Master's Thesis

DEVELOPMENT OF CHEMICAL BIOLOGY TOOLS FOR STUDYING UNSTABLE PROTEIN PHOSPHORYLATION

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Development of chemical biology tools for studying unstable protein phosphorylation

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Development of chemical biology tools for studying unstable protein phosphorylation

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Abstract

Protein phosphorylation is one of the key protein post-translational modifications (PTMs), and it regulates biological process by dynamic phosphorylation and dephosphorylation. Especially, phosphorylation is involved in many signal transduction pathways, and some are related to human diseases. For this reason, kinase and phosphatases have been actively investigated as drug targets. Stable O-phosphorylations, such as phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) are well-studied, and many tools are available. However, unstable phosphorylations, such as phospholysine(pLys), phosphohistidine (pHis) and phosphoaspartate (pAsp) are still much less explored due to their chemical instability and the lack of tools. In this thesis, my progress towards the development of chemical tools for studying pLys and pAsp.

Chapter I describes our strategies and progress toward the development of phospholysine-specific antibodies, including the design and synthesis of the antigen, the affinity purification of the crude antisera, and the validation of the purified antibodies.

In chapter II, the chemistry of pAsp and its biological functions are briefly reviewed. Then our efforts for covalent labeling of pAsp and subsequent detection are described. Our approach was validated with tandem mass spectrometry. Ongoing studies for the affinity enrichment of labeled peptides is also briefly summarized.

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List of Abbreviations

ADP	Adenosine diphosphate
Asp	Aspartic acid
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ETD	Electron-transfer dissociation
HA	Hydroxylamine
HAD	Haloacid dehalogenase
His	Histidine
HK	Histidine Kinase
Hse	Homoserine
IMAC	Immobilized Metal Affinity Chromatography
KLH	Keyhole Limpet Hemocyanin
LC	Liquid Chromatography
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
pArg	Phosphoarginine
pAsp	Phosphoaspartate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pHis	Phosphohistidine
pLys	Phospholysine
PPA	Potassium phospharamidate
pSer	Phosphoserine
pThr	Phosphothreonine
PTM	Post-translational Modification
pTyr	Phosphotyrosine
RR	Response Regulator
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
TBS	Tris-Buffered Saline

TCS	Two component system
TOF	Time of flight
UV	Ultraviolet

Chapter 1. Development of pLys-specific antibody

1. Introduction

1.1. Protein phosphorylations

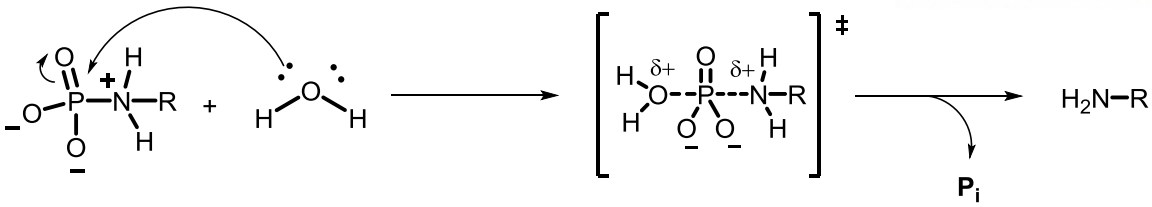
Protein phosphorylation is one of the key post-translational modifications (PTMs) in cells. It regulates lots of physiological processes in diverse biological systems. Especially, protein phosphorylation and dephosphorylation mediates signal transductions, catalyzed by kinase or phosphatase. Regulation of cellular signal transduction mechanism is also associated with diseases, so protein kinases have been drug targets in pharmaceutical field.[1]

Among many phosphoamino acids, *O*-phosphoylations, such as pSer, pThr, pTyr are well known and studied, due to their stability in acids. It was possible to develop the tools for phosphoprotein or peptide analysis, such as antibody and IMAC. However, protein *N*-phosphorylations, such as phosphohistidine (pHis), phosphoarginine (pArg) and phospholysine (pLys) are unstable under acidic conditions, and this instability hindered the elucidation of biological roles of N-phosphorylations.

Despite the difficulties of classical biological tools, due to the development of tools with chemical approaches enabled the researches on non-conventional phosphoamino acid. First pan-specific pHis antibody was developed by Kee *et al.*, using synthetically prepared pHis analog.[2] Similar with this approach, pArg antibody was also discovered. But still, pLys antibody is not reported yet.[3,4]

1.2 Chemistry of pLys

Among phosphoamino acids, pLys is especially labile in acidic conditions. The phosphoramidate of pLys was completely hydrolyzed within 15 min at room temperature in 1 M hydrochloric acid. At pH below 9, nitrogen atom of pLys is protonated, and it makes the amino group a better leaving group and lengthens the P-N bond. These facilitate the hydrolysis of pLys (Scheme 1). It is known that the rate constant of pLys monoanion (pH 7) hydrolysis is 16.4 min^{-1} while the rate constant of neutral N-butylphosphoramidate (acidic pH) is 142 min^{-1}.[5,6] However. pLys is quite stable in basic conditions. It undergoes 11 % hydrolysis in 9 M potassium hydroxide at 100 ^{0}C even after 9 hours. This is due to the loss of leaving group ability of the amino group, since at basic pH it stays deprotonated.



Scheme 1. Hydrolysis of pLys in physiological pH.

1.3 pLys in biological systems

So far, histone H1 is the only protein reported to contain pLys *in vivo,* isolated from regenerating rat liver nuclei. It was observed that histone H1 is more phosphorylated in a regenerating rat liver than in a normal rat liver.[7] But its biological functions are still not clear. But a number of lysine kinase activities and pLys phosphatase have been reported. Smith reported a kinase that can phosphorylate Histone H1 in Walker-256 carcinosarcoma. They partially purified and studied the properties of kinase, which was found in the regenerating rat liver.[8] Hiraishi discovered that inorganic pyrophosphatase from the bovine liver can dephosphorylate pLys and pHis. Additionally, LHPP and PHPT1, which are known as pHis phosphatases, are also reported to dephosphorylate chemically phosphorylated lysine.[9] Still, pLys specific phosphatases are yet to be discovered.

1.4 Current methods for studying pLys

Previously, the putative Lys kinase activity was obtained from nuclear extracts and its kinase activity toward Histone H1 was observed. This was analyzed by ^{32}P radiolabeling and paper chromatography to identify pLys.[8,10] One of the major hurdles in the studies of pLys has the preparation of the pLys-containing peptides and proteins. Traditionally, chemical phosphorylation of Lys residues was performed with potassium phosphoramidate (PPA), as reported by Pia et al.[9] Lysine residues of Histone H1 and polylysine was phosphorylated *in vitro*. Phosphorylation was analyzed by Malachite green phosphate assay, which analyze the amount of hydrolyzed phosphate group, and LC-MS/MS. They also performed amino acid analysis, but the Lys phosphorylation level of histone H1 was quite low.

In 2014, the Hackenberger group reported a synthetic method for site-specific pLys incorportation in peptides.[11] They introduced an azidonorleucine in place of a Lys in a histone-derived peptide by SPPS, and through Staudinger phosphite reaction, the site-specific incorporation of a protected pLys was successfully achieved. They employed a phosphite with base- or UV- cleavable protecting group, and this phosphite were reacted with azide and finally deprotected with UV or base, providing the phospholysine peptide. The site-specific pLys incorporation was confirmed by electron-transfer dissociation (ETD) mass spectrometry. In fact, these pLys peptides were valuable in the studies of ionization and fragmentation behaviors of the pLys-peptides. But its preparation requires a long synthesis and its yields were low. And the protecting group should be deprotected right before use.[11]

2. Development of pLys-specific antibody

2.1 Design and strategy

As described above, pLys was discovered in histones, but its detail biological functions are still unknown. One of the reasons for this lack of knowledge is that there were no essential and fundamental tools for studying pLys in proteins due to its chemical instability. Phosphorylation-specific antibodies have been among the most powerful tools for studying protein phosphorylation. If we can obtain pLys-specific antibody, we can discover and isolate pLys-containing protein, and this can be extended to study pLys functions in biological systems. pLys is a phosphoramidate, which can be hydrolyzed easily. Therefore, it cannot be used as the hapten to generate pLys-specific antibodies. Instead, we designed a stable analog of pLys, which substituted the N-P bond with a more stable C-P bond (Figure 1). Similar synthetic hapten strategies have been successfully employed for the generation of pHis- and pArg-specific antibodies. Our analog contains similar charge state of pLys at physiological pH, so we expected that this analog (pLys*) can mimic pLys.

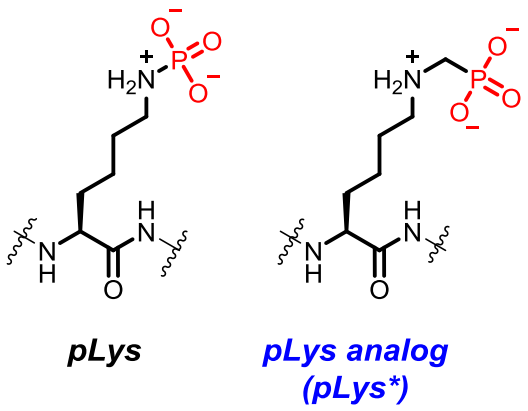


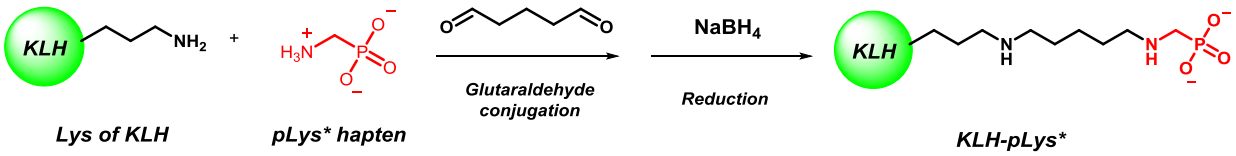
Figure 1. Structure of pLys and pLys analog.

