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Modeling Thip;ate Enzyme Chemistry with Cysteine at a High pH

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MODELING THIOLATE ENZYME CHEMISTRY WITH CYSTEINE AT A HIGH pH

by

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Under the Direction of Kevin Williams

ABSTRACT

Thiolate is a highly reactive species critical to the function of many enzymes. We attempted to model the chemistry of two of these enzymes with cysteine in solution at a high pH. To study cysteine proteases, we increased the pH of a cysteine and 4-nitrophenylacetate solution to create a visible color change when thiolyzed that was monitored through UV-Vis spectroscopy to determine rate constants. To study transferases, we attempted to model two reactions. The first was a methyl transfer from guanine to cysteine which mimicked O^6-alkylguanine-DNA alkyltransferase. The second was activation of halogenated hydrocarbons by a mechanism similar to glutathione S-transferase. Samples were analyzed by NMR spectroscopy. Results showed change in rate constants with the addition of cysteine in the protease reactions and possible formation of products in the activation of halogenated hydrocarbons. Attempts to model methyl transfer were not successful.

INDEX WORDS: Thiolate, Cysteine, Cysteine protease, Halogenated hydrocarbon, O^6-alkylguanine-DNA alkyltransferase, Glutathione S-transferase

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INTRODUCTION

The amino acid cysteine ($C_3H_7O_2NS$) is a critical component of proteins. Cysteine is synthesized in the body from serine and methionine. The presence of a thiol, or sulfur-hydrogen bond, in its side chain makes cysteine a highly reactive, nucleophilic amino acid. In an enzyme the pKa of cysteine is below physiological pH, so it is in its most active form—known as a thiolate—at most times in the body. Cysteine is critical as a structural component of proteins due to its ability to form disulfide bonds with other cysteine residues, a necessary component to maintaining secondary structure and enzyme function. Cysteine is most abundant in the proteins in hair, hooves, and in keratin in the skin. Cysteine's reactivity also gives it antioxidant properties. (Berg et al., 2007)

Cysteine proteases are a class of enzymes that contain a cysteine residue and a histidine residue in their active site. The imidazole of histidine deprotonates the thiol of cysteine creating a thiolate, a strong nucleophile that attacks the substrate. (Berg et al., 2007) Cysteine proteases have numerous vital functions. They are responsible for protein turnover occurring in lysosomes and proper functioning of the immune system. They are also common participants in digestion, apoptosis, and coagulation. Recent research has found a link between cysteine proteases and lung functioning in diseases like emphysema. (Chapman et al., 1997)

Alkyltransferases are another class of enzymes that use a nucleophilic thiolate pathway to repair alkylated DNA bases. O^6-methylguanine is repaired by the protein O^6-alkylguanine-DNA alkyltransferase (AGT). A histidine residue hydrogen bonds with a cysteine residue, which aids in the deprotonation and formation of a thiolate. Again, the

thiolate acts as a nucleophile and displaces the methyl group attached to the guanine base. (Pegg 2000)

Alkyltransferases are of particular interest due to their ability to repair the negative effects of many common alkylating agents like halogenated hydrocarbons, which cause mutations, cancerous growth, and cell death. 1,2-Dibromoethane (DBE) is one example of an alkylating agent that has been studied. It was used as a pesticide and fumigant until the EPA banned most of its uses in 1984. Studies have shown it to be carcinogenic in rats and mutagenic in many species. It was expected that expression of AGT would repair the damage done by this alkylating agent. However, researchers have found that the overexpression of AGT has an unexpected effect on the toxicity of DBE: it increases the toxic effect of the compound. Thus, cells containing excess amounts of the repairing agent actually witness increased cell death. (Liu et al. 2002)

It is known that the tripeptide glutathione (GSH) when conjugated to DBE causes mutagenic adducts through a process catalyzed by glutathione S-transferase (GST) (Guengerich, 1992). It forms a glutathionyl half-mustard that then undergoes dehalogenation forming an episulfonium ion. It then reacts quickly with DNA or water. GST should have been repairing the damage, but it actually increases cytotoxicity when overexpressed in E. coli, as well as other species including humans. Because of the similar result witnessed in AGT, it is believed that these two occur via comparable mechanisms. (Liu et al., 2002; Liu et al., 2004; Liu et al., 2007)

In the experiments discussed here, a simple model was sought that would simplify the study of these reactions involving cysteine residues. Most studies of these pathways required complex procedures. In an attempt to simplify this process, I focused only on the

cysteine residue. It was expected that at a high pH that would deprotonate the cysteine residue, enhanced activity would be witnessed in the model as is evidenced in reactions with the aforementioned enzymes.

