The Accessibility of Cys-120 in $CheA_S$ Is Important for the Binding of CheZ and Enhancement of CheZ Phosphatase Activity†

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ABSTRACT: The *cheA* gene of *Escherichia coli* encodes two proteins from in-frame tandem translation start sites. The long form of CheA ($CheA_L$) is the histidine kinase responsible for phosphorylating the response regulator, CheY. The short form of CheA ($CheA_S$) is identical in domain structure to $CheA_L$ except that it is missing the first 97 amino acids. Reduced $CheA_S$ bound to and enhanced the activity of the phosphatase of phospho-CheY, CheZ. Oxidized $CheA_S$ was unable to interact with CheZ. Oxidized $CheA_S$ formed covalent dimers, whereas $CheA_L$ did not. This property was believed to be the result of an intermolecular disulfide bond. The CheA proteins contain three cysteine residues, one of which likely lies within the CheZ binding region of $CheA_S$ and is exposed to solvent. We identified the CheZ binding domain of $CheA_S$ by testing the various fragments of $CheA_S$ that contain cysteine residues for CheZ binding activity in an ELISA-based $CheA_S$−CheZ binding assay. Fragments of $CheA_S$ lacking the truncated P1 domain of $CheA_S$ ('P1) were unable to bind CheZ. We also found that a fusion of the first 42 amino acids of $CheA_S$ ('P1 domain) to GST bound CheZ and enhanced its activity. The interaction between the GST−CheA[98−139] fusion protein and CheZ was dependent on the accessibility of a cysteine residue (Cys-120) located in the 'P1 domain.

The *cheA* gene is central to the regulation of chemotaxis in *Escherichia coli* (*1*−*4*). The *cheA* gene encodes two polypeptides from in-frame tandem translational start sites (*5*). The long form of CheA ($CheA_L$) is a histidine kinase responsible for the phosphorylation of the response regulator CheY (*6*). $CheA_L$ exists as either a homodimer or a heterodimer with the short form of CheA ($CheA_S$) (*7*, *8*). $CheA_L$ is coupled to the chemoreceptors, and its activity is regulated by these receptors in response to the chemical environment. The receptors normally stimulate $CheA_L$ activity, which in the presence of attractants inhibit this activity, and repellents appear to enhance the stimulation of $CheA_L$ activity (*1*). When $CheA_L$ becomes stimulated, it hydrolyzes ATP and transfers the γ-phosphate from ATP to a histidine residue (His-48) in the N-terminal phosphotransfer domain, P1, of $CheA_L$ (*9*−*12*). The phosphate group is then transferred to a CheY molecule that is docked at the P2 domain of $CheA_L$ (*6*, *13*, *14*). Phospho-CheY (CheY∼P)[1] then leaves $CheA_L$ and binds to the flagellar motor switch complex. This interaction increases the probability of counterclockwise to clockwise reversal of flagellar rotation, thus leading to a tumble event that allows the bacteria to change the direction in which that are swimming (*1*, *3*).

$CheA_S$ is identical to $CheA_L$, except that it lacks the first 97 amino acid residues of $CheA_L$, which includes the site of autophosphorylation, His-48 (*5*, *15*, *16*). Therefore, $CheA_S$ is unable to undergo autophosphorylation and subsequent phosphotransfer to CheY. Besides the P1 domain, $CheA_S$ retains all of the functional domains present in $CheA_L$. $CheA_S$ has been shown to be capable of transphosphorylating $CheA_L$ subunits that have a defective kinase domain, and it can also complement $CheA_L$ subunits that lack the C-terminal receptor coupling domain (*17*−*19*). Reduced $CheA_S$ was shown to interact with and enhance the activity of the CheY∼P phosphatase, CheZ (*20*). Under the conditions that were tested, CheZ binding activity was not observed with $CheA_L$. The oxidized form of $CheA_S$ failed to interact with CheZ and enhance its activity. $CheA_S$ formed a covalent dimer via intermolecular disulfide bonds upon oxidation, which was believed to occlude CheZ from its binding site on $CheA_S$ (*20*). These results suggested there is a solvent-exposed cysteine within the region of $CheA_S$ that binds CheZ. There are three cysteine residues in both CheA proteins. $CheA_L$ failed to undergo the oxidation-dependent covalent dimerization, therefore suggesting the cysteines in $CheA_L$ are not accessible or in the correct conformation for disulfide bond formation to occur (*20*).

One possibility for the difference between $CheA_S$ and $CheA_L$ in CheZ binding is that the first 97 amino acids of $CheA_L$ mask the CheZ binding site found in $CheA_S$. The masking could occur within the P1 domain itself, or somewhere else in $CheA_L$ through interactions between P1 and the other domains of the CheA proteins. The masking of the CheZ binding site could also mask the reactive cysteine residue that is responsible for the oxidation-dependent inhibition of CheZ binding activity of $CheA_S$. We tested the

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[1] Abbreviations: CheY∼P, phospho-CheY; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; ORF, open reading frame; GST, glutathione *S*-transferase; PICM, protein interaction by cysteine modification; $t_{1/2}$, half-life; 5-FM, fluorescein 5-maleimide; IAM, iodoacetamide.