2.2 Preparation of pLys-specific antibody

2.2.1 Design and preparation of KLH-pLys analog (pLys*) immunogen

The pLys* hapten, aminomethyl phosphonic acid, was conjugated to Keyhole Limpet Hemocyanin (KLH) by the glutaraldehyde-mediated conjugation followed by the imine reduction with sodium borohydride (Scheme 2). KLH is a well-known carrier protein for the generation of antibodies, due to its large size and abundant functional groups on the surface. The conjugation of pLys* hapten was confirmed by ^{31}P NMR analysis.

The pLys*-reactive antisera from rabbits were obtained by immunizing KLH-pLys* to two rabbits (performed at Young In Frontier). After each boost immunization, the test bleeds were checked by ELISA to monitor the titer to the pLys* hapten (BSA-pLys*). After five boosts, the rabbits were sacrificed, and the final bleeds were collected.



Scheme 2. Conjugation of pLys* hapten to carrier protein KLH.

2.2.2 Affinity purification of pLys*-specific antibodies

To purify the pLys*-binding antibodies from the crude antisera, affinity purification was carried out. BSA-pLys* was coupled to the iodoalkyl moiety of Sulfolink-agarose® resin via its Cys residues. The crude antisera were affinity-purified using this BSA-pLys* agarose resin, but its specificity towards BSA-pLys* over other BSA-pX was still low. We hypothesized that the background binding antibodies may have bound to the glutaraldehyde linker between pLys* and the Lys sidechains of the antigen. So we performed the affinity depletion, which conjugates BSA-Linker without pLys* to the Sulfolink-agarose resin to remove antibodies that binds to the non-specific part of antigen (Figure 2). After the two-step affinity purification, pLys*-specific antibodies were obtained.

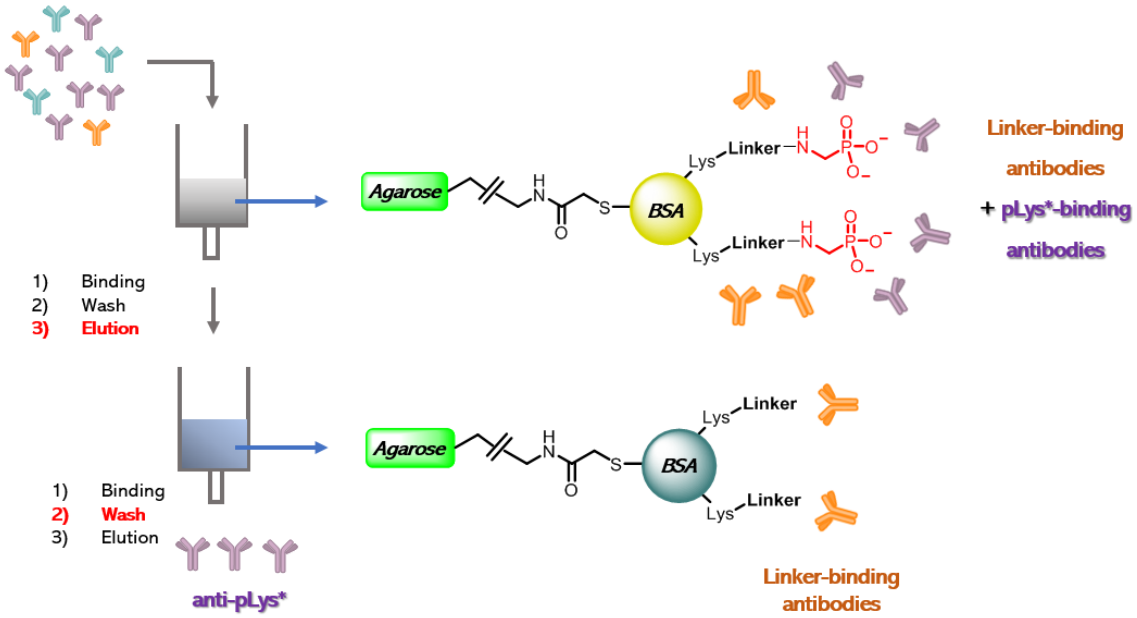


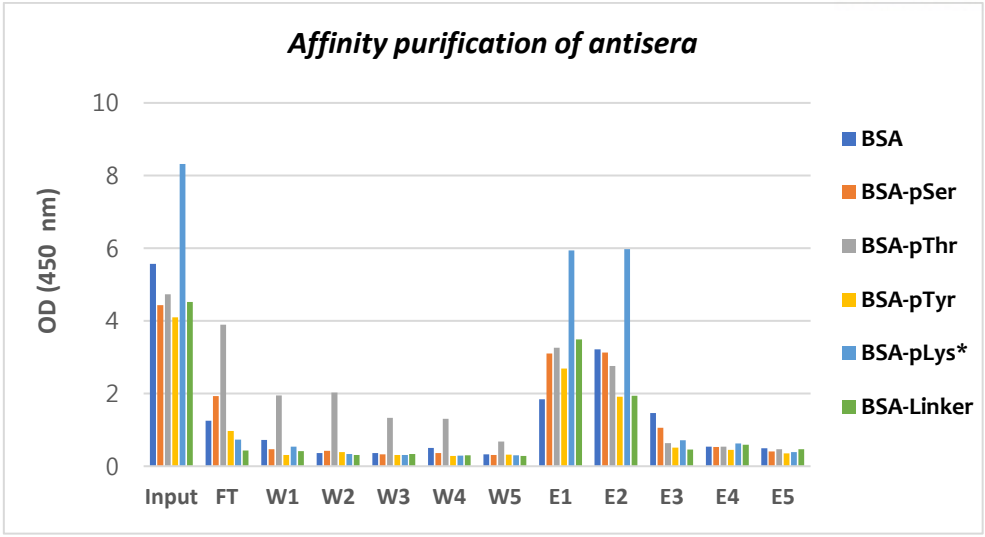
Figure 2. Two-step purification of the pLys*-specific antibodies.

2.3. Validation of the pLys-specific antibody

2.3.1 ELISA studies

The binding specificity of the purified antibodies toward pLys* was checked with ELISA. The binding of antibodies to BSA-pLys* and BSA-X (X = pSer, pThr, pTyr) were measured. We also used BSA and BSA-linker (no phosphoamino acid) to see the background non-specific binding.

We confirmed that expected antibody was successfully obtained by immunization and two-step purifications described above. First, the affinity purification was performed with Sulfolink-BSA-pLys* resin. Although the desired antibody was mostly obtained in E1 and E2 fractions, other antibodies, which binds to the BSA-pX were not removed well, compared to the input fraction (Figure 3, upper panel). So we expected that these unwanted antibodies were binding to the linker of antigen, and synthesized a Sulfolink-BSA-Linker resin to performe the affinity depletion. After the affinity depletion, the desired antibody was observed in FT fraction with much better specificity. Other non-specific antibodies, which seems to bind to the glutaraldehyde linkers, were eluted in elution fractions (Figure 3, lower panel).



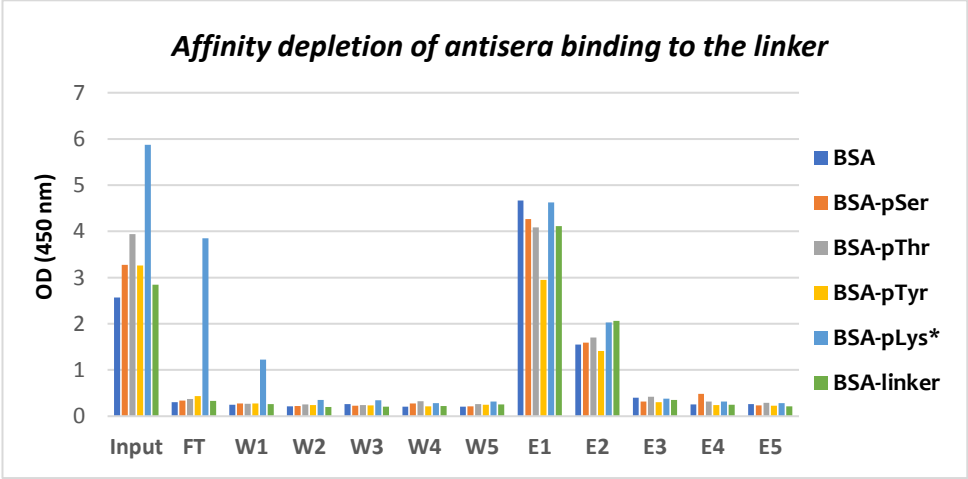


Figure 3. Two-step affinity purification of antisera and ELISA results.

2.3.2 Dot blot and Western blot studies

2.3.2.1 Poly-L-lysine and Histone H4 H18A H75A

Poly-L-lysine was phosphorylated *in vitro* with potassium phosphoramidate (PPA) according to the literature.[9] We tried western-blot analyses of the phosphorylated poly-L-lysine first, but due to abundant lysine groups, the bands were severely smeared on SDS-PAGE gels. Next, dot-blot analyses were performed for poly-L-lysine (Figure 4), but it showed strong non-specific binding to the non-phosphorylated substrate. Therefore, we had to use different proteins substrates, but PPA-mediate phosphorylation can also phosphorylate His residues. So we switched to Histone H4 H18A H75A, in which all histidine residues were mutated into alanine, and we expected to see the phosphorylation of lysine only by PPA in pH 10. But both results showed anti-pLys* antibody binds to the non-

phosphorylated poly-L-lysine and Histone H4 H18A H75A (Figure 5).

