CRYSTALLIZATION OF PHOSPHONO-CHEY FROM *THERMOTOGA MARITIMA*

Cory James Bottone

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Approved by

Advisory Committee

_____Paulo Almeida_____   _____Pamela Seaton_____

_____Christopher Halkides_____
Chair

Accepted by

_____
Dean, Graduate School

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ABSTRACT

An analog to the active form of the signal transduction protein CheY is produced, purified, and crystallized herein. The analog, which was named phosphono-CheY, is synthesized to mimic the *in vivo* active form of CheY known as P-CheY; P-CheY only has a half-life of ~30 seconds, making it extremely difficult to study. P- CheY is produced by the phosphorylation of an aspartate residue on CheY by the histidine kinase CheA. Subsequently, P-CheY binds to the flagellar motor protein FliM and switches the flagella's rotational direction. P-CheY becomes dephosphorylated by the phosphatase CheZ (in *E. coli)* or the phosphatases CheC/D, CheX, FliY/N (in *T. maritima*) to change the flagella rotation back to its previous state. The binding and release of P-CheY from FliM causes the bacteria to exhibit periods of smooth swimming and tumbling motions which drive the bacterium out of harsh environments and into nutrient rich ones; these events define bacterial chemotaxis. phosphono-CheY is synthesized in order to replace the labile P-O bond in P-CheY with a stable P-C bond; this will allow for crystallographic data and binding assays to be performed without degradation of the protein. phosphono-CheY is produced by reacting CheY with phosphonomethyltriflate (PMT) in the presence of 3.0 equivalents of triethylamine and 125 mM Ca^{2+}. Typically, this reaction will result in 45-70% conversion of CheY to phosphono-CheY, meaning a purification step must be performed subsequently. Purification was successful using cation exchange HPLC with a 50 mM sodium acetate buffer at pH = 5.3. The gradient was run from 0 to 11.7% mobile phase B over 47 minutes and pure phosphono-CheY eluted at 31 minutes and CheY at 23 minutes. Crystallization trials modeled after those for unmodified CheY from *T. maritima* were applied and diffraction quality crystals were

grown in wells that contained the following: PEG 3400 (26%)/100 mM HEPES (pH =

7.0)/.2 M $(NH_4)SO_4$/15 mM $MgCl_2$, PEG 3400 (28%)/100 mM acetate (pH = 4.5)/.2 M

$(NH_4)SO_4$/15 mM $MgCl_2$, PEG 4000 (26%)/100 mM HEPES (pH = 7.0)/.2 M

$(NH_4)SO_4$/15 mM $MgCl_2$, and PEG 4000 (28%)/100 mM acetate (pH = 4.5)/.2 M

$(NH_4)SO_4$/15 mM $MgCl_2$.

ACKNOWLEDGEMENTS

DEDICATION

Everything I have ever done and will ever do is because of my wonderful family; there are no words to describe them and what they have done for me. Mom, Dad (I know you're still watching), and Grandma, I will never be able to pay you back so I hope you accept this blurb in my thesis as a beginning. To the rest of my family, Brian, Chris, Eddie, Lisa, Kaci, Donna, and my nieces and nephews (this list would be longer than my thesis), thanks for everything, even though most of you busted my chops for being in school so long (more some than others) I know it was in jest and I couldn't ask for a better, more "entertaining" family. I would also like to dedicate this to the friends that have been with me for a long time, Adam, James, Corey, Mykel, Ashley, Sarah, Darcie, Kathryn, Old Chicago, Buffalo Wild Wings, and Sticky Fingers, I love you guys.

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INTRODUCTION

Bacterial Signal Transduction and Two-Component Systems

The ability of bacteria to sense small environmental changes with high sensitivity is important for bacterial survival. Environmental changes provoke response from bacteria by the relay of information about the environment from extracellular receptors on the surface of the bacteria to their ultimate target downstream to an intracellular target protein. This relay of information is known as signal transduction, where an external signal is processed and passed between various intracellular signaling proteins while being amplified for increased sensitivity (1). Bacteria can regulate a number of important processes by sensing external stimuli arising from various environmental changes; such processes as growth and development, metabolic regulation, and chemotaxis are controlled this way (2).

Signaling proteins in a signal transduction pathway are regulated both covalently and non-covalently; however, both serve the same purpose, to modify the protein in such a way an alteration in its structure occurs. This conformational change is crucial in the activation/deactivation of certain proteins which will allow the signal to either continue along the pathway or stop at a particular point.

The most prevalent signal transduction scheme in bacteria is described as a two-component system (1). These systems all share a common mechanism in which a high-energy phosphoryl group is transferred from a histidine autokinase protein to a conserved aspartate residue of a response regulator protein (3,4). Response regulator proteins interact directly with their effector protein, and they are turned on and off by phosphorylation/dephosophorylation. Again, the structural change in the protein upon

phosphorylation will allow the protein to interact with its effector in a way which its unphosphorylated structure cannot (3,4).

A typical example of this mechanism lies within the signal transduction pathway that constitutes osmoregulation in bacteria. This system contains a histidine kinase protein EnvZ which transfers a phosphoryl group to the response regulator protein OmpR, therefore activating it. Upon activation of OmpR, a conformational change in the protein enables it to bind to DNA and regulate the transcription of genes that encode for the proteins OmpC and OmpF (5,6). These proteins are outer membrane porin proteins which control water movement into and out of the cell (5,6). The most widely studied response regulator however, lies within the two-component system which regulates chemotaxis in bacteria, this response regulator is CheY.

Bacterial Chemotaxis

CheY is the response regulator protein directly responsible for chemotaxis in bacteria; when it becomes activated, it interacts directly with its effector protein FliM. Chemotaxis is the phenomenon which explains the movement bacteria exhibit in response to an external chemical gradient; it is characterized by periods of smooth swimming and tumbling motions the bacteria show as they rotate their flagella either in a clockwise (CW) or counterclockwise (CCW) direction.

The flagella motor (Figure 1) is a complex structure made up of >20 different proteins and five main rings named the L,P,M,S, and C rings. The L and P rings are made up of the hydrophobic membrane proteins FlgH and FlgI which serve as bushings. The S and M rings (commonly referred to together as the MS-ring) are formed from ~26 copies

of the protein FliF and is located in the cytoplasmic membrane. The 5th ring, which is associated to the MS-ring by the multidomain protein FliG, is called the C-ring, and is composed of FliM and FliN. Together, FliM, FliN, and FliG form a switch complex which is essential for torque generation, binding of CheY-P, and motor rotation (7,8).

During chemotaxis, a bacterium will swim toward an area which is rich in a chemoattractant (such as glucose), and tumble away from one which is rich in a chemorepellant (such as phenol). In bacteria such as *Escherichia coli*, CheY is phosphorylated at Asp57 by the histidine kinase CheA in the absence of chemoattractant and dephosphorylated by the phosphatase CheZ when chemoattractant is present. The transition of the phosphoryl group from CheA to CheY involves a His-Asp phosphate transfer mediated by chemoacceptors or methyl-accepting chemotaxis proteins (MCPs) on the surface of the bacteria; the MCPs are methylated by CheR and demethylated by CheB. *E.coli* cells contain five chemoreceptors: Tsr, Tar, Tap, Trg, and Aer which sense attractant and repellant stimuli (9). Attractant stimuli interact with the chemoreceptors to inhibit CheA activity by demethylation of the MCPs by CheB, while a repellant stimulus enhances CheA activity by methylation of MCPs by CheR to allow for phosphate transfer to CheY (7,8). Phosphorylated CheY (CheY-P) will subsequently bind to FliM on the flagella motor to promote clockwise (CW) rotation of the flagella. The CW rotation will switch the bacteria from a smooth swimming state to a state in which the bacterium exhibits a tumbling motion. As the bacteria enters a more attractant rich environment, binding of attractant to surface receptors on the bacteria causes blockage in the signaling pathway leading to CheY phosphorylation. As this happens, P-CheY is hydrolyzed with help of the phosphatase CheZ and the adapter protein CheW, causing P-CheY

concentration to drop and the bacteria to swim smoothly. Overall, the bacteria exhibit a biased random walk towards conditions where there is an attractant-rich environment (10,11).

In other bacteria, such as *Bacillus subtilis* and *Thermotoga maritima*, bacterial chemotaxis works somewhat differently. For one, chemoattractants cause the phosphorylation of CheY by CheA; two, CheY is phosphorylated at Asp54 homologous to Asp57 in *E. coli*; and three, the binding of CheY-P to the flagella motor induces a CCW rotation rather than CW rotation (12). Another difference, and perhaps the most important, is the presence of 3 proteins not present in the *E.coli* chemotaxis system. These 3 proteins are the phosphatases CheC/D, CheX, and FliY/N (12,13). CheC in this case is of particular importance because of its high sequence identity to the bacterial motor protein FliM which P-CheY binds to (13). Because CheC is suspected to be more soluble than full-length FliM, a CheY/CheC complex would be easier to crystallize and at the same time will give insight on the CheY/FliM complex.

Because CheY-P is labile (its half-life on the order of only a few seconds) (14), synthesis of a stable analog is desired. In order for a stable analog of CheY to be synthesized, the methods used to create phosphono-CheY from a mutant CheY in *E. coli* (15) will be applied to a mutant form of CheY from *T. maritima* (D54C/C81S) in order for crystallization and binding studies. In this instance the non-active Asp54 residue is mutated to a cysteine to introduce a nucleophilic site for modification. This mutation allows a formation of a stable S-C bond, which replaces the labile C-O bond in wild type P-CheY. Modification is done by nucleophilic attack of the sulfhydryl group of sulfur on the alkylating agent phosphonomethyltriflate (PMT) in the presence of triethylamine and

metal ion. Figure 2 shows the schematic of both wild type phosphorylation (in vivo) and in vitro phosphorylation of the mutated form of CheY. It should be noted that the cysteine residue at position 81 is mutated to a serine in order to prevent modification at both Cys54 and Cys81. Triethylamine is used to counteract PMT protons and metal ion is used for several reasons; it perturbs the pK_a of the cysteine downwards and it is believed to bring PMT closer in proximity to the active site. By bringing PMT closer to the active site, easier nucleophilic attack of sulfur can occur. In some enzymes, phosphoryl groups have high affinity for metal ion and will chelate them; in our case, metal ion will bring PMT to the active site of the protein because of its phosphoryl group.

Phosphono-CheY is a stable analog of CheY-P which was successfully used for crystallization studies as well as binding studies of phosphono-CheY to peptides mimicking CheZ in *E. coli* (16,17). Binding studies were carried out using fluorescence and isothermal titration calorimetry (ITC) to give information on dissociation constants between phosphono-CheY and CheZ (18).
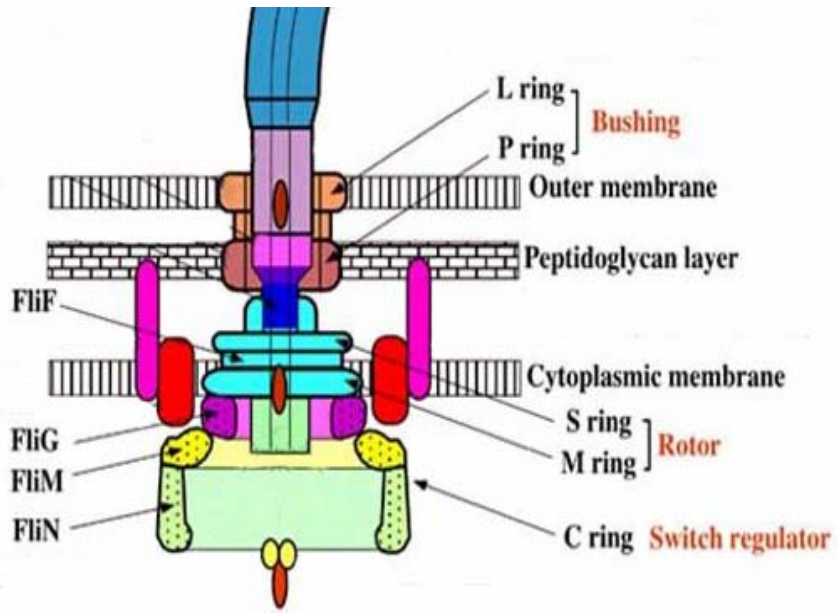
Figure 1. Structure of the Flagella motor.
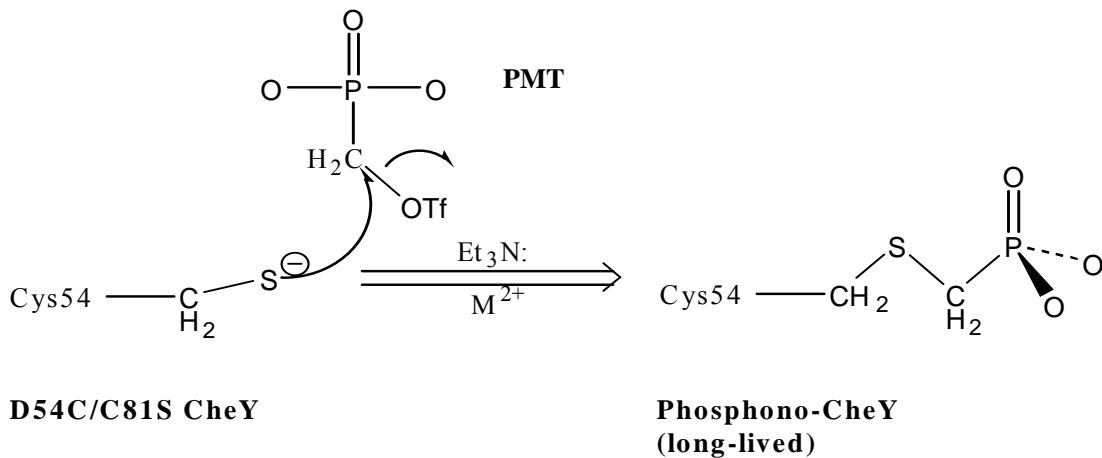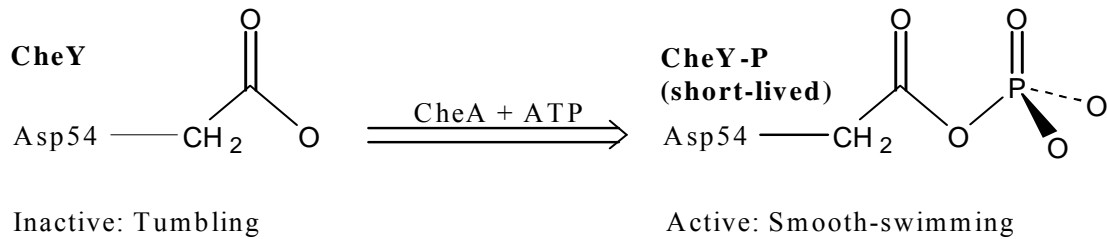
Figure 2. Schematic of CheY phosphorylation both wild type and in vitro. The top mechanism represents in vivo phosphorylation of CheY. Here CheA transfers the phosphoryl group obtained by ATP to the Asp54 of CheY; this forms a labile C-O bond which has a half life of ~ 30s. The bottom mechanism represents the chemistry in the phosphonomethylation reaction performed with PMT. Here the mutated active site Cys54 attacks PMT at the carbon next to the triflate leaving group forming a stable S-C bond. The result of this reaction is a structure which is analogous to wild type P-CheY.

MATERIALS AND METHODS

Production and Purification of D54C/C81S CheY from *Thermotoga Maritima*

T. maritima D54C/C81S CheY cloned into a pet28a(+) vector encoded with an N-terminal hexahistidine-tag (his-tag) was provided by the laboratory of Brian Crane at Cornell University. The plasmid was transformed into *E. coli* cell line B834 (DE3). The his-tag on CheY in this case is a sequence of 6 histidine residues at the N-terminus of the protein; it is used for purification purposes which will be discussed below.

First, 50 mL of Terrific Broth (TB) (Appendix 1) solution is inoculated overnight with D54C/C81S CheY cells from the BE834 (DE3) cell line. 15 µg/mL kanamycin (syringe filtered as 30 mg/mL stock) is added to the solution and it was incubated at 37 °C while shaking at $\geq$ 200 RPM. The next morning two 1 L TB solutions are inoculated with 25 mL from the overnight cultures; again 15 µg/mL of kanamycin is added. The growth is incubated at 37 °C while shaking at $\geq$ 200 RPM. Throughout the growth, the optical density (OD) of the solution is monitored by taking the absorbance of the solution at 600 nm. Using DI water as reference, absorbance at 600 nm is monitored until OD_{600} reaches 1 – 1.5. At that point, the solutions are induced with 0.2 mM IPTG (syringe filtered as 1 M stock) and continue shaking at 37 °C for 4 – 7 hrs. The cells are then harvested at 11,468 RCF x g for 20 minutes at 4 °C, followed by pouring off the supernatant. At this point the cell pellets can either be frozen on dry ice and kept at -80 °C or they are prepared for sonnication.

The cells are gently suspended in $\leq$ 5 mL of lysis buffer (Appendix 1) per 1 gram of wet cell weight and 1mM PMSF, $\leq$ 10mM 2-mercaptoethanol (or $\leq$ 1 mm dithiothreitol), and 1 µM leupeptin and pepstatin are added to this solution. Sonnication

of the cells is then done in a stainless steel beaker while chilling the cells to 2 – 3 °C in an ice water/salt bath between bursts. The cells are sonnicated until the solution is homogenous and an obvious change in color is seen (light to dark); the cells are checked under a light microscope at 1000x magnification to ensure sonnication worked properly. Typically live cells are rod shaped and "buzz" around in solution while dead cells are more round and lack any type of movement. When the cells appear to be broken, the lysate is poured into centrifuge tubes and spun at 38,828 RCF x g for 65 min at 4 °C. The supernatant is now the soluble fraction and the pellet is the insoluble fraction. Both the soluble fraction and pellet are checked for the presence of CheY by SDS-PAGE before proceeding; molecular weight determination is determined as compared to broad range standards from Novagen.

After centrifugation, a 5 mL (10 mL of a 50% slurry) Ni^{2+}-NTA (nitriloacetic acid) column by Novagen is used to purify His-tagged CheY. The column is equilibrated with 10 column volumes of lysis buffer first before syringe filtering the sonnication supernatant onto the Nickel column. After loading the lysate, the column is washed with 2 – 5 column volumes of wash buffer; the elution from the column is collected and checked via SDS-PAGE (this usually contains some CheY and can be rerun over the Nickel column). CheY is then eluted with ~ 10 mL of elution buffer (imidazole competes with the His-tag for nickel chelation sites). The elution volume is kept as small as possible (3 % of the volume of the size exclusion column used in the final step). It is easy to follow the path of the protein on the Nickel column, as the color changes with each step. As the protein binds, the column changes from green to white. Washing the column produces a purple color at the top. Elution turns the white column back to green-

blue and collection of the elution is stopped shortly after all the white color is gone. The elute from the column is then checked via SDS-PAGE to ensure the presence of CheY before proceeding. The nickel column is regenerated after every five uses, or when the resin turns a brownish/yellow color; the regeneration protocol can be seen in Appendix B.

Protein concentration is determined by Bradford assay (using a standard curve constructed from known stock concentrations of egg albumin) first before thrombin digestion. Thrombin digestion is employed to cleave the his-tag off the protein. Using high purity grade thrombin from MP Biomedicals, 1:2000 w:w (thrombin:protein) or 0.5 activity units (NIH units) per mg of protein is added to the protein along with 2.5 mM $CaCl_2$ and 5 mM $MgCl_2$. The reaction is left to proceed overnight (16-22 hours) while shaking at room temperature. After ~16 hours the digestion is checked via SDS-PAGE to check for completion; this is compared to a small sample of CheY taken before digestion to have a band to compare the digest to.

After digestion is completed, a Sephadex G-50 size exclusion column is used to separate thrombin, CheY and his-tag. The molecular weight cutoff of G-50 is slightly below the molecular weight of thrombin so it will elute in the void volume, and sufficient separation between pure CheY and the his-tag (2 KDa) is achieved. The column used is 95 cm high and 2.5 cm in diameter (466 mL); it is poured under flowing conditions as uniformly as possible at 4° C. For best separation, the volume of the sample is kept at less than 3% of the column volume, or 14 mL in this case (19). Prior to loading the column, 1 L of phosphate buffer (pH 6.8 - 7.2) with 25 mM β-mercaptoethanol and 0.02% azide is run through the column to equilibrate it. Once equilibration is complete the protein is loaded to the top of the column bed while being careful not to disturb the

gel. After the sample is loaded, a few mL of elution buffer is used to rinse the sides of

the column. A fraction collector is set up to collect 7 min fractions off the column while

the flow rate is slightly less than 1 mL/min to give fractions of ~6 mL. The fractions are

collected overnight and analyzed by A_{280} nm the following morning. As absorbance is

monitored three peaks in absorbance are observed, eluting at approximately 150 mL, 250

mL, and 500 mL of buffer. Analysis of the fractions by SDS-PAGE and UV-VIS

spectroscopy confirms the 2nd peak as CheY. The fractions are then pooled based on the

above analysis methods and is concentrated using an Amicon concentrator with a YM3 or

PLBC 3,000 molecular weight cutoff membrane (Millipore). The protein is concentrated

to ~7.5 mg/mL and is aliquoted out in 1 mL fractions and quick frozen to store at -80 ^{o}C.

Preparations of CheY were denoted as A, B, C, etc. in order of when they were prepared.

For example, protein prepared from two 1 L growths preformed side by side at the

beginning of a new year is called prep. "year"A.

Phosphonomethylation of D54C/C81S CheY from *T. maritima*

D54C/C81S CheY stored in 50 mM phosphate, pH 6.8, is taken out a night before

a reaction is to be performed. The aliquot is thawed and 10 mM 2-mercaptoethanol, 1

mM DTT or 10 mM bis-2-mercaptoethylsulfone (BMS) is added to allow for full

reduction overnight; all of these reducing agents worked equally well in this case. The

following day the protein is concentrated in a Centriprep (Millipore) or Nanosep (Pall

Life Sciences) concentrator, both with molecular cutoff weights of 3,000. The protein is

concentrated to 250-300 µL for buffer exchange via 2 Penefsky spin columns (20)

performed back-to-back. For volumes 150 µL and less 1 Penefsky column is adequate,

though one can always ensure proper buffer exchange by testing with pH paper. The protein is exchanged into 250 mM TAPS buffer, pH 8.25 and diluted up to 750 μL for a final protein concentration around 8.5 mg/mL as confirmed by Bradford assay or UV scan. A molar absorptivity of 2560 cm^{-1}M^{-1} is used for the A$_{280}$ obtained via UV scan. This molar absorptivity value was calculated from experiments done with Matthew Haas, and data can be seen in his Masters thesis (21). An assay using 5,5-dithiobis(2-nitrobenzoic acid), or DTNB, is used to measure protein free thiol content (22), and the A$_{410}$ of the protein is monitored over 40 minutes. It is desirable for the protein free thiol content to be 75% or greater. The experiment is setup in a 1 mL plastic cuvette containing 950 μL of 0.100 M potassium phosphate buffer (pH = 7.2) and 50 μL 20 mM DTNB solution. The UV spectrophotometer is equilibrated at 25°C and the blank (water) and DTNB solution are placed in the cell changer to allow for temperature equilibration. After ~10 minutes of temperature equilibration a time drive assay is performed to monitor the change in absorbance of the DTNB solution over time after protein is added. A proper amount of protein is added to allow for a change of absorbance approximately equal to 0.100 AU.

Once the DTNB assay has ensured substantial reduction of the protein, the protein is split into 3-250 μL aliquots and 125 mM of BaCl$_2$, CaCl$_2$, or SrCl$_2$ is added. Trivalent metals such as Tb^{3+}, Lu^{3+}, and Nd^{3+} were also used and will be discussed later. 120 mM phosphonomethyltriflate (PMT) is weighed out on a 5-place balance as quickly as possible to avoid water hydrolysis. 3.0 equivalents of dried and distilled Et$_3$N is added to remove the two protons from PMT and the one from the sulfhydryl group on the cysteine residue. Half the volume of Et$_3$N needed of EtOH is added to PMT and mixed until PMT

is dissolved. As quickly as possible, the Et₃N is added to the PMT/EtOH mixture and transferred into the protein/metal mixture. This solution is mixed and left to stand for 30-45 minutes at room temperature; typically a precipitate will form upon addition of PMT mixture to protein. pH is monitored with pH paper to ensure the pH does not drop too low upon the addition of PMT. After the alkylation is complete, the protein is centrifuged in a microcentrifuge to remove any precipitate and it is buffer exchanged into a buffer which is used in cation-exchange chromatography (described later) via a PD-10 desalting column (23). A DTNB assay is preformed to reevaluate the percentage of free thiol after the modification is done. A percentage decrease of at least 50% is desirable. A sample is also taken for HPLC analysis at this point (described below). If the protein is not to be purified immediately, 10 mM 2-mercaptoethanol is added and the protein is quick frozen and stored at -80 $^{\circ}$C.

      Many different conditions were used in phosphonomethylation reactions, though the conditions used above were the most consistent and reliable. Other methods attempted will be discussed in the results section.

Reversed Phase HPLC analysis of Phosphonomethylation Reactions

      Reversed Phase HPLC (RP-HPLC) is the preferred method for analyzing the extent of modification to our protein. Both HP1050 and HP1100 HPLC systems are used; each contains a quaternary pump, degasser, and column heater, while the 1050 contains a variable wavelength detector and the 1100 a diode array detector. Absorbance at 215 nm is read with both the HP1050 and HP1100, as this is the amide bond absorbance region. Additionally, the absorbance at 280 nm is read on the HP1100 with the diode array

detector for absorbance of aromatic amino acids. Two different columns are used in

analysis; one a 4.6 mm ID x 250 mm Vydac C18 Protein and Peptide column with a 300

Å pore size, and the other a 2.1 mm ID x 250 mm column of the same packing. A 2

micron pre-column and the appropriate guard column are also used on both instruments.

The 2.1 mm ID column is preferred in most cases since it saves on both mobile phase and

sample. A flow rate of 0.200 mL/min is used for the 2.1 mm ID column (giving pressures

between 56 and 70 bar), while a 1.00 mL/min flow rate was used for the 4.6 mmID

column (giving pressures between 115 and 121 bar). The mobile phases are acetonitrile

(ACN) and water with triflouroacetic acid (TFA) used as an ion-pairing agent. Mobile

phase A (MPA) is 95% H_2O/ACN with 0.1% TFA, mobile phase B (MPB) is 80%

ACN/H_2O with 0.08% TFA, and mobile phase C (MPC, used for column storage) is 50%

ACN/H_2O with no TFA. For the 2.1 mm ID column, 15 µg of protein is loaded, while 45

µg is loaded for the 4.6 mm ID column. The gradients used employ an increasing

percentage of ACN; for the 2.1 mm ID column the gradient runs from 47% MPB to 54%

MPB over 50 minutes (though variations were used), and from 45% MPB to 53% MPB

over 50 minutes for the 4.6 mm ID column. CheY elutes toward the end of the gradient

at ~45 minutes and P-CheY elutes earlier (more polar) at ~27 minutes. If the HPLC

analysis shows a clean reaction (meaning no more than 2 major peaks) and at least 40%

modification, cation-exchange HPLC will be used to purify the sample (see below).

Purification of Phosphono-CheY from *T. maritima*

Purification is carried out using cation-exchange HPLC (CEX-HPLC); again, HP

1050 and 1100 HPLC systems are used. A PolyCAT A 9.4 mm ID x 200 mm 1000 Å

weak cation-exchange column is used for preparative work, while a 4.6 mm ID x 200 mm 1000 Å column of the same type is used for analytical work. A flow rate of 3.00 mL/min is used for the 9.4 mm ID column, while a 1.00 mL/min flow rate is used for the 4.6 mm ID column. 100 μg is sufficient for the 4.6 mm ID column, while up to 3 mg of a crude phosphono-CheY mixture is sufficient for the 9.4 mm ID column. The buffers used for CEX must be about 2 pH units lower than the theoretical pI value of CheY, which is calculated to be 8.68. MPA is one of low salt while MPB is one of high salt; a gradient of increasing salt is used to elute bound proteins. Four different mobile phases were used in purification of phosphono-CheY, they are as follows: 10 mM potassium phosphate buffer, pH 6.20, 25 mM sodium acetate buffer, pH 5.00, 25 mM MES buffer, pH 5.8, and 50 mM sodium acetate buffer, pH = 5.3. All MPA buffers contained only buffer salt, while all MPB buffers contained buffer salt plus 500 mM NaCl. Prior to sample injection, the sample is always buffer exchanged into the appropriate MPA buffer which is to be used in the purification. Certain problems were present in each buffer, and these will be discussed in the Results section. Appendix C further explains cation exchange HPLC techniques.

