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Title: Ligand Binding in Carbonic Anhydrase Protein Investigated by Nuclear Magnetic Resonance Methods

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Ligand Binding in Carbonic Anhydrase Protein Investigated by Nuclear Magnetic Resonance Methods

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Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

Departmental Distinction in Biochemistry

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Ligand Binding in Carbonic Anhydrase Protein Investigated by Nuclear Magnetic Resonance Methods

Senior Thesis, Aida Oumou Diouf

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1. Abstract:

During fall 2015 and interim 2016, Aida Diouf and Dr. Pamela Artz used Nuclear Magnetic Resonance (NMR) spectroscopy to study protein ligand interactions. The investigations consisted of using fluorine NMR to observe the stoichiometry and temperature effects using a model system of bovine carbonic anhydrase I (BCA) and its inhibitor, 4-fluorobenzenesulfonamide. Additionally, the saturation transfer difference (STD) spectroscopy and spin lattice relaxation times (T_1) were obtained. Another inhibitor of the enzyme, 3,5-difluorobenzenesulfonamide, was also investigated with fluorine NMR. The results were comparable to the results for human carbonic anhydrase interactions with the same inhibitors from (Dugad and Gerig (1988) and Veenstra and Gerig (1998)) showing that the bovine carbonic anhydrase is an acceptable substitute for the human enzyme for use for potential experimentation in biophysical chemistry. During the spring semester of 2016, progress was made toward obtaining a 19F-1H Heteronuclear Overhauser Effect Spectroscopy (HOESY) data acquisition. The HOESY experiment is desirable for the study the conformation of the bound ligand (fluorinated inhibitor) within the BCA protein.

2. Introduction:

For this research project, the preliminary work of a previous student, Gwen Fesmire (class of 2014), was expanded by Dr. Artz and Aida Diouf. The goal was to use fluorine Nuclear Magnetic Resonance (19F-NMR) spectroscopy to examine the interactions between bovine carbonic anhydrase (BCA) and 2 fluorinated inhibitors as a model system for protein/ligand interactions useful for studies in the biophysical chemistry laboratory and possibly for other applications in the study of protein inhibitor binding. These experiments use 19F-NMR to understand the interaction of known inhibitors with the enzyme. The relationships investigated include: stoichiometry, the kinetics of binding, and temperature effects. The NMR methods used involve 1D 19F NMR; saturation transfer; the nuclear Overhauser effect; and spin-lattice relaxation (T_1). Inhibition of carbonic anhydrase has many therapeutic uses. Carbonic anhydrase is a marker of hypoxia, because in hypoxic conditions there is an upregulation of carbonic anhydrase (Dudutiene, *et al.*, 2014). This condition, in turn, makes carbonic anhydrase a marker for cancer cells (Dudutiene, *et al.*, 2014). Since hypoxia causes a positive regulatory effect on carbonic anhydrase, inhibition is needed to produce a negative regulatory effect. In addition, glaucoma, a buildup of fluid pressure in the eye that can lead to blindness is linked to the function of carbonic anhydrase (Bucolo, *et al.*, 2015). Carbonic anhydrase inhibitors can be used to decrease the pressure build-up in the eye. This project proposes to understand the interaction of known inhibitors with their target proteins in the model system of BCA and two fluorinated inhibitors. These methods possibly could be expanded to consideration of proposed inhibitors with target proteins. For now, these investigations represent an expansion and collective of some known techniques that have been applied to study aspects of protein/ligand interaction for human carbonic anhydrase and the same fluorinated inhibitors (Dudutiene, *et al.*, 2014; Bucolo, 2015; Dugad & Gerig, 1988; Veenstra & Gerig, 1998; Craik, *et al.*, 1991; Clore & Gronenborn, 1982 & 1983). This model system with bovine carbonic anhydrase was selected as an economical approach to

these studies in the biophysical chemistry curriculum using commercially available materials to formulate the samples for these studies.

The literature uses human carbonic anhydrase, including two isozymes, carbonic anhydrase I and II, which catalyze the same reaction but are distinct, zinc-containing, metalloenzymes (Dugad & Gerig, 1988; Veenstra & Gerig, 1998). For test purposes, we are using bovine carbonic anhydrase which is commercially available from Sigma Aldrich at a reasonable price. We hypothesize that it can react in a similar fashion to human carbonic anhydrase with the inhibitors mentioned above and experience similar interactions with the same inhibitors as human carbonic anhydrase which is more expensive to purchase directly or would require overexpression and purification.

