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Detecting Extracellular Carbonic Anhydrase Activity Using Membrane Inlet Mass Spectrometry

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Abstract

Current research into the function of carbonic anhydrases in cell physiology emphasizes the role of membrane-bound carbonic anhydrases, such as carbonic anhydrase IX that has been identified in malignant tumors and is associated with extracellular acidification as a response to hypoxia. We present here a mass spectrometric method to determine the extent to which total carbonic anhydrase activity is due to extracellular carbonic anhydrase in whole cell preparations. The method is based on the biphasic rate of depletion of ¹⁸O from CO₂ measured by membrane inlet mass spectrometry. The slopes of the biphasic depletion are a sensitive measure of the presence of carbonic anhydrase outside and inside of the cells. This property is demonstrated here using suspensions of human red cells in which external carbonic anhydrase was added to the suspending solution. It is also applied to breast and prostate cancer cells which both express exofacial carbonic anhydrase IX. Inhibition of external carbonic anhydrase is achieved by use of a membrane impermeant inhibitor that was synthesized for this purpose, p-aminomethylbenzenesulfonamide attached to a polyethyleneglycol polymer.

Keywords

carbonic anhydrase; mass spectrometry; carbon dioxide; stable isotope; enzyme activity

The zinc metalloenzyme carbonic anhydrase (CA) catalyzes the hydration of carbon dioxide to form bicarbonate and a proton [1,2]. There are 16 isozymes of CA in the α -class of mammalian carbonic anhydrases that play a role in respiration, formation of secretory fluids, acid-base balance, and other physiological functions [3]. The isozyme CA IX is of particular interest since it is found in few normal tissues but its expression is induced in certain tumor cells by hypoxia [4,5]. CA IX is a transmembrane protein the catalytic site of which is external to the cell and is shown to play a role in extracellular acidification associated with cell

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proliferation and metastasis [6–9]. Its inhibition may be of benefit in anticancer therapies for several types of cancer, including breast and prostate cancer.

The inhibition of carbonic anhydrases by sulfonamides is well studied in terms of its biochemical, physiological, and medical aspects [3,10,11]. The inhibition of CA IX by classic sulfonamides is comparable to that of CA II [12,13], an expected result since their active sites are comparable in structure [13]. Many of the sulfonamides that inhibit CA readily pass across membranes. For the purpose of selective inhibition of extracellular CA, sulfonamides of large molecular weight have been developed by covalently attaching sulfonamides to large nontoxic polymers, such as polyethylene glycol (PEG) [14]. In this study, p-aminomethylbenzenesulfonamide was chemically attached to polyethyleneglycol resulting in PEGpAMBS, a high-molecular weight (average MW 3546) inhibitor of carbonic anhydrase.

The purpose of this work is to demonstrate membrane inlet mass spectrometry (MIMS) as a method of detecting extracellular activity of carbonic anhydrase in whole cell suspensions. This is often not straightforward since intracellular CA is most likely also present and many inhibitors are permeable to cell membranes. The MIMS method is based on the use of a small tube of silicon rubber permeable to uncharged molecules of low molecular weight that is immersed in the extracellular solution. Molecules passing across the membrane enter a mass spectrometer to provide sensitive and real time detection. This method has been used to detect and quantify dissolved gases [15–17]. In this report, the depletion of ^{18}O from CO_2 measured by MIMS is taken as a measure of catalysis by carbonic anhydrase [16]. The detection of external carbonic anhydrase activity is achieved by a quantitative measure of the biphasic depletion of ^{18}O from CO_2 . We show that such experiments are capable of detecting extracellular CA activity in the presence of intracellular CA.

Materials and Methods

Materials

Polyethylene glycol (MW 3350) (Sigma, St. Louis, MO), p-aminomethylbenzenesulfonamide (Sigma, St. Louis, MO), N,N'-dicyclohexylcarbodiimide (Fluka, St. Louis, MO), N-hydroxybenzotriazole (Aldrich, St. Louis, MO), and DEAE Sephadex, (Sigma, St. Louis, MO) were used in inhibitor synthesis. Human carbonic anhydrase II (HCA II) was expressed in BL21 plys DE3 *E. coli* [18] and purified by affinity chromatography [19].