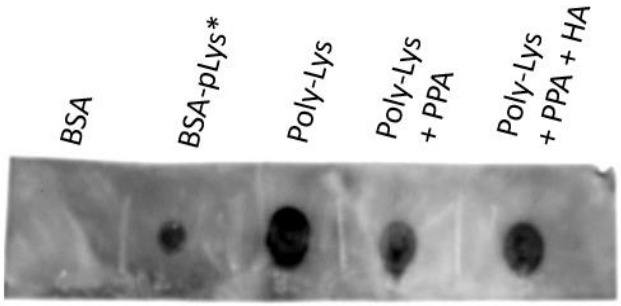


Figure 4. Dot-blot analysis of phosphorylation of polylysine.

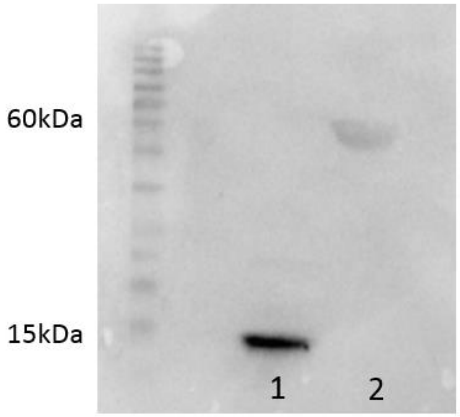


Figure 5. Western-blot analysis of nonphosphorylated Histone H4 H18A H75A with anti-pLys*. (Lane 1: Histone H4 H18A H75A Lane 2: BSA-pLys* (a positive control))

2.4 Conclusion and Future directions

We designed the mimic of pLys and successfully obtained antibodies using the pLys analog as the hapten. Through two-step affinity purifications, pLys*-specific antibodies were purified. We tried to validate the antibody with pLys, but antibody bound to non-phosphorylated polylysine and Histone H4 H18A H75A.

Now, we are trying to find pLys-containing protein from mammalian cell lysates, such as HEK293T cell and HeLa cells through western-blots. We are also trying to do siRNA knockdown of pLys phosphatases to increase the chances to detect pLys containing proteins.

3. Experimental methods

General materials

(Aminomethyl)phosphonic acid and bovine serum albumin were purchased from Sigma Aldrich (St. Louis, MO). 1X TMB ELISA substrate was from eBioscience (San Diego, CA). Goat anti-rabbit IgG HRP conjugate was purchased from Biorad (Hercules, CA). Tris base salt was purchased from Alfa Aesar. SulfoLink® Coupling Resin was purchased from Thermo Scientific (Waltham, MA).

General methods

^{31}P NMR spectra were recorded on a Bruker 400 AVANCE III HD (Bruker BioSpin, Billerica, MA). ^{31}P NMR chemical shifts are reported in parts per million (ppm). Absorbance in microplate was measured with SpectraMax i3x (Molecular devices, Sunnyvlae, CA). Protein concentration was measured at 280 nm by NanoDrop™ 2000/2000c (Thermo Scientific, Waltham, MA). Chemiluminescence from western blot and dot-blot analysis was measured with ChemiDoc™ XRS+ System (Biorad, Hercules, CA).

General protocol of BSA-pX conjugation

To a 2 mL of BSA solution (4 mg/mL), 10 mg of a phosphoamino acid was added in a conical tube. 1 mL of glutaraldehyde solution (20 mM in PBS) was added dropwise. Upon addition, the reaction mixture started turning yellowish, and it was incubated until the yellow color fully developed. Then, 250 μL of $NaBH_4$ solution (10 mg in 1 mL of PBS) was added dropwise. The mixture was incubated at room temperature until the bubbling subdued, by which time the mixture was colorless. The conjugate was dialyzed twice in 450 mL of PBS buffer with 3500 MWCO SnakeSkin® Dialysis Tubing for 8 hours. Finally, pH was adjusted to 7.5 and result was analyzed by ^{31}P-NMR. (Figure 6)

^{31}P NMR data of BSA-pLys* conjugation

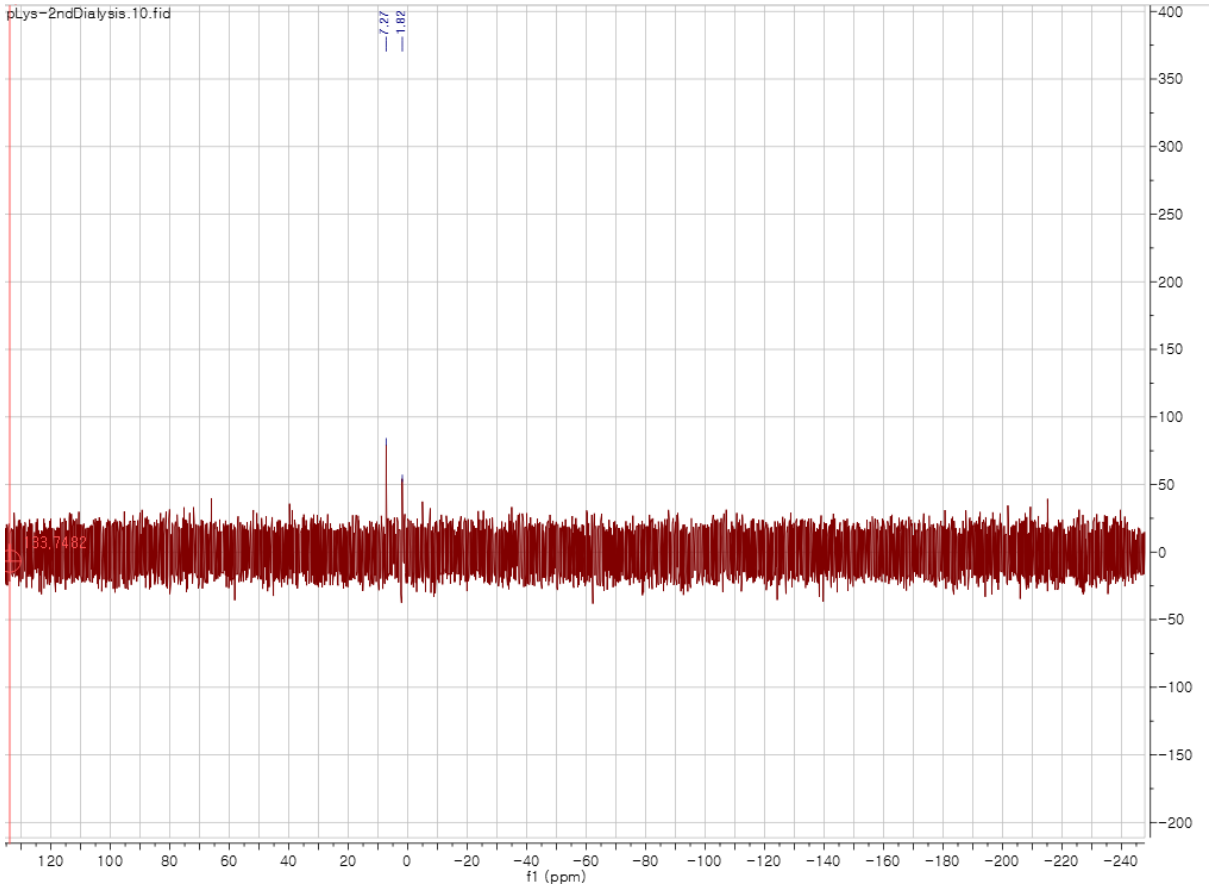


Figure 6. ^{31}P NMR of BSA-pLys* after dialysis

^{31}P NMR (162 MHz, D_2O) δ 7.27 (BSA-pLys*), δ 1.82 (PBS buffer)

Preparation of Sulfolink-agarose-BSA-pLys* and BSA-Linker

Overall procedures were according to the manufacturer's instruction. DTT (2 mg) was added into 2 mL of BSA-pLys* or BSA-Linker solution. Dialysis was followed, twice in 300 to 400 mL of resin coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5) for 4 hr each. SulfoLink® Coupling Resin (1 mL) was loaded to a biospin column after stirring for suspension. Then, the column was washed with the resin coupling buffer (1 mL, each, more than 5 times) and drained. The BSA-linker or BSA-pLys* solution (1 mL) was loaded into the resin column and incubated on a rocker for 30 min, then column was kept standing up for additional 30 min at RT without mixing. The remaining protein concentrations were monitored by absorbance at 280 nm with Nanodrop®. After the coupling, the remaining reactive sites of resin were blocked by incubation with the quenching buffer (1 mL, 50 mM L-cysteine

hydrochloride in the coupling buffer) for 15 min with mixing, and reaction was incubated by standing the column upright without mixing for additional 30 min. To remove the quenching buffer, column was drained and washed with the wash solution (1 M NaCl, 5 x 1 mL). The column was finally washed with the storage buffer (PBS) and stored store at 4 °C upright.

Preparation of the antisera

Production of polyclonal rabbit antisera of KLH-pLys* was requested to Young In Frontier (Seoul, Republic of Korea). Two New Zealand White Rabbit were immunized 5 times with the requested antigen. After each boost immunization, the titer of the crude antisera towards BSA-pLys* was checked with ELISA. After the 5th boost, the final bleed was obtained to provide the crude antisera.