The first inhibitor investigated was 4-fluorobenzenesulfonamide (4-FBS). The inhibitor was prepared at a 50mM concentration in buffered D_2O to run a standard fluorine NMR experiment to determine the chemical shift in parts per million (ppm) relative to the standard 4-fluorophenylalanine. These results were compared to those of Dugad and Gerig (1988). After, the 4-FBS inhibitor was made, stoichiometric, temperature, inversion recovery, saturation transfer studies were performed.

The second inhibitor was 3,5-difluorobenzenesulfonamide (3,5-DFBS). This inhibitor was prepared at an 11.3mM concentration in buffered D_2O. Stoichiometric and temperature studies were investigated.

3. Methods:

Making the Solutions

Bovine carbonic anhydrase (BCA) protein solution was made in buffered D_2O, pH 7.2 and scanned in a UV-Vis spectrometer to determine the absorbance and, consequently, the concentration based on a molar absorptivity at 280 nm of $5.7 \times 10^4 \frac{\text{L}}{\text{mole cm}}$ for BCA (Chen & Kernohan, 1967). Around 17.4mg of lyophilized BCA (Sigma-Aldrich) was dissolved in 750 μL of D_2O and 50 μL of 16X buffer (800 mM potassium phosphate buffer, pH 7.2, 1600mM KCl). The enzyme solution was found to be 0.547mM in BCA for the complex with 4-fluorobenzenesulfonamide. 12.4 μL of 50 mM, 4-fluorophenylalanine standard was added to the solution to provide a reference fluorine peak in the NMR spectra. A second BCA enzyme solution was made in a similar fashion. Using the ^{280}A reading and the molar absorptivity, the enzyme solution was found to be 0.737mM for the complex made with 3,5-difluorobenzenesulfonamide. Similarly, 12.4 μL of the 50 mM 4-fluorophenylalanine standard was added to this mixture.

Stoichiometry Performance (Figures 1 & 4)

A 1D fluorine NMR spectrum was collected for each listed ratio of enzyme to inhibitor. The volume of the 50mM inhibitor solution that was prepared was calculated to create a 1:0.5, 1:1, 1:2, 1:3, and 1:4 stoichiometric ratio of enzyme to inhibitor (Figure 1). The inhibitor was titrated

into the BCA solution in stoichiometric amounts. After each addition, a 1D 19F NMR spectrum was obtained, in order to analyze the stoichiometry of protein inhibitor binding. For the data collection, the number of scans was set to 1024, delay time to 1second, pulse angle to 90° (calibrated for 19F), temperature to 25° Celsius, and spin to 20 Hz.

Inversion Recovery (T1 measurement) (Table 1)

This experiment was collected at a fluorine frequency of 376 MHz, while Dugad and Gerig (1988) ran this experiment at 282 MHz. The pw90 calibration was set as a regular 19F NMR experiment. It was run with a delay time of 5s and the number of scans set to 64. The spin was set to 0. The arrayed pulse width was set to 8 values between $360^{o} - 2 \ldots 360^{o} + 2$. The 360° pulse was determined and the value was divided by 4 to determine the measured 90°pulse. After measuring the pulse length corresponding to 90°, the 90^{o} value for pw and pw90 and the 180^{o} value for p1 were determined. The values were inserted in a new experiment that was set as INVREC. The delay time was set to 20s with 300 scans at a 90^{o} pulse angle. The relaxation delay was set up with the default arrayed values. After acquiring the data with different delays, the last spectrum, with the longest delay, was phased so all peaks were completely up and the data was analyzed using the macro program provided in VNMRJ to determine the T_1 measurement. This measurement was done for the inhibitors without protein and after the addition of protein to 4 equivalents. The presence of the protein substantially changes the T_1 value for the inhibitor due to interactions of the inhibitor with the protein.

