A PAS DOMAIN BINDS ASPARAGINE IN THE CHEMOTAXIS RECEPTOR MCPB IN
BACILLUS SUBTILIS
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Running head: Chemotaxis Receptor PAS Domain Binds Asparagine
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During chemotaxis toward asparagine by Bacillus subtilis, the ligand is thought to bind
to the chemoreceptor McpB on the exterior of the cell and induce a conformational change.
This change affects the degree of phosphorylation of the CheA kinase bound to
the cytoplasmic region of the receptor. Until recently, the sensing domains of the B. subtilis
receptors were thought to be structurally similar to the well-studied Escherichia coli four-
helical bundle. However, sequence analysis has shown the sensing domains of receptors
from these two organisms to be vastly different. Homology modeling of the sensing
domain of the B. subtilis asparagine receptor McpB revealed two tandem PAS domains.
McpB mutants having alanine substitutions in key arginine and tyrosine residues of the upper
PAS domain, but not in any residues of the lower PAS domain, exhibited a chemotactic
defect in both swarm plates and capillary assays. Thus binding does not appear to occur
across any dimeric surface but within a monomer. A modified capillary assay designed
to determine the concentration of attractant where chemotaxis is most sensitive showed that
when Arg-111, Tyr-121 or Tyr-133 is mutated to an alanine, much more asparagine is
required to get an active chemoreceptor. Isothermal titration calorimetry experiments
on the purified sensing domain showed a $K_D$ to asparagine of 14 $\mu$M, with the three mutations
leading to less efficient binding. Taken together, these results reveal not only a novel
chemoreceptor sensing domain architecture but also, possibly, a different mechanism for
chemoreceptor activation.

Many species of bacteria are able to sense their proximal chemical environment and move
towards more favorable locations through a process known as chemotaxis. At the heart of all
known bacterial chemotaxis pathways is a two component signal transduction system involving
the CheA histidine kinase and the CheY response regulator. In the Gram-positive bacterium
Bacillus subtilis, the CheA kinase forms a stable complex with the chemotaxis receptors, also
known as methyl-accepting chemotaxis proteins (MCP’s), and the CheW and CheV adaptor
proteins (1). When the receptors sense attractant molecules, they enhance the rate of CheA
autophosphorylation, which in turn causes CheY-P concentrations to increase (2-4). In B. subtilis,
the binding of CheY-P to the cytoplasmic face of the flagellum increases the likelihood of smooth runs
(5). By biasing the duration and frequency of run and tumble swimming events, the bacterium is
able to migrate up gradients of attractant molecules. In addition to this core signaling
transduction module, the B. subtilis pathway also possesses two CheY-P phosphatases and a number
of regulatory proteins involved in sensory adaptation (6,7). One of the remarkable features
of the chemotaxis system is its ability to adapt the bacteria to their surrounding environment so that
relative changes in the chemical concentrations can be sensitively detected.

The chemotaxis receptors have previously been shown to be long, $\alpha$-helical homodimers with
multiple well-defined domains, shown in Figure 1 (8-10). The amino-terminal, sensing domain is
responsible for binding extracellular attractants and repellents (11). When bound with a ligand or
intermediary binding protein, the sensing domain undergoes a conformational change that is
propagated through the membrane to the HAMP (histidine kinase, adenyl cyclase, methyl-accepting
chemotaxis protein and phosphatase) domain (12).
and, then, the methylation region. In *B. subtilis* this region interacts with the regulator proteins, CheR, CheB and CheD, and is the site for post-translation modifications involved in sensory adaptation (8). The distal tip of the receptors, commonly referred to as the highly-conserved domain (HCD), interacts with the CheA kinase (13). A simple two-state model has been successfully used to describe the receptor complex, where attractants are thought to bias the receptor into a CheA-activating “on” state and repellents are thought to bias it into a CheA-inhibiting “off” state. In addition to attractants and repellents, regulatory proteins such as CheD and covalent modifications such as methylation and deamidation can also alter bias between the two states (1,14).