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Table 1: Plasmids and Primers Used in This Study

plasmid	description	source
pET24a(+)	Kan^r, overproduction of proteins from P_{T7}	Novagen
pGEX-6p-1	Pen^r, overexpression of GST fusion proteins	Amersham
pDV4[a]	Kan^r, $CheA_L$/CheW overproduction	lab collection
pDM011[b]	Kan^r, CheY(WT) overproduction	lab collection
pTM22	Pen^r, CheA[124−257] overproduction	J. S. Parkinson
pTM54	Pen^r, GST−CheA[252−654] overproduction	J. S. Parkinson
pSN58[a]	Cam^r, *cheA*(C120S)	J. S. Parkinson
pAR1.cheA$_S$[a]	Pen^r, $CheA_S$ overproduction	A. Wolfe
pOC100[b]	Kan^r, CheZ overproduction	this study
pOC300[b]	Kan^r, $CheA_S$ overproduction	this study
pOC339[b]	Kan^r, $CheA_S$[98−315] overproduction	this study
pOC803[c]	Pen^r, GST−$CheA_S$[98−315]	this study
pOC804[c]	Pen^r, GST−$CheA_S$[98−139]	this study
pOC805[c]	Pen^r, GST−$CheA_S$[98−150]	this study
pOC806[c]	Pen^r, GST−$CheA_S$[98−247]	this study
pOC808[c]	Pen^r, GST−$CheA_S$[98−117]	this study
pOC809[c]	Pen^r, GST−$CheA_S$[98−127]	this study
pOC810[c]	Pen^r, GST−$CheA_S$C120S[98−139]	this study

primer	sequence
CJ1	5′-CCCCCCCATATGCAAGAACAGCTCGACGCTTAT-3′
CJ2	5′-CCCCCCAAGCTTACTCATTCAGGCGGCGGTGTTC-3′
AsGST-1	5′-CCCCCCGGATCCCATATGCAAGAACAGCTCGACGCTTAT-3′
AsGST-2	5′-CCCCCCCTCGAGACTCATTCAGGCGGCGGTGTTC-3′
GST-P1	5′-CCCCCCCTCGAGACTCACACTTAATCGGGTCAC-3′
GST-L1	5′-CCCCCCCTCGAGACTCTGCTCATCTTGCGGTTC-3′
GST-P2	5′-CCCCCCCTCGAGACTCCGCTCCACGCGGCCGGT-3′
P1−21stop	5′-CCAGCTTCGATTAAATCTGCCAGGC-3′
P1−21stopR	5′-GCCTGGCAGATTTAATCGAAGCTGG-3′
Z-1	5′-GAGGTAGCGCATATGATGCAACCA-3′
Z-2	5′-CATCAGGCATCTCGAGTCAAAATCC-3′

[a] These plasmids were used primarily as templates for PCR. [b] pET24a(+) derivative. PCR products were cloned into the *Nde*I−*Hin*dIII sites of pET24a(+). [c] pGEX-6p-1 derivative. PCR products were cloned into the *Bam*HI−*Eco*RI sites of pGEX-6p-1.

various fragments of $CheA_S$ that contain cysteine residues for their ability to bind CheZ. Both the CheZ binding and activity enhancing functions of $CheA_S$ were located in its truncated P1 domain ('P1). We also show that the accessibility of the cysteine residue (Cys-120) in 'P1 is important for the interaction between 'P1 and CheZ.

MATERIALS AND METHODS

Strains and Materials. E. coli strain BL21(λDE3) (Novagen) was used in this study for cloning and overexpression of proteins. It contains a gene encoding T7 polymerase under the control of the *tac* promoter and is *che*$^-$. Cells were grown in Luria broth (LB) at 25 °C for protein overexpression of $CheA_S$ and CheA fragments. Isopropyl β-D-thiogalactopyranoside (IPTG) (Anatrace) was used at a concentration of 1 mM for protein overexpression. Antibiotics were purchased from Sigma and used at the following concentrations: penicillin (100 μg/mL) and kanamycin (50 μg/mL). Fluorescein 5-maleimide (Pierce) at a final concentration of 415 μM and various concentrations of iodoacetamide (Sigma) were used for PICM experiments (see below).

Plasmids. Plasmids and primers used in this study are listed in Table 1. The pET24a(+) plasmid (Novagen) was used for cloning and overexpression of $CheA_S$ proteins. The polymerase chain reaction (PCR) was used to generate *cheA*$_S$ open reading frames (ORFs) for cloning into the appropriate vectors. The template used for wild-type *cheA*$_S$ and *cheA*$_S$ fragments was the *cheA* gene located in pDV4. The *cheA*$_S$-wt ORF was created by PCR using primers CJ1 and CJ2 and cloned into the *Nde*I−*Hin*dIII site of pET24a(+) to create pOC300. Plasmid pOC339 (*cheA*[98−315]) was created by amplifying the *cheA*$_S$ ORF, using primers CJ1 and CJ2, from a derivative of pAR1.*cheA*$_S$ that contains an amber mutation at codon 316 (we number the codons for *cheA*$_S$ on the basis of the sequence of *cheA*$_L$ for simplicity). For *cheA*$_S$ alleles that contained the cysteine to serine mutation at *cheA*$_S$ codon 120, we used the pSN58 plasmid, which contains *cheA*-C120S (gift from J. S. Parkinson) as the template for PCR.

We created glutathione *S*-transferase (GST) fusions of wild-type $CheA_S$ and $CheA_S$ fragments by cloning *cheA* PCR products into the *Bam*HI−*Xho*I site of pGEX-6p-1 (Amersham Pharmacia). Primers AsGST-1 and AsGST-2 were used to amplify the wild-type *cheA*$_S$ ORF, and this product was cloned into pGEX-6p-1 to create pOC800 (see Table 1). For generation of GST fusions to fragments of $CheA_S$, the AsGST-1 primer was used in combination with the following primers (in bold) to create the following derivatives of pGEX-6p-1 (in parentheses): **GST-P1** for GST−CheA[98−139] (pOC804) and GST−CheA[98−139]C120S (pOC810), **GST-L1** for GST−CheA[98−150] (pOC805), and **GST-P2** for GST−CheA[98−247] (pOC806). Plasmid pOC808 (GST−CheA[98−117]) was created by site-directed mutagenesis of pOC800. Codon 118 was changed to a stop codon using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions using primers P1−21stop and P1−21stopR.

The P2 domain of CheA and the GST−CheA[252−654] fusion protein were expressed from plasmids pTM22 and pTM54, respectively (gifts from J. S. Parkinson). Plasmid

pDV4 overproduces $CheA_L$ from a p_{trp} promoter. CheY was overexpressed from pDM011 which is a pET24a(+) derivative containing *cheY*. Plasmid pOC100 is a pET24a(+) derivative that contains *cheZ*; the gene was cloned in the same way as $cheA_S$ for the plasmids mentioned above. A PCR product of *cheZ* was created by using primers Z1 and Z2 using pRL22 as the template, and was cloned into the *Nde*I−*Xho*I site of pET24a(+).