Crystallization of Phosphono-CheY from *T. maritima*

Crystallization conditions of phosphono-CheY are modeled after known conditions for unmodified CheY from *T. maritima*; hanging drop diffusion is the preferred method of crystallization (24). Wells contain 500 μL of solution, (precipitant plus buffer and metal) while silanized glass cover slips contain 2 μL of protein plus 2 μL of well solution. Protein is concentrated to ~8.5 mg/mL and buffer exchanged via a

Penefsky spin column into pH 7.8 buffer containing 10 mM triethanolamine, 25 mM NaCl, 1 mM EDTA, and 1 mM NaN_3. The wells contain 200 mM ammonium sulfate and various PEG molecular weights and concentrations for the precipitant. 15 mM $MgCl_2$ is always present, and 100 mM buffer is used. After all solutions are present in the well, the plate is stirred on a rotating plate for ~20 minutes to allow for homogenization of the well solution. Before glass slips are suspended above the well solution, the plates are pre-greased with petroleum jelly or some other sealing lubricant.

All pipette tips used are autoclaved and all solutions are made up in Mill-Q water and filter sterilized with 0.25 μm filters with the exception of PEG solutions, which are filtered with 1.2 μm filters under vacuum. Buffers ware made up as 1 M stock solutions and PEGs were made up as a 50 % w/v solution.

Synthesis of Phosphonomethyltrichlorosulfonate

A full reaction scheme can be seen in Figure 3 with each molecule numbered. This procedure involves sulfinylation of our starting alcohol, dibenzyl hydroxymethyl phosphonate (dibenzyl-HMP) (**1**), using trichloromethansulfinyl chloride ($ClSOCCl_3$), followed by oxidation with a sodium periodate/ruthenium system as used by Sharpless *et. al.* (25,26).

Sulfinylation of the starting alcohol is carried out at room temperature under $N_2(g)$ atmosphere, A solution of 2.3 eq. of $ClSOCCl_3$ in CH_2Cl_2 is combined with a stirred solution of starting alcohol (**1**) with 2.5 eq. 2,6-lutidine in CH_2Cl_2 by dropwise addition. The reaction is left to stir for 30 minutes (27), a small amount of precipitate formed and the solution turned slightly orange. The reaction is diluted up with CH_2Cl_2

and washed twice with 4% $CuSO_4$. The original protocol calls for a 2N H_2SO_4 wash,

however this was modified in order to prevent the use of strong acid (26). The organic

layer is dried with $MgSO_4$, filtered, and solvent removed by rotary evaporation. Sulfinate

ester (**2**) is produced as confirmed by ^{1}H NMR and will be discussed in the results

section.

Sulfonylation is carried out at room temperature by adding 2.0 eq. of $NaIO_4$ and

0.2% mol of $RuCl_3$ to a stirred solution of sulfinate in a 5:1 acetone/H_2O solvent system

(A 3:2 ACN/water solvent system was also used successfully). The reaction is left to stir

for two hours; typically the reaction will turn a dark green due to a ruthenium species

(27). The reaction is then diluted with CH_2Cl_2 and washed twice with water. The organic

layer is dried, filtered, and solvent removed by rotary evaporation to yield a solid light

brown product. In some cases it may be necessary to employ a small silica column to

remove the green ruthenium precipitate that forms during the reaction. Sulfonate ester

(**3**) is produced as confirmed by ^{1}H NMR and will be discussed in the results section.

The final step is hydrogenation of the benzyl groups connected to the alcohol.

The sulfonate (**3**) is dissolved in dry ethanol and has Pd/C catalyst added to it; it is then

put under hydrogen gas (~40 psi) for 1 hour. Removal of the Pd/C is done by vacuum

filtration through celite and volatiles are removed via rotary evaporation. The final

sulfonate ester (**4**) is produced and confirmed by ^{1}H NMR analysis.

1
Dibenzyl-HMP

2
Dibenzyl-phosphonomethyltrichloromethylsulfinate

2
Dibenzyl-phosphonomethyltrichloromethylsulfinate

3
Dibenzyl-phosphonomethyltrichloromethylsulfonate

3
Dibenzyl-phosphonomethyltrichloromethylsulfonate

4
Phosphonomethyltrichloromethylsulfonate
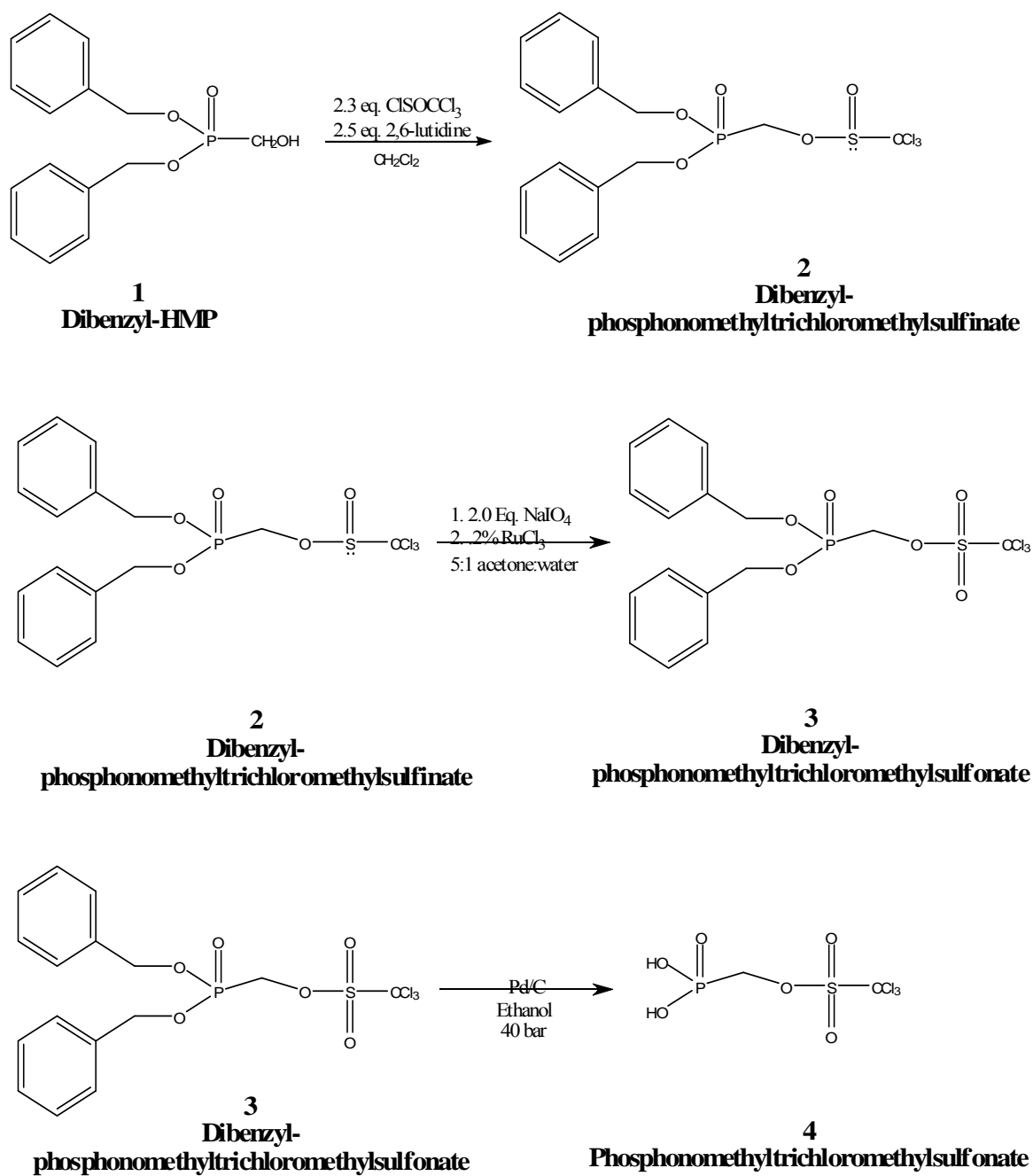
Figure 3. Full reaction scheme for the production of phosphonomethyltrichloromethylsulfonate.

RESULTS

Purification of D54C/C81S CheY from *thermotoga maritima*

SDS-PAGE is used to check each step of the nickel column to monitor CheY. If the CheY band is strong in the SDS-PAGE of the load the lysate, it is re-run back over the nickel column. Washing and eluting steps of the column are also monitored by SDS-PAGE to identify any possible CheY left behind. Figure 4 shows an SDS-PAGE gel of each step on the nickel column purification. Here there are 4 different lanes used in the gel; the 1st lane, containing the lysate directly after sonnication, is rich in protein at all molecular weights. The second lane, containing the load of the lysate onto the nickel column contains mostly high molecular weights impurities. The 3rd lane, which is the low imidazole wash, contains several impurities at low concentration. The 4th lane is a broad range molecular weight standard, and the 5th lane is the wash with the high imidazole buffer, this lane contains ~99% his-tagged CheY as compared to molecular weights in the standard.

Digestion of his-tagged CheY is also monitored by SDS-PAGE time trials until completion. Figure 5 shows an SDS-PAGE gel of a thrombin digestion. Here Lane 1 is CheY before digestion as taken off the nickel column. Lane 2 represents a 15 minute timepoint; from this point on 3 bands will be present; the first highest molecular weight band represents his-tagged CheY, the second band represents non his-tagged CheY, and the third band represents the his-tag. Lane 3 is a 30 minute timepoint, again, notice that the his-tagged CheY band is getting weaker when comparing to the non his-tagged band. Lane 3 is a broad range standard. Lane 5 is a 1-hour timepoint, lane 6 a 2-hour timepoint,

lane 7 an 8-hour timepoint, lane 8 a 16-hour timepoint, and lane 9 a 24-hour time point

where we have essentially all non his-tagged CheY.

Digested CheY is passed through a Sephadex G-50 column and fractions are

collected and monitored via A_{280}. A plot of the fraction number vs. the A_{280} of the

fractions yields 3 peaks corresponding to thrombin, CheY, and his-tag respectively

(Figure 6). Pooled CheY is analyzed by RP-HPLC and a single peak is desirable (Figure

7) though sometimes a second peak ~3 minutes before the main peak is present. Addition

of 1 mM DTT to the protein with the second peak causes the peak to disappear,

consistent with the peak representing oxidized CheY (Figure 8).

Leupeptin and pepstatin (1 μM), along with the presence of 1 mM PMSF (except

during thrombin digestion) and 25 mM 2-mercaptoethanol throughout preparation of

CheY are crucial for phosphono-CheY purification. Preparations not containing these

steps have been unable to produce pure phosphono-CheY. This will be discussed further

in the modification of CheY section.
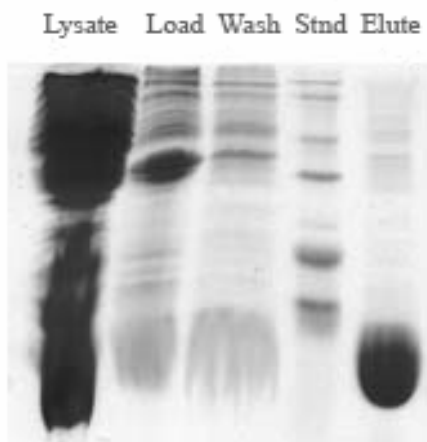
Figure 4. SDS-PAGE of fractions from the Ni^{2+}-NTA column.
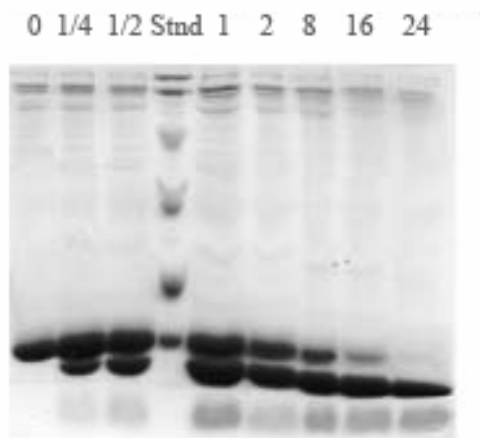
Figure. 5. SDS-PAGE of thrombin digestion timetrials.
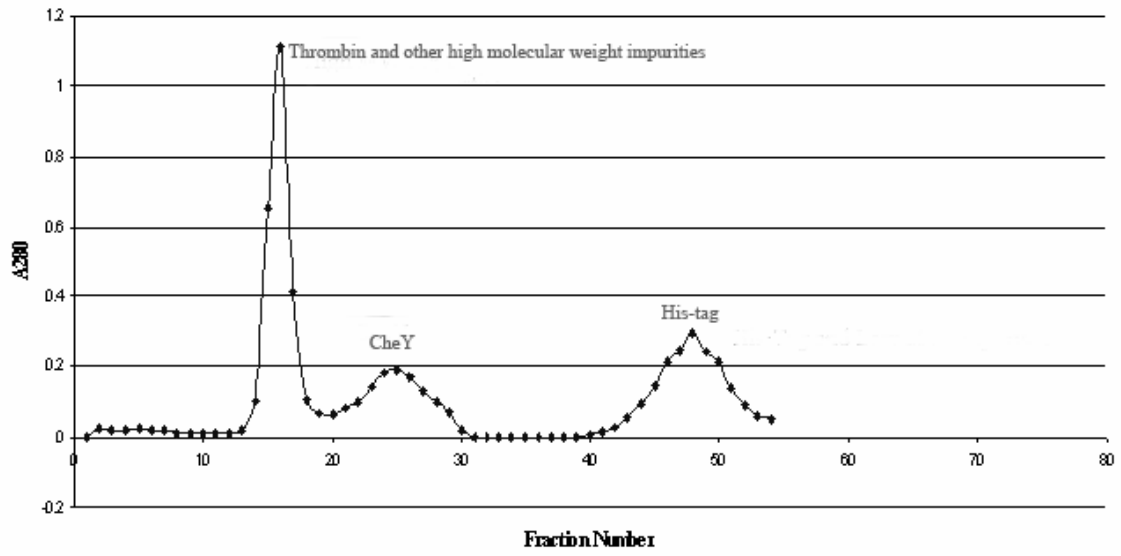
Figure 6. Peaks recorded from Sephadex G-50 size exclusion column.
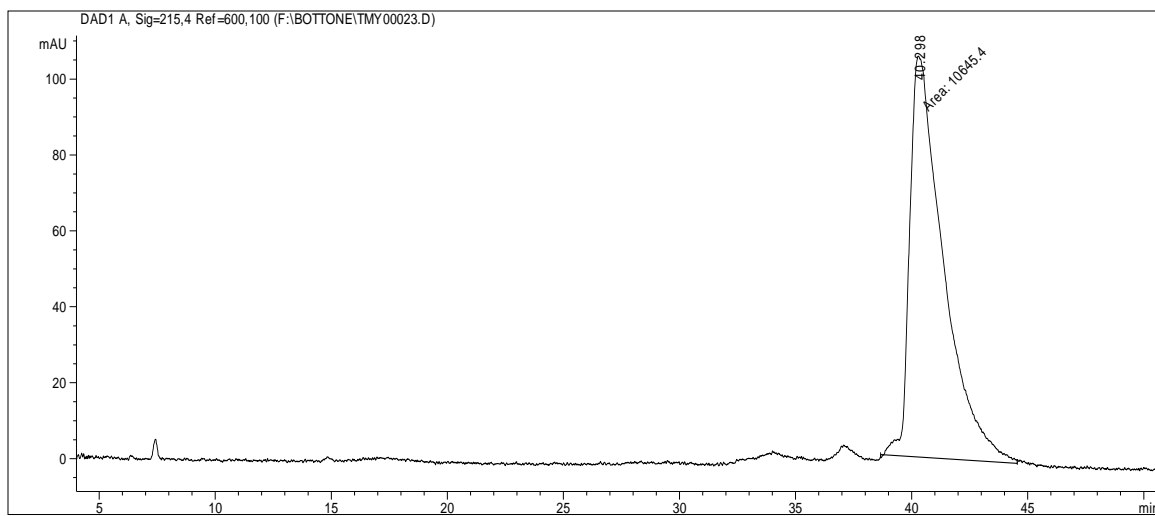
Figure 7. RP-HPLC chromatogram of unmodified CheY after his-tag removal
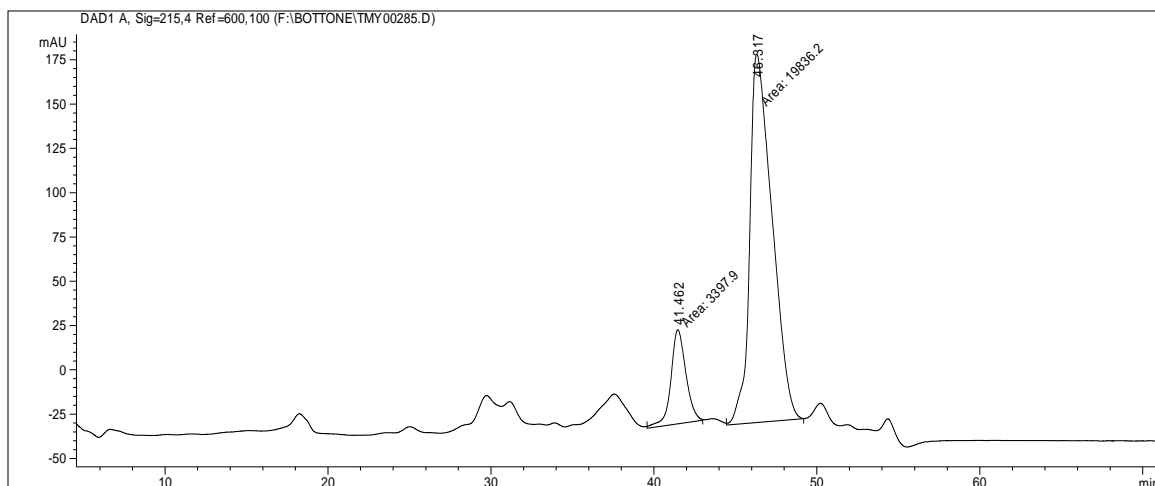
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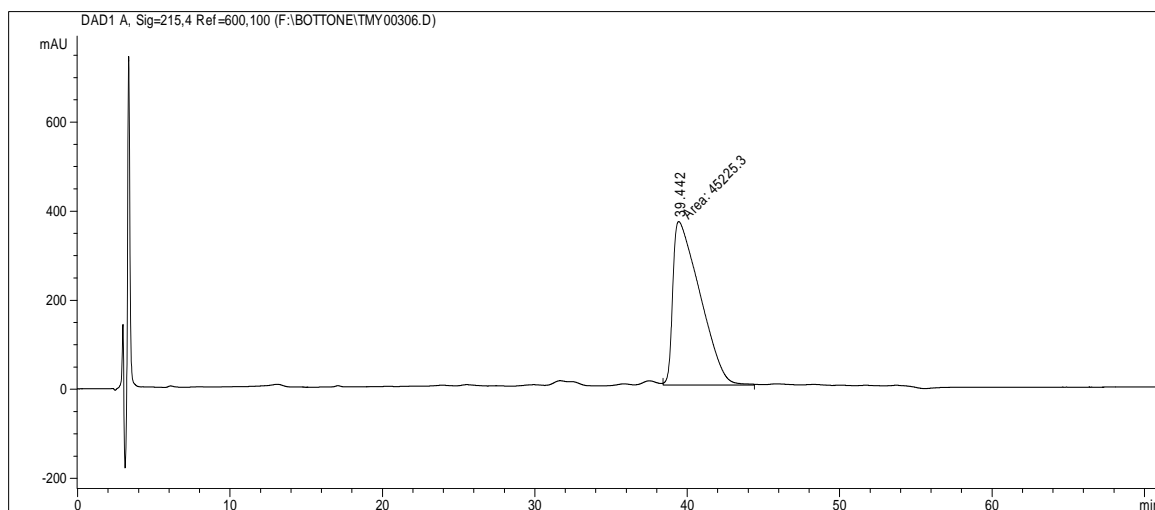
B.

Figure 8. a.) RP-HPLC chromatogram of CheY which is presumed to be slightly oxidized. b.) RP-HPLC chromatogram of the same CheY aliquot with the addition of 10 mM DTT.

Production of Phosphono-CheY from *thermotoga maritima* D54C/C81S CheY

When modifying CheY, two important criteria are present; one being the percent conversion of CheY to phosphono-CheY, and the other being protein recovery. A variety of conditions were tested in order for optimal results to be achieved; those methods will be explained below. Factors which pertain to alkylation variation are CheY preparation conditions (mentioned above), buffer choice, base used, and modification agent used.

Optimal alkylation conditions involve the following method. An aliquot of protein containing ~6.5 mg of CheY is taken out and defrosted about 30 minutes prior to buffer exchange. A DTNB assay is performed on the protein to ensure proper reduction, 75% or greater is recommended; Figure 9 shows a typical DTNB graph. The amount of free thiol is calculated using the equation below:

$$(\Delta A/\ \varepsilon_{410})(13500\ \text{gmu})(x\ \text{mL}/1.0x\ \text{mL})$$

In this equation $\varepsilon_{410} = 14150\ \text{cm}^{-1}\text{M}^{-1}$, 13500 is the molecular weight of CheY, and x is the amount of protein used in the assay. When the amount of free thiol is calculated by using the equation above, it is divided by the total amount of protein (obtained by UV scan or Bradford/BCA assay) to obtain a percent of free thiol.

After DTNB reaction, CheY is alkylated consistent with methods mentioned in the experimental section. This method of alkylation yields 45-70% conversion of CheY to P-CheY as based on DTNB and RP-HPLC while maintaining protein recovery (~5.5 mg recovered out of 6.5 mg at the start). Figure 10 shows a DTNB assay after alkylation, and Figure 11 shows RP-HPLC chromatogram of a typical alkylation from these conditions. Notice the ΔA of the protein after alkylation is less than before modification due to phosphonomethylation of cysteine.

Protein Modification Preparation Dependency

As mentioned above, phosphono-CheY from different preparations of CheY did not always purify via cation exchange HPLC. Comparisons of preparations G and H to preparations C and I show a difference in phosphono-CheY quality upon purification. The differences in these two preparations are as follows: preparations G and H did not contain 1 μM Leupeptin and Pepstatin, nor did they contain 1 mM PMSF and 25 mM 2-mercaptoethanol throughout the preparation, while preparations C and I did. Not one factor can be pointed out as the key to the preparation success, but it should be noted that these conditions should always be employed. RP-HPLC chromatograms of alkylations from these different preparations look virtually identical (under the same alkylation conditions) (Figure 12), however, upon purification via cation exchange HPLC (discussed in the next section), a difference is seen (Figure 13). It also should be noted that while success in purification is consistent in "good" preparations, percent conversion of CheY to phosphono-CheY can be affected. Alkylations of preparation C for instance gave conversion on the order of 80-85%, while alkylation using the same conditions on protein from preparation I gave conversion on the order of 45-60%. (Figure 14).

Buffer/pH Choice for Modification

Buffer/pH choice is important for at least two reasons; the cysteine residue on the protein needs to be in the correct protonation state (R-S⁻ vs. R-SH) for optimal modification, but pH must also stay safely away from the pI of the protein to avoid possible precipitation. Since the pKa of cysteine is different in a protein than it would be free (pKa = 8.3), an exact value is not know. Since this is the case, a range of pH values was tested against the protein; pH values of 8.0-9.50 were tested. All buffers were 250

mM, TAPS was used from 8.00-9.00, whereas AMPSO was used from 9.00-9.50.

Alkylations at different pH values looked very similar in RP-HPLC analysis under

otherwise exact conditions (Figure 15); however, there was one major difference, protein

recovery was substantially lower at higher pH. Alkylation of a 6.5 mg aliquot at pH 9.25

would yield ~2.5 mg of protein (CheY and P-CheY together) after buffer exchange, while

alkylation of a 6.5 mg aliquot at pH 8.25 would yield ~5.5 mg of protein after buffer

exchange. Also noticeable at higher pH values was the large amount of precipitation

seen after the addition of the PMT/EtOH/Et$_3$N mixture. This is most likely due to M^{2+}-

hydroxymethylphosphonate coming out of solution as solubility of the metal ion gets

lower at higher pH values. As the buffer pH is lowered, less precipitation is seen after

addition of the above PMT mixture, with virtually none at pH 8.25.

Metal Ion and Alkylation

Three metals were commonly employed during alkylation; Ca^{2+}, Ba^{2+}, and Sr^{2+}.

As mentioned above, Ca^{2+} gave the most consistent results during alkylation. Sr^{2+} and

Ba^{2+} have both worked successfully, yet would sometimes yield a chromatogram of high

heterogeneity, (Figure 16); the reason for this is unknown. That particular example was

one aliquot of CheY split into three 250 μL reactions; all other conditions were kept

constant. Other metals ions are discussed in Appendix B

Protein Modification Agents Used

Though PMT has been successful in modification of both *E. coli* and *T. maritima*

CheY, other modification agents were tested against *T. maritima* to see if yield of

phosphono-CheY could be increased. Three other modification agents used were the

tresylate (triflouroethanesulfonate) homolog of PMT (phosphonomethyltresylate),

phosphonomethylpentaflourobenzene (PMFB), and phosphonomethyltrichlorosulfonate; the structure of these compounds can be seen in Figure 17. These molecules all contain the desired phosphonomethyl group, but contain different leaving groups as to vary the reactivity of the phosphonomethylation reaction. The first two molecules have been used successfully to modify a cysteine residue on VHR phosphatase, so there was reason to believe they would work in this case. Upon alkylation with these reagents, the protein precipitated completely out of solution. A possibility is that these reagents were not stable at the pH of CheY modification. If so, the molecules could have precipitated from solution while subsequently decreasing pH, causing CheY to precipitate. Reaction with phosphonomethyltrichloromethanesulfonate was more successful than the previous two reagents, however, was not advantageous over PMT. Figure 18 shows RP-HPLC chromatograms of timepoints taken during this reaction. The reaction was performed in TAPS pH = 8.25, 125 mM $CaCl_2$ and 3.0 equivalents of Et_3N. Initially the reaction looked favorable, but started to become worse as time went on. The one minute timepoint contained very little phosphono-CheY, while the 10 minute timepoint began to look favorable with ~35% conversion so far. The 30 minute timepoint contained a large peak with a retention time consistent with phosphono-CheY, but was very broad with several side peaks. By the end of the reaction at a 1 hour timepoint, protein did not retain on the column. The cause for the results seen on the 1 hour timepoint could be overmodification or denaturation of the protein.