In part one of the experiments, I attempted to model the activity of cysteine proteases quantitatively. The rate of this reaction can be determined using 4-nitrophenylacetate (4-NPA) as the substrate by spectrophotometric analysis (Fig. 1). (Street et al., 1985) As the 4-NPA is cleaved by the thiolate it produces a yellow product, 4-nitrophenolate, which can be measured quantitatively with UV-Vis spectroscopy. Once the rate constants were defined, a relationship between cysteine concentration and rate of thiolysis could be observed. It was expected that rate constants would increase as cysteine concentration in the samples increased.

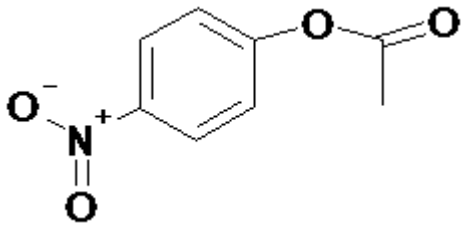

Figure 1: 4-Nitrophenylacetate

In part two of the experiments, I attempted to model the activity of AGT (Fig. 2). I used ^{1}H NMR spectroscopy to observe the spectra of the reactants, O^{6}-methylguanine and cysteine, and the expected product if the alkyl transfer was successful, S-methylcysteine. Again, it was expected that at high pH the thiolate would displace the alkylated guanine through the proposed chemical pathways. This transfer would be

visible upon comparison of the experimental sample with the prepared spectra of the expected products.

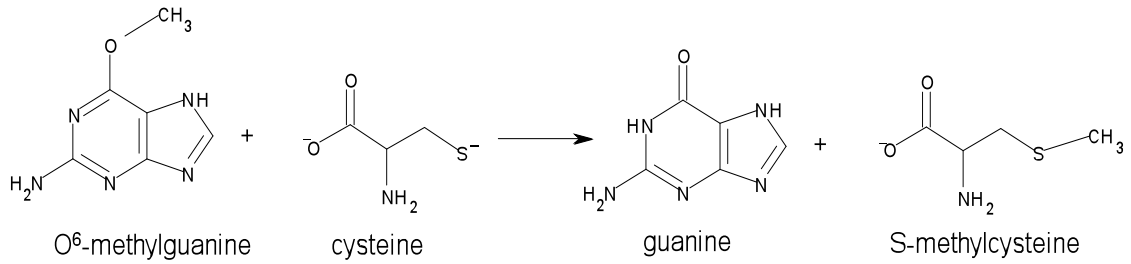

Figure 2: Methyl Transfer Reaction-The thiolate form of cysteine shown above is formed in the body when the sulfur is deprotonated by a histidine residue in the active site of the enzyme. This research sought to create the same reaction with cysteine in solution at a high pH.

In part three of the experiments, I used various forms of cysteine to model the pathway that causes transferases like GST and AGT that should be repairing alkyl damage to instead become cytotoxic. I used three forms of cysteine: normal cysteine, cysteamine (Fig. 3a), and N-acetylcysteine (Fig. 3b) to determine which form created the desired product. I created the experimental sample by mixing the form of cysteine with DBE. To determine if the chemical pathways occurring in the experimental sample were those previously described, I also prepared a sample with the same form of cysteine and 2-bromoethanol (2-BE). This product would show similar NMR signals as that of the expected product in the experimental sample (Fig. 4). Again, NMR spectroscopy was used to determine if the formation of the desired product occurred. It was hypothesized that the expected product could be formed by the thiolate created by deprotonating the various forms of cysteine.

Nuclear magnetic resonance (NMR) spectroscopy is a commonly used tool when identification of compounds is desired. This technique requires a minute amount and does

not harm the sample. NMR works by utilizing the nuclear spin that occurs in atoms with an odd atomic number. The nuclear spin of the proton creates a magnetic field similar to a bar magnet. When a larger external magnetic field is applied to these atoms, the protons will move so their magnetic field is aligned with the external field or against the external field. Alignment with the field is a lower energy state and is therefore favored. If the atom absorbs enough energy, however, it can flip to the higher energy state. When this occurs it is known as resonance. NMR spectroscopy measures this energy and plots it on a spectrum. This spectrum can be used to identify compounds. (Wade, Jr., 2006)

In molecules, many factors affect the energy required to create resonance. The electrons that surround the affected proton, for example, create a condition known as shielding. The electron cloud rotates as the external magnetic field is applied and generates a magnetic field that is opposed to the external field. Therefore, the proton experiences a weaker external magnetic field and is thus shielded. This increases the necessary field strength in order to produce resonance and shifts the position of the proton's signal on the spectrum. Nearby atoms also affect shielding in a similar way. This can create complex signals that are split into doublets, triplets, and so forth. This will be witnessed later. (Wade, Jr., 2006)

Figure 3: Cysteamine (a) and N-acetylcysteine (b)

