INVESTIGATION OF THE ROLES OF CHED IN THE \textit{Bacillus subtilis} 
CHEMOTACTIC SIGNAL TRANSDUCTION

BY

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DISSERTATION
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Abstract

The two-component based chemotaxis signal transduction system allows flagellated bacteria to sense their surrounding chemical environment and move towards more favorable conditions. The attractant signals can be sensed by transmembrane chemoreceptors, and then transmitted to the histidine kinase CheA. Once activated, CheA interacts with the response regulator CheY through phosphorelay, which causes a change in the rotation of the flagella. The direction of flagella rotation determines whether a cell swims straight or just tumbles. Cells also need adaptation to respond to a change in chemical concentrations, and return to their prestimulated level. Adaptation in the *B. subtilis* chemotaxis system is achieved by three coordinated systems: the methylation system, the CheC/CheD/CheY-p system and the CheV system. CheD, the previously identified receptor deamidase, was shown to be critical to the ability of *B. subtilis* to perform chemotaxis and is the main focus of this study.

This study started from characterization of the enzymatic mechanism of CheD. Results showed that CheD deamidase uses a cysteine hydrolase mechanism. The catalytic triad consisting of Cys33-His50-Thr27, and Ser27 is essential for receptor recognition and binding.

In addition, in this study CheC was found to inhibit CheD’s deamidase activity. Through mutant screening, Phe102 on CheD was found to be the essential site to interact with CheC. Furthermore, the CheD/CheC interaction is necessary for the robust chemotaxis *in vivo* as demonstrated by the cheD (*F102E*) mutant, which lacks the ability to swim on swarm plates.
Despite its deamidase activity, we hypothesized that CheD’s main role is its involvement in the CheD-CheC-CheY-p negative feedback pathway during adaptation. In particular, CheD is likely to help stabilize the transient kinase-activating state through binding to receptors. When CheY-p level is increased, CheC-CheY-p complex may attract CheD away from receptors. In this study, CheC-CheD binding kinetics with CheY or CheYp presence was successfully obtained by a series of SPR experiments. The increased affinity of CheD for CheC in presence of CheYp but not CheY makes likely the hypothesis that CheC-CheD-CheY interact as part of a negative feedback pathway during adaptation.

Last, the interaction between CheD and chemoreceptor McpC was studied in order to better understand the role of CheD in adaptation. Results showed that Q304 and Q305 on McpC are essential to recruit CheD. Additionally, the reduced levels of CheD in mcpC (Q304A) or (Q305A) mutants suggested that the dynamic interaction between CheD and receptors is vital to maintain the normal CheD level.

These findings suggest more complicated roles of CheD than its previously identified function as a receptor deamidase, and will lead to a clearer picture of the coordination of the three adaptational systems in the B. subtilis chemotactic sensory transduction system.
Acknowledgements

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# Table of Contents

## Chapter 1: Introduction

- Bacterial Movement and Chemotaxis ........................................................... 1
- The Two-Component Signal Transduction System ....................................... 2
- The Chemotaxis Signal Transduction System ............................................. 3
- *B. subtilis* Chemotactic Sensory Transduction System .......................... 9

## Scope of This Study................................................................................. 10

## Figures and Tables.................................................................................. 12

## Chapter 2: Materials and Methods

- Media and Solutions .................................................................................... 23
- Strains and Plasmids .................................................................................... 24
- Protein Purification ...................................................................................... 25
- Western Blot Sample Preparation ............................................................... 27
- Western Blot.................................................................................................. 28
- Receptor Deamidation Assay ....................................................................... 28
- Swarm Plate Assay ....................................................................................... 29
- Tryptic Digestion and HPLC Peptide Separation ......................................... 29
- Surface Plasmon Resonance (SPR) ............................................................... 30
- SPR Data Analysis ........................................................................................ 31
- Structural Modeling ...................................................................................... 32
- Tables ............................................................................................................. 33

## Chapter 3: CheD is a Cysteine Hydrolase

- Introduction .................................................................................................. 35
- Putative CheD Active Site Mutations Show a Chemotactic Defect ............ 35
- Putative CheD Active Site Mutations Show a Deamidase Defect .............. 36
- CheD is Not a Serine Hydrolase ................................................................. 37
- Structural Modeling Analysis of CheD ......................................................... 38
- Cysteine Protease Inhibitors Stop CheD Deamidation ................................. 39
- Ser32 is Responsible for Receptor Binding .................................................. 39
- Discussion .................................................................................................... 40
- Figures and Tables......................................................................................... 44

## Chapter 4: CheC Inhibits CheD Deamidase Activity

- Introduction .................................................................................................. 58
- CheC Inhibits CheD Deamidase Activity .................................................... 58
- Phe102 on CheD is Required for CheC Interaction .................................... 59
- The CheD/CheC Complex is Important for Chemotaxis ......................... 60
- Discussion .................................................................................................... 61
- Figures and Tables......................................................................................... 63
Chapter 1

Introduction

Bacterial Movement and Chemotaxis

Microorganisms living in nature do not always face harmonious living conditions. Often they have to face a variety of hostile and challenging environmental conditions, e.g. lack of food, competition for nutrients, chemical toxin attacks, constantly changing temperatures, osmotic stress, etc. To survive and thrive in harsh environments, microorganisms developed the ability to move during evolution. Bacterial motility was first discovered in 1676 by Antoni van Leeuwenhoek [27]. Motility lets a microorganism swim towards a nutrient source and away from hostile environments. This process is known as chemotaxis, which has been extensively studied since discovered by Julius Adler in 1965 [1, 2] (Figure 1.1).

Bacterial movement is achieved by different mechanisms, including flagella [4, 15, 65], pili [102, 121], and gliding [69, 88]. The core chemotactic signal transduction systems are similar and relatively conserved among bacteria and archaea. They are all based on the two-component system, albeit flagella in the archaea shows no homology to flagella in bacteria [113].

Even in bacteria, the flagella number and position vary among different species. The gram-positive bacterium *Bacillus subtilis* and the gram-negative bacterium *Escherichia coli* have 6-12 flagella, which can form a flagella bundle during swimming mode [3, 126]. *Thermotoga maritima* has a single polar flagellum [35], and the spirochetes have several flagella located at each of two ends of the cell [14]. Despite using different mechanisms for
flagella-mediated motility, all bacteria use a similar core two-component system for chemotaxis.

The Two-Component Signal Transduction System

Two-component signal transduction systems widely exist in bacteria and archaea and rarely in the eukaryotes. They are the predominant mechanism of signal transduction in bacteria and archaea [106]. The two-component systems enable bacteria to respond and adapt to a wide range of environmental signals, including nutrients, toxins, stressors, cellular redox state, changes in osmolarity, quorum signals, and antibiotics [59]. Different bacteria and archaea may have different numbers of two-component systems that need to be tightly regulated to avoid unnecessary cross-talk. Some bacteria can contain up to as many as 200 two-component systems [59]. *Synechocystis sp.* has as many as 80 two-component systems, while *Mycoplasma genitalium* does not contain any two-component systems [75, 73]. *E. coli* has 30 two-component systems and the *B. subtilis* has 70 two-component systems [28, 74].

Two-component systems mainly consist of a sensor histidine kinase (HK) and its cognate response regulator (RR). Upon activation, the HK catalyses its own autophosphorylation on a conserved histidine residue followed by transfer of the phosphoryl group to a conserved aspartate on the response regulator, which can induce many downstream biological effects. Phosphorylation induces a conformational change in its output domain. Thus, the RR is activated (Figure 1.2). The output domains of RR are usually DNA-binding domains. But in chemotaxis, CheY (RR) is not a DNA-binding protein. Instead, upon phosphorylation it will bind the switch proteins in the flagella base body and alter the direction of flagellar rotation (Figure 1.2). The system can be re-set by
dephosphorylation of RR. Therefore, bacteria can constantly sense the external signals. The activated RR can act as quickly as several seconds (half-life of RR-p), so that cells can response to the environmental signals quickly and efficiently [106].

Interestingly, there is some cross-talk between different two-component systems. For example, the NtrB-NtrC pathway is important in nitrogen assimilation, and studies on the NtrB-NtrC and CheA-CheY pathways found that both NtrB and CheA kinases can phosphorylate NtrC [80]. Furthermore, overexpression of the histidine kinase NtrB in a cheA strain of E. coli showed smooth swimming behavior, indicating the cross-phosphorylation of CheY by NtrB. It has also been verified by in vitro NtrB-CheY phosphorelay assay [80]. In our case, it will be interesting to investigate potential cross-talks between CheA-CheY pathway and other two-component pathways. It will be more biologically meaningful as cells may be facing with different growth or environmental signals at a certain time point.

**The Chemotaxis Signal Transduction System**

Flagellated bacteria can move towards favorable environment by controlling the direction of flagella rotation. When rotating counterclockwise (CCW), due to the inherent structural chirality, the flagella form into a bundle at a pole of the B. subtilis cell. Then the cell swims smoothly. When rotating clockwise (CW), the flagella bundle falls apart, and the cell tumbles. In an isotropic environment, a bacterium will have a 55% of CCW rotation (smooth swimming) and 45% CW rotation (tumbling), resulting a random 3-D walk and little net migration [11, 64, 68, 112]. When an attractant signal is sensed, the flagella bundle will increase the frequency of CCW rotation resulting a net swimming towards higher concentrations of attractant (Figure 1.3). Once there is no change of chemical gradient
sensed, the bacterium will adapt to the environment and return to its pre-stimulus 55/45 bias [53, 90].

Over the past 40 years, *E. coli* has been extensively studied as the model organisms for bacterial chemotaxis. The chemotaxis signal transduction system is based on the modified two-component system. In this system, CheA acts as the histidine kinase and CheY is its cognate response regulator. These two proteins are the core proteins of the chemotaxis two-component system. However, there are some differences from traditional two-component systems. The HK CheA is not a transmembrane protein, but a cytosolic protein that lacks a sensing domain. Therefore, it does not directly interact with an extra-cellular ligand. In this case, chemoreceptors, methyl-accepting chemotaxis proteins (MCPs), bind the external ligand [55, 84, 93, 124]. The binding will induce conformational changes on MCPs, which activates CheA autophosphorylation via a highly conserved domain (HCD) with the aid of the coupling protein CheW [5]. Once CheA is phosphorylated, it will promote CheY phosphorylation at the conserved aspartate residue. The activated CheY (CheYp) will then interact with switch proteins at the flagella base motor, which induces the change of flagella rotation (Figure 1.4). The whole chemotaxis cycle includes two steps, excitation and adaptation, which will be discussed further later.

Beside CheA-CheY core proteins, there are many more Che proteins involved for fully functioning chemotaxis. Individual proteins will be discussed next and summarized in Table 1.1.

A. The Core Proteins CheA and CheY
The histidine kinase CheA is a 74.5-kDa protein with five different domains (Figure 1.5). It is a homodimer with different domains connected to one another via flexible linkers [16]: (a) The P1 (histidine phospho-transfer or HPT) domain is a four-helix bundle containing the conserved histidine residue that can be phosphorylated by the kinase domain [77]; (b) The P2 domain is responsible for CheY binding [70, 76, 82, 105]; (c) The P3 domain is responsible for CheA dimerization [37, 100]; (d) The P4 domain is the catalytic domain, and, along with the P3 domain, forms the core common to all histidine kinases [16, 17]; and (e) the P5 domain is responsible for interacting with both CheW and the tip of MCPs, and also shares considerable homology with CheW [16, 19, 20]. Due to its large size, the detailed mechanism of CheA autophosphorylation is still not very clear. The P4 domain has several conserved regions including the N-box, G1-box, F-box, G2-box, and GT block, which are essential for catalysis and are thought to be involved in positioning of ATP into the active site [40].

Unlike CheA, the response regulator CheY is a small protein. It is a one-domain, 13 kDa protein with a length of about 120 amino acids. CheY can catalyze both its autophosphorylation and autodephosphorylation. CheZ in *E. coli*, CheC and FliY in *B. subtilis* increase the rate of CheY-p dephosphorylation [39, 100]. Several conserved residues are important for CheY activity, including two adjacent N-terminal aspartate residues that bind Mg$^{2+}$, the phosphorylatable aspartate located in the center of the protein, and a threonine and lysine located at the carboxy-terminus involved in catalysis of phosphorylation [62, 117, 127, 128]. Once phosphorylated, CheYp interacts with FliM in the flagella switch complex that promotes a change in flagellar rotation [96, 101, 115, 118]. In addition, CheYp has a very fast turnover rate compared to other RR$s$, and its half life varies from 10s to 30s in
different organisms. The fast CheYp turnover rate ensures cells to response quickly to the changing environment [94].

B. The Chemoreceptors

The chemoreceptors are responsible for ligand binding and transferring the signal to the downstream HK CheA via conformational changes mediated by scaffolding proteins CheW and CheV.

