Articles

The Response Regulators CheB and CheY Exhibit Competitive Binding to the Kinase CheA†

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Received June 20, 1995; Revised Manuscript Received September 11, 1995⊗

ABSTRACT: The autophosphorylating kinase CheA of the bacterial chemosensory signaling pathway donates a phosphoryl group to either of two response regulator proteins, CheY or the receptor methylesterase (CheB). With isothermal titration calorimetry, it was demonstrated that CheA and a CheA fragment composed of amino acid residues 1−233 ($CheA_{1-233}$) bound to CheY with similar dissociation constants of 2.0 and 1.2 μM at 298 K, respectively, indicating that the CheY binding site is wholly within the 1−233 amino acid locus. CheB bound to $CheA_{1-233}$ with a K_D of 3.2 μM, and also bound to intact CheA with the same affinity. CheY was found to compete with CheB for binding to $CheA_{1-233}$, in spite of the low level of sequence identity between CheY and the regulatory domain of CheB. The competitive nature of CheY and CheB binding was determined in two independent sets of experiments: titration experiments in which either a CheB−$CheA_{1-233}$ complex was titrated with CheY or CheB was titrated with a CheY−$CheA_{1-233}$ complex, and competitive affinity chromatography experiments that used Ni−NTA-chelating resin as an affinity matrix for complexes of the histidine-tagged $CheA_{1-233}$ fragment and CheY or CheB. The effects of phosphorylation, binding-site mutations, and active-site mutations were also studied to probe the influence of conformational changes in CheY as a regulatory mechanism of CheY−CheA interactions. Phosphorylated CheY, in the presence of excess EDTA, was found to have a 2-fold lower affinity for $CheA_{1-233}$, and 6 mM Mg^{2+} further reduced the affinity of phosphorylated CheY for $CheA_{1-233}$ (ca. 3-fold), although Mg^{2+} on its own had no effect on the interactions of either CheB or CheY with $CheA_{1-233}$. The data thus indicate that phosphorylated CheY has a significantly lower affinity for CheA under physiological conditions. The idea that phosphorylation may induce a significant conformational change, reducing the strength of the CheY−CheA interactions, is supported by the relative values of the association constants measured for CheY active-site and binding-site mutants. A binding-site mutation (A103V) in CheY, which is remote from the site of phosphorylation produced a 10-fold reduction in K_a, whereas active-site mutations produced a modest (2-fold) reduction.

As it swims through solution *Escherichia coli* continuously samples the local concentrations of nutrients and toxins, which are identified as attractants or repellents by their specific binding interactions to integral membrane receptor proteins and by the behavioral responses that are subsequently generated. Detection of chemoeffector concentration *versus* time permits the bacterium to respond positively to gradients of attractants and negatively to repellents using a signal transduction pathway that couples changes in chemical concentration to the motility apparatus of the cell [reviewed in Bourret et al. (1991); Stock et al. (1992), and Alex and Simon (1994)]. The histidine kinase CheA plays a central role in this pathway, as it is involved in the response and adaptation to all of the chemoeffectors processed through the methyl-accepting chemotaxis proteins, which are a thoroughly characterized class of integral membrane receptor proteins in prokaryotes [reviewed in Hazelbauer (1992)]. Changes in receptor occupancy result in behavioral responses that occur rapidly in comparison to the process of adaptation, which is mediated by changes in the level of receptor covalent modification. Both the receptor occupancy and the level of covalent modification influence the autophosphorylation rate of CheA by ATP through direct protein interaction between the kinase and chemoreceptors in a complex also containing CheW (Gegner et al., 1992; Schuster et al., 1993). Phosphorylated CheA kinase is the phosphate donor for CheY and CheB (Hess et al., 1988), and signal amplification is generated by repeated cycling through the autophosphorylation and the phosphotransfer reactions. CheY and CheB, 14 and 37 kDa, respectively, are members of the response regulator family of signal transduction

† This research was supported by U.S. Public Health Service Grants AI-19296 (M.I.S.) and GM-42636 (R.M.W.) and a Young Investigator Award from the Beckman Foundation (R.M.W.). R.V.S. was supported by a National Research Service Award Fellowship (GM-14767).

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

0006-2960/95/0434-14626$09.00/0

proteins (Stock et al., 1985). Where CheY is composed only of the response regulator domain, CheB has in addition a catalytic domain at the carboxyl terminus facilitating the hydrolysis of methyl esters on the receptor protein (Stock & Koshland, 1978; Lupas & Stock, 1989; Stewart et al., 1990). Both phosphorylated CheY and CheB are the active forms; phosphorylated CheY interacts with the motor switch proteins, thereby promoting clockwise motor rotation in *E. coli* and *Salmonella typhimurium*, a close relative of *E. coli* (Welch et al., 1993), and phosphorylated *S. typhimurium* CheB has over 10-fold higher methylesterase activity *in vitro* relative to the unphosphorylated form (Lupas & Stock, 1989).

Since the elucidation of the basic biochemical (phosphorylation and methylation) reactions in the chemotaxis pathway, the focus of this field has shifted more toward identifying the noncovalent protein−protein interactions in the signal transduction cascade with emphasis on the formation and characterization of behaviorally relevant multiprotein complexes (McNally & Matsumura, 1991; Gegner & Dahlquist, 1991; Gegner et al., 1992; Long & Weis, 1992; Swanson et al., 1993; Schuster et al., 1993; Welch et al., 1993; Blat & Eisenbach, 1994). CheA is centrally positioned in the signal transduction chain, it interacts both with the receptor and the response regulators, CheY and CheB. CheA has been shown to form a high-affinity complex with CheY (McNally & Matsumura, 1991; Swanson et al., 1993). Fragments of CheA also bind to CheY, allowing the binding domain on CheA to be localized between residues 98 and 233 (Swanson et al., 1993; Morrison & Parkinson, 1994).

In this study, the interactions of CheY and CheB with CheA have been characterized using ITC,[1] which provides the first complete report of all the thermodynamic parameters in the binding reactions. The competitive nature of CheB and CheY binding was also established, and the effects of point mutations in CheY and phosphorylation of CheY were measured to determine the influence of conformation on the strength of CheY−CheA interactions, which is a matter of physiological significance for signal amplification. The relative affinities of CheB and CheY, the arrangement of the binding site, and the effect of phosphorylation are all important in developing a quantitative understanding of the signal transduction process through modeling studies (Bray et al., 1993; Hauri & Ross, 1995). Finally, this binding study provides information relevant to the analysis of molecular recognition within the large family of histidine kinases and response regulators.

MATERIALS AND METHODS

Protein Purification. CheB was purified from the *E. coli cheA* strain KO685 (Hess et al., 1987) containing the expression vector pCW/*cheB*, a generous gift from A. F. Roth and F. W. Dahlquist (University of Oregon). After reaching an apparent absorbance at 600 nm (OD_{600}) equal to ca. 0.8 (approximate conversion factor: 10^9 cells/mL/absorbance unit), 1-L LB cultures (typically three or four) of KO685 transformed with pCW/*cheB* at 37 °C were induced to express CheB by the addition of IPTG to 1 mM. Incubation was maintained for 2−3 h more before the cells were harvested by centrifugation (4800*g* for 10 min). The high level of expression from this plasmid made possible a simplified purification scheme adapted from the procedure of Simms et al. (1985). Frozen cells were thawed and resuspended in Tris/Mg buffer (25 mM Tris, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, pH 7.5, 10% glycerol) containing 0.1 mg of RNaseA/mL, 0.1 mg of DNase I/mL, and 0.5 mM PMSF. The suspended cells were lysed by sonication and the resulting lysate was clarified by centrifugation for 30 min at 123 000*g*. The cleared lysate was fractionated by adding 29.6 g of $(NH_4)_2SO_4$ per 100 mL while stirring on ice. After 30 min on ice, the solution was centrifuged (27000*g* for 30 min), the supernatant was discarded, and the pellet was resuspended and dialyzed against Tris/Mg buffer. The dialyzed solution was passed over a DEAE−cellulose column (2.5 cm i.d. × 6 cm) equilibrated in Tris/Mg buffer. The column flow-through and effluent from one column volume wash were pooled and concentrated either by centrifugation (3000*g*) in a Centriprep-10 concentrator (Amicon, Beverly, MA) or by precipitation with 49 g of $(NH_4)_2SO_4$ per 100 mL. Concentrated protein was dialyzed against 50 mM $NaPO_4$, 5 mM $MgCl_2$, pH 7, 1 mM 2-mercaptoethanol. The final purification step was either FPLC gel filtration performed at 0.5 mL/min using a Superdex-75 HR 10/30 column equilibrated in 25 mM Tris, 50 mM KCl, 5 mM $MgCl_2$, pH 7.5, or alternatively chromatography on a 2.5 cm i.d. × 4 cm Affi-gel Blue column. Affi-gel Blue chromatography was performed by binding the protein to the column and washing with the dialysis buffer containing 25 mM NaCl. The protein was then eluted with the same buffer containing 1 M NaCl.

