Catalysis and pH Control by Membrane-associated Carbonic Anhydrase IX in MDA-MB-231 Breast Cancer Cells*

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Carbonic anhydrase IX (CAIX) is a membrane-bound, tumor-related enzyme whose expression is often considered a marker for hypoxia, an indicator of poor prognosis in the majority of cancer patients, and is associated with acidification of the tumor microenvironment. Here, we describe for the first time the catalytic properties of native CAIX in MDA-MB-231 breast cancer cells that exhibit hypoxia-inducible CA IX expression. Using $^{18}$O exchange measured by membrane inlet mass spectrometry, we determined catalytic activity in membrane ghosts and intact cells. Exofacial carbonic anhydrase activity increases with exposure to hypoxia, an activity which is suppressed by impermeant sulfonamide CA inhibitors. Inhibition by sulfonamide inhibitors is not sensitive to reoxygenation. CAIX activity in intact cells increases in response to reduced pH. Data from membrane ghosts show that the increase in activity at reduced pH is largely due to an increase in the dehydration reaction. In addition, the kinetic constants of CAIX in membrane ghosts are very similar to our previous measurements for purified, recombinant, truncated forms. Hence, the activity of CAIX is not affected by the proteoglycan extension or membrane environment. These activities were measured at a total concentration for all CO$_2$ species at 25 mM and close to chemical equilibrium, conditions which approximate the physiological extracellular environment. Our data suggest that CAIX is particularly well suited to maintain the extracellular pH at a value that favors the survival fitness of tumor cells.

The carbonic anhydrase (CA) family of proteins are metalloenzymes that catalyze the reversible hydration of carbon dioxide: CO$_2$ + H$_2$O $\rightleftharpoons$ H$^+$ + HCO$_3^−$. This reaction is fundamental to a variety of biological processes, including respiration, renal tubular acidification, and fluid secretion (1). The mammalian CAs belong to a single gene family that is referred to as the $\alpha$-CAs. There are 16 isozymes or CA-related proteins in this group that differ in their kinetic and inhibitory properties, cell and tissue distribution, and function (2). Two of the catalytically active members of this family, carbonic anhydrase IX (CAIX) and carbonic anhydrase XII, are specifically tumor-related (3). Of these two, CAIX has garnered more interest because of its limited normal expression (4, 5) and its apparent role in cell proliferation and migration (6), cell adhesion (7), and pH control (8–10). CAIX is a transmembrane glycoprotein whose catalytic domain is oriented toward the extracellular milieu (11). In breast cancer, CAIX is a marker for hypoxic regions of tumors (12), is associated with poor prognosis (13, 14), and is linked to acidification of the tumor microenvironment (10) that favors cancer cell survival and resistance to chemotherapeutic agents (15). CAIX expression has also been linked to the basal B, triple-negative phenotype (16, 17), an aggressive breast cancer for which there are few treatment options. Thus, CAIX may represent a new and valuable target in the visualization, diagnosis, and treatment of cancer.

CAIX is expressed as a 49.7-kDa protein (459 amino acids) that is truncated during processing to a mature protein of 45.7 kDa (residues 38–459) (18). This mature form contains a short intracellular segment (residues 436–459), a transmembrane hydrophobic segment (residues 415–435), an exofacial catalytic domain homologous to CAII (residues 113–414), and an N-terminal proteoglycan-like extension (residues 38–112). CAIX is cotranslationally glycosylated on a single asparagine, Asn-346 (19, 20). Several groups have utilized soluble forms of CAIX to gain insight into catalysis (19–21). These data collectively show that catalysis by CAIX of the hydration of CO$_2$ has a rapid turnover more like CAII than slower isozymes.