Saturation Transfer Difference (Table 2 and Figure 2)

A 1D 19F NMR experiment was run in order to determine the on- and off-resonance frequencies for the 19F peaks that correspond to the free and bound fluorinated inhibitors. The parameters consisted of a delay time of 1s, 1024 scans, and no spinning. After acquiring, the cursor was placed at the middle of the resonance corresponding to the fluorine from the bound inhibitor and 'sd' was typed to obtain the on-resonance frequency. The same was done with the peak corresponding to the 19F frequency of the free inhibitor. To obtain the off-resonance frequency, the cursor was applied, at the same distance as the distance between the free and bound fluorinated inhibitor peaks, on either side of the bound and free peak with placement where no peak was detected. These frequency values, two on resonance (or on 19F peak) and two off resonance, were used in four separate experiments, using the experiment cyclenoe without subtraction. Two of the experiments were the control experiments whose control and "satfrq" values were the off-resonance frequencies. These control experiments were compared to the on resonance experiments.

Temperature Study (Figures 3 and 5)

Five separate 1D 19F NMR experiments were set with the number of scans to 512, delay time to 1s, and spin to 20 Hz. Each experiment was set to a temperature of 25, 20, 12, 7, 1 degrees Celsius. The pre-acquisition delay was set to 480s for each experiment to allow for pre-equilibration at the selected temperature. These spectra were collected sequentially starting at 25°C and progressing down in temperature ultimately to 1°C.

4. Results/Discussion:
   a) 4-fluorobenzenesulfonamide inhibitor (4FBS)

Stoichiometry Performance (Figure 1)

Our samples include the standard, 4-fluorophenylalanine, in order to reference the chemical shifts of the fluorine peaks of the free and bound inhibitor. In the 1:1 ratio, both the free inhibitor and bound inhibitor peaks were visible and shown to be 6 ppm apart. Although the published fluorine spectra for human carbonic anhydrase bound to 4FBS show that the free and bound inhibitor peaks are 6 ppm apart, a fluorine peak for the free inhibitor becomes visible at the 1:2 ratio (Dugad and Gerig, 1988). This difference in stoichiometry could be due to the use of BCA instead of human carbonic anhydrase. It can also be due to the difference in activity of the enzyme. More investigation is needed but the results are reproducible in investigations done with different lots of protein prepared and purchased at different times. These results also agree with the results obtained by Gwen Fesmire (2014 interim and spring).

Inversion Recovery (T1 measurement) (Table 1)

The inversion-recovery experiment was performed to measure the T_1 of the enzyme/inhibitor complex (Table 1). First, the fluorine 90^o pulse was calibrated and used to measure the T_1. The T_1 measurement was done for the 1:4 stoichiometric ratio of enzyme to inhibitor; therefore, the relaxation time was measured for both the free and bound fluorinated inhibitors (Table 1). The results were close, but not identical to the T_1 measurements found in the literature (Dugad and Gerig, 1988). The small differences in these results could be due to the difference in resonance frequencies of the NMR spectrometers and also due to the difference in interaction of the bovine carbonic anhydrase with the inhibitor as opposed to the human isozyme. The inversion-recovery needs to be repeated using the 300MHz NMR spectrometer at a fluorine frequency of 282 MHz which was the frequency used for the human carbonic anhydrase/inhibitor binding experiments.

Saturation Transfer Difference (Table 2 and Figure 2)

The saturation transfer difference (STD) spectroscopy (Figure 2) was obtained in order to get more information on the interactions between enzyme and ligand by looking at the saturation (irradiation) transferred between the bound and free inhibitors. Ultimately, this experiment gives information about the exchange between the bound and unbound inhibitor. In order to perform this experiment, the off- and on-resonance frequencies were determined (Table 2). The bound peak and the free peak were both separately irradiated with the intention of saturating the signal at the frequency of choice making it disappear. As a result, there is a change in size of the fluorine peaks (Figure 2) for both the irradiated peak and the unirradiated peak due to the exchange of inhibitor molecules between the bound and free states. Depending on the set-up, the irradiated peak was either from the 19F of the bound or free inhibitor. The results showed that there is exchange in the free and bound species of the inhibitor. When the bound inhibitor was excited, there was a change in the peaks of both the free and bound species. Using the results from figure 2, the rate constants

of free to bound and of bound to free can be calculated to determine the exchange rate between the free and bound inhibitor species (Dugad and Gerig, 1988). The kinetics is not straightforward for this protein inhibitor binding for the human carbonic anhydrase (Dugad & Gerig, 1988) or for the bovine carbonic anhydrase.

