Expression of CheA Fragments Which Define Domains Encoding Kinase, Phosphotransfer, and CheY Binding Activities†

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ABSTRACT: The histidine protein kinase CheA is a central component of the *Escherichia coli* chemotaxis system. The autophosphorylation activity of CheA is controlled by membrane-bound chemoreceptors and by the CheW coupling protein. CheA phosphorylates the CheY and CheB proteins which respectively control the direction of flagellar rotation and the level of receptor adaptation, thereby regulating the cells' chemotactic response. Genes encoding three polypeptide fragments of CheA were constructed and expressed in order to better define the functional organization of the wild-type protein. These fragments allowed the identification of regions of the protein responsible for CheY binding, phosphotransfer, and kinase activity. The kinase domain was expressed as a 30-kDa polypeptide corresponding to the central portion of the wild-type protein which contains sequences homologous to other histidine kinases. It was able to phosphorylate a 15-kDa amino-terminal phosphotransfer domain which was separately expressed and purified. This latter domain is capable of phosphotransfer to CheY despite the fact that it lacks the ability to stably bind CheY. CheY was immobilized to a dextran matrix through a single cysteine residue which was introduced into the protein at a position far removed from the active site. A stable binding site for CheY was mapped to a segment between the site of autophosphorylation and the kinase domain by using surface plasmon resonance to detect binding to the immobilized CheY. The region of the kinase which tightly binds the unphosphorylated substrate may play an important role in regulating the specificity of the signal transducing system.

Escherichia coli is able to respond to gradients of chemical stimuli by altering its swimming behavior to effect net migration toward attractants and away from repellents [for reviews of bacterial chemotaxis, see Bourret et al. (1991) and Stewart and Dahlquist (1987)]. This process is controlled by a variety of ligands which bind to the periplasmic domains of a family of four membrane-spanning receptors [recently reviewed by Hazelbauer (1992)]. These receptors form ternary complexes with the protein kinase CheA and the small coupling protein CheW (Gegner et al., 1992), both of which are located within the cytoplasm. Changes in ligand occupancy of the receptors are thought to be transmitted from the periplasmic domain to the cytoplasmic domain by transmembrane conformational changes (Milburn et al., 1991), and the ligand binding state of the receptor controls the autophosphorylation rate of CheA within the complex (Borkovich et al., 1989; Borkovich & Simon, 1990; Ninfa et al., 1991). CheA is dephosphorylated by phosphotransfer to two different proteins, CheY and CheB (Hess et al., 1988a; Wylie et al., 1988). Phospho-CheY is thought to interact with the flagellar switch and influence the direction of flagellar rotation, thereby coupling receptor ligand binding to swimming behavior. CheB is a protein methylesterase (Stock & Koshland, 1978) which is activated by phosphorylation (Hess et al., 1988a; Lupas & Stock, 1989) and functions in a receptor adaptation pathway. The receptors are methylated at specific glutamyl residues by CheR (Springer & Koshland, 1977; Stewart et al., 1990) and are demethylated by phosphorylated CheB. Since methylated receptors are efficient activators of CheA, this negative feedback loop functions to desensitize the receptors in the presence of a constant level of ligand (Borkovich et al., 1992).

CheA, CheY, and CheB share homology with components of other bacterial regulatory pathways (Bourret et al., 1991; Stock et al., 1985, 1988; Parkinson & Kofoid, 1992; Kofoid & Parkinson, 1988), termed two-component regulatory systems; these circuits consist of proteins which contain a histidine kinase domain and proteins which contain a response regulator domain (Kofoid & Parkinson, 1988). CheY and CheB are members of the response regulator class of proteins. CheY, 129 amino acids in length, consists solely of the regulator domain, whereas CheB contains a regulator domain in addition to a methylesterase domain. Both CheY and CheB are phosphorylated at a conserved aspartyl residue by CheA.

CheA contains a 250 amino acid kinase homology domain, but unlike most known members of this family it is a soluble protein whose activity is controlled by interactions with membrane-bound receptors. In addition to sequence homology, genetic studies beginning with the observation of intragenic complementation between alleles containing missense mutations in the 5′ and 3′ segments of the gene have suggested that CheA is composed of distinct functional domains (Smith & Parkinson, 1980). Oosawa et al. (1988) elaborated on the idea of separate functional domains by demonstrating that mutations affecting phosphotransfer to CheY and CheB were localized to the amino-terminal domain while those affecting autophosphorylation were localized to the central region of the protein. This result was consistent with the work of Hess et al. (1988b), who showed that the site of autophosphorylation in CheA lies in the amino-terminal portion of the protein outside the kinase domain sequences and that limited proteolysis of the phosphorylated protein yields a stable amino-terminal fragment capable of phosphotransfer to CheY. In addition to the phosphotransfer and kinase domains, Bourret et al. (1993) have shown that deletion of the carboxyl terminus of CheA results in a protein which

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is capable of autophosphorylation and phosphotransfer but which is not regulated by receptor and CheW.