We have recently adapted the $^{18}$O exchange between CO$_2$ and water measured by membrane inlet mass spectrometry (MIMS) to detect exofacial CA activity in cancer cells (22) and herein apply this technique to characterize CAIX in its native state. We have selected the MDA-MB-231 breast cancer cells for our studies for several reasons. MDA-MB-231 cells represent a triple-negative cell line and exhibit hypoxic-dependent expression of CAIX yet show no expression of the other membrane-associated CA family members (17). Importantly, they express ample levels of CAII (17), which allows an evaluation of the relationship between intracellular and extracellular CA activities that has not been determined before. Here, we show for the first time a significant difference in the rates of catalyzed hydration of CO$_2$ in intact cells exposed to hypoxic versus normoxic conditions, an activity which is strongly associated with the expression of CAIX. Hypoxia-induced activity in intact cells can be blocked by addition of impermeant sulfonamide CA inhibitors. In addition, we show that low extracellular pH adjusted to mimic the tumor microenvironment increases

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§ The abbreviations used are: CA, carbonic anhydrase; CAIX, carbonic anhydrase IX; CAII, carbonic anhydrase II; MIMS, membrane inlet mass spectrometry; hCAII, human carbonic anhydrase II; PG, proteoglycan.

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CAIX activity. Experiments with membrane ghosts prepared from hypoxic cells demonstrate that the kinetic properties of native CAIX are similar to those of the soluble catalytic domain and show that low pH favors the catalysis in the direction of CO₂ production. These findings are discussed in the context of the role of CAIX in regulating the tumor microenvironment.

EXPERIMENTAL PROCEDURES

Cell Culture—The MDA-MB-231 breast cancer cell line was provided by Dr. Kevin Brown (University of Florida). Cells were plated at a density of 10,000 cells/ml DMEM containing 10% fetal bovine serum (s11450, Atlanta Biologicals). Experiments were initiated when cells achieved 75% confluence (day three post-plating). Cells were then incubated under normal conditions or exposed to hypoxia in modulator incubator chambers (MIC-101) from Billups-Rothenberg, Inc. (1% O₂, 5% CO₂, and balanced N₂) for 16 h at 37 °C. In some experiments, cells were also exposed to 100 μM desferoxamine mesylate, an iron chelator that mimics hypoxia. To quantitate cell number, cells were washed twice with warmed PBS (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate salts (pH 7.4)) and dissociated from plates by incubation with Gibo™ cell dissociation buffer (Invitrogen) for 10 min at 37 °C. After trituration, aliquots of cell suspensions were mixed in Isoton® II diluent (Beckman Coulter) in Accu-vette cups (Beckman Coulter). Cell number was measured by a Coulter Counter® ZM (Beckman Coulter).

Lysate Preparation—Cells were washed three times with ice-cold PBS and then extracted in lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM phosphate salts (pH 7.4)) and dissociated from plates by incubation with Gibo™ cell dissociation buffer (Invitrogen) for 10 min at 37 °C. After trituration, aliquots of cell suspensions were mixed in Isoton® II diluent (Beckman Coulter) in Accu-vette cups (Beckman Coulter). Cell number was measured by a Coulter Counter® ZM (Beckman Coulter).

Membrane Ghost Preparation—MDA-MB-231 cells were exposed to hypoxia for 16 h. Cells were washed three times with cold PBS (2.7 mM KCl, 10 mM phosphate salts, 120 mM NaCl (pH 7.4)) and then exposed to hypotonic buffer (1 ml/plate of a solution containing 2.7 mM KCl, 10 mM phosphate salts (pH 7.4)) in the presence of protease inhibitors (Roche) for 15 min at 4 °C. Cells were washed twice with warm PBS and then centrifuged at 16,300 × g for 15 min at 4 °C. Clarified supernatants were collected, and aliquots were stored at −20 °C. Protein concentration was determined using the Markwell modification of the Lowry procedure (23).

SDS-PAGE and Western Blotting—One-dimensional SDS-PAGE was performed essentially as described by Laemmli et al. (24). Protein samples were mixed with a 4X sample dilution buffer (8% SDS, 4% β-mercaptoethanol, 0.15 mg/ml bromphenol blue, 40% glycerol, 200 mM Tris-base (pH 6.8)). Gels were typically run overnight at room temperature at ~45 V in a Hoefer S.E. 600 electrophoresis unit. Protein samples were electro-transferred from SDS-PAGE gels to nitrocellulose membranes in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol) at 200 mA for 2 h at 4 °C. Western blotting was accomplished as previously described (17).