$CheA_{1-233}$ was purified from *E. coli* strain M15 containing plasmids pREP4 and pRS1−4 by chromatography on a Ni−NTA column as described previously (Swanson et al., 1993), followed by DEAE ion-exchange chromatography which consisted of a linear gradient from 0 to 0.7 M NaCl in 50 mM sodium phosphate, 5 mM EDTA, 2 mM β-mercaptoethanol, pH 7.5. CheA was purified according to published procedures (Hess et al., 1988) in the *E. coli* strain RP3808 transformed with the plasmid pDV4 (a gift from P. Matsumura; McNally & Matsumura, 1991) for CheA expression. CheY was purified according to published protocols (Matsumura et al., 1985; Sanders et al., 1989) from the *E. coli* strain RP3808 transformed with the CheY expression plasmid pCW/*cheY*, a gift from F. W. Dahlquist. $CheY_{Y51C}$, $CheY_{D13K}$, $CheY_{D12E,D57E}$, and $CheY_{A103V}$ were purified as previously described (Swanson et al., 1993).

Determination of Protein Concentrations. To measure protein concentrations precisely, several methods were used to determine the molar extinction coefficient at 280 nm (ϵ_{280}) of CheA, $CheA_{1-233}$, CheB, and CheY. The proteins used in the determination of molar extinction coefficients were judged to be >95% pure by scanning densitometry of samples resolved on Coomassie-stained polyacrylamide gels. Absorbance spectra for each of the proteins were recorded from 250 to 340 nm using a Cary 14 UV−visible spectrophotometer, and the absorbance difference between 280 and 320 nm was taken as A_{280}. The masses of protein samples were determined by amino acid analysis on an ABI model 420/130A derivatizer/analyzer (Applied Biosystems, Foster City, CA), and also, using independent samples, determined

[1] Abbreviations: ITC, isothermal titration microcalorimetry; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IPTG, isopropyl thio-β-D-galactoside; PMSF, phenylmethylsulfonyl fluoride; LB, Luria broth; GFC, gel filtration chromatography; Ni−NTA, nickel−nitrilotriacetic acid.

by the BCA assay (Smith et al., 1985). The masses of CheY and $CheA_{1-233}$ were also determined by the method of dry weights (Kupke & Dorrier, 1978). Values for the molar extinction coefficient obtained by the different methods were found to be in good agreement. The averaged unit absorbances at 280 nm in a 1 cm path cuvette were calculated to be 2.9, 4.9, 1.7, and 1.3 mg/mL of CheA, $CheA_{1-233}$, CheB and CheY, respectively. Using molecular weights of 73, 27, 37, and 14 kDa for CheA, $CheA_{1-233}$, CheB, and CheY, the respective ϵ_{280} values of 25 000, 5 500, 22 000, and 11 000 $(M\ cm)^{-1}$ were computed.

Phosphorylation of CheY. To test the effect of CheY phosphorylation on CheA−CheY interactions, CheY was phosphorylated using the protocol developed by Lukat et al. (1992) using acetyl phosphate (Sigma, St. Louis, MO, Catalog No. A0262) as the phosphodonor. The extent of phosphorylation was determined by the quenching of tryptophan fluorescence as described previously (Lukat et al., 1992) using a PTI QM-1 spectrofluorometer. CheY was incubated in reaction buffer (20 mM sodium phosphate, 20 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, 20 mM acetyl phosphate, pH 7.4) for 20 min at room temperature. Phosphorylated CheY was used immediately in the calorimetry experiments described below. In some cases the reactions were stopped by the addition of EDTA to a final concentration of 20 mM.

Isothermal Titration Calorimetry. All titrations were carried out using a MicroCal MCS ultrasensitive titration calorimeter (MicroCal Inc., Northampton, MA) with Observer software for instrument control and data acquisition. Instrumentation, software, and data analysis have been described elsewhere (Wiseman et al., 1989; Brandts et al., 1990; Lin et al., 1994). Before a titration experiment, protein samples designated for the reaction cell (1.343 mL) and the syringe (100 or 250 μL) were dialyzed against buffer (0.5 L, 20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, pH 7.4) in the same flask overnight at 4 °C. Titrations were carried out in the same buffer over a range of temperatures (8.2−38.5 °C), using a syringe-stirring speed of 500 rpm. Typical titration experiments consisted of 10−30 injections in which the individual injections were 5−10 μL each and were made every 3−4 min. The calibrated cell feedback signal (μcal/s) was collected at 2-s intervals. In most cases the areas derived from the first injection were not used in the analysis since anomolously small values were observed, presumably due to sample dilution during the pre-equilibration step. Experimental data were corrected for buffer mismatch by subtracting a value similar in magnitude to the heat of injection after the system had reached saturation, which was determined as the average heat of the last three injections. The Origin software supplied with the instrument was used to analyze data according to a single-set-of-sites model from which values for the binding stoichiometry, n, the enthalpy of binding, ΔH, and the association constant, K_a, were obtained. The n value is defined as the molar binding ratio of the molecule in the syringe to the molecule in the reaction cell, and binding enthalpies are expressed in kilocalories per mole of injectant. The values of K_a, ΔH, and n returned by the fits were used to compute $\Delta S°$ and $\Delta G°$ at the experimental temperature (T). Whenever possible the binding reactions were carried out at either two or more temperatures, so that the change in the heat capacity (ΔC_p) accompanying the association reaction could be determined and used to compute values of the thermodynamic parameters at the standard temperature (298 K, referred to hereafter as T_0) which were reported as average values for the association reactions summarized in Table 1. To check the stability of the proteins during a titration, samples were compared by SDS−PAGE before and immediately after titration. There was no proteolysis evident in the samples reported in this study.

In experiments with phosphorylated CheY and $CheA_{1-233}$, fluorescence quenching was measured before and after titration experiments to demonstrate that the level of phosphorylation was unchanged during the course of the experiment. Since $CheA_{1-233}$ has no Trp residues, it does not contribute significantly to the tryptophan emission spectrum. Fluorescence quenching was also used to monitor the extent of CheY phosphorylation over the course of a typical titration experiment (ca. 90 min) to evaluate the extent of CheY phosphorylation in titration experiments where the phosphorylation and dephosphorylation reactions had not been stopped by the prior addition of EDTA. In these experiments, phosphorylated CheY was added to both the sample cell and the reference cell to balance the effects of the phosphorylation and hydrolysis heats of reaction.

Competitive Binding Assay with Ni−NTA Affinity Resin. Experiments were carried out using Ni−NTA resin (Qiagen Corp., Chatsworth, CA) to determine the competitive nature of CheY and CheB binding by an independent method. 500 pmol His-tagged $CheA_{1-233}$ was captured on Ni−NTA resin (ca. 10 mg of resin, providing an excess of Ni−NTA binding sites) in a mixture that contained either 250 pmol of CheY or 380 pmol of CheB in phosphate buffer (50 mM sodium phosphate, 150 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5). In some tubes either an additional 3.8 nmol of CheB or 2.5 nmol of CheY was added. The total volume was adjusted to 50 μL with buffer, and the samples were equilibrated on ice for 5 min. The Ni−NTA resin was then separated from the supernatant by passing the sample through 1.5 mL micropure separators (Amicon Corp., Beverly, MA) at 16,000g for 4 min. Protein that remained bound to the Ni−NTA resin was collected by resuspending the resin in 40 μL of phosphate buffer that also contained 250 mM imidazole, and the supernatant was collected by centrifugation after a 5 min incubation period. 5-μL aliquots of the supernatants were analyzed by SDS−PAGE.