The chemoreceptors are typically membrane bound α-helical coiled-coil homodimers organized in five domains (Figure 1.6) [50, 99, 108]. The amino-terminal sensing domain is responsible for ligand binding, and is flanked by two trans-membrane regions, TM1 and TM2. In *E. coli*, the sensing domain is a four-helix bundle. Ligand binding occurs across the dimer interface, and binding-protein interactions occurs on the outer helix [31, 72]. The conformation signal is transferred through the HAMP domain (histidine kinase, adenyl cyclase, methyl-accepting chemotaxis protein and phosphatase). HAMP domains, usually a 50-residue relatively conserved motif, are found in many signaling proteins in bacteria and archaea. They are thought to converts the signal from sensory input modules to output modules. HAMP domains are four α-helices homodimers. In each monomer, there are two short amphipathic α-helices (12 –residue of AS-1 and 15-residue of AS-2) joined by a flexible connector [7-9, 43]. The signal is transferred to two methylation helices [10, 43, 61, 108]. This region has conserved glutamate or glutamine residues that can be post-translationally modified by CheB and CheR. These modifications have been demonstrated to be important during adaptation [36, 54, 86, 114]. Finally, the signal is
transferred to the HCD domain (the Highly Conserved Domain, also called as signaling domain). The HCD domain interacts with CheA and CheW to control CheA activity [108].

There are a number of chemoreceptors allowing bacteria to sense various attractants like amino acids and sugars. For example, there are five chemoreceptors in *E. coli*, and ten chemoreceptors in *B. subtilis*, with size ranging from 35 kDa to 72 kDa [3, 13]. In addition, these receptors have been reported to localize to cell poles in both *E. coli* and *B. subtilis* [51, 66, 67]. They are organized in vast arrays that can magnify even the smallest of signals over a large concentration range ($10^{-6}$ to $10^{-1}$ M) [34]. In *E. coli*, the chemoreceptors are believed to have a trimer-of-dimers organization based on crystallographic evidence from the carboxy-terminal structure of the Tsr receptor [107] and on genetic evidence [6]. However, based on recent crystal structure of the carboxy-terminal cytoplasmic region of the *Thermatoga maritima* TM1143 [83], *B. subtilis* most likely uses “hedgerow of dimers” to organize its chemoreceptors (Figure 1.7).

C. Other Chemotaxis Proteins in *E. coli*

*CheR*, a 32 kDa protein, is a methyltransferase placing a methyl group on particular glutamate residues via the universal methyl donor S-adenosyl methionine (SAM) [26, 103, 119]. *CheB* works as a methylesterase [120, 125]. CheB is also a response regulator that can be phosphorylated by CheA. This phosphorylation increases the methylesterase activity of CheB by roughly 100 fold, and the activated CheB restores the glutamate residue for additional cycles of methylations [104]. Interestingly, the half-life of CheB phosphate is very short, less than a second, so that adaptation to negative stimulus is rapid [104]. Both CheR and CheB modify conserved glutamate and glutamine residues in the methylation helices.
MH1 and MH2 of chemoreceptors. These modifications are important to regulate receptor conformation, which is important during adaptation, and will be further discussed in Chapter 5.

**CheW**, a 32-kDa scaffold protein, couples CheA to the receptors and relays the conformational change signals from chemoreceptors to the downstream HK CheA [71]. The CheW null mutants showed no accumulation in capillary assays and a smooth-swimming bias in the tethered-cell assays [18, 71, 95]. Therefore, it is an essential bridge in the MCP-CheW-CheA complex.

**CheZ**, an 18 kDa protein, works as a CheY phosphatase [39]. However, CheZ does not exist in most chemotactic bacteria outside of the γ- and β- proteobacteria, such as *B. subtilis* [3, 109]. This will be discussed in more detail in Chapter 5. Bringing CheY-p level to its pre-stimulus levels is vital for adaptation, as bacteria need to reset their response to the constantly changing environmental conditions. Once getting into a new environment, they have to adapt to it and quickly get ready to sense new changes.

**The Flagella Switch** is the final output of the chemotactic signal transduction system. It controls the motor at the flagellar base, and is responsible for changing directions of flagella rotation. In *E. coli* there are three sub-components in the flagellar switch, including FliM, FliN and FliG (Figure 1.8) [49]. Deletion of any above three will prevent flagellar formation, indicating that all of these three proteins are essential to motility [122, 123]. After the phosphorelay, CheY-p will interact with FliM, and induce a change in the direction of flagellar rotation [21]. The switch is a multimeric complex consisting of roughly 35 copies each of FliM and FliG and 110 copies of FliN. Note that in *B. subtilis* FliN is substituted by FliY, but the arrangement is assumed to be similar.
**B. subtilis Chemotactic Sensory Transduction System**

During the last 30 years’ studies, the chemotaxis community has started to realize that *E. coli* might not be the ideal paradigm model for chemotaxis study, mostly because it misses several key components found in most other chemotactic organisms. *B. subtilis* has emerged as a more suitable paradigm organism to study bacteria chemotactic signal transduction. Its CheA, CheY, CheW, CheB and CheR proteins have similar structures and functions as in *E. coli* [3, 112]. The core of chemotactic signal transduction in *B. subtilis* is similar to that of *E. coli*: first, MCPs sense ligand signals; then through phosphorelay the HK CheA and CheY is phosphorylated, respectively; CheY-p then binds with the switch proteins in the flagellar basal body. However, *B. subtilis* has three unique proteins, CheV, CheC and CheD, which are not in *E. coli*, but found in most other chemotactic bacteria studied to date [3, 112]. *B. subtilis* lacks CheZ, but possesses the flagellar switch protein FliY, a dual-domain protein with a CheC-like region and a FliN-like region [3, 111]. All of these made *B. subtilis* the best model to study bacterial chemotactic signal transduction (Figure 1.9).

**CheV**, a 35 kDa protein, is the third response regulator in *B. subtilis* chemotactic pathway in addition to CheY and CheB. It has a C-terminal response regulator domain that can be phosphorylated by CheA, and an N-terminal CheW-like output domain serving as a scaffolding protein for CheA [30, 89]. Both cheW and cheV mutants could still have an adequate chemotactic response, however, a cheWcheV double mutant is very tumbly [46]. This indicated that either CheW or CheV can mediate signal transferring from MCPs to CheA as a scaffolding protein, and they are necessary for the normal MCP-CheA complex.
CheD and CheC interact with each other, and both have more than one function. They are believed to be more important in chemotactic adaptation, which will be discussed in more details in Chapter 4-6.

Scope of This Study

The main theme of this study focuses on the B. subtilis CheD. The cheD knockout mutant shows a chemotactic defect, is less sensitive to attractants, more tumbly, and has poorly methylated chemoreceptors, indicating that CheD is an essential protein in B. subtilis chemotactic signal transduction [52, 56, 90]. It has been reported that CheD can deamidate chemoreceptors as a deamidase [56], and CheD interacts with CheC with unknown functions [90, 91].

The first goal of this work was to characterize CheD enzymatic mechanism as a deamidase, and what active sites it has. As described earlier, CheR can methylate chemoreceptors on the conserved Glu residues in B. subtilis. In some instances, the modified site is encoded as Gln rather than Glu. In order to be methylated, the amine group of Gln needs to be removed before it is methylated. K. Kristich has reported that CheD can deamidate chemoreceptors, McpA, McpB, and McpC through an unknown mechanism [56]. By understanding its enzymatic mechanism, we can further study the relationship of deamidation and receptor activation, and how exactly CheD helps tune the downstream kinase activation.

The second goal was to investigate CheD-CheC interaction, whether CheC activates or inhibits CheD in terms of CheD deamidase function, and potential sites on CheD that interact
with CheC. By this part of study, we can further understand the biological meaning of CheD-CheC interaction.

The third goal was to investigate why CheD-CheC interaction is important in adaptation. In detail, we believe that once CheY is phosphorylated, the CheYp-CheC complex will attract more CheD from chemoreceptors, which makes receptors less active. This will affect the CheA-CheY phosphorelay, bringing the CheYp level back to its pre-stimulus level. To verify this hypothesis, I used the Surface Plasmon Resonance (SPR) to exam CheD-CheC association/dissociation kinetics under different situations, like without CheY, with CheY, or with CheY-P.

The fourth and final goal of this study was to investigate the interaction between CheD and chemoreceptor McpC. McpC, the sole proline receptor, responds to a variety of attractants, and is the only CheD-dependent chemoreceptor. It is a good model to study receptor-CheD interactions. Addressing how McpC and CheD interact with each other can help us understand this special CheD requirement and how CheD makes receptors more active. In particular, I mutated the full-length McpC at the AS-1 region of the HAMP domain, then screened and tested mutants for potential interaction sites.
Figure 1.1 Bacteria chemotaxis. Bacteria accumulate around a capillary as an attractant diffuses out of the capillary opening into the pool of bacteria. This experiment was one of the first to show the chemotactic ability of microorganisms. This figure is a reproduction from the original study [92].
Figure 1.2 The two-component systems. The top part shows general schemes of two-component systems, and the bottom part shows a basic two-component frame in chemotaxis. The two-component pathway is a prevalent control system found in many bacteria. The histidine kinase senses an environmental stimulus, causing autophosphorylation on a conserved histidine residue. Once excited, the cognate response regulator protein associates with the HK and the phosphoryl group is transferred to the RR on a conserved aspartate residue. The active, phosphorylated RR then elicits the desired response by interacting with it target through the interactions of its output domain. This response is often achieved by controlling gene expression. But in bacteria chemotaxis, phosphorylated RR will bind with the switch protein in flagella base body to increase the CCW rotation.
Figure 1.3 Chemotactic response of flagellated bacteria. Flagellated bacteria can move towards favorable environment by controlling their flagella rotating. When rotating counterclockwise (CCW), due to the inherent chirality, the flagella form into a bundle at a pole of the cell. Then the cell has a straight and smooth swimming. When rotating clockwise (CW), the flagella bundle falls apart, and the cell tumbles. In both swimming and tumbling, it is just a matter of ratio of CCW/CW. In an isotropic environment, a bacterium will have a 55% of CCW rotation (swimming) and 45% CW rotation (tumbling), resulting a random 3-D walk and little net migration. When an attractant signal is sensed, the flagella bundle will increase the CCW ratio resulting a net swimming towards higher concentrations of attractant.
Figure 1.4 *E. coli* Chemotaxis Model. The chemoreceptors interact with an attractant ligand in their sensing domain, causing a conformational change that activates the HK CheA, which is coupled to the receptors with the aid of the CheW coupling protein. Once activated, CheA transfers its phosphoryl group to the RR CheY, which interacts with the flagellar switch proteins. CheY subsequently gets de-phosphorylated with the aid of CheZ. The receptors can also get post-translationally modified by the proteins CheR and CheB.
Table 1.1 Chemotaxis Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity or Role</th>
<th>Comment</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CheA</td>
<td>Histidine kinase</td>
<td>Autophosphorylates on histidine residue and transfers phosphate to CheY, CheB or CheV.</td>
<td>See Figure 1.5 below</td>
</tr>
<tr>
<td>CheB</td>
<td>Methyl esterase</td>
<td>Demethylates methylglutamate residues on MCP’s: role in adaptation. Has RR domain.</td>
<td></td>
</tr>
<tr>
<td>CheC</td>
<td>Adaptational protein coupling with CheD</td>
<td>Role in adaptation. Shown to bind CheD. Inhibits CheD deamidase activity.</td>
<td></td>
</tr>
<tr>
<td>CheD</td>
<td>Adaptational protein coupling with CheC</td>
<td>Deamidates glutamine residues on the MCP’s. Makes receptors more active. Enhances CheC phosphatase activity.</td>
<td></td>
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<tr>
<td>CheR</td>
<td>Methyl transferase</td>
<td>Methylates conserved glutamate residues on MCP’s.</td>
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<tr>
<td>CheV</td>
<td>Coupling and adaptational protein</td>
<td>CheY-CheW fusion. Role in adaptation as well as a coupling protein.</td>
<td>No structure available.</td>
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Table 1.1 cont.