The sensing domain of the *Escherichia coli* chemotaxis receptors Tar and Tsr have long served as the model for not only other bacterial chemotaxis receptors but also for two-component sensor kinases. The crystal structure of the Tar aspartate receptor revealed a four-helical bundle architecture that can bind aspartate across the dimer interface (11). Three arginine residues (R64, R69 and R73) are directly responsible for binding the aspartate, and mutation to any one of these residues leads to a defect in taxis towards this attractant (15). Comparisons of the apo- and holo-sensing domain structures, as well as extensive cross-linking and electron paramagnetic resonance (EPR) experiments, have elucidated the conformational change that occurs upon aspartate binding. This change involves a vertical piston-like shift in the second trans-membrane helix (TM2), linking the sensing domain to the cytoplasmic region (8,16,17). Recent work in *B. subtilis* has shown that a different mechanism – a rotation of the first trans-membrane helix (TM1) without a piston movement – occurs upon attractant binding, illustrating a fundamental difference in the mechanism for receptor activation in these two species (18).

The sensing domains for the canonical *B. subtilis* receptors McpA, McpB, and McpC do not share any homology with corresponding domains for the *E. coli* chemotaxis receptors, as shown in the alignment in Figure 2. Rather, a Cache domain (Ca^2+ channels and chemotaxis receptors) is found as the core ligand-binding element in McpA, McpB and McpC (19). This domain is not found in the *E. coli* chemotaxis receptors, providing further evidence that there is a fundamental structural difference between the *E. coli* and *B. subtilis* sensing domains. Further analysis of the Cache domain also revealed the possible presence of a structural PAS (named after the Period circadian, Ah receptor nuclear translocator, and Single-minded proteins) domain and identified a conserved histidine residue as possibly being important for function (19). Moreover, genomic sequence analysis revealed that the *E. coli* four-helix bundle, initially defined in the Tar sensing domain, was not conserved in the chemotaxis receptors of many Gram-positive organisms, including the *B. subtilis* receptors McpA and McpB (20). In addition to these studies, recent structures of the periplasmic binding domains of both the *Klebsiella pneumoniae* CitA citrate sensor and the *E. coli* DcuS fumarate sensor showed that an extracellular PAS domain was involved in ligand binding for these two-component sensor kinases (21,22). Finally, the crystal structures of the *Vibrio harveyi* LuxQ sensor kinase sensing domain (23), the putative sensory box/GGDEF family protein from *Vibrio parahaemolyticus* (24), the *Vibrio cholerae* DetB sensing domain (25,26), and the sensing domain of the *Vibrio cholerae* McpN chemoreceptor (27) revealed a novel architecture for the sensing domains of both bacterial two-component sensor kinases and chemoreceptors. These crystal structures revealed a dual PAS domain architecture and suggest that this architecture is a more common sensing module than the *E. coli* four-helical bundle.

This current study aims to elucidate the architecture of the sensing domain of the asparagine receptor McpB. In particular, current evidence suggests that the sensing domain of the *B. subtilis* chemotaxis receptors has a different architecture than that of the *E. coli* chemotaxis receptors. The lack of a crystal structure for the McpB sensing domain led to the development of a homology-based structural model for this domain. This model predicts that a dual PAS domain structure is found in the McpB sensing domain, as well as in other *B. subtilis* chemoreceptors. Furthermore, mutational analysis reveals the residues that are important for ligand binding and overall chemotactic ability and, thus, provides
support for this structural model of the McpB sensing domain.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Plasmids –* All *B. subtilis* strains are derived from the chemotactic strain (Che+) OI1085 (28). All cloning and plasmid propagation were performed in *E. coli* strains TG1 (GE Healthcare Life Sciences). Recombinant protein overexpression was done in *E. coli* strain BL-21, and pGEX-6P-2 (GE Healthcare Life Sciences), a GST-fusion vector, was used for protein expression and purification.

The *B. subtilis* strains were created by using Quickchange (Stratagene) mutagenesis on the pAIN750 plasmid, which contained the full-length *mcpB* receptor gene under control of its native promoter. These single-site mutated receptors were then integrated back into the amyE locus of the Δ10*mcp* strain OI3545 (29), which is missing all ten *B. subtilis* chemoreceptors. Expression was confirmed using Western blots, and expression levels were found to be similar to wild-type McpB.

The GST-fusion pGEX-6P-2 plasmids were made by amplifying the desired receptor fragment (spanning residues 35 to 279) from a pAIN750::mcpB plasmid containing the appropriate mutation by PCR with engineered 5’ EcoRI and 3’ NotI sites. This fragment was then ligated into pGEX-6P-2, resulting in the protein McpBS.