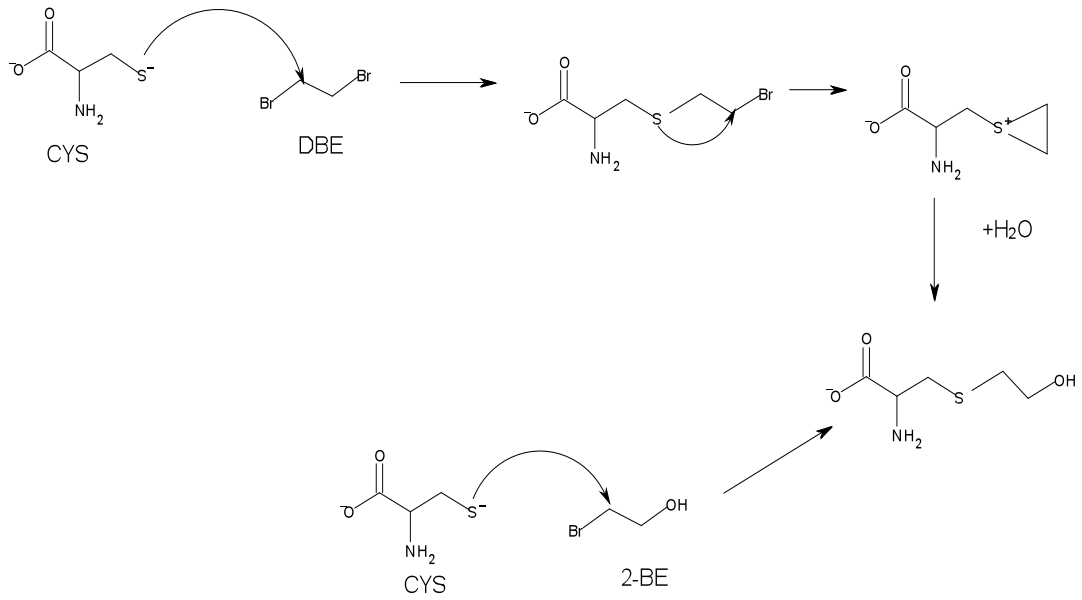

Figure 4: Mechanism for Halogenated Hydrocarbon Reactions-This reaction mechanism shows the reaction between cysteine and DBE that we attempted to model. Because the reaction between cysteine and 2-BE forms the same product, we were able to use this reaction to compare the spectra and determine if the desired product was formed. The mechanism is comparable for cysteamine and N-Acetylcysteine.

METHODS

Cysteine Proteases

4-NPA stock solution was prepared by dissolving 5 mg 4-NPA in 10 mL H_2O and stirring vigorously until dissolved. Cysteine stock was prepared by dissolving 3 mg cysteine in 10 mL H_2O and stirring vigorously until dissolved. Samples were prepared by removing aliquots of the 4-NPA stock and adding various volumes of cysteine to a vial. The samples were diluted to a volume of 1 mL with a 1 mM phosphate buffer pH 7. The vials were capped and inverted and immediately transferred to a cuvette. Absorbencies were measured at 400 nm using a Shimadzu UV-1501 spectrophotometer.

A new cysteine stock was prepared by dissolving 5 mg cysteine in 10 mL H_2O and stirring vigorously until dissolved. 4-NPA stock was prepared as above. Samples were prepared by removing 10 μL aliquots of 4-NPA and adding various volumes of cysteine (0 μL, 30 μL, 60 μL, 100 μL, 200 μL) to a vial. The samples were diluted to a volume of 1 mL with Acros pH 10 buffer. Each vial was capped and inverted and immediately transferred to a cuvette. Absorbencies were measured at 400nm using a Shimadzu UV-1501 spectrophotometer.

Rate constants were determined using the software DynaFit (BioKin, Ltd.). The following mechanism was used:

S + NPA --> SA + NP : kc

S = Cys (total), NPA = nitrophenylacetate, SA= S-AcCys, NP = p-nitrophenylate (color)

NPA --> A + NP : kh

A = acetate (this is base catalyzed reaction)

O^6-methylguanine Transfer

13.5 mg of cysteine and 1.65 mg O^6-methylguanine were dissolved in 1 mL of D_2O each. A sample was prepared by mixing the two solutions and the pH was raised to 10 using sodium deuteroxide (NaOD). A JEOL Eclipse 500 MHz NMR instrument was used to characterize the sample. Samples of S-methylcysteine, cysteine, and O^6-methylguanine were prepared for comparison of NMR spectra at similar concentrations.

Halogenated Hydrocarbons

12.1 mg cysteine was dissolved in 1 mL of D_2O. 8.6 μL of DBE was added. NaOD was added to bring the pH to 10. For comparison, 12.1 mg cysteine was dissolved in 1mL of D_2O and 7.1 μL of 2-BE was added. NaOD was added to bring the pH to 10.

7.71 mg cysteamine was dissolved in 1 mL of D_2O. 8.6 μL of DBE was added. NaOD was not needed because the pH was already 10. For comparison, 7.71 mg cysteamine was dissolved in 1 mL of D_2O and 7.1 μL of 2-BE was added. Again, no NaOD was added because the pH was already 10.