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<th>Activity or Role</th>
<th>Comment</th>
<th>Structure</th>
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<td>CheW</td>
<td>Coupling protein</td>
<td>Couples CheA to the receptors.</td>
<td><img src="image" alt="CheW structure" /></td>
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<td>CheY</td>
<td>Primary response regulator</td>
<td>When phosphorylated, interacts with the flagella switch to induce CCW flagellar rotation.</td>
<td><img src="image" alt="CheY structure" /></td>
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<tr>
<td>MCP’s</td>
<td>Receptors</td>
<td>Bind chemoeffectors and transduces signal to CheA. Are methylated and demethylated on glutamate residues.</td>
<td><img src="image" alt="MCP’s structure" /></td>
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**Non-B. subtilis**

<table>
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<th>Activity or Role</th>
<th>Comment</th>
<th>Structure</th>
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<tbody>
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<td>CheX</td>
<td>CheY-P phosphatase</td>
<td>Truncated form of CheC found in close relatives of <em>B. subtilis</em>.</td>
<td><img src="image" alt="CheX structure" /></td>
</tr>
<tr>
<td>CheZ</td>
<td>CheY-P phosphatase</td>
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(Note: The collection of 3-D structures in this table was from G. Glekas Ph.D. thesis.)
Figure 1.5 CheA Architecture. The histidine kinase CheA has five distinct domains (P1-5), that each has a separate function as shown in the figure. Crystal structures for certain domains have been solved, and are shown above.
Figure 1.6 Chemoreceptor architecture. The receptors are long α-helical homodimers with five distinct regions, shown above in both a 3-D model of the *E. coli* Tsr receptor (left) and a schematic diagram (right). The sensing domain binds a ligand, inducing a conformational change in the receptor that is transmitted through the transmembrane domain to the HAMP domain and down into the highly conserved domain (HCD), which interacts with the CheA kinase. The methylation domain can be post-translationally modified on conserved glutamine and glutamate residues, which makes receptors more or less active.
Figure 1.7 Higher order chemoreceptor structure. (A) The crystal packing of both the *T. maritima* and *E. coli* receptors. (B) The proposed hedgerow model of the chemoreceptor signaling array proposed for *T. maritima* [83].
Figure 1.8 The flagellar switch. Electron microscope images of 60 individual *S. enterica* flagella basal bodies [29]. In *B. subtilis* FliN is substituted by FliY, but the arrangement is assumed to be similar.
Figure 1.9 B. subtilis chemotaxis model. The chemoreceptors (MCPs) interact with a ligand in their extracellular sensing domain, inducing an increase in the level of the HK CheA activation. CheA then interacts with the RR CheY, which can bind the flagellar switch, causing a change in flagellar rotation. The receptor modifying enzymes CheB, CheR and CheD post-translationally modify conserved residue on MH1 and MH2. CheW and CheV are involved in coupling the HK to the chemoreceptor array. CheC and FliY can aid in the rapid dephosphorylation of the RR.
Chapter 2
Materials and Methods

Media and Solutions

**Blocking buffer**: 20 mM Tris, pH 7.5, 250 mM NaCl, 5% (w/v) milk powder, 0.05% Tween 20

**Buffer B**: 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8

**Buffer C**: 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 6.3

**Buffer E**: 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 4.5

**CAMM (Capillary assay minimal media)**: 50 mM K₂HPO₄/KH₂PO₄, pH 7.0, 1.2 mM MgCl₂, 0.14 mM CaCl₂, 1 mM (NH₄)₂SO₄, 0.01 mM MnCl₂, 20 mM sorbitol, 50 µg/ml histidine, methionine, and tryptophan

**CAMM+ (Capillary assay minimal media + TBr)**: 50 mM K₂HPO₄/KH₂PO₄, pH 7.0, 1.2 mM MgCl₂, 0.14 mM CaCl₂, 1 mM (NH₄)₂SO₄, 0.01 mM MnCl₂, 0.2% (v/v) TBr, 20 mM sorbitol, 50 µg/ml histidine, methionine, and tryptophan

**Chemotaxis Buffer**: 10 mM K₂HPO₄/KH₂PO₄, pH 7.0, 0.14 mM CaCl₂, 0.3 mM (NH₄)₂SO₄, 0.1 mM EDTA, 5 mM sodium lactate, 0.05% (v/v) glycerol

**Elution buffer**: 50mM NaH₂PO₄, 300mM NaCl, 250mM imidazole, pH 8.0

**GEB (Glutathione elution buffer)**: 50 mM Tris, pH 8.0, 10 mM glutathione

**LB**: 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 0.5% (w/v) NaCl

**Lysis buffer**: 50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH 8.0

**PBB (Prescission Protease Buffer)**: 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris, pH 8.0
**Protoplast buffer:** 25 mM K$_2$HPO$_4$/ KH$_2$PO$_4$, pH 7.0 20% (w/v) sucrose, 10mM MgCl$_2$, 1 mM EDTA, 30 mM sodium lactate

**SDS solubilizer buffer (1X):** 50 mM Tris, pH6.8, 10% (v/v) glycerol, 5% β-mercaptoethanol, 2% SDS

**SPR charging buffer:** 0.5 M NiCl$_2$ in SPR running buffer

**SPR regeneration buffer:** (I) first round regeneration: 0.35 M EDTA in SPR running buffer
(II) second round regeneration: 0.1% SDS

**SPR running buffer:** TKMD buffer without glycerol

**TBAB:** 1% (w/v) Tryptone, 0.3% (w/v) Beef extract, 0.5% (w/v) NaCl, 1.5% Agar

**TBr:** 1% (w/v) Tryptone, 0.5% (w/v) NaCl

**TKMD buffer:** 50 mM Tris, pH 8.0, 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM dithiothreitol (DTT), 10% (v/v) glycerol

**Transfer buffer:** 50mM Tris, 40mM Glycine, 0.15mM SDS, 20% (v/v) Methanol

**Wash buffer:** 50mM NaH$_2$PO$_4$, 300mM NaCl, 20mM imidazole, pH 8.0

**Strains and Plasmids**

All of the strains and plasmids used in this study are noted in Table 2.1. All of the *B. subtilis* strains are derived from the chemotactic strain (*che+*) OI1085. All cloning and plasmid propagation were performed in *E. coli* strains TG1. Recombinant protein overexpression was done in *E. coli* strains BL-21 DE3. Plasmids used for protein purification were expressed in either pUSH1 or pGEX-6P-2.

The 6xHis fusion pUSH plasmids that contained the cytoplasmic receptor fragments were made by amplifying the carboxy-terminal fragment from OI1085 genomic DNA, or
from pAIN750 derivatives containing the corresponding receptor, by introducing BamHI restriction sites. This resulting fragment was then ligated into the pUSH1 plasmid. The pEB112 derivatives for co-expression of the receptor fragment and cheD were made as described [56]. Briefly, plasmids containing the receptor and CheD in pEB112 were digested with NotI and BamHI, and the 1100 bp fragment that contained the receptor fragment gene was ligated into the bigger pEB112 derivative.

The GST-fusion pGEX-6P-2 plasmids were made by amplifying the desired receptor fragment from a pAIN750 plasmid containing the appropriate receptor and mutation by PCR and engineering a 5’ EcoRI and 3’ NotI site. This fragment was then ligated into pGEX-6P-2.

All mcpC mutants were created by using Quickchange (Stratagene) mutagenesis on pAIN750 variants that contained the full-length receptor gene. These plasmids were then transformed into the amyE locus of the desired B. subtilis background, and selected for spectinomycin resistance.

**Protein Purification**

All purified proteins used in this study originated from B. subtilis. The chemoreceptor carboxy-terminal fragments used in Chapter 3 were purified as 6xHis-fusions as previously described [56]. Both 6xHis-fusion expression plasmids (pUSH1) and the GST-fusion expression plasmids (pGEX-6P-2 variants) were expressed in the E. coli BL21 DE3 strain. A 5 ml Hi-Trap Chelating column or a 5 ml GSTrap columns (GE Healthcare) was used with an AKTA Prime FPLC system (GE Healthcare) for purification per manufacturer’s instructions.
The 6xHis-McpAc was purified under denaturing conditions. A 6-liter culture of LB plus 10 µg/ml chloramphenicol was inoculated 1:100 with an overnight culture of RP3098 harboring pAIN620 (for the unmodified McpA c-fragment) and grown at 37°C with agitation (250 rpm) to an A$_{600nm}$ = 0.8. IPTG was added to 1 mM, incubation was continued as before, and cells were harvested by centrifugation at 5000 x g for 5 min after 3 h. Cell pellets were frozen at -80 °C. Thawed cell pellets were resuspended in Buffer B and incubated at room temperature for 1 h with mixing. The supernatants were clarified by two serial centrifugations (7000 x g, 5 min; 40,000 x g, 40 min) and loaded onto the Ni-charged Hi-Trap column. The column was washed with 10 volumes Buffer B, followed by 10 washes with Buffer C. Elution was performed with 25 mL Buffer E. These samples were dialyzed against three changes of 25 mM NH$_4$HCO$_3$ at 4 °C, and aliquots were frozen at -80° C.

To purify the GST-fusion proteins, pGEX-6P-2 with the assorted chemotaxis proteins was cloned in the multiple cloning site. A saturated overnight culture was diluted 1:100 into three of 2 liters LB with 100 µg/ml ampicillin and grown at 37 °C (250 rpm) to A$_{600}$ of 0.6. IPTG was then added to 1 mM. GST-CheC, GST-6xHis-CheD, and GST-CheA cultures were incubated at 200 rpm overnight at room temperature, and GST-CheY culture was incubated at at 15°C (200 rpm) for 3 days. Cells were spinned down at 8,000 x g, 8 min and frozen for storage. Thawed cell pellets were resuspended in PBS (pH 7.3) buffer followed by sonication. The supernatants were clarified by two rounds of centrifugations (9,000 x g, 15 min; 65,000 x g, 40 min), and applied to 5-ml GSTrap columns pre-washed with 10 column volumes of PBS buffer. Protein bound columns were then washed with at least 15 column volumes of PBS buffer, and GST tagged proteins was eluted with using 10 ml of elution buffer (50 mM Tris, 5 mM glutathione, pH 8). To remove the GST tag, the
purified GST-CheC or GST-CheY was cleaved by PreScission protease, as specified by the supplier (Amersham Biosciences), and applied to another 5 ml GSTrap column. The flow-through was collected and concentrated to ~5 ml using a cellulose ultrafiltration membrane (Millipore) in an Amicon ultrafiltration cell. Then the purified proteins were dialyzed in TKMD buffer without glycerol and aliquots were stored at -80°C.

The cell lysate was then passed through a 5 ml GSTrap column (GE Healthcare) and washed with at least 5 bed volumes of TBS. The fusion proteins were eluted from the column with 20 ml GEB. This was dialyzed against six changes of 800 ml PPB. The GST tag was removed by digestion with 100 units of PreScission protease for 12 h at 4°C. This solution was again passed over the GSTrap column to remove the GST and protease. The pure Che protein flow-through was collected and dialyzed against four changes of 1 L TKMD and then stored at -80°C.

**Western Blot Sample Preparation**

Bacterial strains that were used for quantitative western blot analysis (Δ10 background strains in this study) were streaked out on TBAB plates and incubated overnight at 30°C. Colonies from this plate were used to start a 50 ml CAMM+ culture with an initial $A_{600nm} = 0.02$. Cells were then incubated at 37°C with aeration until reaching mid-exponential phase (approximately 10 hrs.). Cells were then diluted 1:10 (v/v) into 50 mls CAMM, then put back in the incubator until reaching mid-exponential phase (approximately 10 hrs.). Cells were then diluted with CAMM to $A_{600nm} = 0.01$ and were incubated until they reached mid-exponential phase (approximately 14hrs.). The culture was then diluted 1:10 (v/v) with CAMM into multiple flasks (to a total volume of 50 ml) and
were returned to the incubator until they reached $A_{\text{600nm}} = 0.6$. Cells were then pelleted. The pellet was then washed once with 5 ml of Protoplast buffer and diluted to $A_{\text{600nm}} = 1$ in 10 ml Protoplast buffer with 250 g/ml chloramphenicol and 4 mg/ml lysozyme and incubated at 37°C for 30 min. Protoplasts were collected by centrifugation and resuspended in 500 µl of SDS solubilizer buffer (1X). The number of cells loaded per pane of SDS-PAGE was 5.2E+07 cells per lane.

**Western Blot**

Samples were separated by SDS-PAGE and then transferred to PDVF membrane (Millipore) by semi-dry transfer. Membrane was blocked for 4 h with Blocking buffer; then the primary antibody was added for overnight incubation. Dilutions for primary antibodies were: 1:20,000 anti-McpAc, 1:400 anti-CheD. The membranes were then washed with water at least four times. Then they were incubated with a 1:20000 dilution of the secondary antibody (HRP-conjugated goat-anti-rabbit-IgG, Pierce) in Blocking buffer for 2 h. Membranes were again washed four times with water and then treated with ECL Plus signal solution as per the manufacturer’s specifications (GE Healthcare). Visualization was achieved via LabWork photostation. Bands were quantified with LabWork software or ImageQuant software.

**Receptor Deamidation Assay**

Deamidation reactions were performed as previously described [56]. Briefly, 1 µM of McpA c-fragment was incubated with equal concentration of CheD or mutated CheD at room temperature in reaction buffer (50 mM Tris, pH 7.5, 0.1 mM dithiothreitol, 1 mM MgCl₂,
0.5 mM EDTA). At the indicated times, samples (10 µl) were removed and mixed with 10 µl of 2× SDS loading buffer. Immunoblotting was performed as described above. To investigate CheC’s inhibition, 2 µM of CheC and 2 µM of CheD were used in the incubation of McpAe.

**Swarm Plate Assay**

The swarm plate assay allowed for quick characterization of an overall chemotactic phenotype comparing to a wild type strain and was performed as described [57, 56]. In this study, proline swarm plates were used to identify cheD or mcpC mutants’ phenotypes. Briefly, strains were grown on a TBAB plate at 30ºC for 16 h. Individual colonies were spotted onto low nutrient low agar swarm plates (0.2 mM proline, 0.1x Spizizen’s salts, 0.7mM sorbitol, 5 µg/ml required amino acids, 0.25% agar) and incubated at 37ºC for 10 h. As the bacteria metabolized the nutrient, they started swimming outwards and created an attractant gradient. This behavior led to the formation of a characteristic ring. The diameter of the ring was measured and compared with the wild type strain, which was present on each swarm plate as a positive control. All strains were observed in at least three duplicate plates.