**Swarm Plate Assay –** The swarm plate assay (30,31) was used to quantitatively measure the chemotactic ability of various strains. Briefly, strains were grown overnight at 30°C on TBAB plates. Cells were then scraped from the plate and resuspended to $A_{600nm} = 0.014$ in capillary assay minimal media (50 mM K$_3$PO$_4$, pH 7.0, 1.2 mM MgCl$_2$, 0.14 mM CaCl$_2$, 1 mM (NH$_4$)$_2$SO$_4$, 0.01 mM MnCl$_2$, 20 mM sorbitol and 0.02% tryptone, supplemented with 50 µg/ml histidine, methionine, and tryptophan). The cultures were grown to $A_{600nm} = 0.4$ at 37°C and 250 rpm shaking, after which 50 µl of a 5% glycerol-0.5 M sodium lactate solution was added, and then the cells were incubated a further 15 min. The cells were then washed three times with chemotaxis buffer (10 mM K$_3$PO$_4$, pH 7.0, 0.14 mM CaCl$_2$, 0.3 mM (NH$_4$)$_2$SO$_4$, 0.1 mM EDTA, 5 mM sodium lactate, 0.05% (v/v) glycerol (32)) and diluted to $A_{600nm} = 0.001$. The cells were aliquoted into 0.3 ml ponds on a temperature-controlled plate at 37°C. Closed-end capillary tubes filled with the appropriate chemoattractant (asparagine) were inserted into the pond. After 30 minutes, the cells in the capillaries were harvested and transferred to 0.5 ml top agar (1% (w/v) tryptone, 0.8% (w/v) NaCl, 0.8% (w/v) agar, 0.5 mM EDTA) and plated onto TB (1% (w/v) tryptone, 0.5% (w/v) NaCl) agar plates. These plates were incubated at 37°C for 16 h at which point colonies were counted to derive the data. Experiments were performed in triplicate and on two different days to assure reproducibility.

The sensitivity capillary assays were performed as previously described (33,34). In these assays, each capillary contained 3.16-times as much attractant as found in its corresponding pond. The experiments were performed over a range of concentrations spanning eight orders of magnitude. The number of bacteria that enter the capillary in such an assay is proportional to the difference in number of receptors titrated with attractant at the two end points – the capillary concentration and the pond concentration. The numbers of bacteria per capillary are graphed as a function of the geometric mean of the concentrations of attractant in the capillary and in the pond. The center of symmetry of the peak in this corresponding graph is the concentration at which chemotactic activity is most potent and, hence, provides an apparent dissociation constant for the receptors (34).
**Protein Purification** – To purify the GST-fusion proteins (McpBS), strains harboring pGEX-6P-2 with the receptor fragment cloned in the multiple cloning site were grown in 2 L LB cultures with 100 µg/ml ampicillin at 37°C and 250 rpm shaking until A<sub>600nm</sub> = 0.8. Expression was then induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown at 25°C with 250 rpm shaking for 12 hrs. Cells were collected by centrifugation at 7000 x g for 10 min. The cell pellet was resuspended in 3 ml TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.5) + 1% Triton X100 + 1mM DTT for every 1 g of cell pellet. The cells were then disrupted by sonication (5 x 10 second pulse). The cell debris was removed by centrifugation at 10,000 x g for 10 min., and the lysate was further centrifuged for 1 h at 40,000 x g.

The cell lysate was then loaded onto 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 (glutathione elution buffer: 50 mM Tris, 10 mM glutathione, pH 8.0). The GST-fusion protein was dialyzed against six changes of 800 ml PPB (Prescission protease buffer: 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris, pH 8.0). The GST tag was removed by digestion with 100 units of PreScission protease (GE Healthcare) for 12 h at 4°C. This solution was again passed over the GSTrap column to remove the GST and protease.

The pure receptor fragment flow-through was collected and dialyzed against six changes of 1 L TKMDmod buffer (50 mM Tris, 50 mM KCl, 5 mM MgCl₂, pH 8.0), concentrated using an Amicon Ultra spin concentrator with a 10,000 MW cut-off (Millipore), resulted in a final concentration of 2.0 µM. Data acquisition was performed using software provided by JASCO, and the raw data files were uploaded to the DICHROWEB online server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) for structural analysis (35,36).

**Structural Modeling** – Structural modeling was performed on the amino-terminus of the B. subtilis chemoreceptors using the Phyre (37), LOMETS and I-TASSER (38,39) modeling servers. The region of sequence between TM1 and TM2, which spans residues 35 – 279, for each receptor was submitted, and the resulting pdb files were consequently examined. These servers use homology modeling to produce a structural match, although the I-TASSER server also used de novo modeling to achieve a final model. Each server determines a score for each model it produces, and only models within the published threshold were used. The model produced by the Phyre server showed 100% confidence. The model generated using the LOMETS server showed high confidence (Z-score 26.67), and the model from the I-TASSER server had a C-score of 1.06 and a TM-score of 0.86 +/- .07.