For the detection of CAIX, the M75 monoclonal antibody was used at a dilution of 1:1000. This antibody was originally made by Pastoreková et al. (25) and was a gift from Dr. Egbert Oosterwijk (Department of Urology, University Hospital Nijmegen, Nijmegen, The Netherlands). For CAII detection we used a rabbit polyclonal antibody made against the entire protein (Novus Biologicals). Actin was used as a loading control. ECL was used according to the manufacturer’s directions (GE Healthcare (no. RPN2106)). Band intensity was quantified using Un-Scan-It (Silk Scientific, Inc.) in the linear range of the film.

Catalysis by Carbonic Anhydrase—Catalysis was measured by the exchange of 18O from species of CO₂ into water (26) determined by MIMS. The apparatus consists of a silicon rubber probe that is permeable to dissolved gases and is submerged in the reaction solution. The probe is connected by glass tubing to a mass spectrometer (27). In the first of two independent stages of catalysis, the dehydration of labeled bicarbonate has a probability of transiently labeling the active site with 18O (Equation 1). In the second stage, the protonation of the zinc-bound, 18O-labeled hydroxide results in the release of H₂18O, which is essentially infinitely diluted by H₂16O in the solvent (Equation 2).

\[ \text{HCO}_3^- + \text{E} + \text{ZnH}_2\text{O} \rightarrow \text{E} + \text{ZnCO}_3^- + \text{H}_2\text{O} \]  
\[ \text{H}^+ + \text{His}^+ + \text{E} \rightarrow \text{H}^+ + \text{His}^- + \text{E} \]  

This method is used to obtain catalytic rates \( R_1 \) from the 18O exchange catalyzed by carbonic anhydrase (26, 28). \( R_1 \) is the rate of interconversion of CO₂ and HCO₃⁻ at chemical equilibrium, is shown in Equation 3.

\[ R_1/[E] = k_{cat}^{exch}[\text{CO}_2]/(K_{eff}^{COH} + [\text{CO}_2]) \]  

The ratio \( k_{cat}^{exch}/K_{eff}^{COH} \) is, in theory and in practice, equal to \( k_{cat}/K_{eq} \) for hydration obtained by steady-state methods (29). The rate constants \( k_{cat}^{exch}/K_{eff}^{COH} \) and \( k_{cat}^{exch}/K_{eff}^{COH} \) are related by the equilibrium constant for catalysis (Haldane relation) and can both be obtained from the data.

We have recently described the application of this method in demonstrating exofacial carbonic anhydrase activity in cancer cells (22). To decrease inaccuracies arising from 13C-containing CO₂ in cell preparations, we used 13C- and 18O-enriched CO₂/HCO₃⁻ and measured the rate of depletion of 18O in 13C-containing CO₂. We measured specifically the atom fraction of 18O in extracellular 13C-containing CO₂ using peak heights from the mass spectrometer: \( 18O \) atom fraction = \( [45/(45 + 49)]/[[45] + (47 + 49)] \), where the numbers in parentheses represent the peak heights of the corresponding masses. Mass spectra were obtained on an Extrel EXM-200 mass spectrometer using electron ionization (70 eV) at an emission current of a 1 mA. Source pressures were ~1 × 10⁻⁶ torr. Each of the peaks...
monitored at masses 45, 47, and 49 were well separated and resolved with a return of ion current (detector response) to the baseline between each mass peak.

MDA-MB-231 cells were collected from culture plates using cell release buffer (Gibco). Cells were extensively washed with bicarbonate-free DMEM buffered with 25 mM Hepes (pH 7.4) and counted. Cells (5 x 10^5 cells/ml) were added to a reaction vessel containing 2 ml of buffered, bicarbonate-free DMEM at 16 °C in which was dissolved 18O-enriched 13CO₂/H13CO₃⁻ at 25 mM total 13CO₂ species. We chose 16 °C for the reaction temperature (in contrast to the traditional 25 °C) for two reasons. First, the lower temperature slows the enzyme-mediated reactions to better contrast the intracellular and extracellular CA activities. Second, we sought to prevent endocytotic events during the course of the experiment, which is accomplished at temperatures slightly below the phase transition for lipids in the plasma membrane. The membrane inlet was immersed in the medium in the reaction vessel and used to detect the atom fraction of 18O in extracellular 13CO₂. This activity was measured after addition of cells (exposed to normoxic or hypoxic conditions) in medium at specific pH values and with specific CA inhibitors. In some experiments, hypoxic cells were isolated (released from plates after hypoxic exposure) in buffered DMEM from which oxygen was removed by nitrogen gassing. Cells prepared in this way were assayed for CA activity in buffered DMEM that had been flushed with helium to remove oxygen. Thus the cells were isolated and assayed under anoxic conditions.