Affinity purification and depletion

For the affinity purification, a BSA-pLys* conjugated agarose resin was used, and a BSA-Linker conjugated agarose resin was used for affinity depletion. The conjugated agarose resin was washed with 1 mL of the wash buffer (TBS, 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 8.5) for 5 times. The crude antiserum (100 μL) was diluted with 900 μL of TBS-Tween (0.1 % Tween in TBS) and loaded on the BSA-pLys* column. After gentle mixing, the column was incubated on a rocker for 1 hour at room temperature. The flow-through was collected and column was washed with 1 mL of TBS for 5 times. Each wash fraction (total 5 fractions) was collected separately. The antibodies were eluted with 900 μL of the elution buffer (0.1 M Glycine, pH 2.5). Each eluted fraction was neutralized immediately to pH 7 with 50~100 μL of the neutralization buffer (1 M Tris, pH 8.6). The flow-through, wash fractions, and elution fractions were diluted into 1:50 for affinity purification and 1:20 into TBS-Tween for depletion, and they were analyzed by ELISA.

General procedure of ELISA

BSA-conjugated pX was dissolved in the ELISA coating buffer (0.1 M Sodium carbonate, pH 9.6) and diluted into 50 μg/μL. An immunoplate (SPL, Cat. No.: 32296) was coated with 50 μL of each BSA-conjugated pX solution for 1 or 2 hours on a rocker at RT. The wells were washed with TBS-T (TBS pH 8.5, 0.1 % Tween 20) 3 times, followed by blocking with the blocking buffer (3 % BSA in TBS-T) for 1 hour on a rocker at RT. After the removal of the blocking solution, the crude or purified antisera were diluted into 1:2000 in TBS-T. The diluted antisera solution (50 μL) was added to the wells and incubated on a rocker at least for 1.5 hr at RT. The wells were washed 3 times with TBS-T. Goat anti-rabbit IgG

HRP conjugate (Biorad, Cat.No.: 1706515) was diluted 1:5000 in TBS-T, and 50 μL of this secondary antibody solution was added to each well and incubated for 30 min at RT on a rocker. After washing with the washing buffer 3 times, 50 μL of 1X TMB ELISA substrate (eBioscience, Cat. No.: 00-4201-56) was added to each well, and it was incubated on rocker for 10 min, by which time blue green color developed. Then, 50 μL of 2 N sulfuric acid was added to stop the reaction, and the wells immediately turned yellow. The optical density was measured at 450 nm by a plate reader.

General procedure of Western blots

Western blots were performed according to the following procedures. Samples were loaded on a 12 % SDS-acrylamide gel with 4 X loading buffer (pH 8.5). After running the electrophoresis, transfer was performed to an Immun-Blot PVDF Membrane (Biorad, Cat.No.: #162-0177), at 100 V for 1.5 hr at 4 °C. After the transfer, the membrane was blocked with 3% BSA in TBS-T for 1 hr. Subsequently, the membrane was incubated with 1° antibody (purified pLys*-specific antiserum, diluted 1:2000 diluted into TBS-T) for 1.5 hr at RT. The membrane was washed 3 times with TBS-T. After incubation with the secondary antibody (goat anti-rabbit IgG HRP conjugate) for 30 min at RT, the membrane was washed 3 times with TBS-T 3 times. Finally, the membrane was incubated with ECL chemiluminescence substrate, D-Plus™ ECL Pico System, (Dongin LS, Cat.No.: ECL-PS100) for 5 min and the chemiluminescence was analyzed with ChemiDoc.

4. References

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Chapter 2. Chemical biology tool for study Bacterial TCS

1. Introduction

1.1 Bacterial TCS

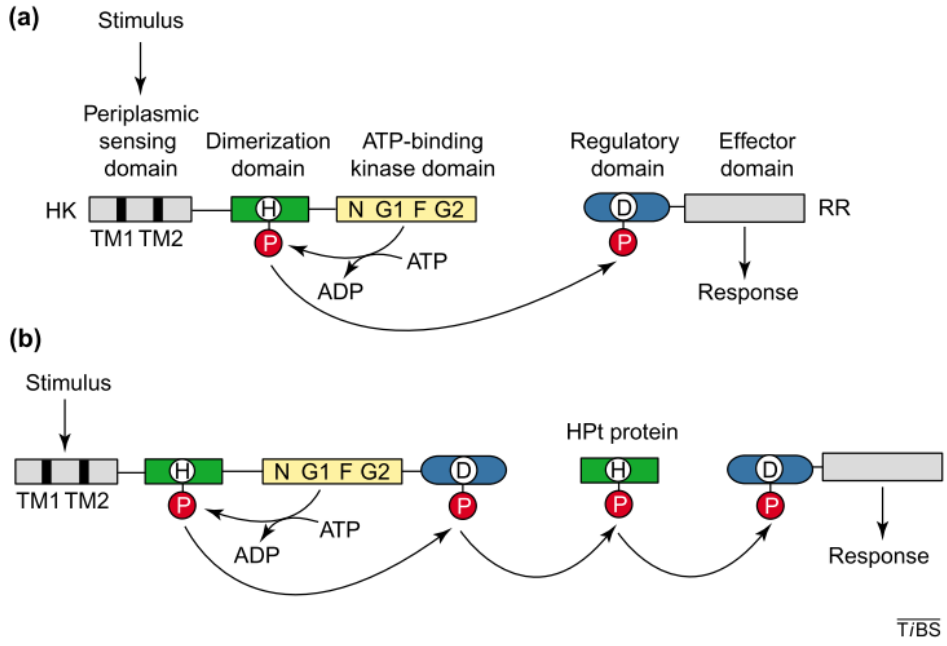


Figure 7. Mechanistic diagram for bacterial TCS (a) and phosphorelay (b) systems.

(adopted from Reference 1, Copyright reserved)

Two-component systems (TCSs) are key sensory systems found in bacteria and plants. They sense a variety of external physical and chemical stimuli, such as temperature, pH, antibiotics, and regulate the cellular responses accordingly.[1,2] In the TCS, phosphorylation of unconventional amino acid residues, such as histidine (His) and aspartic acid (Asp) are crucial for the signaling cascade. The histidine kinase (HK), which is embedded in the cell membrane, is the sensor of TCS. Most HKs are homodimeric proteins consisting of the N-terminal sensing domain and the C-terminal cytoplasmic kinase domain. If extracellular stimuli activate the sensing domain of a HK, the HK autophosphorylates its specific His residue on the dimerization domain (DHp domain). Then, its cognate response regulator (RR) catalyzes the transfer of the phosphoryl group from phosphohistidine (pHis) of the HK to a specific Asp residue in its regulatory domain. This phosphorylation on regulatory domain activates the effector domain of the RR. The activated RR can act as transcriptional factor, regulating the target gene

expression and the resulting physiological responses to the stimuli.[1] A phosphorelay system, a more complex variation of TCS, consists of multiple transphosphorylation steps. A hybrid histidine kinase autophosphorylates its His site and the phosphoryl group is intramolecularly transferred to an Asp residue. Then, the histidine-containing phosphotransfer (HPt) protein transfers this phosphoryl group of pAsp to its own His and eventually to an Asp residue of the cognate RR (Figure 7).

Some HKs also have RR phosphatase activity. Not only HKs, but some RRs also contains autophosphatase activity, and these phosphatase activities shorten the lifetime of pAsp *in vivo* and make studies of pAsp more difficult.

1.2 Biological functions and significance of TCS

In bacteria, TCSs regulate various physiological responses to external physical and chemical stimuli. TCSs are involved in important phenomena such as chemotaxis, biofilm formation, and antibiotic resistance. In plants, TCSs regulate the perception and signaling of cytokinin and ethylene signaling.[3]

To date, over 80,000 TCSs have been found in various prokaryotes.[4] However, for the vast majority, the cognate signal for the individual TCS is not known. Even for the cases where their cognate input signals are known, the detailed sensing mechanisms are still unclear. So far, in *E.coli*, about 30 TCS have been discovered, but their functions are only partially revealed. Only some external signals and its correlated TCS have been discovered since 1987.[5]

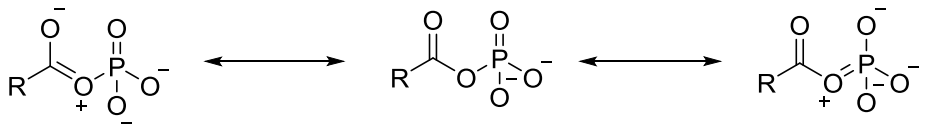
In general, a HK and its target RR has a one-to-one relationship, but some TCSs are known to have cross-talks between multiple HKs and RRs. With classical methods such as knockdown studies, however, it is difficult to analyze the complicated network between TCSs.

Characterization of the signal-TCS relationship can promote our understanding of bacterial signaling systems, which is fundamentally important for basic microbiology. Additionally, its application to the development of biosensors based on the bacteria will have tremendous practical significance. For example, if new TCSs that recognize heavy metals or toxins are elucidated, these can be potentially used as cell-based biosensors and bioswitches.