**RESULTS**

**Structural Modeling of the Amino-terminus Sensing Domain.** To obtain a structural model of the McpB sensing domain, the Phyre and LOMETS meta-servers were initially utilized (37,38). These servers align a sequence to those with known Protein Data Bank structures and attempt to produce structural models, assigning confidence scores based on the model’s accuracy. In order to obtain the best possible structural predictions, the I-TASSER online server was also utilized (39-41). The I-TASSER models matched the predictions from both the Phyre and LOMETS servers very well, and were used for further investigation of a possible amino acid binding site.

The resulting McpB sensing domain structural model, shown in Figure 3, aligned very well with the sensing domain of the MepN chemoreceptor from V. cholerae and the putative sensory box/GGDEF family protein from V. parahaemolyticus. In addition, the model aligns
well with the *V. harveyi* LuxQ and the *V. cholerae* DctB sensor kinase sensing domains. All four of these structures show a dual-PAS domain architecture. Each PAS domain of the McpB sensing region aligned, structurally, with the structures of the periplasmic binding domains of both the *K. pneumoniae* CitA citrate sensor and the *E. coli* DcuS fumarate sensor.

The sensing domain structural model of an McpB monomer clearly shows two tandem PAS domains. After the region for TM1, there is a long α-helix, presumably the site of dimerization (based on the structure of the McpN and LuxQ dimers), followed by the upper, membrane-distal, PAS domain. Next is the lower, membrane-proximal, PAS domain, followed by the TM2 helix spanning the membrane. The *V. parahaemolyticus* putative sensory box was crystallized with a small molecule, acetic acid, bound in the upper PAS domain, and the DctB and McpN structures also have a small molecule ligand bound in the upper PAS domain. This putative binding site is very similar to the CitA and DcuS binding pockets, and could, possibly, be the area responsible for binding asparagine in McpB.

Using the Phyre and LOMETS servers, comprehensive structural modeling of all ten *B. subtilis* chemoreceptors was performed (data not shown). It appears that five of the receptors, McpA/B/C and TlpA/B, all have a similar dual PAS domain structure in the sensing region. Interestingly, the sensing domain of the *B. subtilis* YvaQ chemoreceptor seems to contain a Tar-like four-helix bundle sensing domain.

**Identification of Putative Asparagine Binding Residues in McpB.** The putative binding pockets in both PAS domains of the McpB sensing domain were compared to the known binding areas in the PAS domains of the McpN, DetB, the putative sensory box, CitA, and DcuS sensing domains, both visually and using multiple sequence alignments. The following residues were identified as possibly being involved in asparagine binding: Arg111, Tyr121, and Tyr133 in the upper PAS domain, and Phe205, Tyr213 and His216 in the lower PAS domain. These residues were selected both because of their alignments with known PAS domain binding sites and because they have been shown to be involved in binding in other amino acid receptors, such as the glutamate and LAO receptors (42-45). They are identified on the McpB sensing domain homology model shown in Figure 3B. To test whether these residues are involved in asparagine binding, they were all mutated to alanines. An alanine residue would not be able to form hydrogen bonds with a small ligand, and is also not bulky enough to cause structural disruption in the putative binding pockets. It should be noted that His216 was previously identified as a possible active site residue in the Cache domain, and Phe205 is highly conserved in all Cache domains (19).

In addition to these residues, Glu142 was mutated to an alanine because it aligns with the *E. coli* Tar binding site, and Thr168 was mutated to an alanine because it is highly conserved in the *B. subtilis* sensing domains. These mutations in the upper PAS domain could also be considered negative controls, as they fall outside of the putative McpB binding pocket and would not be expected to have an effect on asparagine binding. In the lower PAS domain, Ile206 was also mutated to an alanine because it is also highly conserved in the Cache domain (19). Lastly, Tyr133 was also mutated to a phenylalanine, thus removing its hydrogen-bonding –OH group.

