Nuclear Magnetic Resonance Studies of Protein-Ligand Interaction Models

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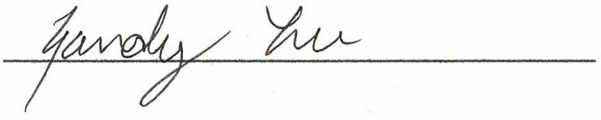
Signature of Author:  Date: 4/20/2018

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Nuclear Magnetic Resonance Studies of Protein-Ligand Interaction Models

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1. Abstract:

Nuclear magnetic resonance (NMR) was used to study various models of protein and ligand interactions as these systems are highly relevant in drug design and discovery. The first model consists of yeast alcohol dehydrogenase (YADH) with adenosine monophosphate (AMP) or adenosine triphosphate (ATP). The second model consists of firefly luciferase with ATP. One and two-dimensional NMR experiments such as ^{1}H NMR, ^{31}P NMR, correlation spectroscopy (COSY), and nuclear Overhauser effect experiments were performed using these model systems. A clear transfer NOE was observed due to through space interactions between the saturated sugar protons and the H8 proton of AMP and ATP, confirming the anti-conformation for both ligands when interacting with YADH (Craik 1991). A clear transfer NOE was also observed between the saturated sugar protons and the H8 proton of ATP when interacting with luciferase, confirming the anti-conformation of the ligand when bound to the protein. Significant changes in linewidth from ^{31}P NMR spectra were observed between bound and unbound ligand for YADH with AMP or ATP. It was determined that NMR is a viable method to study interactions and determine optimal conformations between proteins and ligands.

2. Introduction:

The study of protein and ligand interactions can be applied to several fields of science and is important for understanding several concepts amongst these fields. One major application of this research is in the field of drug design and discovery. This includes the screening of several potential compounds that can serve as pharmacological drugs. Proteins are often found to be drug targets that play an important role in some biological pathways that may potentially be part of a disease-causing mechanism. Studying how different ligands bind to these proteins can lead to discovery of new drugs that may stimulate or inhibit these proteins, which would then have some effect on the disease-causing mechanism. However, it is often difficult to directly analyze how certain ligands bind and interact with proteins.

One method that can be used to study protein and ligand interactions is nuclear magnetic resonance (NMR). One dimensional (1D) NMR experiments were used in the research of protein and ligand interactions. Proton and phosphorus NMR were chosen based off the available nuclei present in the samples. Chemical shifts may be utilized to study the chemistry and environment of the molecules. Typically, the chemical shift will change when there is a change to the chemical environment of the ligand. The transverse relaxation rate (related to linewidth) can also be observed between the free and bound states. This difference depends on dipole-dipole interactions that tumble in solution. Different tumbling rates will be observed from the change in mass of the two states (Clarkson 2003).

Two-dimensional (2D) NMR experiments are also utilized in the research of protein and ligand interactions. Correlation spectroscopy (COSY) was used to assign signals to their

respective protons by observing neighboring protons. This correlation can be seen when the protons undergo spin-spin coupling. The nuclear Overhauser effect (NOE) is when one nucleus undergoes a change in population due to another magnetic nucleus nearby that is saturated by decoupling. The effect takes place through space via dipole interactions, distinct from J coupling that occurs through bonds of the molecules. The NOE was used to determine the most active conformation of ligands when bound to a protein. When an NOE is present, the intensity of specific peaks will change when others are irradiated as seen in figure 1. This intensity depends on the distance of the protons involved in the interaction. The parameters are also influenced by the rate of exchange between the free and bound states that can be termed fast or slow. Separate resonances are observed when the system is in slow exchange. However, only a single averaged resonance can be observed if the system is in fast exchange. The build-up rate of the NOE is slower for free-ligand resonances compared to bound resonances (Craik 1991).

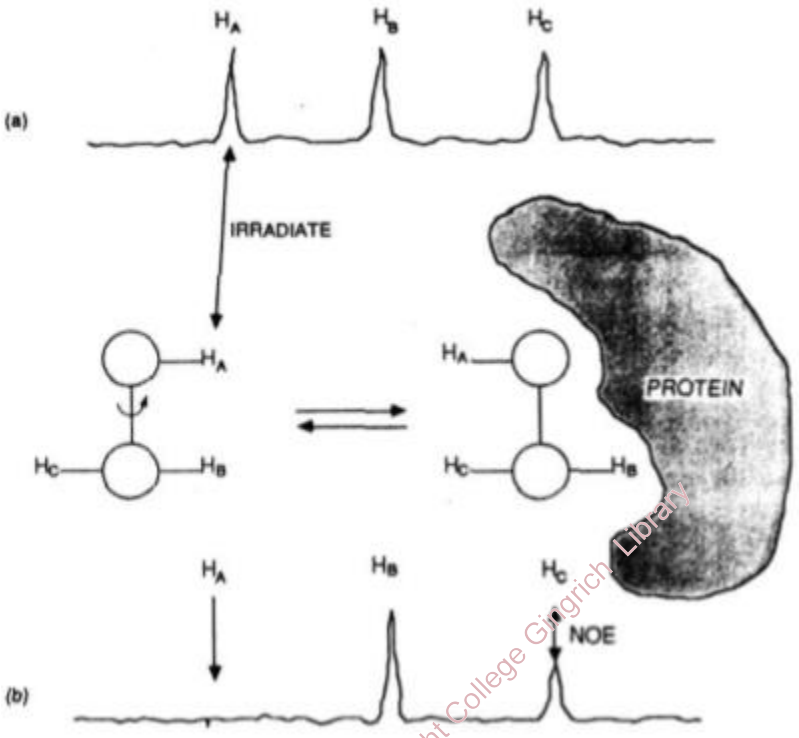

Figure 1: Schematic of NOE experiment (Craik 1991)

Yeast alcohol dehydrogenase (YADH) and adenosine monophosphate (AMP), seen in figure 2, were used as a model system because this interaction has previously shown a clear NOE by David Craik *et al.*(1991). Luciferase with adenosine triphosphate (ATP) was selected to be studied due to its importance in several biological assays as well as due to its easy accessibility. Both systems serve well as models to test the method of using NMR to study protein and ligand interactions.