RESULTS

Titration Calorimetry of CheY and $CheA_{1-233}$. A series of titration experiments were carried out which verified that $CheA_{1-233}$ bound to CheY in a 1:1 ratio. The top data trace in Figure 1a shows six 10-μL control injections, spaced at 200-s intervals, of a 0.51 mM $CheA_{1-233}$ solution into buffer at 11.5 °C. Titrations of $CheA_{1-233}$ into solutions of CheY at 11.3 and 28.5 °C are shown in the middle and lower traces of Figure 1a, respectively. Experimental parameters, including protein concentrations and titration temperatures, are summarized in Table 1. The large exothermic peaks recorded in the middle and lower curves were due to the specific binding of $CheA_{1-233}$ to CheY. The injection heats displayed a steady decrease in exothermicity as higher mole ratios of $CheA_{1-233}$ to CheY were produced. The heats became endothermic and displayed little change in area once

Table 1: Thermodynamic Parameters for Titrations Involving CheA with CheY or CheB[a]

expt no.	protein (concn) reaction cell (μM)	syringe (mM)	temp (°C)	n	K_a ($M^{-1} \times 10^{-5}$)	ΔH (kcal/mol)	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)	ΔC_p (kcal/mol·°C)	mean values at 298 K (±sd) K_{a298} ($M^{-1} \times 10^{-5}$)	$\Delta H°_{298}$ (kcal mol)	$\Delta S°_{298}$ (eu)
1	CheY (37)	$CheA_{1-233}$ (1.13)	10.3	0.95	17.8	−8.4	−8.1	−1.1				
2	CheY (38)	$CheA_{1-233}$ (0.85)	11.3	0.94	16.7	−9.3	−8.1	−4.1	−0.22	8.1 ± 0.8	−12.0 ± 0.5	−13 ± 2
3	CheY (40)	$CheA_{1-233}$ (1.13)	28.0	0.98	7.4	−12.4	−8.1	−14.4				
4	CheY (43)	$CheA_{1-233}$ (0.80)	28.5	0.90	6.3	−12.3	−8.0	−14.2				
5	CheY (47)	$CheA_{1-233}$ (0.83)	28.5	0.92	6.7	−13.5	−8.0	−18.0				
6	$CheA_{1-233}$ (28)	CheY (0.67)	10.2	1.04	13.9	−7.6	−8.0	1.2				
7	$CheA_{1-233}$ (35)	CheY (1.29)	10.5	1.00	19.5	−9.2	−8.2	−3.8	−0.23	9 ± 2	−11.7 ± 0.7	−12 ± 2
8	$CheA_{1-233}$ (28)	CheY (0.80)	28.0	0.97	8.5	−12.2	−8.2	−13.3				
9	$CheA_{1-233}$ (33)	CheY (1.29)	28.5	0.90	8.8	−12.8	−8.2	−15.3				
10	CheA (25)	CheY (0.67)	10.1	1.02	8.8	−7.2	−7.7	1.7	−0.40	5 ± 1	−13.1 ± 0.1	−17.9 ± 0.6
11	CheA (31)	CheY (0.80)	28.0	0.87	5.1	−14.3	−7.9	−21.5				
12	CheY−Mg^{2+} (40)[b]	$CheA_{1-233}$ (1.13)[b]	28.0	1.01	6.9	−12.2	−8.0	−13.9				
13	CheY−P (21)[c]	$CheA_{1-233}$ (0.70)	8.2	0.95	6.4	−7.1	−7.5	1.4	−0.29	4 ± 1	−12.0 ± 0.1	−14.8 ± 0.6
14	CheY−P (50)[c]	$CheA_{1-233}$ (0.60)	28.2	1.01	3.8	−12.9	−7.7	−17.4				
15	$CheY_{A103V}$ (43)	$CheA_{1-233}$ (0.85)	11.5	0.91	1.4	−6.5	−6.7	0.86	−0.15	0.81 ± 0.01	−8.4 ± 0.1	−5.6 ± 0.3
16	$CheY_{A103V}$ (43)	$CheA_{1-233}$ (0.83)	28.5	0.93	0.69	−8.8	−6.7	−7.2				
17	$CheY_{Y51C}$ (37)	$CheA_{1-233}$ (1.13)	10.8	0.96	17.2	−8.4	−8.1	−1.04	−0.37	6.6 ± 0.4	−13.6 ± 0.1	−18.9 ± 0.1
18	$CheY_{Y51C}$ (35)	$CheA_{1-233}$ (1.13)	28.0	0.92	4.8	−14.7	−7.8	−22.9				
19	CheB (59)[d]	$CheA_{1-233}$ (0.71)	8.5	nd	nd	0	nd	nd				
20	CheB (38)	$CheA_{1-233}$ (0.59)	18.8	0.96	3.2	−5.3	−7.4	6.9	−0.45	3.1 ± 0.5	−8.0 ± 0.8	−2 ± 3
21	CheB (36)	$CheA_{1-233}$ (0.59)	28.0	0.94	2.7	−10.1	−7.5	−8.7				
22	CheB (30)	$CheA_{1-233}$ (0.59)	38.2	0.88	1.7	−13.2	−7.4	−18.5				
23	CheB−Mg^{2+} (80)[b]	$CheA_{1-233}$ (1.13)[b]	28.0	0.94	2.96	−8.2	−7.5	−2.1				

[a] n, K_a, and ΔH were obtained from the single-set-of-sites fit to the data. The other parameters were calculated as follows: $\Delta G° = -RT \ln K_a$, $\Delta S° = (\Delta H - \Delta G°)/T$, $\Delta H°_{298} = \Delta H + \Delta C_p(298 - T)$, $\Delta S°_{298} = \Delta S° + \Delta C_p \ln(298/T)$, $K_{a298} = \exp[-\Delta H°_{298}/298R + \Delta S°_{298}/R]$, where ΔC_p was determined as the slope of least-squares fit of ΔH *versus* temperature. Standard deviations were calculated from the averages of K_a, $\Delta H°$, and $\Delta S°$ at 298 K. The buffer in all experiments was 20 mM sodium phosphate, 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, pH 7.4, unless indicated otherwise. [b] 6 mM $MgCl_2$ was added to the titration buffer. [c] CheY−P: CheY was phosphorylated with 20 mM acetyl phosphate in the titration buffer plus 6 mM $MgCl_2$ and quenched with 20 mM EDTA as described in Materials and Methods. [d] nd, not determined. Neither n nor K_a could be determined from the data since $\Delta H \approx 0$.

the binding sites on CheY for $CheA_{1-233}$ were saturated. The endothermic peaks (which point upward from the base line) seen in the control and the last several injections in the titrations were due to the buffer mismatch between the solution in the syringe and the reaction cell. In several control experiments, where the $CheA_{1-233}$ fragment was injected into buffer, the observed heats were nearly constant as a function of the injection number (e.g., see Figure 1a). The magnitudes of the heats in different control experiments were found to be proportional to the difference in the sample dialysis temperature prior to the experiment (4 °C) and the experimental temperature (ranging from 8.5 to 38.5 °C). This mismatch was attributed to the differences in the temperature dependence of the buffer pH and the pH of the protein solution, and was subtracted as background.

The background-corrected, integrated heats are plotted in Figure 1b as kcal per mol of $CheA_{1-233}$ injected *versus* the molar ratio of $CheA_{1-233}$ to CheY. The open circles are the integrated heats of titration at 11.3 °C, and the open squares are the integrated data obtained at 28.5 °C. Fits of the data to a single-set-of-sites model (Wiseman et al., 1989) were drawn through the data in Figure 1b and provided estimates of the binding stoichiometry (n), the enthalpy of binding (ΔH) and the association constant (K_a). An n value of 0.94 was obtained from the fit of the 11.3 °C data. This n value agreed well with the average value (0.94 ± 0.03) determined from five titrations (Table 1, expts 1−5), indicating that $CheA_{1-233}$ and CheY interacted in a 1:1 ratio. The values K_a and ΔH were determined from the data plotted in Figure 1b were 16.7×10^5 M^{-1} and −9.3 kcal/mol, respectively, at 11.3 °C and 6.7×10^5 M^{-1} and −13.5 kcal/mol at 28.5 °C. ΔC_p of binding was found to be −0.22 kcal/mol/deg using the values ΔH determined from five experiments (Table 1, expts 1−5) including those determined from the data plotted in Figure 1b. K_{a298} and ΔH_{298} were determined to be $8.1 \times 10^5 \pm 8 \times 10^4$ M^{-1} and −12.0 ± 0.5 kcal/mol, respectively, from the same five experiments, using ΔC_p to adjust the values obtained from individual experiments to T_0 so that they could be averaged. Finally, the average value of the entropy change ($\Delta S°_{298}$) accompanying CheY−$CheA_{1-233}$ binding was calculated to be −13 ± 2 eu.