**Putative McpB Binding Site Mutations Show a Chemotactic Defect.** To initially test whether the various sensing domain mutations had an effect on overall chemotactic ability, low-agar swarm plates were utilized, and the size of the resultant chemotactic ring was analyzed. These asparagine swarm plate results showed that, when *mcpB* is expressed as the sole receptor in a Δ10mcp strain lacking all chemoreceptors, the R111A and Y133A mutations had a severe impact on the formation of a chemotactic ring, when compared to the wild-type McpB receptor (Figure 4A). McpB(Y121A) and mcplB(F205A) also showed a slight defect, while all other mutations seemed to have no effect. These results implicated residues R111 and Y133 as possibly being critical for asparagine binding. Western blots on all the mutants showed that McpB was expressed at wild-type levels, indicating that mutations did not affect expression (data not shown).

While swarm plates give a good indication of overall phenotypic chemotactic ability, capillary assays provide a more precise approach for measuring chemotactic ability. Thus, capillary assays were performed on all the McpB sensing
domain point mutants to better assess chemotactic ability. Asparagine concentrations of 10^4 and 10^5 M were used, and the accumulated colonies were counted. These results mirrored the swarm plate results, in that mcpB(R111A) and mcpB(Y133A) showed virtually no chemotactic accumulation in the capillary tubes at these asparagine concentrations (Figure 4B). Furthermore, mcpB(Y121A) also showed a significant decrease in accumulation, while mcpB(F205A) did not. Taken together, these results show that an alanine substitution at the following residues – Arg111, Tyr121 and Tyr133 – shows a defect in chemotaxis towards the amino acid attractant asparagine.

Putative McpB Binding Site Mutations Show an Increase in “K_D”. Sensitivity capillary assays have been used to determine the apparent dissociation constants (K_D) of both the E. coli and B. subtilis receptors (33,34). Utilizing this assay, the apparent K_D of wild-type B. subtilis McpB for asparagine has been approximated at 50 µM. This sensitivity capillary assay was used to examine asparagine binding in the wild-type McpB and the R111A, Y121A, Y133A and Y133F McpB mutations, all in the Δ10mcp background. These results, shown in Figure 5, indicated a dramatic shift in apparent K_D for the R111A and Y133A mutations, a less pronounced shift for the Y121A mutation, and no apparent change in sensitivity for the Y133F mutation. By determining the center of symmetry of the peaks, the apparent K_D’s could be determined, the results of which are shown in Table 1. McpB(R111A) and mcpB(Y133A) showed dissociation constants 19-24 times higher than wild-type McpB. As before, mcpB(Y121A) showed a less severe K_D shift, 5 times higher than wild type, and mcpB(Y133F) showed no difference from wild-type McpB. It should be noted that accumulation in the capillary tubes for all these mutants was at wild-type levels, indicating that the chemotaxis machinery is functioning normally.

Finally, there was a difference between the apparent K_D of the wild-type O11085 strain (80 µM) and the wild-type mcpB allele in the Δ10mcp background (500 µM). These results seemed to indicate that the presence of the other nine chemoreceptors increases the proportion of McpB in the active conformation, confirmed by the fact that the wild-type exhibits a random bias, while the Δ10mcp amyE::mcpB strain is tumbly (30).

Isothermal Titration Calorimetry of the McpB Sensing Domain. To further characterize the physical binding properties of the McpB chemoreceptor and asparagine, the amino-terminal sensing domain was overexpressed and purified. The wild-type sensing domain (McpBS) proved to be soluble and easily over-expressed. Three of the previously tested mutations – R111A, Y121A, and Y133A – were introduced into the sensing domain and the corresponding proteins were also purified.

The physical binding of McpBS to asparagine was assayed using isothermal titration calorimetry (ITC), a technique that can measure the thermodynamics of a ligand-protein interaction. These results, summarized in Table 2, showed an endothermic reaction for the wild-type McpB-asparagine binding reaction and a K_D of 14 µM, which is slightly lower than the K_D as determined by the sensitivity capillary assay. This difference would reflect the greater tendency of the free domain to adopt the conformation that binds asparagine in comparison to the whole receptor complexed with other proteins like CheA and CheW.

McpBS-Y121A, as expected, showed a decrease in its binding affinity for asparagine, as the K_D for that reaction was only 280 µM. As with the wild-type, stronger binding was seen with the soluble receptor fragment than in the sensitivity assay. McpBS-R111A and McpBS-Y133A showed no binding using ITC. However, this technique may be unable to measure binding for these two proteins because, if the K_D is in a similar range to the sensitivity assays, the insolubility of asparagine at concentrations over 100 mM makes it difficult to test such high K_D values.