Other methods employed in alkylation which were not exhausted and have only brief data can be seen in Appendix D.
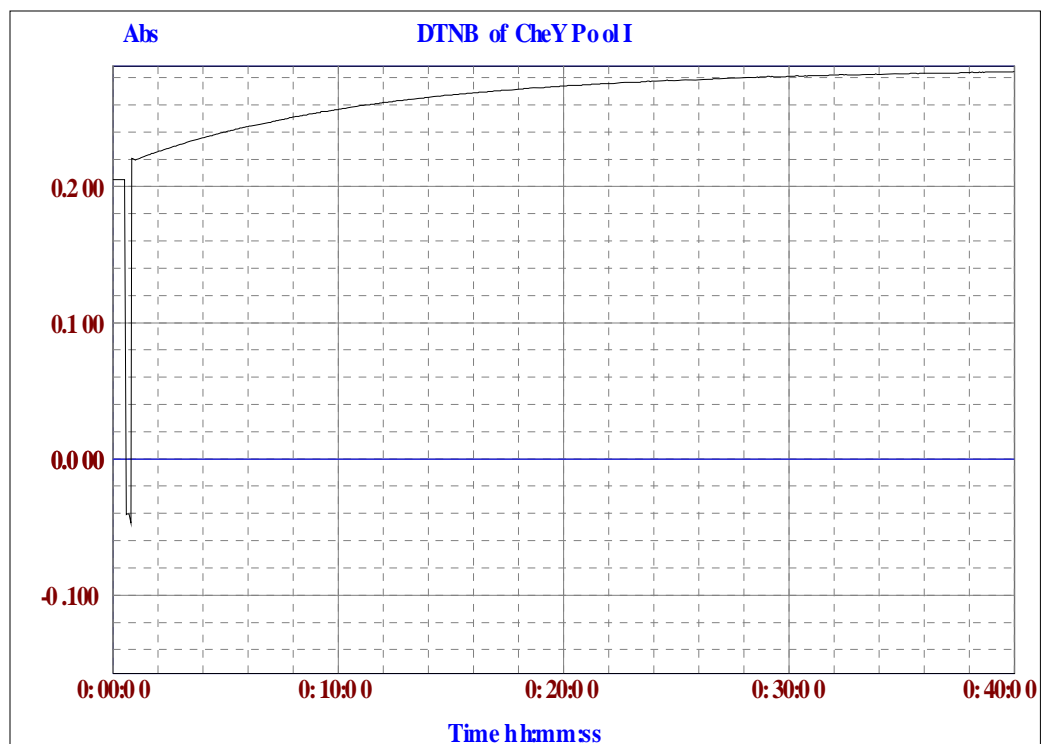
Figure 9. DTNB assay of CheY. The absorbance at 410 nm is monitored over a 40 minute period as the DTNB reacts with the –SH group of cysteine.
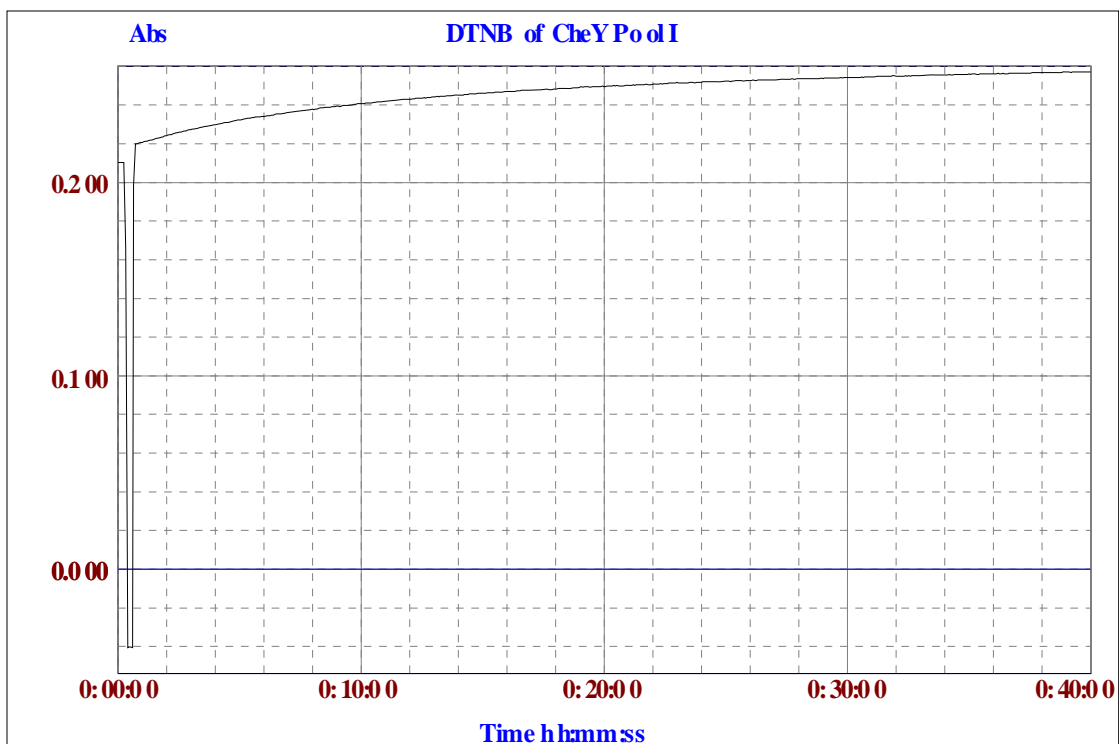
Figure 10. DTNB assay of CheY after phosphonomethylation. The change in absorbance here is lower than the previous DTNB due to less free thiol content.
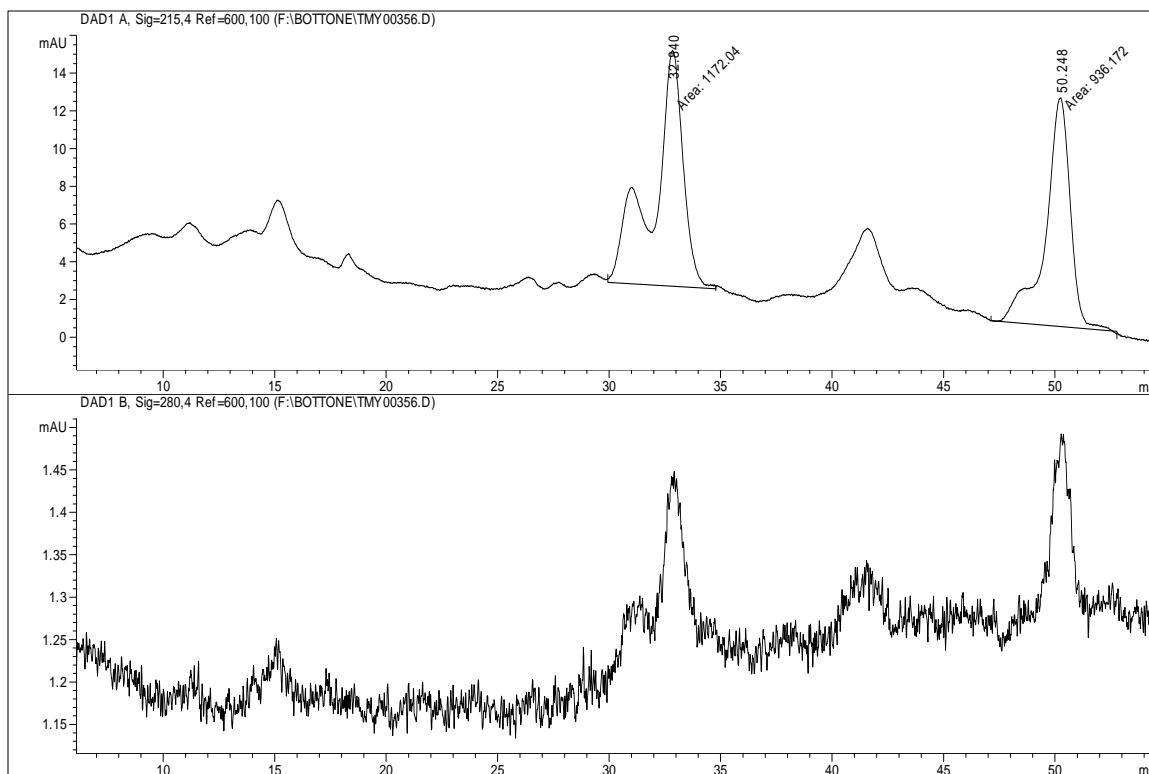
Figure 11. RP-HPLC chromatogram of a high yield/high purity alkylation. The 32 minute peak represents phosphono-CheY, and the 50 minute peak unmodified CheY.
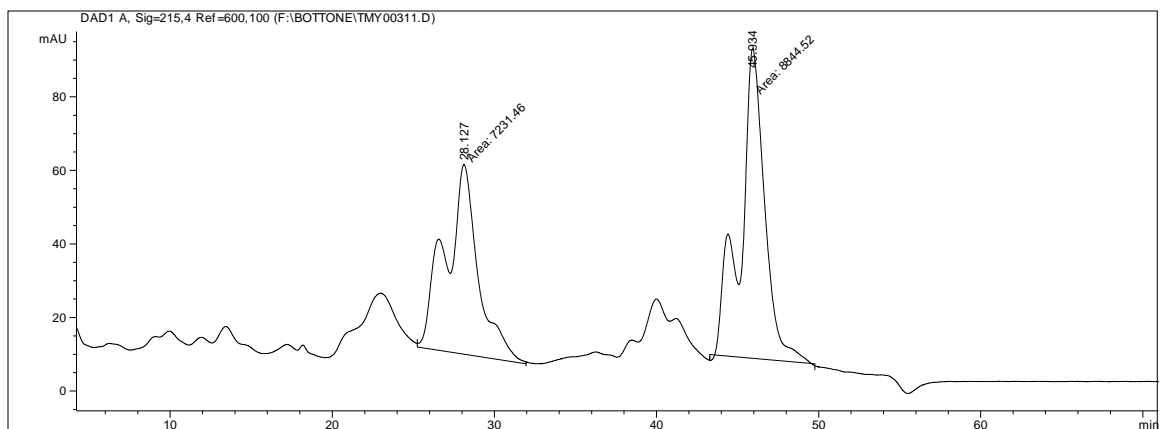
A.
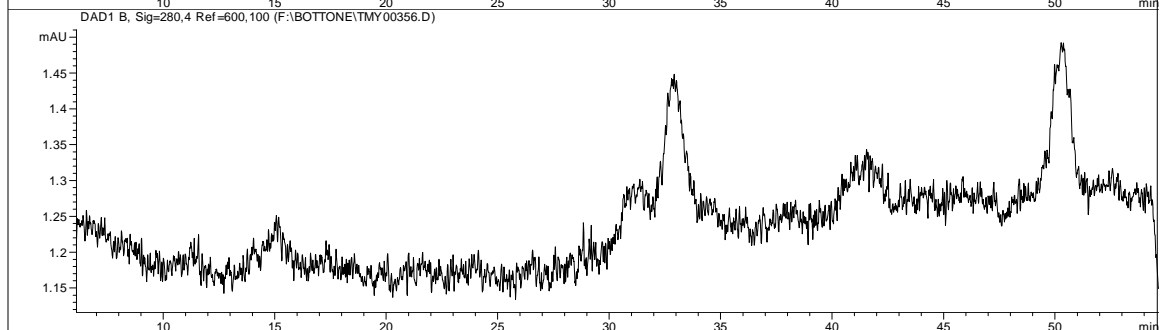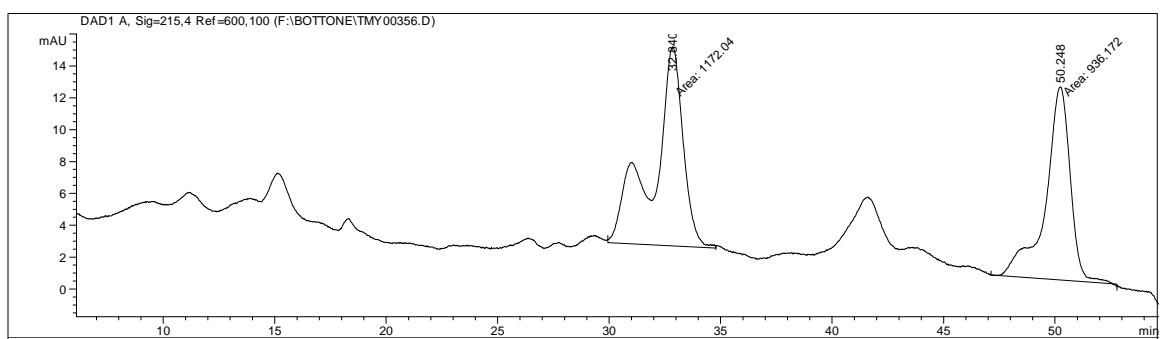
B.

Figure 12. a.) RP-HPLC chromatograms of alkylations from A) Preparation H and b.) Preparation I. The gradients of these two are slightly different, explaining the slight difference in retention time.
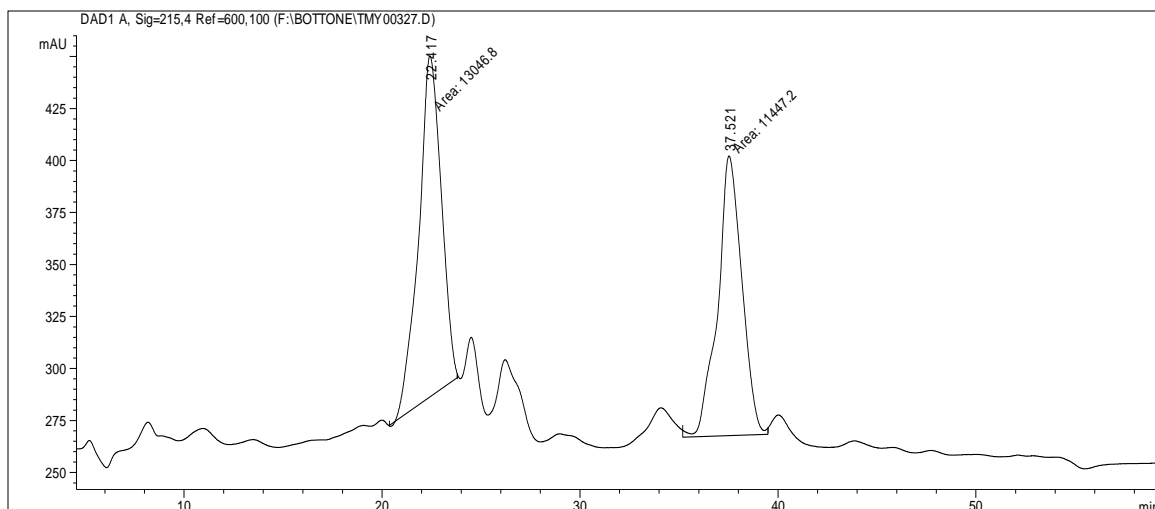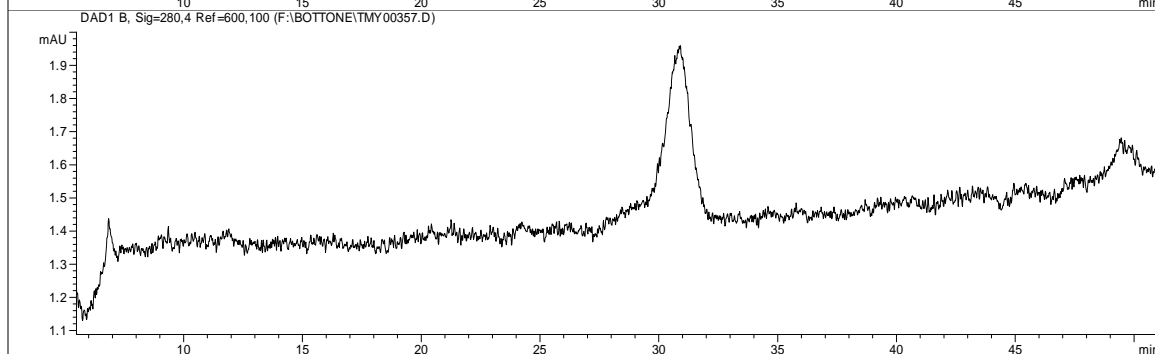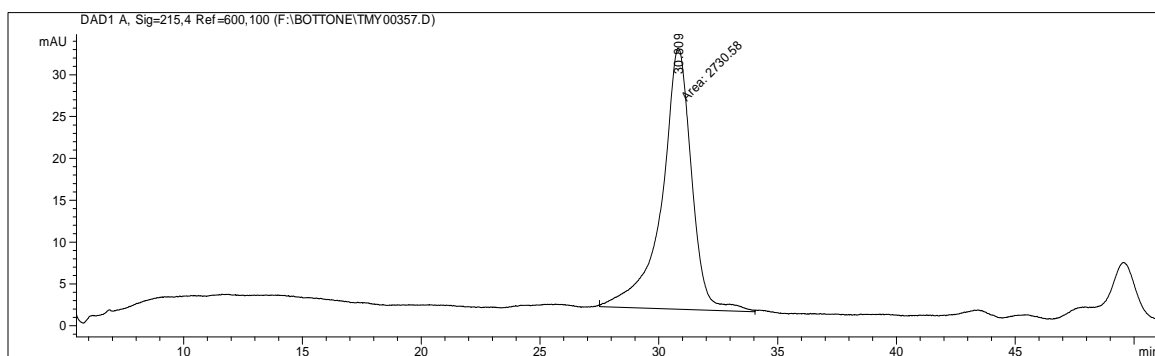
Figure 13. a.) RP-HPLC analysis of purified phosphono-CheY from the alkylation shown in Figure 10a. b.) RP-HPLC analysis of purified phosphono-CheY from the alkylation shown in Figure 10b. These two were purified via the same sodium acetate cation exchange (will be described in the next section).
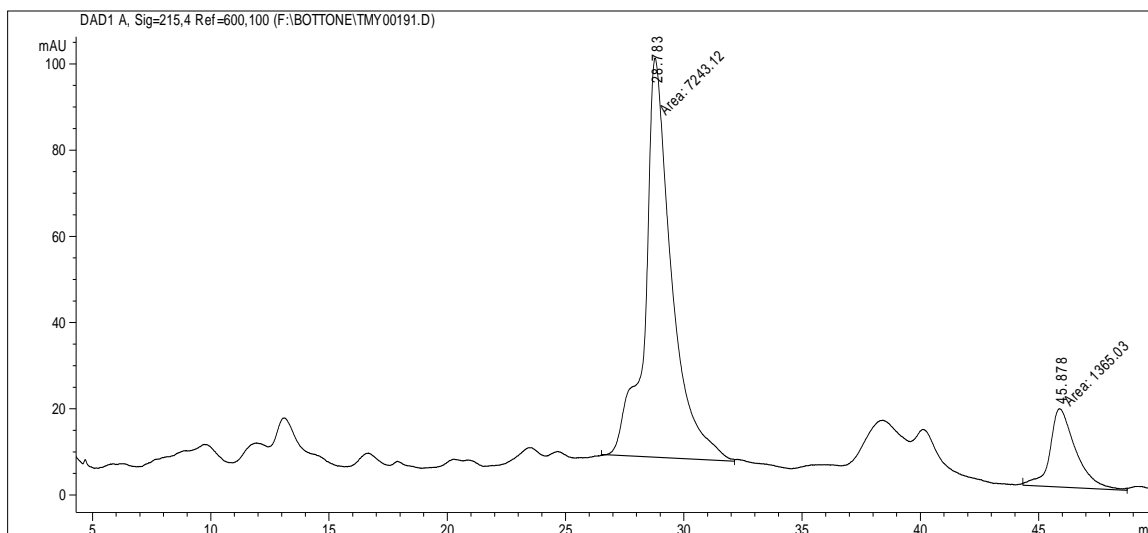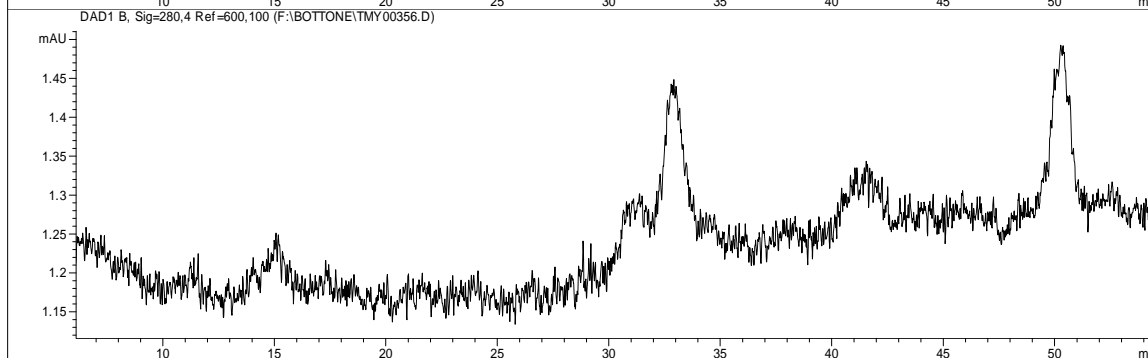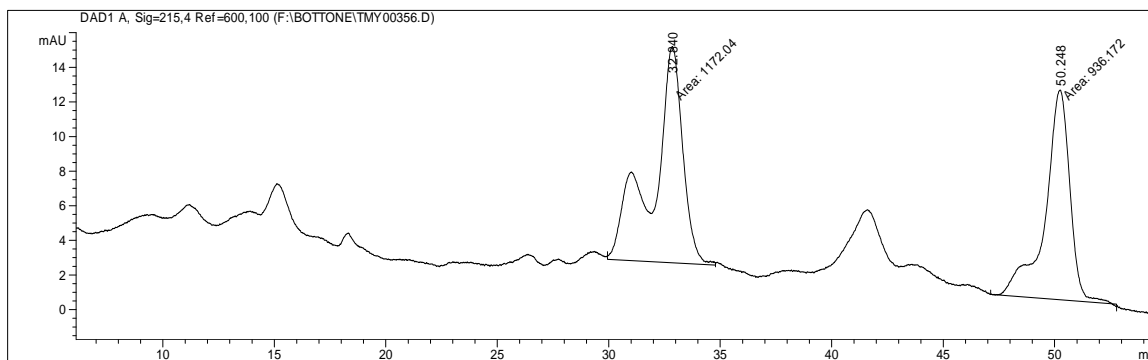
Figure 14. a.) RP-HPLC analysis of a typical alkylation from preparation C. b.) RP-HPLC analysis of a typical alkylation from preparation I. These alkylations were done under the same alkylations conditions (250 mM TAPS pH = 8.25, 120 mM PMT, 125 mM Ca^{2+}).
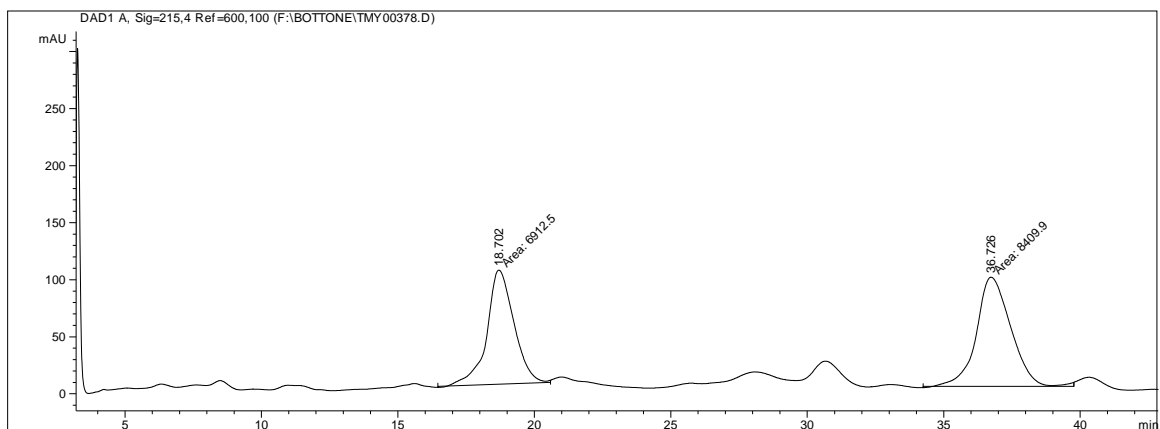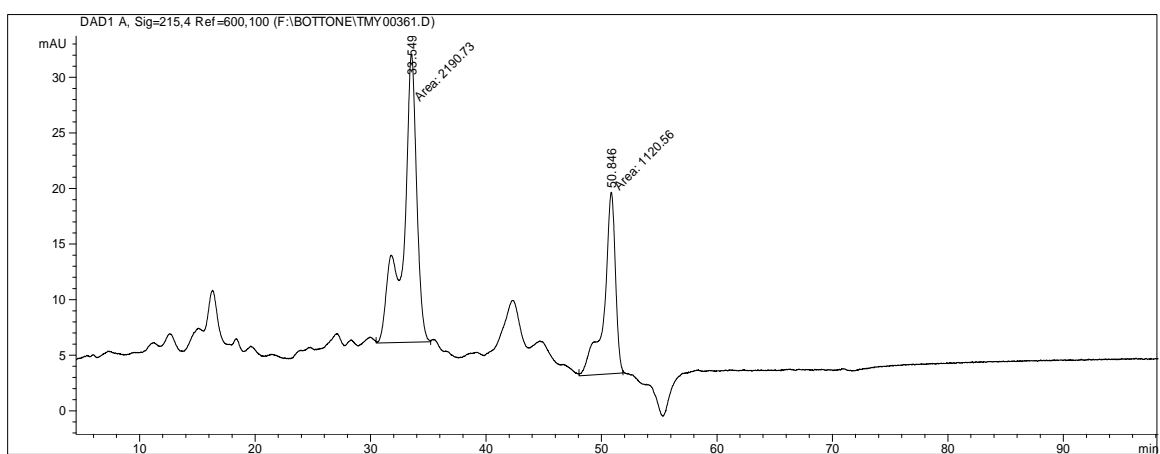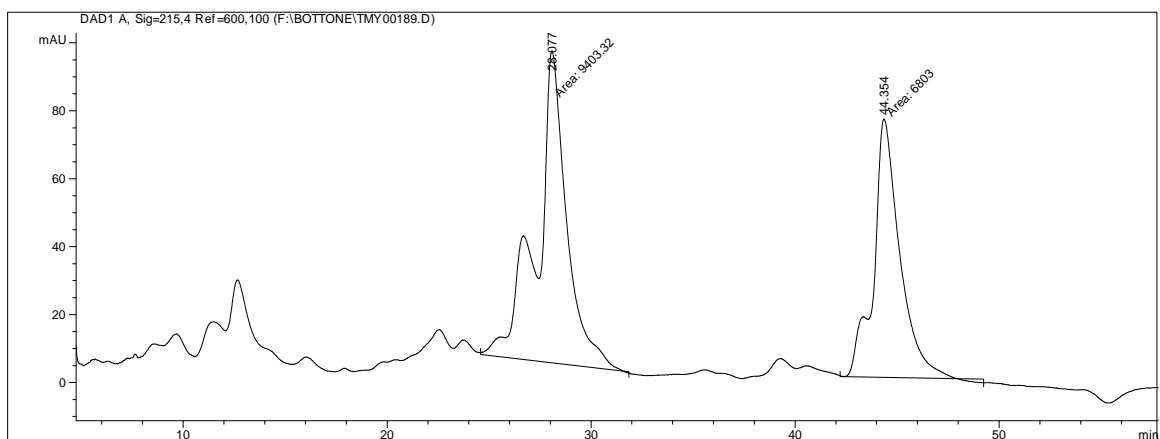
Figure 15. RP-HPLC analysis of a.) Alkylation of pool I at pH = 8.0. b.) alkylation of pool I at pH = 8.25. c.) alkylation of pool I at pH = 9.25.
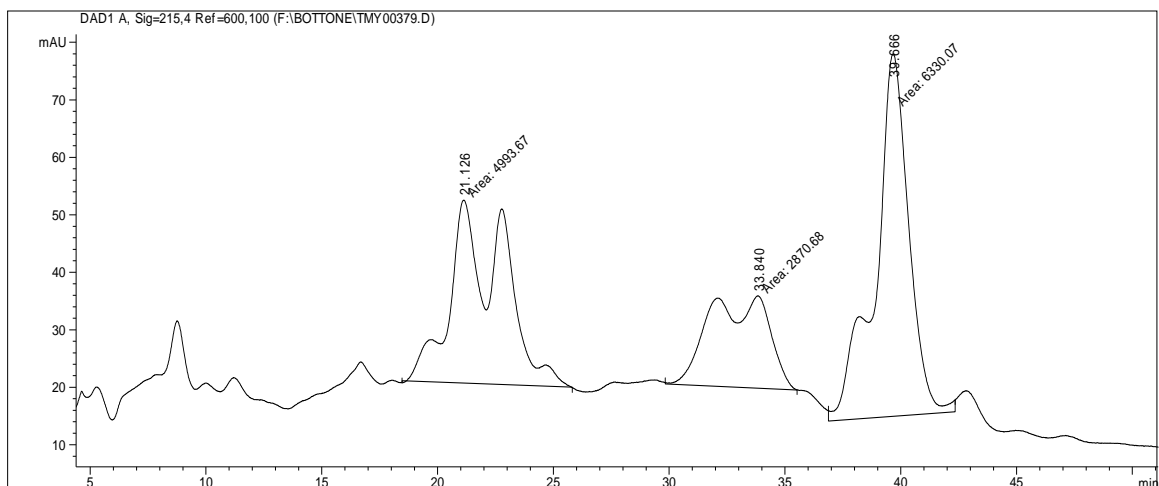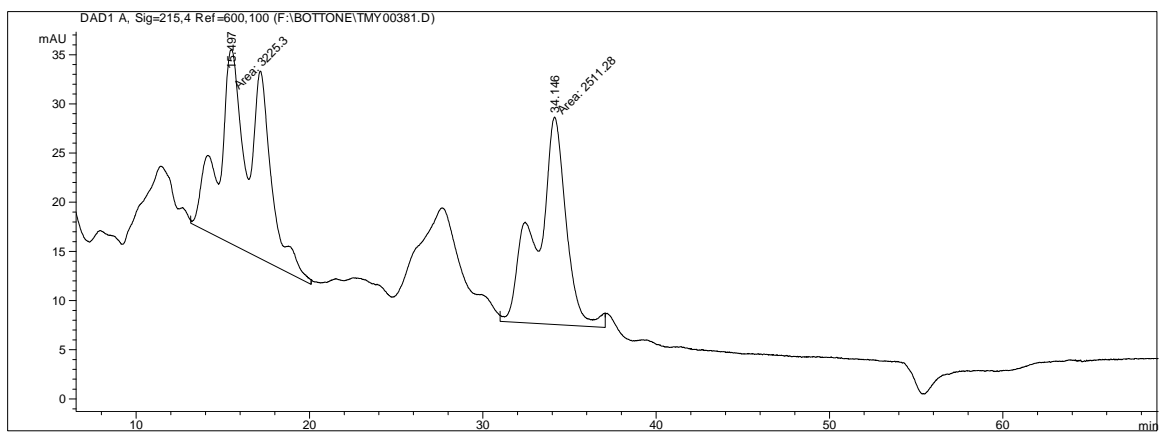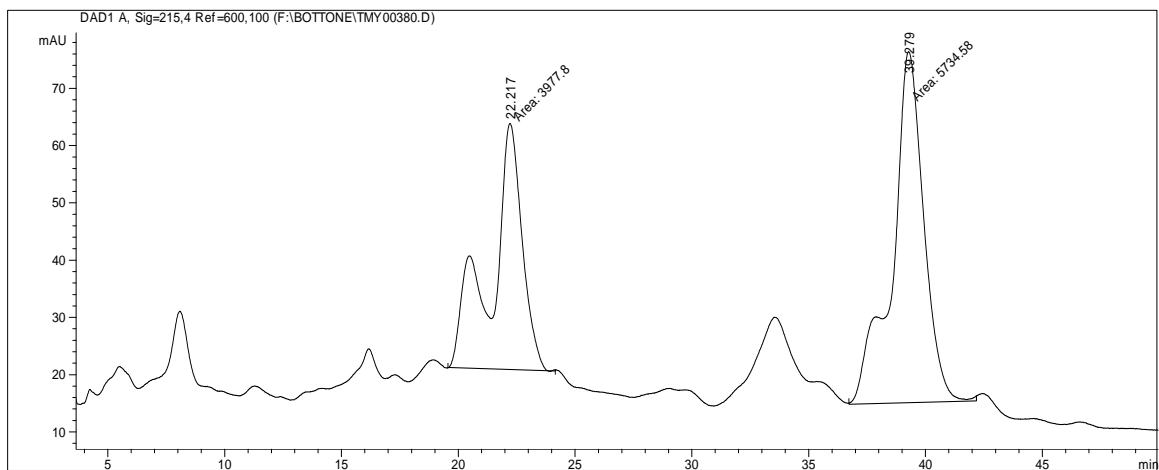
Figure 16. RP-HPLC analysis of a single aliquot of CheY from preparation I separated into 3 reactions. a.) Alkylation containing 125 mM Sr^{2+}. b.) Alkylation containing 125 mM Ba^{2+}. c.) Alkylation containing 125 mM Ca^{2+}.
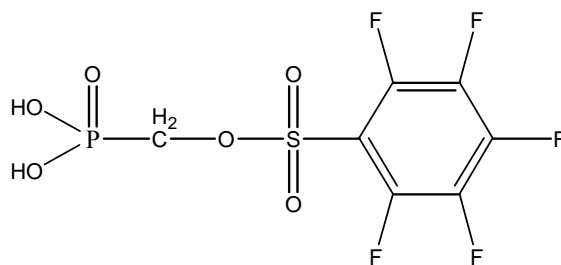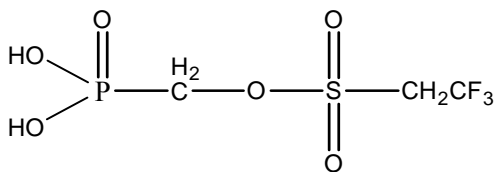
Figure 17. Structures of a.) Phosphonomethylpentaflourobenzenesulfonate and b.) Phosphonomethyltriflouroethanesulfonate.
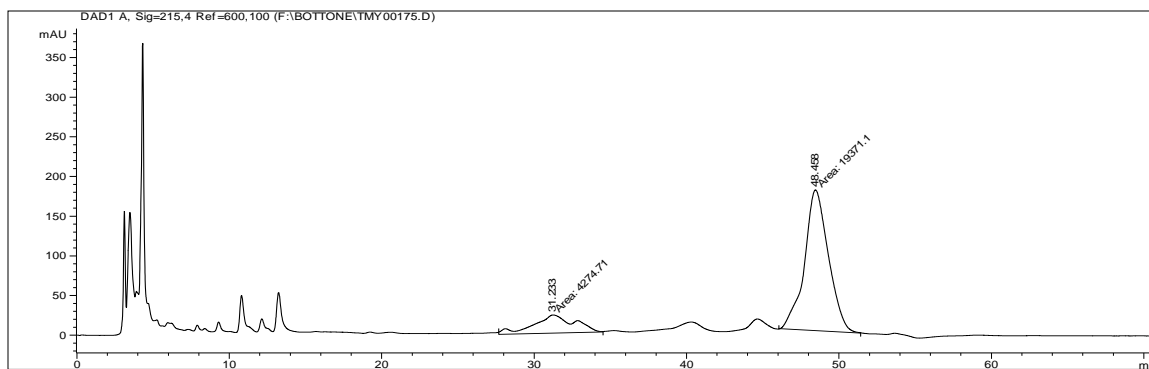
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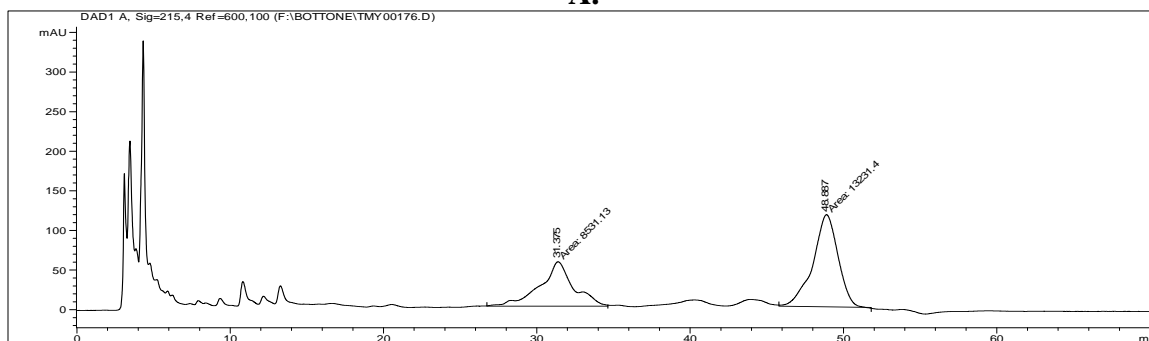
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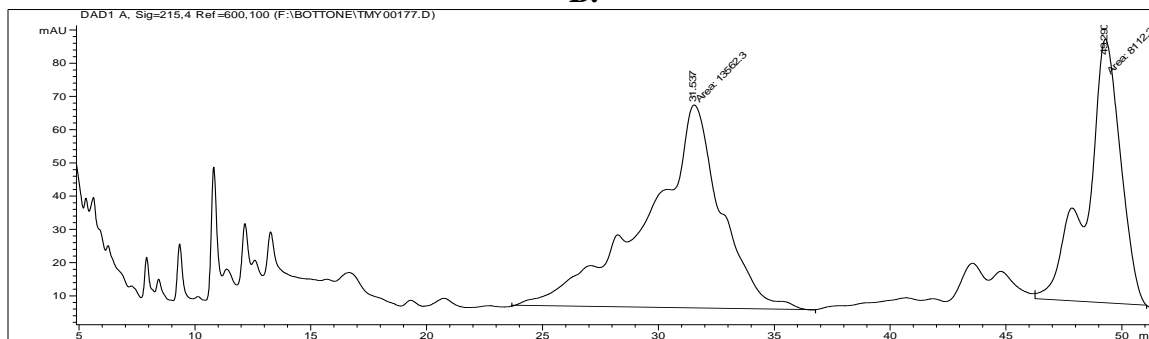
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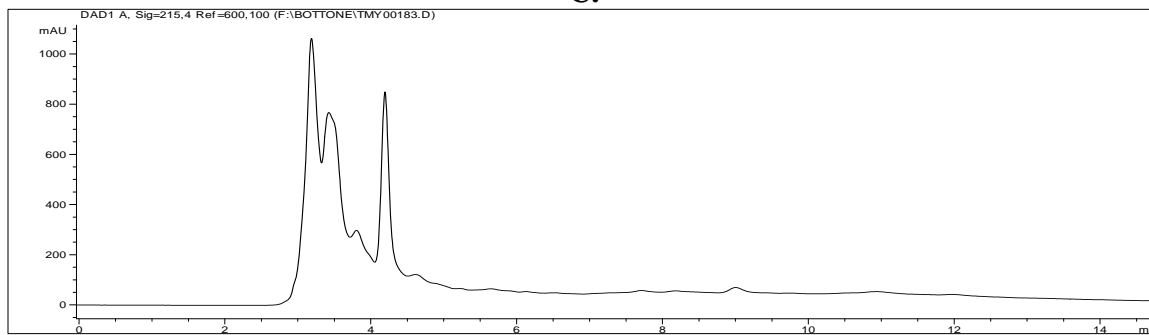
D.