Oxygen-18 and carbon-13 labeled bicarbonate was prepared by dissolving $\text{KH}^{13}\text{CO}_3$ (99% ^{13}C) in enriched water (up to 90% ^{18}O enrichment). This solution was allowed to come to isotopic equilibrium overnight, after which water was removed by vacuum distillation. The resulting product was stored at room temperature in a sealed vessel over desiccant.

Synthesis of the membrane impermeable PEGpAMBS

The following synthesis was a modified combination of methods used by Fishman et al. [20] for the simple preparation of PEG-carboxylates by direct oxidation, Johansson et al. [21] for the synthesis of a polymer used in affinity partitioning of synaptic membrane domains, and Conroy et al. [14] for covalent linkage reagents. Polyethylene glycol (5.00 g, 1.49 mmol) and H_2SO_4 (32 mL) were dissolved in 100 mL of deionized water and cooled in an ice-water bath. A solution of CrO_3 (1.00 g, 5.0 mmol) in 20 mL deionized water was added to the PEG solution in one portion, and the resulting orange solution was stirred at room temperature for 22 hours. The deep green colored reaction mixture was diluted with 300 mL water, and extracted with dichloromethane (3×200 mL). The organic layers were combined, washed with 100 mL of saturated NaCl, then dried over anhydrous magnesium sulfate. Most of the dichloromethane was removed by rotary evaporation, and the solution was rapidly stirred while adding diethyl

ether to precipitate out the PEG product. The precipitate was collected by filtration, air dried, and then dried in vacuo to yield 3.92 g (78%) of PEG-carboxylic acid (Figure 1). The carboxyl content of PEG-carboxylic acid was estimated by monitoring pH during titration of a 0.50 g aliquot of product with 1 M NaOH, and found to be ~80% dicarboxylic acid.

A reaction mixture of p-aminomethylbenzene sulfonamide hydrochloride (pAMBS-HCl, 0.042 g, 0.19 mmol) and PEG-dicarboxylic acid (0.80 g, 0.24 mmol) was prepared in 6.0 mL of pyridine, to which was added N-hydroxybenzotriazole (0.026 g, 0.19 mmol) and N,N'-dicyclohexylcarbodiimide (0.078 g, 0.38 mmol). The mixture was stirred for 18 hours at room temperature. It was then concentrated by rotary evaporation to remove pyridine. Dichloromethane was added to the residue, which was washed with 0.1 M aq. HCl (2 × 50 mL) and saturated NaCl (50 mL). After drying over anhydrous magnesium sulfate, concentration of the dichloromethane solution afforded an off-white solid product (0.86 g) (Figure 1).

The product was dissolved in ~ 3 mL of water, and its pH was adjusted to 7 with 0.1 M NaOH. The product was centrifuged to separate the remaining precipitate. The supernatant was applied to a 2.5 × 40 cm column of Sephadex G-50-80. Fractions were spot-tested on UV-indicating silica TLC plates to identify fractions containing product. Candidate fractions were further analyzed by UV-VIS spectroscopy. Fractions with $A_{274} > 0.30$ (eluted volume 84–160 mL) were combined and concentrated. $^1\text{H-NMR}$ analyses (D_2O) confirmed the structure of the final compound, PEGpAMBS. Mass spectral analysis showed that the average molecular weight of PEGpAMBS was 3546 (calculated 3548 for $\text{HO}_2\text{C-PEG-pAMBS}$).

Human erythrocytes

Human blood was freshly obtained and red cells were washed in isotonic buffer (sodium phosphate (50mM), sodium chloride (78mM), and potassium chloride (2.7mM) at pH 7.4).

Cancer cells

PC3 cells, a cellular model of advanced human prostate cancer, were obtained from the American Type Cell Culture Collection, cultured in a Ham's F12 media containing 2 mM L-glutamine, and supplemented with 10% fetal bovine serum, 100 kU/L penicillin-streptomycin [22]. These cells have not been transformed or transfected with any construct. Ivanov et al. [23] conducted Northern blot analysis of mRNAs from over 90 human cancer cell lines, and the human prostate cancer PC-3 cell line was found to express CA IX. Our published data [6] show that the PC-3 cell line expresses CA IX in culture using the M75 monoclonal antibody, as well as in xenograft tumors grown in mice. The cells washed, and resuspended in a phosphate buffer (0.1M) containing sodium chloride (140mM), potassium chloride (2.7mM), dibasic sodium phosphate (1.4mM) and potassium phosphate (8.4mM) at pH 7.3.