Secondary Structure Estimation. To test whether these mutants showed a change in structure, the secondary structures of the wild-type sensing domain, McpBS, and the previously characterized mutants were analyzed by far-UV CD spectroscopy. The resultant spectra, shown in Figure 6, indicated that the three mutations have very little effect on the overall secondary structure of the purified sensing domain. When the raw data was uploaded onto the DICHROWEB online server, the wild-type sensing domain was shown to have an α-helical content of roughly 49%, and a β-sheet content of approximately 23%. These results also correlated very well with the homology
model, which showed an α-helical content of roughly 44% and a β-sheet content of approximately 24%. The three mutants, McpBS-R111A (46% α-helix, 26% β-sheet), McpBS-Y121A (49% α-helix, 23% β-sheet) and McpBS-Y133A (46% α-helix, 27% β-sheet), showed very similar structural content. These results showed that the mutant purified proteins have no trouble correctly folding into the same conformation as the wild-type protein.

**DISCUSSION**

Bacterial chemoreceptors are essential for the sensing of external ligands and initiating the associated two-component signal transduction cascade. Extensive work in *E. coli* and *S. typhimurium* has elucidated the structure and function of the chemoreceptor sensing domain, and the four-helical bundle was long thought to be the paradigm of not only bacterial chemoreceptors, but also other two-component sensor kinase sensing domains (11). However, recent work on CitA, DcuS and DctB has revealed a different sensing architecture, namely small ligand binding to a PAS domain (21,25,26).

The sensing domain of the *B. subtilis* chemoreceptors seems to be vastly different from that of the *E. coli* chemotaxis receptor, as first revealed from sequence analysis (19,20). The data presented here showed that a dual PAS domain structure is, most likely, the functional fold of the McpB sensing domain, quite unlike the Tar four-helix bundle. Although no crystal structure of a *B. subtilis* sensing domain is yet available, the *B. subtilis* sensing domains were found to be easy targets for all three modeling programs, indicating a high probability of an accurate homology-based model (37-39). The in vivo sensitivity assays and in vitro ITC experiments further verified the modeling results, as the putative asparagine-binding residues predicted by these models showed an effect on amino acid binding and chemotaxis. The ITC experiments performed here showed, for the first time, that the *B. subtilis* receptor McpB can directly bind an amino acid ligand. Finally, circular dichroism spectra correlated very well to the predicted structure and showed that point mutations in the sensing domain do not affect the secondary structure.

In the McpB receptor sensing domain, the Arg111 and Tyr133 residues seemed to play a very important role in binding the asparagine ligand. The sensitivity capillary assays and the ITC experiments both showed that an McpB receptor with one of these residues mutated to an alanine had a drastically lower affinity for asparagine. The third residue that has an effect on binding is Tyr121, although it is not nearly as dramatic of an effect as Arg111 and Tyr133.

Capillary assays performed on *E. coli* Tar mutants in which one of the three arginine residues responsible for ligand binding were mutated showed a decrease in sensitivity of two-to-three orders of magnitude (46,47). In addition, in vitro assays, using a coupled methylation system that examines receptor methylation after ligand binding, showed an increase in $K_D$ from 3 μM to 35 mM when one of the arginine residues is mutated, with the R64A mutation having the most drastic effect (46). Isothermal titration calorimetry experiments on the *E. coli* Tsr receptor, using serine as a ligand, show a $K_D$ of 27 μM, similar to the range of binding found in the McpB receptor (48). The *B. subtilis* and *E. coli* receptors show a similar range of attractant sensing, so it is not surprising that the results presented here are similar to data generated for the *E. coli* receptors.

The Tar receptor, as well as the CitA and DctB sensors, has key residues present in the binding pocket that can hydrogen bond with the ligand (15,21). Assuming the *B. subtilis* sensing domains use a similar mechanism, it is possible that both Arg111 and Tyr121 may be able to hydrogen bond with the asparagine ligand. When one of these residues is mutated to an alanine, a residue that would not be able to form a hydrogen bond with the ligand, the sensitivity of the receptor decreases. It then takes a higher concentration of asparagine to get the receptor into an active conformation, which is why there was a shift in the apparent dissociation constant.

The Tyr133 residue in the McpB sensing domain may play a different role in asparagine binding. McpB(Y133A) showed a similar phenotype to mcpB(R111A) in every experiment. The Tyr133 residue was also mutated to a phenylalanine, to determine if the –OH group on the tyrosine residue, or its inherent bulk, facilitated asparagine binding. Chemotactic activity, as well as asparagine binding, seemed to be normal in
suggesting that the size of the tyrosine residue was important in asparagine binding, not possible hydrogen-bonding with the \(-OH\) group.