1.3 Chemistry of pAsp

Among phosphoamino acids, pAsp is probably the most difficult one to study since it is an acyl phosphate, which is unstable both in acidic and basic conditions as well as in neutral pH if certain nucleophiles are present. It is thermodynamically and kinetically unstable. According to the literature,

standard hydrolysis free energy of acetyl phosphate, a model acyl phosphate, is -10.3 kcal/mol. Compare to the hydrolysis energy of ATP to ADP, which is -7.3 kcal/mol, acetyl phosphate is thermodynamically more poised towards hydrolysis.[6] This can be explained with two reasons. First, due to the delocalization of lone pair on the bridging oxygen to the phosphorus (Scheme 3, right structure). Resonance form of acyl phosphate implies a positive charge on bridging oxygen. Therefore, these resonance structures cannot contribute to stabilize acyl phosphate. The second reason is that the hydrolysis products of pAsp can be more readily ionized and more strongly solvated than the starting material, leading to large free energy of hydrolysis of acyl phosphate.[7] Kinetic stability of pAsp was measured and reported. At 30 ˚C, pH 4 to 10, 30 % hydrolysis of pAsp was observed within 30 min.[8]

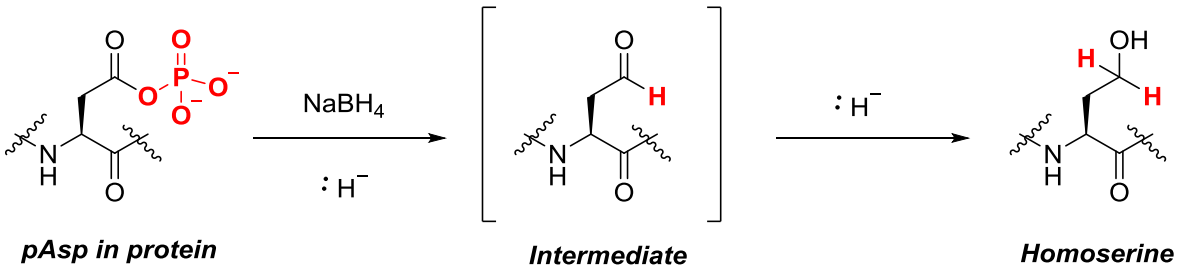


Scheme 3. Resonance structures of acyl phosphates.

1.4 Current detection methods for pAsp

Several papers describe the detection methods for pAsp in proteins. A classical method is the radioactive labeling using [γ-^{32}P] ATP to measure the radioactivity of the phosphorylated target protein.[9] This is a very sensitive method for detection of phosphoamino acids in protein. But it expensive and dangerous since it uses radioactive material.

Another method is to convert pAsp into homoserine (Hse). pAsp can be selectively reduced and transformed into Hse with $NaBH_4$ (Scheme 4). Sometimes tritium-labeled NaB^3H_4 was used to detect the labeled Hse by radioactivity. This reduction method is frequently used to confirm the pAsp site in protein, combined with tandem mass spectrometry.[10,11]



Scheme 4. Conversion of pAsp residue into Hse by $NaBH_4$.

2. Covalent-labeling of pAsp in proteins

As described above, it would be extremely useful to identify which specific TCSs are activated under certain external stimuli. To observe it, it is essential to analyze and quantify the pAsp-containing RRs in the proteomes. But since pAsp is easily hydrolyzed, it is difficult to analyze the pAsp-containing proteins in classical phosphoproteomics which require acidic condition in elution of phosphopeptide. Therefore, we aim to develop new methods to specifically label pAsp residues in proteins. In the following sections, our progress in this labeling strategy using *in vitro* model systems is described. We expect that this methodology will also be very useful to investigate other pAsp-containing proteins than the study of TCS.

2.1 Strategy and work flow of pAsp labeling in RR

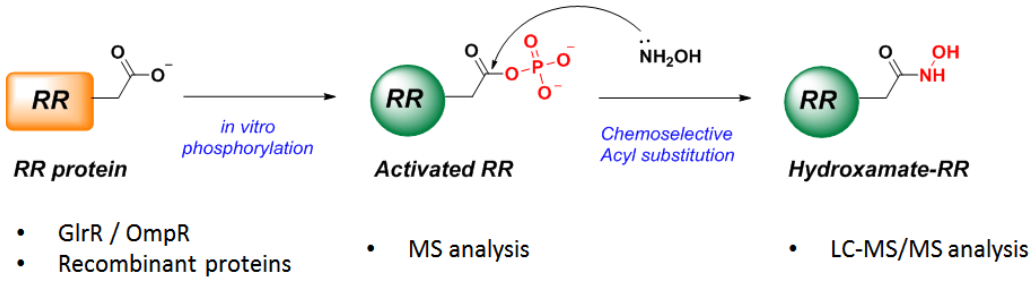
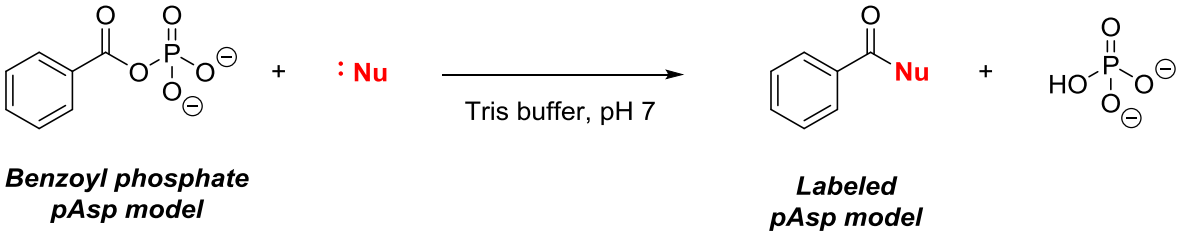


Figure 8. Scheme of hydroxamate based covalent pAsp labeling.

Our project aims to analyze TCS through development of specific covalent labeling methods for pAsp in proteins (particularly activated RR). This way, it would be possible to identify which specific TCSs are activated under certain stimuli. Hydroxylamine is used as nucleophile for pAsp. Since acyl phosphate reacts with hydroxylamine in aqueous solution, pAsp will be labeled into the Asp-hydroxamate (Figure 8). Our design is based on the fact that other amino acids in proteins have low reactivity toward nucleophiles, and this labeling method is expected to be specific to pAsp. Then, we also plan to develop methods that can selectively purify the hydroxamate-labeled pAsp. Through this methodology, combined with proteomic analyses through tandem mass spectrometry, we will be able to detect various TCS activation at the same time in response to certain stimuli. In addition, this methodology does not require any genetic manipulation, as in reporter gene assays.

2.2 Nucleophiles for acyl phosphate

Previously, Wenjie Ma in our research group tested various nucleophiles on acyl phosphate, using benzoyl phosphate as a pAsp model. Several water-soluble nucleophiles were tested. α–Nucleophiles have high nucleophilicity due to the delocalization of lone pair electrons of the adjacent heteroatom to the nucleophilic center.[12] A series of α–nucleophile including hydroxylamine, hydrazine and their derivatives, such as oxime, hydrazone and hydroxamic acids were synthesized and tested in pH 7, Tris buffer (**Scheme 5**). Reactions were monitored by HPLC and based on the data, reaction rate was calculated. The result showed that hydrazine and hydroxylamine were most reactive, and they exhibited enhanced reactivity in presence of magnesium ion. Several substituted derivatives of hydroxylamine or hydrazine were also tested, but still hydroxylamine showed best reactivity toward benzoyl phosphate.[13]



Scheme 5. Test of α-nucleophiles to benzoyl phosphate.

When hydroxylamine reacts with an acyl phosphate, it forms a hydroxamate. It is known that hydroxamates have high affinity toward various metals and metal oxides, such as Fe^{3+} and TiO_2. If pAsp residues in proteins and peptides can be covalently labeled as the hydroxamate, we expect that using immobilized metal affinity chromatography (IMAC), selective enrichment of hydroxamate-labeled proteins and peptides will be possible.

2.3 In-vitro covalent labeling of RR

2.3.1 Phosphorylation of RR

2.3.1.1 Phosphorylation by small molecules

It is known that some bacterial response regulators (CheY, OmpR, PhoB, etc.) can be phosphorylated at specific Asp residues *in vitro* by small-molecule phospho-donors, such as acetyl phosphate, phosphoramidate, and carbamoyl phosphate. In the case of CheY, all three phospho-donors successfully phosphorylated it.[14, 15] This small-molecule phosphorylation was identified with radiolabeled acetyl [^{32}P] phosphate, in the presence of magnesium ion. CheY phosphorylation was also monitored by its intrinsic tryptophan fluorescence change after phosphorylation since it has only one Trp residue and its fluorescence was quenched by the nearby pAsp.

To repeat this phosphorylation in our hands, recombinant CheY was expressed and purified. Firstly, phosphorylation with acetyl phosphate was performed with 4 μM of CheY in 0.1 M Tris pH 7.0 with 8.8 mM of $MgCl_2$. To our delight, after addition of acetyl phosphate, decrease of the Trp fluorescence (295 nm excitation and 360 nm emission) was observed. Decrease of the fluorescence after the phosphorylation was around 40 % in 20 to 40 mM acetyl phosphate, which is consistent with the literature (Figure 9).[16] CheY phosphorylation was also analyzed by MALDI-TOF MS and ESI-MS. Both analyses showed an +80 Da increase in the CheY protein mass after phosphorylation (Figure 10).

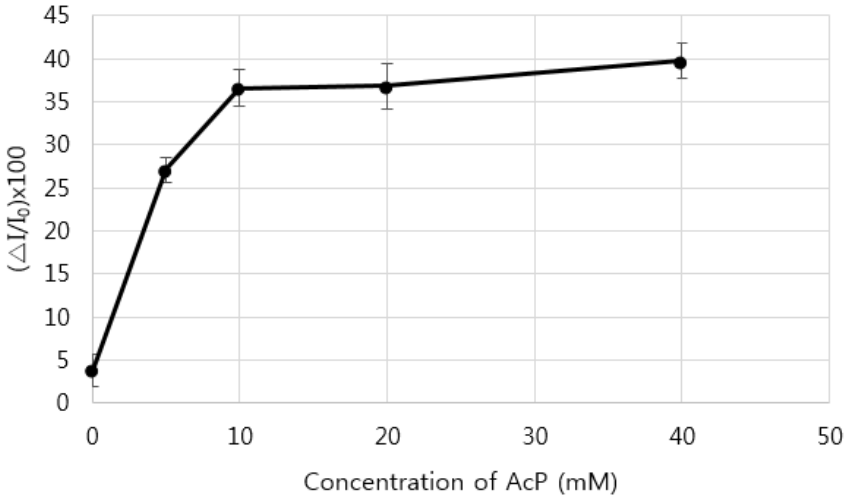


Figure 9. CheY fluorescence quenching by the phosphorylation with AcP.