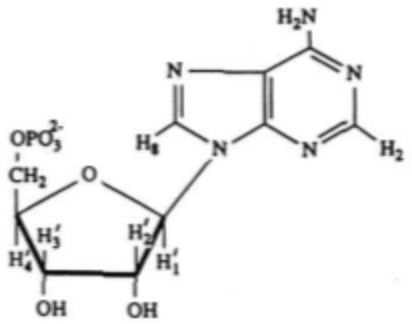

Figure 2: Structure of 5'-AMP in the anti-conformation around the glycosidic linkage (Craik, *et al.* 1991)

All solutions were prepared in deuterated solvents. To account for differences between pD and pH measurements, the conversion pD = pH + 0.4 was taken into consideration. To obtain solutions with a pD of 8.0, the measured pH was optimized to 7.6 (Krezel and Bal 2004).

3. Methods:

Solution Preparation:

Table 1: Components of solutions analyzed using NMR

Solution	D_2O (µL)	AMP (mM)	ATP (mM)	YADH (mM)	Phosphate Standard (mM)	10x Tris Buffer, pH 7.6 (µL)	pH
1	750	20	0	0	0	0	8.85
2	750	3.33	0	0.1	0	0	6.99
3	750	0	20	0	0	0	2.88
4	750	3.33	0	0.0333	0	0	7.05
5	750	1.1	0	0.0333	0	0	7.10
6	500	3.33	0	0.1	0	0	7.21
7	750	100	0	0	100	0	9.25
8	750	3.33	0	0.1	100	0	8.45
9	0	0	20	0	50	750	7.00
10	0	0	3.33	0.1	50	750	7.73

Solution	D_2O (µL)	AMP (mM)	ATP (mM)	YADH (mM)	Luciferase (mM)	1x Phosphate Buffer, pH 7.6 (µL)	pH
11	0	0	20	0	0	750	6.37
12	0	0	3.33	0.1	0	750	7.17
13	0	0	3.226	0	0.09677	500	7.21

Tris buffer preparation:
0.59278 g Tris HCl, 0.15120 g Tris base, 0.29283 NaCl, and 0.06011 $MgSO_4$ were dissolved in 10 mL D_2O. The pH was adjusted to 7.6 using 1.0 M DCl.

Potassium phosphate buffer preparation:
9.89 mg KH_2PO_4, 74.69 mg K_2HPO_4, 29.225 mg NaCl, and 6.0185 mg $MgSO_4$ were dissolved in 10 mL D_2O.

1D ^{1}H NMR:
Proton spectra were obtained for solutions 1-6 and 11-13 using the Varian Inova 400 MHz NMR spectrometer. 8 scans were obtained for each sample at 25 °C with a spin of 20 Hz.

1D ^{31}P NMR:
Phosphorous spectra were obtained for solutions 7-12 using the 400 MHz NMR spectrometer. 1024 scans were obtained for solutions 7-9 and 11 and 16384 scans for solutions 10 and 12. Each sample was tested at 25 °C with a spin of 20 Hz.

2D Correlated Spectroscopy (COSY):
COSY spectra were obtained for solutions 1 and 3 using the 400 MHz NMR spectrometer. 8 scans were obtained for each sample at 25 °C with no spin.

2D Nuclear Overhauser Effect (NOE):
NOE spectra were obtained for solutions 1-6 and 12-13 using the 400 MHz NMR spectrometer. For each corresponding proton spectrum, the decouple nucleus was set to hydrogen. A control frequency was obtained where no protons were present using the "set decouple" function to obtain the control frequency and chemical shift. The delta of the saturation frequency was found around the sugar proton signals from AMP or ATP. The function "set decouple" was also used to obtain the frequency and chemical shifts. A "cyclenoe" experiment was created and was arrayed using "satfrq" as the parameter to be arrayed with a total of 30 steps to be arrayed in increments of 10. The control frequency was entered under the "control" function. 32-64 scans were obtained for the samples at 25 °C with no spin.

4. Results and Discussion:

As seen in figure 3, the sugar proton signals can be seen around 3.8-4.6 ppm. These signal frequencies were to irradiate the corresponding sugar protons and to generate an NOE to the aromatic protons resulting in resonances, which appear around 8-9 ppm. Even with the addition of the protein, YADH, the sugar protons and aromatic protons signals can still be distinguished as seen in figure 4. Changing the concentration of AMP or YADH as well as changing the volume of the samples did not have much effect on the proton NMR spectra. The proton NMR spectra for solutions 4-6 resembled that for solution 2. As expected, the proton NMR spectra for ATP was very similar to that for AMP, as seen in figure 5. This is

expected as the proton environment does not drastically change between the two molecules. The sugar proton signals can be seen around 4.0-4.6 ppm which were again the frequencies used to irradiate the sugar protons and generate the through space affect to the aromatic protons generating and NOE in the aromatic range if there is proximity through space as a result of the bound conformation of the ligand ATP or AMP interacting with YADH. These NOE peaks are still distinguishable after the addition of the protein, luciferase, as seen in figure 6 in an analogous fashion to those in the AMP or ATP with YADH.

All phosphorous NMR spectra were obtained using sodium phosphate dibasic heptahydrate as a standard, referenced to 0 ppm. As seen in figure 7, the expected phosphorus peak for AMP was observed between 0 and 2 ppm that reflects the one phosphorous atom in the molecule. The addition of YADH to AMP resulted in figure 8. There was a minimal increase in chemical shift after the addition of the protein. However, there was an approximately 28% increase in linewidth after the addition of protein. This change in linewidth potentially correlates to the transition between free and bound ligand. As seen in figure 9, the expected phosphorus peaks for ATP was observed as three individual peaks between -7 and -23 ppm that reflect the three phosphorus atoms in the molecule. The addition of YADH to ATP resulted in figure 10. There was a minimal decrease, to a larger negative value, in chemical shift after the addition of the protein. However, there was an approximately 34%, 26%, and 3% increase in linewidth after the addition of protein. This change in linewidth, again, potentially correlates to the transition between free and bound ligand.