All the other mutants of the McpB sensing domain studied did not show any impact on chemotaxis, suggesting they do not play a role in asparagine binding. These results showed that the lower PAS domain, which includes the Cache-domain conserved Phe205, Ile206 and His216, did not appear to bind asparagine. The most plausible conclusion is that only the upper PAS domain is responsible for ligand binding in the \textit{B. subtilis} McpB chemoreceptor, similar to small-ligand binding in both the McpN and DetB structures (25-27).

The McpN sensing domain structure was solved as a homodimer, and dimerization was shown to be similar to the LuxQ sensor domain (23,27). Likely, the sensing regions of the \textit{B. subtilis} chemoreceptors dimerize in a similar fashion. This mode of dimerization, along the long \(\alpha\)-helix at the amino-terminal of the sensing domain following TM1, would orient the PAS domains outwards. Such an orientation would facilitate ligand binding, and is important in the LuxQ dimer and its interactions with LuxP, as well (23). This structure shows the possibility of the \textit{B. subtilis} sensing domain being conserved throughout many two-component chemoreceptors, much more widely than the \textit{E. coli} chemoreceptor four-helix bundle, as has been postulated by others (49,50).

The importance of the ligand-binding event in the membrane-exterior domain of the chemoreceptor is that it stabilizes an allosteric conformation of the chemoreceptor in which CheA, bound to a very distant helical loop (the HCD), is very active. In \textit{E. coli}, an inactivating conformational shift upon ligand binding is thought to involve a piston-like movement of the TM2 helix (8). In the McpB structural model presented here, the TM2 helix comes after the lower PAS domain in the protein sequence. In \textit{B. subtilis}, no such piston shift has been found. Instead, TM1 is the helix that moves, as shown by disulfide cross-linking experiments, and that movement is a rotation of the helix (18,51). Moreover, the ligand binds \textit{within} a monomer, in the upper PAS domain, not across the dimeric surface as in the \textit{E. coli} receptors (11).

The LuxQ crystal structure provided an even clearer picture of the mechanism of activation of this type of two-component sensor. The LuxQ dimer has been crystallized with its binding partner, LuxP, in both the apo and bound forms (23,52). LuxP senses the extracellular molecule AI-2, which mediates quorum-sensing in many bacterial species. Upon binding AI-2, LuxP was shown to induce a global conformational change in the LuxQ dimer, by binding sites in the LuxQ PAS domains, resulting in a 140° rotation of one of the LuxQ domains in the dimer (23). This rotation produces an asymmetry in the quaternary structure of the LuxQ dimer, which induces kinase activation (23,53). While the binding of a small molecule might not produce such a dramatic rotation in the \textit{B. subtilis} receptors, any perturbation in the symmetry of the sensing domain dimer upon ligand binding may result in chemoreceptor activation.

Additional work in LuxQ showed that mutations at the top or bottom of the long dimerization helix could produce a severe phenotype in which the kinase is constitutively activated (23). If the ligand is binding in the upper PAS domain in the sensing domains of McpB, it is possible that it could change the dimerization helix, which is attached to TM1. This movement could possibly be responsible for affecting the TM1-TM1’ interactions, and might lead to kinase activation. Such a model would also explain why, upon attractant sensing, movement is seen only in TM1 of the McpB receptor, not in TM2 as in \textit{E. coli}.

Obviously, much more work is needed to examine these possibilities. However, binding of a ligand to the upper PAS domain could fit into a model of TM1 movement being responsible for chemotactic activation in \textit{B. subtilis} and possibly other organisms. Further structural insight into the \textit{B. subtilis} sensing domain, and the possible effects of ligand binding, would lead to a much better understanding of the mechanism of chemoreceptor activation.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Schematic representation of the domain arrangement of the B. subtilis McpB chemoreceptor and the key residues within McpBS examined in this study. Abbreviations: TM, trans-membrane helix; HAMP, acronym formed from the names of the proteins in which the characteristics for this sensing module are found (12).
Fig. 2. Sequence alignments of chemoreceptor sensing domains. Amino-acid sequences for select chemoreceptors, spanning the TM1 and TM2 helices. Conserved residues not aligned with the *E. coli* sequences are shown in red boxes. Organism names: Vc is *Vibrio cholerae*, Bs is *Bacillus subtilis*, Ec is *Escherichia coli*.

