Activation of the CheA kinase by asparagine in Bacillus subtilis chemotaxis

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Past experiments have shown that CheA and CheY are required to generate smooth swimming signals in Bacillus subtilis chemotaxis. This study, as anticipated from in vivo experiments, demonstrates in vitro that an attractant-bound chemoreceptor leads to an increase in CheA activity, which in turn leads to an increase in the CheY-P pool that ultimately causes a behavioural change in the bacteria. Asparagine has been found to increase the rate of CheY-P formation in the presence of McpB-containing membranes, CheA, and an excess of CheY. This asparagine effect requires the presence of both CheA and McpB, the latter of which has been shown to be the sole receptor for this attractant. Utilizing membranes from a number of B. subtilis null mutant strains, insight has also been gained into the potential roles of a number of unique chemotaxis proteins in the regulation of CheA activity in the presence and absence of this attractant.

Keywords: Bacillus subtilis, signal transduction, kinase, bacterial chemotaxis

INTRODUCTION

Bacteria respond to a wide array of environmental stimuli. The molecular mechanism employed to control these responses consists of an autophosphorylating histidine kinase and a response-regulator protein. With the exception of CheA, NRIII, SpoIIiJ, DegS and FrzE, all of the histidine kinase proteins so far examined are transmembrane (Bourret et al., 1991; Stock et al., 1989). It is hypothesized that this group of proteins may contain an extracellular domain that senses an environmental ligand(s) and regulates the activity of the intracellular kinase domain. In the case of the cytoplasmic kinases, it is hypothesized that regulation may be achieved through a separate transmembrane receptor protein (Stock et al., 1989; Forst & Inouye, 1988).

Many of these histidine kinases have been shown to autophosphorylate in vitro and to transfer phosphoryl groups to their response-regulator protein. The majority of these regulator proteins alter the expression of specific sets of genes to allow the bacteria to cope with the environmental challenge that it is presented with (Bourret et al., 1991; Stock et al., 1989). Several of these response regulators have even been shown to be important in bacterial pathogenesis (Arico et al., 1989; Miller et al., 1989a, b). Although many of the components of the numerous bacterial two-component regulatory systems have been identified and characterized, there is no in vitro biochemical evidence confirming that small environmental (extracellular) ligands are capable of upregulating bacterial histidine kinase activity, either through direct binding of the transmembrane kinase or through binding of a separate receptor protein. What has been shown for several of these systems, however, is that some intracellular ligands can modulate the relative kinase/phosphatase activity that a particular kinase has toward its response-regulator protein (Ninfa & Magasanik, 1986; Makino et al., 1985; Igo et al., 1989).

Chemotaxis is a process that allows motile bacteria to migrate toward more favourable environments. By controlling swimming and tumbling events (produced by counterclockwise and clockwise flagellar rotation, respectively), the bacterium is able to travel toward increasing attractant concentrations and decreasing repellent concentrations.

Extensive study of the Escherichia coli and Salmonella typhimurium chemotaxis systems has greatly advanced our understanding of how this signal transduction system operates. In vitro, E. coli CheA has been shown to autophosphorylate by attaching the γ-phosphate of ATP to His-48 (Hess et al., 1987, 1988a). CheA-P has also been shown to donate phosphoryl groups to CheY (Hess et al., 1988b). CheY-P is thought to interact with the switch complex at the base of the bacterial flagella to...
cause clockwise rotation, or tumbling (Bourret et al., 1991; Stock et al., 1989). *Bacillus subtilis* CheA has also been shown to autophosphorylate on a histidine residue in *vitro*. Unlike its enteric counterpart, however, *B. subtilis* CheA is able to achieve its maximum phosphorylation potential at very low ATP concentrations (10 μM ATP for *B. subtilis* as opposed to 0.3 mM ATP for *E. coli* CheA). In addition, *B. subtilis* CheA-P is remarkably stable in the presence of ADP and other observations). In contrast to *E. coli* and *S. typhimurium*, CheA and CheY-P in *B. subtilis* are required for smooth swimming rather than tumbling (Bischoff & Ordal, 1991; Fuhrer & Ordal, 1991). In other words, cheA and cheY null mutants in the enterics are smooth swimming, while cheA and cheY null mutants in *B. subtilis* are tumbly.