MDA-MB-231 breast cancer cells were plated at a density of 1000 cells/cm² in DMEM supplemented with 10% FBS (Valley Biomedical, #BS3033) [6]. MDA-MB-231 cells were grown to about 75% subconfluence and then exposed to 1% oxygen for 16 hours to mimic hypoxia [6]. The MDA-MB-231 cells were washed and resuspended in Krebs Ringer Phosphate Buffer with sodium chloride (128mM), potassium chloride (4.7mM), magnesium sulfate (1.25mM), calcium chloride (1.25mM), and sodium phosphate salts (10mM), at pH 7.4.

^{18}O depletion from CO_2 measured by MIMS

This mass spectrometric method is based on the measurement of the depletion of ^{18}O from CO_2 as catalyzed by carbonic anhydrase. It is based on the redistribution of ^{18}O at chemical equilibrium from CO_2 to water. In the first stage of catalysis, the dehydration of labeled bicarbonate has a probability of transiently labeling the active site with ^{18}O (Eq.1). In a

subsequent step, the protonation of the zinc-bound, ^{18}O -labeled hydroxide results in the release of H_2^{18}O to the solvent (Eq. 2). The labeled water is essentially infinitely diluted by H_2^{16}O .

In classic studies, Mills and Urey used the rate of depletion of ^{18}O from CO_2 to determine rate constants for hydration of CO_2 and dehydration of bicarbonate [24]. In solutions containing carbonic anhydrase, this first-order rate of depletion measures k_{cat}/K_m for hydration and dehydration [25]. In suspensions of cells the depletion of ^{18}O from CO_2 is observed to be biphasic. This is due to the different permeabilities of CO_2 and bicarbonate across the red cell membrane. Carbon dioxide passes rapidly across the membrane and has rapid access to intracellular carbonic anhydrase and is rapidly depleted of label; this is observed in an initial rapid phase of ^{18}O depletion. Bicarbonate has a much slower access and its rate of depletion of ^{18}O is accordingly slower. Simulations based on simple models of these processes are able to derive the intracellular catalytic activity of carbonic anhydrase [26,27].

The atom fraction of ^{18}O in ^{13}C -containing CO_2 was determined by MIMS in suspensions of red cells. This method uses a membrane permeable to dissolved gases to measure this atom fraction in CO_2 in extracellular solution. The rate of depletion or decrease in atom fraction of ^{18}O from CO_2 is a measure of carbonic anhydrase activity [2,16]. To decrease inaccuracies arising from ^{12}C -containing CO_2 in the cell preparations, we used ^{13}C - and ^{18}O -enriched $\text{CO}_2/\text{HCO}_3^-$ and measured rate of depletion of ^{18}O in ^{13}C -containing CO_2 . We measured specifically the atom fraction of ^{18}O in extracellular ^{13}C -containing CO_2 using peak heights from the mass spectrometer: ^{18}O atom fraction = $[\frac{1}{2}(47) + (49)]/[(45) + (47) + (49)]$. Here 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 1 mA. Source pressures were approximately 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.

Cells were added to a reaction vessel containing about 2.5 ml of buffer in which was dissolved ^{18}O -enriched $\text{CO}_2/\text{HCO}_3^-$ at 25 mM total CO_2 species. The membrane inlet was immersed in the suspension in this vessel and used to detect the atom fraction of ^{18}O in extracellular $^{13}\text{CO}_2$ [2]. This activity was measured after addition of cells to solutions containing different concentrations of various inhibitors, including the PEGpAMBS, ethoxzolamide, and acetazolamide. There was no pre-incubation of inhibitors with cells prior to measurements. Similarly, red cells were added to solutions containing HCA II to initiate measurements.

Results

Inhibition of HCA II by PEGpAMBS

The inhibition of purified HCA II with PEGpAMBS was determined using ^{18}O exchange between CO_2 and water measured by MIMS to determine the catalyzed rate of interconversion of CO_2 and bicarbonate [16]. This is the catalyzed rate of hydration of CO_2 determined from the rate of depletion of ^{18}O from CO_2 obtained after subtracting the uncatalyzed rate. Catalyzed rates were determined at concentrations of PEGpAMBS from 1 to 20 μM (data not shown).