Figures 11 and 12 show the COSY spectra for AMP and ATP, which were utilized to assign all protons in each molecule. This two dimensional spectrum assisted in assigning the peaks in the proton NMR spectra of protein and ligand to the respective protons of AMP and ATP. From figure 11, it was determined that the peak at 3.93 ppm correlates to H4’ on the sugar ring, 4.27 ppm correlates to H3’ on the sugar ring, and 4.41 ppm correlates to the H2’ on the sugar ring of AMP. These peak frequencies were irradiated to generate the transfer NOE observed on the peaks at 8.09 and 8.43 ppm which correlate to H2 and H8 on the aromatic ring, respectively. From figure 12, it was determined that the peak at 4.13 ppm correlates to H4’ on the sugar ring, 4.31 ppm correlates to H3’ on the sugar ring, and 4.47 ppm correlates to the H2’ on the sugar ring of ATP. These peak frequencies were irradiated to generate the transfer NOE the peaks for which are observed at 8.13 and 8.39 ppm which correlates to H2 and H8 on the aromatic ring, respectively.

As seen in figure 13, a clear NOE was observed for the H8 proton at the three chemical shift frequencies that were irradiated. No NOE was observed for the H2 proton. This signifies that the magnetic environments between the sugar protons and the H8 proton are interacting, confirming that the anti-conformation is the optimal conformation for AMP binding to YADH. As seen in figure 14, an NOE was observed for the H8 proton at one of the irradiated frequencies appearing around 4.43 ppm. The absence of the NOE at the other irradiated frequencies may be because ATP is not the true ligand of YADH. This situation may leave more ATP in the unbound state, resulting in a lower intensity or nonexistent NOE. However,

since there is some transfer seen for the H8 proton and none for the H2 proton, it can be hypothesized that the anti-conformation is also the optimal conformation for ATP binding to YADH. As seen in figure 15, a clear NOE was observed for the H8 proton at two of the chemical shift frequencies that were irradiated. The absence of the NOE at the other irradiated frequency may be due to low concentrations of the protein, leaving more unbound ATP in solution. There is also a small NOE seen for the H2 proton that again, may be from unbound ATP. However, since the NOE intensity is greatest for the H8 proton, it can be hypothesized that the anti-conformation is also the optimal conformation for ATP binding to luciferase.

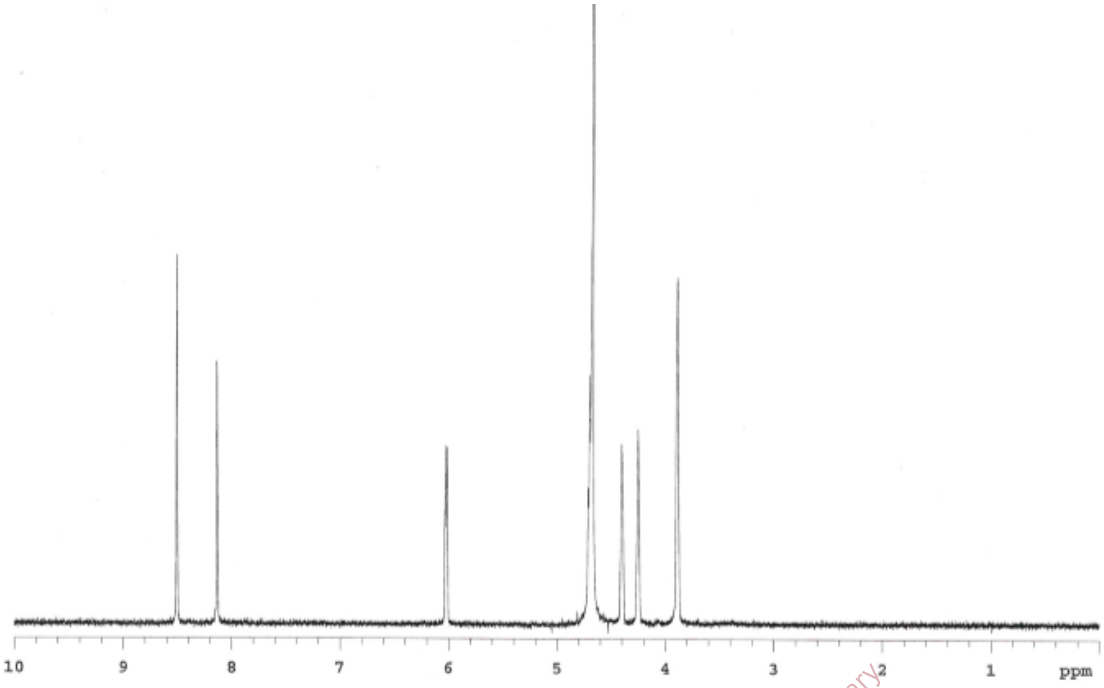

Figure 3: Proton NMR spectrum of solution 1 (20 mM AMP in D_2O)

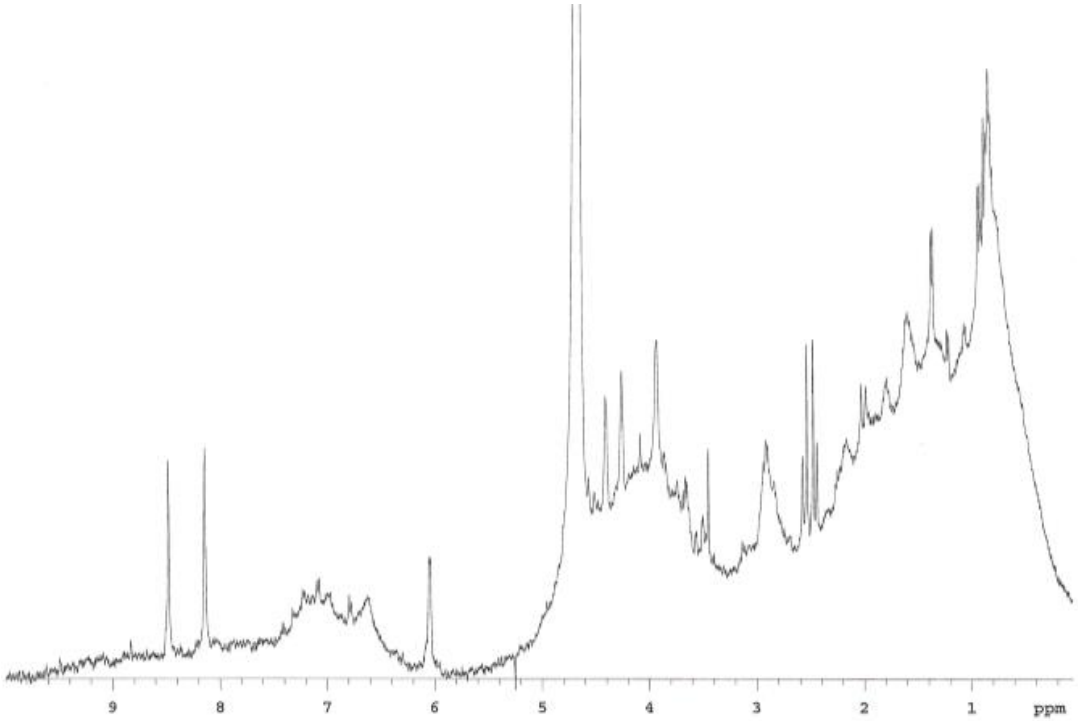

Figure 4: Proton NMR spectrum of solution 2 (3.33 mM AMP and 0.1 mM YADH in D_2O); irradiated sugar proton peaks between 3.81 and 4.5 ppm (solutions 4-6 and 11 displayed similar spectra)