In this study we have analyzed the architecture of CheA by determining the functions of individually expressed fragments of the protein. Expression of individual domains in functional form provides conclusive evidence for the modular nature of CheA. In conjunction with the conclusions of previous studies, the results presented here demonstrate that individual functions of CheA, including kinase activity, phosphotransfer ability, CheY binding, and receptor coupling, are encoded within separable domains. These results are consistent with the observed evolutionary diversity of the two-component systems that bacteria have adapted to a variety of functions. The kinase and the regulator domains are placed in different contexts to provide inputs for diverse stimuli and to generate information processing circuits with different output responses that function on a variety of time scales.

MATERIALS AND METHODS

Strains and Plasmids. Two *E. coli* strains were used for the expression of CheA derivatives, M15 (Villarejo & Zabin, 1974) and KO685 which carries a *cheA* deletion (Hess et al., 1987). Strains which carry pQE12 (Qiagen) or its derivatives also carry pREP4 [*neo*, *lacI*] (Qiagen) to confer tight regulation on the inducible promoter of pQE12. Derivatives of pQE12 were constructed by cloning polymerase chain reaction (PCR) (Sakai et al., 1988) products amplified from the *cheA* gene of the expression plasmid pDV4 (Stader et al., 1986). The primers used for amplification were A501 (5′-CCGGAATTCATTAAAGAGGAGAAATTAACT-ATGAGCATGGATATAAGCGATTTT-3′), A502 (5′-CGG-GATCCGAATCCACCAGCATCCGTGTA-3′), A304 (5′-GGAAGATCTGGATATTTTTGGCGAGACTTC-3′), A305 (5′-CGGGATCCGGGTTGCAGTGATTCCATAAC-3′), and A306 (5′-GGAAGATCTTACGTTTCGCCTTTCGCTTC-TAA-3′). PCR was performed in a 50-μL volume containing 50 ng of pDV4, 50 pmol of each primer, as indicated, 200 μM each dNTP, 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer). The reaction mix was overlaid with mineral oil. Amplification was carried out using 25 step cycles of the sequence 1 min at 92 °C, 1 min at 57 °C, and 2 min (3 min for A502–A305) at 72 °C in a Perkin-Elmer thermal cycler. pRS1–6 was constructed by amplification with the primers A501 and A306. Following amplification, the fragment was precipitated with ethanol and digested with *Eco*RI and *Bgl*II. pQE12 was also digested with *Eco*RI and *Bgl*II and treated with calf intestinal alkaline phosphatase. Both the PCR product and the vector were purified by gel electrophoresis following digestion. The fragments were ligated with T4 DNA ligase and transformed into *E. coli* strain M15/pREP4. Amplicillin-resistant transformants were selected and screened for inducible protein production by SDS–PAGE after induction with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 1–2 h. pRS1–4 was constructed as above except that the primers A501 and A304 were used for amplification. pRS2–5 was constructed by a similar scheme, using primers A502 and A305; amplification was followed by digestion with *Bam*HI and ligation into pQE12 which had been digested with *Bam*HI and treated with phosphatase. After identification of a transformant which produced an inducible protein of the predicted size, plasmid preparations containing both pREP4 and pRS2–5 were transformed into KO685 and amp^r kan^r transformants selected.

Expression and Purification of CheA1–134. M15/pREP4/pRS1–6 was cultured in LB media at 37 °C. The cultures were induced upon reaching an OD_{600} of 0.7–0.9 by the addition of IPTG to a final concentration of 2 mM. Incubation was continued under the same conditions for an additional 3–5 h. Cells were collected by centrifugation and stored at −70 °C. Frozen cells were thawed on ice and resuspended in 50 mM Tris, pH 7.5/5 mM EDTA/2 mM 2-mercaptoethanol (buffer A) containing 0.1 mg mL^{-1} lysozyme. The cell suspensions were lysed by brief sonication. Lysates were centrifuged at 123000*g* for 30 min. Pelleted material was discarded, and the supernatant was precipitated with ammonium sulfate at 50% saturation, the pellet was discarded, the supernatant was brought to 60% saturation, and the pellet was collected by centrifugation. Precipitated protein was resuspended in, and dialyzed against, buffer A. Dialyzed protein was applied to an FPLC Q-Sepharose column and eluted with a linear gradient from 0 to 1 M KCl in buffer A. Fractions were assayed by SDS–PAGE, and those containing CheA1–134 judged to be pure were pooled and concentrated using a Centriprep-10 concentrator (Amicon).