Protein Purification. To overexpress wild-type and variant $CheA_S$ proteins, BL21(λDE3) cells containing the $cheA_S$ plasmids described above were grown in LB to an OD_{600} of 0.4−0.6 at 27 °C. To induce protein overproduction, 1 mM IPTG was added and cultures were allowed to grow for an additional 6−8 h. Cells were collected and resuspended in TEDG [50 mM Tris (pH 7.9), 1 mM EDTA, 2 mM DTT, and 10% glycerol]. Cells were disrupted by sonication, and cellular debris was cleared from the lysate by centrifugation at 15 000 rpm for 30 min. S-30 lysates were stored at −80 °C. Wild-type $CheA_S$ was purified as previously described (*20*). GST fusions to various $CheA_S$ fragments were purified by affinity purification on a glutathione−agarose affinity column. After being extensively washed, the $CheA_S$ fusion proteins were eluted from the column with 30 mM glutathione in 50 mM Tris (pH 8.0). Proteins were dialyzed against 50 mM Tris (pH 7.5) to remove free glutathione, and protein purity was assessed by SDS−PAGE.

CheZ was overexpressed in BL21(λDE3) cells containing pOC100, and was purified as previously described with some minor modifications (*20*). Cells were grown to an OD_{600} of 0.4−0.6 at 37 °C. CheZ was induced by the addition of 1 mM IPTG, and cells were allowed to grow for 4 h at 37 °C. Cells were collected and resuspended in 50 mM Tris (pH 7.9), and S-30 lysates were made as mentioned above. CheZ was partially purified by DEAE column chromatography by eluting proteins from the column with a 800 mL NaCl gradient (from 0.2 to 0.4 M) in Bis-Tris buffer. Fractions containing CheZ were pooled and concentrated. CheZ was further purified by size exclusion chromatography on a G-50 Sephadex column.

CheY was overexpressed from pDM011 as described above for CheZ and purified as previously described (*21*).

ELISA-Based $CheA_S$−CheZ Binding Assay. To detect binding of CheZ to wild-type and various $CheA_S$ peptides, an ELISA-based assay was used. This assay was similar to the CheA−CheY binding assay described by Shukla and Matsumura (*22*). Briefly, Maxisorp 96-well microtiter plates (Nunc) were coated with 2-fold serially diluted monoclonal anti-CheA antibodies (starting concentration of 1 μg/well) and incubated overnight at 4 °C. The antibodies that were used were from monoclonal clones CA1.42 and CA1.177. CA1.42 binds to the P4 domain of CheA and does not block the interaction between $CheA_S$ and CheZ as previously described (*20*). CA1.177 is specific for the P2 domain of CheA and does not block the interaction between $CheA_S$ and CheZ. After being coated overnight, plates were blocked overnight in blocking buffer (1% BSA in PBS). Proteins were added to blocked plates in sequential order: wild-type and variant $CheA_S$ proteins (10 μg/well), CheZ (1 μg/well), polyclonal rabbit anti-CheZ (100 ng/well), and anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution). Each addition of protein was followed by a 1 h incubation at room temperature. Complexes were detected by adding 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma), and the reaction was stopped with 1 N HCl. Plates were read with a Bio-Tek Instruments microplate reader at a wavelength of 450 nm.

GST−$CheA_S$ Coprecipitation Assay. To assay CheZ binding by small $CheA_S$ fragments (i.e., CheA[98−139]), we used a GST coprecipitation assay similar to the assay described by Tang et al. (*23*). Briefly, lysates (1−2 mg/mL) containing overexpressed GST−$CheA_S$ proteins were mixed with lysates (1−2 mg/mL) containing overexpressed CheZ and incubated on ice for 1 h. The proteins were then mixed with 100 μL of a 50% suspension of glutathione and agarose (sigma) and allowed to incubate for 30 min on a Labquake rotating shaker at room temperature. After the 30 min incubation, the glutathione−agarose suspension was pelleted by centrifugation at 3000 rpm for 5 s. The supernatant was decanted, and the glutathione−agarose beads were washed three times with 1 mL of TEDG containing 25 mM NaCl to eliminate nonspecific interactions with the fusion proteins. The proteins were then eluted from the glutathione−agarose suspension with 50 μL of elution buffer [30 mM glutathione in 50 mM Tris (pH 8.0)]. Eluted complexes were analyzed via SDS−PAGE on 20% acrylamide gels. Proteins were observed after the gels had been stained with Coomassie blue stain and/or Western analysis using anti-CheZ polyclonal antibodies.

Oxidation of GST−CheA[98−139]. We used a method for oxidizing GST−CheA[98−139] similar to that used for Hsp33, with some modifications (*24*). Purified GST−CheA-[98−139] or GST−CheA[98−139]C120S was oxidized by incubating proteins in the presence of 12 mM H_2O_2 for 1 h at 37 °C. Proteins were then mixed with SDS sample buffer with or without β-mercaptoethanol (β-ME). Proteins were analyzed via SDS−PAGE and Coomassie blue staining. To assess the ability of oxidized GST−CheA[98−139] to interact with CheZ, lysates containing overexpressed GST−CheA[98−139] or GST−CheA[98−139]C120S were incubated under reducing and/or oxidizing conditions with or without CheZ (final concentration of ∼2 mg/mL). Protein mixtures were incubated at 37 °C for 1 h. Proteins were then mixed with glutathione−agarose beads and analyzed as described above (see GST−$CheA_S$ Coprecipitation Assay).