16 mg N-acetylcysteine was dissolved in 1 mL of D_2O. 8.6 μL of DBE was added. NaOD was added to bring the pH to 10. For comparison, 16 mg N-acetylcysteine was dissolved in 1 mL of D_2O and 7.1 μL of 2-BE was added. NaOD was added to bring the pH to 10.

Each of the previous samples was prepared using 9.81 μL bromomethylacetate instead of DBE. A sample of acetic acid was prepared for comparison.

An aliquot of each sample was placed in an NMR tube and measured over time using a JEOL Eclipse 500 MHz NMR instrument.

RESULTS

Cysteine Proteases

At pH 7, the reaction between 4-NPA and cysteine showed no significant increase in absorbencies. Thus, reliable rate constants could not be determined.

At pH 10, the measured absorbencies from the range of cysteine concentrations ranged from 0.120 to 0.225 over a time period of ten minutes. These absorbencies increased over time with total increases ranging from 0.05 to 0.09. The second order rate constants (units: $mM^{-1}\ s^{-1}$) obtained from the samples containing various amounts of cysteine are in Table 1.

Table 1: Rate Constants and Cysteine Concentrations

Cysteine amount	Rate constant, k ($mM^{-1}\ s^{-1}$)
0 μL	2.00×10^{-4}
30 μL	1.00×10^{-6}
60 μL	0.474
100 μL	0.0461
200 μL	0.0684

O^6-methylguanine Transfer

The ^{1}H NMR spectrum of O^6-methylguanine (Fig. 6) showed the typical guanine signals with a large signal at 4.1 ppm for the methyl group attached at O^6. The spectrum for cysteine (Fig. 5) showed a doublet of doublets at 2.95 ppm, another at 2.7 ppm and another at 3.46 ppm. The spectrum of S-methylcysteine (Fig. 7), which was the expected product if the methyl transfer was successful, showed the typical cysteine signals mentioned above but shifted downfield and the methyl signal at 2.1 ppm.

The ^{1}H NMR spectrum of our experimental sample (Fig. 8) containing O^6-methylguanine and cysteine showed no peak at 2.1 ppm which would have signaled the transfer of the methyl group to the cysteine. NMR spectra were taken over seven days to insure change had not occurred.