RESULTS

Effect of Hypoxia on Exofacial CA Activity in MDA-MB-231 Breast Cancer Cells—We have previously shown that hypoxia increases the expression of CAIX in MDA-MB-231 cells and that this is associated with elevated CA activity in isolated plasma membrane fragments (17). Using the 18O exchange method, which directly measures CA activity, we demonstrate that exofacial CA activity increases in intact MDA-MB-231 cells in response to hypoxia. This conclusion is based on the significant changes to the rates of depletion of 18O from CO₂ in whole cell suspensions of MDA-MB-231 cells, an experimental system thoroughly characterized in red blood cells (22, 28, 30, 31).

In the biphasic depletion, the first phase (the first 20–40 s after addition of cells in Fig. 1A) represents a rapid diffusion of CO₂ into cells where catalytic cycles deplete 18O followed by efflux of CO₂ from the cell. There is little change in the slope of this phase between normoxic and hypoxic cells, although the length of time in this phase is shortened. The second phase (from 200–500 s in Fig. 1A) is dominated by hydration-dehydration activity of CO₂/HCO₃⁻ outside of the cells (28, 30). This is confirmed by experiments in Fig. 2 in which purified CAII was added to the extracellular solution (described below).

The data in Fig. 1A show that the first-order rate constant of the second phase is greater for hypoxic cells (2.5 x 10⁻⁵ s⁻¹) compared with normoxic cells (1.1 x 10⁻⁵ s⁻¹). This indicates a higher rate of CO₂ hydration/dehydration on the extracellular face of hypoxic cells, which we associate with enhanced expression and activity of exofacial CAIX. We have previously described the synthesis and characterization of an impermeant, polymeric sulfonamide CA inhibitor (N-3500) (22). Here we show that N-3500 has only a limited effect on either phase 1 or phase 2 of the progress curve in normoxic cells (Fig. 1A), consistent with minimal exofacial CAIX expression. Hypoxic cells showed a different pattern. The first order rate constant of the second phase in hypoxic cells in the presence of N-3500 is significantly less than in its absence, at 0.5 x 10⁻⁵ s⁻¹, identical to that of normoxic cells in the presence of N-3500 (0.4 x 10⁻⁵ s⁻¹) (Fig. 1A). The calculated Kᵢ of N-3500 for phase 2 in hypoxic cells was 12.6 ± 3.2 μM (supplemental Fig. S1).

The similar slopes of the first phase in the progress curves between normoxic and hypoxic cells in Fig. 1A suggest that the expression of CAII is nearly equivalent between the two conditions. To evaluate this, we examined multiple protein concentrations from cell lysates by SDS-PAGE and immunoblotting (Fig. 1B). Consistent with our previous data (17), hypoxia significantly up-regulated the expression of CAIX without affecting CAII expression.

Estimating External CA Activity—To demonstrate that exofacial CA activity in whole cell suspensions contributes to the
slope of phase 2 of the biphasic $^{18}$O depletion, we have carried out experiments in which purified human CAII (hCAII) was added to suspensions of normoxic and hypoxic MDA-MB-231 cells. hCAII, the soluble and widespread CA isozyme, does not enter the cells and its overall catalytic efficiency ($k_{cat}^{exch}/K_{eff}^{CO_2}$ of 120 $\mu M^{-1}s^{-1}$) is comparable with that of soluble, catalytic domain CAIX fragments ($k_{cat}^{exch}/K_{eff}^{CO_2}$ of 55 $\mu M^{-1}s^{-1}$) (32)). Fig. 2 demonstrates that addition of increasing concentrations of hCAII to suspensions of hypoxic MDA-MB-231 cells increases the slope of the second phase of the biphasic depletion of $^{18}$O from CO$_2$ (there was no significant change in the first phase). For ease of presentation in Fig. 2B, the absolute slopes are shown as positive increases although the slopes increase in a negative direction. These data show that the rate of depletion of $^{18}$O from CO$_2$ in the second phase is a linear function of the external carbonic anhydrase activity in suspensions of MDA-MB-231 cells. Data for normoxic cells are shown in supplemental Fig. S2.