CheY∼P Dephosphorylation Assay. To examine the activity of CheZ with $CheA_S$ and GST−CheA[98−139], CheY∼P dephosphorylation assays were used as previously mentioned (*20*). Briefly, CheY (initial concentration of 0.25 mg/mL) was phosphorylated by ^{32}P-labeled CheA conjugated to Sepharose beads. Equal amounts of phosphorylated CheY were incubated alone or in combination with CheZ (4 nM) with or without $CheA_S$ (1 nM) or GST−CheA[98−139] (1 nM). Reactions were run at 4 °C in the presence of 100 μM $MgCl_2$. The conditions of this assay prolong the half-life of CheY∼P, allowing for the assessment of $CheA_S$ enhancement of CheZ activity. Samples were collected every 30 s, and the reactions were stopped by addition of SDS sample buffer. Samples were run on 20% acrylamide gels, which were dried and exposed to a Phosphorimager screen (Molecular Dynamics). Exposed screens were analyzed with the Storm 860 Phosphorimaging system (Molecular Dynamics), and phospho-CheY bands were quantified with ImageQuaNT (Molecular Dynamics).

Protein Interaction by Cysteine Modification (PICM) Assay. PICM assays were carried out as described by Bass

and Falke (*25*). Briefly, lysates containing overexpressed GST−CheA[98−139] were mixed with 100 μL of glutathione−agarose beads as described above. After proteins were bound, the beads were washed four times with TE buffer [50 mM Tris and 1 mM EDTA (pH 7.5)] containing 25 mM NaCl. The beads were washed three times with TE buffer to remove any residual salt from the samples. Proteins were resuspended in 1 mL of TE buffer with or without fluorescein 5-maleimide (5-FM, Pierce). The protein beads were mixed with or without 5-FM for 30 min at room temperature on a rotating shaker. After being labeled, beads were washed three times with TE buffer to remove any unbound 5-FM. Lysates containing overexpressed CheZ were then added to the protein beads and incubated for 1 h at room temperature on a rotating shaker. Unbound proteins were washed from beads with TE buffer containing 25 mM NaCl. Protein complexes were eluted and analyzed as described above.

GST−CheA[98−139] was also labeled with iodoacetamide (IAM) as previously described for aconitase (*26*). Purified GST−CheA[98−139] (2 mg/mL) was mixed with various concentrations of IAM for 20 min at room temperature. After incubation with IAM, GST−CheA[98−139] was mixed with CheZ (2 mg/mL), and coprecipitation assays were performed as described above.

RESULTS

$CheA_S$ Proteins Require the Partial P1 Domain for Interaction with CheZ. It was hypothesized that a solvent-exposed cysteine residue in $CheA_S$ was responsible for the oxidation-dependent covalent dimerization of $CheA_S$ (*20*). This dimerization was further implicated in occluding CheZ from its binding determinants on $CheA_S$ (*20*). We reasoned that one of the domains of $CheA_S$ that contains a cysteine residue was a likely candidate for being the CheZ-binding region. Since loss of $CheA_S$ produces no detectable phenotype in standard chemotaxis assays (*27*), a genetic approach to studying $CheA_S$ function seemed not to be feasible. To identify the region of $CheA_S$ that is responsible for CheZ binding, we tested the various fragments of $CheA_S$ that contain cysteine residues for CheZ binding activity. An ELISA-based binding assay, similar to the one used for studying the CheA−CheY interaction (*22*), was used to detect $CheA_S$−CheZ interactions. Microtiter plates were coated with monoclonal anti-CheA antibodies specific for the P4 domain (CA1.42) and P2 domain (CA1.117). There are three regions of CheA that contain a single cysteine residue, and these were used for the ELISA (see Figure 1 for schematic of proteins used and Table 1 for plasmids encoding these proteins): GST−CheA[252−654], CheA-[124−257], and CheA[98−315]. Full-length $CheA_S$ and $CheA_L$ were used as positive and negative controls for CheZ binding activity, respectively. We determined the relative CheZ binding activity of each CheA fragment by normalizing the data for each fragment to those of wild-type $CheA_S$ (Figure 2). As previously shown by co-immunoprecipitation (*20*), we detect binding with $CheA_S$, but not with $CheA_L$ (Figure 2). The CheA fragment lacking the P1 and P2 domains (GST−CheA[252−654]) was unable to interact with CheZ, whereas CheZ binding was observed with a truncated form of $CheA_S$ (CheA[98−315]) (Figure 2). This variant of $CheA_S$ contains the truncated P1 ('P1) and P2 domains, as well as part of the P3 domain (see Figure 1). The P2 domain of CheA (CheA[124−257]) exhibited no appreciable binding to CheZ. Taken together, these data suggest that the 'P1 domain of $CheA_S$ is essential for CheZ binding.

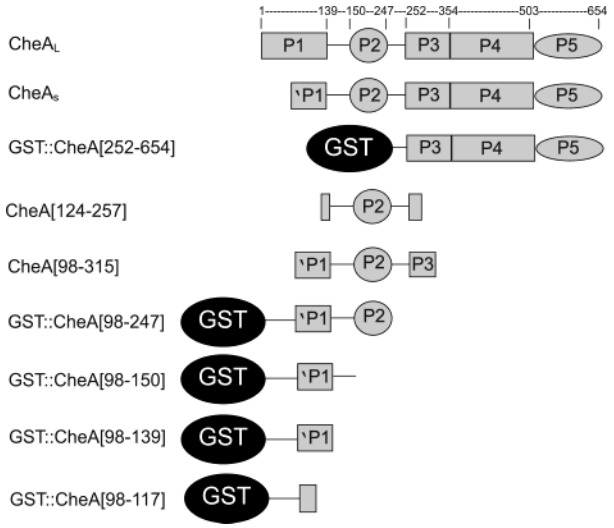

FIGURE 1: CheA proteins used in this study. The various CheA proteins used in this study are depicted here. Amino acid boundaries for each domain are listed relative to $CheA_L$. The plasmids encoding these proteins are listed in Table 1.