**Tryptic Digestion and HPLC Peptide Separation**

50 µM of CheD was incubated with 1 mM of dansyl fluoride (DNSF) at room temperature for 30 min. Then the DNSF was removed by passing through the Sephadex™ G-25 column as per the manufacturer’s specifications (GE Healthcare). Purified CheD was then digested in approximately a 25:1 molar ratio of protein to trypsin for 24 hours at 37ºC. Samples were then injected into a Shimadzu VP Series HPLC, incorporating a Waters Symmetry 300 Reversed Phase C18 column, and running Shimadzu VP-EZStart software,
which was subsequently used for all HPLC analysis. A 0-55% gradient of water:acetonitrile, both with 0.1% trifluoroacetic acid, run over 45 min, at 1 ml/min and 35°C, was used to elute tryptic peptides. Peptides were visualized with a UV detector set at 220 nm. Peaks were collected and sent for MS/MS analysis, which was performed at the UIUC Center for Top-Down Proteomics under the direction of Andy Forbes.

**Surface Plasmon Resonance (SPR)**

SPR is a good tool to study protein-protein interaction. In addition to $K_D$, we can also measure the binding and dissociation kinetics by SPR. In this study SPR was performed using the Biacore 3000 system and NTA sensorchips (GE Health). The sensorchip was activated according to the manufacturer’s instructions, and the buffers used in the microfluidic system were as follows: running buffer (TKMD without glycerol), regeneration buffer (0.35 M EDTA in running buffer), and nickel solution (0.5 M NiCl$_2$ in running buffer). The flow rate 20 µl/min was used through all experiments.

The chip was pre-cleaned by injection of 20 µl regeneration buffer I and II, and then charged by 20 µl of nickel solution. 20µl of 0.1% BSA was then injected to remove background binding. Then 500 response units (RU) of GST-6xHis-CheD was immobilized on three different flow cells (2, 3, and 4), while the flow cell 1 was used as a mock immobilized, blank control. After immobilization, 80 µl of CheC at different concentration was injected, respectively, to study CheD-CheC binding kinetics.

To study CheD-CheC-CheY binding kinetics, different from the above, 20 µM of CheY in TKMD (without glycerol) was used as the running buffer and through all the experiment. During injection of CheC, the same concentration of CheY was also added.
Similarly, to study CheD-CheC-CheYp binding kinetics, 5 mM of acetyl phosphate and 20 µM of CheY in TKMD (without glycerol) was used as the running buffer. The same concentrations of Ace-P and CheY were also added while injecting CheC.

After each round of binding and dissociation, the NTA chip was regenerated and charged again for the next round of study. The SPR curves were then processed and analyzed using BIAevaluation software as per the manufacturer’s specifications (GE Healthcare).

**SPR Data Analysis**

Sensorogram base lines were normalized to zero response units (RU) prior to analysis. Samples were run over mock-derivatized flow cell 1 to determine background RU values due to bulk refractive index differences between solutions. SPR data were globally fit to a 1:1 Langmuir binding model:

$$ A + B \rightleftharpoons AB $$

In this equation, $[A]$ is the concentration of analyte CheC injected, and $[B]$ is the immobilized ligand GST-6xHis-CheD.

Because the biosensor data did not fit to a simple 1:1 model (the derivatives of the binding response and the dissociation were nonlinear), the binding data were evaluated by linear transformation analysis [23, 97]. For a simple bimolecular interaction (Equation 1), the dissociation and association process are described, respectively, as:

$$ RU = RU_0 e^{(-k_{on} t)} $$

and

$$ dRU/dt = k_{on}[A]RU_{max} - (k_{on}[A] + k_{off})RU $$

In these equations, $k_{on}$ and $k_{off}$ are the association and dissociation constants, respectively; $RU$ is the relative response at time $t$, and is proportional to the amount of the
AB complex formed (CheC-CheD complex in this case); \(RU_{\text{max}}\) denotes the amount of ligand CheD immobilized on chip; and \(RU_0\) is the response at the beginning of the dissociation.

For a single exponential process (Equation 2), a plot of \(\ln(RU)\) vs time will yield a straight line with a slope of \(-k_{\text{off}}\). For Equation 3, set \(k_s = -(k_{\text{on}}[A] + k_{\text{off}})\). By plotting \(dRU/dt\) vs \(RU\), \(k_s\) (the slope) was determined. With the previously calculated \(k_{\text{off}}\) and a known \([A]\) value, \(k_{\text{on}}\) was then calculated. The equilibrium dissociation constant (\(K_D\)) was calculated from the equation \(K_D = k_{\text{off}}/k_{\text{on}}\).

**Structural Modeling**

Structural modeling was performed on CheD, CheC, and the HAMP domain of McpC using the SWISS-MODEL modeling server [12, 48, 85]. Related sequences were submitted, and the resulting PDB files were consequently studied using PyMol or VMD softwares [44]. WinCoot software was used to superimpose related protein structures from different species in order to see any potential difference.
### Tables

#### Table 2.1 Strains and plasmids used in this study

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Chapter 3

CheD is a Cysteine Hydrolase

Introduction

CheD, an 18 KDa protein, is one of the unique chemotaxis proteins that do not exist in *E. coli*. The cheD knockout mutant shows a chemotactic defect in every assay in which they were tested, which indicates that CheD must play important roles in chemotaxis [52, 56, 91].

As described in Chapter 1, CheR can methylate chemoreceptors on the conserved Glu residues in *B. subtilis* [38, 129]. In some instances, the modified site is encoded as Gln rather than Glu. In order to be methylated, the amine group of Gln needs to be removed before it is methylated. In *E. coli*, this deamidation is carried out by CheB through a serine hydrolase mechanism, then subject to cycles of reversible methylation [47]. In *B. subtilis*, K. Kristich has reported that CheD can deamidate chemoreceptors, McpA, McpB, and McpC [56]. Specifically, in K. Kristich’s work he incubated McpAc-term with CheD in vitro. After immunoblot assays, band shifts were found. By peptide sequencing it turned out that the band shifting were due to deamidation of McpAc-term. Meanwhile, V. Cannistraro also found that CheD can deamidate the soluble receptor YfmS (V. Cannistraro Ph.D. thesis).

But the enzymatic mechanism of this deamidation was still unknown. The focus of this chapter is on the identification of CheD enzymatic mechanism as a deamidase.

Putative CheD Active Site Mutations Show a Chemotactic Defect

In order to verify CheD active sites, the sequence of CheD in *B. subtilis* was aligned with CheD from 17 other related species. Some 100% conserved residues were
point-mutated into Ala, including Thr27, Ser32, Cys33, Asp40, and His50 (Figure 3.1). Those pEB112 derived plasmids containing mutated cheD were transformed into the cheD null strain. The mutants were then analyzed on swarm plates, and examined whether these mutants could form chemotactic rings towards proline.

From the swarm plate assay, the cheD complemented strain was capable of supporting chemotactic ring formation, whereas the cheD-null strain could not. Interestingly, Ser32, Cys33, His50, Thr27, and Asp40 mutants did not form a clear chemotactic ring, and only diffused within a small region as cheD-null did (Figure 3.2 A and B). Therefore, these sites are essential for the normal CheD function, and some of them can be within CheD active sites. Other mutated strains like cheD(S56C) or cheD(S134C) could still swim as the positive control (data not shown).

To make sure that these mutated CheD proteins could be expressed in vivo, strains were grown under minimum media as described in Chapter 2. The cell lysates were then immunoblotted against the anti-CheD antibody. Results showed that these mutated CheD’s could be expressed in vivo (Figure 3.3).

**Putative CheD Active Site Mutations Show a Deamidase Defect**

The swarm plate assay showed overall phenotypes of cheD mutants. In order to verify their effects on CheD deamidation, the above mutated cheD’s were subcloned into pGEX-6p-2 vector for protein expression. After protein purifications, receptor deamidation assays were performed by incubating McpAc-term with equal concentrations of CheD or mutated CheD, respectively.
The immunoblot results showed that there was no deamidation for mutated CheD’s Cys33, Thr27, His 50, Ser28, and Asp40 (Figure 3.4), which indicated that these sites are possibly in the active sites, or related to receptor binding, or maintain the normal CheD structure.

**CheD is Not a Serine Hydrolase**

Both Ser32 and Cys33 are 100% conserved residues. CheD can possibly be a serine or cysteine hydrolase. To test if it is a serine hydrolase, I used a serine protease inhibitor, dansyl fluoride (DNSF). DNSF is a small ser-type suicide inhibitor, which covalently binds to the serine active site of enzymes. After incubation of GST-CheD with or without DNSF, DNSF was removed by passing through Sephadex™ G-25 protein desalting column to prevent impairing subsequent digestion by trypsin. Then proteins were digested by trypsin in solution followed by rp-HPLC purification. By comparing the spectrum difference, I picked up vial #72 from the DNSF+ elution samples, which did not appear in DNSF- elution spectrum. DNSF is a fluorescent inhibitor that can be monitored at A\textsubscript{360}. A peak was detected at Vial#72 position under A\textsubscript{360} monitoring (Figure 3.5 A). Vial#72 was then sent for tandem MS Spec peptide sequencing.

From the tryptic digestion spectrum of GST-CheD, Ser32 should be in the peptide of TSGLGSCVGLVLYDK (position 254-268). But the MS Spec results showed that the selected peptide is EQLSLFNPIIISEDTGSSGR (position 350-370) (Figure 3.5 B). This result indicated that CheD is not likely a serine protease, because the DNS derivatizing site is not in the proposed peptide. If Ser32 were the active site, it should have much more readily
formed a DNS adduct than any other Ser residues in EQLSLFNPIIISEDGTGSSGR, and gave us a stronger signal during HPLC elution.

**Structural Modeling Analysis of CheD**

The Crane lab successfully obtained CheC-CheD co-crystal structure from *T. maritime* (2.5Å, R factor 21%) [24], which greatly helps us to better understand CheD functions (Figure 3.6). Structure homology modeling was performed to get CheD structure in *B. subtilis* (Figure 3.7). The structure was obtained from SWISS-MODEL database, and was based on the template 2f9z, the same structure that the Crane lab got (Sequence identity: 38%). From the structure, *B. subtilis* CheD has a three-fold $\alpha/\beta/\beta$ sandwich-like structure.

To see how much CheD structures from *B. subtilis* and *T. maritime* overlap with each other, two structures were superimposed with using WinCoot software and viewed in PyMol software (Figure 3.8). From the superimposed structure, the two CheD structures overlay very well (Core rmsd achieved: 0.310487Å). The only notable difference may be the TNDL loop (107-110 in *B. subtilis*), which is extended further in space comparing with CheD in *T. maritime*. Overall, CheD structure from the Crane lab is a very good starting model to study CheD structure in *B. subtilis*.

When enlarging the CheC-CheD interaction area, we can see that Cys33 is closer to His50 and Thr27 compared with Ser32, and is more likely to be in the active site. And Asp40 is located at the end of $\beta$-sheet far away from the active site, and is unlikely in the active site (Figure 3.9). Cys33-His50-Thr27 is likely to be the catalytic triad of CheD through a cysteine hydrolase mechanism.
Cysteine Protease Inhibitors Stop CheD Deamidation

To further verify that CheD is through a cysteine hydrolase mechanism, a CheD double mutation (D86152) was made by mutating Cys86 and Cys152 into Ala. In this mutant, Cys33 is the only cysteine in CheD.

The purified D86152 were then used in the receptor deamidation assay. In this case, McpAc-term was incubated with D86152 and with or without cysteine protease inhibitors followed by anti-McpA immunoblot. Two cysteine proteases that were used are n-ethylmaleimide (NEM) and iodoacetamide (IA). The results showed that D86152 could still deamidate receptor, albeit its deamidase activity was lower than the wild-type CheD from a separate assay (data not shown). After two above cys inhibitors were added, respectively, the deamidations were stopped (Figure 3.10). It indicated that CheD is most likely to be a cysteine hydrolase.

Ser32 is Responsible for Receptor Binding

Previous data have shown that both Cys33 and Ser32 mutant had no deamidase activity. Although Cys33 is most likely to be in the active site based on the last section’s result, unanswered questions still remain, “what is Ser32 function?” and “why did Ser32A mutant show no deamidation”?

To address these questions, the GST pulldown assay followed by immunoblot was performed. In this assay, McpAc-term was incubated with wild-type GST-CheD or GST-CheD mutants (S32A or C33A) with presence of glutathione beads. The anti-McpA antibody was used in the following western blot. The immunoblot result showed that GST-CheD could pull down McpAc-term (where the signal had to be amplified by western
blot); GST-CheD (S32A) had no McpAc-term binding signal; and C33A mutations would not influence McpAc-term binding (Figure 3.11). These interesting results indicated that Ser32 is most likely plays an important role in receptor binding, and Cys33 is the in the active site. Since S32A mutation destroyed CheD-McpA binding, it is therefore reasonable that the enzyme became inactive.

Discussion

It has been reported that CheD deamidates 'Gln-593' and 'Gln-594' of the chemoreceptor McpA, and deamidates other chemoreceptors including McpB and McpC [56]. Additionally, CheD is required for the generation of wild-type prestimulus CheA autophosphorylation levels shown by the cheD null phenotypes [56, 90]. Elucidating of CheD deamidase mechanism can potentially help us understand CheD’s role better, especially how CheD makes chemoreceptors more active.

Originally, we thought CheD’s deamidase activity is through a serine hydrolase mechanism, which usually has a Ser-Asp-His catalytic triad. It was based on two facts: (a) CheB, which deamidates receptors in E. coli, is through a serine hydrolase mechanism [47]; (b) Ser32 is a 100% conserved residue, which indicates that it may be essential to CheD’s function.