Hypoxia-dependent CAIX Activation—The procedure for suspending hypoxic (and normoxic) MDA-MB-231 cells prior to $^{18}$O exchange analysis utilizes normoxic buffer and medium and takes approximately 2 h. We have observed no change in CAIX expression in hypoxic cells over this time frame (data not shown), consistent with the 38-hour half-life of CAIX (33). However, several reports have suggested that oxygen availability (re-exposure) for only a short time reduces CAIX activity, perhaps by changing the conformation of CAIX (10, 34, 35). To test this, we devised an alternative method for preparing the cells using anoxic buffers, as described under “Experimental Procedures.” We then compared the activity of hypoxic cells prepared in this manner with hypoxic cells prepared in normoxic buffers. Fig. 3A shows that the activity of CAIX associated with phase 2 was higher in cells prepared in anoxic buffers versus normoxic buffers (first order rate constants of 3.7 $\pm$ 0.13 $\times$ 10$^{-5}$s$^{-1}$ versus 2.2 $\pm$ 0.01 $\times$ 10$^{-5}$s$^{-1}$, respectively; $p \leq 0.001$). However, the $K_i$ values for N-3500 did not change (12.6 $\pm$ 3.3 $\mu M$ for the normoxic preparation of cells (supplemental Fig. S1) versus 13.1 $\pm$ 3.7 $\mu M$ for the anoxic preparation (data not shown)). Because earlier reports have suggested that the affinity of CAIX for an impermeant fluorescent sulfonamide (Cpd 5c) decreases in response to oxygen availability (10, 34, 36), we synthesized this compound as described (36) and

![FIGURE 2. Scaling of extracellular activity by addition of carbonic anhydrase. A, normoxic and hypoxic MDA-MB-231 cells were prepared as in Fig. 1A. CA activity was determined at pH 7.4 using MIMS in the presence of purified hCAII added to the extracellular solution at the indicated concentrations. B, slopes of phase 2 ($\times 10^{-5}$) were determined and plotted against the hCAII concentration. Note that these values are shown as positive numbers for ease of representation. Normoxic cells (○); hypoxic cells (□). Data are representative of duplicate experiments.](image1)

![FIGURE 3. Effect of anoxia on carbonic anhydrase activity in MDA-MB-231 cells. A, MDA-MB-231 cells were exposed to hypoxic conditions for 16 h. Cells were then separated into two groups. One group was isolated and assayed for activity under normoxic conditions, similarly to experiments described in Figs. 1 and 2. The second group was isolated and assayed under anoxic conditions. Here, medium was deoxygenated with nitrogen (for cell harvesting) and helium (for the CA assay). Activity data were collected for both groups at pH 7.4. The data represent triplicate experiments from which the slopes for phase 2 activity were used to determine the first order rates constants (see text). B, cells prepared as in A were exposed to Cpd 5c at the indicated concentrations for the duration of the assay, which was conducted under normoxic or anoxic conditions. The $K_i$ values shown on the figure are representative of three separate experiments.](image2)
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FIGURE 4. Effect of pH on carbonic anhydrase activity in MDA-MB-231 cells. A, normoxic and hypoxic MDA-MB-231 cells, prepared as in Fig. 1A, were assayed for carbonic anhydrase activity at pH 6.8, 7.4, and 7.9 using MIMS. B, first order rate constants were determined for phase 2 activity. These data represent duplicate experiments.

FIGURE 5. pH profile of activity by membrane-associated CAIX. A, membrane ghosts were isolated from hypoxic MDA-MB-231 cells. Membrane-associated CAIX and purified CAII, were assayed for carbonic anhydrase activity at pH 6.8, 7.4, and 7.9 using MIMS. B, first order rate constants were determined for phase 2 activity. These data strongly support the hypothesis that the pH values associated with the tumor microenvironment specifically increase CAIX activity.