To check the consistency of the parameters determined from the experiments described above, $CheA_{1-233}$ and CheY were exchanged so that $CheA_{1-233}$ (in the reaction cell) was titrated with CheY (in the syringe) under otherwise identical conditions. Figure 2a includes plots of the raw data of the injection series of CheY into a solution of $CheA_{1-233}$ at 10.5 °C (top) and 28.5 °C (middle). The raw data were subjected to the same data treatment described above, resulting in the integrated data and fit curves plotted in Figure 2b of normalized binding heat per mole of CheY injected as a function of the mole ratio of CheY to $CheA_{1-233}$. The open circles and squares represent the data obtained at 10.5 and 28.5 °C, respectively. The data were described very well by a simple model that fit the data to a single set of sites. Values for n, K_a, ΔH, and ΔC_p obtained from the titrations of $CheA_{1-233}$ with CheY were in good agreement with the parameters obtained from the inverted titrations, i.e. CheY titrated with $CheA_{1-233}$. The average value of n determined from four experiments (Table 1, expts 6−9) was 0.97 ± 0.06.

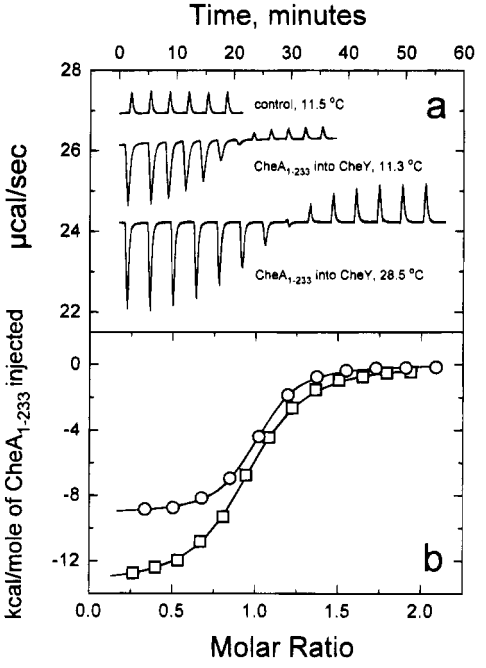

FIGURE 1: The results of titration calorimetry of CheY with $CheA_{1-233}$. The top curve in panel a is a control experiment consisting of 10-μL injections of the $CheA_{1-233}$ fragment into buffer (as described in Materials and Methods) at 11.5 °C. The second and third curves in panel a are titrations of CheY with $CheA_{1-233}$ at 11.3 and 28.5 °C, respectively, using 10-μL injections and the protein concentrations specified in Table 1 (expts 2 and 5). Panel b shows the integrated areas for the titrations between $CheA_{1-233}$ and CheY at 11.3 (○) and 28.5 °C (□). The lines plotted with the data are the best-fit curves described by the values of n, K_a, and ΔH listed in Table 1.

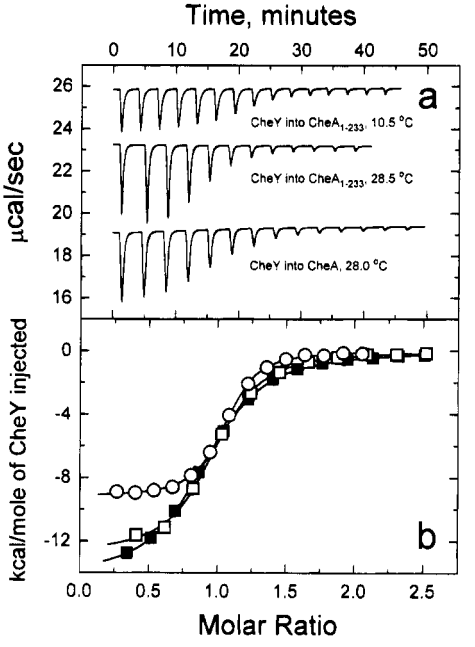

FIGURE 2: Results of titration calorimetry of $CheA_{1-233}$ and intact CheA with CheY. The upper and middle titration curves in panel a are titrations carried out with $CheA_{1-233}$ in the reaction cell and CheY in the syringe at 10.5 and 28.5 °C, respectively. The bottom curve is data from a titration between intact CheA in the reaction cell and CheY in the syringe at 28 °C. Protein concentrations given in Table 1 (expts 7, 9, and 11). Panel b plots the integrated areas for the titrations between $CheA_{1-233}$ and CheY at 11.3 (○) and 28.5 °C (□), and for the titration between intact CheA and CheY (■). The lines plotted through the data are the best-fit curves described by the values of n, K_a, and ΔH listed in Table 1.

Also from the same four experiments ΔC_p was determined to be −0.23 kcal/mol/deg using the ΔH values. K_{a298}, ΔH°_{298}, and ΔS°_{298}, obtained from the titrations of $CheA_{1-233}$ with CheY ($9 \times 10^5 \pm 2 \times 10^5$ M^{-1}, -11.7 ± 0.7 kcal/mol, and -12 ± 2 eu) were in agreement with the corresponding values from titrations of CheY with $CheA_{1-233}$ (see Table 1). Since the titrations of $CheA_{1-233}$ with CheY (Figure 2a) exhibited a small buffer mismatch (and slightly exothermic), the possibility that the mismatch observed in titrations of CheY with $CheA_{1-233}$ (Figure 1a) could have significantly affected the results was eliminated.

Intact CheA and $CheA_{1-233}$ Bind to CheY with Similar Affinities. Most titrations were carried out using the $CheA_{1-233}$ fragment since it was readily purified in high yield and did not precipitate at the high concentrations required in the syringe. However, a few titrations were carried out between intact CheA and CheY to determine what effect, if any, the deletion of amino acids 234−654 had on CheA−CheY interactions. Raw data from one such titration (Table 1, expt 11) carried out at 28.0 °C is plotted in Figure 2a (bottom curve) which after curve-fitting with the single-set-of-sites model resulted in a value for the association constant of 5.1×10^5 M^{-1}. The value of K_a determined for CheY and intact CheA is ca. 60% of the value determined for $CheA_{1-233}$. The modest difference in K_a measured by ITC contrasts sharply with the relative values determined using SPR (Schuster et al., 1993; Swanson et al., 1993), where CheY was observed to bind 12-fold more tightly to intact CheA relative to $CheA_{1-233}$. The opportunity for multivalent interactions to form between intact (dimeric) CheA and (surface-attached) CheY but not with (monomeric) $CheA_{1-233}$ may be largely responsible for the difference in binding constants observed by SPR (see Discussion). In contrast, the ITC experiments (Figures 1 and 2; Table 1) measured single-site interactions, which indicated that the majority of the binding interactions between CheY and CheA resided in the first 233 amino acids. The modest decrease in the affinity of the interactions between CheY and intact CheA may be experimentally significant. Two plausible explanations include either a small (negative) contribution of the carboxyl tail of CheA to binding or a weak negative cooperativity between sites in the CheA dimer.

Effects of Mg^{2+}, Phosphorylation, and Mutation on CheA−CheY Interactions. Several factors that might influence the interactions between CheA and CheY were explored: the presence of divalent cation (Mg^{2+}), which is required for the phosphotransfer reaction; the influence of mutations near the active site and the putative CheA−CheY-binding surface, and phosphorylation of aspartate-57 in the active site. Figure 3 plots representative results of some of these experiments. 5 mM Mg^{2+} was observed to have no significant effect on the interaction of CheY with $CheA_{1-233}$. The values of K_a and ΔH determined from titrations in the presence of Mg^{2+} were, within experimental error, the same as the values obtained in its absence. (Compare expt 12 in Table 1 with expts 3−5.)

