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Divalent Metal Ion Binding to the CheY Protein and Its Significance to Phosphotransfer in Bacterial Chemotaxis†

Gudrun S. Lukat, Ann M. Stock, and Jeffry B. Stock*
Department of Biology and Chemistry, Princeton University, Princeton, New Jersey 08544
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ABSTRACT: Signal transduction in bacterial chemotaxis involves transfer of a phosphoryl group between the cytoplasmic proteins CheA and CheY. In addition to the established metal ion requirement for autophosphorylation of CheA, divalent magnesium ions are necessary for the transfer of phosphate from CheA to CheY. The work described here demonstrates via fluorescence studies that CheY contains a magnesium ion binding site. This site is a strong candidate for the metal ion site required to facilitate phosphotransfer from phospho-CheA to CheY. The diminished magnesium ion interaction with CheY mutant D13N and the lack of metal ion binding to D57N along with significant reduction in phosphotransfer to these two mutants are in direct contrast to the behavior of wild-type CheY. This supports the hypothesis that the acidic pocket formed by Asp13 and Asp57 is essential to metal binding and phosphotransfer activity. Metal ion is also required for the dephosphorylation reaction, raising the possibility that the phosphotransfer and hydrolysis reactions occur by a common metal-phosphoprotein transition-state intermediate. The highly conserved nature of the proposed metal ion binding site and site of phosphorylation within the large family of phosphorylated regulatory proteins that are homologous to CheY supports the hypothesis that all these proteins function by a similar catalytic mechanism.

Phosphoryl group transfer between regulatory proteins plays a central role in the control of gene expression and cell motility in bacteria [for a recent review, see Stock et al. (1989a)]. Two families of homologous regulatory proteins are involved. Generally, a histidine protein kinase (HPK) catalyzes the transfer of ATP γ-phosphoryl groups to an imidazole nitrogen in one of its own histidine side chains; this group is then transferred to an aspartic acid side chain in a response regulator (RR) protein. Finally, this phosphoaspartyl mixed anhydride is hydrolyzed to restore the response regulator to its dephosphorylated state:

$$\text{ATP} + \text{HPK-His} \rightleftharpoons \text{ADP} + \text{HPK-His-P}$$

$$\text{HPK-His-P} + \text{RR-Asp} \rightleftharpoons \text{HPK-His} + \text{RR-Asp-P}$$

$$\text{RR-Asp-P} + \text{H}_2\text{O} \rightarrow \text{RR-Asp} + \text{P}_i$$

The phosphorylated response regulators control the activities of effector components such as the flagellar motor apparatus or RNA polymerase. Environmental signals elicit responses by regulating the rate of autophosphorylation, phosphotransfer, and/or dephosphorylation.

Here we report the effects of divalent cations such as Mg^{2+} on the phosphorylation and dephosphorylation of CheY, the response regulator that controls motor behavior during chemotaxis in *Escherichia coli* and *Salmonella typhimurium*. The histidine kinase of the chemotaxis system, CheA, has been purified as a soluble homomultimer of approximately 250000 molecular weight composed of 73000 molecular weight subunits (Stock et al., 1988b). When incubated with $MgATP^{2-}$, CheA is phosphorylated at a histidine residue to levels approaching one phosphoryl group per monomer (Hess et al., 1988a; Stock et al., 1988a). Phosphoryl-CheA, isolated free of nucleotide, will act as a phosphodonor for CheY (Hess et al., 1988b; Wylie et al., 1988). CheY has been purified as a 128-residue monomeric protein. Its three-dimensional structure has been determined by X-ray crystallography to be

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* Address correspondence to this author.

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a single domain with a doubly wound five-stranded parallel β sheet surrounded by five α helices (Stock et al., 1989). The phosphoacceptor site, Asp57 (Sanders et al., 1989), is located within a cluster of aspartic acid residues that form an acidic pocket between the C-terminal ends of two adjacent β strands. Our results indicate that metal binding is required for both the phosphoacceptor and phosphatase activities of the CheY protein. Metal binding causes a dramatic quenching of the intrinsic fluorescence of the only tryptophan residue in the protein, which is adjacent to the acidic pocket (Trp58). Oligonucleotide-directed mutagenesis of aspartate residues within the pocket (Asp13 and Asp57) to asparagines appears to interfere with metal binding. These results indicate that the acidic pocket provides a site where divalent metals bind to CheY and play an essential role in the catalytic activities mediated by this protein.

Materials and Methods

Protein Preparation and Analysis. Salmonella typhimurium CheY was isolated and purified (>95%) as described previously (Stock et al., 1985) and stored in aliquots at −20 °C. CheY mutants were generated by oligonucleotide-directed mutagenesis as reported previously (Sanders et al., 1989). The mutant CheY proteins were purified by using the same procedures as those used to purify the wild-type protein. CheA was isolated by the procedure of Stock and co-workers (Stock et al., 1988b) with slight modification. The final purification steps included a phenyl-Sepharose column and a gel filtration column. Phosphotransfer was monitored via reaction of purified phospho-CheA with CheY. The phospho-CheA was generated by reaction of [γ-^{32}P]ATP with CheA in the presence of Mg^{2+} ion. During purification of phospho-CheA, ATP^{4-} and Mg^{2+} were removed by passage over a G-100 column equilibrated in 50 mM phosphate buffer/0.5 mM $EDTA^{4-}$, pH 7.5. CheZ was isolated as described elsewhere (Stock & Stock, 1987).