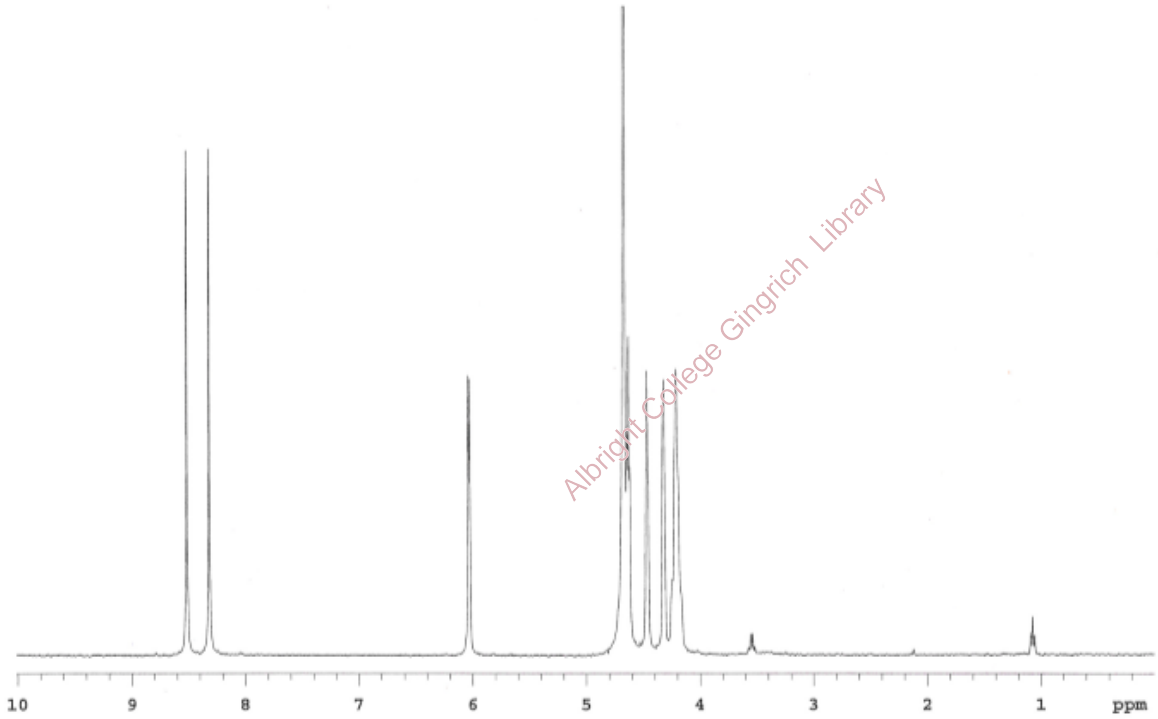

Figure 5: Proton NMR spectrum of solution 3 (20 mM ATP in D_2O)

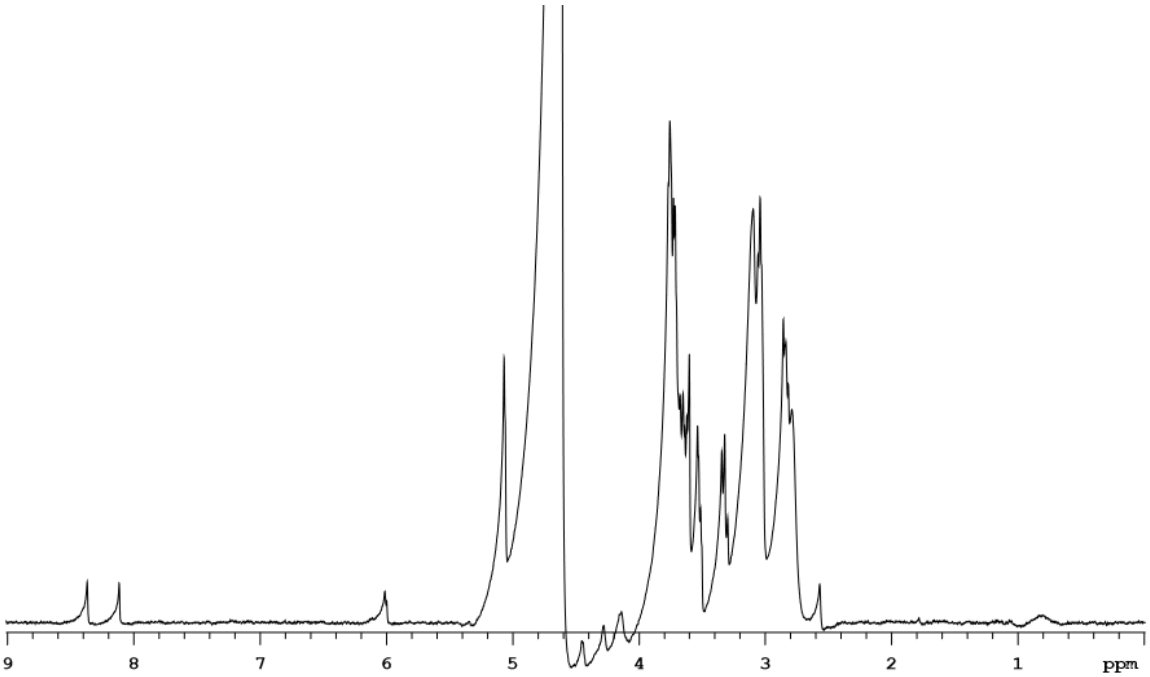

Figure 6: Proton NMR spectrum of solution 13 (3.226 mM AMP and 0.097 Luciferase in phosphate buffer); irradiated sugar proton peaks between 4.0 and 4.6 ppm