Expression and Purification of CheA1–233. M15/pREP4/pRS1–4 were grown at 37 °C in LB until an OD_{600} of 0.7–0.9 was reached. Cultures were then induced by the addition of IPTG to a final concentration of 2 mM. Incubation was continued for an additional 3–5 h, and cells were harvested by centrifugation and frozen. Cell pellets were thawed and resuspended in buffer B (50 mM sodium phosphate/300 mM NaCl/2 mM 2-mercaptoethanol/1 mM imidizole) containing 0.1 mg mL^{-1} lysozyme and lysed by sonication. Lysates were cleared by centrifugation at 123000*g* for 30 min. The fusion protein was found in the soluble fraction and was loaded directly onto a Ni-NTA column (Qiagen) preequilibrated in buffer B. The column was washed with buffer B and eluted with buffer B containing 250 mM imidizole.

Expression and Purification of CheA260–537. KO685/pREP4/pRS2–5 cells were grown at 30 °C in LB until an OD_{600} of 0.6 was reached. Cultures were than induced by the addition of IPTG to a final concentration of 50 μM. Incubation was continued for an additional 1 h, and cells were harvested by centrifugation and frozen. Purification of the histidine-tagged protein was carried out as described above for CheA1–233. The protein was dialyzed against 50 mM Tris/0.5 mM EDTA/2 mM DTT, pH 7.5/10% glycerol (TEDG) and stored at −70 °C.

Phosphorylation Assays. Purified CheY used in phosphorylation assays was a gift from Lisa Alex. CheA470GK was purified as previously described (Swanson et al., 1993). Phosphorylation assays were performed at room temperature in a 10-μL final volume. Reactions contained 50 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.5. Protein concentrations were as follows: CheA260–537, 5 μM; CheA1–233, 10 μM; CheA, 10 μM; CheA470GK, 2 μM; CheY, 18 μM. Reactions were initiated by the addition of [γ-^{32}P]ATP to a final concentration of 0.1 mM (specific activity 11 Ci $mmol^{-1}$). Reactions were terminated by the addition of 10 μL of SDS–PAGE sample buffer after 1 min. Aliquots of samples were analyzed by electrophoresis on a 15% SDS–PAGE gel, followed by staining as described (Borkovich & Simon, 1991), drying and autoradiography.

Surface Plasmon Resonance Measurements. CheY51YC was expressed in the Δ*cheY* strain KO641 (Bourret et al., 1990) containing the plasmid pRBB40.51YC (supplied by R. B. Bourett). A 1-L culture was grown to an OD_{600} of 0.5, induced with 100 μg mL^{-1} 3-β-indoleacrylic acid for 3 h, and then harvested by centrifugation. The cell pellet was resus-

pended in 20 mL of cold TEDG buffer and lysed by sonication. Cell debris was removed by a low-speed centrifugation (10 min, 6000g). The supernatant was centrifuged at 4 °C for 35 min at 100000g. The resulting supernatant was loaded onto a 30-mL Blue Sepharose column. The column then was washed with 60 mL of TEDG. The bound CheY was eluted with 1 M NaCl in TEDG (50 mL) and concentrated by filtration in a Centriprep-10 concentrator. The concentrated sample was loaded onto a Superdex 75 HR 10/30 FPLC sizing column equilibrated in TEDG buffer. The FPLC fractions were examined by SDS–PAGE; fractions containing CheY51YC were pooled and used without further purification. CheY51YC was coupled to a CM5 sensor chip (Pharmacia Biosensor) equilibrated in 10 mM Hepes/0.15 M NaCl/0.005% Tween 20, pH 7.4, by the following procedure. Carboxyl groups within the dextran layer of the sensor chip were converted to *N*-hydroxysuccinimide esters by exposure to 10 μL of 0.2 M *N*-ethyl-*N*'-[(dimethylamino)propyl]-carbodiimide hydrochloride (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS) in water. Reactive disulfide groups were introduced by exposure to 45 μL of 50 mM 2-(2-pyridinyldithio)ethanamine hydrochloride (PDEA) in 100 mM sodium borate buffer, pH 8.5. Following the PDEA, 35 μL of ethanolamine was injected to block unreacted activated esters. CheY51YC (22 mg mL^{-1}) was coupled by injection of 45 μL in 50 mM formic acid, pH 2.5. Excess sulfhydryl groups were removed by exposure to 20 μL of a solution containing 100 mM cysteine and 100 mM NaCl in 50 mM formic acid, pH 4.5. The flow rate during the coupling procedure was 5 μL min^{-1} for all steps except for the CheY51YC injection when it was lowered to 2 μL min^{-1}. Surface plasmon resonance measurements of the binding of CheA and CheA fragments were performed at 5 μL min^{-1} in 50 mM Tris, pH 7.5/50 mM KCl/5 mM $MgCl_2$/0.005% Tween 20. Injections of 35 μL were performed with 0.2 mg mL^{-1} protein solutions. $CheA_S$ was purified as previously described (Swanson et al., 1993), and purified wild-type CheA was a gift from Lisa Alex and Juan Davagnino. Control injections were also performed by activating and coupling a chip as above except that buffer was substituted for the CheY51YC solution. All surface plasmon resonance measurements were performed using a BIAcore instrument (Pharmacia Biosensor).