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Evaluation of its inhibitory properties. Using the recombinant soluble catalytic domain of CAIX and purified CAII, we measured $K_i$ values of 9.4 ± 1.3 and 15.5 ± 2.0 nm, respectively (supplemental Fig. S3). Applied to intact cells, Cpd 5c exhibited the same impermeant behavior as did N-3500 (data not shown), demonstrating its selectivity for CAIX at least for the duration of the MIMS assay. Fig. 3B shows that the apparent $K_i$ values calculated from the slope of the second phase of the progress curves for Cpd 5c did not change between hypoxic cells assayed under normoxic versus anoxic conditions ($K_i = 85.3 ± 19.4$ versus $91.6 ± 35.1$ nm, respectively), although the increase in CAIX activity persisted in cells prepared and assayed with the anoxic buffers compared with normoxic buffers.

pH-dependent CAIX Activity—There is substantial data to show that CAIX plays a role in regulating intracellular and extracellular pH in intact cells (8–10, 37–39). For the most part, these data measured CAIX activity indirectly by determining the effect of CAIX expression on extra- and intracellular pH. We demonstrate here the effect of extracellular pH on catalysis by CAIX in intact breast cancer cells using $^{18}$O exchange (Fig. 4). We note that the steep slope of phase 1 of $^{18}$O depletion in normoxic or hypoxic cells is largely unaffected by extracellular pH (Fig. 4A). However, the slopes of phase 2 in the progress curves of $^{18}$O depletion show higher exofacial CA activity at pH values that resemble the tumor microenvironment (pH 6.8) than either physiological (pH 7.4) or even higher values (pH 7.9) in both the normoxic and hypoxic cells (Fig. 4A). The resulting changes in the first order rate constants for phase 2 are shown in Fig. 4B. Indeed, the enhancement of $^{18}$O depletion from CO$_2$ in the second phase at pH 6.8 for hypoxic cells compared with hypoxic cells at pH 7.9 corresponds to a 5-fold increase in the first order rate constant. These data strongly support the hypothesis that the pH values associated with the tumor microenvironment specifically increase CAIX activity.

Catalytic Activity of Membrane-associated Carbonic Anhydrase—We isolated membrane ghosts from hypotonically treated MDA-MB-231 cells to determine the influence of pH on CAIX-mediated hydration versus dehydration reactions. Extensive washing was performed to remove cytosolic CAII, which has been shown to interact with the inner surface of membrane-bound proteins like the anion exchange transporter family members (40), and assured by Western blotting (Fig. 5A). Here, we compared two washing procedures: one which used Na$_2$CO$_3$ at a high pH, and one which utilized PBS. Although both were effective at removing CAII, the Na$_2$CO$_3$-treated membrane ghosts were devoid of CA activity (data not shown). Thus, we utilized the PBS-washed membrane ghosts for analyzing CAIX catalytic activity. The catalytic activity in suspensions of ghosts was measured by the $^{18}$O exchange method and the resulting depletion of $^{18}$O from CO$_2$ was monophasic (data not shown), as expected for non-compartmentalized...
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mented CA. Activity was titrated with the tight-binding sulfonamide inhibitor ethoxzolamide, and the effective concentration of CA in the suspension (1.3 nm) was determined by the method of Henderson (41). The catalytic rate constant for the interconversion of CO₂ and bicarbonate, \( R_1/[E] \) (Fig. 5B, inset), was determined directly from the rates of ¹⁸O depletion (described in 26). Equation 3 (see “Experimental Procedures”) was fitted to the values of \( R_1/[E] \), which revealed a catalytic efficiency \( k_{cat}^{exch}/k_{eff}^{CO₂} \) of 62 ± 5 μM⁻¹s⁻¹ with an apparent \( pK_a \) of 6.2 ± 0.2 in the hydration direction. We make the reasonable assumption that these kinetic constants pertain specifically to CAIX. Interestingly, these data are in close agreement with the values of \( k_{cat}^{exch}/k_{eff}^{CO₂} \) of 55 ± 1 μM⁻¹s⁻¹ and a \( pK_a \) of 6.3 ± 0.1 determined for a soluble, recombinant fragment of CAIX corresponding to full-length CAII (21). It is noteworthy that the dehydration reaction (production of CO₂) increases by over two orders of magnitude from pH 9 to pH 6, whereas the hydration reaction is considerably less sensitive to pH (Fig. 5B). These data show that the rate constant for the catalytic hydration reaction is faster than for the dehydration at physiological pH 7.4. At pH 6.8, the rate constants for hydration and dehydration are essentially equal. At pH values lower than pH 6.8, the dehydration reaction is faster.