The rates of hydration of CO₂ in the presence of inhibitors were fit to a simple Langmuir binding isotherm giving an inhibition constant K_i of $3.4 \pm 1.0 \mu\text{M}$. PEGpAMBS was determined to be membrane impermeant by the observation of no change in the rates of ¹⁸O depletion from CO₂ determined by MIMS over a period of 2 hours in a suspension of red cells containing a concentration of 4 μM of this inhibitor (data not shown).

Extracellular carbonic anhydrase in cell suspensions

The rate of change of the atom fraction of ¹⁸O in CO₂ in red blood cell suspensions is biphasic with a steep loss of ¹⁸O in the initial 20 seconds followed by a second phase of much slower depletion of ¹⁸O from CO₂ (Figure 2). In this experiment, red cells are added at time zero to a solution containing CO₂/HCO₃⁻ with or without extracellular carbonic anhydrase. The aim was to determine the effect of extracellular CA activity on the biphasic depletion. Figure 3 presents the progress curves for m/z 45, 47, and 49 from which the isotopic contents of Figure 2 are derived. Figure 2 demonstrates that the presence of HCA II in extracellular solution alters the slopes in the biphasic depletion. Specifically, the slope of the second phase decreased (became more negative). That is, as the concentration of extracellular HCA II increased, the pattern of ¹⁸O depletion approached the monophasic rate observed in solutions of cell free carbonic anhydrase [16]. The slope of the slow second phase is a linear function of the concentration or activity of extracellular CA (*see insert*, Figure 2).

These examples with red cells help to elucidate the pattern of ¹⁸O depletion from CO₂ in suspensions of MDA-MB-231 breast cancer cells and PC3 prostate cancer cells. These cells express an external, membrane-bound CA IX as well as intracellular CA II [6]. The biphasic nature of the rate of ¹⁸O depletion was more exaggerated upon addition of the membrane-impermeant CA inhibitor PEGpAMBS; that is, the slope of the second phase increased (Figures 4,5). The active-site structure of CA IX is very similar to that of HCA II [13,28], and we anticipate that the inhibition constant of PEGpAMBS is similar between these enzymes, as is the inhibition constants of other prominent inhibitors [12,13]. Addition of 100 μM of PEGpAMBS is sufficient to nearly completely inhibit the external CA IX, based on the value of K_i given above, and the observed depletion in this experiment is more strongly biphasic than in the absence of inhibitor (Figure 4). The same comments pertain to the presence of acetazolamide, an inhibitor that is slowly permeable across membranes [29], as is shown specifically for red cell membranes by the data of Figure 2. Ethoxzolamide, which readily crosses the membrane, blocks all CA activity in these experiments (Figure 4). This pattern of ¹⁸O depletion is also observed in PC3 prostate cancer cells that also express an extracellular, membrane-bound CA IX. Here, again, the biphasic nature of the depletion is enhanced upon addition of PEGpAMBS and the slope of the second phase is increased (Figure 5).

Discussion

The depletion of ¹⁸O from CO₂ in the presence of erythrocytes is exhibited in two phases. This property has been well studied in previous reports [26,27,30]. In these methods, the membrane inlet is immersed in cell suspension or in contact with cell suspension and measures the ¹⁸O content of extracellular CO₂. In the initial stage, for example from 0 to 20 s in Figure 2, the isotope exchange demonstrates a very rapid depletion rate that is mainly determined by the rapid flux of C¹⁸O₂ across the red cell membrane and its subsequent loss of ¹⁸O by contact with intracellular carbonic anhydrase [26,27]. This is followed by a much slower second phase ($t > 50$ s) in which ¹⁸O depletion is determined mostly by the rate of interconversion of CO₂ and bicarbonate outside the cells. This second phase is a linear function of extracellular CA with a slope near unity ($1.6 \text{ ms}^{-1} \text{ nM}^{-1}$; Figure 2, *inset*). In addition, it is this slope of the second phase that decreases and is sensitive to addition of the membrane impermeant CA inhibitor PEGpAMBS (Figures 4,5). The extracellular CA activity could be estimated by a titration with

impermeant inhibitor. However, it is important to note that the slope of this second phase although dominated by the extracellular CA activity is a complicated function also of the flux of CO_2 and HCO_3^- across the membrane and the intracellular activity as well.