(I_0: Initial fluorescence intensity (before phosphorylation), I: Fluorescence intensity after phosphorylation, $\triangle I = I - I_0$)

To this Asp-phosphorylated CheY, covalent labeling with hydroxylamine was attempted. Indeed, the fluorescence recovery was observed when 1 M hydroxylamine was added to the phosphorylation reaction mixture, suggesting that pAsp was converted to a different product, either a hydroxamate or an Asp (Figure 11). Unfortunately, the +15 Da difference between the nonlabeled Asp and the hydroxamate product was difficult to be distinguished with ESI-MS or MALDI-TOF MS. Therefore, we analyzed the hydroxylamine-treated CheY by LC-MS/MS, but the +15 Da increase on desired Asp residue was not observed. We attribute this lack of labeling to the competing reaction between the remaining acetyl phosphate and hydroxylamine, but the exact reason is still unclear. So we decided to carry out the phosphorylation without acetyl phosphate, via transphosphorylation of the RR by its cognate HK.

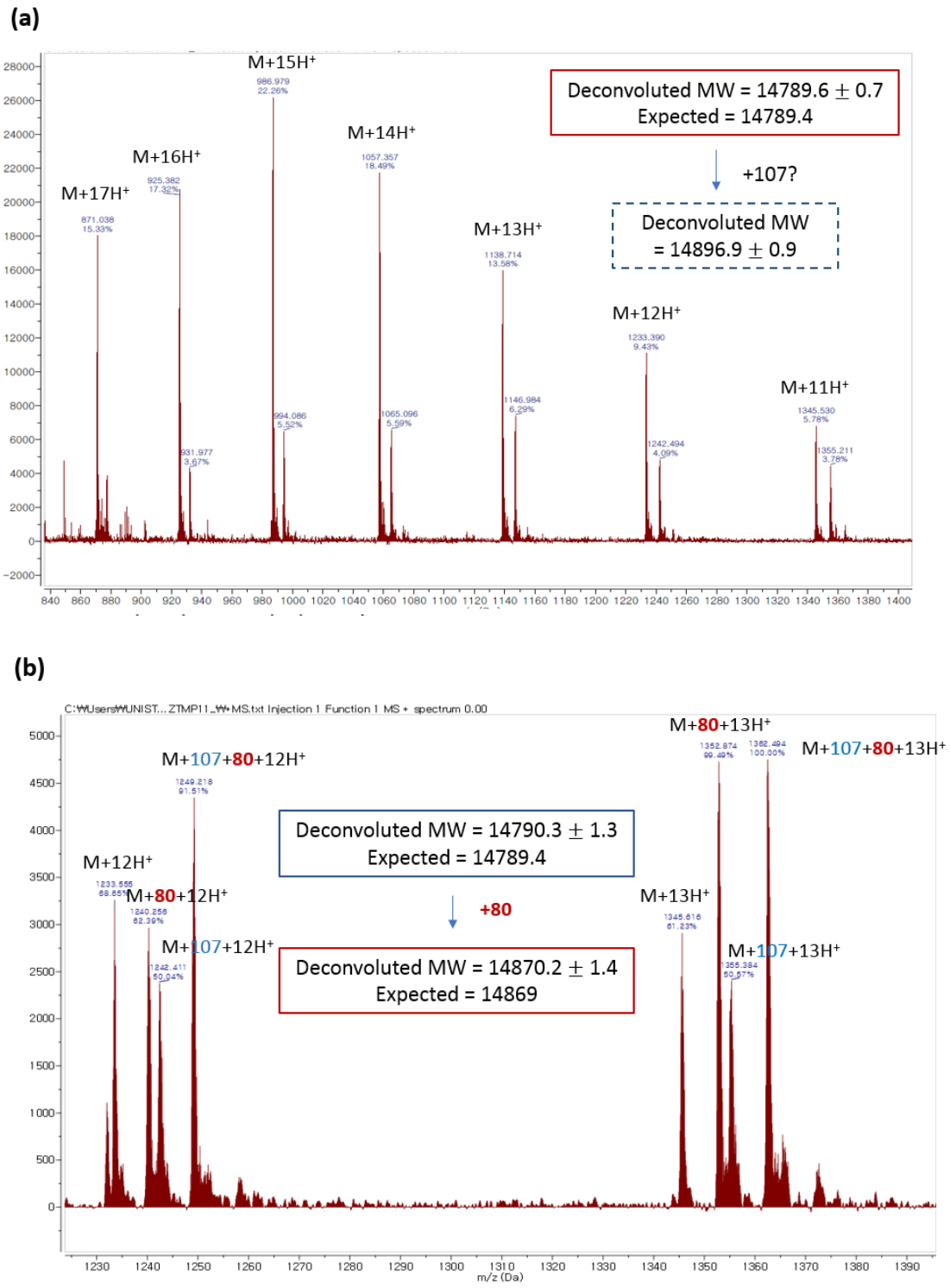


Figure 10. ESI-MS result of (a) CheY and (b) pCheY (positive mode).

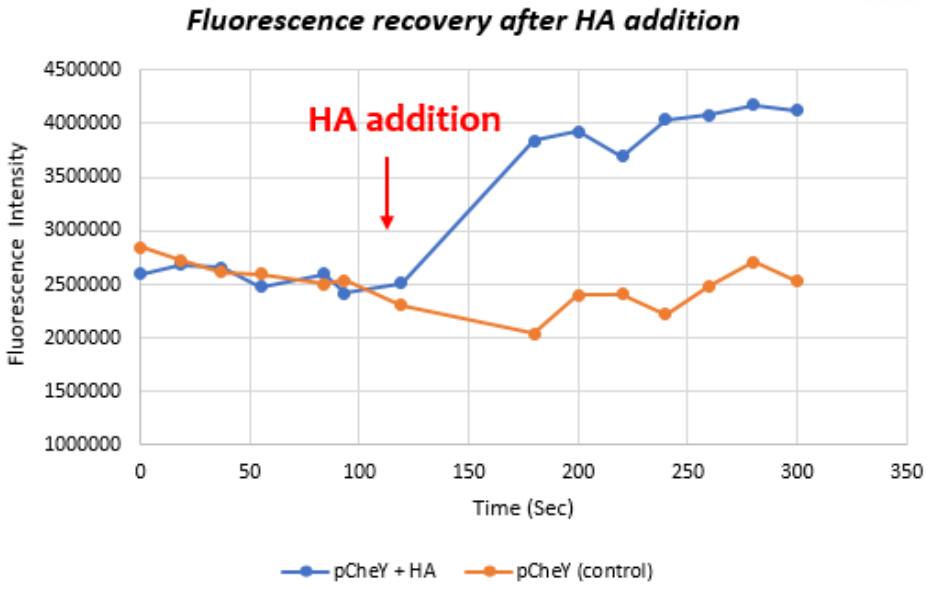


Figure 11. Fluorescence recovery after HA addition.

2.3.1.2 Transphosphorylation of a RR by its cognate HK

In a TCS, if a signal from outside activates the sensor HK, the HK phosphorylates its own His residue. The phosphoryl group from the pHis is then transferred to a specific aspartate residue of the cognate RR. Ishihama group characterized the trans-phosphorylation of RRs by HKs with various combinations of TCS system from *E.coli*.[17] They expressed and purified 30 HKs and 34 RRs including putative HK/RR candidates. Their phosphorylation was monitored by *in vitro* radiolabeling assays with [γ-^{32}P] ATP. The levels of the autophosphorylation of the HKs and the trans-phosphorylation the RRs were analyzed by measurement of the intensity of ^{32}P radioactivity.

Encouraged by this precedence, we tested the transphosphorylation of GlrK-GlrR and EnvZ-OmpR HK-RR pairs. His_6-tagged HKs and RRs were recombinantly expressed and purified. After purification, the autopho^{s}phorylation of the HK and the subsequent trans-phosphorylation to the RR was performed using non-radioactive ATP as the substrate.

Generally, an RR act as a transcriptional factor, regulating the target gene expression. But in the case of CheY, a chemotaxis protein of *E.coli*, it binds and regulates the activity of its target protein FliM. Attractants and repellents bind to the cell surface domain of protein CheA, a receptor histidine kinase, and CheA phosphorylates CheY. When phosphorylated, CheY binds to the N-terminal segment of FliM via conformational change. FliM constitutes the switch complex of the flagellar motor and involved in

clockwise rotation.[18]

OmpR is involved in the regulation of osmolarity of *E.coli* by modulate porins. OmpR act as a transcriptional factor of OmpF and OmpC, which are outer membrane proteins that form pores to allow the diffusion of small molecules. The level of phosphorylation of OmpR is determined by its cognate HK, EnvZ. In high osmolarity growth media, the phosphorylation level of OmpR was increased.[19]

2.3.2 Covalent labeling of phosphorylated RRs

After the trans-phosphorylation, hydroxylamine hydrochloride solution (pH 7) was added to a final concentration of 2 M. The reaction was performed at RT for 1 h, followed by desalting with reversed-phase R2 poros resin to remove excess hydroxylamine before the protein digestion. Subsequently, the protein digestion was performed with trypsin. Since OmpR has long tryptic peptides around the known pAsp sites, a double-digestion was carried out with Glu-C endoproteinase.