Calculation of Binding Constants. The kinetics of the interaction of CheA1–233 with CheY were analyzed using the procedure described by Karlsson et al. (1991). The interaction curve of CheA1–233 was analyzed by plotting dR/dt vs R, where R is resonance units. dR/dt and R values are continually calculated and displayed by the BIAlogue software (Pharmacia Biosensor). The slope values obtained from several concentrations of CheA were plotted against the concentration. The association constant (k_a) was obtained from the slope of the fitted line. The dissociation rate (k_d) was obtained from the slope of the $\ln(R_{A1}/R_{An})$ vs $(t_n - t_1)$ plot, where dissociation began at time t_1 at a level R_{A1}. The affinity constant of the CheA/CheY interaction was calculated from $K_D = k_d/k_a$.

FPLC–Size Exclusion Chromatography. Gel filtration was performed on a Superdex 75 HR 10/30 FPLC column (Pharmacia) maintained at 4 °C. The column was equilibrated in 50 mM Tris, pH 7.5/150 mM KCl/5 mM $MgCl_2$. Injections consisting of 150 μL were performed, and the column was developed at a flow rate of 0.5 mL min^{-1}. Protein concentrations were adjusted by using extinction coefficients at 280 nm of 4080 for CheA1–233 and 8250 for CheY. Extinction coefficients were calculated by the method of Gill and von Hippel (1989) from the predicted amino acid sequences. The column was calibrated using bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa) as standards.

RESULTS

Design, Cloning, Expression, and Purification of CheA Fragments. The sequences which encompass the kinase homology domain, as defined by Kofoid and Parkinson (1988), extend from amino acid 261 to amino acid 507 within CheA. In addition, residues 1–97 (Swanson et al., 1993) and residues 538–654 (Bourret et al., 1993) of CheA were shown to be dispensable for kinase activity. We subcloned a portion of the *cheA* gene corresponding to residues 260–537 into pQE12 using the polymerase chain reaction. pQE12 is a vector designed to express histidine-tagged fusion proteins from an IPTG-inducible promoter. The predicted gene product from this plasmid, pRS2–5, is composed of four vector-encoded amino acids at the amino terminus, residues 260–537 of CheA, and ten vector-encoded residues at the carboxyl terminus including six histidines. The protein fragment CheA260–537 was expressed in soluble form at 30 °C after induction with IPTG and was rapidly purified by chromatography on Ni-NTA resin (Janknect et al., 1991). When the fragment was purified from strain M15/pREP4, we observed copurification of a protein which migrates with CheA on SDS–PAGE and becomes phosphorylated upon incubation with [γ-^{32}P]ATP. Therefore, we expressed CheA260–537 in a Δ*cheA* host, strain KO685 (Hess et al., 1987), to avoid possible contamination of the purified protein with wild-type CheA. The contaminant was not present when the protein was expressed in KO685, which suggests that CheA260–537 forms heterodimers with full-length CheA *in vivo*.

The site of autophosphorylation in CheA is located at histidine 48 (Hess et al., 1988b). Amino-terminal fragments of approximately 16–18 kDa derived from limited trypsin digestion of phosphorylated CheA can be isolated and are functional when assayed by phosphotransfer to CheY (Hess et al., 1988b). A comparison of the deduced amino acid sequences of CheA from *E. coli* (Kofoid & Parkinson, 1991) and *Salmonella typhimurium* (Stock et al., 1988) was made to determine the extent of the region of homology that could encode a functional phosphotransfer domain. While the sequences of the two proteins are 85% identical overall, many of the differences are concentrated in two highly divergent regions (Figure 1). On the basis of the amino-terminal proximal border of the first nonconserved region, we selected Thr-134 as the carboxyl-terminal residue. The polymerase chain reaction was used to amplify the CheA sequence corresponding to the first 134 amino acids. The resulting PCR product was cloned into pQE12 to produce pRS1–6. In order to facilitate future structural studies CheA1–134 was not expressed as a histidine-tag fusion protein. A stop codon was included in the sequence of the downstream primer to prevent the formation of a fusion protein; therefore, pRS1–6 encodes a protein consisting solely of the first 134 amino acids of CheA. The protein was expressed at 37 °C after induction with IPTG and was purified in a two-step procedure consisting of ammonium sulfate precipitation followed by anion-exchange chromatography.