CheY was phosphorylated using acetyl phosphate, which has been shown to be able to transfer phosphate to CheY (Lukat et al., 1992). The quenching of intrinsic tryptophan fluorescence was used to estimate that the extent of CheY phosphorylation was over 90% using the reaction conditions described in Materials and Methods. Titrations were carried

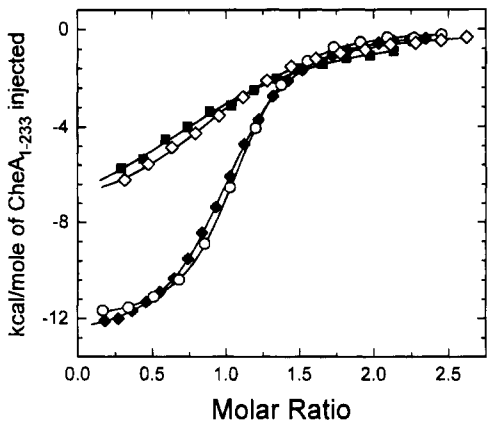

FIGURE 3: Injection heats from titrations between $CheA_{1-233}$ and various CheYs. CheY (reaction cell) was titrated with $CheA_{1-233}$ (syringe) under different conditions: CheY in the presence of 6 mM $MgCl_2$ (O), phosphorylated CheY in the presence of excess EDTA (◆), and the A103V mutant of CheY (■) according to the specific conditions summarized in Table 1, experiments 12, 14, and 16, respectively. In the titration of phosphorylated CheY in the presence of 6 mM $MgCl_2$ (◇), 40 μM CheY was titrated with 1.05 mM $CheA_{1-233}$ in reaction buffer using 8-μL injections at 28 °C, as described in Materials and Methods.

out both on phosphorylated CheY (CheY−P) in which the phosphorylation and dephosphorylation reactions had been quenched by an excess of EDTA prior to the experiment and also in the presence of excess Mg^{2+} and acetyl phosphate. K_a values obtained from experiments where the phosphotransfer reaction was first quenched with EDTA were ca. 50% of the values obtained with unphosphorylated CheY. Figure 3 shows the integrated heats and fits to a titration (Table 1, expt 14) between $CheA_{1-233}$ and CheY−P carried out at 28.2 °C that produced an association constant of 3.8 × 10^5 M^{-1}. Although the value for K_{a298}, determined with CheY−P was ca. 50% of K_{a298} determined with CheY, the values of $\Delta H°_{298}$ were the same within experimental error (12.0 ± 0.1 kcal/mol).

Titration experiments performed with $CheA_{1-233}$ and CheY in which both acetyl phosphate and Mg^{2+} were present (i.e., in the absence of EDTA to quench the reaction) produced values of K_a that were ca. 6-fold lower than unphosphorylated CheY. Integrated heats from a titration carried out at 28.0 °C between 1.05 mM $CheA_{1-233}$ (syringe) and 40 μM CheY (reaction cell) are plotted in Figure 3. The experiment consisted of sixteen 8-μL injections in a buffer [20 mM sodium phosphate, pH 7.4, and 20 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10% glycerol (v/v)] that also contained 6 mM $MgCl_2$ and 20 mM acetyl phosphate. Under these conditions the phosphorylation reaction was estimated to be ca. 90% complete by fluorescence quenching (data not shown). K_a, ΔH, and n were determined from the data and found to be 1.5 × 10^5 M^{-1}, −7.9 kcal/mol and 0.95, respectively. A second experiment carried out in the same buffer system but at 7.5 °C with 0.58 mM $CheA_{1-233}$ and 60 μM CheY produced respective values for K_a, ΔH, and n of 3.0 × 10^5 M^{-1}, −9.0 kcal/mol, and 0.98. From these data it would appear that binding of Mg^{2+} at the active site and phosphorylation at aspartate-57 of CheY together have a significant effect on the interaction of CheA with CheY.

Four mutants of CheY were tested for altered affinity to CheA. The most significant decrease in affinity was detected with an alanine-103 to valine (A103V) point mutant of CheY which yielded titration data with values for K_{a298} that were 10-fold lower than wild type CheY (Figure 3 and Table 1). Alanine-103 of CheY is proposed to be near to the CheY−CheA binding interface, a conclusion based on the analysis of changes in the ^{15}N-^1H correlation spectrum of CheY which are observed to occur upon binding of $CheA_{1-233}$. On this basis, $CheY_{A103V}$ was investigated and shown to be defective in binding $CheA_{1-233}$ (Swanson et al., 1995). The active-site point mutant aspartate-13 to lysine (D13K), which produces the tumble phenotype in cells without being phosphorylated (Bourret et al., 1990), and the D12E, D57E nonphosphorylatable double mutant of CheY were both found to bind to $CheA_{1-233}$ with an affinity ca. 2-fold lower than wild type CheY (data not shown). Finally, the tyrosine-51 to cysteine (Y51C) point mutant that was used in SPR studies of CheA−CheY interactions (Schuster et al., 1993; Swanson et al., 1993) was found to yield values of K_a (Table 1, expts 17 and 18) similar to wild type CheY. Observations from this small sample of CheY mutants were consistent with the interpretation that the effects of the mutations tended to be local, i.e., the largest effects on binding were observed when the mutations were made close to the CheA−CheY binding interface.

Interaction of CheB with $CheA_{1-233}$ Observed by GFC. Gel filtration chromatography was used to qualitatively characterize the interaction between $CheA_{1-233}$ with CheB. As shown in Figure 4a, top trace, CheB eluted from the gel filtration column with a volume that was consistent with the molecular mass of 37 kDa (upper trace) and agreed well with the predicted mass of the monomer on the basis of sequence data (Mutoh & Simon, 1986). As described by Swanson et al. (1993) and shown in Figure 4a (bottom trace), $CheA_{1-233}$ eluted with a retention time characteristic of a 45 kDa globular protein, which was significantly larger than the predicted M_r of 27 kDa based on sequence. $CheA_{1-233}$ was a monomer nonetheless since it eluted with a retention volume that was significantly smaller than the smallest possible elution volume based on a globular dimer of $CheA_{1-233}$ (54 kDa). When a mixture of $CheA_{1-233}$ and CheB (1.5-fold molar excess of $CheA_{1-233}$) was coinjected (Figure 4a, middle trace), the CheB was found to elute with a larger apparent mass, as determined by PAGE analysis of the column fractions (Figure 4b), suggesting that the two proteins formed a 1:1 complex.

Titration Calorimetry of CheB and CheA. Titrations of CheB in the reaction cell with $CheA_{1-233}$ in the syringe were carried out under conditions similar to those of CheY with $CheA_{1-233}$ at four different temperatures, 8.5, 18.8, 28.0, and 38.2 °C, according to the concentrations of $CheA_{1-233}$ and CheB listed in Table 1 (expts 19−22). The heat of binding was nearly zero at 8.5 °C (expt 19), which precluded estimates of K_a and n being made at that temperature. Nevertheless, those data were still useful in determining ΔC_p, where a plot of ΔH *versus* temperature indicated a linear relationship and produced a value of −0.45 kcal/mol/deg by linear regression (R = 0.993). Using ΔC_p, the association constant for $CheA_{1-233}$ and CheB (K_{a298}) was estimated as 3.1 × 10^5 M^{-1}, which was 40% of the value determined for $CheA_{1-233}$-CheY interactions. "Inverted" titrations with $CheA_{1-233}$ in the reaction cell and CheB in the syringe were not carried out because the solubility limits of CheB prevented its use in the syringe.