Regulation of kinase activity in bacterial two-component signal-transduction systems is not fully understood. One goal of these studies is to understand how CheA activity is regulated according to the signalling state of the various methyl-accepting chemotaxis proteins (MCPs, attractant- or repellent-bound). The enteric CheA has been shown to have greatly increased activity in the presence of unliganded MCPs and CheW (Borkovich & Simon, 1990; Ninfa et al., 1991). Studies on the enteric chemotaxis system have also shown that CheA activity is decreased in the presence of attractant-bound MCPs and CheW (Borkovich & Simon, 1990; Ninfa et al., 1991). This result is consistent with the current model for the enteric chemotaxis system: as the MCP binds the attractant, CheA activity is decreased. Subsequently, the bacteria undergo a period of smooth swimming as CheY-P levels decrease.

What has not been reported for the enteric chemotaxis system is that a repellent-bound MCP leads to an increase in CheA activity greater than that seen in the presence of an unliganded MCP. One may predict such an increase to explain the increase in tumbling seen upon the addition of a repellent. Therefore, the question still remains: can MCPs bind with an extracellular ligand increase the activity of CheA in the bacterial chemotaxis system?

We have long hypothesized that attractant-bound MCPs lead to an increase in CheA activity in *B. subtilis*, in turn leading to an increase in the CheY-P pool that ultimately causes a behavioural change in the bacteria (Garrity & Ordal, 1995). Here, we report the development of an *in vitro* system that has allowed us to demonstrate this hypothesis. Utilizing membranes from a number of *B. subtilis* null mutant strains, we have gained insight into the potential roles of various chemotaxis proteins in the regulation of CheA activity in the presence and absence of attractants.

**METHODS**

**Materials.** Bacterial growth media were purchased from Difco. Disodium ATP was obtained from Sigma. [γ-32P]ATP (5000 Ci mmol⁻¹; 185 TBq mmol⁻¹) and the ECL Western blot kit were purchased from Amersham Life Sciences.

**Preparation of bacterial membranes.** Overnight cultures (20 ml) of various *B. subtilis* strains were grown at 37 °C in the presence of the appropriate antibiotic (5 μg chloramphenicol ml⁻¹ or 10 μg kanamycin ml⁻¹) in Lbr (1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl). The next morning, these cultures were diluted to 1 litre in fresh medium with drug and grown with shaking at 37 °C to late-exponential phase to ensure maximal expression of MCPs (~10 h). The cultures were immediately harvested and washed once in 200 ml PCME buffer (100 mM sodium phosphate pH 7.0, 250 μg chloramphenicol ml⁻¹, 1 mM EDTA, 2 mM KCl). The cells were pelleted by centrifugation at 8000 g for 15 min at 4 °C and washed in 200 ml TKMD buffer (50 mM Tris/HCl pH 8.0, 5 mM MgCl₂, 50 mM KCl, 62 mM dithiothreitol, 10%, w/w, glycerol). The cells were pelleted as before and resuspended in 20 ml of fresh TKMD buffer with 1 mM PMSF. The cell suspension was lysed by sonication, and cell debris was removed by centrifugation at 10000 g for 30 min at 4 °C.

Membranes were isolated by centrifugation of the resulting supernatant at 50000 r.p.m. overnight at 4 °C in an L-5 75 ultracentrifuge (Beckman) using a 70-Ti rotor. Membranes were resuspended in 3 ml fresh TKMD/PMFS buffer with mild homogenization and stored in small aliquots at −70 °C.

**Quantification of CheA in membrane fractions.** The concentration of total protein in the various membrane preparations was determined with the Coomassie Protein Assay Reagent (Pierce) using bovine serum albumin as a standard.