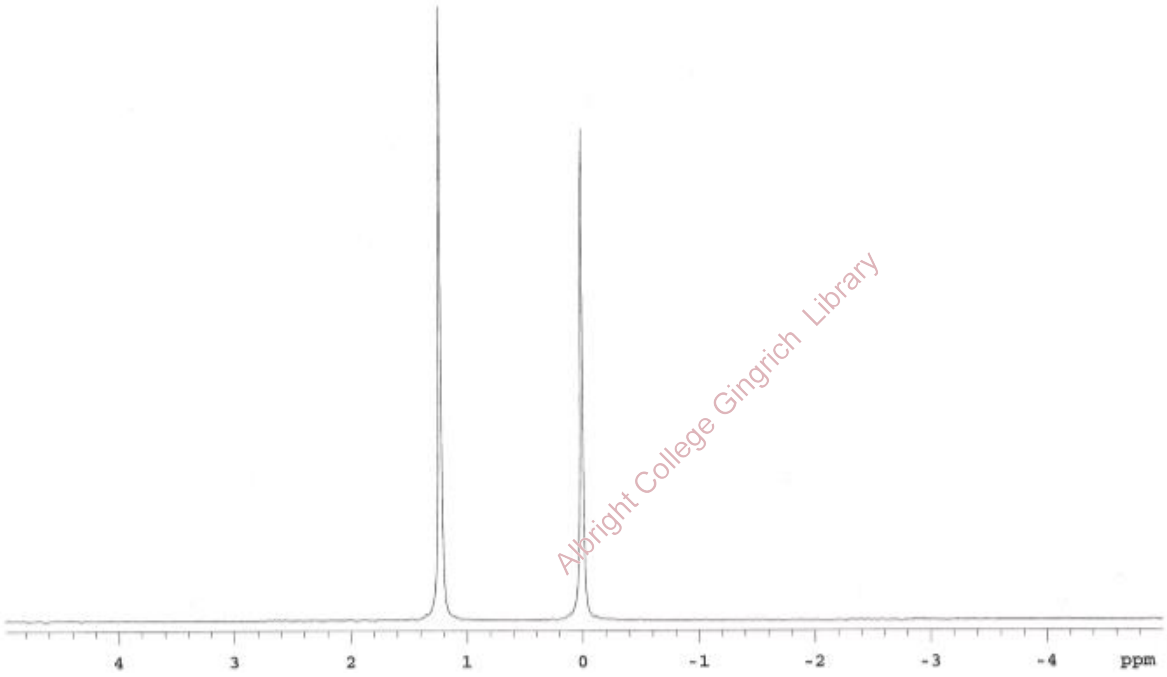

Figure 7: Phosphorus NMR spectrum of solution 7 (100 mM AMP with 100 mM phosphate standard in D_2O); 1.229 ppm, linewidth = 3.26084 Hz signal correlates to AMP

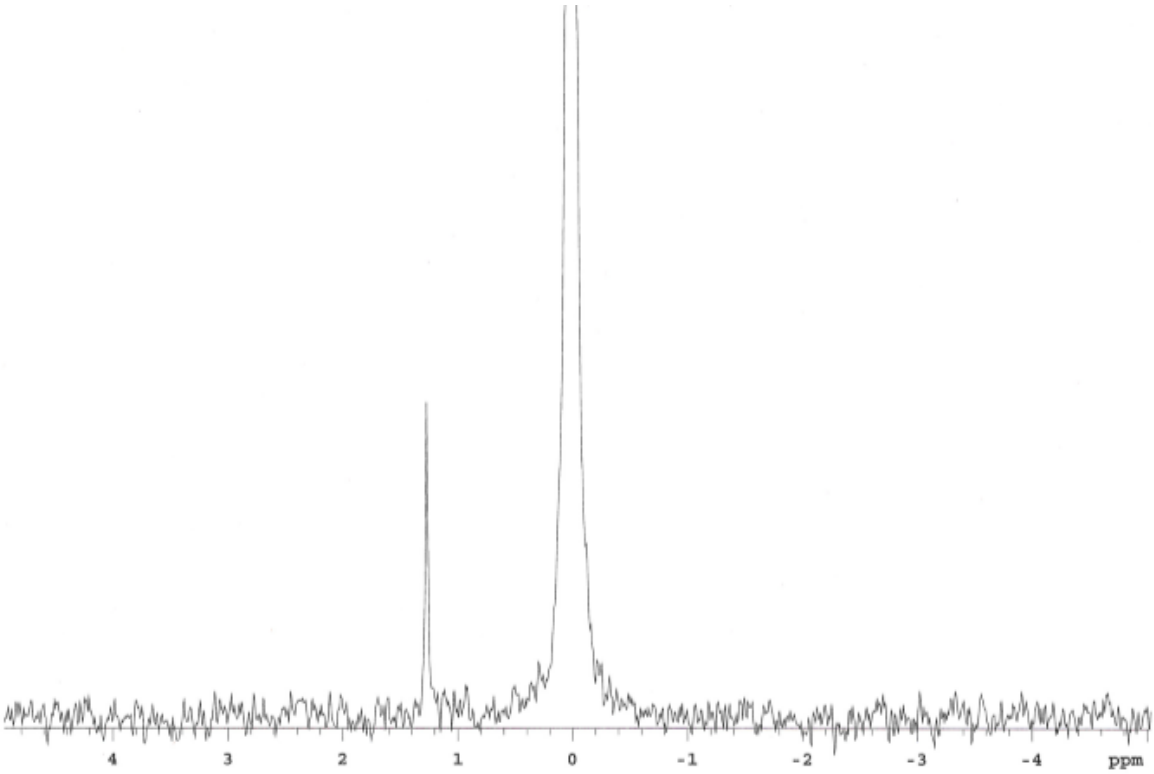

Figure 8: Phosphorus NMR spectrum of solution 8 (3.33 mM AMP and 0.1 mM YADH with 100 mM phosphate standard in D_2O); 1.2667 ppm, linewidth = 4.18183 Hz signal correlates to AMP