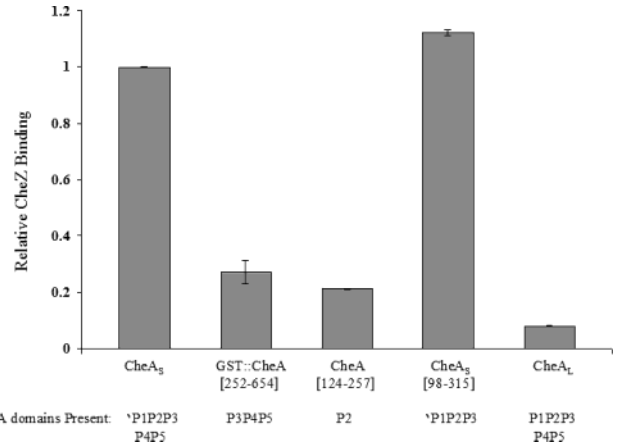

FIGURE 2: $CheA_S$ peptides require 'P1 for interaction with CheZ. The ability of various CheA fragments to bind CheZ was assayed in the ELISA-based $CheA_S$−CheZ binding assay. Microtiter plates were coated with monoclonal CheA antibodies specific for P4 or P2 domains. Lysates containing overexpressed CheA proteins were mixed with lysates containing overexpressed CheZ, and complexes were detected as mentioned in Materials and Methods. Each bar represents results for each sample that was normalized to the binding of CheZ to $CheA_S$. The particular CheA peptide that was tested is listed below its representative bar. Error bars represent the standard error of the mean ($n \geq 3$).

Since the monoclonal antibodies used in the ELISAs do not detect the 'P1 domain, GST coprecipitation assays were used to demonstrate that 'P1 is the CheZ-binding domain (Figure 3). GST fusions to C-terminally truncated $CheA_S$ peptides were incubated with CheZ and coprecipitated with glutathione−agarose beads, and complexes were analyzed by SDS−PAGE. As shown in Figure 3A, all but the shortest GST fusion protein coprecipitated with a protein with a molecular weight similar to that of CheZ (lanes 1−3). Western blots confirmed these bands as CheZ (Figure 3B). The 'P1 domain contains a cysteine residue (Cys-120), so we further truncated 'P1 to remove the region containing

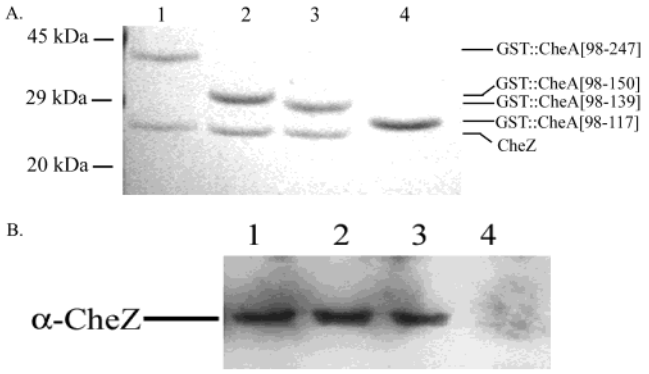

FIGURE 3: 'P1 is the CheZ binding domain of $CheA_S$. (A) Coomassie-stained 20% acrylamide gel and (B) anti-CheZ Western blot of protein eluents from GST coprecipitation assays. Proteins were run on SDS−PAGE and stained with Coomassie blue to visualize the GST−$CheA_S$ peptides that were released. The presence of CheZ was verified by running Western blots of the samples with a polyclonal anti-CheZ antibody. Lane numbers correspond to the following samples in both panels A and B: lane 1, GST−CheA-[98−247]−CheZ coprecipitate; lane 2, GST−CheA[98−150]−CheZ coprecipitate; lane 3, GST−CheA[98−139]−CheZ coprecipitate; and lane 4, GST−CheA[98−117]−CheZ coprecipitate.

Cys-120. No CheZ binding was seen with CheA[98−117] (Figure 3, lane 4). It was concluded from these results that residues 117−139 are essential for binding and could represent either binding determinants or elements needed for proper structure of the binding site(s). We also tested the CheZ binding activity of a truncated variant of 'P1, CheA-[98−127]. The CheA[98−127] fragment contained Cys-120, but was missing a significant portion of the region we suspect is important for CheZ binding. We could not detect CheZ binding with this fragment (data not shown). Although Cys-120 was present in this fragment, other essential CheZ-binding determinants could have been missing. The loss of residues 128−139 could also have an effect on the overall structure of the CheZ binding face in 'P1.

The 'P1 Domain Enhances CheZ Activity. It has been previously shown that $CheA_S$ enhances the CheY∼P phosphatase activity of CheZ (*20*). The mechanism and domain(s) of $CheA_S$ responsible for this effect are currently unknown. Since the 'P1 domain was capable of binding CheZ, we wanted to test if it could enhance CheZ phosphatase activity as well. We performed CheY∼P dephosphorylation assays as previously described (*20*), in the presence of full-length $CheA_S$ or GST−CheA[98−139] (GST−'P1), to determine if 'P1 alone could enhance CheZ activity. Both $CheA_S$ and GST−'P1 enhanced CheZ-mediated dephosphorylation (Figure 4). The half-lives ($t_{1/2}$) of CheY∼P in the presence of CheZ with $CheA_S$ and GST−'P1 were shown to be nearly identical ($t_{1/2}$ = 63 and 58 s, respectively) (Figure 4). The $t_{1/2}$ values for CheY∼P alone and CheY∼P with CheZ were considerably longer ($t_{1/2}$ = 204 and 147 s, respectively) under the conditions of these experiments (at 4 °C in the presence of 100 μM $MgCl_2$). CheY∼P dephosphorylation was not enhanced by GST−'P1 alone (data not shown). These results show that the 'P1 domain is responsible for both the binding of CheZ and the enhancement of CheZ activity.