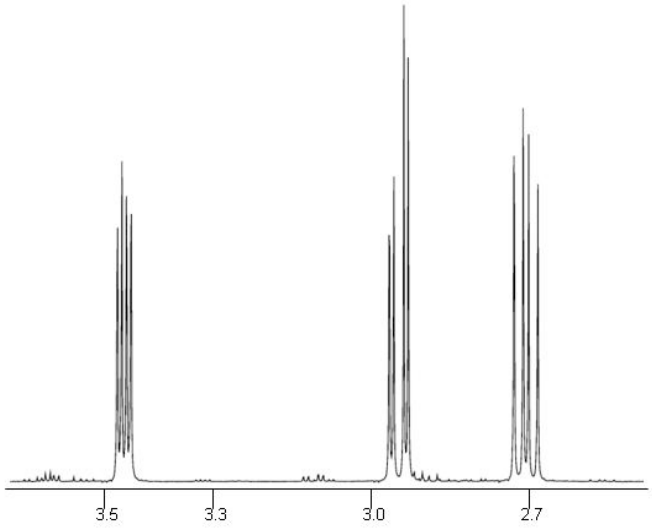

Figure 5: Cysteine at High pH

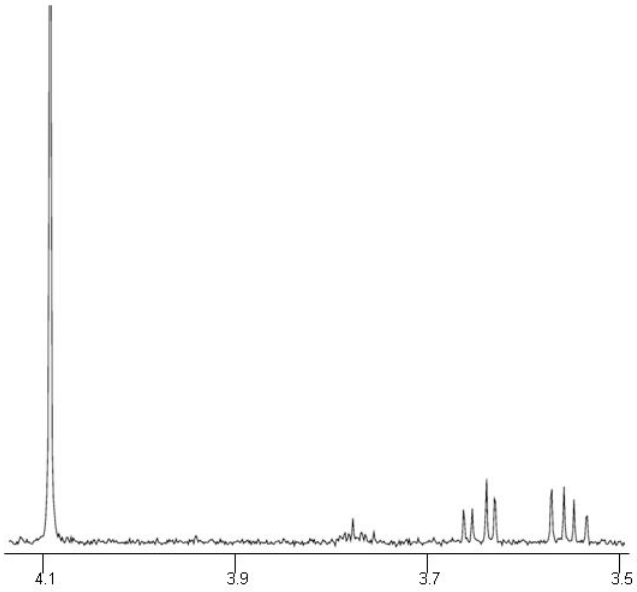

Figure 6: O^6-methylguanine at High pH

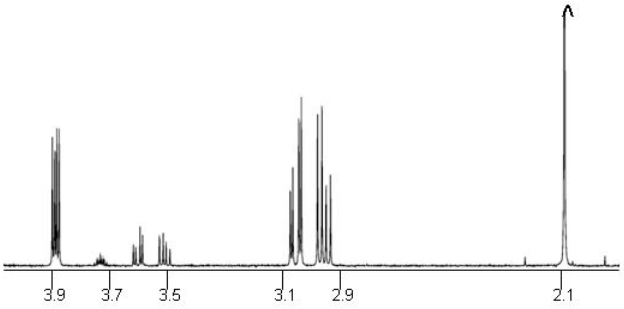

Figure 7: S-methylcysteine

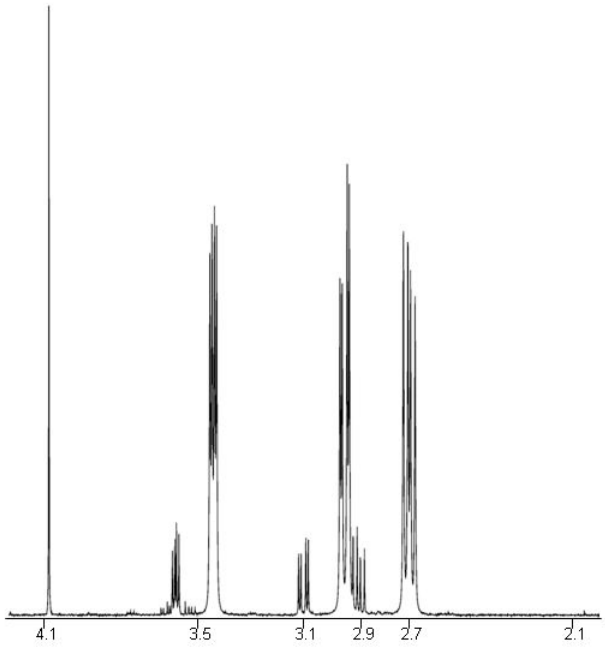

Figure 8: O^6-methylguanine and Cysteine

Halogenated hydrocarbons

After seven days at high pH, the ^{1}H NMR spectra of cysteine and 2-BE showed signals of the expected product (Fig. 9). These include the α-H at 3.85 ppm and two doublets of doublets at 3.0 ppm and 3.1 ppm possibly from the β-H. The two signals at 3.75 ppm and 2.75 ppm were triplets in the initial spectra but showed more complex splitting as time passed. It is thought these are the hydrogens between the sulfur atom and hydroxyl group in the expected product.

However, the ^{1}H NMR from the cysteine and DBE sample showed a large singlet at 2.85 ppm signaling the formation of a cysteine dimer (Fig. 10). This dimer forms a singlet due to its symmetrical nature. The four hydrogens between the sulfide atoms are all equivalent, so they display a single signal. Further sampling showed a reduction in the

formation of this dimer when cysteine concentrations were decreased to one tenth the original concentration. The large peak at 3.75 ppm is unreacted DBE.