DISCUSSION

We have recently taken advantage of ¹⁸O exchange between CO₂ and water to demonstrate the existence of exofacial CA activity in breast and prostate cancer cells (22). We have significantly extended these studies to explore for the first time the catalytic properties of full-length CAIX in its native membrane environment under several specific conditions. This was possible because, in our hands, the MDA-MB-231 breast cancer cells do not express other forms of membrane-bound CAs (17). This was possible because, in our hands, the MDA-MB-231 breast cancer cells do not express other forms of membrane-bound CAs (17). This allowed us to associate exofacial CA activity with hypoxic-dependent expression of CAIX. Importantly, our data reflect the activity of CAIX in the context of a cancer cell line that displays a robust glycolytic phenotype which requires acid-base balance both intra- and extracellularly, processes in which CA family members are traditionally involved (39).

There have been measurements of the catalytic activity of several solubilized, recombinant fragments of the CAIX protein corresponding just to the catalytic (CA) region (19–21) and to the catalytic region with the proteoglycan-like N-terminal extension (PG-CA) (19, 20). This report contains the first detailed measurement of the catalytic properties of the native enzyme in its membrane environment. Interestingly, we found that the specific activity of the membrane-bound CAIX is similar to that of the E.coli-derived, recombinant CA domain reported by Wingo et al. (21). We conclude from this comparison that neither the proteoglycan domain, nor the membrane environment, significantly influence the catalytic properties of native CAIX. This was not necessarily expected based on data comparing the catalytic properties of the insect-derived, soluble recombinant CAIX CA domain with that of the PG-CA domain (20). That study reported a significant difference in the \( pK_a \) values between these two constructs: 7.0 for the CA construct and 6.5 for the PG-CA construct. The authors concluded that the proteoglycan domain confers higher activity at low pH. Because the \( pK_a \) of the zinc-bound water can be influenced by the conditions of the assay, it is not surprising that each group derives somewhat different values from their data.

In our hands, the proteoglycan domain and possibly the membrane environment do appear to influence the inhibitory properties of at least one sulfonamide. For Cpd 5c, we have measured the inhibition of the recombinant CAIX CA domain \( K_i = 9.4 \text{ nm, supplemental Fig. S3} \) and CAIX in intact MDA-MB-231 cells \( K_i = 85.3 \text{ nm, Fig. 3B} \). Thus, the apparent \( K_i \) value derived from cells is about nine times higher than that observed for purified CAIX fragments. Interestingly, the inhibition of CAIX by Cpd 5c in membrane ghosts appears to be even less efficacious than in intact cells \( K_i = 172 \text{ nm} \) (data not shown). For N-3500, we do not have data for the recombinant CAIX but assume that the value will be close to that for purified CAII \( K_i = 3.4 \pm 1.0 \text{ μM} \) (21). The \( K_i \) value of N-3500 for CAIX in intact MDA-MB-231 cells is 12.6 μM (supplemental Fig. S1B). This comparison suggests a 4-fold reduction in the efficacy of the pegylated inhibitor for full-length CAIX in its membrane environment. These differences could result simply from nonspecific interactions of the inhibitors with the membrane, reducing the effective concentration of the inhibitor in solution. Alternatively, CAIX could be in an environment that creates a barrier for the inhibitors, such as the extracellular matrix.