Temperature Study (Figure 3)

A temperature test, monitoring 1D 19F NMR spectra, was performed in order to investigate how the interactions of enzymes and their ligands change due to temperature. Figure 3 shows the alteration in chemical shift and linewidth as the temperature changes. The bound peak became broader and shifted towards the peak representing the free inhibitor due to the decrease in temperature. This illustrates that at lower temperatures, the rate of exchange between the free and bound inhibitor species is lower (Dugad & Gerig, 1988).

b) 3,5-difluorobenzenesulfonamide inhibitor (3,5-DFBS)

The interaction of bovine carbonic anhydrase and the inhibitor 3,5-difluorobenzensulfoanimde (3,5-DFBS) was also investigated. This inhibitor was not as soluble as the previous inhibitor and; therefore, was diluted to 11.3mM with D_2O. The enzyme was found to be 0.737mM. A 1D fluorine spectrum and a temperature study were obtained.

Stoichiometry Performance (Figure 4)

In the 1:0.5 ratio of enzyme to inhibitor, only one peak is visible, although the inhibitor is contains two symmetric fluorines. This result was also seen in previous experiments. This overlap of peaks indicates that the rotation in the bound inhibitor is fast, thus, the magnetic environments of the two fluorine atoms are very similar, which produces overlapping resonances. In the 1:1 ratio, both peaks representing the free and bound species are present, which shows that the inhibitor binds in a 1:1 ratio. The stoichiometric results published by Gerig and his colleagues (1988) are in accordance with this result.

Temperature Study (Figure 5)

The effects of temperature on the interaction between the enzyme and 3,5-DFBS were similar to that of the enzyme:4-FBS complex. As the temperature decreased, the peak representing the free inhibitor species moved further upfield (right) and the peak representing the bound inhibitor species became broader. At 1 degree Celsius, it seems like both peaks merged into one peak. This indicates that there is a slower rate of exchange between free and bound inhibitors as the temperature is decreased resulting in less discrete chemical shifts for the 19F atoms that are in the bound and free condition.

Table 1. ^{19}F spin lattice relaxation (T_1) values measured using the 1:4 complex of bovine carbonic anhydrase with 4-fluorobenzenesulfonamide inhibitor.

	Spin Lattice Relaxation, T_1 (s)	Error
Free 4-FBS inhibitor	4.204	0.052
Bound 4-FBS inhibitor	0.5729	0.086