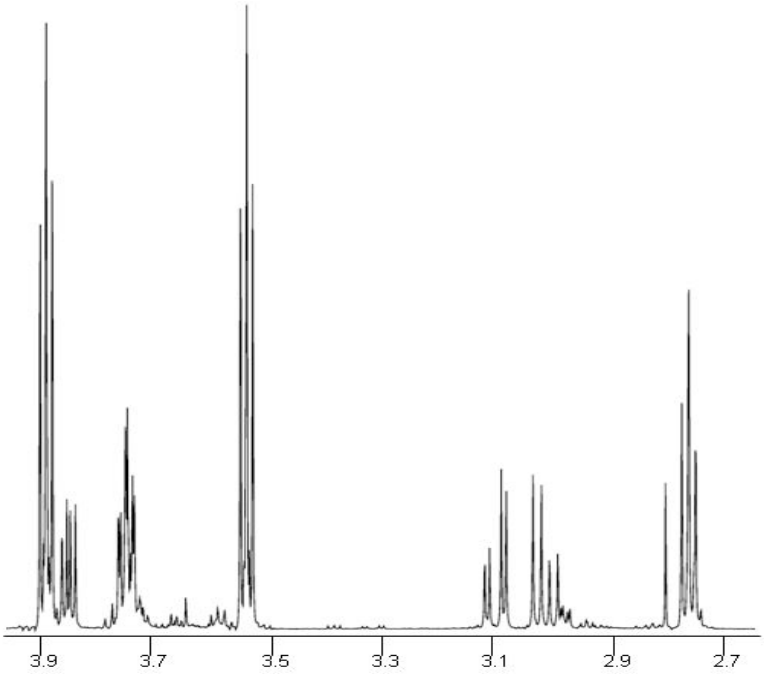

Figure 9: Cysteine and 2-BE

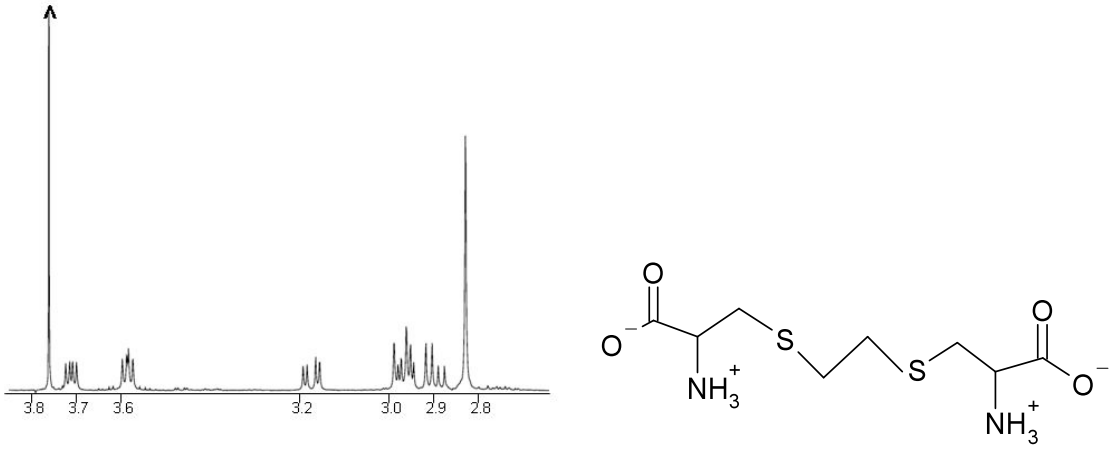

Figure 10: Cysteine and DBE--NMR Spectra and Dimer

After seven days at high pH, the ^{1}H NMR from the cysteamine and DBE sample (Fig. 11) showed the possible formation of two products. One possible product—a

cysteamine ring structure— would signal two triplets, and another—a cysteamine chain structure— would show 2 triplets and a singlet (Fig. 11a and 11b). These signals could be the triplets present at 2.83 ppm and 2.67 ppm with another triplet and singlet combining their signals at 2.7 ppm. The sample of cysteamine and 2-BE (Fig. 12) showed the characteristic signals of residual 2-bromoethanol as triplets at 3.4 ppm and 3.75 ppm as well as residual cysteamine signals at 2.88 ppm and 2.52 ppm. Six triplets were present at 3.6 ppm, 3.0 ppm, 2.9 ppm, 2.75 ppm, 2.65 ppm, and 2.6 ppm which are evidence of the formation of two products. When temperature of the sample was held at 25°C only four triplets were present (Fig. 13).

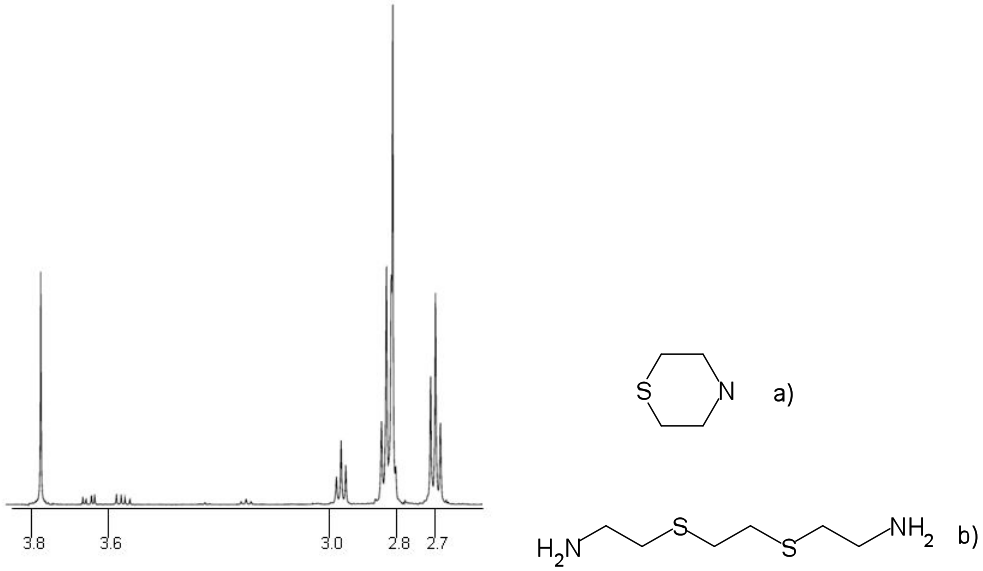

Figure 11: Cysteamine and DBE with Possible Products (a) and (b)

