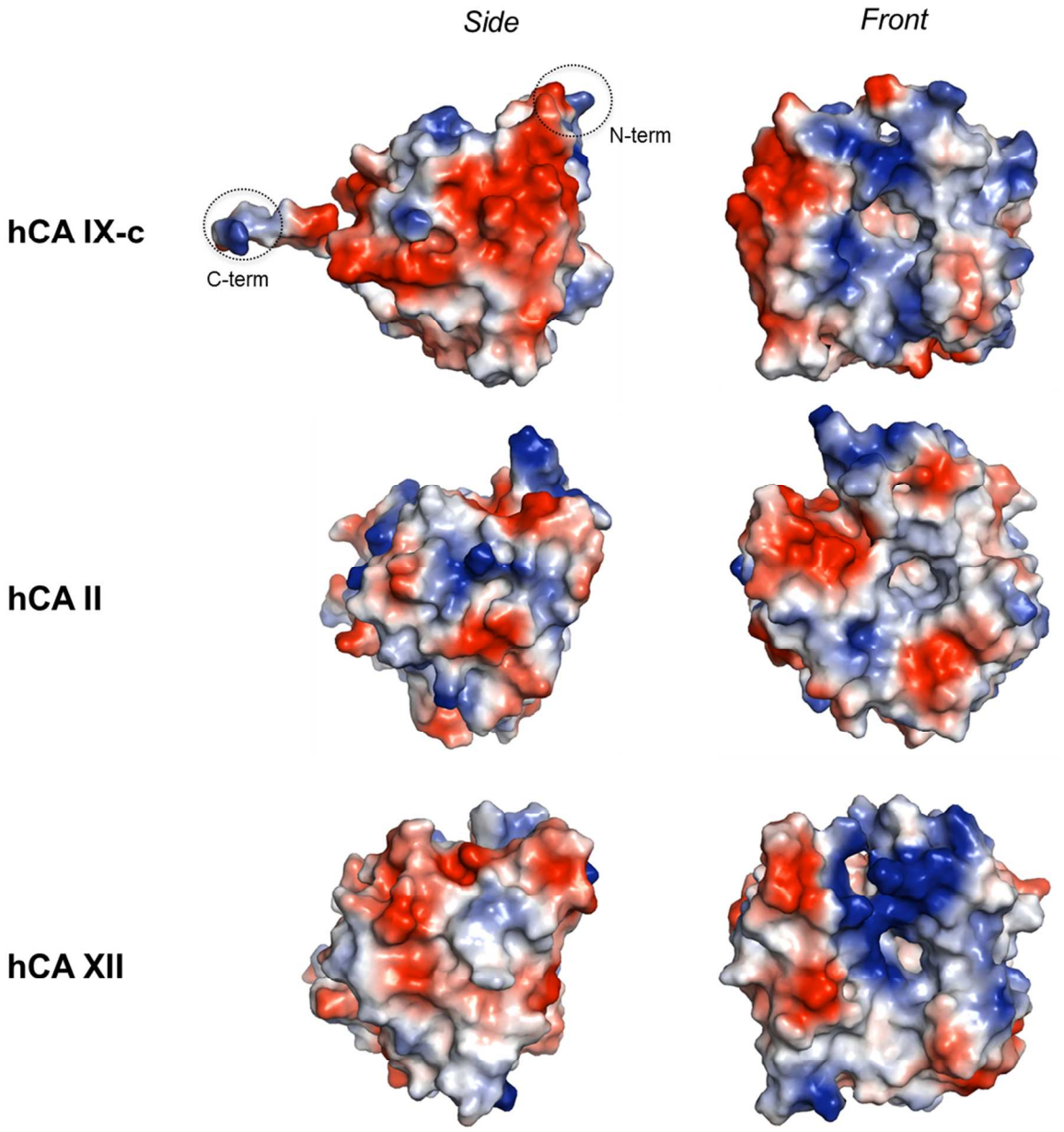



**Figure S4.** Electrostatic potential of the enzyme surface of hCA IX-c, hCA II, and hCA XII in both *Front* (face of the active site) and *Side* ($90^{o}$ rotation about the z-axis) views. Relative surface charges are given by red (negative), blue (positive), and grey (non-polar) based on amino

acid side-chain. Highlighted are the positions of the N- and C-terms in hCA IX-c, which also correspond to postions in hCA II and XII. Figure was generated using *PyMol*.[1]