Indeed, cheD(Ser32A) mutation did not show deamidation bands in the receptor deamidation assay (Figure 3.4 A), and cheD(Ser32A) did not swim well on proline swarm plates (Figure 3.3 A). But other mutations including Cys33A, His50A, Thr27A, and Asp40A caused similar phenotypes (Figure 3.3 and 3.4). It is possible that these sites are in the
deamidase active sites, or related to CheD-receptor interaction, or important to maintain the normal CheD structure.

To further investigate CheD active sites, a series of experiments were performed. I firstly tested effect on CheD of serine protease inhibitors, like DNSF and AEBSF. Both inhibitors are small suicide inhibitors that derivatize the serine residue of serine proteases. With addition either of these two inhibitors, CheD deamidase activity was stopped (data not shown). In addition, AEBSF seems to unspecifically derivatize McpAc-term as it caused faster migration on SDS-PAGE gel. The deamidated receptors should migrate slower in gel. Therefore, AEBSF is not a good inhibitor in this case.

To get direct evidence of DNSF derivatization, GST-CheD was incubated with DNSF followed by removal of DNSF, tryptic digestion, reverse phase-HPLC separation, and tandem MS-Spec peptide sequencing. If Ser32 were in the active site, DNS should derivatize it and be detected by tandem MS-Spec peptide sequencing. But the result showed that no Ser32-containing peptide was detected. Instead, another peptide (position 350-370) was derivatized (Figure 3.3). Therefore, CheD is unlikely to be a serine hydrolase. Otherwise, the peptide containing Ser32 should have been the most likely to have been derivatized.

With emergence of the CheC-CheD co-crystal structure from the Crane lab, we had a better tool to understand CheD’s mechanism and functions [24]. CheD of B. subtilis overlays that of T. maritime very well, except that the TNDL loop (position: 107-110) is more extended in B. subtilis (Figure 3.8). Therefore, the CheD structure from T. maritime is a very sound template to study CheD from B. subtilis.

From the CheD structure, we can see that Ser32 is farther away from His50 and Thr27 center compared to Cys33 (Figure 3.9). B. Crane proposed that CheD resembles a class of
bacterial toxins represented by the cytotoxic necrotizing factor 1 (CNF1) [22, 24]. CNF1 causes alteration of the host cell actin cytoskeleton and promotes bacterial invasion of blood-brain barrier endothelial cells. CNF1 also has a three-layered $\alpha/\beta/\beta$ sandwich-like structure. The position of the catalytic Cys residue is located at the base of a deep pocket restricting access to potential substrates, which can bring the high specificity of binding to target proteins. The putative active site of CheD in *B. subtilis* is also surrounded by $\alpha$ helices, $\beta$ sheets, and an extended loop. Additionally, B. Crane thought that CheD also resembles a class of proteins of unknown function represented by YfiH from *B. subtilis* (Figure 3.12). CheD, CNF1, and YfiH have common topologies with an $\alpha/\beta/\beta$ sandwich core surrounded by different peripheral loops. They all have Cys-His in the catalytic triad, while the third active residue varies. The coordinates of the CheD putative active site is closer to CNF1. In fact, the coordinates of the CheD active site in *B. subtilis* is also similar to that in *T. maritima* (Figure 3.13).

To get the direct evidence that CheD is a Cys-type hydrolase, I did two more experiments. First, I made CheD (C86A C152A) double mutant and purified the protein. There are only three Cys residues in wild-type CheD. By mutating other two Cys (positioned at 86 and 152) into Ala, the potential alternative derivatizing sites was removed. Theoretically, only Cys33 can derivatize with a cysteine protease inhibitor if CheD is a cysteine hydrolase. Then the mutated protein was used in receptor deamidation assay with or without cysteine protease inhibitors like NEM or IA. Indeed, both NEM and IA stopped its deamidation. Therefore, CheD is most like a cysteine hydrolase.

Furthermore, it turns out that S32 plays an important role in receptor recognition and binding (Figure 3.11). Once mutated into Ala, CheD will be no longer able to interact with
receptors. That is why CheD (S32A) showed a defect in deamidation. In addition, CheD (D40A) also showed deamidation defect. The reason is still unknown. Asp40 is located at the end of a β sheet, far away from the active center. The defect on deamidation could possibly due to the need for Asp40 to maintain the normal CheD structure, so that cheD(Asp40A) was unable to function as a deamidase.

Like most other cysteine proteases, CheD involves a nucleophilic cysteine thiol in the catalytic triad. The first step is deprotonation of Cys27’s thiol group by adjacent His50 with a basic side chain. Next, the deprotonated cysteine’s anionic sulfur will take nucleophilic attack on the substrate carbonyl carbon (receptor). Thr27 would polarize and stabilize the whole catalytic triad intermediate structure. After a fragment of substrate with an amino terminus is released, the thioester intermediate is formed linking the Cys27 thiol to the new carboxy-terminus of the substrate. Next, the thioester bond is hydrolyzed to release the remaining substrate fragment. Meanwhile, the free Cys hydrolase, CheD, is regenerated for the next round of digestion (Figure 3.14).

Overall, CheD deamidase activity is through a cysteine hydrolase mechanism, instead of a serine protease mechanism. The catalytic triad consists of Cys33-His50-Thr27. And Ser32 is essential for receptor recognition and binding. We may further study the relationship of deamidation and receptor activation, and how exactly CheD helps tune the downstream kinase activation.
Figures and Tables

Figure 3.1 Sequence alignments of CheD from *B. subtilis* and 17 other species. The alignment was performed with Clustal X. CheD putative active sites (T27-C33-H50) and S32 were boxed in black. Species abbreviations: Aful, *Archeoglobus fulgidus*; Atum, *Agrobacterium tumefaciens*; Bbur, *Borrelia burgdorferi*; Bhol, *Bacillus halodurans*; Bsub, *Bacillus subtilis*; Cace, *Clostridium acetobutylicum*; Ccre, *Caulobacter crescentus*; Cthe, *Clostridium thermocellum*; Dhaf, *Desulfitobacterium hafniense*; Neur, *Nitrosomonas europaea*; Oihe, *Oceanobacillus iheyensis*; Paer, *Pseudomonas aeruginosa*; Phor, *Pyrococcus horikoshii*; Rsph, *Rhodobacter sphaeroides*; Smel, *Sinorhizobium meliloti*; Tmar, *Thermotoga maritime*; Tten, *Thermoanaerobacter tengcongensis*; Xcam, *Xanthomonas campestris*.
Figure 3.2 Swarm plate screening of putative CheD active site mutants. Strains contain pEB112 derived plasmids with mutated cheD in cheD-null background. Swarm plates were composed of minimal medium supplemented with proline as the attractant. All strains were assayed a minimum of four independent times. Results from a representative experiment are shown. The image was processed using LabWork to maximize contrast. (A) cheD S32A, D40A, H50A, and C33A mutants. (B) cheD T27A mutant. Strain analyzed are: (1) cheD-null, negative control; (2) cheD complemented strain, positive control; (3) cheD (S32A) in cheD-null; (4) cheD (D40A) in cheD-null; (5) cheD (H50A) in cheD-null; (6) cheD (C33A) in cheD-null; (7) cheD (T27A) in cheD-null.
Figure 3.3 In vivo protein expression assays of cheD putative active site mutants. Strains contain pEB112 derived plasmids with mutated cheD were grown as described in Chapter 2, then immunoblotted with anti-CheD.
Figure 3.4 Receptor deamidation assays with CheD and its putative active site mutants. All reactions contained 1 µM of McpAc-term and equal concentrations of CheD or mutated CheD. Samples were incubated at RT for 1 hr, and reactions were terminated by addition of 2x SDS loading buffer.
Figure 3.5 HPLC and tandem MS spec analysis of tryptic digested GST-CheD with or without DNSF. (A) Separation of tryptic digested CheD with or without DNSF by rp-HPLC. (B) Tandem MS spec results of the selected peptide peak from (A).
Figure 3.6 CheD-CheC cocrystal structure (*T. maritima*). The 3-D structure was regenerated with VMD based on the Crane lab work [24].
Figure 3.7 Structure modeling of *B. subtilis* CheD. The 3-D structure of *B. subtilis* CheD was obtained from SWISS-MODEL database,
Figure 3.8 Superimposing of CheD structure from *B. subtilis* and *T. maritima*. The 3-D structure of *B. subtilis* CheD was obtained from SWISS-MODEL database, and then superimposed by WinCoot software. The red color represents CheD from *B. subtilis*, and the cyan color represents CheD from *Thermotoga maritima*. 
Figure 3.9 Structure of CheD putative active sites. Only CheC-CheD interaction area was shown. From the structure, C33 is closer to H50 and T27 comparing with S32, and is more likely to be in the active site.
Figure 3.10 Immunoblot of receptor deamidation assay with cysteine protease inhibitors. Two cysteine protease inhibitors that were used are N-ethylmaleimide (NEM) and iodoacetamide (IA). D86152 represents CheD (C86A C152A) mutant removing all other Cys sites except C33 in CheD. Anti-McpA antibody was used to detect modifications.
Figure 3.11 Binding of McpAc-term and GST-CheD and its mutants by pulldown assay followed by immunoblot. Equal concentrations of GST-CheD or its mutants were incubated with McpAc-term using glutathione beads. After pulldown assay, anti-McpA antibody was used in the following immunoblot. Lane 1: McpAc-term + GST-CheD; lane 2: McpAc-term + GST-CheD (S32A); lane 3: McpAc-term + GST-CheD (C33A); lane 4: GST-CheD only; lane 5: McpAc-term alone without incubating with glutathione beads (M.W. marker).
Figure 3.12 The structures of CheD, CNF1 catalytic domain, and 1XAF. Folds and active site structures for (A) CheD, (B) CNF1 catalytic domain, and (C) *Shigella flexneri* YfiH. The three classes of proteins have common topologies (analogous regions in orange) but different peripheral loops and inserted regions (gray). CheD and CNF1 have similar active sites; YfiH incorporates an additional His ligand that allows zinc coordination. This figure is from B. Crane’s published paper [24].
Figure 3.13 3-D coordinates of CheD active site in B. subtilis. The catalytic triad (Cys33-His50-Thr27) was viewed and drawn by PyMol software.
Figure 3.14 The cysteine protease mechanism. The picture was from http://en.wikipedia.org/wiki/File:Cysteinprotease_Reaktionsmechanismus.svg.
Chapter 4

CheC Inhibits CheD Deamidase Activity

Introduction

When the present study was initiated, we knew that CheD interacts with CheC, but the physiological significance of this interaction was unknown [91]. Since the cheD null mutant showed a chemotaxis defect, CheD was thought to be important for chemotactic signal transduction in B. subtilis.

The 22kDa protein CheC was initially identified as a possible chemotaxis protein by the presence of cheC gene in the B. subtilis major Che/Fla operon just like cheD [90]. The cheC mutant had a prestimulus rotational bias and excitation response similar to the wild-type, but was unable to adapt to attractant, and also the receptors in this mutant were hyper-methylated [90]. This indicated that CheC is important during adaptation. In addition, CheD was demonstrated to interact with CheC by the yeast two-hybrid experiment and the GST pulldown assay [91]. Interestingly, CheC regulates methylation of the chemoreceptors in B. subtilis by an unknown mechanism [90, 91]. Not related to this study, but interesting to note that CheC may have a weak interaction with CheA and MepB [52].

Based on above facts, I started to focus on CheC among all other chemotaxis proteins that could potentially regulate CheD. The focus of this chapter is on the importance of CheD/CheC interaction to CheD function and chemotaxis. The CheD site interacting with CheC was also investigated.
CheC Inhibits CheD Deamidase Activity

To test the effect of CheC on CheD deamidase activity, the chemoreceptor deamidation assay was employed (Figure 4.1). In this experiment, receptor McpAc-term was incubated with CheD and with or without CheC. The same amount of reaction mixture was taken out and terminated by addition of 2x SDS loading buffer. As shown in Chapter 3, the wild-type CheD deamidates McpAc-term resulting in migrated bands on the western blot membrane. Another batch of CheD was pre-incubated with CheC, and then McpAc-term was added. CheC turned off CheD deamidase activity quickly. By 1 min, no McpAc-term deamidation bands were observed (Figure 4.1). This indicated that CheC can work as an inhibitor of the deamidase CheD.

Phe102 on CheD is Required for CheC Interaction

CheC is involved in adaptation and interacts with CheD. It seemed reasonable that the interaction might play a role. I wanted to investigate the site of interaction on CheD as part of the process of exploring for such a role.

As shown in Figure 4.2, bacteria having CheD could be rooted into two classes based on CheD sequences. One class has both CheD and CheC, and the other has only CheD but not CheC. It is reasonable to hypothesize that CheD/CheC interaction sites are conserved in CheD+CheC+ strains, but not conserved in CheD+CheC- strains. Without the CheC binding requirement, interaction sites might have been lost during evolution. This was a clue to search for CheD/CheC binding sites.
Based on above hypothesis, the sequence of CheD in *B. subtilis* was aligned with CheD from 17 other related species (Figure 4.3). Those residues that are only conserved in CheD+CheC+ strains but not in CheD+CheC- strains were picked up (Figure 4.3, in black boxes), followed by the site-directed mutagenesis. Mutations were based on the features of original residues. The mutated cheD was transformed into the pGEX-6p-2 vector, respectively, and then proteins were purified.