Oxidized 'P1 Does Not Interact with CheZ. Formation of a complex between $CheA_S$ and CheZ has been shown to be dependent on the redox state of $CheA_S$; oxidized $CheA_S$ was unable to bind to CheZ (*20*). The redox regulation of formation of the $CheA_S$−CheZ complex was believed to be due to intermolecular disulfide bond formation (*20*). Since we have found both CheZ binding and phosphatase enhancing functions within the 'P1 domain, we reasoned that Cys-120 could be responsible for the oxidative inhibition of the $CheA_S$−CheZ interaction. We tested the 'P1 domain for the same oxidation-dependent regulation of CheZ binding activity. To determine if oxidized GST−'P1 (o-GST−'P1) could interact with CheZ, we incubated GST−'P1−CheZ complexes in the presence or absence of H_2O_2. This experiment showed that o-GST−'P1 interacted only weakly with CheZ (Figure 5A, lane 2). We conclude that the redox regulation of the $CheA_S$−CheZ interaction occurs within the 'P1 domain. To determine if Cys-120 was responsible for the redox regulation of the $CheA_S$−CheZ interaction, we tested the ability of GST−'P1C120S to interact with CheZ under oxidizing and reducing conditions. The C120S mutation does not interfere with the interaction of GST−'P1 with CheZ, because GST−'P1C120S still bound CheZ (Figure 5A, lane 3). Under oxidizing conditions, GST−'P1C120S also bound CheZ (Figure 5A, lane 4), indicating that Cys-120 is responsible for the redox-dependent regulation of the $CheA_S$−CheZ interaction.

Purified GST−'P1 was oxidized by incubating the purified protein in the presence of H_2O_2. After being treated with H_2O_2, o-GST−'P1 was mixed with SDS sample buffer with or without the reducing agent, β-mercaptoethanol. When o-GST−'P1 was mixed with nonreducing sample buffer, a dimer of GST−'P1 was observed (Figure 5B, lane 2). When o-GST−'P1 was mixed with reducing sample buffer, dimerization did not occur (Figure 5B, lane 1). These results suggested that GST−'P1 might be responsible for the oxidation-dependent dimerization of $CheA_S$. o-GST−'P1C120S fails to undergo oxidation-dependent dimerization (Figure 5B, lane 4). The dimerization shown in this experiment was not as profound as that seen with full-length $CheA_S$. We conclude oxidative dimerization via Cys-120 may lead to the loss of CheZ binding. However, we must concede that other modifications, such as S-hydroxylation, at Cys-120 could play a role.

The PICM Assay Reveals that the Accessibility of Cys-120 Is Important for the Interaction between CheZ and 'P1. The redox regulation of the $CheA_S$−CheZ interaction implies that Cys-120 is involved in CheZ binding. Modification of Cys-120, such as a disulfide linkage between two adjacent Cys-120 residues, could potentially lead to decreased CheZ binding activity, thus implying that Cys-120 is surrounded by the CheZ-binding determinants of 'P1. There is also the possibility that Cys-120 also contribues to the interaction between 'P1 and CheZ. To determine if sulfhydryl modifications at Cys-120 affect the binding of CheZ to 'P1, we reacted GST−'P1 with fluorescein 5-maleimide (5-FM) and tested the ability of the labeled proteins to coprecipitate with CheZ. 5-FM treatment disrupted the interaction between GST−'P1 and CheZ (Figure 6A, lane 2), suggesting that Cys-120 is close to the CheZ binding site(s) in 'P1. To verify that the inhibition of the interaction of GST−'P1 with CheZ was due to specific modification of Cys-120, we repeated the PICM test with GST−'P1C120S and found that 5-FM failed to block the interaction between GST−'P1C120S and

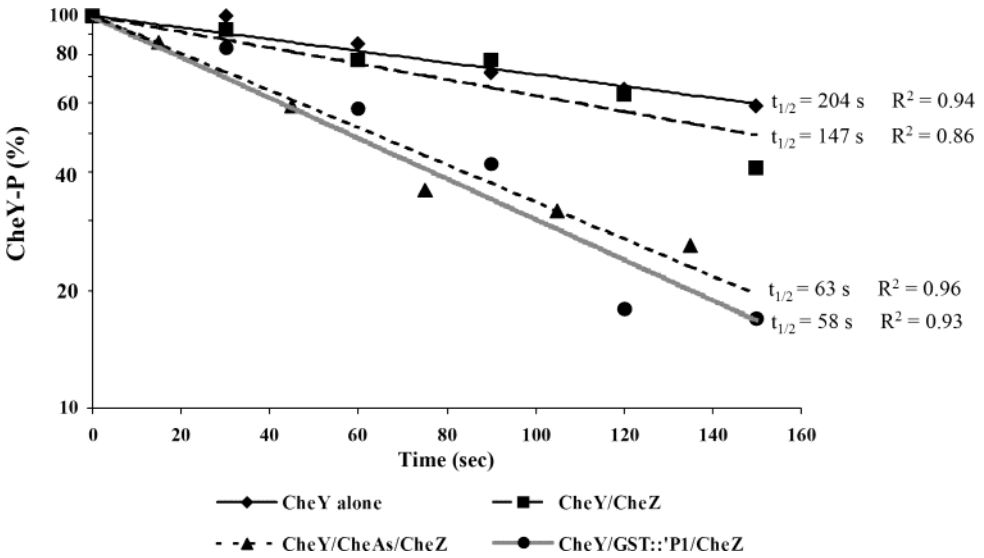

FIGURE 4: 'P1 enhances CheZ activity. A time course of phospho-CheY dephosphorylation was followed in the presence of CheZ alone or in complex with $CheA_S$ or GST−CheA[98−139] (GST−'P1) as previously described (*20*). The graph represents the percentage of CheY∼P as a function of time, and the half-life ($t_{1/2}$) was calculated with the equation $t_{1/2}$ = ln 2/slope. The trend lines represent the exponential regression for each sample. Correlation coefficients are given as R^2 values and are listed next to their appropriate samples.

CheZ (Figure 6A, lane 4). These results indicate that the effect of 5-FM on the binding of GST−'P1 to CheZ was due to modification of Cys-120 and that Cys-120 probably lies on the face of 'P1 that interacts with CheZ.