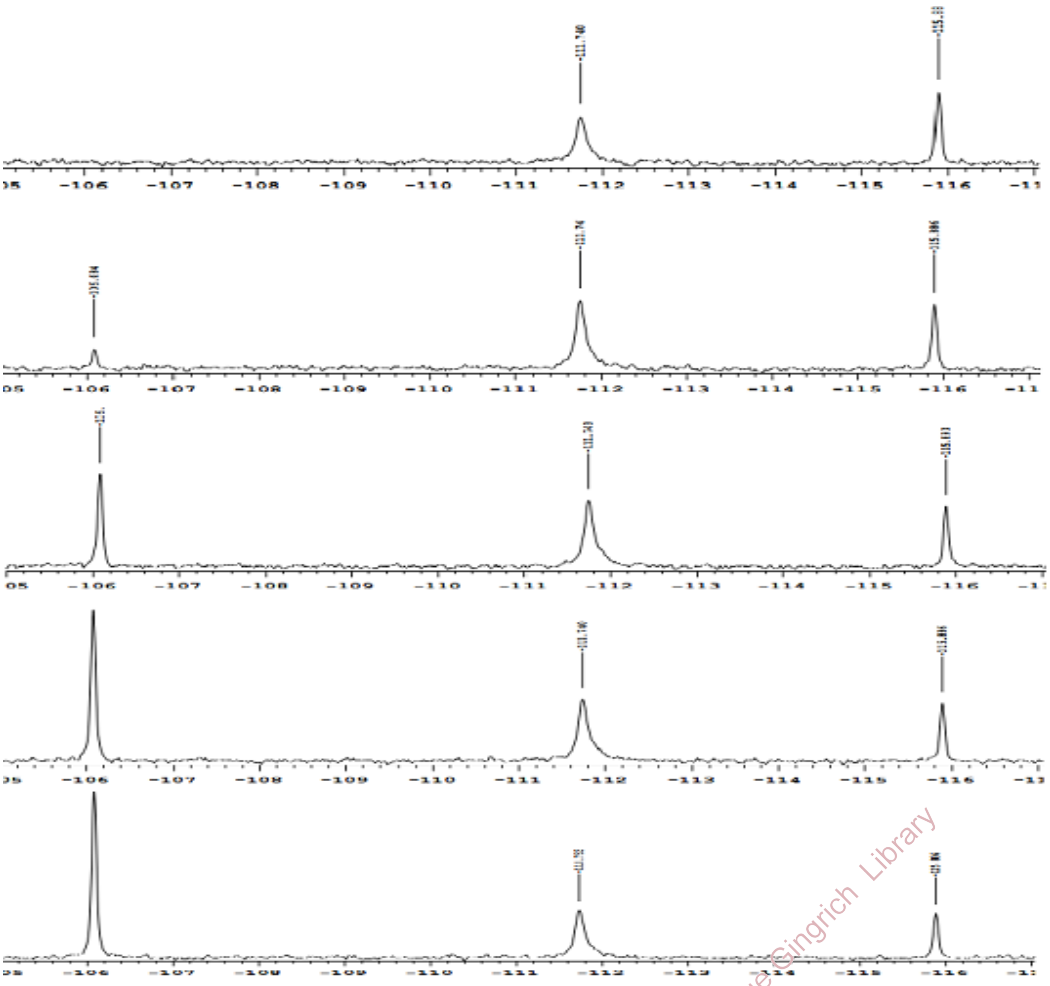

Figure 1. Titration data for 0.547 mM bovine carbonic anhydrase (BCA) measured with (from top to bottom) 0.5, 1, 2, 3, and 4 equivalents of the inhibitor 4-fluorobenzenesulfonamide (4-FBS). Spectra are referenced to the fluorine peak chemical shift from 4-fluorophenylalanine which appears the most upfield. The data appears to indicate that the protein binds in a ratio of 1:1 with the inhibitor. After that, the fluorine peak for the free inhibitor which is the most downfield increases with each additional equivalent of 4-FBS.

Table 2. The on- (irradiating bound and irradiating free) and off- (control bound and free) frequencies for the bound and free inhibitor species.

Experiments	Control - Bound	Irradiating - Bound	Control - Free	Irradiating - Free
Control	-2307.3	-2307.3	4079.3	4079.3
Satfrq	-2307.3	-177.8	4079.3	1949.5