2.3.3 LC-MS/MS analysis

Two hydroxylamine (HA)-treated samples were prepared, both with phosphorylated OmpR (pOmpR-HA) and non-phosphorylated RR(OmpR-HA). After the double digestion using trypsin and Glu-C, LC-MS/MS analysis was performed by Prof. Jeong-Kon Seo at UCRF, UNIST. The target peptides harboring the known pAsp site (D55) were observed in both samples, but not all amino acids in the target peptide were read in LC-MS/MS. To our delight, the amino acids around the known pAsp site (D55) was read and 15 Da increase was observed in pOmpR-HA, supporting that it had been converted to a hydroxamate, presumably *via* pAsp (). Additionally, no hydroxamate (D+15 Da) was observed in the corresponding site in OmpR-HA.

Following the same procedure, pGlrR-HA and GlrR-HA were prepared and analyzed by LC-MS/MS. Consistent with the result of pOmpR-HA and OmpR-HA, only phosphorylated GlrR showed the +15 Da modification on Asp on D66 (Figure 13). So, our covalent labeling approach was validated in two TCS systems *in vitro*.

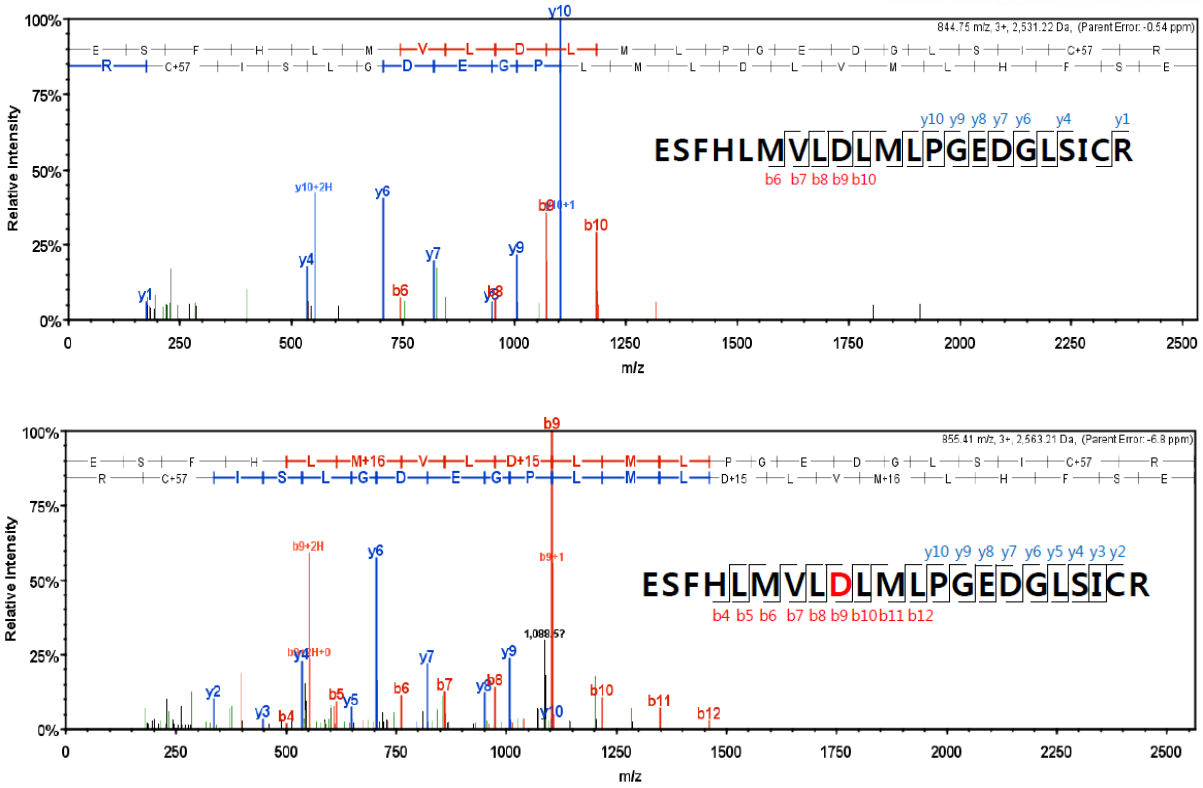


Figure 12. LC-MS/MS analysis of OmpR-HA and pOmpR-HA. The presence of pAsp in Asp55 (b8 and b9) was observed.

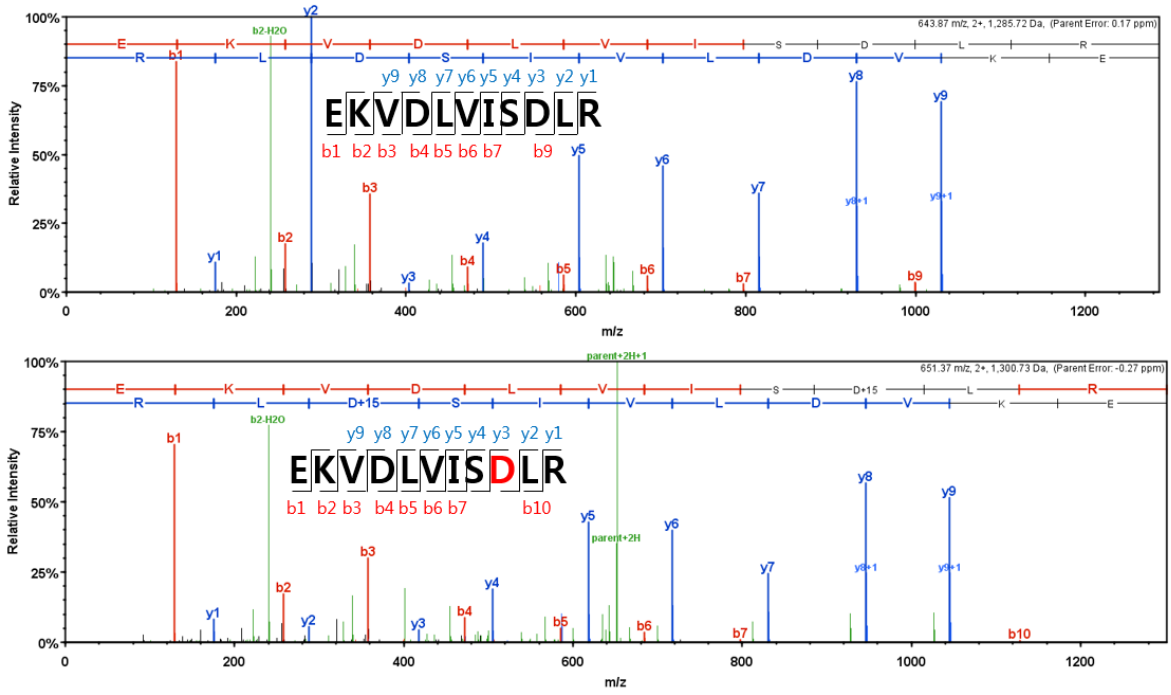


Figure 13. LC-MS/MS analysis of GlrR-HA and pGlrR-HA. The presence of pAsp in Asp56 (y2 and y3) was observed.

2.4 Conclusion and future directions

We demonstrated that the covalent *in vitro* labeling of pAsp in RR proteins with hydroxylamine after enzymatic phosphorylation and transphosphorylation in two TCS pairs, EnvZ-OmpR and GlrK-GlrR. The LC-MS analysis clearly indicated the successful phosphorylation of CheY, but the subsequent covalent labeling was not observed in this case. Now we are focusing on the reproducibility of our labeling esperiments and the subsequent enrichment of the hydroxamate-labeled peptide with IMAC approaches.

During the preparation of this thesis, a quite similar covalent labeling strategy for pAsp in RRs using an alkoxyamine nucleophile was reported.[20] However, in our own studies, such nucleophiles showed very low reactivity to acyl phosphates. So it should be tested if such strategies can be applied to the detection of low-level pAsp in RRs. Head-to-head comparison of our hydroxylamine-based method and their method should be helpful.

3. Experimental method

General materials

Trypsin and Glu-C were purchased from Thermo Scientific (Pittsburgh, PA). All biological reagents (unless other noted) were purchased from Bio-Rad (Hercules, CA). Adenosine 5'-diphosphate sodium salt (ATP) and lithium potassium acetyl phosphate were purchased from Sigma-Aldrich (St. Louis, MO), and magnesium chloride*hexahydrate ($MgCl_2$) was purchased from Biosesang (Seongnam, Republic of Korea). Tris base salt was purchased from Alfa Aesar. All other salts in buffer solution and hydroxylamine hydrochloride were purchased from Daejung (Siheung, Republic of Korea). All reagents used in LC-MS/MS analysis were provided by Prof. Jeong-Kon Seo.

General methods

Mass spectra were measured with Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, MA), by Prof. Jeong-Kon Seo at UCRF, or with Ultraflex III-MALDI-TOF/TOF (Bruker BioSpin, Billerica, MA). Fluorescence was measured with SpectraMax i3x (Molecular devices, Sunnyvale, CA). Fluorescence of CheY protein was measured at excitation wavelength 295 nm and emission wavelength at 360 nm. PCR was performed with T100™ Thermal Cycler (Thermo Scientific, Waltham, MA). DNA or protein concentration was measured by

NanoDrop™ 2000/2000c (Thermo Scientific, Waltham, MA).