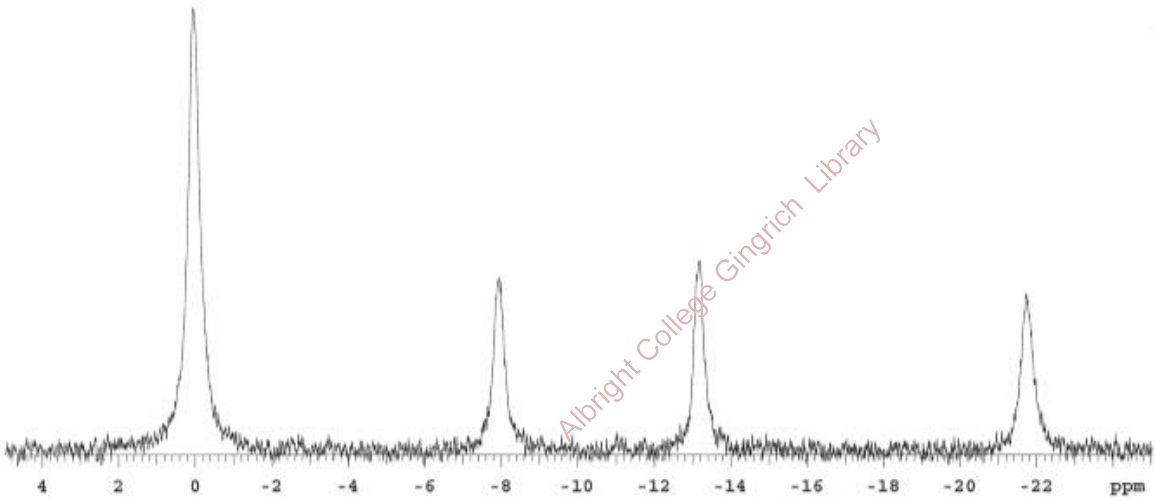

Figure 9: Phosphorus NMR spectrum of solution 9 (20 mM ATP with 50 mM phosphate standard in Tris buffer); -7.951 ppm, linewidth = 47.3124 Hz signal correlates to γ-phosphate; -13.209 ppm, linewidth = 45.5538 Hz signal correlates to α-phosphate, -21.725 ppm, linewidth = 55.1177 Hz signal correlated to β-phosphate

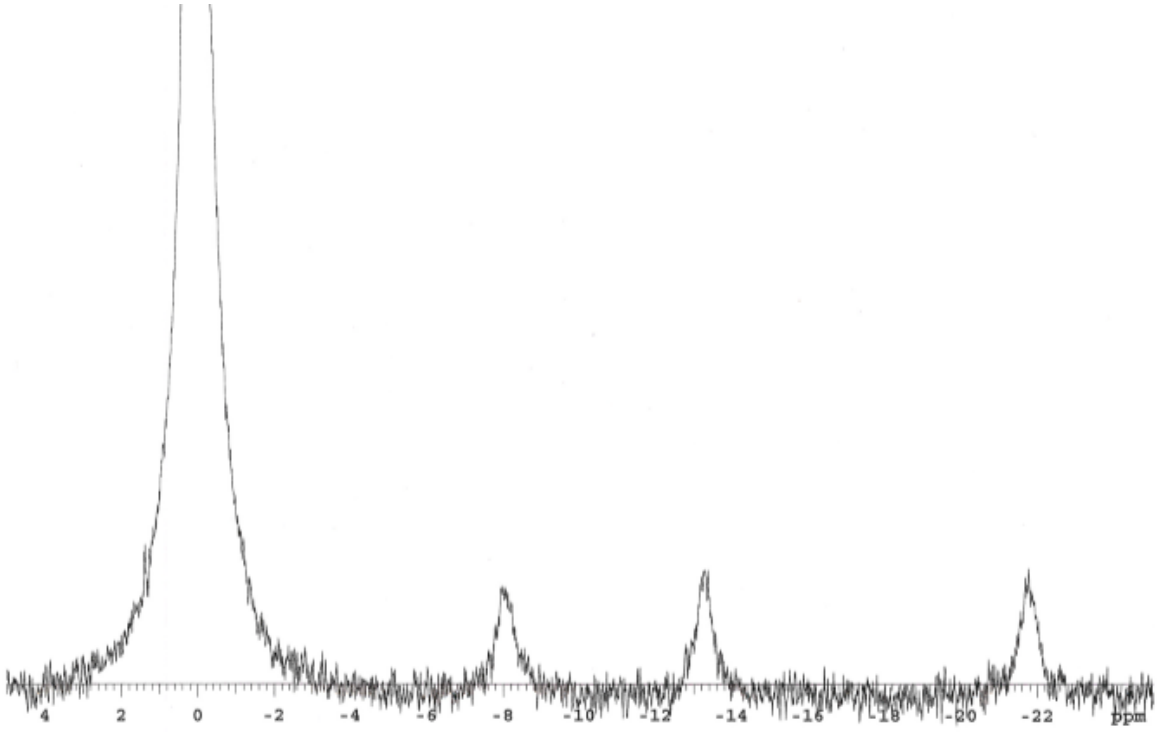

Figure 10: Phosphorus NMR spectrum of solution 10 (3.33 mM ATP and 0.1 mM YADH with 50 mM phosphate standard in Tris buffer); -7.966 ppm, linewidth = 63.6275 Hz signal correlates to γ-phosphate; -13.377 ppm, linewidth = 57.1766 Hz signal correlates to α-phosphate, -21.771 ppm, linewidth = 56.9169 Hz signal correlated to β-phosphate