The mutated CheD proteins were used in the chemoreceptor modification assay. In this assay, McpAc-term was incubated with mutated CheD with or without CheC presence (all at 2 µM) (Figure 4.4). The wild-type CheD still deamidated McpAc-term and could be inhibited by CheC. Without addition of CheC, both CheD(F102A) and CheD(F102E) caused McpAc-term deamidation. With addition of CheC to CheD(F102A), there was some receptor deamidation mediated by CheD, but not when CheC was added to CheD(F102E). This indicated that Phe102 on CheD is required for CheC interaction.

**The CheD/CheC Complex is Important for Chemotaxis**

To observe the CheD/CheC interaction’s effect *in vivo*, it was necessary to disrupt the interaction without affecting the individual proteins’ functions. CheD (F102E) still deamidated McpAc-term, indicating that mutation on Phe102 has destroyed CheD deamidase function. To observe its *in vivo* effect, the proline swarm plate assay was performed. Expression of the *cheD(F102E)* gene in the ∆cheD strain caused a swarm with a similar size as the ∆cheD itself, which was much smaller than the cheD complemented strain (Figure 4.5). This swarm plate assay showed that the association of CheD and CheC is critical for the normal chemotaxis in *B. subtilis*. 
Discussion

Besides its deamidase function, CheD also interacts with CheC. Impairing of CheD/CheC interaction showed defective chemotaxis (Figure 4.5), indicating that CheD/CheC interaction is critical for a robust chemotaxis.

Initially, this study was to look for protein(s) that could bind or regulate CheD. Besides CheC, other proteins like CheA, CheA-P, CheY, and CheY-P were also tested for their potential bindings to CheD by the chemoreceptor deamidation assay (data not shown). Interestingly, CheC, and only CheC, showed the inhibition of CheD deamidase activity. With equal concentrations of CheD and CheC, CheD deamidase activity was completely inhibited (Figure 4.1). Phe 102 on CheD was subsequently identified as a site to interact with CheC. More recently, CheD has also been shown to increase the CheY-P phosphatase activity of CheC [111]. Asp 149 on CheC is a site to interact with CheD. Ala or Lys mutations on this site disrupted CheD binding, but did not influence its CheY-P phosphatase activity [24, 87].

The CheC-CheD co-crystal structure from the Crane lab greatly improved our understanding of the CheD/CheC interaction [24]. Based on their structure, Phe 102 lies on the outside loop of CheD interacting with the α2′ helix of CheC (Figure 4.6). This is consistent with Phe 102 being on essential site to interact with CheC. The deamidase catalytic triad of CheD, C33-H50-T27, is right behind Phe 102 (Figure 4.7). Once CheC is recruited by Phe 102, it will block CheD active sites from chemoreceptors’ approaching so that no receptor deamidation can occur. By mutating the hydrophobic and bulky Phe 102 into a charged glutamate residue, CheD/CheC interaction was disrupted, but CheD deamidase
function was retained. Therefore, with or without CheC, it still deamidated receptors (as shown in Figure 4.4 B). In addition, since the same surface side on CheD is required for binding to CheC or receptor access, B. Crane also proposed that CheC mimics the receptor substrate in binding to CheD [24]. More details of CheD/receptor binding will be discussed later in Chapter 6.

Overall, the work presented in this chapter studied the CheD/CheC interaction, and represents the identification of a molecular role of the protein CheC as an inhibitor of the deamidase CheD in the chemotaxis system. Further, the CheD/CheC interaction is necessary for the robust chemotaxis in vivo as demonstrated by the \textit{cheD (F102E)} mutant, in which CheD(F102E) lacks the ability to bind CheC.

These results are exciting. CheD can deamidate receptors and enhance CheY-P phosphatase activity of CheC; and CheC, on the other hand, can inhibit CheD deamidase activity and possesses CheY-P phosphatase activity. Putting all above into the whole \textit{B. subtilis} chemotactic signal transduction background (Figure 1.9), there seems to be a feedback mechanism from CheY(p) to receptors mediated by CheD/CheC coupling proteins. This will be further discussed in the next chapter.
Figures and Tables

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Figure 4.1 CheC inhibits CheD deamidase activity shown by the immunoblot of receptor deamidation assay. All reactions contained 1 µM of McpAc-term and equal concentrations of CheD or CheC. Time-course reactions were terminated by addition of 2x SDS loading buffer.
Figure 4.2 Unrooted tree of species with either CheD+CheC+ or CheD+CheC-. Alignment was made based on CheD sequences using ClustalW [25].
Figure 4.3 Sequence alignments of CheD from B. subtilis and 17 other species either with both CheD and CheC or with CheD alone. Species with CheD alone (CheC−) are in the blue box. Residues only conserved in CheD+CheC+ strains but not in CheD+CheC− strains are in black boxes. F102 is the site on CheD required for interaction with CheC. The alignment was performed with Clustal X. Species abbreviations: Aful, Archaeoglobus fulgidus; Atum, Agrobacterium tumefaciens; Bbur, Borrelia burgdorferi; Bhol, Bacillus halodurans; Bsub, Bacillus subtilis; Cace, Clostridium acetobutylicum; Ccre, Caulobacter crescentus; Cthe, Clostridium thermocellum; Dhaf, Desulfotomaculum hafniense; Neur, Nitrosomonas europaea; Oihe, Oceanobacillus iheyensis; Paer, Pseudomonas aeruginosa; Phor, Pyrococcus horikoshii; Rsph, Rhodobacter sphaeroides; Smel, Sinorhizobium meliloti; Tmar, Thermotoga maritima; Tten, Thermoanaerobacter tengcongensis; Xcam, Xanthomonas campestris.
Figure 4.4 Phe102 on CheD is required for interaction with CheC shown by the chemoreceptor deamidation assay. (A) Chemoreceptor deamidation assay with mutated CheD and with or without CheC presence. Without addition of CheC, both F102A and F102E showed deamidation migration. With addition of CheC, there was small increasing of deamidation for F102A. When mutating the hydrophobic F102 into a charged glutamate residue, CheC no longer inhibited CheD. (B) A separate chemoreceptor deamidation assay showing that F102E mutation released CheC inhibition to CheD deamidase activity.
Figure 4.5 The CheD-CheC interaction is critical for the robust chemotaxis in *B. subtilis*. This swarm plate assay demonstrates that the *cheD(102E)* mutant gene could not complement a *cheD*-null strain. Thus, the association of CheC and CheD is necessary for the robust chemotaxis.
Figure 4.6 CheD/CheC interaction in *B. subtilis*. The 3-D structure was based on the CheC-CheD co-crystal structure from *Thermotoga maritima* [24]. F102 lies on the face of CheD that binds to the α2’ helix of CheC.
Figure 4.7 Coordinates of CheD active sites and CheC interacting residue Phe 102. The 3-D structure was based on the CheC-CheD co-crystal structure from *Thermotoga maritima* [24]. Phe 102 was in red color; and CheD deamidase catalytic triad C33-H50-T27 is labeled in magenta.
Chapter 5
The CheC-CheD-CheY-p Adaptation System

Introduction

As mentioned in previous chapters, CheC and CheD in B. subtilis are unique proteins that are not present in E. coli [89, 90]. CheC is a CheY-p phosphatase [110, 111], and inhibits CheD’s deamidase activity (Chapter 4). The cheC mutant had a prestimulus rotational bias and excitation response similar to the wild-type, but was unable to adapt to attractant, and also the receptors in this mutant were hyper-methylated [90]. This indicated that CheC is important during adaptation. In addition, in the cheC (N120A, N121A) mutant CheC could bind CheYp but without phosphatase activity, and the strain was able to perform chemotaxis at ~50% wild-type levels. It seems that it is more important that CheC bind to CheY-p or CheD than dephosphorylate CheY-p [78, 87].

CheD, on the other hand, can enhance CheC’s phosphatase activity [111], and deamidate receptors by converting conserved glutamine residues to glutamates. CheD also interacts with receptors in the AS-1 region of receptor HAMP domains (Chapter 6), and makes receptors more active [56, 58]. cheD mutants have largely inactive receptors as they are very tumbling [52].

Based on above results, if we put receptor, CheD, CheC, and CheY-p into one frame, it is reasonable to hypothesize that there may be a signal feedback mechanism from CheY-p to receptors through CheC-CheD coupling proteins. It could be a pathway for the cell to achieve adaptation. In particular, we hypothesize that once CheY is phosphorylated, the CheC-CheY-p complex will attract more CheD from receptors to make receptors less able to
activate CheA kinase [87]. Previously it has been reported that from the pulldown assay there was an increase of 50-70% CheC binding to CheD with CheY-p compared with CheY alone [78]. But this assay did not show the direct evidence of affinity change between CheC and CheD and was very qualitative. In order to quantitate the kinetics of affinity change between CheD and CheC in different environments, we chose the Surface Plasmon Resonance (SPR).

Surface Plasmon Resonance is a very powerful technique to study interactions among different molecules, e.g. protein-protein interaction. It provides a non-invasive, label-free means of recording interactions between an injected analyte and an immobilized molecule in real time. This technique has been widely used for many different purposes, including affinity analysis, kinetic analysis, concentration assays, binding stoichiometry, thermodynamic analysis, drug screening, and more [97]. The theory of this technique is that if the analyte binds the ligand, the addition of mass causes a proportional increase in refractive index, which results into a shift in the resonance angle (Figure 5.1). The change of the SPR angle can be monitored in real-time by detecting changes in the intensity of the reflected light, producing a sensorgram (Figure 5.2). By monitoring the shift vs. time, molecular binding events can be monitored and kinetics of the binding events can be studied without labels. In particular, from the sensorgram we can calculate the association and dissociation constants $k_{on}$ and $k_{off}$. Then the equilibrium dissociation constant ($K_D$) can be calculated from the equation $K_D = k_{off}/k_{on}$ (Chapter 2).

The sensor chip used in this study was Sensor Chip NTA (BiaCore), which is designed to bind histidine-tagged biomolecules for interaction analysis in BiaCore systems. The
surface of the chip has a carboxymethylated dextran matrix pre-immobilized with nitrilotriacetic acid (NTA) for capture of histidine-tagged molecules via Ni$^{2+}$-NTA chelation. The focus of this chapter is to analyze affinity changes of CheC-CheD binding under different situations, including CheC-CheD alone, CheC-CheD-CheY, or ChC-CheD-CheY-p. We want to see whether phosphorylation of CheY can affect CheC-CheD binding or not, which may help give credence to our previous hypothesis of the CheC-CheD-CheYp adaptation system.

**Pulldown Assay with GST-6xHis-CheD**

In order to immobilize CheD onto the surface of SPR chips, we needed to introduce a tag that can interact with functional groups of the surface. The sensor chip NTA was chosen in my SPR assays, which binds histidine-tagged biomolecules. Therefore, the 6xHis tag should be attached to CheD. Previous studies showed that the solubility of CheD was very poor with the 6xHis tag at either the N-terminal or the C-terminal end (personal communications with H. Szurmant and T. Muff). To increase its solubility, different versions of CheD with fusion tag proteins were constructed, including GST-6xHis-CheD and GST-CheD-6xHis in the pGEX-6p-2 vector, and 6xHis-GST-CheD in the pUSH1 vector. GST-6xHis-CheD (GHD) had a high yield after protein purification that is similar to that for GST-CheD; whereas the other two yielded little purified protein (data not shown).

To test whether the GST-6xHis-CheD construct can actively bind CheC, a pull-down assay was performed (Figure 5.3). The NTA beads were used in this assay. Results have shown that GST-6xHis-CheD could clearly pulldown CheC, and is a good ligand protein
interacting with CheC in the SPR kinetic studies. In addition, when CheY-p was added, about 1.9 fold more CheC could be pulled down compared to that with unphosphorylated CheY.

**SPR Analysis of CheC-CheD Interaction**

The SPR experiment was first performed to analyze CheC-CheD interaction. By this experiment, we could know: (1) if the SPR technique is workable for CheC-CheD kinetic analysis; (2) the range \((K_D)\) of CheC-CheD interaction for downstream SPR assays.

In this SPR experiment, 500 RU of GST-6xHis-CheD was immobilized on the NTA chip, and different concentrations of CheC were allowed to associate to CheD with 4 min of association and 2 min of dissociation. Results have shown that with increasing concentration of CheC, the binding signal (Response Unit, RU) was also increasing proportionally. It indicated that SPR is a suitable technique to investigate CheC-CheD interaction, especially in potentially different environments, for example, with addition of CheY or CheYp. In addition, \(K_D\) of CheC-CheD interaction was \(4.1 \pm 0.3 \mu\text{M}\) calculated by the Biacorevaluation software. These preliminary data implied a reasonable range of CheC concentration for the downstream CheC-CheD-CheYp experiments.