A gene encoding a third fragment, CheA1–233, was also constructed by PCR amplification from *cheA* and cloning into pQE12. This fragment encompasses the first 233 amino acids of CheA fused at the carboxyl terminus to eight amino acids encoded by pQE12 including the histidine tag. CheA1–

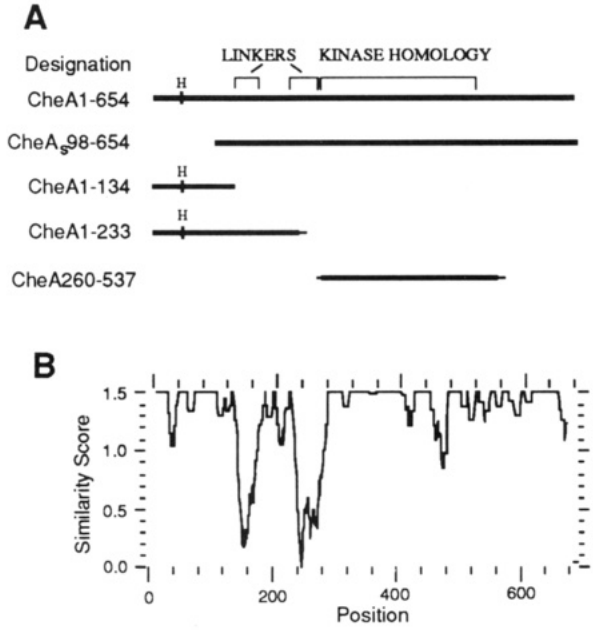

FIGURE 1: (A) Schematic diagram depicting the relationship of the CheA fragments described in this study to the linear structure of CheA. The regions of similarity to kinase domain sequences are shown above the fragments as are the regions of greatest divergence between the *S. typhimurium* and *E. coli* proteins. Histidine 48, which is the site of autophosphorylation, is denoted as H. CheA1–134 consists solely of the first 134 amino acids of CheA. CheA1–233 contains a short additional sequence at the carboxyl terminus encoding the histidine tag. CheA260–537 contains four vector-encoded amino acids at the amino terminus in addition to the carboxyl-terminal histidine tag. (B) Plot of the similarity between the aligned sequences of CheA from *S. typhimurium* (Stock et al., 1988) and *E. coli* (Kofoid & Parkinson, 1991). A higher score indicates a greater similarity. The sequences were aligned using the program PileUp, and the plot was generated from this alignment using the program PlotSimilarity with the comparison window set at 10 residues. Both programs are part of the GCG package of sequence analysis software.

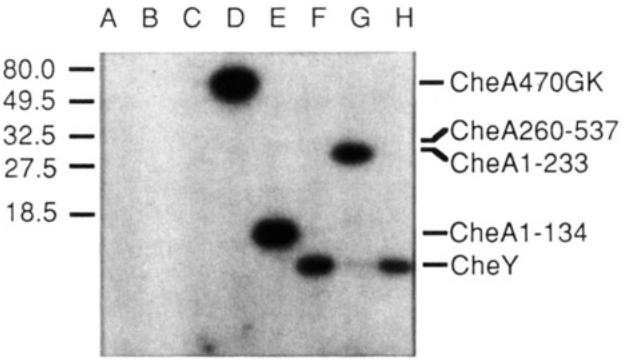

FIGURE 2: Autoradiograph of phosphorylation reaction products after separation on 15% SDS–PAGE: lane A, CheA260–537; lane B, CheA1–134; lane C, CheA1–233; lane D, CheA260–537/CheA470GK; lane E, CheA260–537/CheA1–134; lane F, CheA260–537/CheA1–134/CheY; lane G, CheA260–537/CheA1–233; lane H, CheA260–537/CheA1–233/CheY. The relative mobility (in kilodaltons) of prestained molecular mass markers is indicated on the left. The migration of the proteins used in the assays is indicated on the right.

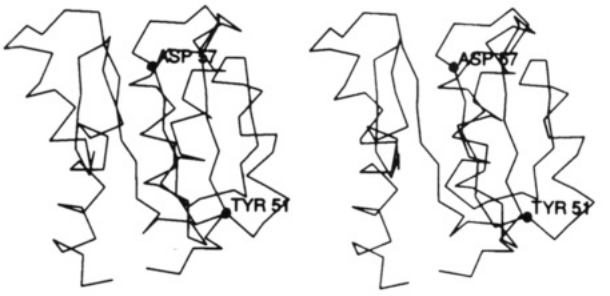

FIGURE 3: Stereoview of the backbone trace of *E. coli* CheY from the crystal structure determined by Volz and Matsumura (1991). The location of the active site of CheY is indicated by the position of the site of phosphorylation, Asp 57 (Sanders et al., 1989). The mutant CheY51YC was selected for immobilization because the introduced cysteine is removed from the active site.