**Assay for CheA activity.** CheA-containing membrane fractions were incubated with 50 μM [γ-32P]ATP (~15000 c.p.m. pmol⁻¹) and 200 pmol CheY in the presence or absence of various concentrations of asparagine at room temperature. Reaction volumes ranged from 50 to 75 μl (see figure legends). At various times, aliquots of 20 μl were removed and added to 10 μl SDS/EDTA buffer (0.25 M Tris/HCl pH 6.8, 8% SDS, 20% glycerol, 25 mM EDTA) to stop the reactions. Reactions were run on 20% SDS polyacrylamide gels and washed briefly in 250 ml phosphate-buffered saline (2 mM NaH₂PO₄, 18 mM Na₂HPO₄, 0.15 M NaCl, pH 7.3) to reduce background. The gels were dried and exposed to X-ray film for 4 h.

**Preparation of GST-CheA-bound glutathione beads.** An overnight culture of strain O13426 (E. coli Tgl [K12, Δ(lac-pro) supE thi hsdD5/ftr4D36 proA' B' lacI2Δ215]) expressing the full-length CheA protein fused at the C-terminus with the C-terminal portion of glutathione S-transferase, GST) was grown at 37 °C in Lbr with 100 μg ampicillin ml⁻¹. The culture was diluted 1:10 in fresh Lbr with ampicillin and grown at 37 °C for 1 h. IPTG was added to a final concentration of 0.1 mM and the culture was harvested 4 h later. The cells were resuspended in MTPBS (150 mM
NaCl, 16 mM NaH_2PO_4, 4 mM NaHPO_4, pH 7.3) and lysed by sonication. Triton X-100 was added to 0.1% and the cell lysate was clarified by centrifugation at 10000 g for 30 min at 4 °C. A 50% glutathione bead slurry in MTPBS was added to the supernatant and the CheA-GST was allowed to bind to the beads for 30 min with gentle rocking at room temperature. The glutathione beads were pelleted by centrifugation at 500 g for 5 min and washed three times in 50 ml MTPBS. Following the last centrifugation step, the GST-CheA-bound beads were resuspended in 1 ml TKMD/PMFS buffer.

Binding/activity assay for GST-CheA. Glutathione beads containing 30 pmol bound GST-CheA were incubated with 45 µg whole cell lysate (extract and membranes) from several B. subtilis chemotaxis null mutant strains in TKMD/PMFS buffer. Binding was allowed to proceed for 1 h at room temperature with gentle rocking to keep the beads resuspended. The beads were centrifuged for 10 s, and the supernatant containing unbound protein was drawn off with a Pasteur pipette. The beads were washed with 1 ml TKMD buffer and resuspended in 50 µl fresh TKMD/PMFS buffer. [γ-^32P]ATP was added (0.6 mM, ~ 8000 c.p.m. pmol^-1) and the reactions were stopped by adding 4 x SDS/EDTA buffer. The reactions were run out on a 12.5% SDS polyacrylamide gel and exposed to X-ray film for 3 h. GST-CheA-P was quantified by scintillation counting after being excised from the gel.

RESULTS

Quantification of CheA in membrane fractions

Past experiments revealed that B. subtilis CheA had affinity for the membrane, since significant amounts of the kinase were detected even in salt-washed membrane preparations (unpublished results). With this in mind, we felt that we may be able to isolate a membrane-CheA complex capable of activation by attractants if care was taken not to remove membrane-associated proteins. Chemiluminescent Western blots revealed that membrane fractions from a number of different strains (except for ΔcheA) contained roughly the same amount of CheA per mg membrane protein (Fig. 1, Table 1). The mean value was 1268 ± 348 ng CheA (mg membrane protein)^-1.