**SPR Analysis of CheC-CheD-CheY-p Adaptation System**

In this study, the affinity changes of CheC-CheD binding were studied with SPR under different circumstances, CheC-CheD alone, CheC-CheD-CheY, or CheC-CheD-CheY-p. Through all the experiments, 500 RU of GST-6xHis-CheD was immobilized on the NTA chip, and all experiments were repeated in three individual days.
In the study of CheD-CheC binding kinetics, 5 µM of CheC was injected (Figure 5.5). In the study of CheD-CheC-CheY binding kinetics, 5 µM of CheC and an excess amount of CheY (20 µM) were injected with the same concentration of CheY in the running buffer (Figure 5.6), which removed CheY background effect during buffer switching, but still let CheY be present to interact with CheC. The same scheme was used in the study of CheD-CheC-CheY-p except 5 mM of acetyl phosphate was added during injection and in the running buffer (Figure 5.7). Results were reasonably reproducible.

Putting data from all three conditions together (Figure 5.8 – 5.10), we can compare the CheD-CheC affinity changes. The association ($k_{on}$) and dissociation constants ($k_{off}$), and the equilibrium dissociation constant ($K_D$) were calculated according to methods in Chapter 2, and listed in Table 5.1. Results showed that $K_D$ of CheD-CheC binding alone was $(3.95 \pm 1.06) \times 10^{-6}$ mol/L. When unphosphorylated CheY was also added, $K_D$ for CheD-CheC was $(7.01 \pm 0.53) \times 10^{-6}$ mol/L. And when phosphorylated CheY was added, $K_D$ was $(5.46 \pm 0.33) \times 10^{-7}$ mol/L. We can see that after CheY was added, CheD-CheC affinity decreased by half compared to CheD-CheC alone. In addition, if phosphorylated CheY was present (in CheC-CheD-CheY-p study), CheD-CheC affinity was greatly increased by 12.8 fold compared to that of CheD-CheC-CheY. In addition, when CheY-p was added, $k_{on}$ changed from $(1.06 \pm 0.12) \times 10^4$ M$^{-1}$S$^{-1}$ to $(1.79 \pm 0.13) \times 10^5$ M$^{-1}$S$^{-1}$ compared to adding of unphosphorylated CheY, and $k_{off}$ changed from $(7.43 \pm 0.79) \times 10^{-2}$ S$^{-1}$ to $(9.76 \pm 0.57) \times 10^{-2}$ S$^{-1}$.

**Discussion**

Adaptation is the ability to respond to a change in input stimulus, and return to its prestimulated output level. Despite its deamidase activity, we hypothesize that CheD’s main
role is its involvement in the CheD-CheC-CheY-p negative feedback pathway during adaptation. In particular, CheD is likely to help stabilize the transient kinase-activating state through binding to receptors. When CheY-p level is increased, CheC-CheY-p complex may attract CheD away from receptors. In order to understand this negative feedback pathway, in this study a series of SPR experiments were performed to detect CheC-CheD binding kinetics with CheY or CheY-p present.

When the unphosphorylated CheY was added to CheD/CheC, the affinity of CheD/CheC decreased by half; and when the phosphorylated CheY was added, the affinity increased by 12.8 fold. If looking at the top of association curves, which is close to reaching the equilibrium of CheD-CheC association and dissociation, the RU change from CheD-CheC-CheY-p was 5.4 fold higher than that of CheD-CheC-CheY. In other words, when CheY is phosphorylated, 5.4 fold more CheC binds to CheD compared to when CheY is unphosphorylated. In addition, the half life (t_{1/2}, equals 0.69302/ k_{off}) of dissociation from above data is ranged from 7.1 S to 12.3 S, which is compatible with the observed adaptation rate. It showed that these data are reasonable from another point of view.

In sum, above results show that, CheD-CheC coupling proteins work to facilitate adaptation in bacterial chemotaxis through a negative-feedback mechanism involving CheYp, a result consistent with our recent hypothesis [87]. To summarize, the scheme of adaptation through CheD-CheC-CheY-p: (a) The interaction between CheD and chemoreceptors makes the receptors more active. (b) When cells are exposed to attractant, CheAp and CheYp levels increase; (c) More CheY-p then binds to CheC. This complex attracts more CheD away from the receptors. (d) Then CheYp level is decreased, and the receptor becomes less active. (e) When the cell is exposed to the repellent or the attractant is removed, it goes the opposite
way. In this situation, less CheAp and CheYp are produced, and less CheC-CheYp complexes are formed then. Therefore, more CheD binds the chemoreceptors and make receptors more active (Figure 5.11).

Besides the CheC-CheD-CheYp adaptation system, the methylation system and the CheV system are also responsible for adaptation of *B. subtilis* chemotaxis, as proposed by Drs. C. V. Rao and G. W. Ordal [87].

In the methylation system, methyl groups are shuttle between different sites on the receptor in response to the addition or removal of attractant, while the net level of methylation of the *B. subtilis* receptors appears to be fairly constant [41]. In addition, the receptors are rapidly demethylated (1 min) and then slowly remethylated (20 min) upon both addition and removal of attractant [42, 43]. Another unique feature of the *B. subtilis* methylation system is that the glutamate residues get selectively methylated, which can either stimulate or inhibit the CheA kinase [96]. For example, McpB has three methylation sites located at Glu371, Glu630 and Glu637. Upon addition of attractant, the cell adapts by the demethylation of residues 371 and 630. Upon removal of attractant, sites 630 and 637 get demethylated, so that the cell adapts and the receptors to return to pre-stimulus levels [96]. Homology modeling shows that these three methylation sites on McpB form a tight cluster on the outward face of McpB. Charge-charge repulsion between them likely to affect receptor stability and associated kinase activity [87].

Similar to CheC-CheD-CheYp system, the CheV system is also through a negative feedback loop. CheV has two domains, an N-terminal CheW-like coupling domain and a C-terminal response-regulator domain that can be phosphorylated by CheA. Upon
phosphorylation, it could yield a conformational change that inhibits CheA kinase activity, likely by disrupting the receptor complex [46, 87].

It is still unclear yet how these three adaptation systems are coordinated. Further studies are required in each adaptation system before we can integrate all three systems.

It has been reported that despite the diverse adaptation pathways in biological systems, there are only a finite set of solutions for robustly achieving adaptation [63]. In their study, all possible three-node enzyme network topologies were computationally searched to identify those that could perform adaptation. Only two major core topologies have emerged as robust solution: a negative feedback loop with a buffering node and an incoherent feedforward loop with a proportioner node [63]. In our case, the CheC-CheD-CheYp system and the CheV system belong to the classic negative feedback loop pathway. The CheC-CheD coupling proteins and CheV serve as a buffering node that can potentially fine tune the output. The methylation system in *E. coli* also belongs to the classic negative feedback pathway with CheR and CheB affected methylation level serving as buffering nodes. The methylation system in *B. subtilis* is more complicated, since the net methylation level during adaptation is almost constantly same and the (de)methylation is selective. We may treat it as a modified negative feedback system with the special pattern of combinations of methylation/demethylation as the buffering node, which can be affected by CheR/CheB as in *E. coli*.

Overall, in the study I successfully obtained CheC-CheD binding kinetics with CheY or CheYp presence by a series of SPR experiments. The increased affinity of CheD for CheC in presence of CheYp but not CheY makes likely the hypothesis that CheC-CheD-CheY interact as part of a negative feedback pathway during adaptation.
Figures and Tables

Table 5.1 Kinetic parameters of CheC-CheD binding from SPR experiments.

<table>
<thead>
<tr>
<th>Unit</th>
<th>$K_s$</th>
<th>$k_{off}$</th>
<th>$k_{on}$</th>
<th>$K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CheC-CheD1</td>
<td>0.1468</td>
<td>0.0613</td>
<td>17100</td>
<td>3.58E-06</td>
</tr>
<tr>
<td>CheC-CheD2</td>
<td>0.1213</td>
<td>0.0466</td>
<td>14940</td>
<td>3.11E-06</td>
</tr>
<tr>
<td>CheC-CheD3</td>
<td>0.1234</td>
<td>0.0626</td>
<td>12160</td>
<td>5.15E-06</td>
</tr>
<tr>
<td>Average</td>
<td>0.1305</td>
<td>0.0568</td>
<td>14733</td>
<td>3.95E-06</td>
</tr>
<tr>
<td>SD</td>
<td>0.0141</td>
<td>0.0089</td>
<td>2476</td>
<td>1.06E-06</td>
</tr>
<tr>
<td>CheC-CheD-CheY1</td>
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<td>0.0829</td>
<td>11300</td>
<td>7.34E-06</td>
</tr>
<tr>
<td>CheC-CheD-CheY2</td>
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<td>0.0724</td>
<td>11320</td>
<td>6.40E-06</td>
</tr>
<tr>
<td>CheC-CheD-CheY3</td>
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<td>0.0675</td>
<td>9260</td>
<td>7.29E-06</td>
</tr>
<tr>
<td>Average</td>
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<td>0.0743</td>
<td>10627</td>
<td>7.01E-06</td>
</tr>
<tr>
<td>SD</td>
<td>0.0129</td>
<td>0.0079</td>
<td>1184</td>
<td>5.30E-07</td>
</tr>
<tr>
<td>CheC-CheD-CheY-p1</td>
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<tr>
<td>CheC-CheD-CheY-p2</td>
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<td>CheC-CheD-CheY-p3</td>
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<tr>
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<tr>
<td>SD</td>
<td>0.0702</td>
<td>0.0057</td>
<td>13284</td>
<td>3.31E-08</td>
</tr>
</tbody>
</table>
Figure 5.1 Surface plasmon resonance detection unit. L: light source, D: photodiode array, P: prism, S: sensor surface, F: flow cell. The two dark lines in the reflected beam projected on to the detector symbolise the light intensity drop following the resonance phenomenon at time = t1 and t2. Adapted from http://www.astbury.leeds.ac.uk.
Figure 5.2 Schematic of SPR kinetic analyses. Adapted from Biacore web site (www.biacore.com).
Figure 5.3 GST-6xHis-CheD pull-down assay. The Coomassie stained SDS-PAGE shows a pull-down experiment demonstrating the interaction of GST-6xHis-CheD with CheC (lane 2), with CheC and CheY (lane 3), and with CheC and CheY-p (lane 4). Lane 1 is a control, which had GST-6xHis-CheD alone. The NTA beads were used in this assay. Results have shown that GST-6xHis-CheD could pulldown CheC, and is a good ligand protein that can be potentially used in the SPR experiments. In addition, when CheY-p was added, about 1.9 fold more of CheC could be pulled down compared to that with unphosphorylated CheY.
Figure 5.4 SPR analysis of the interaction between CheD and CheC. Different concentrations of CheC were allowed to associate to GST-6xHis-CheD on the NTA chip with 4 min of association and 2 min of dissociation.
Figure 5.5 SPR analysis of the interaction between CheD and CheC. 5 µM of CheC was allowed to associate to GST-6xHis-CheD on the NTA chip with 4 min of association and 2 min of dissociation. Data were from three independent dates.
Figure 5.6 SPR analysis of CheC-CheD interaction with CheY presence. 5 µM of CheC and an excess amount of CheY (20 µM) were injected and allowed to associate to GST-6xHis-CheD on the NTA chip with 4 min of association and 2 min of dissociation. The same concentration of CheY was added in the running buffer. Data were from three independent dates.
Figure 5.7 SPR analysis of CheC-CheD interaction with CheYp presence. 5 µM of CheC, an excess amount of CheY (20 µM), and 5 mM of acetyl phosphate were injected and allowed to associate to GST-6xHis-CheD on the NTA chip with 4 min of association and 2 min of dissociation. The same concentrations of CheY and acetyl phosphate were added in the running buffer. Data were from three independent dates.
Figure 5.8 CheY-p increased CheD-CheC affinity shown by SPR (day 1). Blue color: CheD-CheC; green color: CheD-CheC-CheY (unphosphorylated); and red color: CheD-CheC-CheY-p (phosphorylated).
Figure 5.9 CheY-p increased CheD-CheC affinity shown by SPR (day 2). Blue color: CheD-CheC; green color: CheD-CheC-CheY (unphosphorylated); and red color: CheD-CheC-CheY-p (phosphorylated).
Figure 5.10 CheY-p increased CheD-CheC affinity shown by SPR (day 3). Blue color: CheD-CheC; green color: CheD-CheC-CheY (unphosphorylated); and red color: CheD-CheC-CheY-p (phosphorylated).
Figure 5.11 Model for the CheC-CheD-CheY-p adaptation system in *B. subtilis*. (a) The interaction between CheD and chemoreceptors makes the receptors more active; (b) When cells are exposed to attractant, CheAp and CheYp levels are increased sequentially; (c) More CheY-p then binds to CheC. This complex attracts more CheD away from the receptors. (d) Then CheYp level is decreased, and the receptor is less active. (e) When the cell is exposed to the repellent or the attractant is removed, it goes to the opposite way. In this situation, less CheAp and CheYp are produced, and less CheC-CheYp complexes are formed then. Therefore, more CheD binds the chemoreceptors and make receptors more active. This figure was adapted from reference [87].
Chapter 6
Interaction between CheD and McpC

Introduction

The cheD knockout mutant shows a chemotactic defect, is less sensitive to attractants, more tumbling, and has poorly methylated chemoreceptors [52, 56, 90]. Through an unknown mechanism, CheD was reported to interact with receptors [32, 90, 91]. Therefore, we assume that CheD can make receptors more active either directly or indirectly.