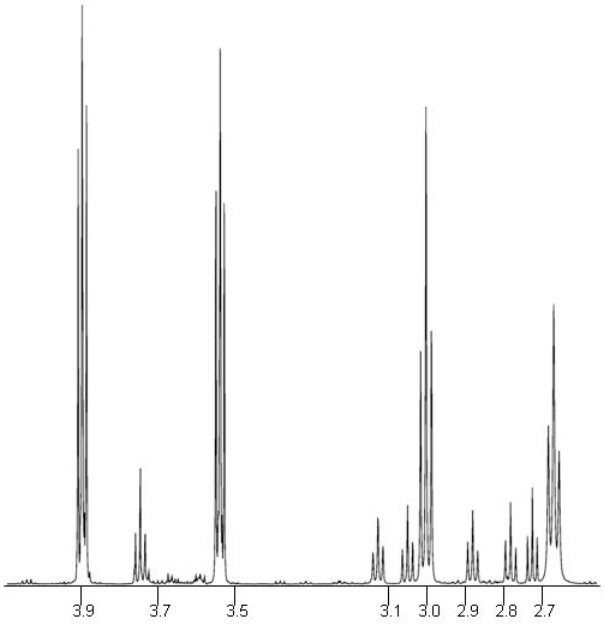

Figure 12: Cysteamine and 2-BE

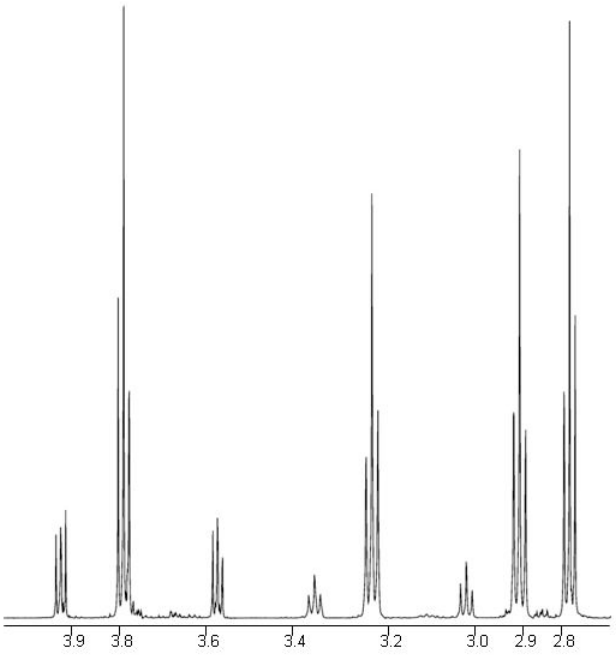

Figure 13: Cysteamine and 2-BE--Temp 25°C

After seven days at high pH, the experimental and expected product samples using N-Acetylcysteine showed very similar signals. Three signals at 4.35 ppm, 3.05 ppm, and 2.90 ppm are similar in both the expected product sample with 2-BE (Fig. 15) and the experimental sample containing DBE (Fig 14). The signals appearing at 2.1 ppm correspond to the acetyl group.

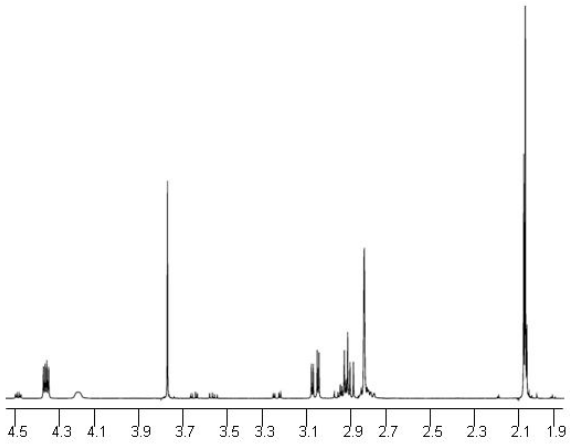

Figure 14: N-acetylcysteine and DBE

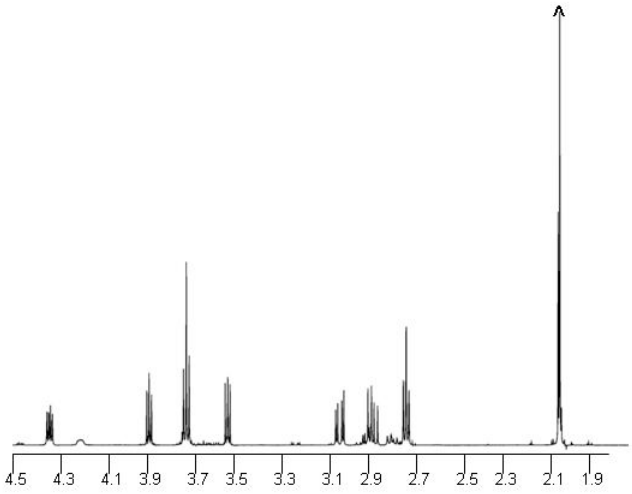

Figure 15: N-acetylcysteine and 2-BE

The samples prepared with bromomethylacetate would have shown a clear acetic acid signal on the spectra when thiolyzed simplifying the interpretation. However, this was inconclusive because the acetate group was immediately hydrolyzed when the pH was increased and thus it was not possible to detect the displacement of the acetate by the thiolate separately.