**Supplemental Table S1**. Comparison of active site residues between hCA IX-c, II, and XII.

| *Residue # | hCA II | hCA IX | hCA XII |
|---|---|---|---|
| **7 (145)** | Tyr | Tyr | Tyr |
| **62 (198)** | Asn | Asn | Asn |
| **64 (200)** | His | His | His |
| **65 (201)** | **Ala** | **Ser** | **Ser** |
| **67 (203)** | **Asn** | **Gln** | **Lys** |
| **69 (205)** | **Glu** | **Thr** | **Asn** |
| **91 (227)** | **Ile** | **Leu** | **Thr** |
| **92 (228)** | Gln | Gln | Gln |
| **94 (230)** | His | His | His |
| **96 (232)** | His | His | His |
| **119 (251)** | His | His | His |
| **121 (253)** | Val | Val | Val |
| **131 (262)** | **Phe** | **Val** | **Ala** |
| **135 (266)** | **Val** | **Leu** | **Ser** |
| **141 (272)** | Leu | Leu | Leu |
| **143 (274)** | Val | Val | Val |
| **170 (302)** | **Lys** | **Glu** | **Lys** |
| **198 (330)** | Leu | Leu | Leu |
| **199 (331)** | Thr | Thr | Thr |
| **200 (332)** | Thr | Thr | Thr |
| **201 (333)** | Pro | Pro | Pro |
| **202 (334)** | Pro | Pro | Pro |

| | | | |
|---|---|---|---|
| **204 (336)** | **Ala** | **Ser** | **Asn** |
| **209 (341)** | Trp | Trp | Trp |

**Residues in parenthesis indicate full-length hCA IX numbering.*

Supplemental Table S2.

**Table S2**: Average $T_M$ relative to pH for hCA IX-c and II determined from DSF.

| pH | $T_M$ (°C) | |
|---|---|---|
| | hCA IX-c | hCA II |
| 11.0 | 55.5 ± 0.6 | 55.2 ± 0.5 |
| 9.5 | 57.0 ± 0.3 | 55.5 ± 0.7 |
| 8.0 | 56.5 ± 0.4 | 55.4 ± 0.5 |
| 7.0 | 56.0 ± 0.3 | 54.5 ± 0.3 |
| 6.0 | 52.0 ± 0.2 | 52.6 ± 0.5 |
| 5.0 | 49.1 ± 0.5 | 41.1 ± 0.6 |
| 4.0 | 42.5 ± 0.4 | - |
| 3.0 | 37.0 ± 0.6 | - |

*-Experiments performed in triplicate.*

Supplemental Figure S3.