McpC, the sole proline receptor, responds to a variety of attractants, and is probably the most important receptor among the MCP chemoreceptor family. It responds to all amino acids except asparagine, and many PTS sugars [57, 79, 81]. Interestingly, McpC is the only chemoreceptor that absolutely requires CheD for the normal chemotaxis. Other receptors like McpB can still have appreciable but subnormal taxis without CheD [52, 58]. Addressing how McpC and CheD interact with each other can help us understand this special CheD requirement and how CheD makes receptors more active.

Particularly, we recently thought that there are three adaptation systems in B. subtilis, and CheD is involved in the CheD-CheC-CheYp negative feedback pathway [87]. Once CheY is phosphorylated, the CheC-CheYp complex can attract more CheD from receptors, which will make receptors and thus CheA less active. Elucidating the interaction between McpC and CheD can help us better understand the role of CheD in adaptation.

Previously C. Kristich in our lab made several McpB-McpC receptor chimeras including the one swapping the AS-1 region of McpC HAMP domain by that of McpB (Figure 6.1). The new McpCBC, interestingly, became CheD-independent, whereas on
swarm plates McpB\textsubscript{324}C and McpC\textsubscript{317}B had phenotypes similar to McpB and McpC, respectively. By his work, he narrowed down that AS-1 region could contain the information that McpC requires CheD [58].

The focus of this chapter is on identification of the sites on McpC that are required to recruit CheD. Some additional protein expression and protein modeling work have also been done to validate those sites that were discovered.

**Strategy of Identification of Putative McpC Sites that Recruit CheD**

Based on C Kristich’s work the HAMP domain of McpC, especially the AS-1 region, most likely contains the information that recruits CheD. AS-1 region is a small 12-residue region. Like most other protein-protein interactions, CheD-McpC interaction may occur through hydrophobic, polar or charged residues. Therefore, six sites on McpC AS1 region were selected including P302, Q304, Q305, K309, T310, and K311 (Figure 6.1), and then were mutated into alanine on the full-length McpC in pAIN750 vector. After transformation into *B. subtilis*, the mutant phenotypes were screened on the swarm plates. Those that were defective in taxis were identified.

**Q304 and Q305 on McpC AS-1 Region are Required to Recruit CheD**

After site-directed mutagenesis, the plasmids were then transformed into the amyE locus of Δ10 strain, which lacks all 10 chemoreceptors, and then selected for spectinomycin resistance. The selected mutants were then analyzed on swarm plates, and examined whether these mutants could form a chemotactic ring towards proline in the presence of CheD activity.
The mcpC complemented strain in Δ10 background was capable of supporting chemotactic ring formation in the presence of CheD, whereas Δ10 could not. Surprising to us, in the presence of CheD, the Q304A and Q305A mutants in the AS-1 region did not form a clear chemotactic ring, and only diffused within a small region as Δ10 did (Figure 6.2). Thus, in either of these mutants, CheD could no longer activate McpC. Therefore, Q304 and Q305 on McpC are most likely the sites required to bind CheD. More supporting evidence will be discussed at follows.

Expression of CheD in McpC Q304 and Q305 Mutants

Recently V. Cannistraro in the lab found that the expression level of CheD seems to be tuned to give the functional ratio of protein need to participate in adaptation. When the binding counterparts of CheD are deleted (cheC and all 10 receptors), the CheD level drops significantly. In detail, CheD protein levels in the Δ10 background are about 19% of wild-type, and 32% of wild-type in the cheC null (V. Cannistraro Ph.D. thesis). Therefore, we would like to see if CheD levels have any change when it is no longer recruited by McpC in Q304A and Q305A mutants as evidenced by reduced total levels of CheD.

For this purpose, 8 different strains with Δ10 background were grown in the minimum medium, and western blots were then performed with anti-CheD antibody to the cell lysates. These strains included Δ10, Δ10ΔcheD, Δ10mcpB, Δ10mcpC, Δ10mcpCBC, Δ10mcpC(Q304A), and two isolates of Δ10mcpC(Q305A). The Δ10 receptor background can remove all potential background of CheD-receptor interactions. Only those complemented or mutated receptors could possibly interact with CheD.
The bands corresponding to CheD were later quantified using LabWork software (Figure 6.3). The CheD levels in Δ10mcpC(Q304A) and Δ10mcpC(Q305A) are approximately 52.8% and 69.8% as in Δ10mcpC, respectively (Figure 6.3). The reduced CheD level may suggest that CheD is more exposed to proteolysis when unbound or protected from receptors. In addition, the CheD levels in Δ10mcpB and Δ10mcpCBC are similar to Δ10mcpC.

**CheD May Need the Receptor Homodimer Structure to be Recruited**

To investigate CheD/McpC interaction, I firstly used McpC C-terminal part as the mutation target. After mutating different sites mentioned before, each mutated protein was purified. Then NTA beads pulldown assay and SPR were used for screening purpose. But surprisingly, no obvious binding was observed on CheD with even McpCc-term (although G. Glekas in the lab could see the binding by the GST pull-down assay). In pulldown assays, McpCc-term with CheD had a similar level of “binding” as without CheD. Especially, SPR is a very sensitive tool to study protein-protein interaction. But even at the high concentration of McpCc-term (20µM), no obvious binding was found with CheD.

Another source of evidence could be from using of ProFound protein interaction mapping kit (PIERCE) to search for McpCc-term and CheD interaction. The theory is that when one protein is bound by another, the interaction area will be protected from cleavage reagents, which can be revealed by western blots. With this kit I used McpCc-term and CheD, but no protection areas were found, and the McpCc-term was completely digested (data not shown). This suggested that there is no obvious binding between CheD and McpCc-term.
Based on above evidence, the c-terminal part of receptor monomer may not be enough to recruit CheD. Remember that in *B. subtilis* chemoreceptors are long α-helical homodimers [83], I assume that the quaternary homodimer structure of receptors is needed to recruit CheD, and the packed four helices in HAMP domain together may be required for CheD recognition. Alternatively, the structure of the HAMP domain is different in the full-length receptor than in the C-terminal receptor.

**Structure Homology Modeling of QQ sites in McpC AS-1 Region**

The four-helix arrangement of receptor HAMP domain is a parallel coiled coil that can have two alternative conformations influenced by binding of attractant in the region outside the membrane [7]. By this way the upstream extra-cellular signal information can be transferred to the downstream highly conserved domain (HCD), which interacts with the CheA kinase. It is possible that alanine substitutions of Q304 and Q305 could alter the internal structure of McpC and let it mis-folded after expression. To remove this possibility, protein structure homology modeling of McpC HAMP domain was performed in SWISS-MODEL database (Figure 6.4)[12].

The modeling was based on the matching template 2asxB, the HAMP domain of the receptor AF1503 from *Archaeoglobus fulgidus* [43], error rate 3.20E-11, 28.302% sequence identity. This is a very good matching.

From the model picture, we can clearly see that Q304 and Q305 are facing towards outside of the HAMP domain. It is very unlikely that substitutions of these two residues into alanine will alter its four helices homodimer structure. The signal flux through helix rotating
should not be influenced significantly. Therefore, these two mutated McpC should still be able to fold properly.

On the other hand, the polar groups of Q304 and Q395 put McpC into a good position to interact with CheD. These glutamine polar groups are facing extruded of the helices homodimer, and are more convenient to connect with CheD, presumably at S32 site on CheD according to Crane’s model [24].

Discussion

Despite its deamidase activity, we hypothesize that CheD’s main role is its involvement in the CheD-CheC-CheYp negative feedback pathway. In particular, CheD is likely to help stabilize the transient kinase-activating state through binding to receptors. When CheYp level is increased, CheC-CheYp complex may attract CheD away from receptors [87]. In order to understand this negative feedback pathway, it is critical to address how CheD interacts with receptors and how this interaction tunes the downstream CheA kinase activation. Since we knew that McpC absolutely requires CheD for its normal taxis, it is reasonable to start from the CheD/McpC interaction.

Previous studies from McpB/McpC receptor chimeras showed that the AS-1 region of McpC HAMP domain most likely has the determinant sequence recruiting CheD. In this study, two point mutations have been made in the AS-1 region of McpC HAMP domain to study this interaction.

HAMP domains are a relatively conserved domain, widely existing in many signaling proteins in bacteria and archaea, which converts the signal from sensory input modules to output modules. It is usually a 50-residue motif located right after the transmembrane
domain (TM) and immediately inside the cytoplasmic membrane. It is named for its presence in histidine kinases, adenylyl cyclases, chemoreceptors (methyl-accepting chemotaxis proteins, MCPs), and some phosphatases. HAMP domains consist of 4 α-helices homodimers. In each monomer, there are two short amphipathic α-helices (12-residue of AS-1 and 15-residue of AS-2) joined by a flexible connector [7-9, 43]. Although the structure of HAMP domain has been resolved by NMR [43], the detail mechanism how HAMP transfers signal from the upstream sensing module to the downstream output module is yet to be explained.

In this study, I found that Q304 and Q305 in the AS-1 region of McpC HAMP domain are likely part of the determinant sequences that recruit CheD. When viewing them in the 3-D structure, these two residues are clearly facing towards outside of helix rotating axis. This is important, because the polar side group of glutamine extruded from the helix axis can potentially interact with other proteins, like CheD.

It is noteworthy to notice that CheD deamidase activity is not required for McpC mediated intramolecular signal transduction. K. Kristich mutated Q304 and Q305 into glutamate (in fact, CheD may not deamidate these two glutamine at all), and found that the mutants still need CheD for normal taxis on swarm plates [58]. Therefore, CheD’s main role in regard to the receptor HAMP domain is not deamidation, but is probably to facilitate CheA activation.

We were also interested to see if CheD levels have any change when it is no longer recruited by McpC in Q304A and Q305A mutants. For this purpose, CheD levels were tested from 8 different strains with Δ10 background. The CheD levels in Δ10mcpC(Q304A) and Δ10mcpC(Q305A) are approximately 52.8% and 69.8% as in Δ10mcpC, respectively (Figure
6.3). The reduced CheD level may suggest that CheD is more exposed to proteolysis when unbound or protected from receptors (or CheC as in V. Cannistraro thesis). Therefore, the dynamic interaction among CheD, receptors and CheC are vital to maintain the normal CheD level. It has been reported that CheW is subject to Clp and ClpX-mediated proteolysis during glucose starvation in \textit{B. subtilis} [33]. This may be the similar situation. It will be interesting to see if CheC levels vary in a similar way. Potentially in vitro enzyme protection time-course experiments can be done to verify this hypothesis with incubating of purified CheD, receptor, or CheC under a general digestion enzyme background.

Surprisingly, the CheD levels in \(\Delta 10\text{mcpB}\) and \(\Delta 10\text{mcpCBC}\) are similar to \(\Delta 10\text{mcpC}\). We originally thought that without McpC AS-1 region, the CheD levels would be lower in \(\Delta 10\text{mcpB}\) and \(\Delta 10\text{mcpCBC}\), since CheD is not necessary to couple with receptor in these cases. These results may suggest that besides being recruited by McpC, CheD may have general roles on all chemoreceptors through protein association. It is not necessary to be deamidation because most likely deamidation is only a small part of CheD functions. The deamidation of receptors occurs only once, and it is very costly to synthesize receptors again. McpC absolutely requires CheD in order to carry out taxis, but somehow CheD may work on all receptors to make them more active, as evidenced by that \(\Delta 10\text{cheD mcpB}\) has less efficient taxis than \(\Delta 10\text{mcpB}\) (G. Glekas Ph.D. thesis).

Overall, Q304 and Q305 are essential for CheD recruitment to McpC. CheD’s structure association, instead of deamidation function, is important to McpC mediated signal transduction. With McpC Q304A, Q305A, and CheD S32A, C33A mutants, we may further study how exactly CheD helps activate receptors, and how CheD helps tune the downstream kinase activation.
Figures and Tables

Figure 6.1 Schematic of the HAMP regions of wild-type and chimeric receptors. Sequences derived from McpB are in plain type, whereas sequences derived from McpC are presented on a gray background [58]. This figure is a reproduction from the reference.
Figure 6.2 Swarm plate screening of McpC AS-1 mutants. Swarm plates were composed of minimal medium supplemented with proline as the attractant. All strains were assayed a minimum of six independent times. Results from a representative experiment are shown. The image was processed using LabWork to maximize contrast.
Figure 6.3 Expression of CheD in McpC Q304 and Q305 mutants. In both figure A and B, 1: Δ10mcpB; 2: Δ10mcpC; 3: Δ10mcpCBC; 4: Δ10; 5: Δ10 cheD; 6: Δ10mcpC(Q304A); 7: Δ10mcpC(Q305A), strain 1; 8: Δ10mcpC(Q305A), strain 2; 9: 10 pmol of purified CheD loaded as the molecular weight marker. (A) Anti-CheD Western blot picture in Δ10 background strains. The whole cell lysate from 5.2E+07 cells were loaded per lane, and 1:400 anti-CheD antibody was added for each blot. (B) Quantified band density on above western blot picture.
Figure 6.4 Structure modeling of McpC HAMP Domain. The 3-D structure was obtained from SWISS-MODEL database. Two Q304 and Q305 residues facing towards outside α-helical homodimers are in purple color.
References