![Fig. 1. Quantification of CheA in membrane fractions. Western blots were performed as described in Methods. (a) Pure CheA standards: 100 ng (lane 1), 75 ng (lane 2), 50 ng (lane 3), 25 ng (lane 4). (b) Membranes from various null mutant strains. Lane designations: wild-type (1), ΔcheA (2), ΔmcpB (3), Δ(mcpA-mcpB-tlpA-tlpB), ameE::mcpB (4), ΔcheD (5), ΔcheC (6), ΔcheW (7), ΔcheV (8), ΔcheR (9), ΔcheB (10), Δ(cheW-cheV) (11).](image-url)
Table 1. CheA activation in null mutant membrane fractions

Membranes from the various strains containing 1-65 pmol CheA were incubated with 50 μM [$\gamma$-32P]ATP (~18000 c.p.m. pmol$^{-1}$) and 200 pmol CheY with or without 1 mM asparagine in a total of 75 μl TKMD buffer at room temperature. At 10 s intervals, 20 μl of each reaction was removed and stopped in 10 μl 4x SDS/EDTA buffer. CheY-P was quantified by SDS-PAGE and scintillation counting. The fold increase in CheA activity (CheY-P$^+$/CheY-P$^-$) was determined by dividing CheY-P (c.p.m.) in the reaction with asparagine by CheY-P (c.p.m.) in the absence of asparagine for each membrane at each timepoint. The data are means of two experiments. The maximum CheY-P (c.p.m.) observed in the presence and absence of asparagine (columns 6 and 5, respectively) is the mean value of CheY-P produced at the final timepoint (30 s) for each membrane.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
<th>CheA</th>
<th>Max. CheY-P (c.p.m.)</th>
<th>Max. CheY-P (c.p.m.)</th>
<th>CheY-P$^+$/CheY-P$^-$</th>
</tr>
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<tbody>
<tr>
<td>OI1085</td>
<td>che+ hisH2 metC trpF7</td>
<td>Ordal et al. (1977)</td>
<td>780</td>
<td>1474</td>
<td>2844</td>
<td>1.93±0.36</td>
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<tr>
<td>OI3056</td>
<td>ΔmcpB</td>
<td>Hanlon &amp; Ordal (1994)</td>
<td>1660</td>
<td>552</td>
<td>563</td>
<td>*</td>
</tr>
<tr>
<td>OI3184</td>
<td>Δ(mcpB-mcpA-tpA-tpB) amyE::mcpB</td>
<td>This work</td>
<td>1340</td>
<td>769</td>
<td>1707</td>
<td>2.22±0.13</td>
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<tr>
<td>OI2934</td>
<td>ΔcheD</td>
<td>Rosario et al. (1995)</td>
<td>1400</td>
<td>466</td>
<td>1327</td>
<td>2.85±0.62</td>
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<tr>
<td>OI3135</td>
<td>ΔcheC</td>
<td>Rosario et al. (1995)</td>
<td>1700</td>
<td>1530</td>
<td>2693</td>
<td>1.76±0.22</td>
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<tr>
<td>OI2737</td>
<td>ΔcheW</td>
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<td>840</td>
<td>864</td>
<td>867</td>
<td>*</td>
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<tr>
<td>OI3155</td>
<td>ΔcheV</td>
<td>Rosario et al. (1994)</td>
<td>960</td>
<td>1064</td>
<td>1608</td>
<td>1.51±0.20</td>
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<td>OI2652</td>
<td>ΔcheR</td>
<td>Kirsch et al. (1993b)</td>
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<td>953</td>
<td>1516</td>
<td>1.59±0.28</td>
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<tr>
<td>OI2836</td>
<td>ΔcheB</td>
<td>Kirsch et al. (1993b)</td>
<td>1700</td>
<td>1127</td>
<td>1725</td>
<td>1.53±0.22</td>
</tr>
<tr>
<td>OI3157</td>
<td>Δ(cheW, cheV)</td>
<td>Rosario et al. (1994)</td>
<td>1140</td>
<td>918</td>
<td>908</td>
<td>*</td>
</tr>
</tbody>
</table>

* No detected increase in CheY-P formation in the presence of asparagine.