233 was designed to investigate the function of a conserved region of CheA bordered by the two highly divergent stretches (Figure 1). This protein was purified by Ni-NTA chromatography after expression at 37 °C.

The Central Kinase Domain Is Active and Can Phosphorylate the Amino-Terminal Domain in an Intermolecular Reaction. CheA260–537 cannot autophosphorylate (Figure 2, lane A), but it is active as a kinase when assayed with CheA470GK as a substrate (Figure 2, lane D). CheA470GK is a missense mutant which is defective in kinase activity and lacks the ability to autophosphorylate (Oosawa et al., 1988) but can be phosphorylate by CheA$_S$ or CheA48HQ (Swanson et al., 1993; Wolfe & Stewart, 1993), both of which lack the site of autophosphorylation. These phosphorylation reactions occur through an intersubunit reaction within a dimer (Swanson et al., 1993), suggesting that CheA260–537 is both active as a kinase and is capable of dimerization.

CheA260–537 can phosphorylate both CheA1–134 and CheA1–233 in an intermolecular reaction (Figure 2, lanes E and G). In addition, both of these fragments can transfer the phosphoryl group to CheY (Figure 2, lanes F and H). This result is consistent with previous observations of phosphoryl transfer by proteolytically derived fragments (Hess et al., 1988b).

Sequences Mediating Stable Binding to CheY Are Localized between Residues 98 and 233 of CheA. McNally and Matsumura (1991) have described the binding of CheA/CheW complexes to CheY immobilized on a column. We immobilized CheY on a BIAcore sensor chip to investigate the interaction between CheA and CheY using surface plasmon resonance (Jönsson et al., 1991). We did not observe any binding to CheY immobilized through primary amino groups to the sensor chip via carbodiimide coupling (data not shown). Therefore, we prepared a mutant CheY in which a single cysteine residue had been introduced, CheY51YC. The mutation has no effect on CheY function in chemotaxis as assayed on swarm plates (R. B. Bourret, personal communication). This particular mutant protein was chosen because the location of the introduced cysteine is on the opposite face of the molecule from the active site (Figure 3). Wild-type CheY does not contain any cysteine residues; therefore, the mutant protein can be immobilized on the dextran matrix through a single bond by derivitization of the unique sulfhydryl. CheY51YC was coupled to the sensor chip through Cys-51 after the surface was activated with carbodiimide and then reacted with the activated disulfide reagent PDEA (Figure 4A). When CheA was passed through the CheY matrix, it bound strongly to CheY independent of the presence of CheW (Figure 4B).

We expected that CheA1–134 would bind to CheY since they are capable of interacting in the phosphotransfer reaction; however, no significant binding was observed when a 0.2 mg mL^{-1} solution of CheA1–134 was exposed to the CheY sensor chip (Figure 4C). In contrast, CheA$_S$ exhibited binding to CheY which was not significantly different from that observed with full-length CheA (Figure 4C), despite the fact that it lacks the site of autophosphorylation. The fragment CheA1–233 also bound to CheY (Figure 4C). The apparent K_D for