Figure 18. RP-HPLC chromatograms of CheY modification with phosphonomethyltrichlorosulfonate. a.) 1 minute timepoint. b.) 10 minute timepoint. c.) 30 minute timepoint. d.) 1 hour timepoint.

Purification of Phosphono-CheY via Cation-Exchange HPLC (CEX-HPLC)

Due to the substantial change in charge of CheY upon addition of the phosphonomethyl group, CEX- HPLC seemed to be a suitable purification tool. Because our protein is positively charged, (theoretical pI = 8.68) cation, rather than anion exchange was desirable. Mobile phases with varying salts, salt concentrations, and pH values were tested against CheY. All peaks were collected and re-analyzed via RP-HPLC to check the purity of the cation exchange peaks. Development of the final gradient came about after several initial conditions were tested. The preferred method of purification is discussed first, followed by other methods which led up to the final method.

The system that gave both optimal separation and highest recovery of protein was composed of a 50 mM acetate buffer at a pH = 5.3; the gradient ran from 0 to 11.7% MPB over 47 minutes. Injection of an alkylation (Figure 19) gave us a chromatogram composed of two major peaks and several smaller peaks (Figure 20.). Upon analysis of the first cation exchange peak onto RP-HPLC, a single peak corresponding to phosphono-CheY was observed (Figure 21). 5.5 mg of protein typically gave us about 700 µg of phosphono-CheY in return. Since most of our alkylations showed 50% conversion to phosphono-CheY according to RP-HPLC, and a standard injection was ~5.5 mg, this number seems low, yet has given us the highest recovery out of all the other methods used.

Initially, a 10 mM potassium phosphate buffer (pH = 6.8) was used. CheY was buffer exchanged into MPA and run on a gradient which went from 0 to 30% MPB over 40 minutes. An alkylation from preparation C (figure 22) was buffer exchanged into

MPA and run over the same gradient. The resulting chromatogram (figure 22) showed

three main peaks with some other minor shoulders and peaks. Each peak was collected

and concentrated down to a reasonable concentration, usually ~2-3 mg/mL, and run on

RP-HPLC for analysis. Reinjection of the 1st CEX-HPLC peak gave one main peak at 29

minutes (figure 23) which is consistent with phosphono-CheY, and one minor peak at 47

minutes corresponding to unmodified CheY. Reinjection of the 2nd peak, which was a

double peaked species at 13.5 and 14 minutes, gave a mixture of mostly unmodified

CheY with a peak a few minutes earlier, possibly oxidized CheY (figure 24). Reinjection

of the 3rd peak at 16 minutes (figure 25) gave two species as well, one consistent with

unmodified CheY and the other ~4 minutes earlier, consistent with possible oxidized

CheY. Besides lack of resolution, this method seemed to have two major flaws, the yield

from the column was very low, and optimal purity was not achieved, as CheY was still

present at ~20%. A single injection of an alkylation containing ~5.5 mg of protein would

typically only yield in about 700 μg total protein in return, with only about 100 μg being

P-CheY. It is believed that the ionic strength of the buffer was too low for the protein to

remain in solution. Also, precipitation became a problem when metal plus protein

solution was in phosphate; we believe the phosphate buffer acted as a chelation agent for

the metal and therefore pulled everything out of solution.

Because higher salt was needed, as well as a new buffer salt, the solvent system

was switched to a 25 mM sodium acetate buffer with a pH = 5.0; the gradient ran from 0

to 15% MPB over 30 minutes. Because the pH is lower than in the phosphate buffer, the

protein should stick more tightly to the column, thus require a higher concentration of salt

to elute the protein. Upon injection of a CheY alkylation (Figure 26a), this was found to

be true (Figure 26b). Here the protein came out in a higher salt concentration than it did

in the phosphate buffer. One must remember to take into account both the ionic strength

of the salt (NaCl) and the buffer itself, which had a higher initial concentration in this

method (25 mM vs. 10 mM). The method gave a chromatogram with two main peaks

with sufficient resolution (Figure 26b). A problem however arose when reinjection of the

first CEX peak onto RP-HPLC gave a double peak, one representing phosphono-CheY

and one representing CheY (Figure 27); analysis of the 2nd peak in the chromatogram

looked nearly identical (Figure 28). With this method however, the yield of total protein

did go up, with about 60% of total protein recovered.

Although the 25 mM acetate gradient gave a clean looking chromatogram with

sufficient resolution, a new method was sought because of incomplete purification and

protein recovery that was still less than desirable. The new gradient developed was one

consisting of 25 mM MES buffer at pH = 5.8. The gradient was run from 0 to 16% MPB

over 30 minutes. Figure 29 shows chromatograms of both RP-HPLC analysis of an

alkylation mixture as well as its subsequent CEX-HPLC chromatogram using this

method. Like the phosphate method, 3 main peaks are seen, and each of these was

analyzed via RP-HPLC. The first peak at 21 minutes (Figure 30) showed mainly

phosphono-CheY, but still had ~10% CheY present. Peak 2 at 23 minutes (Figure 31)

was fairly messy and showed 3 main peaks. The only peak which can be positively

identified is the 3rd peak, which corresponds with unmodified CheY; the possibilities of

the other two are oxidized and/or denatured protein. Analysis of the 3rd peak at 27

minutes (Figure 32) showed two main species, corresponding with phosphono-CheY and

unmodified CheY. Although this method gave fairly pure phosphono-CheY, greater

purity was needed; that, plus the expense of MES, gave need to develop another method.
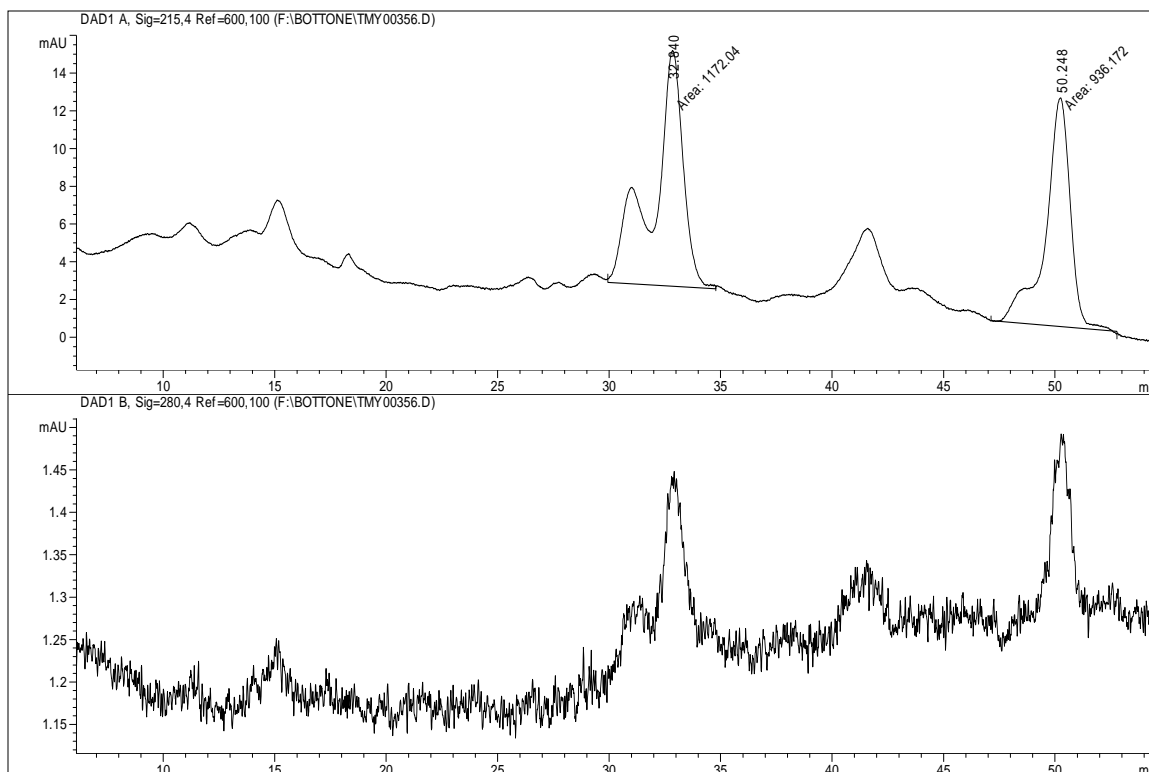
Figure 19. RP-HPLC chromatogram of an alkylation, this alkylation is both high in purity and in yield. The 12 minute is presumed doubly modified CheY, the 28 minute peak is P-CheY, and the 44 minute peak is unmodified CheY.
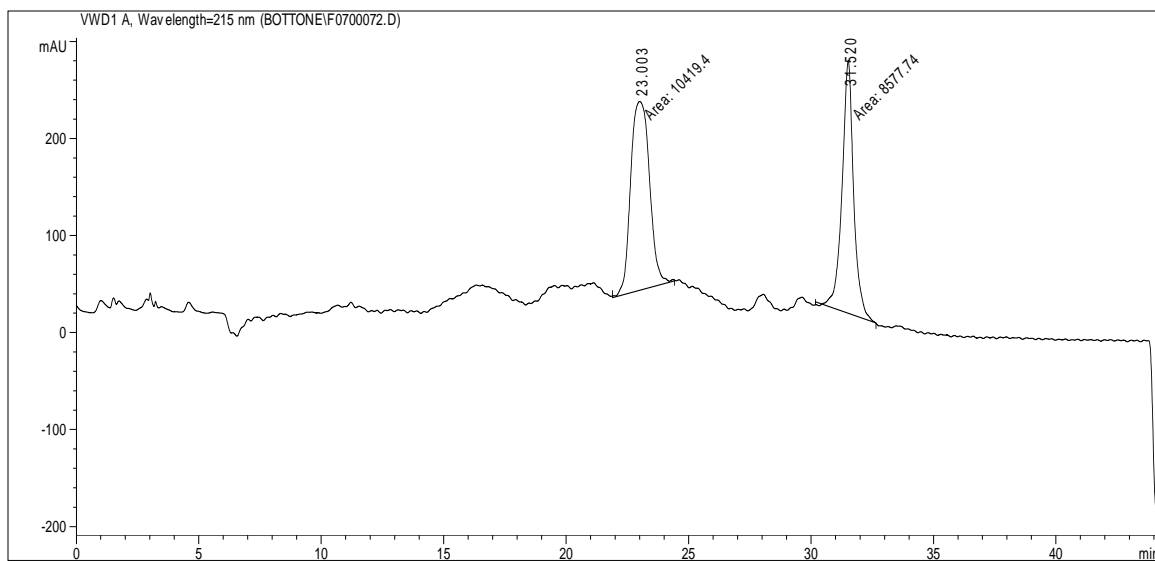
Figure 20. Cation exchange HPLC chromatogram of alkylation seen in Figure 19 using 50 mM sodium acetate pH = 5.3 with a gradient going from 0 to 11.7% MPB over 47 minutes.
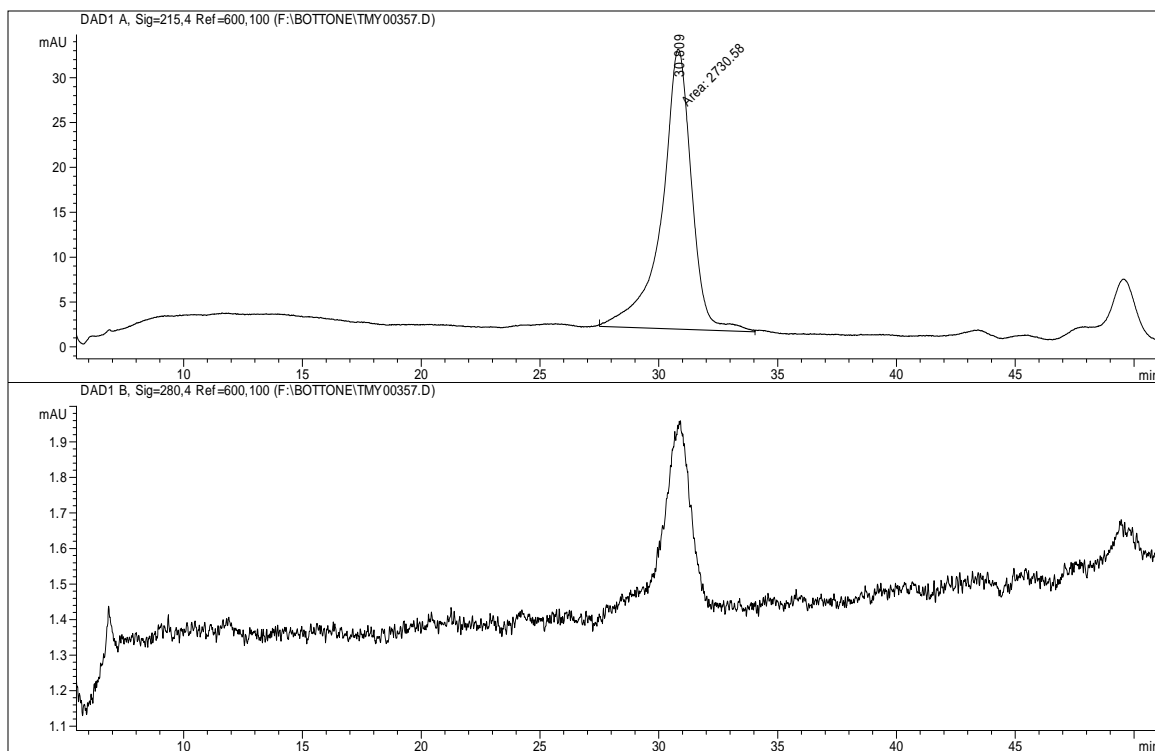
Figure 21. RP-HPLC chromatogram of the 1st cation exchange peak from the 50 mM sodium acetate method. It is a single peak representative of phosphono-CheY.
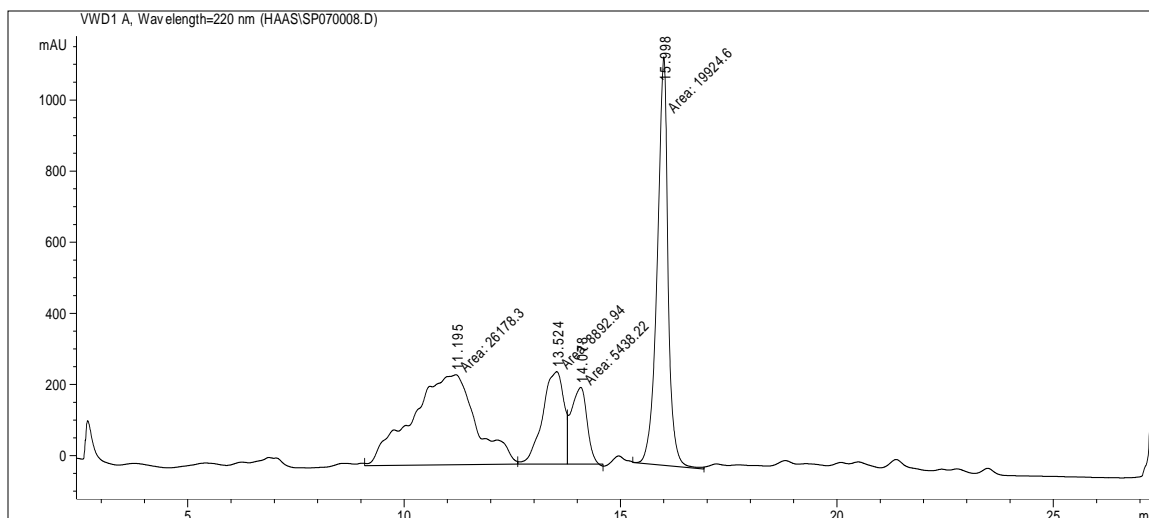
Figure 22. CEX-HPLC chromatogram of the previous alkylation. The gradient run was 0 to 30% MPB over 30 minutes. MPA was 10 mM potassium phosphate, pH = 6.2, and MPB was 10 mM potassium phosphate plus 500 mM NaCl, pH = 6.2.
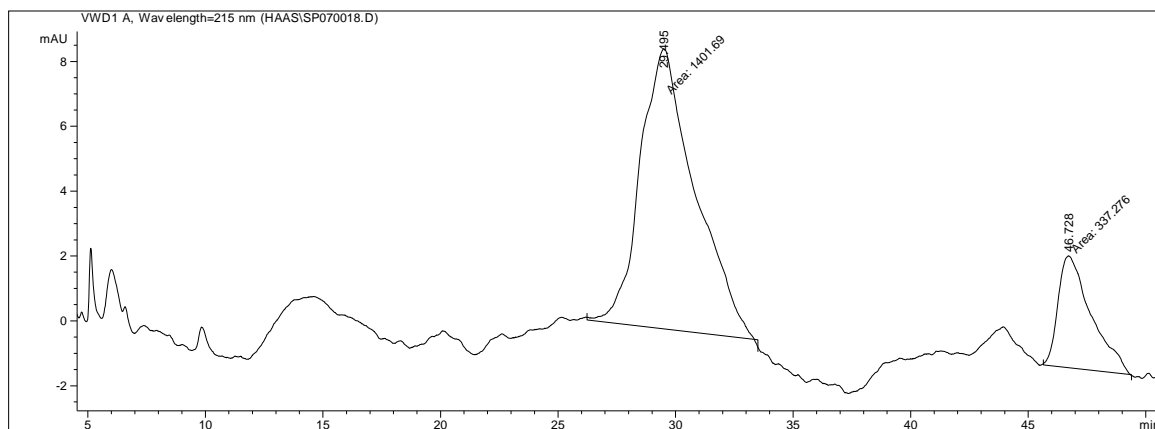
Figure 23. RP-HPLC analysis of the 1st peak in the cation exchange chromatogram shown in Figure 22. The major peak here corresponds with phosphono-CheY; however, there is some CheY still present.
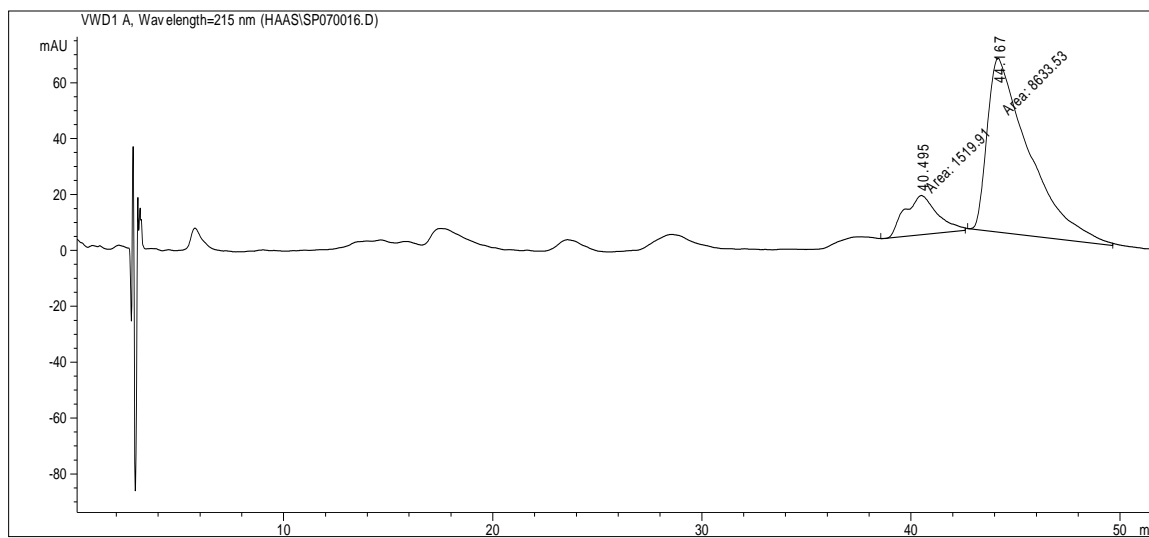
Figure 24. RP-HPLC analysis of the 2nd peak in the cation exchange chromatogram shown in Figure 22. The major peak here corresponds with unmodified CheY.
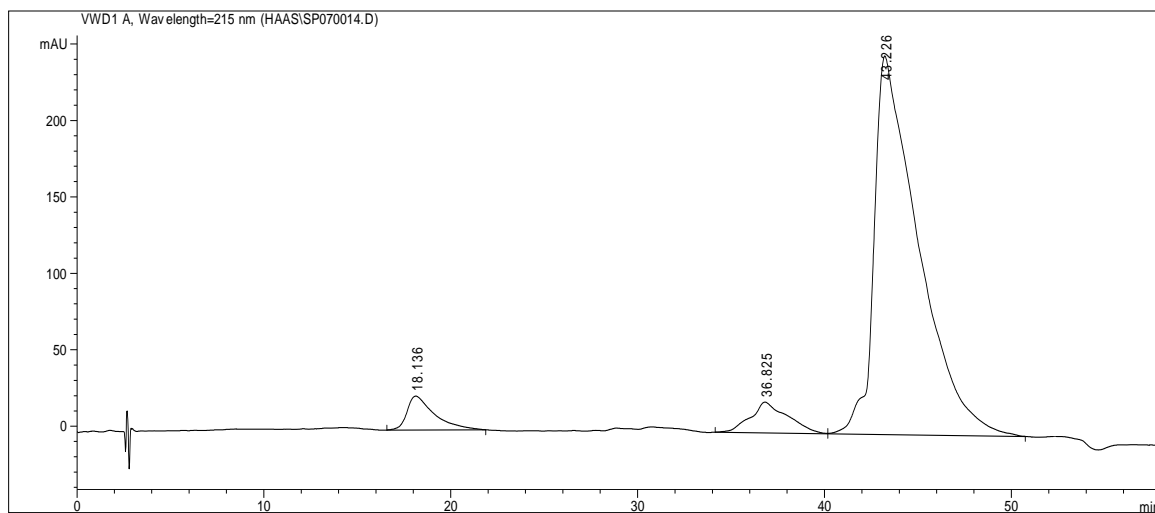
Figure 25. RP-HPLC analysis of the 3rd peak in the cation exchange chromatogram shown in Figure 22. The major peak here corresponds with unmodified CheY.
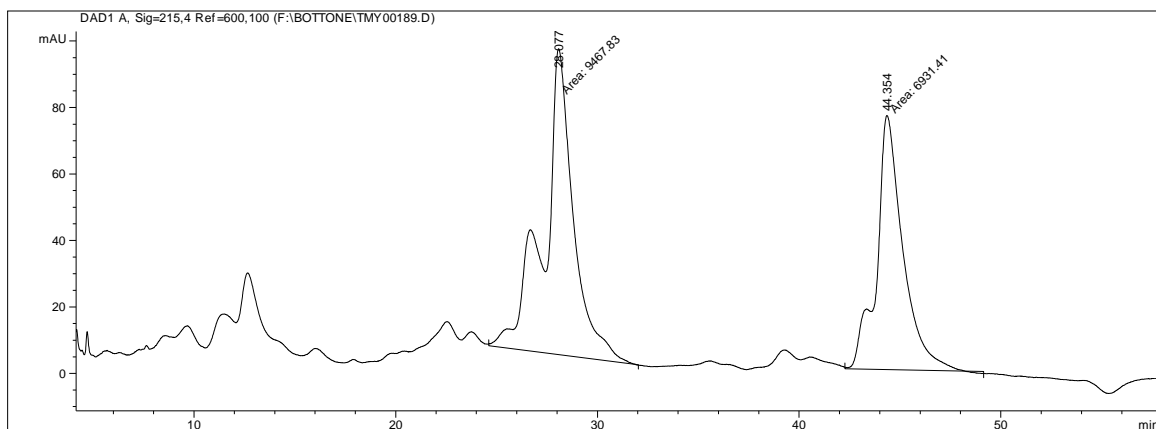
A.
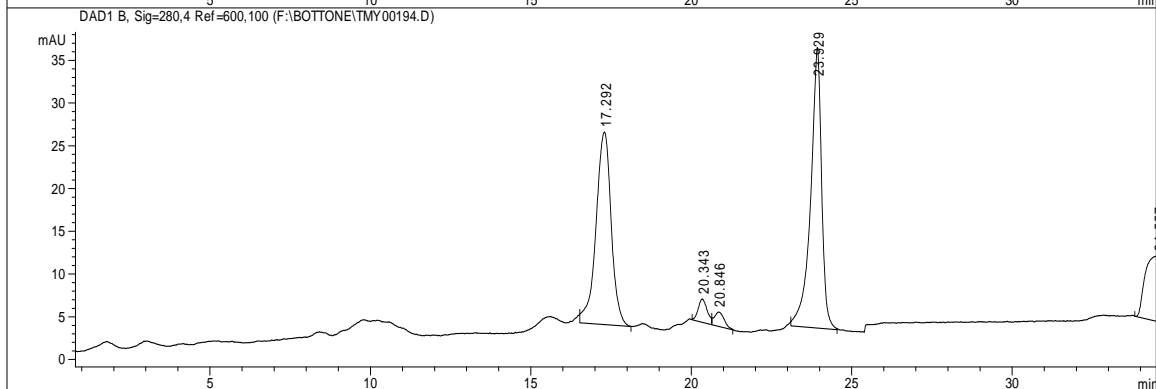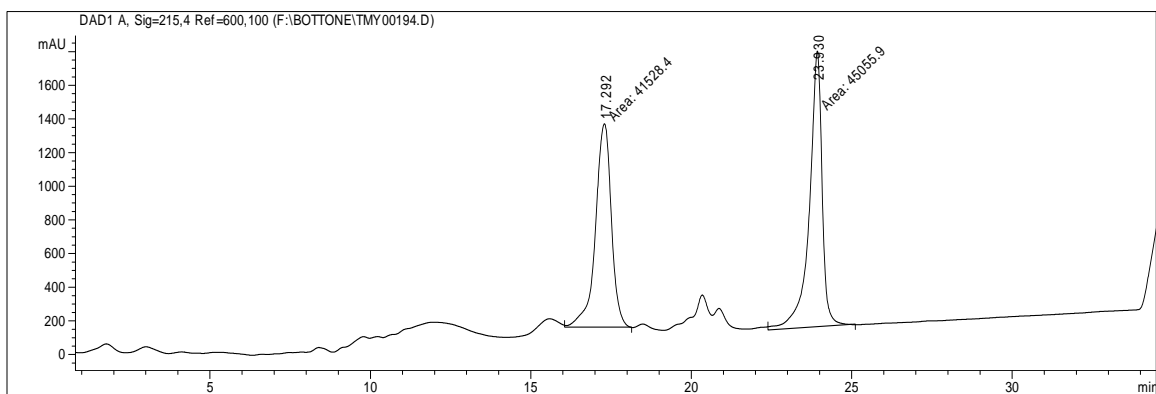
B.