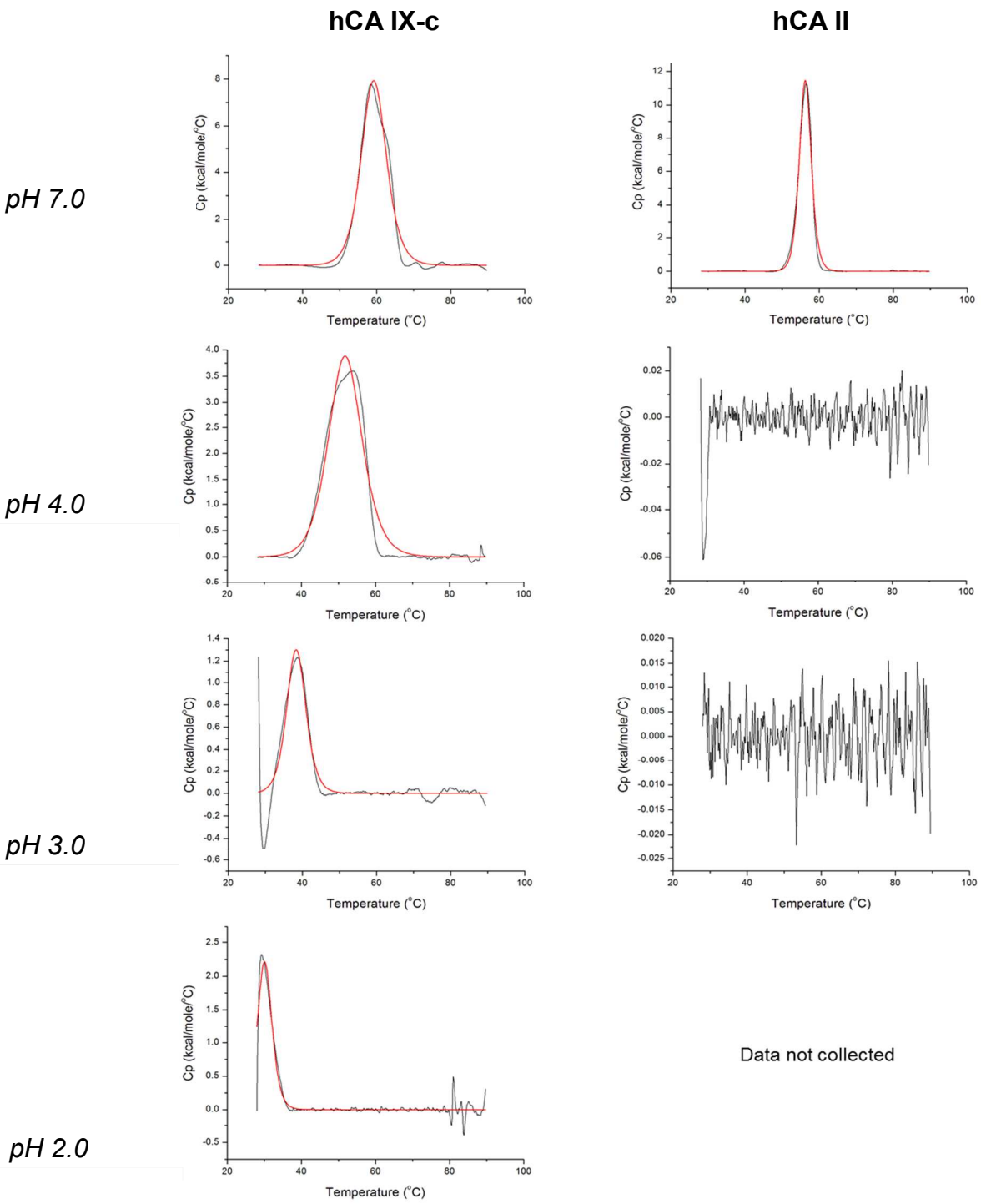
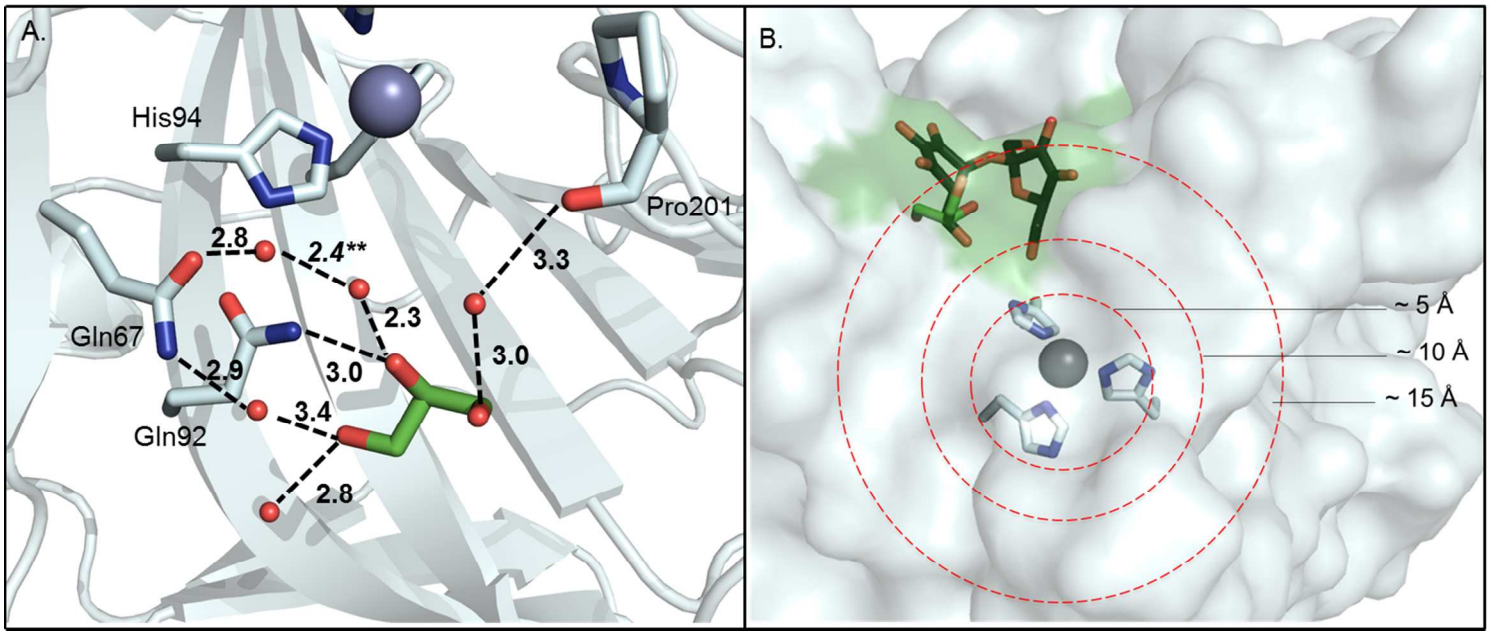