Although the impermeant sulfonamide inhibitors were less efficacious in the membrane setting, our data show that their interaction with CAIX did not depend on oxygen. Studies from several labs have shown that overexpressed CAIX (10, 36) or that induced by hypoxia (34) require the continued exposure to low oxygen to observe Cpd 5c binding (as detected by fluorescence). These investigators clearly showed that CAIX expression was unaffected by short-term re-exposure to oxygen despite the loss of Cpd 5c binding. This observation was interpreted as evidence for a conformational change in the catalytic pocket of CAIX. Although these observations are intriguing, we were unable to show differences in the binding affinity of Cpd 5c or N-3500 for CAIX in hypoxic cells when assayed under conditions where oxygen was reintroduced (or not). Yet, in the anoxic environment, the activity of CAIX was elevated (Fig. 3B). We interpret this to mean that the catalytic site of CAIX is exposed to the inhibitor under the conditions tested and that factors other than stability of the active site are responsible for changes in CAIX activity.

It is also clear that N-linked glycosylation does not influence CAIX activity, by the arguments given above. We have previously shown that membrane-bound CAIX in MDA-MB-231 cells contains high mannose structures (42). Thus, the similarity in kinetic constants between the recombinant CAIX CA domain made in Escherichia coli and membrane-bound CAIX provides evidence that CAIX activity does not depend on glycosylation. This finding is consistent with data comparing CAIX activity in soluble recombinant fragments made in bacterial and insect lines (19).

One of the most striking features of cancer cells is the up-regulation of glycolysis, leading to the development of the glycolytic phenotype (43). The initial trigger for enhanced glycolysis is likely hypoxia, which occurs early in the development of cancer cells (43). A significant consequence of the glycolytic phe-
notype is increased production of lactic acid and extracellular acidification. Although we do not understand the reasons, cancer cells become tolerant to low pH, establishing a new set point, allowing cells to survive and proliferate at pH 6.8, the pH that typifies the tumor microenvironment (44). Our data describing catalysis by CAIX at pH 6.8 provides a mechanism by which cancer cells can control their extracellular pH and prevent an extreme drop in pH. Thus a major role of CAIX is to improve buffering by using the extracellular CO2/bicarbonate system. This buffering is enhanced by the catalytic hydration/dehydration reaction of CAIX, which is maximal at pH 6.8 (as demonstrated by Rf of Fig. 5A). This fits well into the classical view of carbonic anhydrase function in “regulating” pH but differs from the proposal that the principle function of CAIX is in the acidification of the tumor microenvironment (8, 45, 46). In this latter proposal, it is envisioned that intracellular CAII catalyzes a reaction between an intracellular proton (from lactic acid or other metabolic acid) and bicarbonate ion-generating CO2. CO2 is freely permeable to the membrane and is reconverted back to bicarbonate in the extracellular environment by the action of CAIX, releasing a proton in the process. Protons accumulate, causing extracellular acidification. In support of this proposal, spheroid growths of human colon carcinoma cells (HCT116) that were engineered to express CAIX show decreased extracellular pH (and increased intracellular pH) relative to cells transfected with empty vector (39). Indeed, our own data show that at physiological pH, CAIX operates more efficiently in the hydration reaction, which would increase proton accumulation. However, it is important to put CAIX in the context of hypoxia, which causes the induction of CAIX and the increased expression of a number of proteins that specifically transport or extrude protons including the lactate transporter, the sodium-proton exchanger, and the vacuolar ATPase (47–50). In this acidic environment, we propose that a primary role of CAIX is to utilize the interconversion of CO2 and HCO3 to buffer and stabilize the pH at 6.8.

Our data suggest that CAIX activity is well adapted to carry out this function in the tumor microenvironment. The inset in Fig. 5 shows the pH profile of Rf/E defined in Equation 3. The rate constant Rf/E describes the catalyzed interconversion of CO2 and bicarbonate, and it increases as pH is decreased from pH 9, reaching a maximum at approximately pH 6.5. This rate constant is determined at a total concentration of all CO2 species at 25 mM and close to chemical equilibrium, conditions which closely resemble the extracellular environment. Moreover, the pH dependence of the equilibrium between CO2 and bicarbonate itself shifts to favor CO2 as the pH is decreased. This suggests that CAIX may uniquely contribute to maintenance of the new pH set-point of cancer cells in response to the proton load from the intracellular metabolism. In this manner, CAIX contributes to the survival fitness of tumor cells.

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