Figure 26. a.) RP-HPLC chromatogram of alkylation from preparation H. b.) CEX chromatogram of the same alkylation using a 25 mM sodium acetate buffer at pH = 5.0.
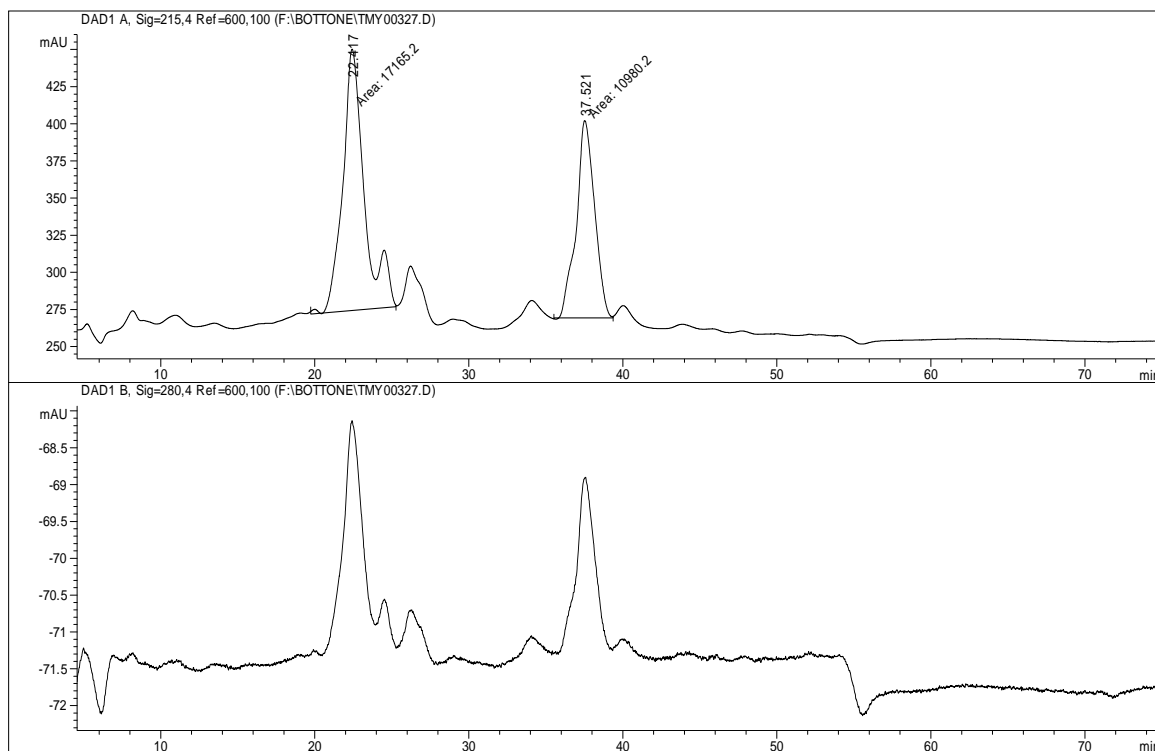
Figure 27. RP-HPLC chromatogram of the 1st cation exchange peak at 17.2 minutes from Figure 26. The main species is phosphono-CheY; however, The CheY peak at 37 minutes is still present in about ~45%.
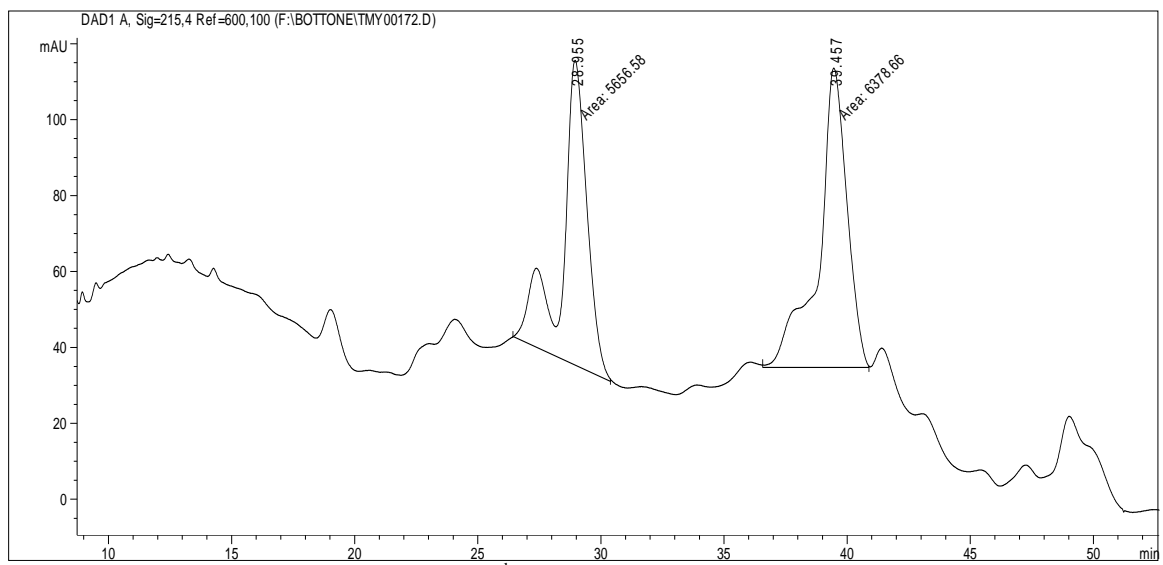
Figure 28. RP-HPLC chromatogram of the 2nd peak in the cation exchange chromatogram shown in Figure 26.
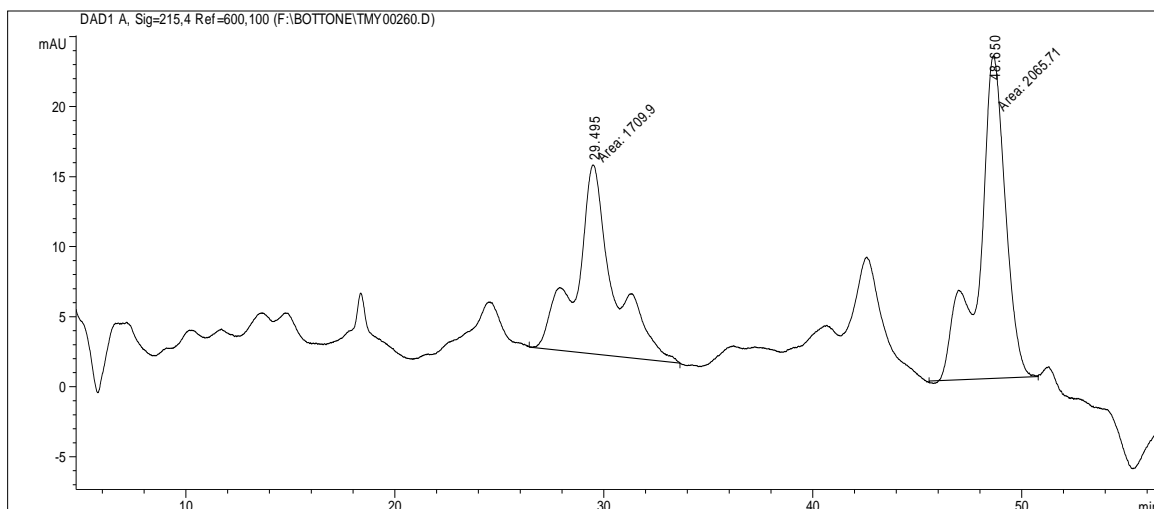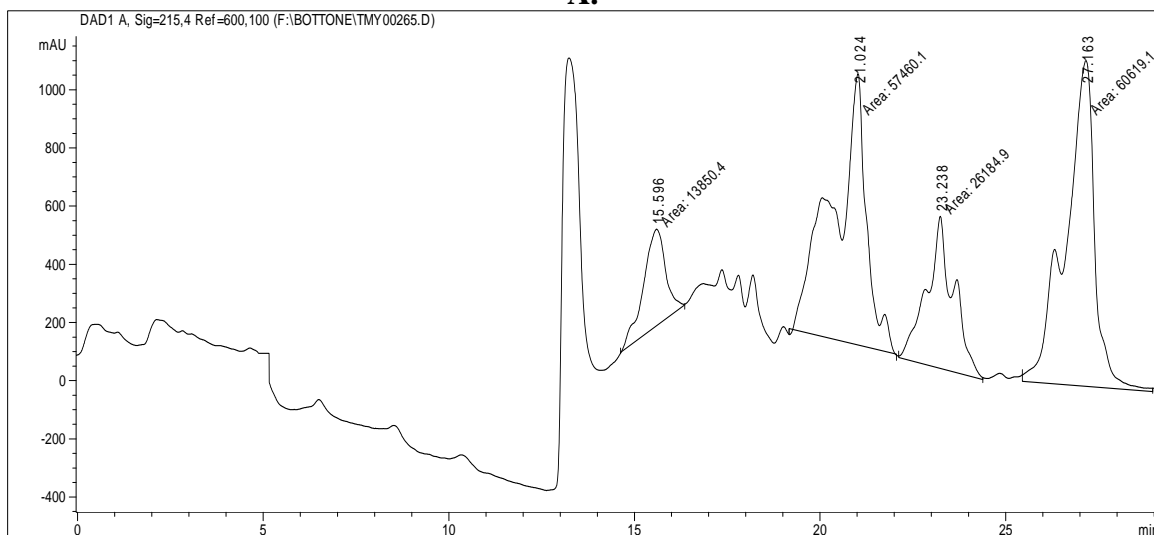
Figure 29. a.) RP-HPLC chromatogram of an alkylation from preparation C. b.) Cation exchange HPLC chromatogram of the same alkylation on a 25 mM MES gradient pH = 5.8.
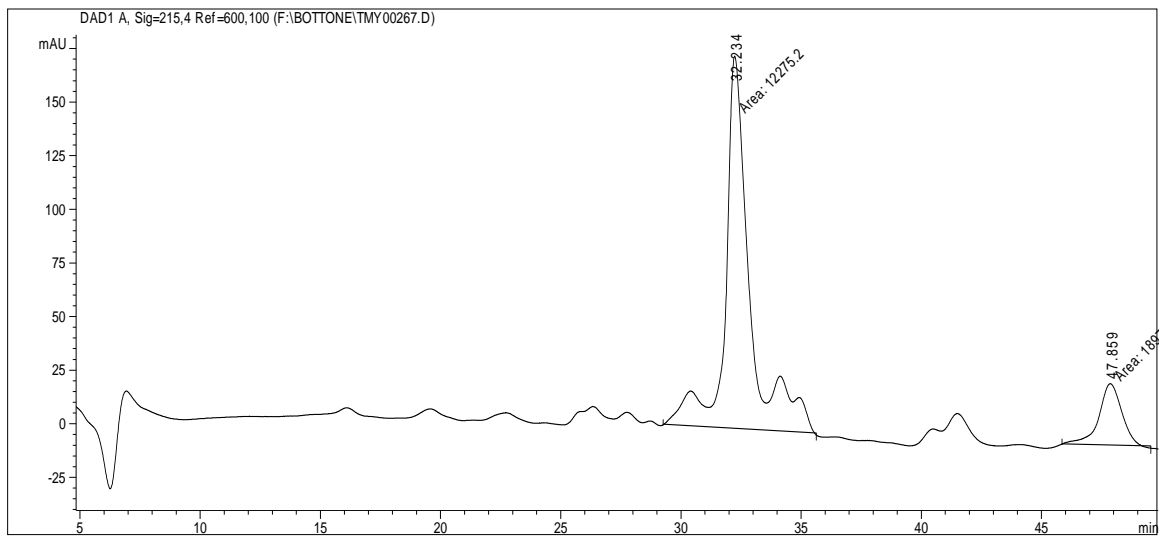
Figure 30. RP-HPLC chromatogram of the 21 minute CEX peak seen in Figure 29. Here there is one major species corresponding to phosphono-CheY, with a little small amount of CheY still present.
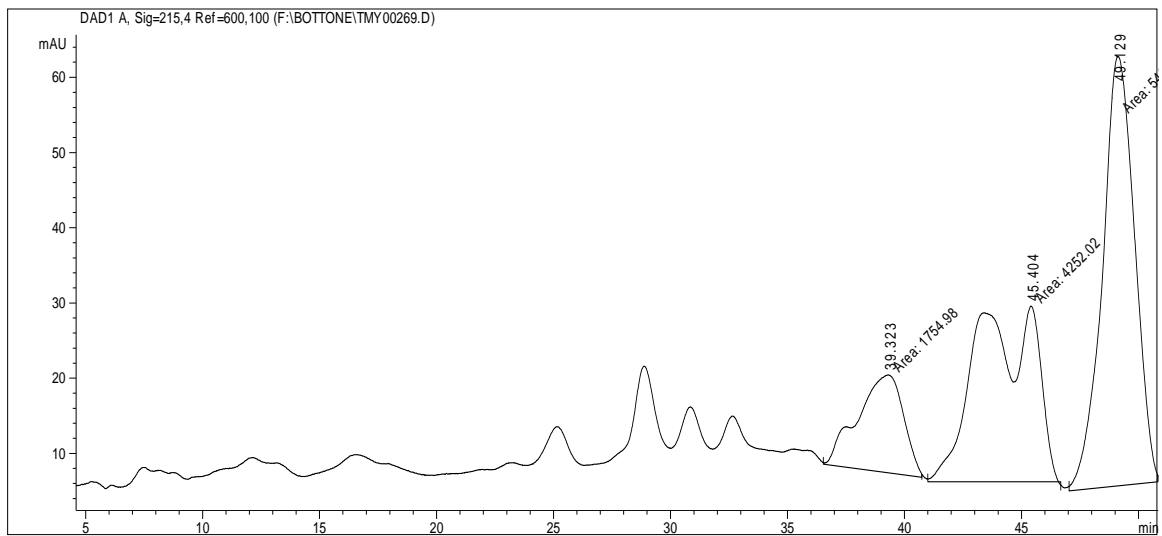
Figure 31. RP-HPLC chromatogram of the 23 minute CEX peak seen in Figure 29. Here there is one major species corresponding to unmodified CheY. Several other peaks are present as well but their identity is only speculative; oxidized and denatured proteins are possibilities.
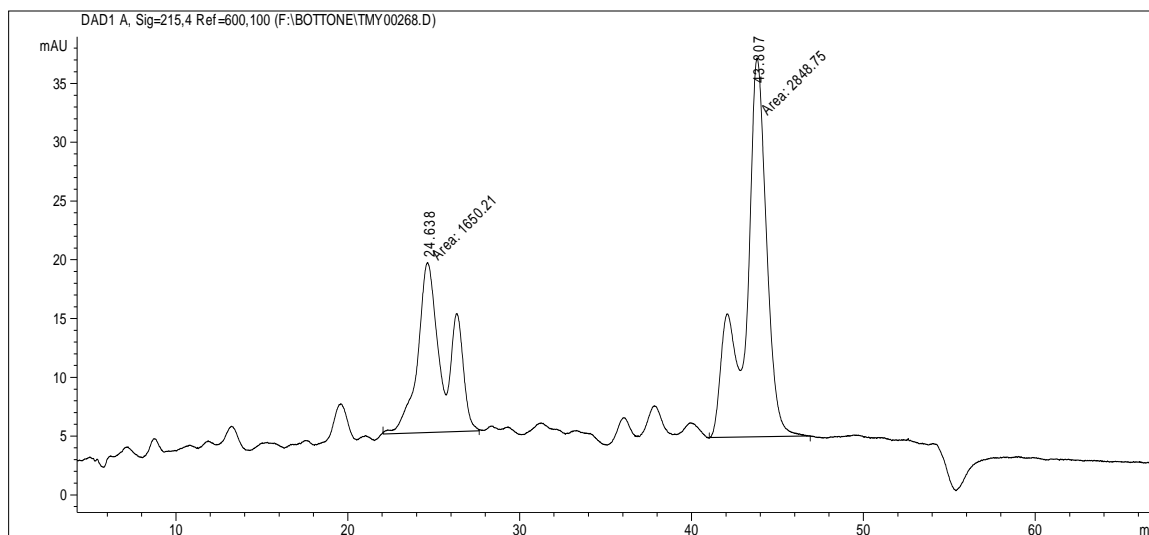
Figure 32. RP-HPLC chromatogram of the 27 minute CEX peak seen in Figure 29. Here there is one major species corresponding to unmodified CheY and another corresponding to phosphono-CheY.

Crystallization of Phosphono-CheY from *T. maritima*

Once a reasonable amount of pure phosphono-CheY was obtained via CEX-HPLC, two crystallization screens were setup. These screens were based on conditions that had worked for *E. coli* phosphono-CheY and for wild type CheY from *T. maritima*. Tables 1 and 2 show the conditions used for the two screens (Note that $MgCl_2$ is used because a crystal structure of the magnesium complex of phosphono-CheY is desirable). While all wells produced crystals of some sort, only a few produced crystals which might be large enough to diffract X-rays. Tables 3-8 show the results of the crystallization trials after a 1 month period, 3 month period, and 5 month period respectively. Wells typically produced one of seven different results, these being the following: clear drops, phase separation, needles, rosettes, single crystals of 0.2 mm or less which were either scattered throughout the drop or centrally "clumped" together, or single crystals of 0.2 mm or greater. The tables mentioned above summarize these results. Desirable crystals are those which are a single crystal of 0.2 mm or greater. Other crystals, such as needles and single or clustered crystals of 0.2 mm or less are usually the result of only a minor issue such as the percentage of PEG present or a minor pH issue (off in pH in the order of only a few tenths of a pH unit). The least desirable of these is a clear drop, in which nothing forms. Phase separation (Figure 33) is an amorphous stage in which crystallization may or may not occur in the future. Needles are one-dimensional growth in which the crystal grows in one direction and very little in the other dimensions (Figure 34). The rosettes were small star like structures (Figure 35) which if bigger, would have been possible candidates for X-ray diffraction; the same can be said of the spherulites, only they are more "weblike" in appearance. A large majority of the crystals came in the form of a

small (<0.1 mm) "x" shape; there were usually very many of these scattered throughout a drop (Figure 36). In some cases small rectangular crystals were seen bunched together in the middle of the drop (Figure 37). Though these crystals would not be suitable for X-ray diffraction, they are a promising result, as crystallization screens can be focused around those particular conditions. They may also be good candidates for gel electrophoresis. These crystals can be washed and dissolved for use on native or SDS PAGE as demonstrated by Michael Harrington and Lindsey Boroughs (unpublished results). Several larger crystals (≥0.2 mm) (examples seen in Figures 38 and 39) which are high candidates for crystallography were also seen. The four larger crystals produced were shaped differently, as two of them were less rigid looking than the other; however, it is impossible to judge whether or not a "pretty" or "ugly" crystal will give suitable diffraction without actually shooting it. The conditions that gave these larger crystals were quite different, which might be the cause of their differences. The nicer looking crystals were both produced in PEG 3400 with one being in HEPES pH = 7.0 and the other in acetate pH = 4.5; their PEG concentrations were 26% and 28% respectively. Interestingly, the less attractive looking crystals were produced under the same conditions, only in PEG 4000.

A1 100 mM Acetate(4.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B1** 100 mM PIPES(6.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C1** 100 mM HEPES(7.0) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D1** 100 mM Tris(8.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A2 100 mM Acetate(4.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B2** 100 mM PIPES(6.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C2** 100 mM HEPES(7.0) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D2** 100 mM Tris(8.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A3 100 mM Acetate(4.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B 3** 100 mM PIPES(6.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C3** 100 mM HEPES(7.0) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D3** 100 mM Tris(8.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A4 100 mM Acetate(4.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B4** 100 mM PIPES(6.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C4** 100 mM HEPES(7.0) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D4** 100 mM Tris(8.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A5 100 mM Acetate(4.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B5** 100 mM PIPES(6.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C5** 100 mM HEPES(7.0) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D5** 100 mM Tris(8.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A6 100 mM Acetate(4.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**B6** 100 mM PIPES(6.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**C6** 100 mM HEPES(7.0) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	**D6** 100 mM Tris(8.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$

Table 1. P-CheY crystal screen I. This tray screened using four different pH values as well as 6 different PEG 4000 concentrations. Ammonium sulphate and magnesium chloride were held constant throughout.

A1 100 mM Acetate(4.5) PEG 3400, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B1 100 mM PIPES(6.5) PEG 3400, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C1 100 mM HEPES(7.0) PEG 3400, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D1 100 mM Tris(8.5) PEG 3400, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A2 100 mM Acetate(4.5) PEG 3400, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B2 100 mM PIPES(6.5) PEG 3400, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C2 100 mM HEPES(7.0) PEG 3400, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D2 100 mM Tris(8.5) PEG 3400, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A3 100 mM Acetate(4.5) PEG 3400, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B 3 100 mM PIPES(6.5) PEG 3400, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C3 100 mM HEPES(7.0) PEG 3400, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D3 100 mM Tris(8.5) PEG 3400, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A4 100 mM Acetate(4.5) PEG 3400, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B4 100 mM PIPES(6.5) PEG 3400, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C4 100 mM HEPES(7.0) PEG 3400, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D4 100 mM Tris(8.5) PEG 3400, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A5 100 mM Acetate(4.5) PEG 3400, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B5 100 mM PIPES(6.5) PEG 3400, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C5 100 mM HEPES(7.0) PEG 3400, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D5 100 mM Tris(8.5) PEG 3400, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$
A6 100 mM Acetate(4.5) PEG 3400, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	B6 100 mM PIPES(6.5) PEG 3400, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	C6 100 mM HEPES(7.0) PEG 3400, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$	D6 100 mM Tris(8.5) PEG 3400, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$

Table 2. P-CheY crystal screen II. This tray screened using four different pH values as well as 6 different PEG 3400 concentrations. Ammonium sulphate and magnesium chloride were held constant throughout.

A1 100 mM Acetate(4.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B1** 100 mM PIPES(6.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A2 100 mM Acetate(4.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**	**B2** 100 mM PIPES(6.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**C2** 100 mM HEPES(7.0) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**D2** 100 mM Tris(8.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A3 100 mM Acetate(4.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**B 3** 100 mM PIPES(6.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C3** 100 mM HEPES(7.0) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D3** 100 mM Tris(8.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**
A4 100 mM Acetate(4.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**B4** 100 mM PIPES(6.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**C4** 100 mM HEPES(7.0) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D4** 100 mM Tris(8.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A5 100 mM Acetate(4.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ – **7**	**B5** 100 mM PIPES(6.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A6 100 mM Acetate(4.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B6** 100 mM PIPES(6.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**D6** 100 mM Tris(8.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)
Table 3 . P-CheY crystal screen I results after 1 month.

A1 100 mM Acetate(4.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B1** 100 mM PIPES(6.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A2 100 mM Acetate(4.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**	**B2** 100 mM PIPES(6.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**C2** 100 mM HEPES(7.0) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**D2** 100 mM Tris(8.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A3 100 mM Acetate(4.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**B 3** 100 mM PIPES(6.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C3** 100 mM HEPES(7.0) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D3** 100 mM Tris(8.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**
A4 100 mM Acetate(4.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**B4** 100 mM PIPES(6.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**C4** 100 mM HEPES(7.0) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D4** 100 mM Tris(8.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A5 100 mM Acetate(4.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ – **7**	**B5** 100 mM PIPES(6.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A6 100 mM Acetate(4.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B6** 100 mM PIPES(6.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**D6** 100 mM Tris(8.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)
Table 4 . P-CheY crystal screen II results after 1 month.