Fig. 3. Determination of the apparent $K_d$ for the asparagine-McpB complex. CheA-containing wild-type membranes (1-1 pmol CheA) were incubated with 50 μM [$\gamma$-32P]ATP (~15000 c.p.m. pmol$^{-1}$), 200 pmol CheY, and varying concentrations of asparagine in TKMD buffer at room temperature in a total volume of 50 μl for 15 s. The reaction was stopped by adding 25 μl 4x SDS/EDTA buffer. Reactions were separated on a 20% SDS polyacrylamide gel and exposed to X-ray film for 4 h. The CheY-P band was excised from the gel and quantified by scintillation counting. The data are means of two experiments. The data points were fitted to a third-order polynomial. The apparent $K_d$ value for the asparagine-McpB complex was judged to be the concentration of asparagine that gave half-maximal CheY-P in the assay.

Fig. 2. Increases in the rate of CheY-P formation require the presence of both CheA and McpB in the membrane fraction. CheA-containing membranes from different strains (1-1 pmol CheA) were incubated with 50 μM [$\gamma$-32P]ATP (~15000 c.p.m. pmol$^{-1}$) and 200 pmol CheY, with (hatched bars) and without (solid bars) 1 mM asparagine for 15 s in a total volume of 50 μl TKMD buffer. SDS/EDTA solubilizer was added to stop the reaction, and CheY-P was quantified by SDS-PAGE and liquid scintillation counting as described in Methods. Background was determined from the same reaction performed on the ΔcheA membrane. The wild-type strain is OI1085. The mutant strain is OI3056 (ΔmcpB). The strain complemented for mcpB is OI3184 Δ(mcpB-mcpA-tpA-tpB) amyE::mcpB.
**Fig. 4.** Inhibition of GST-CheA autophosphorylation by ΔcheW and ΔcheD cell lysates. The GST-CheA binding/activity assay was performed as described in Methods. GST-CheA bound to glutathione beads (~3.5 µg) was incubated with wild-type (○), ΔcheW (△), or ΔcheD (■) cell lysates, or with the same volume (130 µl) of TKMD/PMSF buffer (△) at room temperature for 1 h. The GST-CheA was pelleted, washed, and resuspended in 40 µl TKMD/PMSF buffer. [γ-32P]ATP was added (0.5 mM, ~6000 c.p.m. pmol⁻¹), and 12 µl aliquots were removed at 10 s, 15 s and 20 s and stopped in 10 µl 4 × SDS/EDTA buffer. GST-CheA-P was quantified by SDS-PAGE and liquid scintillation counting as described in Methods. The data are means of two experiments.

**Fig. 5.** Inhibition of GST-CheA autophosphorylation by ΔcheD cell lysate is dose-dependent. GST-CheA-bound glutathione beads (~25 pmol GST-CheA) were incubated with increasing amounts of the ΔcheD cell lysate at room temperature for 1 h. GST-CheA was collected by centrifugation, washed, and resuspended in 22 µl TKMD/PMSF buffer. [γ-32P]ATP (0.6 mM, ~5000 c.p.m. pmol⁻¹) was added and the reaction was stopped 15 s later by adding 12 µl 4 × SDS/EDTA buffer. GST-CheA-P was analysed by SDS-PAGE and liquid scintillation counting as described in Methods.

**ΔcheW and ΔcheD cell lysates inhibit autophosphorylation of GST-CheA**

Although the membrane-associated CheA activation assay just described is useful in measuring relative increases in CheA activity in the presence of asparagine, we could not be sure that the CheA pool in each of the isolated membrane fractions was equally active in the absence of asparagine. Utilizing a slightly different approach to test the effect of various null mutations on CheA autophosphorylation in the absence of chemoeffectors, cell lysates (extract plus membranes) from a number of different *B. subtilis* strains were incubated with GST-CheA-bound glutathione beads. After allowing 1 h for binding, GST-CheA was pelleted, washed, and assayed directly for autophosphorylation activity.