DISCUSSION

It was hypothesized that the activity of thiolate-containing enzymes could be modeled with cysteine in solution at a high pH, specifically that activity would increase in the experiments when the cysteine was deprotonated in reactions similar to that of the enzymes. In the first experiment looking at the activity of cysteine proteases, it was apparent that cysteine addition affected the rate constants--increasing them above that of the base-catalyzed hydrolysis of 4-NPA. However, there was not a concentration based dependency on those rate constants due to unknown causes.

The attempt to model the methyl group transfer accomplished by AGT was not successful. ^{1}H NMR analysis showed there was no transfer of the methyl group from O^{6}-methylguanine to cysteine. This would imply that the activity of these enzymes is based on factors other than the activation of the thiolate alone, and these factors could not be modeled in solution.

In attempting to model halogenated hydrocarbon chemistry, it became apparent that the form of cysteine used had a tremendous effect on the product formed. When cysteine was used in the experimental sample, a large portion of the product formed was a cysteine dimer that was not as prevalent when cysteine concentrations were decreased. This dimer is not relevant to biological studies because its formation would require two proteins to dimerize. In our model the two free cysteine residues could dimerize easily, but this would be difficult to achieve with entire proteins. When cysteamine was used, multiple side-products formed. This could be due to the ability of nitrogen to act as a nucleophile and again the tendency of cysteine to form dimers in solution. Further testing could possibly identify these side products. However, the use of N-acetylcysteine

provided NMR spectra with similar signals, thus proposing a similar product formed in both the experimental and expected products. Though not in high concentrations, the similar signals evidenced between the expected product and experimental samples showed that N-acetylcysteine was the most promising form of cysteine used to model the pathways that cause the activation of transferases.

These experiments show that thiolate enzyme chemistry can be modeled with cysteine at high pH in certain circumstances. However, achieving the desired products in substantial amounts is difficult. Enzymes are highly specialized to function at maximum efficiency, and in solution conditions are too variable to maintain completely. Further studies could examine the use of different buffers to maintain pH sufficiently and more thorough identification of side products to better determine how to prevent their formation.

REFERENCES

Berg, J., Tymoczko, J., and Stryer, L. 2007. Biochemistry. Sixth Ed. W. H. Freeman and Company. New York

Chapman, H., Riese, R., and Shi, G. 1997. Emerging Roles for Cysteine Proteases in Human Biology. Annual Review of Physiology. Vol. 59 pp. 63-88

Guengerich, F. 1992. Metabolic Activation of Carcinogens. Pharmacology and Therapeutics. Vol. 54 pp. 17-61.

Liu, L., Hachey, D., Valadez, G., Williams, K., Guengerich, F., Loktionova, N., Kanugula, S., and Pegg, A. 2004. Characterization of a Mutagenic DNA Adduct Formed from 1,2-Dibromoethane by O^6-Alkylguanine-DNA Alkyltransferase. The Journal of Biological Chemistry. Vol. 279 No. 6 pp. 4250-4259.

Liu, L., Pegg, A., Williams, K., and Guengerich, F. 2002. Paradoxical Enhancement of the Toxicity of 1,2-Dibromoethane by O^6-Alkylguanine-DNA Alkyltransferase. The Journal of Biological Chemistry. Vol. 277 No. 40 pp. 37920-37928.

Liu, L., Watanabe, K., Fang, Q., Williams, K., Guengerich, F., and Pegg, A. 2007. Effect of Alterations of Key Active Site Residues in O^6-Alkylguanine-DNA Alkyltransferase on Its Ability To Modulate the Genotoxicity of 1,2-Dibromoethane. Chemical Research in Toxicology. Vol. 20 No. 1 pp. 155-163.

Pegg, A. 2000. Repair of O^6-alkylguanine by alkyltransferases. Mutation Research Vol. 462 pp. 83-100.

Street, J., Skorey, K., Brown, R., and Ball, R. 1985. Biomimetic Models for Cysteine Proteases. 3. Acylation of Imidazolium—Thiolate Zwitterions by *p*-Nitrophenylacetate as a Model for the Acylation Step and Demonstration of Intramolecular General-Base-Catalyzed Delivery of H2O by Imidazole to Thiol Esters as a Model for the Deacylation Step. Journal of the American Chemical Society. Vol. 107 No. 25 pp.7669-7679

Wade, Jr. L. 2006. Organic Chemistry, Sixth Ed. Pearson Prentice Hall. Upper Saddle River, NJ.