A1 100 mM Acetate(4.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3/4**	**B1** 100 mM PIPES(6.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 4000, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**
A2 100 mM Acetate(4.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**	**B2** 100 mM PIPES(6.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**C2** 100 mM HEPES(7.0) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D2** 100 mM Tris(8.5) PEG 4000, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**
A3 100 mM Acetate(4.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **4**	**B 3** 100 mM PIPES(6.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C3** 100 mM HEPES(7.0) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D3** 100 mM Tris(8.5) PEG 4000, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**
A4 100 mM Acetate(4.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**B4** 100 mM PIPES(6.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C4** 100 mM HEPES(7.0) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D4** 100 mM Tris(8.5) PEG 4000, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**
A5 100 mM Acetate(4.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ – **7**	**B5** 100 mM PIPES(6.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 4000, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**
A6 100 mM Acetate(4.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**B6** 100 mM PIPES(6.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D6** 100 mM Tris(8.5) PEG 4000, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)

Table 5 . P-CheY crystal screen I results after 3 months.

A1 100 mM Acetate(4.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **4**	**B1** 100 mM PIPES(6.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A2 100 mM Acetate(4.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - *****	**B2** 100 mM PIPES(6.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**C2** 100 mM HEPES(7.0) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D2** 100 mM Tris(8.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A3 100 mM Acetate(4.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B 3** 100 mM PIPES(6.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**C3** 100 mM HEPES(7.0) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D3** 100 mM Tris(8.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**
A4 100 mM Acetate(4.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B4** 100 mM PIPES(6.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C4** 100 mM HEPES(7.0) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D4** 100 mM Tris(8.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**
A5 100 mM Acetate(4.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B5** 100 mM PIPES(6.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A6 100 mM Acetate(4.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**B6** 100 mM PIPES(6.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**D6** 100 mM Tris(8.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)
* - Broken Coverslip
Table 6 . P-CheY crystal screen II results after 3 month.

A1 100 mM Acetate(4.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**B1** 100 mM PIPES(6.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 4000, 36% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **3**
A2 100 mM Acetate(4.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **1**	**B2** 100 mM PIPES(6.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **3**	**C2** 100 mM HEPES(7.0) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **3**	**D2** 100 mM Tris(8.5) PEG 4000, 34% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**
A3 100 mM Acetate(4.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **2/5**	**B 3** 100 mM PIPES(6.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**C3** 100 mM HEPES(7.0) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**	**D3** 100 mM Tris(8.5) PEG 4000, 32% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**
A4 100 mM Acetate(4.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**	**B4** 100 mM PIPES(6.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **2/5**	**C4** 100 mM HEPES(7.0) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**	**D4** 100 mM Tris(8.5) PEG 4000, 30% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**
A5 100 mM Acetate(4.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ – **7**	**B5** 100 mM PIPES(6.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 4000, 28% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **3**
A6 100 mM Acetate(4.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**B6** 100 mM PIPES(6.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **7**	**D6** 100 mM Tris(8.5) PEG 4000, 26% .2 M $(NH_4)SO_4$ 15 mM $MgCl_2$ - **3**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)
Table 7 . P-CheY crystal screen I results after 5 months.

A1 100 mM Acetate(4.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B1** 100 mM PIPES(6.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C1** 100 mM HEPES(7.0) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**D1** 100 mM Tris(8.5) PEG 3400, 36% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A2 100 mM Acetate(4.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **1**	**B2** 100 mM PIPES(6.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**C2** 100 mM HEPES(7.0) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**	**D2** 100 mM Tris(8.5) PEG 3400, 34% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A3 100 mM Acetate(4.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**B 3** 100 mM PIPES(6.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C3** 100 mM HEPES(7.0) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D3** 100 mM Tris(8.5) PEG 3400, 32% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**
A4 100 mM Acetate(4.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**B4** 100 mM PIPES(6.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **2/5**	**C4** 100 mM HEPES(7.0) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D4** 100 mM Tris(8.5) PEG 3400, 30% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**
A5 100 mM Acetate(4.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ – **7**	**B5** 100 mM PIPES(6.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C5** 100 mM HEPES(7.0) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **6**	**D5** 100 mM Tris(8.5) PEG 3400, 28% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**
A6 100 mM Acetate(4.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**B6** 100 mM PIPES(6.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **5**	**C6** 100 mM HEPES(7.0) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **7**	**D6** 100 mM Tris(8.5) PEG 3400, 26% .2 M (NH$_4$)SO$_4$ 15 mM MgCl$_2$ - **3**

1 – Clear Drop
2 – Phase Separation
3 – Rosettes or Spherulites
4 – Needles (1D Growth)
5 – Single Crystals (3D Growth <0.2 mm) scattered throughout
6 – Single Crystals (3D Growth <0.2 mm) clumped centrally
7 – Single Crystals (3D Growth >0.2 mm)
Table 8 . P-CheY crystal screen II results after 5 month.
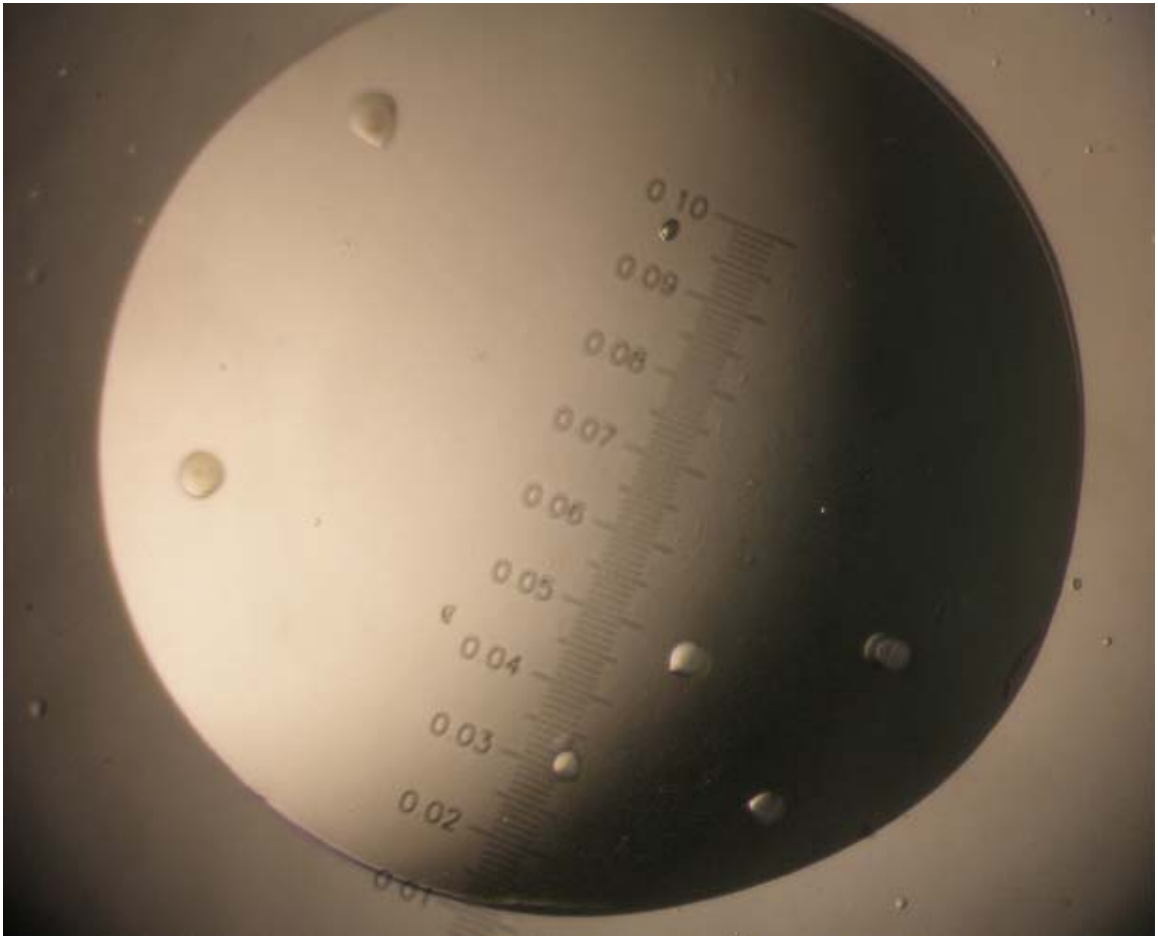
Figure 33. Picture of a crystallization drop showing phase separation.
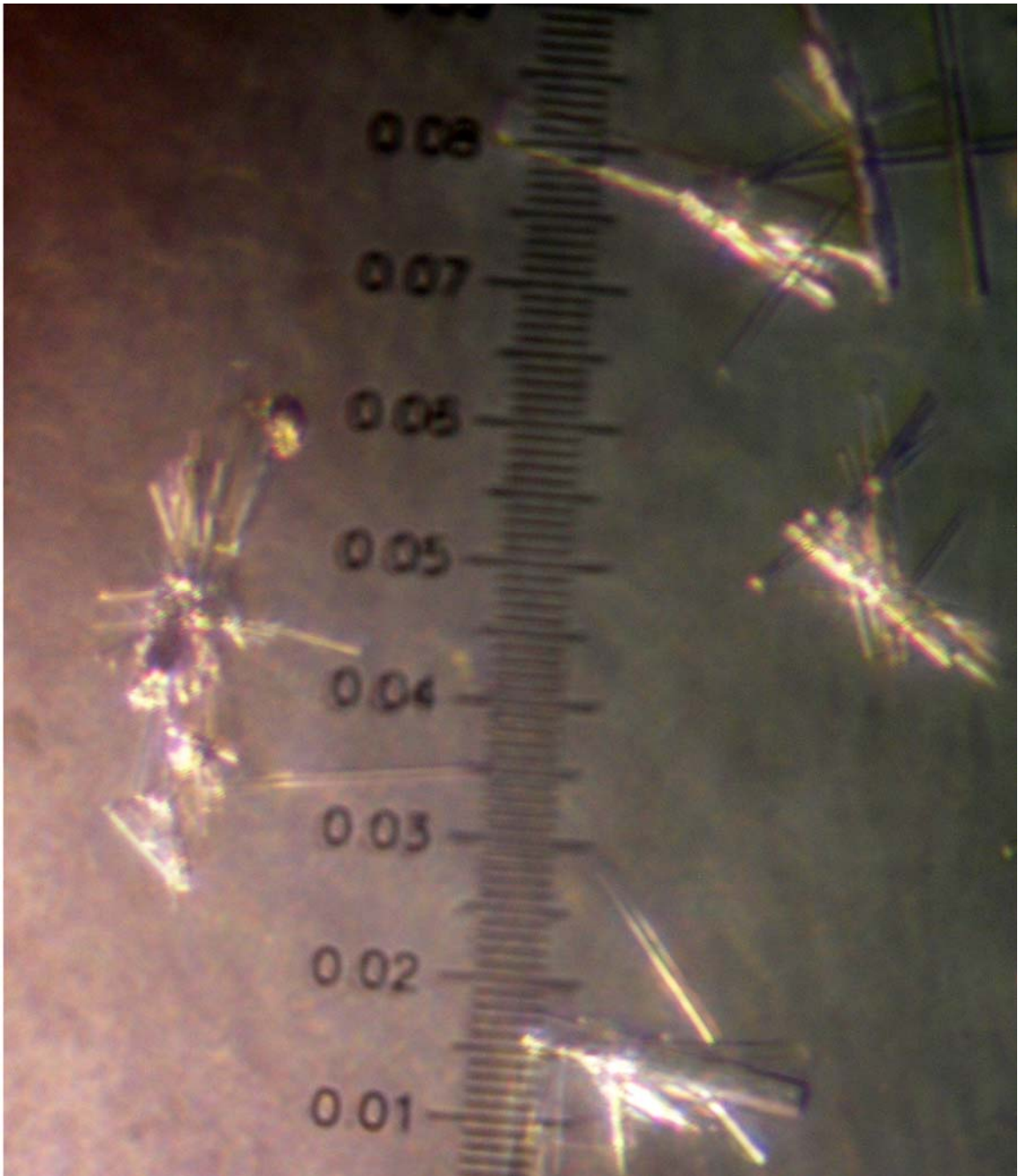
Figure 34. Picture of a crystallization drop showing phase spherulletes.
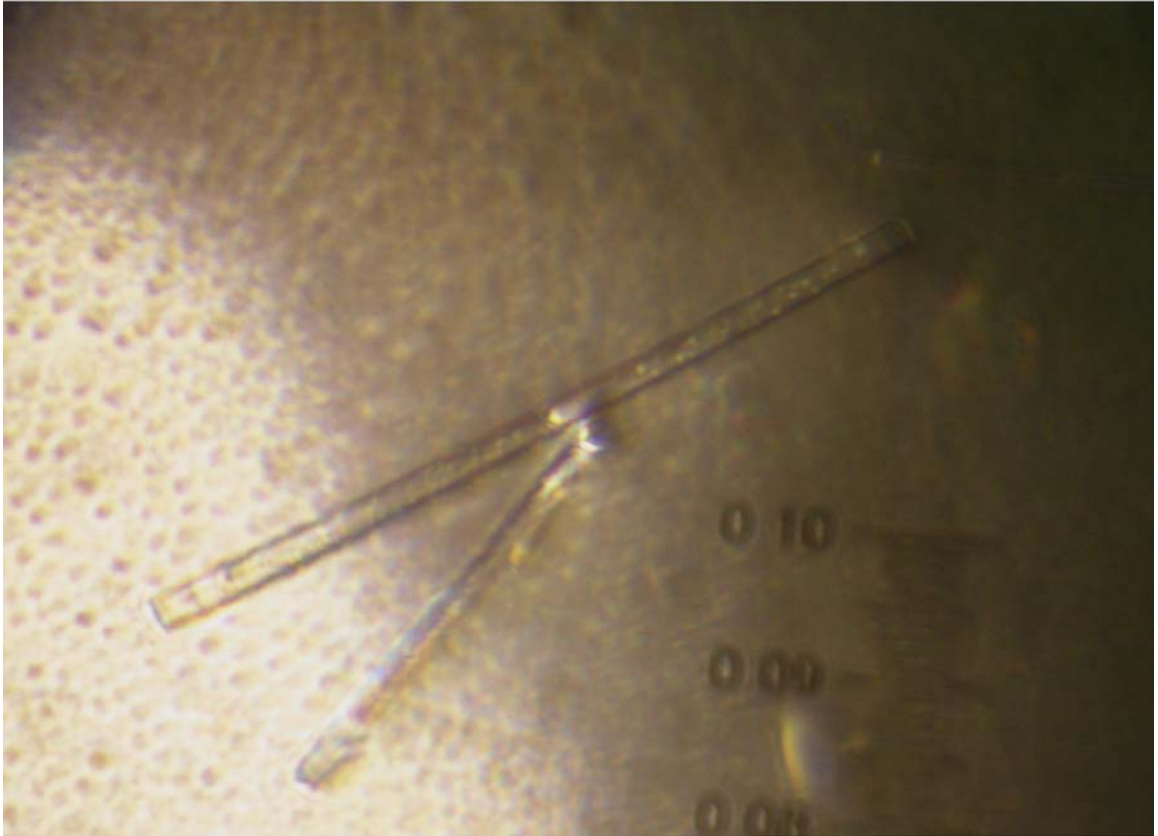
Figure 35. Picture of a crystallization drop showing needles.

Figure 36. Picture of a crystallization drop showing crystals which are <0.2 mm and scattered throughout the drop.
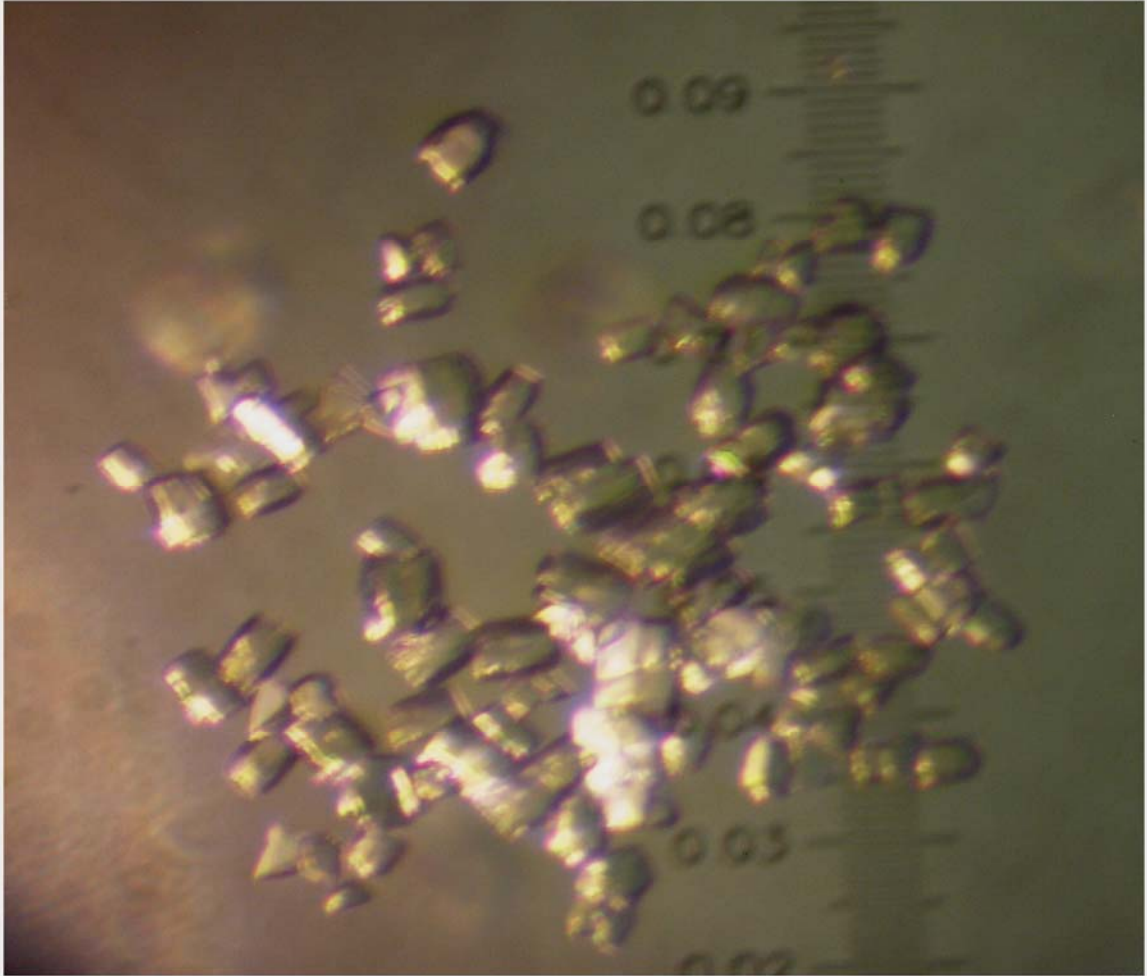
Figure 37. Picture of a crystallization drop showing crystals which are <0.2 mm and clumped centrally in the drop.
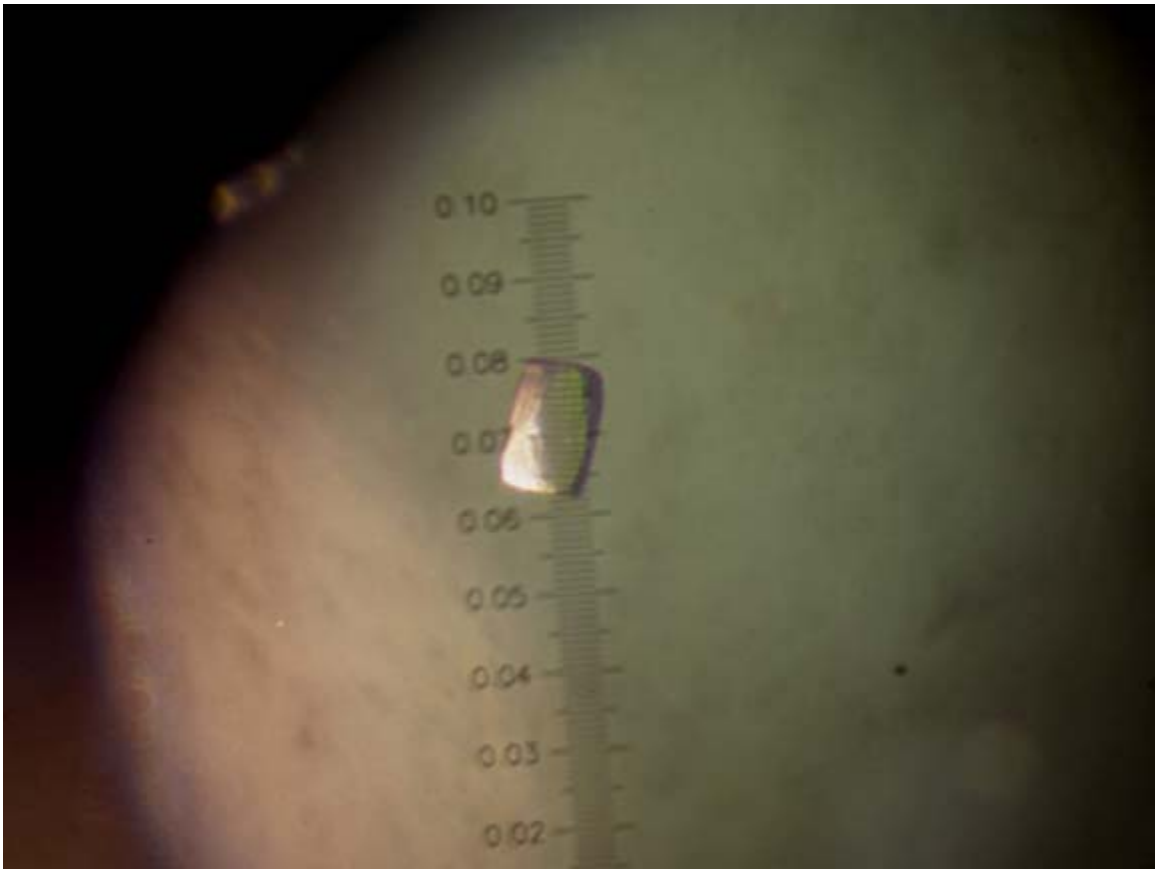
Figure 38. Picture of a crystallization drop showing a single crystal which is >0.2 mm.

Figure 39. Picture of a crystallization drop showing a single crystal >0.2 mm in size.

Synthesis of Phosphonomethyltrichlorosulfonate

As sulfinate ester was formed the solution began to turn orange in color and precipitate slightly. The reaction also gave off gas during the dripping in of the trichlorosulfinylchloride. Following the reaction NMR samples are made up of 5 μL of sample and brought up to ~500 μL with CDCl$_3$. Figure 40 shows the ^{1}H NMR spectra of the starting dibenzyl-HMP and Figure 41 the ^{1}H NMR of the sulfinylation product along with their peak assignments. The starting material contains 3 different sets of protons; the aromatic, the benzylic, and the ones next the –OH group. The triplet at ~3.9 ppm represents the CH$_2$ protons next to phosphorus. Since phosphorus is NMR active, those protons will couple with phosphorus. The multiplet at 5 ppm arises from benzyl protons, and the large peak at ~7.4 ppm is the aromatic region. The changes in the sulfinate ester arise from two factors, one being the stronger electron withdrawing effects contained within the trichlorosulfonate group, and the other arising from the introduction of a stereogenic center at the sulfur atom. The stereocenter causes the CH$_2$ protons to become diastereotopic. Diastereotopic protons are not magnetically equivalent therefore will not couple equally with neighboring molecules. Because of this, an ABX pattern is seen in the spectra. A slightly downfield shift is seen in the CH$_2$ protons which are now spread out between 4.1-4.7 ppm. The benzylic and aromatic protons are unaffected as they are too far away to feel any electron withdrawing effects.

If sulfination is successful, oxidation is carried out. Typically the oxidation reaction will turn dark green and some precipitate will form, the resulting product is a syrupy light green substance. ~5 μL is taken for an NMR sample and added to ~450 μL CDCl$_3$. The ^{1}H NMR spectra of the sulfonation reaction can be seen in Figure 42. The

major difference in the spectra of the sulfonation reaction is the absence of the ABX

pattern. The doublet which replaces the ABX splitting at 4.56 ppm represents the CH_2

protons next to the oxygen. The doublet appears because the added oxygen on the sulfur

atom makes the molecule achrial and the two protons are now magnetically equivalent;

only splitting with phosphorus is seen. The chemical shifts of the benzylic and aromatic

protons again are unaffected.

The final step in the synthesis is the removal of the benzyl groups; this is done by

standard catalytic hydrogenation. The ^{1}H NMR spectra of the compound shows only the

doublet as the aromatic and benzylic protons are absent (Figure 43).
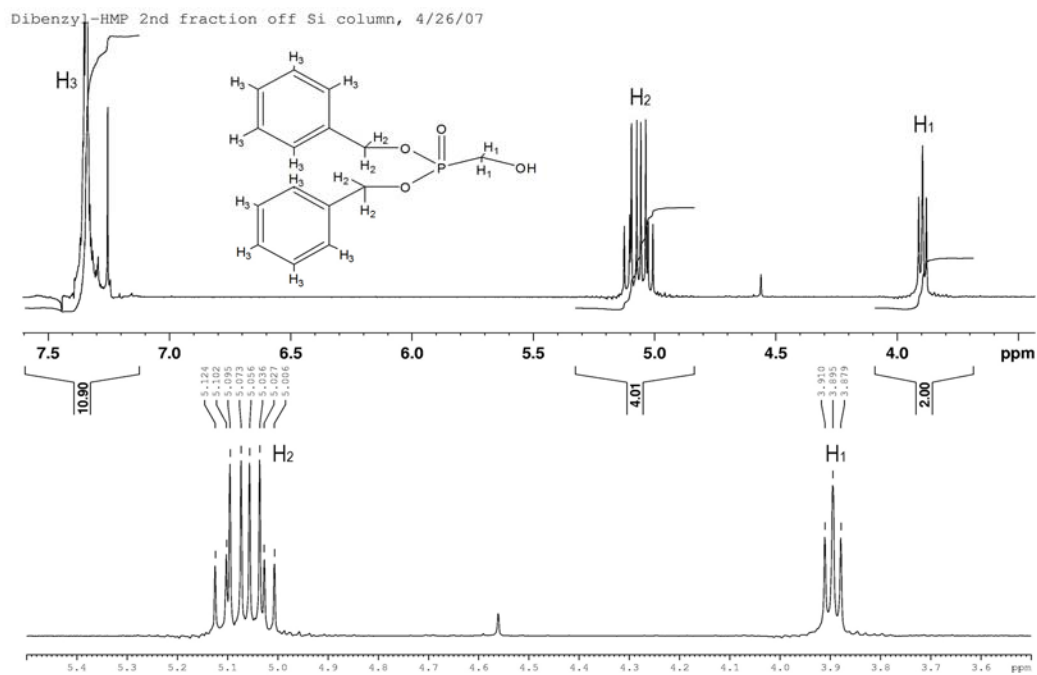
Figure 40. ^{1}H NMR spectra of the starting material, dibenzyl-hydroxymethylphosphonate.
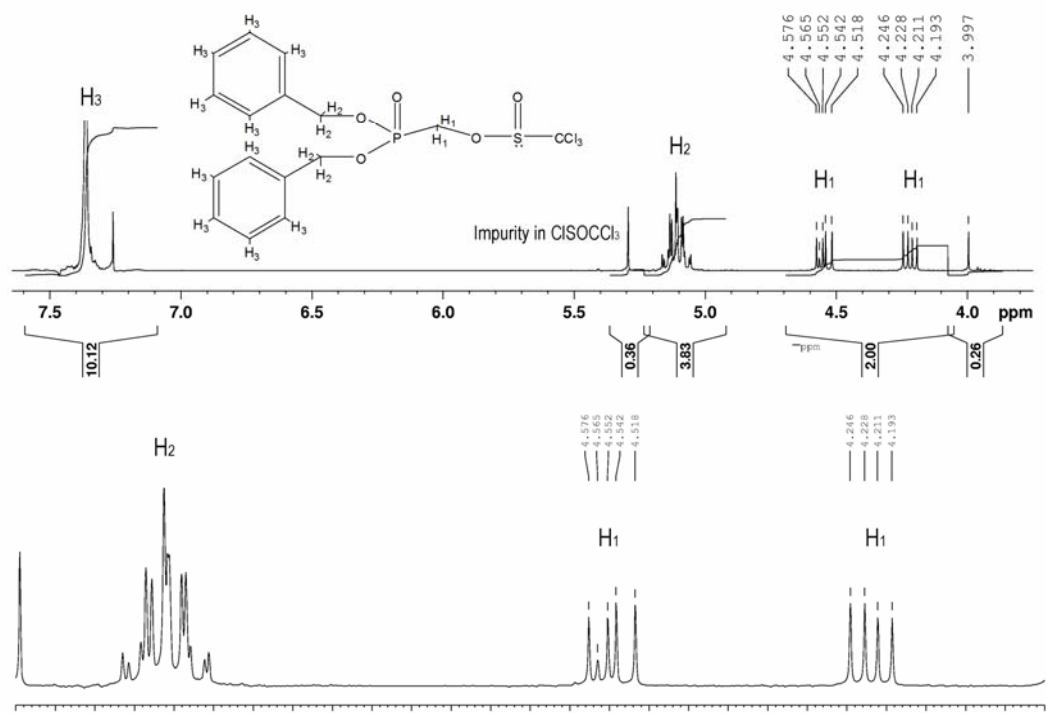
Figure 41. ^{1}H NMR spectra of the sulfinylation reaction.