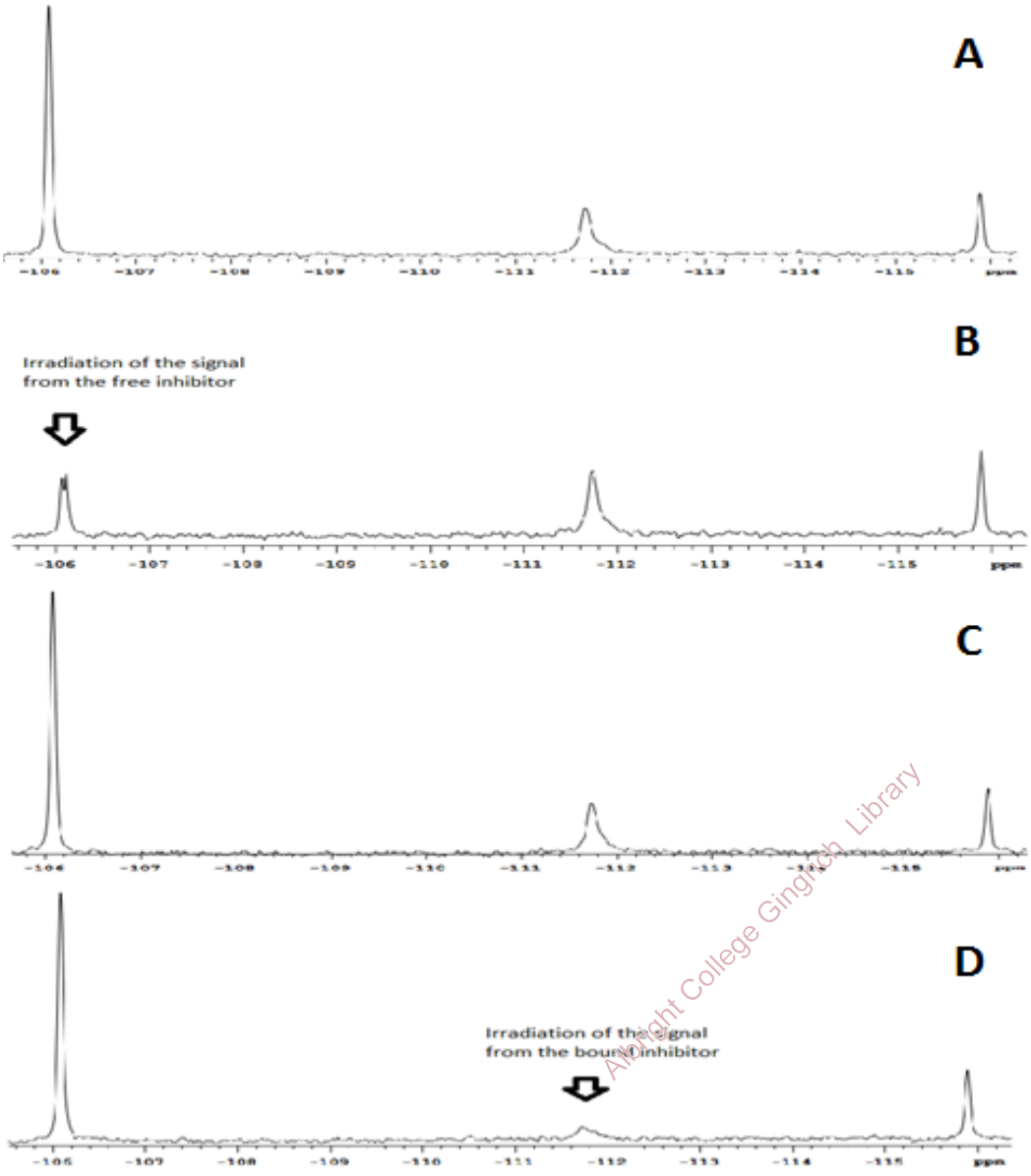

Figure 2. Spectra for saturation transfer experiments showing the irradiation of either the fluorine in the free or bound inhibitor and the change in the other peak resulting from that irradiation. A & C: control (irradiation off resonance). B & D: saturation transfer (irradiation on resonance)