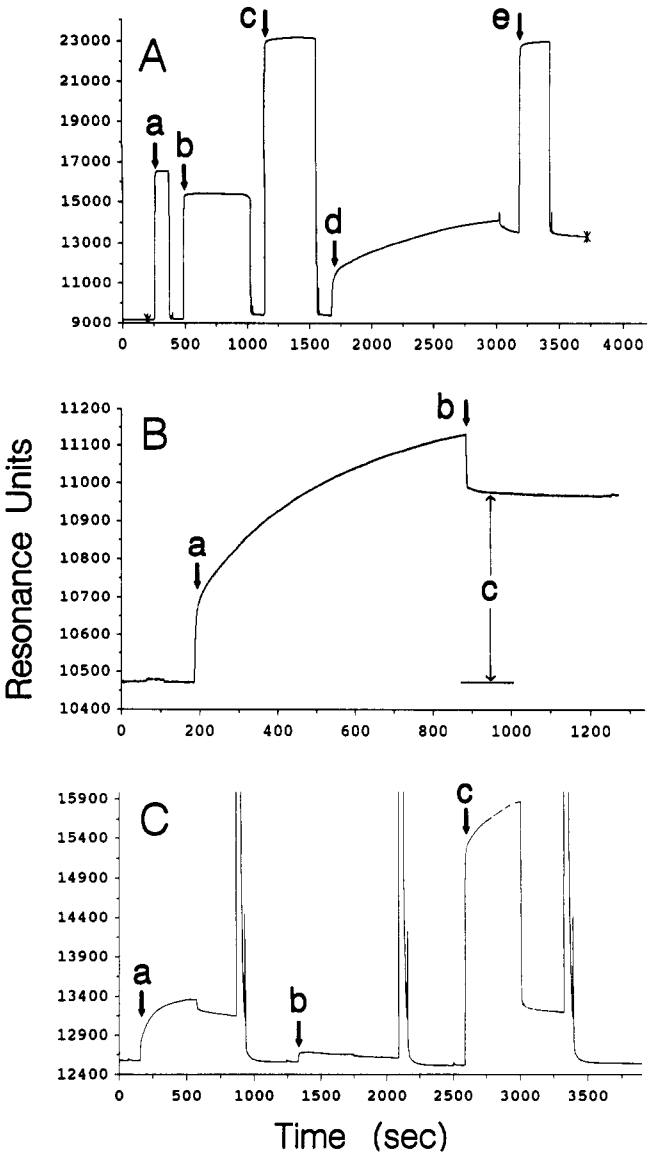

FIGURE 4: (A) Immobilization of CheY to the carboxydextran layer of the CM-5 sensor chip. The beginning of each injection of a reactant is indicated by an arrow. The carboxydextran matrix is first activated by reaction with *N*-hydroxysuccinimide ester/carbodiimide (a). Reactive disulfide groups are then introduced by injection of 2-(2-pyridinyldithio)ethanamine (PDEA) (b). Unreacted *N*-hydroxysuccinimide esters are deactivated by reaction with ethanolamine (c). CheY51YC is immobilized (d). Finally, excess reactive groups are blocked by injection of cysteine (e). (B) CheA binding to CheY detected by surface plasmon resonance. A solution of CheA is injected into a chip containing CheY immobilized as described above. The beginning of the injection is indicated by an arrow (a). The end of the injection is also indicated (b). The increase in resonance units over the course of the injection and the change (c) in baseline after the injection indicate binding of CheA to the CheY surface. CheA does not interact with a surface prepared exactly as in (A) if CheY is omitted. (C) Sensorgram of the interactions of CheA1–233 (a) CheA1–134 (b), and $CheA_S$ (c) with immobilized CheY51YC. Each injection consisted of 35 μL of 200 μg mL^{-1} of the indicated CheA derivative flowed over the CheY sensor chip at a rate of 5 μL/min. The beginning of each injection is indicated by an arrow. The total amount of immobilized CheY on the chip was 2700 resonance units (RU). The large change in bulk refractive index during the $CheA_S$ injection is a result of traces of glycerol in the storage buffer of that sample and does not interfere with the absolute change in RU after the injection. The CheY surface was regenerated after each binding experiment by a short pulse (2 μL) of 3 M guanidine. These injections result in the three narrow, off-scale, peaks. The increase in resonance units (RU) was measured 20 s after the end of each injection. $CheA_S$ increased 755 RU, CheA1–233 increased 646 RU, and CheA1–134 increased 75 RU. Wild-type CheA run on the same sensor chip exhibited a 709-RU increase.

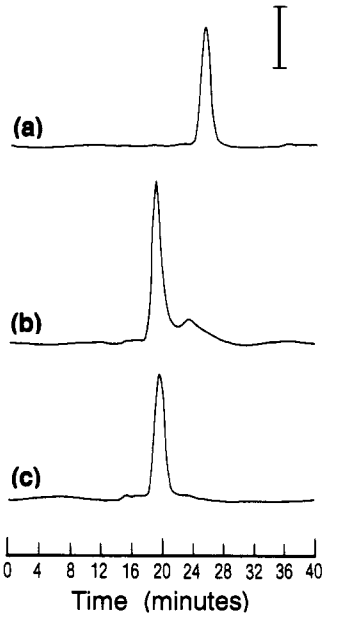

FIGURE 5: Gel filtration chromatography of CheA1–233 and CheY monitored at 280 nm. Each injection consisted of 150 μL of (a) 40 μM CheY, (b) 40 μM CheY and 80 μM CheA1–233, and (c) 80 μM CheA1–233. The bar shown in the upper right is 0.01 absorbance unit.