Figure 42. ^{1}H NMR spectra of the sulfonylation reaction.

Figure 43. ^{1}H NMR spectra of debenzylation reaction.

DISCUSSION

Phosphonomethylation of D54C/C81S CheY from *T. maritima*

Phosphonomethylation was carried out under conditions similar to those used for

D57C CheY from *E. coli*. Because CheY from *T. maritima* is more sensitive to high

ionic strength, buffer concentration as well as metal ion concentration was lowered when

compared to CheY from *E. coli*. For best alkylation, D54C/C81S CheY prepared from a

growth with proper preparation conditions (containing protease inhibitors as previously

mentioned) will be used; proper reduced (>75%) must also be insured by DTNB assay.

CheY is alkylated by placing it in a 250 mM TAPS buffer, pH = 8.25 and modifying it

using 120 mM PMT, and 3.0 equivalents of Et$_3$N; the conversion of CheY to phosphono-

CheY under these conditions averages ~50% by RP-HPLC. Higher yield was obtained at

times, with as much as 85%, though these experiments could not be repeated consistently.

As mentioned earlier, alkylation success varied from preparation to preparation. A given

preparation of CheY would give consistent results under the same alkylation conditions,

however, would not be consistent between one preparation of CheY and another. For

example, alkylations on the order of 80% were achieved in prep. C, but alkylations using

the same conditions only gave conversion on the order of 50% or so in preparation I;

there are several possible reasons for this. Though growth conditions are reproduced as

closely as possible, the exact time between induction (addition of IPTG) of the cells and

harvesting of them varies quite a bit (this time should be kept constant in the future). As

the protocol reads, 4-7 hours is suggested for growth after induction, it might be desirable

to pick an exact time. The most obvious answer for this problem is the reduction sate of

the protein. Protein reduction might vary from preparation to preparation, thus affecting

percent conversion. If the protein is oxidized to an irreversible state such as a sulfonic

acid, reducing agents may not be effective. Although DTNB assay should confirm

reduction, the assay itself is subject to experimental error. One factor of error arises from

the fact that a small amount of reducing agent may be present during the time of the

assay. Although a buffer exchange step is employed before the assay, it is not

unreasonable to expect that a few percent of reducing agent is left behind. The presence

of reducing agent will reduce DTNB, therefore causing reduction of the protein to seem

higher than it actually is.

Other than percent conversion of CheY to phosphono-CheY, protein loss during

modification can become a problem. Loss of protein during modification seems to be the

same throughout all preparations of CheY. Typically, a reaction containing 6.5 mg of

CheY will yield $\leq$ 5.5 mg of protein after alkylation and buffer exchange. Because the

loss of protein is so consistent, regardless of preparation, this problem must be assessed

carefully. Though the reasons stated herein are presented in order to explain protein loss,

many of them can also affect the success of the alkylation as was discussed above.

Because alkylation success varies from preparation to preparation in the first place, it is

difficult to assess how a change in alkylation conditions in one given preparation will

affect CheY modification throughout all preparations of CheY.

First, loss of protein during buffer exchange must be expected, it is not

uncommon to lose ~5% of one's protein during buffer exchange over a PD-10 column.

This, however, is not the major cause of protein loss in this case. One likely reason for

protein loss is precipitation due to pH change. Even though the buffer is supposed to

resist pH change, the PMT will still drive the pH down a few tenths of a pH unit as

measured with narrow range pH paper. When using a buffer with a pH higher than the

protein's pI (8.68), the buffer pH can sometimes fall down just enough to reach the pI of the protein, causing CheY to fall out of solution. If an alkylation is performed below the pI of CheY, loss of protein becomes less of a problem; however, is not irrelevant all together. It is possible that upon addition of PMT there is a large change in pH in the area where PMT is first introduced, due to a high initial concentration of PMT in the reaction before diffusion can occur. If this is the case, protein could fall out of solution due to the sudden pH change. One concern is the increased amount of precipitation seen as the pH of the alkylation buffer is increased. This precipitate can be deceiving, considering metal ion is increasingly insoluble as pH increases. A large majority of precipitate seen in alkylations of pH 9 and above is due to metal precipitation. One must also remember that the pH cannot be lowered too much or the lessened nucleophilicity of the cysteine will decrease as we seen in lower pH alkylations (pH = 8.0).

Another possibility for protein loss arises from the high organic content of the reaction. Organic solvents are commonly used to precipitate proteins out of solution, and the organic content of the modification reaction may just be high enough to cause some of the protein to come out of solution. Alkylation using KOH rather than Et_3N as a base has been employed in the past, however no conclusive results have been made and further work should be carried out in that area.

The final consideration is the one which is the most difficult to control, that factor is technique. Even though careful consideration is taken to repeat exactly how a reaction was performed, it is extremely difficult to be exactly the same every time. Technique can be a factor in how long the PMT/ethanol mixture is left to sit, or how long the PMT and Et_3N are left together, and most importantly, how fast and efficient the PMT/Et_3N

mixture is transferred into the protein solution. It is common for a little bit of the PMT mixture to be left behind while transfer is taking place, and it must be added a few seconds later after the main PMT mixture is delivered. Time is especially important during this transfer since the PMT hydrolyzed quickly and will degrade in the presence of Et_3N if left for too long.

Purification of Phosphono-CheY

Initially, purification of phosphono-CheY from *T. maritima* was modeled after methods which worked for phosphono-CheY from *E. coli*. This method involves biotin/avidin chemistry. Derivatives of biotin such as PEO-Iodoacetyl biotin and PEO-maleimide biotin are typically reactive toward sulfhydryl groups, like the one on cysteine in CheY. Biotin itself has a high affinity for avidin, which is advantageous in the purification step. When purifying phosphono-CheY from *E. coli*, one of the above biotin derivatives is reacted with the phosphono-CheY/CheY mixture causing any unmodified CheY to become biotinylated. The mixture of the biotinylated CheY and phosphono-CheY is run over an avidin column to allow biotinylated CheY to bind and phosphono-CheY to run through, yielding pure phosphono-CheY. This method was highly successful for *E. coli* CheY but has been mostly a failure with *T. maritima* CheY. Phosphono-CheY from *T. maritima* has been purified from this method in the past, however an extremely low yield was obtained, and several biotinylations were needed. Because this method became undesirable, a new method was sought out.

The idea for ion exchange came about after numerous conversations with Dr. Andrew Alpert of PolyLC Inc., and due to prior knowledge of cation exchange use in

separation of hemoglobin. Initial conditions for cation exchange HPLC were suggested

by him and the recommended buffer was 10 mM K_2HPO_4 at pH = 6.8 with Na^+ used as a

counter ion in MPB. The gradient worked out was one which went from 0 to 40% MPB

over 40 minutes. Separation and purification using this gradient worked moderately well;

however, protein recovery was a major issue. Injection of a 5 mg reaction mixture would

only give back a 1 mg or so of protein, with only ~150 μg being phosphono-CheY. Two

possibilities were possible culprits for protein loss, one being that the ionic strength of the

buffer was too low, and the other being that phosphate itself pulled the protein out of

solution. The first course of action was to raise the ionic strength of the phosphate

buffer; it was raised to 25 mM and re-run. At the higher phosphate concentration no

protein had stuck at all, meaning that if this method was to work a pH change in the

buffer would have to take place as well. Because a different pH was needed and a change

in actual buffer identity was desirable, a 25 mM sodium acetate buffer at pH = 5.0 was

used. The gradient used for this buffer ran from 0 to 15% MPB over 30 minutes. This

method initially seemed to work much better than the phosphate method as far as

purification went. The overall yield from the column was much greater and the

chromatogram itself looked much cleaner. Since both ionic strength and buffer identity

was changed from the phosphate system, it's hard to pinpoint which one of the two was

the problem. When reinjection of the first peak gave back two peaks after several runs,

the quality of this gradient was questioned and the method was abandoned. In retrospect,

it is now known that the preparation itself was most likely responsible for this and not the

method of purification.

The following method consisted of 25 mM MES at pH = 5.8, with the gradient going from 0 to 16% MPB over 30 minutes. The MES method came with three main problems; cleanliness, expense, and purity, which was later dismissed as a preparation problem (pertaining to the absence of leupeptin and pepstatin during the growth). Running on this method produced a large baseline drift over time which affected the resolution of the peaks, making it sometimes difficult to discern between two different peaks. That, coupled with the amount of buffer which is used in purification (HPLC grade MES was expensive and being used at a high rate), made this method somewhat undesirable already.

The main problem with the MES method was the purity of phosphono-CheY which eluted from the column. Purification using this method never produced pure phosphono-CheY; however, at the same time this method was being devised, a preparation of CheY which did not contain optimal growth conditions was used (no Leupeptin, Pepstatin, or PMSF). At the time, these "bad" preparations were not thought about, so the problem in purification likely was because of the preparation itself rather than the method used. Because we didn't know about preparation dependency, this actually became the main reason the method was abandoned.

Upon further consideration and after another preparation of CheY (containing the three protease inhibitors), another acetate system was devised. Because acetate had given the best resolution and cleanest chromatograms in the past, a method with slight modification over the previous system was developed in hopes the protein recovery could be increased. The method developed was contained 50 mM sodium acetate at pH = 5.3, with the method running from 0 to 11.7% MPB over 47 minutes. This method gave both

better resolution and higher recovery than all the previous systems. Because higher

recovery was seen again, the ionic strength hypothesis becomes more valid, as this

system contained the highest ionic strength of all the systems. Still though, recovery was

less than expected. Theoretically, ~30 mg of phosphono-CheY should be obtained for

every 100 mg of CheY alkylated. Based on rough calculations, there is an average of

40% conversion of CheY to phosphono-CheY, and an estimated 10% loss for steps such

as buffer exchange and concentration. Currently, methods to improve recovery are under

investigation, these include the use of an organic modifier in the cation exchange buffers

(such as 10% isopropanol), and using a buffer of even higher ionic strength.

It should also be noted that just because the RP-HPLC and CEX-HPLC

chromatograms of a particular alkylation are very similar looking as far as number and

size of peaks (As seen in the Figures 17/18, and 24 and 27), one must still at least analyze

fractions via RP-HPLC after purification because one CEX peak can sometimes

correspond to two or more RP peaks. Why this happens after purification and not before

is unknown.

Crystallization of Phosphono-CheY

Based on conditions used for the crystallization of CheY from *T. maritima* and for

phosphono-CheY from *E. coli*, two screens were set up for phosphono-CheY

crystallization. The majority of the crystallization results were fairly similar to each

other throughout all the conditions tested. All wells in both trays produced at least some

crystals, which in itself is successful as some crystallization trials take dozens of screens

before some crystals are seen. A few wells produced crystals which are candidate for X-

ray diffraction. The most common result was one which produced many small crystals (<0.2 mm) in a single drop. Many of these crystals were either "X" or butterfly shaped, and were most prevalent in wells which contained high PEG concentrations (> 28%, which the majority of the wells contained). Because PEG is the main precipitant during crystallization, the higher the PEG concentration, the more prevalent nucleation will be. Large number of nucleations will create a large number of crystals, while fewer nucleations will create less crystal formation, but usually larger more desirable crystals (as seen in wells with lower PEG concentrations). This suggests that the initial PEG concentrations were a little too high and should be reduced in subsequent trials using these particular PEG molecular weights. One use for these smaller crystals is to seed them to attempt to grow other larger crystals. Seeding is a technique in which crystals from one drop are transferred into another drop and streaked across this new drop (28).

Another less common result was the formation of needles; these did not become prevalent until around 3 months into crystallization trials in the low pH acetate wells. Needles usually suggest that one is not far off from optimal crystallization conditions and only minor changes in pH and PEG concentration should be made (28). After 5 months, many of the low pH wells which contained needles became clear, with no crystals at all present.

The wells which produced the most promising results throughout (those which contained single crystals >0.2 mm or crystals which were between 0.1-0.2 mm) were the same in both PEG molecular weights. HEPES pH = 7.0 at PEG concentrations of 32% and less produced the best crystals; with the largest crystals seen at 26% PEG. The other promising areas were in the low pH acetate wells. At both PEG concentrations, the

acetate wells which contained 30% and 28% PEG concentrations produced very nice looking crystals. Subsequent screens should be focused around these areas.

Because crystals continued to change after 5 months, crystal trays should constantly be analyzed and never given up on. Some suggest that crystal formation so late into the trials is a sign of protein degradation, however, successful X-ray analysis has been performed on crystals which are a year old or more.

REFERENCES

1. Bourret, R. B., and Stock, A. M. (2002). Molecular Information Processing: Lessons from Bacterial Chemotaxis, *J. Biol. Chem. 277*, 9625-9628.

2. Stock, A.M. (2007). Molecular Mechanisms of Signal Transduction, http://www.hhmi.org/research/investigators/stock.html.

3. West, A.H., and Stock, A.M. (2001). Histidine Kinases and Response Regulator Proteins in Two-Component Signaling Systems, *Trends Biochem. Sci.* 26(6), 369-376.

4. Robinson, V.L., Buckler, D.R., and Stock, A.M. (2000). A Tale of Two Components: A Novel Kinase and a Regulatory Switch, *Nat. Struct. Biol.* 7(8), 626-633.

5. Harrison-McMonagle, P., Denissova, N., Martinez-Hackert, E., Ebright, R.H., and Stock, A.M. (1999). Orientation of OmpR Monomers With an OmpR:DNA Complex Determined by DNA Affinity Cleaving, *J. Mol. Biol.* 285(2), 555-566.

6. Cai, S.J., and Inouye, M. (2002). EnvZ-OmpR Interaction and Osmoregulation in *Escherichia coli*, *J. Biol. Chem.* 27, 24155-24161.

7. Park, S.Y., Lowder, B., Bilwes, A.M., Blair, D.F., and Crane, B. (2006). Structure of FliM Provides Insight Into Assembly of the Switch Complex in The Bacterial Flagella Motor. *Proc. Natl. Acad. Sci.* 103, 11886-11891.

8. Hansen, C.H., Endres, R.G., and Wingreen, N.S. (2007). Chemotaxis in *Escherichia coli*: A Molecular Model for Robust Precise Adaptation, *PLoS Comput. Biol.* 4, 0014-0027.

9. Berg, J.M., Tymoczko, J.L., and Stryer, L. (1999). Bacterial Motion, Biochemistry, 6th Edition, 993-996.

10. Homma, M., Shiomi, D., Homma, M., and Kawagishi, I. (2004). Attractant Binding Alters Arrangement of Chemoreceptor Dimers Within its Cluster at a Cell Pole, *Proc. Natl. Acad. Sci*. 101, 3462-3467.

11. Scharf, B., Fahrner, K.A., Turner, L., and Berg, H. (1997). Control of Direction of Flageller Rotation in Bacterial Chemotaxis, *Proc. Natl. Acad. Sci.*, 95, 201-206.

12. Sagi, Y., Khan, S., and Eisenbach, M. (2003). Binding of the Chemotaxis Response Regulator CheY to the Isolated, Intact Complex of the Bacterial Flagellar Motor, *J. Biol. Chem.*, 278, 25867-25871.

13. Park, S., Chao, X., Gonzalez-Bonet, G., Beel, B.D., Bilwes, A.M., and Crane, B. (2004). Structure and Function of an Unusual Family of Protein Phosphatases: The Bacterial Chemotaxis Proteins CheC and CheX, *Mol. Cell*, 16, 563-574.

14. Kirby, J.R., Kristich, C.J., Saulmon, M.M., Zimmer, M.A., Garrity, L.F., Zhulin, I.B., and Ordal, G.W. (2001). CheC is Related to the Family of Flagellar Switch Proteins and Acts Independently from CheD to Control Chemotaxis in *Bacillus subtilis*, *Mol. Micro.*, 42(3), 573-585.

15. Halkides, C.J., Zhu, X., Phillion, D.P., Matsumura, P., and Dahlquist, F.W. (1998). Synthesis and Biochemical Characterization of an Analogue of CheY-Phosphate, A Signal Transduction Protein in Bacterial Chemotaxis, *Biochemistry*, 37(39), 13674-13680.

16. Halkides, C.J., McEvoy, M.M., Casper, E., Matsumura, P., Volz, K., and Dahlquist, F.W. (2000). The 1.9 Å Resolution Crystal Structure of Phosphono-CheY, an Analogue of the Active Form of the Response Regulator, CheY.

17. Halkides, C.J., Bottone, C.J., Casper, E.S., Haas, R.M., and McAdams, K. (2007). Synthesis of a Stable Analogue of the Phosphorylated Form of CheY: Phosphono-CheY. *Methods Enzymol.*, 422, 338-351.

18. McAdams, K., Casper, E., Haas, R.M., Santarsiero, B.D., Eggler, A.L., Mesecar, A., and Halkides, C.J. (under review). The Structure of T87I and T87I/Y106W Phosphono-CheY Help to Explain Their Binding Affinities to the FliM and CheZ Peptides, *J. Bacter.*, Under review.

19. Hirs, C.H.W. (1977). Gel Filtration. *Methods Enzymol.* XLVII, 97-107.

20. Penefsky, H.S., (1977). Reversible Binding of P_i by Beef Hear Mitochondrial Adenosine Triphosphatase, *J. Biol. Chem.*, 252, 2891-2899.

21. Haas, R.M. (2007). Synthesis and Characterization of Phosphono-CheY from *Thermotoga maritima*, Masters Thesis.

22. Wright, S.K., and Viola, R.E., (1998). Evaluation of Methods for the Quantitation of Cysteines in Proteins, *Anal. Biochem.*, 265, 8-14.

23. GE Healthcare. (2007). PD-10 Desalting Columns,

24. Usher, K.C., De La Cruz, A.A., Dahlquist, F.W., Swanson, R.V., Simon, M.I., and Reminton, S.J. (1997). Crystal Structures of CheY from *Thermotoga maritima*, *Protein Science*, 7, 403-412.

25. Carlsen, H.J., Katsuki, T., Martin, V.S., and Sharpless, K.B. (1981). A Greatly Improved Procedure for Ruthenium Tetraoxide Catalyzed Oxidations of Organic Compounds, *J. Org. Chem.*, 46, 3936-3938/

26. Gao, Y., and Sharpless, K.B. (1988). Vicinal Diol Cyclic Sulfates: Like Epoxides Only More Reactive, *J .Am. Soc.*, 110, 7538-7539.

27. Netscher, T., and Bohrer, P. (2002). Towards Highly Activating Leaving Groups: Studies on the Preparation of Some Halogenated Alkyl Sulfonates, *Molecules*, 7, 601-617.

28. Bergfors, T.M., (1999). <u>Protein Crystallization: Techniques, Strategies, and Tips</u>. 134-138.

Appendix A: Buffers For the Growth of CheY from *Thermotoga maritima*

<u>Terriffic Broth (1 L)</u>
Tryptone	12 g
Yeast Extract	24 g
KH_2PO_4	2 g
K_2HPO_4*	12 g
Glycerol	4 mL

<u>PMSF</u>	<u>Concentration</u>	<u>g/mol</u>	<u>10 mL</u>
PMSF	100 mM	174.2	.174g
Ethanol			10 mL

<u>Lysis Buffer</u>	<u>Concentration</u>	<u>g/mol</u>	<u>1 L</u>
HEPES	25 mM	238.3	5.96 g
NaCl	500 mM	58.44	29.22 g
Imidazole	5 mM	68.08	0.3404 g
Azide	0.02%		0.05 g
pH = 7.5			

<u>Wash Buffer</u>	<u>Concentration</u>	<u>g/mol</u>	<u>250 mL</u>
HEPES	20 mM	238.3	1.19 g
NaCl	500 mM	58.44	7.31 g
Imidazole	20 mM	68.08	.340 g
Azide	0.02%		0.05 g
pH = 7.5			

<u>Elute Buffer</u>	<u>Concentration</u>	<u>g/mol</u>	<u>100 mL</u>
HEPES	20 mM	238.3	.596 g
NaCl	500 mM	58.44	2.92 g
Imidazole	200 mM	68.08	1.36 g
pH = 7.5			

*$K_2HPO_4•3H_2O$ maybe be used in place since it is easier to weigh out, however, the weight of water must be taken into account for and corrected.

Appendix B: Regeneration of Nickel Column

Wash the column successively with the following:

1.   2 vol regeneration buffer (6 M Gu-HCl, 0.2 M acetic acid)

2.   5 vol water

3.   3 vol 2% SDS

4.   1 vol 25% ethanol

5.   1 vol 50% ethanol

6.   1 vol 75% ethanol

7.   5 vol 100% ethanol

8.   1 vol 75% ethanol

9.   1 vol 50% ethanol

10.   1 vol 25% ethanol

11.   1 vol water

12.   5 vol 100 mM EDTA, pH = 8.0

13.   10 vol water

14.   Recharge column with 2 vol freshly made 100 mM $NiSO_4$

15.   Wash with 2 vol water

16.   Wash with 2 vol regeneration buffer

17.   Equilibrate in 2 vol of a suitable buffer.

Appendix C: Other Possible Conditions for Phosphonomethylation of CheY

Several methods of alkylation not mentioned in the main text were tested, these methods have not been exhausted and have only minimal data, however; it is highly recommended that further work on the following conditions is put forth.

Because metal ion is important during the alkylation reaction, the possibility of a larger metal ion may be advantageous. Trivalent metals such as Nd^{3+}, Tb^{3+}, and Lu^{3+} were tested against CheY. These metals were used only in a 5-10 mM concentration since their solubilities at higher pH values is low. The results from these alkylations were similar to results obtained from Ba^{2+} and Sr^{2+}; the RP-HPLC chromatograms would sometimes look clean with fairly high P-CheY yield, while others would exhibit high heterogeneity. At the time of these alkylations, the buffer being used was 250 mM AMPSO at pH = 9.25, so it is possible that at lower pH values better results would have been obtained. Because some clean alkylation reactions were observed, this are should be looked into further.

Earlier, it was mentioned that the high organic content of the reaction was a possible cause of protein precipitation. Because of this, Et_3N was replaced with KOH and triethanolamine. The data from these results suggest that alkylation is possible with other bases. Alkylations with KOH showed a large amount of protein loss while alkylations with triethanolamine should similar results as Et_3N. One problem with the triethanolamine was its viscosity; it became very difficult to deliver the triethanolamine efficiently and methods for easier transfer should be looked into and retested.

Two other experiments, which are somewhat related involve the use of different alkylating agents and while as higher temperature. Because our protein is a thermophile

it is able to retain stability at high temperatures where other proteins would not be stable. In fact, a method of purifying CheY is to heat lysate up to $85^{o}C$ and let all other molecules fall out of solution while CheY stays intact and in solution. Higher temperature allows for modification with molecules that are less reactive than PMT such as phosphonomethylpentaflourobenzenesulfonate (PMFB), and phosphonomethyltresylate; at higher temperature, these molecules will become slightly more reactive. Alkylations with these two molecules was carried out at $55^{o}C$ at pH = 9.25. The results of these experiments were the same, all the protein had come out of solution. The temperature is not the cause of this result as the protein sat in the warm water bath for several hours prior to alkylation with no precipitation. Further work needs to be done using different temperatures and pH values.

Appendix D: Ion Exchange HPLC of Proteins

I. Basics of Ion exchange HPLC

The following is written with the assumption that a 4.6 x 200 mm, 5 um, 1000 Å column will be used, PolyCAT A for cation exchange and PolyWAX LP for anion exchange. Proteins can be eluted using either an increasing gradient of salt or pH (decreasing pH in anion exchange).

The purpose of IEC is to separate compounds based on their difference in charge state. This is advantageous when you have a mixture of molecules containing groups such as NH_3^+, COO^-, PO_4^-, or SO_3^{-2}; each can be separated from each other using this technique. IEC has several advantages over RP-HPLC:

- Higher resolving power

- Higher recoveries

- Preformed under non-denaturing conditions (especially advantageous)

When deciding on whether to use cation or anion exchange for a protein a good rule of thumb is as follows:

Typically, a protein will bind to a cation exchange resin if the buffer pH is lower than the isoelectric point (pI) of the protein, and will bind to an anion exchange resin if the pH is higher than the pI.

In other words, if pH of the buffer is below the pI, the protein will have a net positive charge, therefore wanting to exchange with cations. If pH of the buffer is above pI, the protein will have a net negative charge, warranting exchange with anions. This does not necessarily mean that either cation or anion exchange can be used for one protein if one place it in a specific buffer. There are several restrictions:

- Both the cation and anion exchange columns have a pH range, if one goes outside of these ranges the column matrix can be ruined. One would not want to run a cation exchange column at pH 8 for example.

- The protein must be stable at the pH used. A protein may precipitate out of solution if pH is too high or too low, or more importantly (and more likely to happen) if it is at or too near the pI of the protein. Cation exchange using a buffer at 6.20 would not be a good choice for a protein with a pI of 6.1 for example.

- A weak cation or anion exchange column can also become uncharged at the wrong pH.

II. Preparing Samples

Samples should always be exchanged into a buffer that is the same as the starting conditions of the gradient. Usually this means that one's protein will be loaded at low ionic strength and/or pH. If one fails to do this, the protein will likely not stick to the column and the column will become unequilibrated.

Both analytical and preparative work can be performed on this column, and samples should be prepared as follows:

For qualitative work:

A sample that is ~0.5 ug/uL is sufficient (typically 45 ug in 120 uL). 50 uL of sample is loaded into a 20 uL sample loop. Excess is used to ensure any previous sample is washed out of the sample loop.

For preparative work:

As much as 3 mg can be loaded onto a 9.4 mm ID column without losing sufficient resolution (This depends on protein MW however, this is using a protein with MW of

13.5 kDa). Here a 2 mL sample loop is used and multiple injections can be used if needed. If multiple injections are needed, one must unplug the injector from the HPLC so the method does not start when the injector is turned to "load." Also, ensure there is sufficient time between multiple injections to be sure sample is out of the sample loop.

III. Preparing Mobile Phases

The buffer used should have a pH that is at least 2 pH units away from the pI of the protein, this ensures the protein is in its correctly charged form and it is safely away from the pI (avoiding precipitation). In order to pick a useful buffer two things must be taken into consideration:

- The p_{ka} of that buffer must be within the range of pH you are using
- The buffer ions should be the same charge as the ion-exchanger to avoid competition with the sample. For example, an anionic buffer should be used with a cation-exchange column.

Some commonly used buffers for cation exchange are HEPES, Phosphate, Acetic Acid, and Formic Acid. Common anion exchange buffers are *bis*-Tris, Piperazine, and Tris-Cl. The counter-ion used in ion exchange is also an important factor in elution of a protein. Counter-ions are the ions which will ultimately exchange with the resin to elute bound protein. For example, Na^+ ions (from NaCl) eluting a bound positively charged protein. The stronger the counter-ion, the quicker a protein will elute from the column, and vice versa for a weaker counter-ion. Below are some common counter ions in cation and anion exchange and their strengths of displacement:

Cation exchange: $Mg^{2+} > Ca^{2+} > NH_4^+ > Na^+ > K^+$

Anion exchange: $SO_4^{-2} > HPO_4^{-2} > Cl^- > CH_3COO^-$

Once a buffer and pH is chosen, two mobile phases will be made up (denoted MPA and MPB), one of low ionic strength (with low or no salt) and one of higher ionic strength (with higher salt concentrations). For example, a typical cation exchange buffer could be as follows:

MPA: 10 mM KH_2PO_4, pH = 6.20

MPB: 10 mM KH_2PO_4, 500 mM NaCl, pH = 6.20

All reagents must be HPLC grade and made up in Mill-Q water. Filtering of the mobile phases under vacuum are done with Pall GH polypro (0.45 um) filters. It is important that all glassware is acid washed with HNO_3/H_2SO_4 to ensure all detergents are absent.