It was interesting to note that $\Delta H°_{298}$ and $\Delta S°_{298}$ of $CheA_{1-233}$-CheB association (Table 1) were both significantly

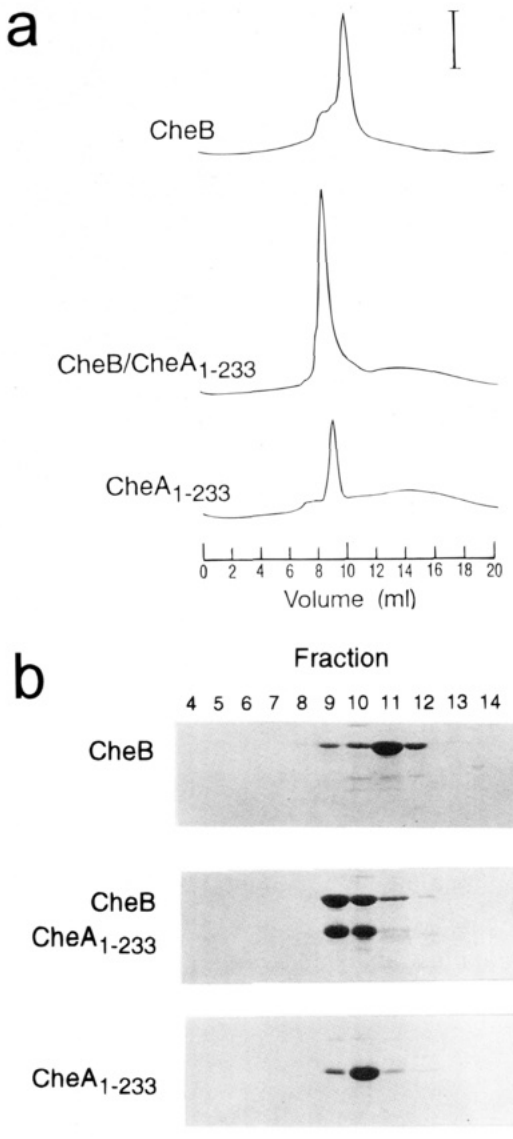

FIGURE 4: Results of GFC experiments with CheB and $CheA_{1-233}$. In panel a, the abosrbance at 280 nm is plotted as a function of elution volume for CheB (top), a mixture of $CheA_{1-233}$ and CheB (middle) and $CheA_{1-233}$ (bottom). Samples were eluted from a Superdex-75 HR10/3 column (Pharmacia) in 25 mM Tris, 50 mM KCl, 5 mM $MgCl_2$, pH 7.5, buffer at a flow rate of 0.5 mL/min. The scale bar is 0.02 absorbance units. In panel b the SDS−PAGE analysis of 1-mL fractions collected from the above GFC trace of the $CheA_{1-233}$/CheB mixture are displayed. The $CheA_{1-233}$ was mixed with CheB in a 1.5 molar excess.

smaller than the values characterizing the interactions between $CheA_{1-233}$ and CheY. The decrease in magnitude of both ΔH°_{298} and ΔS°_{298} had compensating effects on ΔG°_{298}, so the difference in ΔG°_{298} (and thus K_{a298}) for the two processes was modest by comparison. The thermodynamic parameters in Table 1 indicate that the enthalpic and entropic contributions to ΔG°_{298} for the binding of CheY and CheB to $CheA_{1-233}$ are significantly different. Comparison of the entropic contribution ($T_0\Delta S^{\circ}_{298}$) to ΔG°_{298} provides a simple illustration of the difference in the driving force for complex formation. $T_0\Delta S^{\circ}_{298}$ only weakly opposes CheB−$CheA_{1-233}$ complex formation (ca. 0.6 kcal/mol), in comparison to the unfavorably large value for formation of the CheY−$CheA_{1-233}$ complex (3.7 kcal/mol).

From the results of these experiments it was evident that $CheA_{1-233}$ and CheB formed a 1:1 complex. Moreover, a few titrations between intact CheA and CheB resulted in

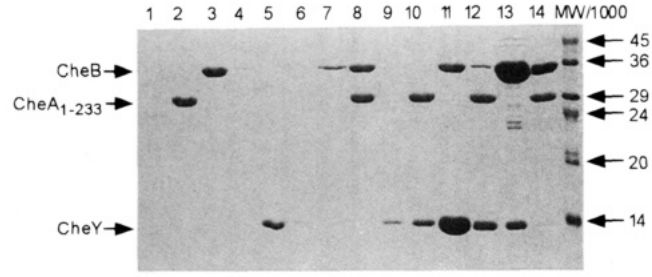

FIGURE 5: Analysis of competitive binding between CheY and CheB with histidine-tagged $CheA_{1-233}$ by Ni−NTA affinity interactions. As described in Materials and Methods, the SDS−polyacrylamide gel (15%) shows the proteins found in the supernatants of Ni−NTA resin previously equilibrated with Che proteins in buffer without imidazole (odd-numbered lanes) followed by supernatants collected from a wash step carried out with buffer containing 250 mM imidazole (even-numbered lanes). The Che proteins (in each of pair of lanes) were 50 pmol of $CheA_{1-233}$ (1 and 2); 38 pmol of CheB (3 and 4); 25 pmol of CheY (5 and 6); 50 pmol of $CheA_{1-233}$ and 38 pmol of CheB (7 and 8); 50 pmol of $CheA_{1-233}$ and 25 pmol of CheY (9 and 10); 50 pmol of $CheA_{1-233}$, 38 pmol of CheB and 250 pmol of CheY (11 and 12); 50 pmol of $CheA_{1-233}$, 380 pmol of CheB, and 25 pmol of CheY (13 and 14). Lane 15 contains molecular weight standards.

values of K_a which were the same as those obtained for CheB and $CheA_{1-233}$ within experimental error (data not shown). These data indicate a similarity in the binding of CheY and CheB to CheA, i.e., residues 234−654 of CheA do not appear to be involved in the binding process.

CheB and CheY Compete for Binding to $CheA_{1-233}$ using a Ni−NTA Binding Assay. CheY binding to CheA was previously localized between residues 135 and 233 of the intact CheA molecule in an SPR binding study (Swanson et al., 1993). The N-terminal regulatory domain of the methylesterase (CheB) is phosphorylated on a conserved aspartate residue but otherwise has a relatively low (23%) amino acid identity with CheY (Mutoh & Simon, 1986). To test whether CheY and CheB recognized the same site on CheA, the competitive binding experiment summarized in Figure 5 was carried out. The gel lanes in Figure 5 report the ability of $CheA_{1-233}$, CheY, and CheB to bind to the affinity matrix (Ni−NTA Sepharose), when the proteins were incubated either individually or in various mixtures. When incubated in phosphate buffer without imidazole, $CheA_{1-233}$ was completely removed from the solution by the Ni−NTA sites on the Sepharose beads (which are in excess) through a high-affinity interaction with the $(histidine)_6$ tag located at the C-terminus of $CheA_{1-233}$ (lane 1). The $CheA_{1-233}$ eluted when the Sepharose beads were washed with phosphate buffer containing 250 mM imidazole (lane 2). Neither CheY nor CheB bound to the Ni−NTA Sepharose when they were subjected to the same treatment (lanes 3−6). When either CheY or CheB was incubated with a stoichiometric excess of $CheA_{1-233}$ and Ni−NTA Sepharose, the majority of protein was bound to the Ni−NTA Sepharose and then coeluted (with $CheA_{1-233}$) in the 250 mM imidazole wash (lanes 7−10). These results demonstrated that CheY and CheB were captured on the affinity matrix via specific interactions with $CheA_{1-233}$. In other experiments, a smaller fragment of CheA (residues 1−134 of CheA followed by the C-terminal-histidine affinity tag) could also be captured by and eluted from the Ni−NTA Sepharose in the same manner as $CheA_{1-233}$, but neither CheY nor CheB could be captured using $CheA_{1-134}$ (data not shown). These results demonstrate that amino acid residues 135−233 are required

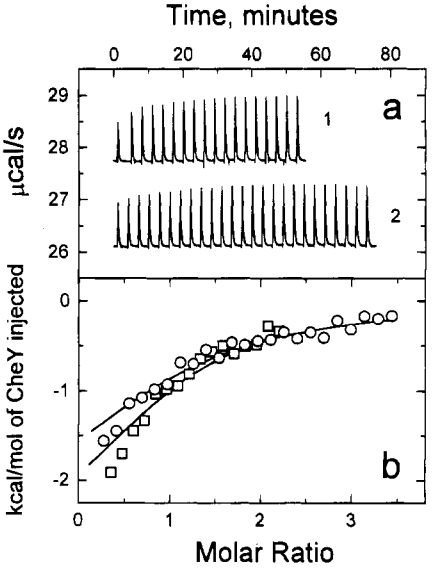

FIGURE 6: Raw data from competitive titration experiments of CheY and CheB binding to $CheA_{1-233}$ at 28.5 °C are plotted in panel a. The upper and lower curves are displacement reactions of the $CheA_{1-233}$–CheB complex in the reaction cell titrated with CheY in the syringe using 5-μL injections and respective concentrations of $CheA_{1-233}$, CheB, and CheY of 26 μM, 51 μM, and 0.83 mM in the upper curve and 35 μM, 110 μM, and 1.29 mM in the lower curve. Integrated areas are plotted in panel b from the upper (open squares) and lower (open circles) curves of panel a. Control heats were subtracted to adjust the data to the simulated binding isotherms. The solid lines are simulated binding isotherms derived from the estimates of K_{app} and $\Delta\Delta H$ as described in the text, with n fixed as 1.

for CheB binding, as demonstrated previously for CheY (Swanson et al., 1993).