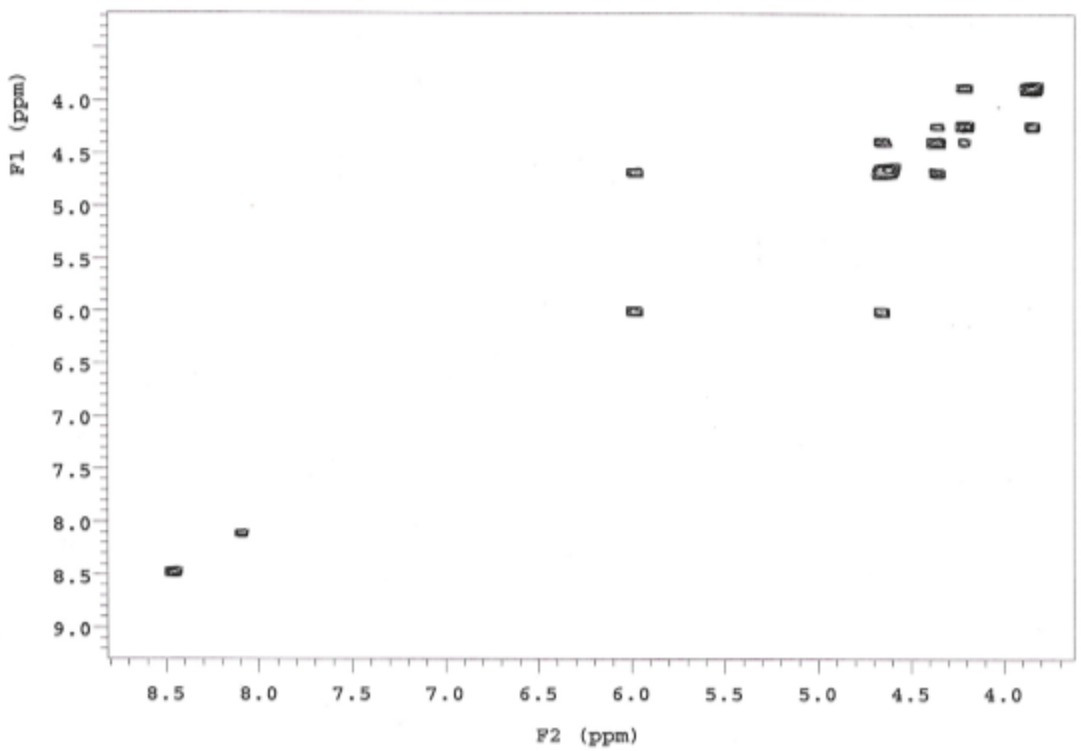

Figure 11: COSY spectrum of solution 1 (20 mM AMP in D_2O)

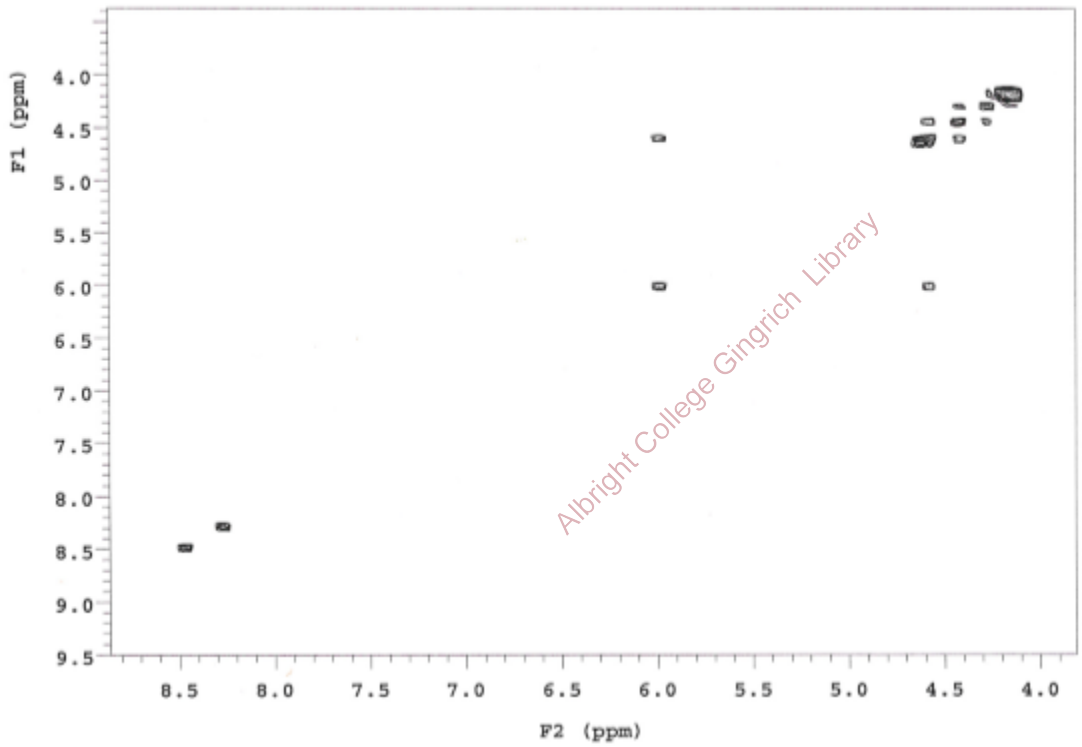

Figure 12: COSY spectrum of solution 3 (20 mM ATP in D_2O)

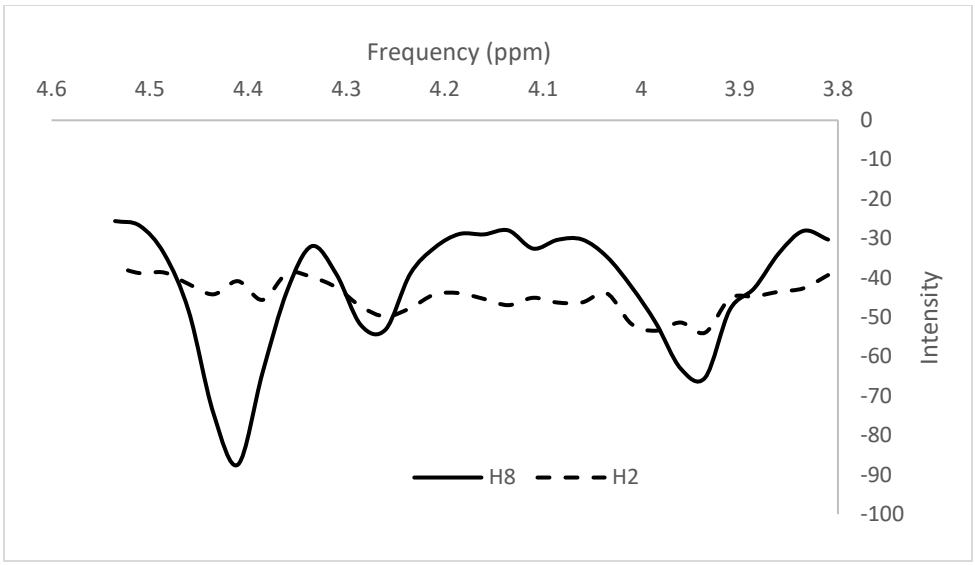

Figure 13: NOE of solution 2 (3.33 mM AMP and 0.1 mM YADH in D_2O)