IV. Method Development

Before a run is preformed on a sample the column must be equilibrated twice, once to convert to the column to the correct salt form, and once to wash away excess salt. With a flow rate of 1 mL/min, 100% of MPB (high salt) will be run through the column for 30 minutes to convert the column to the appropriate form (if using NaCl, converting to Na^+ form for cation exchange or Cl^- form for anion exchange). Following this, 100% MPA is flowed through for 30 minutes to wash away excess salt, if this is not done sample will not bind to the column.

For a new protein, a broad gradient will be run first to find an approximate % of MPB a sample will elute. For example, a first run would be something like 0 → 100% MPB over 60 minutes. If the protein elutes at 30 minutes one can calculate the approximate salt concentration and shallow the gradient for better separation. In the case using the mobile phases listed above, the protein would elute at ~250 mM NaCl. Knowing this, the gradient can be adjusted accordingly to where one wants the protein to

elute in the gradient. Following the run, the column must be requilibrated as stated above if it is to be used again within a day or so. For storage over several days it is preferred to store the column in water to avoid clogging and corrosion from salts. For long term storage over several weeks or more it is preferred to store the column in 50% ACN/H$_2$O in the refrigerator.

Appendix E: Native PAGE

Besides RP-HPLC and CEX HPLC, another method to verify the quality of alkylation was sought out. One method which seemed attractive was the use of Native PAGE, a gel electrophoresis method similar to SDS-PAGE which separates proteins based on molecular weight in non-denaturing conditions.

High pH, neutral pH, and low pH buffer systems were tested for native gel analysis. The low pH system contained an acetic acid-KOH buffer for both stacking (pH = 6.7) and resolving (pH = 4.3) buffers with a β-alanine-acetic acid electrophoresis buffer (pH = 4.5). The neutral pH system contained a Tris-HCl buffer for a resolving buffer (pH = 7.9) and an Imidazole-HCl buffer for the stacking buffer (pH = 6.2) with a Tris-glycine electrophoresis buffer (pH = 7.3). The high pH system contained a Tris-glycine buffer for both stacking (pH = 6.8) and resolving (pH = 8.8) buffers with a Tris-glycine electrophoresis buffer (pH = 8.3). Right before the gel is poured the stacking buffers had 4.5% acrylamide, 0.0005% v/v riboflavin, and 0.00075% v/v TEMED (0.0075% v/v for low pH buffer) added to it. The resolving buffers had 13.5% acrylamide, 0.075% APS, and 0.00075% TEMED (0.0075% for low pH buffer) added to it prior to the pouring of the gel. Both buffers are degassed (without APS and TEMED present) before pouring the gel.

The Resolving gel is poured first to ~3.5 cm from the top of the glass plate, and a few drops of isobutanol are applied to the top of the resolving gel to level the top and rid of any bubbles. The gel is left to sit until polymerization occurs, which is usually around 15 minutes. After polymerization, the isobutanol is poured off and the stacking gel is poured on top of the resolving gel to the top of the glass plate; a comb is put in place for

well formation. Polymerization will occur once the gel is illuminated with UV light from a UV lamp (or any other strong UV source), normal fluorescent light is not sufficient enough for polymerization by riboflavin. The gel is done polymerizing once the stacking gel is no longer glowing bright yellow under the UV light.

Samples should contain 10-50 μg of protein for a single protein, and 50-100 μg for a mixture. All samples should contain 10% v/v glycerol (or sucrose), a 10 fold dilution of the stacking gel buffer used, and 0.0025% v/v tracking dye (methylene green for low and neutral pH, and bromophenol blue for high pH). For the low and neutral pH systems the gels run positive to negative and the gel housing must be put in backwards (red connecting to black and black connecting to red), while the high pH system runs normally, negative to positive.

Results of the gel runs were inconclusive as they were hard to interpret. Because the pI of CheY is relatively high, the low pH system was preferred, though all the systems were tested. The low pH system showed only one band when a reaction mixture was run on the gel. Two explanations can be made for this results, one is that the molecular weight difference between phosphono-CheY and CheY is not great enough to resolve (M.W. difference = 94 amu), and the other is the amount of phosphono-CheY might not be great enough to detect using conventional Commaisse stain. The literature suggests otherwise on both accounts, but because these gels were not precast from a manufacturer, technicalities arising from making them in lab may have affected resolution and detection. Silver staining is a method which can be used to detect smaller amount of protein, and that method may be worth investigating if this technique is

employed in the future. The neutral pH system gave almost the exact same results as the low pH system.

The high pH system did not work at all as should have been expected. Because the stacking gel pH is lower than the pI value of CheY and the resolving gel is higher, there are opposing forces at work. In the stacking gel, CheY would be negatively charged, and in the resolving gel, CheY would be positively charged. This method therefore resulted in no movement of the CheY band.

Appendix F: Oxidation of Amino Acid Derivatives

Amino acid derivatives, focused mostly around modifications to derivatives of cysteine, were synthesized with the idea that they were to be inhibitory analogs to the enzyme aspartate semialdehyde dehydrogenase (ASADH). The majority of the reactions were oxidation reactions on the sulfur of cysteine to create various sulfoxides and sulfones. The oxidations were carried out on four main cysteine derivatives; they are s-methylcysteine, S-allycysteine, phosphonomethylcysteine (PMC), and triflouroethylcysteine (Figure A1). All the compounds are available commercially with the exception of phosphonomethylcysteine, which is synthesized using cysteine and PMT in the presence of 3.0 equivalents of 6 N NaOH, and triflourethylcysteine, which was synthesized and given to us as a gift by Dr. DesMartaeu's lab at Clemson University. Several oxidation methods were tried and others are known but have not yet been attempted. Three of the oxidation agents tested were hydrogen peroxide, sodium periodate, and sodium perborate; TPAP/NMO and MCPBA are oxidation agents which still need to be tested.

Sulfoxide formation was achieved with all of the given molecules above as suggested by ^{1}H, ^{13}C, and ^{31}P NMR. The method by which each was formed however varied from molecule to molecule; sulfone formation was only achieved with one (possibly another as well) of the molecules.

As mentioned above, phosphonomethylcysteine was synthesized by reacting cysteine with PMT in the presence of 3.0 equiv. of 6N NaOH; this however did not produce 100% conversion and the product must be cleaned via Dowex-1 and -50 to rid of free cysteine and the hydrolysis product of PMT. Sulfoxide formation is achieved by

reacting PMC with 1.1 equivalents of H_2O_2 (or 10% v/v of a 30% solution) until the reaction is virtually complete by ^{31}P NMR. Sulfone formation has been achieved with this compound as well by oxidizing with 3.0 equivalents of H_2O_2. PMC reactions were done extensively by Neal Johnson and Billy Kish (unpublished results).

S-allylcysteine (^{1}H NMR spectra, Figure A2) was oxidized to sulfoxide using approximately the same method used for PMC, sulfoxide formation started to occur after the introduction of 1.1 equivalents of 10.6 M H_2O_2. The overnight reaction went very far, but not quite all the way to sulfoxide (Figure A3a.); small peaks corresponding to starting material at 2.85 ppm are still present at ~5%. Sulfoxide formation shows a downfield shift off all sulfide protons between the 2.5 and 4.5 ppm range; the expected shift of ~0.5 ppm downfield is seen here. We note that the change in peak shape as sulfoxide formation introduces another chiral center in the molecule. Because one chiral is already present, the formation of another will add further complexity to the spectra as the protons on either side of the sulfur are magnetically unequivalent; in other words, the two protons on the carbon next to sulfur do not have the same chemical shift. The protons therefore are diastereotopic and 2 ABX patterns are seen. Because the reaction was still somewhat incomplete, the reaction was put at 37°C and left to sit overnight. The next day ^{1}H NMR spectra suggested complete sulfoxide reactivity (Figure A3b.); we note that the small peaks at 2.85 ppm are gone as compared to the previous spectra.

With this s-allylcysteine, a new methodology was employed to test for sulfoxide/sulfone formation as well (H_2O_2 had failed for sulfone attempt), that was the use of sodium periodate. 3.0 equivalents of periodate at .5 M (in H_2O) was added to 167 mM s-allylcysteine and let to sit for 30 hours. In the first 20 minutes crystals had formed,

however they eventually disappeared and the reaction became clear with a slightly yellow

color. After 30 hours, a Dowex-50 (AG 50W-X8 resin from Bio-Rad) cation exchange

column was used to rid of any Na^+ ions and to purify and sulfoxide/sulfone away from

any starting material. The amount of Dowex needed is calculated by the following

equation:

$$\text{mmol of product plus sodium ion } *(1/1.7)(20) = \text{mL of gel warranted}$$

In this equation, the 1/1.7 factor states that 1 mL of gel will bind 1.7 mmol equivalents of

ion and the 20 is for a 20 fold increase of column size which is suggested over amount of

ion present. If a new column is poured it is suggested to rinse the beads with several

washes of ethanol first, to rid the resin of an orange impurity, and follow by rinsing with

several DI water washes.

Once the column is poured, it is first washed with 2 column volumes of 1 M HCl

(to convert to H^+ form) and subsequently washed with water until neutral pH is reached.

The sample is applied once it is diluted to ½ of the column volume, the drip rate should

be ~2 mL/min, and two separate washes are applied. The first wash is 2 column volumes

of water, and the second wash is 3 column volumes of 2 N NH_4OH. In this case the

water wash was an orangey spongy looking substance, while the ammonia wash was a

crystalline/yellowish substance; in this case it was expected that the product would elute

in the ammonia wash. Upon ^1H and ^{13}C analysis of the ammonia wash, a mixture of

sulfoxide and sulfone was suspected. Peaks which agreed with sulfoxide formation as

compared with earlier peroxide oxidations was seen, however, there were new peaks

which were believed to be consistent with sulfone formation. At this point, an additional

2 equivalents of periodate were added to the NMR tube, while the main reaction flask

was left alone. The following day the ^{1}H NMR spectra (Figure A4) suggested mostly sulfone. The major change to note in this spectra its lessened complexity. Addition of a second oxygen atom to the sulfur atom causes sulfur to lose its chirality. Because of this, the protons next to sulfur are now only diastereotopic to one chiral center and not two. TLC was also run on s-allyl-cysteine; it was done on both the reaction flask (no additional periodate) and the NMR tube (additional periodate) using a 70/30 EtOH/H_2O solvent system. Upon analysis, the reaction flask showed a single spot corresponding to sulfoxide formation, while the NMR tube showed two spots, one corresponding to sulfoxide and one with a lower R_f value which is presumed to be sulfone.

S-methylcysteine was oxidized using a variety of different compounds. The first two oxidation systems tested were composed of 1.1 equivalents of 30% H_2O_2 and another of 500 mM sodium periodate. Both of these reactions were done as NMR size reactions (performed in NMR tube) in D_2O as the solvent. The reactions were purified via Dowex-50 as discussed above. Conformation of sulfoxide formation is based on both ^{1}H and ^{13}C NMR. Two other systems were also tested against s-methylcysteine, both used 1.1 equivalents of sodium perborate as the oxidant with one in d-acetic acid and the other in d-methanol. MCPBA was another possibility; however, it was never attempted. Both perborate systems worked somewhat with about a 50/50 mixture of sulfoxide and starting material after an overnight reaction was performed. s-methylcysteine was used more as a test compound since its sulfoxide form is available commercially.

The final compound was one which was synthesized and provided to us by Dr. DesMarteau's lab at Clemson University. Triflouroethylcysteine (^{1}H NMR spectra in Figure A5) was first tested against 1.1 equivalents of H_2O_2; the reaction had not

proceeded at all after 2 days and it was put on reflux. After 1 week in peroxide and 4 days on reflux the reaction still had not proceeded at all and another method was attempted. 1.1 equivalents of sodium periodate in D_2O was tested against the triflouro compound and after a day had seemed to proceed somewhat. Although the ^{1}H NMR spectra was messy (Figure A6), likely due to diastereomers, the chemical shift difference was consistent with sulfoxide formation. In this case it was believed that little sulfide starting material was left and the majority of the product was sulfoxide. In an attempt to clean up the spectra and the compound, a Dowex-50 column (as above) was run on the reaction. Upon analysis of both the water wash and ammonia wash, no product at all was found. It is unknown what could have happened to our compound besides some type of chemical degradation.
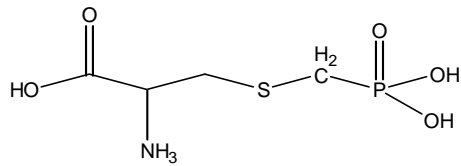
A.
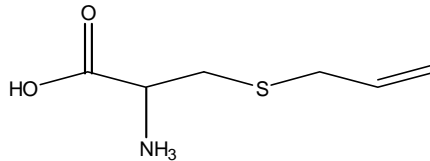
B.
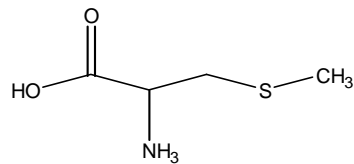
C.
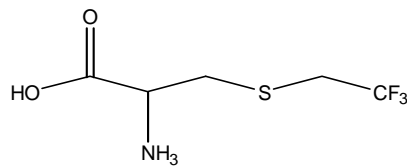
D.
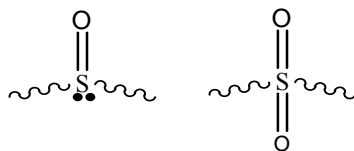
E.

Figure A1. Structures of the following molecules: a.) phosphonomethylcysteine b.) s-allylcysteine c.) s-methylcystein d.) triflouroethylcysteine e.) general sulfoxide and sulfone oxidation states.

s-allyl-L-cysteine

Current Data Parameters
NAME   protonPMT
EXPNO   73
PROCNO   1

F2 - Acquisition Parameters
Date_   20070221
Time   17.34
INSTRUM   spect
PROBHD   5 mm Multinucl
PULPROG   zg30
TD   65536
SOLVENT   CDCl3
NS   8
DS   2
SWH   8250.825 Hz
FIDRES   0.125898 Hz
AQ   3.9715316 sec
RG   128
DW   60.600 usec
DE   6.00 usec
TE   691.1 K
D1   1.00000000 sec
TD0   1

======== CHANNEL f1 ========
NUC1   1H
P1   7.50 usec
PL1   -6.00 dB
SFO1   400.1324710 MHz

F2 - Processing parameters
SI   32768
SF   400.1300060 MHz
WDW   EM
SSB   0
LB   0.30 Hz
GB   0
PC   1.00

Figure A2. ^{1}H NMR spectra of s-allylcysteine.

111

A.
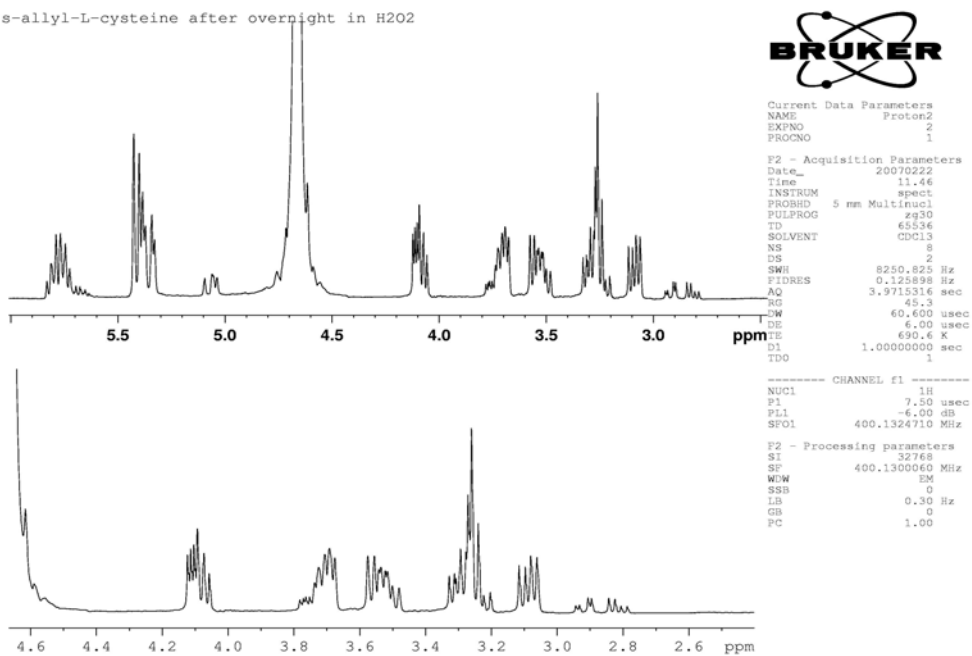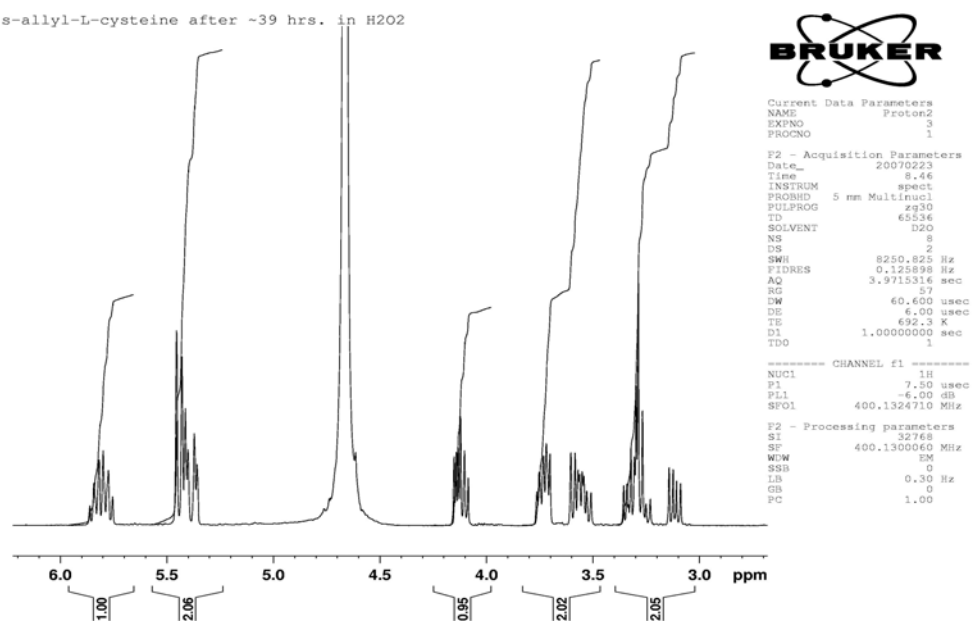
B.

Figure A3. a.) ^{1}H NMR spectra of s-allylcysteine in peroxide overnight; forming mostly sulfoxide. b.) ^{1}H NMR spectra of s-allylcysteine in peroxide for 39 hours and heated up; forming all sulfoxide.

s-allyl-L-cysteine, first sulfone attempt, 4/9/07

BRUKER

Current Data Parameters
NAME   Proton2
EXPNO   26
PROCNO   1

F2 - Acquisition Parameters
Date_   20070412
Time   17.03
INSTRUM   spect
PROBHD   5 mm Multinucl
PULPROG   zg30
TD   65536
SOLVENT   D2O
NS   8
DS   2
SWH   8250.825 Hz
FIDRES   0.125898 Hz
AQ   3.9715316 sec
RG   114
DW   60.600 usec
DE   6.00 usec
TE   691.7 K
D1   1.00000000 sec
TDO   1

-------- CHANNEL f1 --------
NUC1   1H
P1   7.50 usec
PL1   -6.00 dB
SFO1   400.1324710 MHz

F2 - Processing parameters
SI   32768
SF   400.1300060 MHz
WDW   EM
SSB   0
LB   0.30 Hz
GB   0
PC   1.00

Figure A4. ^{1}H NMR spectra of s-allylcysteine with 3.0 equivalents of NaOI$_4$ after 30 hours. Product is believed to be a mixture of sulfoxide and sulfone.

Current Data Parameters
NAME   protonPMT
EXPNO   72
PROCNO   1

F2 - Acquisition Parameters
Date_   20070221
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PULPROG   zg30
TD   65536
SOLVENT   CDCl3
NS   8
DS   2
SWH   8250.825 Hz
FIDRES   0.125898 Hz
AQ   3.9715316 sec
RG   143.7
DW   60.600 usec
DE   6.00 usec
TE   690.8 K
D1   1.00000000 sec
TD0   1

======== CHANNEL f1 ========
NUC1   1H
P1   7.50 usec
PL1   -6.00 dB
SFO1   400.1324710 MHz

F2 - Processing parameters
SI   32768
SF   400.1300060 MHz
WDW   EM
SSB   0
LB   0.30 Hz
GB   0
PC   1.00
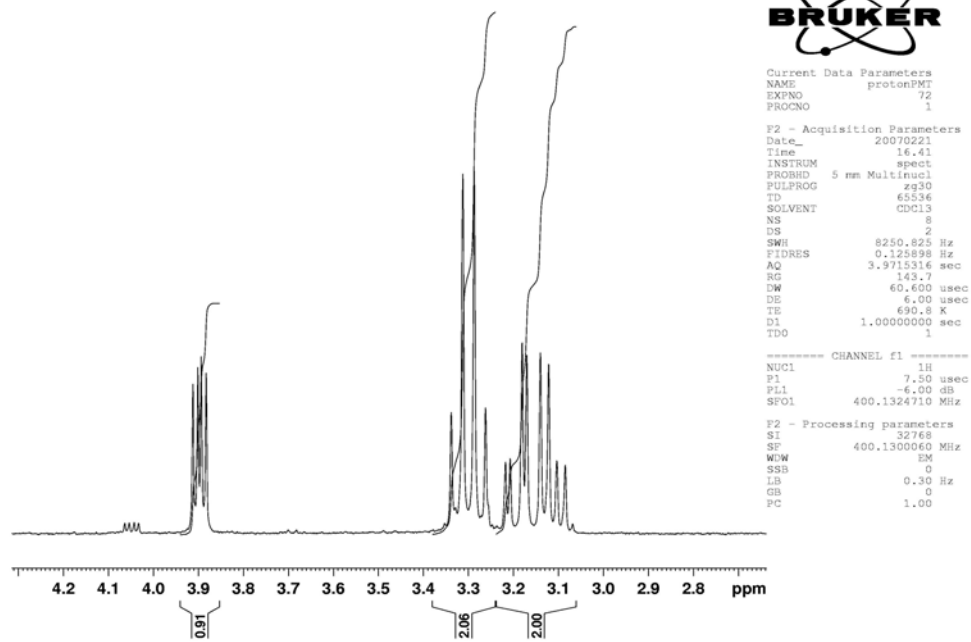
Figure A5. ^{1}H NMR spectra of Triflouroethylcysteine.

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BRUKER

Current Data Parameters
NAME   Proton2
EXPNO   18
PROCNO   1

F2 - Acquisition Parameters
Date_   20070320
Time   13.01
INSTRUM   spect
PROBHD   5 mm TXI 13C Z
PULPROG   zg30
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DS   2
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FIDRES   0.125898 Hz
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TE   295.4 K
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TDO   1

-------- CHANNEL f1 --------
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F2 - Processing parameters
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SF   400.1300052 MHz
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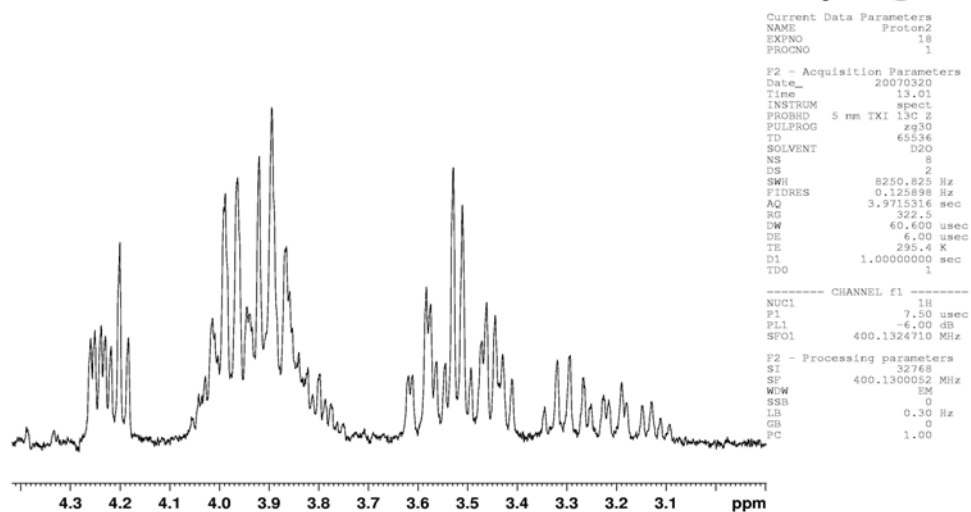PC   1.00

Figure A6. ^{1}H NMR spectra of Triflouroethylcysteine after an overnight in NaOI$_4$.

Appendix G: MALDI-TOF Mass spectrometry of Phosphono-CheY

MALDI-TOF MS was tested on protein purified by early purification techniques involving the 10 mM phosphate method. Phosphono-CheY from the purification seen in Figure 23a was sent to Cornell University for analysis. The protein sent was not pure phosphono-CheY, but only ~ 70%. Figure A7 shows the mass spectrum of this sample. Two masses are represented in this spectrum, one of 13470.7676, and one of 13582.0215. The lower molecular weight corresponds to native D54C/C81S CheY with the added GSH sequence at the N-terminus. The higher molecular weight (111 amu greater) corresponds to the addition of the phosphonomethyl group (+15) and an oxygen (+16). The extra oxygen is suspected to be due to oxidation of methionine.
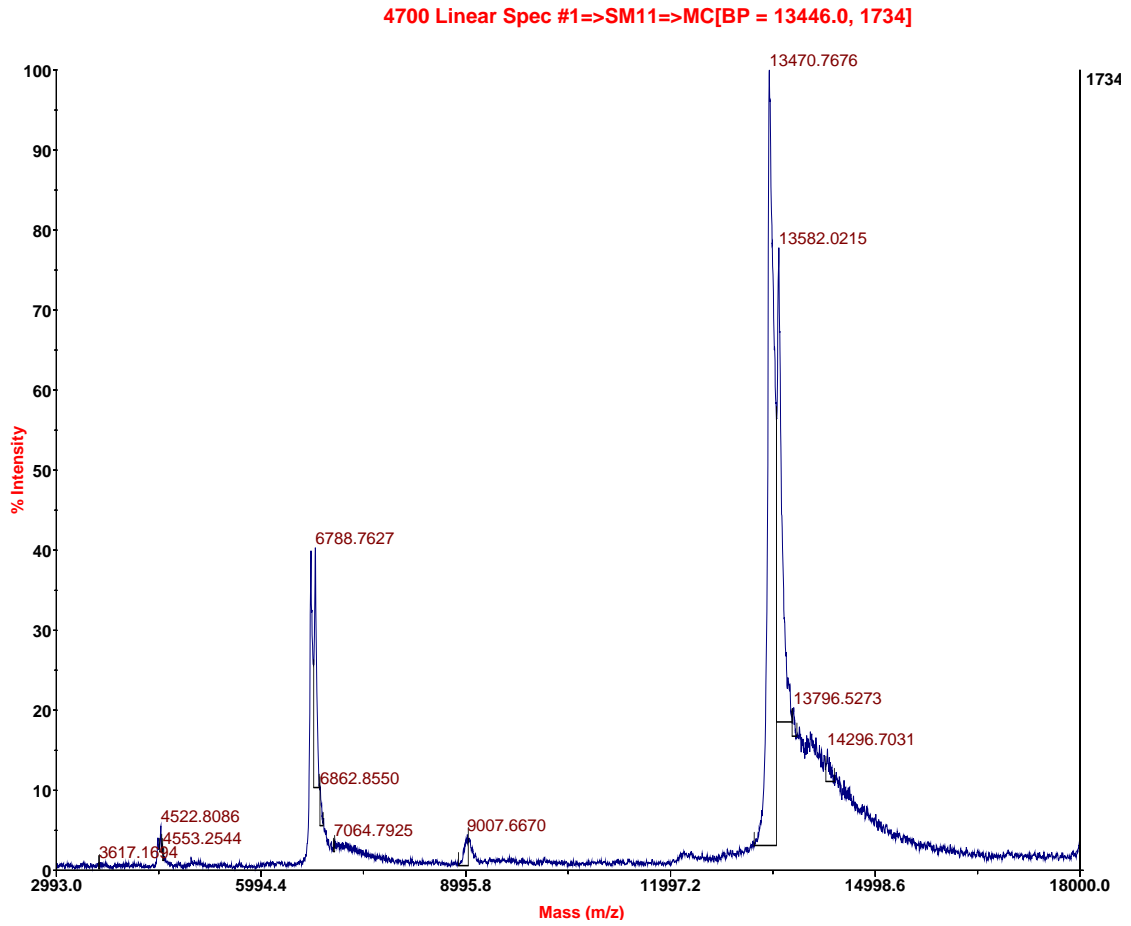
Figure A7. MALDI-TOF mass spectrum of the purified phosphono-CheY seen in Figure 23.