CheB and CheY compete with one another for binding to $CheA_{1-233}$. For example, when a large excess of CheY was added to the mixture of CheB and $CheA_{1-233}$, the majority of CheB was not captured by Ni–NTA Sepharose but remained in the supernatant (Figure 5, lane 11), and CheY was the predominant protein that coeluted with $CheA_{1-233}$ (lane 12). Some CheB also coeluted with $CheA_{1-233}$, but in an amount that was consistent with the relative association constants and concentrations of CheY and CheB used in the experiment. Similar behavior was observed when the relative concentrations of CheY and CheB were reversed. With a large stoichiometric excess of CheB relative to CheY (ca. 15-fold), CheB was the major protein that was observed to coelute with $CheA_{1-233}$ (lanes 13 and 14). These results provide strong evidence for competitive binding of CheB and CheY to $CheA_{1-233}$. The competition for binding to $CheA_{1-233}$ was not a nonspecific effect due to large protein concentrations, since BSA at similar high concentrations did not affect the interaction of either CheY or CheB with $CheA_{1-233}$ (data not shown).

Competitive ITC Experiments with $CheA_{1-233}$, CheY, and CheB. The results of the Ni–NTA affinity binding experiments demonstrate that a large excess of CheY could compete for binding with CheB to $CheA_{1-233}$. Competition between CheY and CheB was also tested in ITC experiments in which the ratio of CheY to CheB was varied continuously. Raw data from two experiments carried out at 28.5 °C are plotted in Figure 6a, where the $CheA_{1-233}$–CheB complex ($CheA_{1-233}$ in an excess of CheB) was titrated with CheY. The integrated heats are plotted in Figure 6b. The overall change in the magnitude of the heats between the first and last injection was small, ca. 2 kcal/mol injectant. This range was 4-fold smaller than the range of heats observed in simple titrations and was consistent with the expected enthalpy change ($\Delta\Delta H$) for the displacement of one Che protein by the other. As described below a more careful analysis of the data demonstrated that the results were consistent with competitive binding between CheB and CheY.

The top injection series in Figure 6a consisted of eighteen 10-μL injections of 0.83 mM CheY into a solution that was 0.026 mM in $CheA_{1-233}$ and 0.051 mM in CheB. The gradual increase in the areas of the endothermic peaks was indicative of an exothermic process. The heats tended toward a steady level, which was assumed to contribute a constant background and was subtracted from the data. This resulted in a series of heats that resembled a low-affinity, weakly-exothermic binding process which was expected for displacement of CheB by CheY (calculated $\Delta\Delta H \approx -3$ kcal/mol at 28.5 °C, see Table 1). The lower injection series of Figure 6a were obtained with a larger proportion of CheB (1.29 mM CheY, 0.035 mM $CheA_{1-233}$, and 0.11 mM CheB). These two experiments produced qualitatively similar results, indicating that CheB was competing with CheY in binding to $CheA_{1-233}$. A second type of competitive titration experiment (not shown), in which CheY was displaced from the $CheA_{1-233}$–CheY complex by CheB, was also consistent with competitive binding. Finally a control experiment (not shown) which consisted of nine 10-μL injections of 0.17 mM CheY into 10 μM CheB gave no indication of an interaction between CheY and CheB.

Data from the competitive titration experiments were found to be in quantitative agreement with the thermodynamic parameters determined from the simple binding studies of CheY and CheB with $CheA_{1-233}$. On the basis of competitive binding, the trends displayed by the titration data in Figure 6b could be explained with computed values for an apparent binding constant, K_{app}, and the difference in the binding enthalpies of CheY and CheB, $\Delta\Delta H$. The latter were estimated using the thermodynamic parameters obtained in the direct binding experiments. An approximate expression for K_{app} of CheY binding to $CheA_{1-233}$ in the presence of CheB is given by

$$K_{app} = \frac{K_{AY}}{(1 + K_{AB}[\overline{B_{free}}])} \quad (1)$$

where K_{AY} and K_{AB} are the association constants between $CheA_{1-233}$ and CheY and between $CheA_{1-233}$ and CheB, respectively, and $[\overline{B_{free}}]$ is the average concentration of free CheB during the course of the titration (see the Appendix). K_{app} was estimated using values for K_{AY} and K_{AB} of 6.5×10^5 and 2.5×10^5 M^{-1}, respectively, and -3.1 kcal/mol was used for $\Delta\Delta H$. $[\overline{B_{free}}]$ was computed as the average of the free CheB concentration at the beginning and the end of the titration. Values for K_{app} of 2.7×10^4 and 5.8×10^4 M^{-1} were estimated from eq 1 using values of 93 and 41 μM for $[\overline{B_{free}}]$, respectively, and were used to generate the theoretical titration curves for the integrated heats plotted in Figure 6b (as circles and squares, respectively). Considering the approximations that were made in deriving eq 1, estimating $[\overline{B_{free}}]$, and taking into account the small values of both K_{app} and $\Delta\Delta H$, the agreement between the simulations and the

data was found to be quite reasonable, and thus these titrations experiments provided additional support for competitive binding of CheB and CheY to the same (or mutually exclusive) site(s) on CheA.

DISCUSSION

Properties of CheY and CheB Binding to CheA. The results of this study demonstrate that the binding of CheY and CheB to the $CheA_{1-233}$ fragment is competitive, that the individual affinities of CheY and CheB for $CheA_{1-233}$ are comparable (differing by a factor of 2), and that truncating CheA has little effect on the strength of the interactions with either CheB or CheY. These results are summarized in Table 1. On first inspection, the similarity in the strength of the binding interaction is unexpected given that *E. coli* CheY and the amino terminal domain of CheB have only a moderate amount of sequence identity, ca. 23% (Mutoh & Simon, 1986). Furthermore, the majority of the conserved residues are found in the region of the active site, which are regarded as part of the "consensus" residues in the CheY superfamily (Volz, 1993). Considering that a seemingly subtle change caused by a single mutation (A103V) near the CheA−CheY binding interface can significantly reduce the association constant (10-fold), one may arrive at the conclusion that, while the specific (favorable and unfavorable) interactions of CheA with CheY may be very different from those of CheA with CheB, the sum of the interactions must be similar for the two cases.

The negative value of ΔC_p is a feature common to CheY and CheB binding to $CheA_{1-233}$, −0.22 and −0.45 kcal/mol/deg, respectively, and is typical of protein association reactions (Hibbits et al., 1994, and references therein). A negative ΔC_p can be interpreted as qualitative evidence for a decrease in the amount of hydrophobic surface in contact with water (Brandts, 1964), although other factors may contribute (Sturtevant, 1977). A comparison of the measured ΔC_p values with structure-based estimates determined from changes in solvent-exposed hydrophobic surface could be carried out were the structures of all of the Che proteins and their complexes known. In other studies where such comparisons have been made, the estimates were significantly smaller in magnitude than the measured values, so that additional factors (e.g., changes in protein conformation and dynamics) were required to explain the decrease in C_p not accounted for by the burial of nonpolar surface (Connelly et al., 1993; Ladbury et al., 1994; Spolar & Record, 1994). In a similar vein, ITC experiments can provide two different estimates of ΔH: (i) ΔH_{cal} as a direct result of the calorimetry experiment and (ii) ΔH_{vH} derived from the temperature-dependence of K_a (van't Hoff analysis). Analysis of the equilibrium constants in Table 1 yielded values for ΔH_{vH} that were consistently smaller than the ΔH_{cal} (average 65 %, range 40−90 %). Although these differences appear to be experimentally significant and have been observed in other ITC studies [e.g., Lin et al. (1994), and J. F. Brandts, personal communication], the reasons for the discrepancy are not apparent.