**Figure S3.** Thermograms from DSC experiments from hCA IX-c and hCA II collected at pH 7.0, 4.0, 3.0, and 2.0.

*Observations of a glycerol binding site in hCA IX-c*

During structural refinements of hCA IX-c, a molecule of glycerol (used as a cryoprotectant) was observed towards the entrance of the active site. This had been previously observed in crystal structures of hCA II with the presence of an inhibitor bound in the active site cleft.[2,3] Here, the glycerol is interacting directly with residues of the hydrophilic face of hCA IX-c, between neighbouring solvent molecules and the Nε2 of Gln67 (203) and 92 (224) (Supplemental Figure S4). In addition, an OH group of glycerol forms a stabilizing interaction between a water molecule that is coordinated with the Oα of the main chain of Pro201 (334) (Supplemental Figure S4). The binding affinity of glycerol to hCA IX is unknown. However, this observation is similar to previous studies that show carbohydrates bind selectively in the hCA IX active site in a similar region.[4] An overlay of the positions of glycerol with a previously determined structure of a variant of hCA II engineered to "mimic" the hCA IX active site (hCA $IX_{mimic}$) in complex with sucrose indicates they occupy a similar pocket in the active site (Supplemental Figure S4B). This is the same region of the hCA IX active site that has been previously defined as the "selective pocket".[5,6]

Supplemental figure S4




**Supplemental Figure S4.** Glycerol binding site in hCA IX-c (pale cyan). A) Specific interactions of glycerol (shown as green sticks) with active site residues (shown as sticks) and neighboring water molecules (shown as red spheres). Labelled are residues and relative bond distance (Å). B) A cross section of the hCA IX-c active site (surface rendition) highlighting the "selective pocket" (green) with glycerol (green sticks) and sucrose (black; PDB ID: 4YWP [7]). In addition, distance shells are shown relative to the active site zinc. Distances were measured by tracing waters in the hCA IX-c active site. Note, that due to the conical shape of the enzyme active site, the circles appear distorted. This is to reflect the actual distances from the zinc. All residues are normalized to hCA II numbering for clarity. Figure was made using *PyMol*.[1]

**References**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Carbonic Anhydrase IX. *Acta Crystallographica Section F Structural Biology and Crystallization Communications* **2015**.