Plasmid construction for recombinant protein

Plasmid containing the HK catalytic domain and the full-length RR were prepared by PCR-based restriction-free cloning method.[21] *E.coli* genomic DNA was used as the template and primers were designed with an online tool (http://rf-cloning.org). Primers were synthesized at Macrogen (Seoul, Republic of Korea). PCR-amplified megaprimer was incorporated into a pET21a(+) vector (with a C-term His_6-tag) by overlap-extension PCR. For overlap-extension PCRs, Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific, Catalog No.: F531S) was used. MAX Efficiency® DH5α™ Chemically Competent Cells (Invitrogen, Catalog No.: 18258-012) was used as the host for transformation. All plasmid sequences were confirmed by DNA sequencing (Macrogen, Korea).

Preparation of the megaprimers for overlap-extension PCR

PCRs were performed according to the literature.[21] Primers (Table 1) were diluted into 100 μM with distilled water. For megaprimer synthesis, 10 μL of 2 X Phusion High-Fidelity PCR Master Mix was added and 2 μL of forward and reverse primer respectively. Then, 1 μL of *E.coli* genomic DNA was added (50 ng/μL). Distilled water was added, and final volume of sample was 20 μL. In case of cloning of HK, 0.8 μL of DMSO was added additionally. PCRs were run using the following parameters (Table 1).

Primers

Gene		Primer sequence
CheY	Forward	5'- CCC TCT AGA ATA ATT TTG TTT AAC TTT AAG AAG GAG ATA TAC ATA TGG CGG ATA AAG AAC TTA AAT TTT TGG -3'
	Reverse	5' -CAG TGG TGG TGG TGG TGG TGC ATG CCC AGT TTC TCA AAG ATT TTG -3'
OmpR	Forward	5'- CCC TCT AGA ATA ATT TTG TTT AAC TTT AAG AAG GAG ATA TAC ATA TGC AAG AGA ACT ACA AGA TTC TGG TG -3'
	Reverse	5' - CAG TGG TGG TGG TGG TGG TGT GCT TTA GAG CCG TCC GGT AC -3'
YfhA(GlrR)	Forward	5'- CCC CTC TAG AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AGC CAT AAA CCT GCG CAT -3'
	Reverse	5'- CAG TGG TGG TGG TGG TGG TGT TCC TTG AAA TCG TTT GCA TCC AG -3'
YfhK(GlrK)	Forward	5'- TAG AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT AAT GAT TAT CGG GCC GG -3'
	Reverse	5'- GTG GTG GTG GTG GTG GTG GCC GCT GCC TTT CGT GTT TTT CGA CGA C -3'
EnvZ	Forward	5' – CCCTCTAGAATAATTTTGTTTAACCTTT AAGAAGGAGATATACATATGCGTATCCAGAACCGACC – 3'
	Reverse	5' – CTCAGTGGTGGTGGTGGTGG TGGCCGCTGCCCCCTTCTTTTGTCGTGC – 3'

Table 1. Forward and reverse primers used in PCR.

EnvZ incorporated in pET21a(+) plasmid was provided by Son Hye Shin.

Step		Temperature	Time
Initial denaturation		98 °C	10 sec
Cycles (25~30 repeats)	Denaturation	98 °C	1 sec
	Annealing	55 °C to 65 °C	5 sec
	Extension	72 °C	15 sec
Final extension		72 °C	2 min

Table 2. PCR method for megaprimer synthesis.

Finally, the megaprimer was purified by 0.8 % agarose gel electrophoresis and AccuPrep® Gel Purification Kit (Bioneer, K-3035). The product megaprimer concentration was measured by Nanodrop®.

Overlap-extension PCR

To a 10 μL of 2 X Phusion High-Fidelity PCR Master Mix, 20~40 ng of vector and 100 ng of megaprimer was added. Final volume was adjusted into 20 μL with distilled water. Overlap-extension PCRs were run using the following parameters (Table 3).[21]

Step		Temperature	Time
Initial denaturation		96 °C	30 sec
Cycles (25~30 repeats)	Denaturation	96 °C	30 sec
	Annealing	55 °C to 65 °C	60 sec
	Extension	72 °C	2-3 min
Final extension		72 °C	7 min

Table 3. Method of overlap-extension PCR.

Final product was treated with FastDigest DpnI (Thermo Scientific, Cat. No.: FD1703) for 5 min at 37 °C according to the manufacturer's instruction to remove the original vector template.

Transformation and mini-prep

The transformation was performed according to the NEB website protocol. To a 50 μL stock of Maxeffciency DH5α™ chemically competent cells (Invitrogen, Cat. No: 1701602), 2 μL of the desired plasmid was carefully added without vortexing. After incubation on ice for 30 min, heat shock was performed for 45 sec at 42 °C, and the cells were immediate kept on ice for at least 2 min for membrane recovery. To this, 450 μL of LB broth without antibiotics was added and the mixture was incubated on a shaker for 3 hr at 200 rpm. Then, the transformed cell stock (50 to 100 μL) was spread on an agar plate containing the desired antibiotic and incubated overnight at 37 °C. On the next day, colonies were picked, and each colony was inoculated in a 5 mL of LB broth which contains ampicillin and incubated on a shaker for 8 to 16 hr at 37°C, 200 rpm.

Mini-prep was done with AccuPrep® Nano-Plus Plasmid Mini Extraction Kit (Bioneer, Cat. No.: K-3111), according to the manufacturer's instruction. The purified plasmids were quantified with Nanodrop® and sequencing was performed at Macrogen (Seoul, Republic of Korea). Sequencing result was confirmed by BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression and purification of recombinant proteins

Expression and purification of recombinant *E. coli* proteins were carried out following the literature.[22] BL21(DE3) pLysS Competent Cells (Promega, Catalog No.: L1191) was used as host, HK-His_6 (cytoplasmic domain) and RR-His_6 (pET21a(+) derivative containing RR and HK from *E.coli*) was used as plasmid. To a 200 mL of LB broth with the antibiotic, 2 mL of the transformed cell stock was inoculated and cultured on a shaker at 37 °C, 200 rpm. At OD_{600} of 0.6~0.7, IPTG stock solution (final 1 mM) was added for induction. After incubation at 37 °C for 3 h, cells were harvested by centrifugation at 4 °C, 4000 rpm for 30 min.

Cell lysis

After centrifugation, cell pellet was resuspended in B-PER™ Complete Bacterial Protein Extraction Reagent (Thermo Scientific, Catalog No.: 89822) with 2 mM PMSF. 1 mM EDTA was added in case of BL21(DE3) competent cell lysis. For a 200 mL culture, 4 mL of B-PER Complete buffer was used. All lysis procedures were carried out with standard protocol according to the manufacturer's instruction. The lysate was centrifuged at 4000 rpm for 30 min at 4 °C and the supernatant was purified by Ni-NTA affinity purification.

Ni-NTA protein affinity purification

From the cell lysate, the His_6-tagged protein was purified by HisPur™ Ni-NTA Resin (Thermo Scientific, Catalog No.:88223). Ni-NTA was performed according to the standard protocol from manufacturer's instruction. To a 3 mL of Ni-NTA resin slurry, 4 to 5 mL of the lysate was added. Lysate was incubated 30 min at RT on a rocker. After incubation, purification was performed. All buffers were prepared with 50 mM Tris-HCl, pH 7.5, and 20 mM imidazole was added to the equilibrium and wash buffers. The elution buffer contained 100 mM or 200 mM of imidazole. Each fraction contains 3 mL of washed or eluted sample. 5 wash fractions and 6 elution fractions were analyzed by SDS-PAGE and its concentration was measured by Bradford assay after dialysis and concentration of protein. Total 9 mL of clean elution fractions were combined and dialyzed three times (for 2 to 4 h each time) at 4 °C in 1 L of 50 mM Tris-HCl, pH 7.5.

Autophosphorylation of HK and transphosphorylation to RR

The recombinant HK was diluted in TBS pH 7.8, into final concentration of 5 μM. Autophosphorylation was performed by addition of ATP and $MgCl_2$, both at final concentration of 5 mM. Autophosphorylation reaction was carried out for 1~2 hr at 37 °C. Then, equimolar amount of RR and additional ATP were added in ice. The transphosphorylation reaction was proceeded at 37 °C for 1 hr.

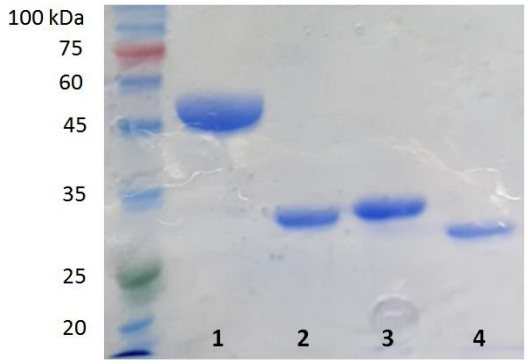


Figure 14. SDS-PAGE of the purified proteins, stained by Coomassie blue. From left, (1) GlrR-His6 (50 kDa), (2) EnvZ-His6 (32 kDa), (3) GlrK-His6 (31 kDa), (4) OmpR-His6 (28 kDa) after dialysis.

In-solution digestion

After desalting of reaction samples, elution fraction was dried by speed-vac. Then, proteins were denatured by addition of 8 M urea. After denaturation, disulfides were reduced with DTT (final 15 mM) at 56 °C for 1 hr and subsequently alkylated by treatment of iodoacetamide (final 55 mM) at 25 °C for 30 minutes. The digestion was done in a total volume of 250 μL, adjusted with 50 mM ammonium bicarbonate, pH 8. Trypsin was added to 1:50 protease to protein mass ratio. After 2 hr digestion at 37 °C, same amount of Glu-C was added, and reaction was kept overnight (13 h) at 37 °C. After the digestion, desalting was done by R2 reversed-phase chromatographic resin. Peptide fragments were eluted and dried under speed-vac. Then, dissolved in 0.1 % formic acid for LC-MS/MS analysis.

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