Cell lysate from two of these strains, ΔcheW and ΔcheD, inhibited GST-CheA autophosphorylation (Fig. 4). The inhibition was dose-dependent, reducing GST-CheA activity by as much as 60% (Fig. 5). Coomassie stains of these gels showed that the reduced activity of GST-CheA in these assays was not due to proteolysis of the fusion protein (data not shown). There was no effect on GST-CheA autophosphorylation by any of the other tested null mutant cell lysates (data not shown).

**DISCUSSION**

Since membranes from strains with varying degrees of MCP methylation contained roughly the same amount of CheA (Table 1), it could be the case that much of the affinity that *B. subtilis* CheA has for the membrane is non-specific (MCP independent). After quantifying the amount of CheA in the various membrane fractions, it was possible to test whether the activity of the kinase was capable of being modified by a chemoeffector. We used the attractant asparagine, taxis to which is known to be mediated solely through McpB (Hanlon & Ordal, 1994). We chose to measure increases in CheA activity through the formation of CheY-P. As long as CheY is in excess in these experiments, CheA autophosphorylation becomes the rate-limiting step in the production of CheY-P. Therefore, changes in the rate of CheY-P production will reflect changes in CheA activity (Ames & Parkinson, 1994).

CheA-containing wild-type membranes showed a doubling in the rate of CheY-P formation in the presence of asparagine. This effect was seen only in membranes containing both CheA and McpB, evidence that asparagine-bound McpB is leading to an increase in CheA activity (Fig. 2, Table 1). The increased level of CheY-P was not due to enhanced CheY-P stability in the presence of asparagine, since the rate of spontaneous hydrolysis of CheY-P was the same in the presence or absence of membranes and the attractant (data not shown). The apparent *Kₐ* for the asparagine–McpB complex appears to be about 20 µM, as judged by the concentration of asparagine required for half-maximal CheY-P production in the assay (Fig. 3). This agrees well with the ‘*Kₐ*’ for the asparagine–McpB complex determined using modified behavioural capillary assays, which appear to reflect true *Kₐ* values (Hazelbauer &
CheA-containing membranes from seven additional B. subtilis null mutant strains were tested for asparagine-induced enhancement of CheA activity. Five of these exhibited this ability to varying degrees, while two were completely deficient in this regard (Table 1). Does the methylation state of McpB affect its ability to increase CheA activity in the presence of asparagine? Membranes from the ΔcheR and ΔcheB strains showed the same relative increase in the rate of CheY-P formation in the presence of asparagine. MCPs in the ΔcheR strain are not methylated, while those of the ΔcheB strain are highly methylated in B. subtilis (Kirsch et al., 1993a, b). Although these mutants have slightly different tumble biases, the ΔcheR, ΔcheB and Δ(cheR-cheB) strains all respond to attractants similarly (Kirsch et al., 1993a, b). It appears, then, that the degree of methylation of McpB does not play a major role in the ability of the receptor to increase CheA activity in the presence of high concentrations of asparagine.

CheC and CheD are chemotaxis proteins unique to B. subtilis. The cheC null mutant has a high smooth swimming bias, which becomes even higher upon addition of attractants (Rosario et al., 1995). This phenotype suggests that CheA activity may be unusually high in this strain in the absence of chemoeffectors. One might therefore expect that CheA activity would be highest in the ΔcheC membrane in the absence of asparagine. Although the basal level (no asparagine) of CheA activity was highest in the ΔcheC membrane, it was not significantly higher than the CheA activity observed in the wild-type membrane in the absence of asparagine (Table 1, column 5). Because of this, we cannot confidently conclude that CheC plays a direct role in downregulating CheA activity in the absence of chemoeffectors. We can conclude, however, that CheC does not affect the ability of McpB to increase CheA activity in the presence of high concentrations of asparagine, since the amount of CheY-P produced by the ΔcheC membrane increased significantly in the presence of asparagine (Table 1).