We also tested whether less drastic modifications of Cys-120 could block the binding of CheZ to GST−'P1. GST−'P1 (wild-type and C120S mutant) was incubated with various amounts of iodoacetamide (IAM). IAM covalently modifies the sulfhydryl group of reactive cysteine residues by adding a carboxyamidomethyl group. As seen in Figure 6B, IAM effectively blocks the binding of CheZ to wild-type GST−'P1 at all concentrations that were tested. As shown with the 5-FM labeling, IAM fails to block the interaction of GST−'P1C120S with CheZ (data not shown).

DISCUSSION

The role of $CheA_S$ in chemotaxis is at present unclear. $CheA_L$ is essential for chemotaxis in laboratory assays, whereas $CheA_S$ is not (*27*). $CheA_L$ is responsible for phosphorylating CheY (*6*), whereas $CheA_S$ can interact with and enhance the ability of CheZ to dephosphorylate phospho-CheY (CheY∼P) (*20*). In this study, we mapped CheZ binding to the remnant of the P1 domain present in $CheA_S$, which we term 'P1. These results are in agreement with those from a recent study looking at the localization of CheZ to receptor complexes (*28*). Fluorescent protein fusions to CheZ did not localize to receptor patches containing CheA proteins that lacked the P1 and P2 domains (*28*). The authors of that study concluded that the loss of localization was due to the absence of the 'P1 domain; however, no evidence of direct interaction between CheZ and the 'P1 domain of $CheA_S$ was presented (*28*). In our study, we have shown that the 'P1 domain confers the biochemical properties associated with the $CheA_S$−CheZ complex. The 'P1 domain binds to and enhances CheZ phosphatase activity. Even though $CheA_L$ contains the 'P1 region, we have not identified conditions that would allow for an interaction between $CheA_L$ and CheZ *in vitro*. Cantwell and co-workers were able to see only CheZ localization with $CheA_S$ and not with $CheA_L$ in their *in vivo* localization studies (*28*). We have not ruled out the possibility that $CheA_L$ has the potential to interact with CheZ under conditions yet to be identified.

The $CheA_S$−CheZ interaction was previously shown to be dependent on the redox state of $CheA_S$. Oxidation of $CheA_S$ led to covalent dimerization and loss of CheZ binding activity. This was also seen with the 'P1 domain and is due to the cysteine residue located in this region, Cys-120. The oxidation-dependent covalent dimerization was not observed with $CheA_L$, which suggested that the cysteine(s) responsible for this property was not exposed to the solvent in $CheA_L$. The P1 domain of $CheA_L$ has five α-helices (α-A−α-E) that form a tightly packed bundle (see Figure 7A). The 'P1 domain is comprised of the C-terminal half of α-helix D (α-D) and all of α-helix E (α-E) found in the crystal structure of P1 from $CheA_L$ (*29*) (Figure 7). As shown in Figure 7, Cys-120 has the potential of being solvent-exposed in $CheA_S$ and not in $CheA_L$.

The amphipathic nature of the α-E helix leads to the packing of the hydrophobic residues of α-E against the four N-terminal helices of the P1 domain in $CheA_L$ (*29*, *30*). It has been proposed that the interaction between $CheA_S$ and CheZ is through hydrophobic interactions (*28*). Hydrophobic residues Trp-97 and Phe-98 of CheZ are specifically necessary for the localization of CheZ to the receptor patches (*28*). Cantwell and co-workers proposed that hydrophobic residues located on the α-E helix of $CheA_S$ might bind to Trp-97 and Phe-98 of CheZ (*28*). Our data suggest that CheZ binding occurs within the hydrophobic face of α-E, which is consistent with their model. Cys-120 lies within this hydrophobic face (Figure 7), and labeling of this cysteine residue with fluorescein 5-maleimide (5-FM) and iodoacetamide (IAM) blocked the 'P1−CheZ interaction. We believe Cys-120 is important for the interaction between 'P1 and CheZ, but not essential. Although 5-FM and IAM had significant effects on the binding of CheZ to 'P1, the C120S mutant was still capable of interacting with CheZ. The Cys-120 residue likely serves as a marker for the location of the CheZ-