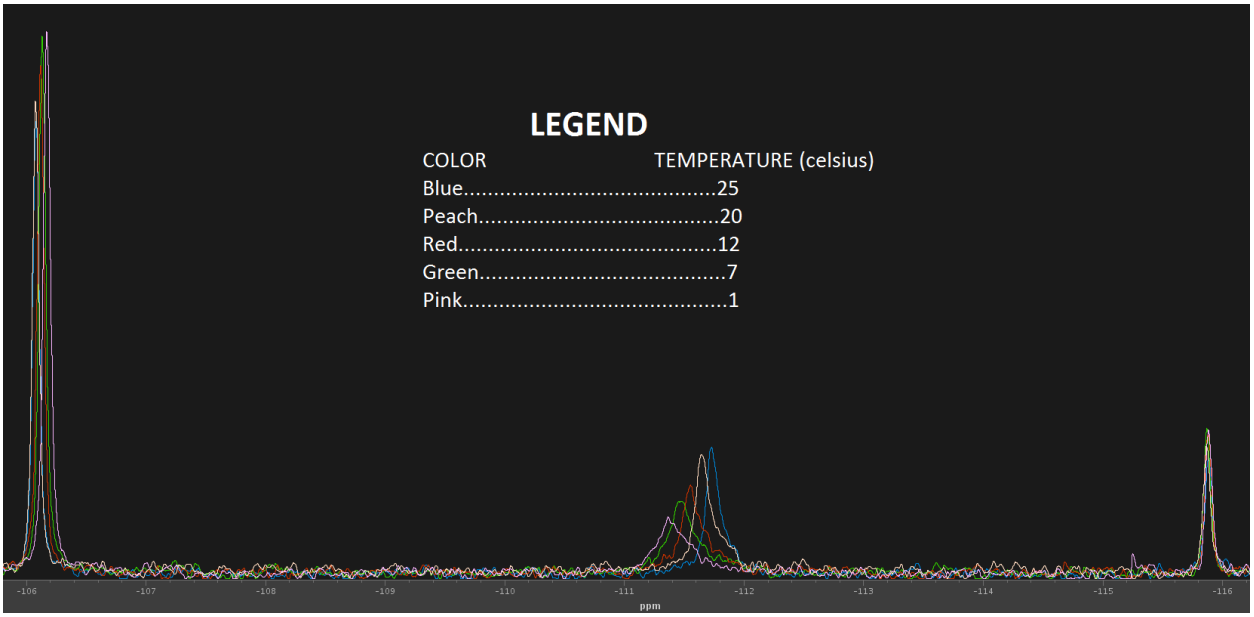

Figure 3. Temperature study of the 1:4 complex of BCA with 4-FBS. The peaks from left to right represent fluorine from the free inhibitor, the protein bound inhibitor, and the 4-fluorophenylalanine standard. Note that the peaks shift toward each other with the decrease in temperature. The peak corresponding to the bound 4FBS also gets broader indicating slow exchange between different bound electronic environments of for the 4FBS inhibitor in the BCA protein.

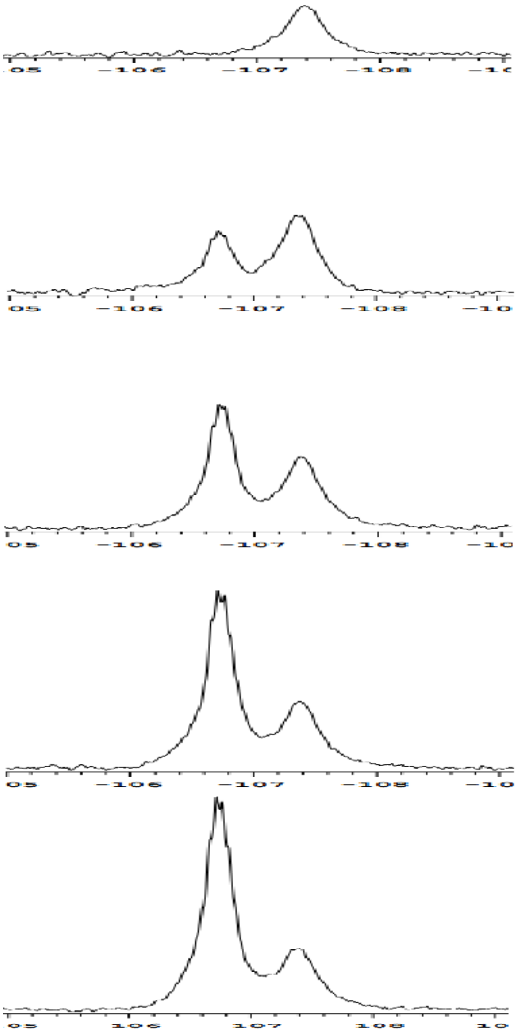

Figure 4. Titration data for 0.737 mM bovine carbonic anhydrase (BCA) measured with (from top to bottom) 0.5, 1, 2, 3, and 4 equivalents of the inhibitor 3,5-difluorobenzenesulfonamide (3,5-DFBS). The data appears to indicate that the protein binds in a ratio of 1:1 with the inhibitor. After that, the fluorine peak for the free inhibitor which is the most downfield increases with each additional equivalent of 3,5-DFBS.