Tethering analysis reveals that the cheD null mutant is very tumbly in the absence of chemoeffectors, suggesting that CheA activity may be unusually low in this strain in the absence of chemoeffectors (Rosario et al., 1995). Not surprisingly, CheA-containing membranes from this mutant were found to produce very little CheY-P in the absence of asparagine, suggesting that CheA may be inhibited in this strain (Table 1). This idea is further supported by the fact that the GST-CheA fusion protein had decreased activity after incubation with a ΔcheD cell lysate (Figs 4 and 5). Yet the ΔcheD membrane showed the highest relative increase in CheA activity in the presence of asparagine (Table 1). This result is consistent with in vivo behavioural studies on this strain which show that it does excite (swim smoothly) upon the addition of asparagine (J. R. Kirby & G. W. Ordal, unpublished). Therefore, it appears that CheD serves to upregulate CheA activity in the absence of chemoeffectors.

CheV is another chemotaxis protein unique to B. subtilis, which shows homology with both CheW and CheY from B. subtilis and E. coli (Rosario et al., 1994). The activity of CheA in the ΔcheV membrane in the presence or absence of asparagine is almost identical to that of the ΔcheR and ΔcheB membranes. Like these MCP methylation mutants, the cheV null mutant strain also responds to attractants (Rosario et al., 1994). The MCPs of the ΔcheV null mutant are methylated at wild-type levels, however, suggesting that CheV does not affect CheA activity by influencing the MCP methylation state (Rosario et al., 1994). Membranes lacking both CheW and CheV, or CheW alone, are incapable of enhancing CheA activity in the presence of asparagine (Table 1). Assays on the GST–CheA fusion protein suggest that the absence of CheW may allow another cellular component to inhibit CheA in vitro (Fig. 4). Unlike the ΔcheD membrane, however, the ΔcheW membrane is incapable of activating CheA in the presence of asparagine (Table 1). This is reminiscent of in vitro studies on the E. coli chemotaxis system, where CheW was found to couple CheA activity to the signalling state of the MCPs (Borkovich & Simon, 1990; Ninfa et al., 1991). These data are also consistent with the behaviour of the B. subtilis Δ(cheW-ΔcheV) mutant: tumbly and unable to respond to attractants (Rosario et al., 1994). Similar to the enteric system, McpB appears to require a ‘coupling’ protein to be able to increase CheA activity. One limitation of this membrane-associated CheA activation assay is that the amount of increase in CheA activity in the presence of asparagine-bound McpB cannot be calculated accurately. Although the amount of CheA in each membrane fraction was quantified, there is no reliable method to determine how much CheA is bound to McpB and, therefore, contributing to the increase in the rate of CheY-P formation. It is possible that only a small percentage of the CheA in these assays is responsible for this, perhaps explaining why CheY-P levels do not increase by an order of magnitude in this in vitro assay in the presence of asparagine. Since the rate of autophosphorylation of the activated form of CheA is thought to be on the millisecond timescale, we do not claim that the 15 s timepoints used in this assay necessarily reflect the initial rate of the B. subtilis CheA autophosphorylation reaction. Rather, these experiments demonstrate that CheA activity is statistically higher in the presence of McpB and its known environmental ligand, asparagine. This is the first in vitro biochemical evidence suggesting that environmental (extracellular) ligands are capable of upregulating bacterial two-component kinase activity. How might asparagine-bound McpB lead to an increase in CheA activity, as judged by an increase in the rate of CheY-P production? Several explanations are possible.

(i) The ligand-bound receptor may increase the rate of CheA autophosphorylation directly. (ii) The ligand-bound receptor may promote the formation of
MCP–CheA complexes that have intrinsically higher activity than the ‘open’ form of CheA, as has been shown for the enterics (Borkovich & Simon, 1990; Ninfa et al., 1991). (iii) The ligand-bound receptor may sequester another component(s) that normally act to inhibit CheA. (iv) The ligand-bound receptor may increase the rate of transfer of phosphoryl groups from CheA-P to CheY. Finally, it is also possible that the ligand-bound receptor sequesters another component(s) that normally works to enhance the rate of CheY-P dephosphorylation. This too would manifest itself as an increase in the rate of CheY-P production in this assay. Further experiments are being conducted to distinguish between these possibilities.

REFERENCES