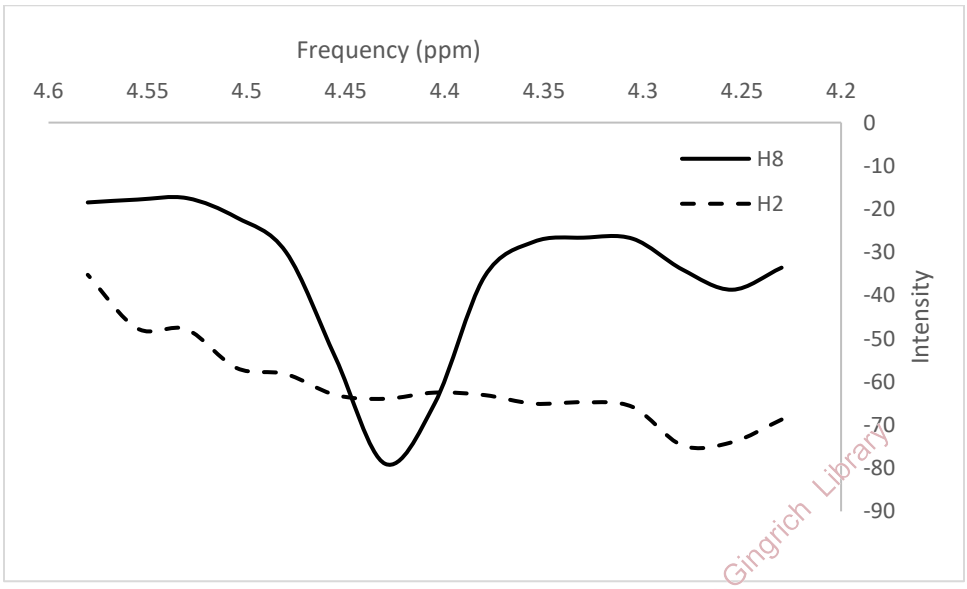

Figure 14: NOE of solution 12 (3.33 mM ATP and 0.1 mM YADH in phosphate buffer)

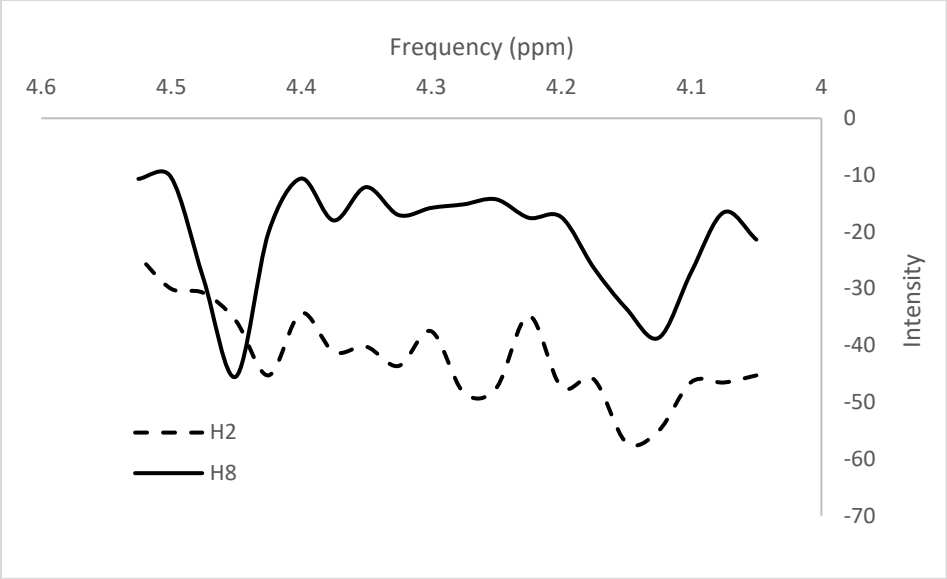

Figure 15: NOE of solution 13 (3.226 mM ATP and 0.09677 mM Luciferase in phosphate buffer)

5. Conclusion:

NMR spectroscopy is a viable method to study protein and ligand interactions. Yeast alcohol dehydrogenase with AMP as well as luciferase with ATP are notable model systems that can be used to investigate protein and ligand interactions using NMR spectroscopy. 1D proton NMR spectroscopy gave insight of the chemical environments of the ligand by itself as well as in the presence of protein. 1D phosphorus NMR spectroscopy revealed changes in the linewidth of free versus bound ligand. 2D COSY experiments allowed for signal to proton designations. Finally, 1D NOE experiments revealed that the H8 proton of AMP and ATP experience the transfer NOE when interacting with YADH as well as with luciferase. This led to the conclusion that AMP and ATP optimally bind to their respective proteins in the anti-conformation. These studies provide a possible method of study and serve a great importance to several scientific problems, including investigation of drug discovery and development.

6. References:

Clarkson, J. and Campbell, I.D. (2003) “Studies of protein-ligand interactions by NMR.” *Biochemical Society Transactions* **31.5**: 1006-1009.

Clore, G.M. & Gronenborn, A.M. (1982) “Theory and Applications of the Transferred Nuclear Overhauser Effect to the Study of the Conformations of Small Ligands Bound to Proteins” *Journal of Magnetic Resonance*. **48:**402-417.

Craik, D. J., Higgins, K. A., Kneen, M. M., Munro, S. L. A., and Waterman, K. J. (1991) "Determining the Conformation of a Ligand Bound to an Enzyme." *Journal of Chemical Education* **68.3**: 258-261.

Krezel, A. and Bal, W. (2004) "A formula for correlating pKa values determined in D_2O and H_2O." *Journal of Inorganic Biochemistry* **98:** 161-166.

Lian, Y., Jiang, H., Wang, X., Hou, X., and Deng, P. (2015) "Direct and simultaneous quantification of ATP, ADP, and AMP by ^{1}H and ^{31}P Nuclear Magnetic Resonance spectroscopy." *Talanta* **150**: 485-492.

Marques, S. M. and Esteves da Silva, J. C. G. (2009) "Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions." *Life* **61.1**: 6-17.

Min K. and Steghens J. P. (2001) "ADP is produced by firefly luciferase but its synthesis is independent of the light emitting properties." *Biochimie* **83**:523-528.

Odai, K., Nishiyana, S., Yoshida, Y., and Wada, N. (2009) "^{1}H NMR spectrum and computational study of firefly luciferin in dimethyl sulfoxide." *Journal of Molecular Structure* **901**: 60-65.

7. Acknowledgements:

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