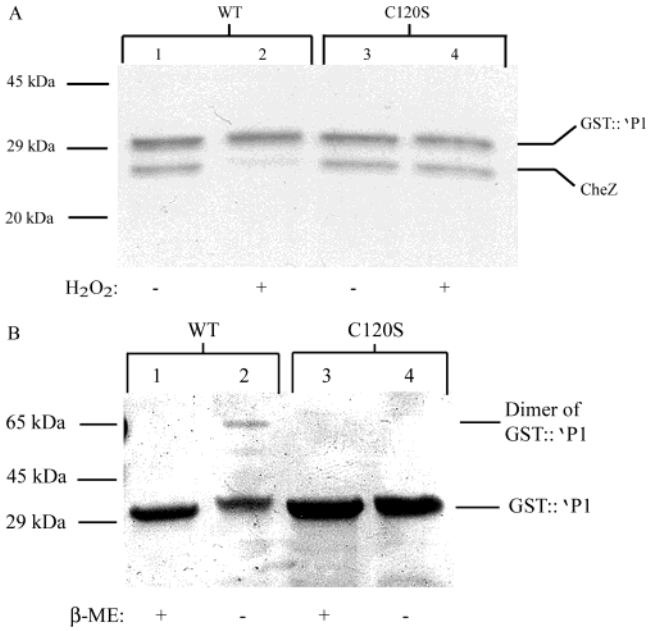

FIGURE 5: Oxidized 'P1 does not interact with CheZ. (A) Lysates containing GST−CheA[98−139] (GST−'P1) and GST−'P1C120S were incubated in the presence of 12 mM H_2O_2 for 1 h at 37 °C. After oxidation with H_2O_2, oxidized GST−'P1 and GST−'P1C120S were mixed with CheZ. As a control, reduced GST−'P1 and GST−'P1C120S were incubated with CheZ. Glutathione−agarose beads were used to precipitate the protein complexes, which were subsequently eluted from beads with 30 mM glutathione. Proteins were mixed with reducing SDS sample buffer and separated via SDS−PAGE (15% acrylamide gel). Gels were stained with Coomassie blue: lane 1, reduced GST−'P1−CheZ coprecipitate; lane 2, oxidized GST−'P1−CheZ coprecipitate; lane 3, reduced GST−'P1C120S−CheZ coprecipitate; and lane 4, oxidized GST−'P1C120S−CheZ coprecipitate. (B) Purified GST−'P1 and GST−'P1C120S were treated as described for panel A. Glutathione−agarose beads were used to precipitate GST−'P1 and GST−'P1C120S. Proteins were eluted from the beads with 30 mM glutathione and dialyzed against TE buffer to remove glutathione. The purified proteins were then mixed with SDS sample buffer with or without β-mercaptoethanol (β-ME). Proteins were separated via SDS−PAGE (15% acrylamide gel) and visualized by Coomassie blue staining: lane 1, GST−'P1 with β-ME; lane 2, GST−'P1 without β-ME; lane 3, GST−'P1C120S with β-ME; and lane 4, GST−'P1C120S without β-ME.

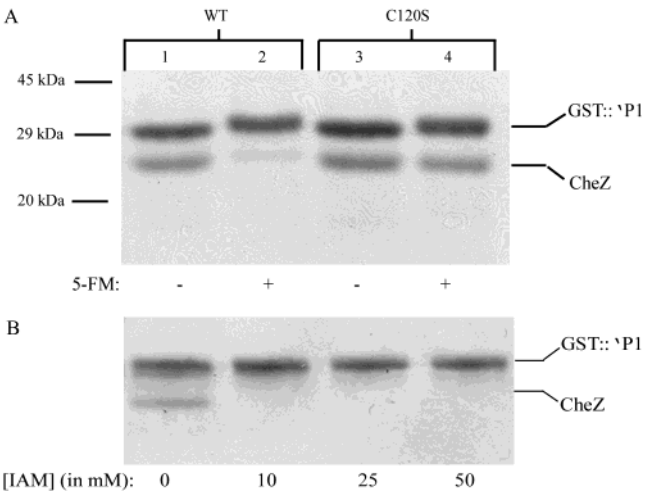

FIGURE 6: PICM analysis of the 'P1−CheZ interaction. (A) Glutathione−agarose beads with bound GST−CheA[98−139] (GST−'P1) and GST−'P1C120S bound to glutathione−agarose beads were incubated with or without fluorescein 5-maleimide (5-FM) and tested for CheZ binding. Protein complexes eluted from the beads were analyzed by SDS−PAGE (15% acrylamide gel) and Coomassie blue staining: lane 1, GST−'P1 unlabeled; lane 2, GST−'P1 labeled with 5-FM; lane 3, GST−'P1C120S unlabeled; and lane 4, GST−'P1C120S labeled with 5-FM. (B) Purified GST−'P1 (2 mg/mL) was incubated at room temperature for 20 min in the presence of iodoacetamide (IAM) at 0, 10, 25, and 50 mM. After incubation with IAM, the fusion protein was mixed with CheZ, and coprecipitation assays were run as described in Materials and Methods. Samples were analyzed via 15% SDS−PAGE, and gels were stained with Coomassie blue.

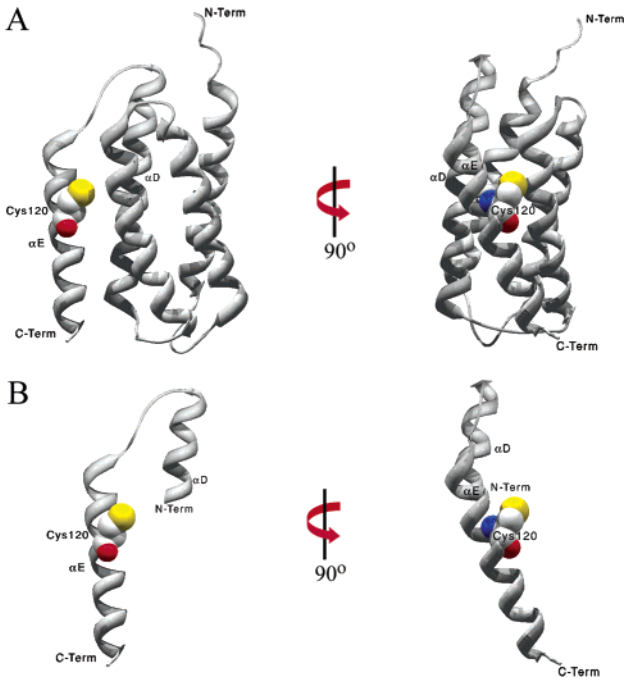

FIGURE 7: Predicted structure of the 'P1 domain. (A) Crystal structure of the P1 domain from *Salmonella* (*30*) and (B) hypothetical structure of the 'P1 domain. The space-filled residue represents Cys-120.

binding site of 'P1. The PICM results with IAM suggest that Cys-120 is fairly close to residues that are important for the CheZ interaction, since the area of steric hindrance would be small compared to that of the bulky fluorescein probe of 5-FM. We must also concede the possibility that Cys-120 makes a small contribution to the interaction between CheZ and 'P1.

In $CheA_L$, the hydrophobic face of α-E is packed in the center of the five-helix bundle, whereas in $CheA_S$, these hydrophobic residues would be solvent-exposed (Figure 7B). As shown in Figure 7A, Cys-120 is masked by the first 97 amino acids present in $CheA_L$. We believe this to be the reason for the difference in CheZ binding between $CheA_L$ and $CheA_S$. We must also include the possibility that the first 42 amino acids of $CheA_S$ could refold into a conformation slightly different from the conformation they assume in $CheA_L$. Whichever is the case, the accessibility of Cys-120 appears to be important for the interaction between $CheA_S$ and CheZ.

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