this interaction was found to be 3.7×10^{-7} M. In order to verify these observations by an independent method, we followed the interaction by chromatography using CheA1–233 and CheY alone and in combination on an FPLC–size exclusion column. The results shown in Figure 5 confirm that these proteins form a stable complex. Although CheA1–233 has a calculated molecular mass of 26.8 kDa, it migrates on this column with an apparent molecular mass of 45 kDa. Because this is less than the mass of 53.6 kDa predicted for a homodimer, CheA1–233 is likely to be a monomer which migrates anomalously large. When chromatographed alone, CheY migrates with an apparent molecular mass of 14 kDa. When CheY is combined with a 2-fold excess of CheA1–233 prior to chromatography, most of the CheY elutes with the CheA1–233, and the peak shifts only slightly to an apparent molecular mass of 50 kDa; this is very close to the predicted mass for a 1:1 complex of CheA1–233 and CheY of 50.9 kDa. Taken together, these results support the interpretation that CheA1–233 is a monomer which migrates anomalously and that the binding stoichiometry is likely to be 1:1, as a 2:1 complex would have mass of 67.6 kDa.

DISCUSSION

We have utilized the polymerase chain reaction to produce genes encoding fragments of CheA. These fragments were purified and assayed for their ability to perform specific functions present in the full-length wild-type protein in order to experimentally define functional domains. Bourret et al. (1993) showed that CheA mutants, such as CheA538Ram, in which the carboxyl-terminal sequences of the protein are deleted, are not regulated by receptor. The present work serves to define four functionally distinct regions within the CheA protein: phosphotransfer, CheY binding, kinase activity, and regulation. All four of these functions are separable in the sense that if CheA538Ram, CheA1–233, and CheA1–134 are viewed as truncations of progressively larger amounts of the carboxy terminus of the protein, each further truncation results in the loss of a single additional activity without eliminating the more amino-terminal functions. Thus,

CheA538Ram lacks only regulation, CheA1–233 lacks regulation and kinase activity but retains both CheY binding and phosphotransfer, and CheA1–134 retains only the phosphotransfer activity. Truncation at the amino terminus, as evidenced by $CheA_S$, results in the loss of a single activity, phosphotransfer, without affecting the other three activities. In addition, kinase activity in CheA260–537, like phosphotransfer activity in CheA1–134, can exist in the absence of the other three functions. There may be even further partitioning of functions in CheA; Parkinson and Kofoid (1992) have cited results which suggest that the regulatory region may be separable into subdomains, one responsible for physical coupling to receptor and a second necessary for activation of CheA. The conclusion that specific functions are segmentally organized along the linear polypeptide chain is important for considering how kinase domains have been modified during evolution to participate in the variety of signaling pathways observed in bacteria today. It is easy to envision the reconfiguration of systems such as these by the shuffling of linearly encoded functions.

We found that a 15-kDa amino-terminal fragment is stable, capable of phosphotransfer to CheY, and can be phosphorylated by a separately expressed kinase domain. This observation suggests that homologous kinases could have evolved which do not autophosphorylate but which phosphorylate specific histidines, or some other residues, on a different protein substrate. One possibility for such a kinase is phytochrome. Phytochrome contains a sequence with homology to the histidine kinase domain (Schneider-Poetsch et al., 1991); however, in many of the phytochrome sequences the conserved histidine corresponding to the presumed autophosphorylation site is replaced by some other residue, suggesting that a different protein or some other residue may act as substrate. A second possibility for such a kinase is the branched-chain α-keto-acid dehydrogenase kinase from mitochondria (Popov et al., 1992). This enzyme is a serine protein kinase which shares homology with the bacterial histidine kinases but no homology with other known serine protein kinases.

We observed a stable binding interaction between CheA and CheY using surface plasmon resonance. Both $CheA_S$ and CheA1–233 bind to CheY, indicating that the major determinants required for binding lie between residues 98 and 233. This stretch includes a region, residues 160–233, which is conserved between the CheA proteins of *E. coli* and *S. typhimurium* but is flanked by nonconserved residues in the wild-type protein (Figure 1). This conserved domain was recognized by Parkinson and Kofoid (1992) but its function was not known. The binding interaction between CheA and CheY may be important *in vivo* for differentiating between response regulators and reducing the level of cross-talk. Stable complex formation has also been documented for AlgR1 and AlgR2, a kinase and response regulator in the signal transduction system controlling alginate biosynthesis in *Pseudomonas aeruginosa* (Roychoudhury et al., 1922). Thus, the existence of stable interactions between kinases and their response regulator substrates may be a more common feature of bacterial signal transduction systems than has been generally recognized.

The surface plasmon resonance technology used here to define the binding site for CheY on CheA offers the potential for rapidly screening missense mutants of CheA and CheY. We anticipate that studies employing mutagenesis will more precisely delineate the specific residues of both CheA and CheY which participate in this interaction as well as the role of this interaction in chemotaxis. In addition, SPR can be used for mapping other protein–protein interactions within the receptor/CheA/CheW signal transduction complex.

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