A quantitative analysis of these data would require computational best fits of the observed ^{18}O depletion rates to models of the cellular system. This has been done for red blood cells assessing intracellular CA activity and flux rates of CO_2 and bicarbonate across the membranes [26,27,30]. The advantage of the ^{18}O depletion method measured by MIMS is that it sensitively measures CA activity in whole cell suspensions, manifests the second phase which is sensitive to extracellular CA activity, and provides a visual demonstration of the inhibition of the extracellular and total CA activity.

We have demonstrated the usefulness of this method with two cancer cell lines, MDA-MB-231 breast cancer cells (Figure 4) and PC3 prostate cancer cells (Figure 5). Each system produces biphasic ^{18}O depletion similar to that of the red cell suspensions of Figure 2. This indicates the presence of compartmentalized CA. In Figures 4 and 5, increasing amounts of impermeant inhibitor increased the slope of the second phase of depletion. The emergence of a strongly biphasic pattern upon addition of impermeant inhibitor confirms the presence of extra- as well as intracellular CA. The biphasic pattern with inhibited extracellular CA can be used to estimate the activity of intracellular CA, as previously shown [26,27]. The capacity to observe intra- and extracellular catalysis in a single experiment using cell suspensions is unique to this method and cannot be achieved by other methods. The Wilbur-Anderson [31] and stopped-flow [32] methods measure pH changes as a result of catalysis by carbonic anhydrase, and their results in cell suspensions depend on more complex steps of proton flux across membranes. In addition, stopped-flow can subject cells to mechanical damage. In contrast, the ^{18}O -exchange method is based on the isotopic content of CO_2 which readily permeates cell membranes. This method is a useful procedure for the experimental study of the physiology of cells and tumors that express a membrane-bound extracellular CA, such as CA IV, CA IX, CA XII, or CA XIV.

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Figure 1.

Key steps in the synthesis of PEGpAMBS. In Step 1, polyethylene glycol was oxidized with chromium trioxide, washed with sodium hydroxide and water, and concentrated under rotary evaporation to yield PEG-carboxylate. In Step 2, PEG-carboxylate was reacted with pAMBS to yield product, PEGpAMBS.

Figure 2.

The progress curve for atom fraction of ^{18}O in $^{13}\text{CO}_2$ in suspensions of human red blood cells (RBCs all at 6.0×10^3 cells/ml) to which is added external HCA II. (*solid line*) RBCs with no extracellular HCA II; (*long dashes*) RBC suspension containing 1.0 nM purified HCA II in the external solution; (*short dashes*) RBC suspension containing 2.0 nM HCA II in the external solution; (*dotted*) RBC suspension containing 3.0 nM HCA II in the external solution. The suspending solution contained 78 mM NaCl, 50 mM sodium phosphate at pH 7.4 with initial concentrations of all species of CO_2 at 25 mM and 25 °C. RBCs were added at time zero.

Inset: a plot of the negative slope in the slow second phase (phase 2; 50 to 250 s) versus the concentration of extracellular HCA II.

Figure 3.

Ion currents representing m/z ratios for CO_2 in a suspension of human red blood cells measured using an immersed membrane inlet to the mass spectrometer. At time zero red cells were added to a solution containing ^{18}O -labeled $^{13}\text{CO}_2$ in equilibrium with bicarbonate. These are the data from which the solid line in Figure 2 is derived.

Figure 4.

The progress curve for atom fraction of ^{18}O in $^{13}\text{CO}_2$ in suspensions of MDA-MB-231 cells (8.3×10^5 cells/ml) to which is added inhibitors of carbonic anhydrase. Data are labeled as: MDA cells with no inhibitor; MDA cells with $25\mu\text{M}$ PEGpAMBS; MDA cells with $1.0\mu\text{M}$ acetazolamide; and MDA cells with $1.0\mu\text{M}$ ethoxzolamide. Other conditions were as described in Figure 2. MDA cells were added at time zero.

Figure 5.

The progress curve for the atom fraction of ^{18}O in $^{13}\text{CO}_2$ in suspensions of PC3 prostate cancer cells (6.3×10^8 cells/ml). Data are labeled as: PC3 cells containing no inhibitor; PC3 cells and 10 μM PEGpAS; and PC3 cells and 25 μM PEGpAMBS (this curve was shifted up on the y-axis for clarity). Cells were suspended in phosphate buffer (110 mM) containing sodium chloride (140 mM) and potassium chloride (2.7 mM) at pH 7.3. PC3 cells were added at time zero.