Fig. 3. Structural homology-based model of the *B. subtilis* McpB sensing domain. *A*, model based on the sensing domain sequence (residues 35 – 279) of the McpB receptor reveals a dual-PAS domain architecture, with a long dimerization helix. *B*, residues substituted with an alanine in the upper and lower PAS domains are highlighted.

Fig. 4. Swarm plate and capillary assay results. *A*, swarm plates results for various sensing domain alanine substitutions. *B*, capillary assays on various sensing domain alanine substitutions. Solid bars are at an asparagine concentration of 1 x 10^{-4} M, striped bars are at an asparagine concentration of 1 x 10^{-5} M. Error bars show standard deviation.

Fig. 5. Sensitivity assay results. Top left panel shows the wild-type (1085) *B. subtilis* strain. All other assays were performed using strains lacking all ten chemoreceptors with the wild-type or mutant mcpB integrated back into the chromosome.

Fig. 6. Asparagine binding to wild-type McpB (WT) and R111A, Y121A and Y133A mutants measured by isothermal titration calorimetry. The upper panel shows the heat changes observed upon injection of 10 µL of a 50 mM asparagine solution in TBS buffer pH 8.0 into a protein solution of 50 µM in the same buffer. The lower panels show the integrated heat changes of each injection plotted against the molar ratio of asparagine to protein.

Fig. 7. Circular dichroism spectra of McpBS and the various McpBS mutants. The measurements were performed with a 2 µM protein solution in 50 mM potassium borate buffer, pH 8.0. Wild-type McpBS, thick line; McpBS R111A, dotted line; McpBS Y121A, thin line; McpBS Y133A, dashed line.
Table 1. Apparent $K_D$ values of $mcpB$ mutants$^a$.

<table>
<thead>
<tr>
<th>Background</th>
<th>$mcpB$ Mutant</th>
<th>“$K_D$” (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OI1085</td>
<td>None - Wild-type $B. subtilis$</td>
<td>84</td>
</tr>
<tr>
<td>$\Delta 10 mcp$</td>
<td>None - Wild-type $mcpB$</td>
<td>460</td>
</tr>
<tr>
<td>$\Delta 10 mcp$</td>
<td>$mcpB_{(R111A)}$</td>
<td>8200</td>
</tr>
<tr>
<td>$\Delta 10 mcp$</td>
<td>$mcpB_{(Y121A)}$</td>
<td>2100</td>
</tr>
<tr>
<td>$\Delta 10 mcp$</td>
<td>$mcpB_{(Y133A)}$</td>
<td>11000</td>
</tr>
<tr>
<td>$\Delta 10 mcp$</td>
<td>$mcpB_{(Y133F)}$</td>
<td>460</td>
</tr>
</tbody>
</table>

$^a$ $K_D$ values determined by capillary sensitivity assays
Table 2. $K_D$ values of wild-type McpBS and McpBS mutants determined with ITC.

<table>
<thead>
<tr>
<th>McpBS Mutation</th>
<th>$K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None - Wild-type McpB</td>
<td>14</td>
</tr>
<tr>
<td>McpB R111A</td>
<td>na&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>McpB Y121A</td>
<td>280</td>
</tr>
<tr>
<td>McpB Y133A</td>
<td>na&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Binding was too low to obtain reliable data
Figure 1

McpBS (245 amino acids, 27.0 kDa)

S_{35}S---R_{111}---Y_{121}---Y_{133}---Q_{142}---T_{168}---F_{205}I_{206}---Y_{213}---H_{216}---KS_{279}
Figure 3

A

Dimerization Domain

90°

TM2    TM1
Membrane

B

Upper PAS Domain

Y121   R111   Y133   Q142

T160

Lower PAS Domain

I205   F205   Y213

H216
Figure 6

![Graph showing the relationship between time (min) and energy (μcal/sec) for different protein variants. The graph compares WT, R111A, Y121A, and Y133A proteins at varying [Asparagine]/[Protein] ratios.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ by guest on January 1, 2018
Figure 7

CD (mdeg)

Wavelength, nm
A pas domain binds asparagine in the chemotaxis receptor MCPB in bacillus subtilis
George D. Glekas, Richard M. Foster, Joseph R. Cates, Jeffrey A. Estrella, Michael J. Wawrzyniak, Christopher V. Rao and George W. Ordal

J. Biol. Chem. published online October 28, 2009

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