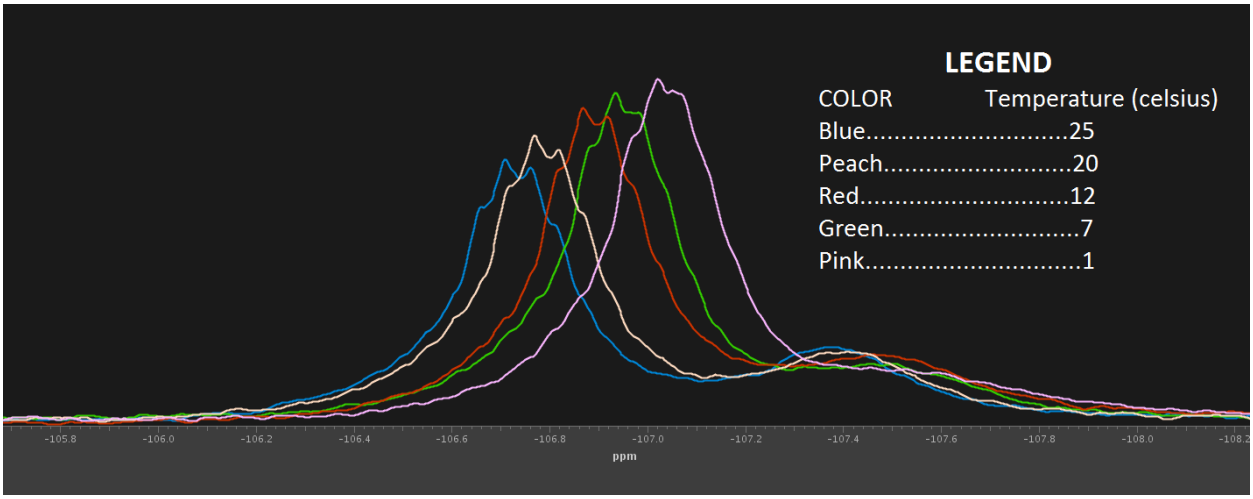

Figure 5. Temperature study of the 1:4 complex of BCA with 3,5-DFBS. The peaks from left to right represent fluorine from the free inhibitor and the protein bound inhibitor. Again, the bound 3,5-DFBS becomes broadened as the lower temperature slows exchange between slightly different electronic environments within the BCA protein. The bound and free inhibitor peaks get closer as the temperature decreases.

5. Conclusion:

^{19}F NMR can be used to study binding in the model system of bovine carbonic anhydrase and its inhibitors. These studies have potential in the development of a biophysical chemistry laboratory due to the commercial availability of BCA at a reasonable price from Sigma-Aldrich. These studies verified the results of single pulse, 1D, ^{19}F NMR used to examine the stoichiometry. Inversion recovery, saturation transfer, and the effects of temperature were also studied and provide comparable results compared to similar experiments with human carbonic anhydrase (Dugad & Gerig, 1988; Veenstra & Gerig, 1998).

The stoichiometry figure for the enzyme:4-FBS complex shows a ratio of 1:1, while the published spectra (Dugad and Gerig, 1988) shows 1:2 for the human enzyme. The figure for the enzyme:3,5-DFBS complex also shows a 1:1 ratio, which is the same ratio found by and Veenstra and Gerig (1998) in studies with human carbonic anhydrase. The results of the T1 measurements for the enzyme:4-FBS complex were close but not identical to the literature values for human enzyme as would be expected for the bovine isozyme of the enzyme which differs in structure from the human isozyme even though they function similarly in the respective species.. When the bound 4-FBS peak was excited for the saturation transfer study, there was a change in peaks for both the bound and free species, which was also shown in the literature, presented by Gerig and his colleagues (1988). However, when the free 4-FBS peak was excited, the peak for the bound species did not change. The bound peaks for both inhibitors became broader and closer to the peaks for the free species with the decrease in temperature, which shows a lower rate of exchange. The same phenomenon was shown in previous work with the human carbonic anhydrase (Dugad & Gerig, 1988; Veenstra & Gerig, 1998).

The differences in the results presented compared to results presented from previous experiment are most likely due to the use of bovine carbonic anhydrase instead of human carbonic anhydrase, the difference in activity of the enzyme, or the difference in frequency of the NMR. Therefore, more analysis of these results will be attempted. Also, more investigation on the 3,5-DFBS inhibitor will be continued.

A Heteronuclear Overhauser Effect Spectroscopy (HOESY) will be conducted for each protein-inhibitor complex. HOESY data for the human carbonic anhydrase/inhibitor complex is cited in both: Dugad and Gerig (1988), and Veenstra and Gerig (1998). The HOESY experiment can be used to investigate spatial interactions and can determine the proximity of 19F nuclei from the inhibitor relative to protons within about 6 angstroms. This experiment can be used to assess the interactions of the fluorine nucleus and the proton nuclei of the bound inhibitor which can lead to information about the conformation of the bound ligand within the protein. The correct parameters of this experiment need to be determined. We were able to gain information relative to using the 4-fluorophenylalanine standard as a test compound for the HOESY experiment and other possible investigations to ultimately make this data collection possible using our instrumental set-up. (Lumsden, Mike, 2014, Dalhousie University; Facey, Glenn, 2014, University of Ottawa; Combettes *et al.*, 2012)

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7. Acknowledgements

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