Comparison of Association Constants Determined by ITC and SPR. In previous SPR studies of CheA−CheY interactions (Schuster et al., 1993; Swanson et al., 1993) the association constants measured at 24 °C for CheY−$CheA_{1-233}$ and CheY−CheA (intact CheA) interactions were found to differ significantly (2.7×10^6 and 3.3×10^7 M^{-1}, respectively), indicating that CheY interacted 10-fold more tightly with intact CheA. In contrast the ITC experiments reported here have produced comparable values of K_a for complexes of CheY and either CheA or $CheA_{1-233}$ (Table 1). This rather significant discrepancy undoubtedly resides in the differences between the two methods of measurement. ITC measures the single-site equilibrium association constant directly between two molecules in solution. SPR experiments often determine K_a indirectly using rates of binding and dissociation between a molecule covalently attached to a polymeric matrix on a sensing chip and a second freely diffusing molecule. Furthermore, CheA−CheY interactions measured by SPR may be influenced by possible multivalent interactions between the intact CheA protein, which is dimeric, and the polymer-bound CheY, which can behave as a multivalent ligand if the density of sites is sufficiently large. $CheA_{1-233}$ has been demonstrated to be a monomer (Swanson et al., 1993; Figure 4) and thus is unable to exhibit the same avidity effect as the intact dimer. A similar 10-fold ratio between the SPR and ITC-determined association constants has been observed in a study of proline-rich peptides (derived from the Ras guanine nucleotide releasing factor, Son of Sevenless) binding to Grb2, which has two peptide binding sites per Grb2 protein (Lemmon et al., 1994). For a discussion of the effects of multivalency on the measured values of K_a, see Ladbury et al. (1995). It thus appears that the major difference in K_a(CheA)/K_a($CheA_{1-233}$) estimated by SPR and ITC may be explained by multivalent interactions. Differences in the absolute values of K_a between our study and the previous studies of CheY−$CheA_{1-233}$ interactions might be due to differences in the buffer composition, although conditions unique to the SPR experiment (e.g., possible effects of the polymer matrix and the determination of K_a using kinetic data) may also be significant.

Effects of CheY Phosphorylation on CheA−CheY Interactions. Phosphorylated CheY generates tumbles of the bacterial cell by enhancing the probability of clockwise motor rotation through direct interaction with a switch complex protein (FliM) on the motor (Welch et al., 1993). The data presented here indicate that phosphorylation lowers the affinity of CheY−CheA interactions (ca. 6-fold in the presence of Mg^{2+}). This may be physiologically significant, since a higher affinity for unphosphorylated CheY will improve the efficiency of the phosphotransfer reaction as a signal amplification process.

Experimentally it was found that mutations in the phosphorylation site of CheY had little effect on CheY−CheA interactions. For example the "activated" $CheY_{D13K}$ mutant, which has been found to produce the same effect on tumble generation as phosphorylated CheY (Bourret et al., 1990), binds to CheA with an affinity comparable to wild type CheY. Yet phosphorylation of CheY resulted in a comparatively large reduction in K_a, and a similar large reduction was produced by the A103V mutation. Alanine-103 residue has been implicated in CheA−CheY interactions by NMR studies (Swanson et al., 1995). These results may be explained by postulating that phosphorylation produces a significant conformational change which propagates from the site of phosphorylation to the CheA−CheY binding interface, and that mutations introduce relatively small perturbations restricted to the region in close proximity to the mutation.

This difference in the sphere of influence that mutations and phosphorylation produce is consistent with the interpretations drawn from ^{19}F NMR studies of CheY conformation (Bourret et al., 1993; Drake et al., 1993). The results of the ITC experiments and the "global" effect of phosphorylation *versus* the "local" effect of a mutation taken together imply that the CheA−CheY binding interface is (at least partly) separate from the site of phosphorylation.

Significance for Information Processing in Bacterial Signal Transduction Systems. The chemosensory pathway requires coordination of the receptor-mediated excitation and adaptation processes, which are both influenced by changes in the concentrations of chemoeffectors. The methylatable receptors in *E. coli* exert their influence on swimming behavior through the kinase CheA (Borkovich et al., 1992; Schuster et al. 1993), whose activity is modulated both by ligand (binding inhibits activity) and receptor covalent modification (increased levels of methylation and/or amidation attenuates inhibition by ligand, Borkovich et al., 1992). The signaling pathway branches at CheA-mediated phosphorylation and directs phosphate along the excitation pathway to CheY and along the adaptation pathway to CheB. Partitioning of phosphate between CheY and CheB in the branchpoint is thus a potential means of controlling the magnitude and duration of the excitation and adaptation signals. An understanding of the interactions between CheA, CheB, and CheY is essential to determine whether control is exerted at this level. The results of this study contribute to this goal by (i) determining the association constants for the formation of complexes between CheA, CheB, and CheY, (ii) finding that binding is competitive, and (iii) finding that phosphorylation of CheY can affect the strength of the interaction with CheA.

E. coli has numerous two-component systems which likely have a high degree of structural homology in the response regulator proteins at the site of phosphorylation (i.e., the CheY superfamily; Volz, 1993). The conservation of structure at the active site is dictated by the chemistry of the phosphotransfer reaction, but this conservation might lead to adventitious phosphorylation of response regulator proteins from a physiologically-unrelated pathway (cross-talk). The results of this study and the NMR study of CheY−CheA interactions (Swanson et al., 1995) suggest that the surface involved in CheA−CheY (and presumably CheA-CheB) interactions includes protein−protein contacts at sites distinct from the site of phosphorylation. This feature of the binding interaction may serve to maintain the specificity of protein interactions and thus reduce unwanted cross-talk between signaling pathways.

APPENDIX

An ITC experiment measures the extent of ligand−receptor complex as a function of the amount of added ligand. With this in mind, the association constant for the formation of the $CheA_{1-233}$−CheY complex (K_{AY}) can be written to reflect the extent of complex formation out of the total number of binding sites (in this example on the $CheA_{1-233}$ protein) and is given by

$$K_{AY} = \frac{[AY]}{(A_{tot} - [AY])[Y]} \quad (A1)$$

where A_{tot} is the total concentration of $CheA_{1-233}$ (and is equal to [A] + [AY]). In the presence of CheB, A_{tot} is given by the sum of all forms of $CheA_{1-233}$, which includes the unbound or free $CheA_{1-233}$ and the complexes with CheY and CheB.

$$A_{tot} = [A] + [AY] + [AB] \quad (A2)$$

Substituting this expression for A_{tot} into eq A1 accounts for the presence of $CheA_{1-233}$−CheB complex and thus becomes an expression for K_{app}, the apparent association constant of CheY and $CheA_{1-233}$

$$K_{app} = \frac{[AY]}{([A] + [AB])[Y]} = \frac{[AY]}{([A] + K_{AB}[A][B])[Y]} = \frac{[AY]}{(1 + K_{AB}[B])[A][Y]} \quad (A3)$$

which, after substitution of $K_{AB}[A][B]$ for the concentration of the $CheA_{1-233}$−CheB complex, [AB], and K_{AY} for [AY]/[A][Y], yields eq 1 in the text, the expression for the apparent association constant of CheY and $CheA_{1-233}$. In the experiments described in the text, the average concentration of free CheB (which changes modestly over the course of the competitive binding experiment) is used to provide an estimate for K_{app}.

It is worth noting that the expression for K_{app} simplifies to $K_a/K_c[C]$ when the product of the association constant for competitor binding (K_c) and the competitor concentration, [C], is much greater than one, i.e., $K_c[C] \gg 1$ (in this specific example $K_{AB}[B] \gg 1$), and thus provides a means to estimate K_a for tight binding ligands if K_c and [C] are known.

ACKNOWLEDGMENT

We thank A. F. Roth and F. W. Dahlquist at the University of Oregon for providing the CheB, CheY, and CheW expression plasmids pCW/*cheB*, pCW/*cheY*, and pCW/*cheW*, and Cheryl Cote in the Molecular and Cellular Biology Program Core Facility at the University of Massachusetts for carrying out amino acid analyses on $CheA_{1-233}$, CheB, and CheY. R.M.W. and J.L. also thank Drs. Lung-Nan Lin and John F. Brandts for helpful discussions.

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BI951395F