Radiolabeled proteins in sodium dodecyl sulfate sample buffer (62.5 mM Tris·HCl, 2% sodium dodecyl sulfate, 10% glycerol, 0.002% bromphenol blue, and 1% 2-mercaptoethanol, pH 6.8) were analyzed by electrophoresis in 15% polyacrylamide–sodium dodecyl sulfate gels (80 × 70 × 0.75 mm) according to the method of Laemmli (1970). The wet, unstained gels were autoradiographed at −70 °C using Kodak X-omat AR5 film and Dupont Cronex Lightening Plus intensifying screens.

Fluorescence Measurements. Steady-state fluorescence measurements were obtained with a Perkin-Elmer MPF66 spectrofluorometer interfaced to a Perkin-Elmer 7500 computer. The temperature of the sample was kept at 20 °C with a thermostated metallic cell holder to a precision of ±0.1 °C. CheY contains two fluorescent residues: Trp58 and Tyr106. The excitation wavelength was set at 295 nm for selective excitation of the tryptophan. In all cases, the solution absorbance was less than 0.1 at the excitation wavelength. L-Tryptophan in water was used as reference for quantum yield (Wiget & Luisi, 1978) determinations of wild-type and mutant CheY.

Tryptophan fluorescence quenching experiments with acrylamide were performed by addition of aliquots of an acrylamide stock solution in the absence and in the presence of saturating magnesium ion concentrations. Since no change in the overall shape and position of the fluorescence spectrum occurred in the presence of acrylamide, changes in the fluorescence intensity at 346 nm were recorded. All data were corrected for dilution and for internal screening due to acrylamide absorption at 295 nm (ϵ = 0.23) (Sellers & Ghiron, 1973; Eftink & Ghiron, 1976). Quenching data were then analyzed according to the Stern–Volmer relationship (eq 1)

$$F_0/F = K_{sv}[Q] + 1 \quad (1)$$

by plotting F_0/F versus [Q], where F_0 and F are the unquenched and the quenched fluorescence intensities, respectively, [Q] is the concentration of the quencher, and K_{sv} is the dynamic quenching constant.

Similar quenching experiments were performed in the form of titration of CheY and the mutant CheY enzymes with various metal ions. CheY metal ion titrations were done in 50 mM Tris·HCl, pH 7.4, unless otherwise indicated. Stock metal ion solutions were prepared at concentrations that allowed complete titration with no more than 2% dilution of the sample. The data were treated according to the Scatchard equation for equilibrium binding (Segel, 1975):

$$v/[S]_f = -(1/K_D)v + n/K_D \quad (2)$$

where $v = [S]_b/[E]_t$ = the ratio of the molar concentration of bound ligand to the total molar concentration of enzyme, $[S]_f$ = the concentration of free ligand, K_D = the substrate dissociation constant, and n = the number of ligand binding sites per enzyme molecule. $1/K_D$ and n/K_D are the slope and intercept of a straight line calculated via a nonweighted linear least-squares analysis.

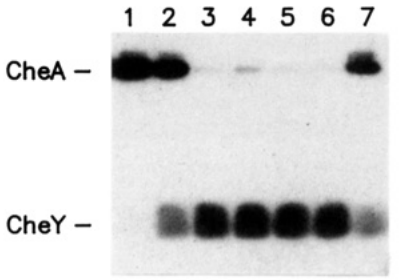

FIGURE 1: Phosphotransfer from phospho-CheA to CheY in the presence of various divalent cations. Reactions were initiated by addition of 5.0 μL of 0.5 μM [^{32}P]phospho-CheA in 50 mM potassium phosphate/0.5 mM $EDTA^{4-}$, pH 7.0, to 15 μL of 0.83 μM CheY in 17 mM sodium phosphate, pH 7.0, containing metal ion as indicated: lane 1, no metal; 2, 0.18 mM $CdCl_2$; 3, 0.22 mM $MnCl_2$; 4, 0.39 mM $ZnCl_2$; 5, 0.68 $CoCl_2$; 6, 1.8 mM $MgCl_2$; 7, 5.3 mM $CaCl_2$. After 15 s at 20 °C, the reaction was quenched by the addition of 7.0 μL of 4× concentrated sodium dodecyl sulfate sample buffer, and the samples were analyzed by gel electrophoresis and autoradiography.

Results

Mg^{2+} is required for phosphotransfer between CheA and CheY. In the absence of Mg^{2+}, CheY will not function as an acceptor of phosphoryl groups from phospho-CheA (Figure 1). Since the generation of phospho-CheA from ATP^{4-} and CheA also requires Mg^{2+} (Hess et al., 1987), care must be taken to remove Mg^{2+} from phospho-CheA prior to the addition of CheY in order to demonstrate the metal requirement for phosphotransfer. Addition of Mg^{2+} to the reaction mixture restores rapid phosphotransfer. The rate is too rapid to measure via radiolabeling monitored by electrophoresis at millimolar concentrations of Mg^{2+} even at 0 °C, but at micromolar concentrations, the rate of phosphotransfer can be shown to vary with Mg^{2+} concentration.

The requirement for metal ion is not specific to Mg^{2+}. All of the divalent metal cations examined supported some degree of phosphotransfer (Figure 1). CheA and CheY tended to precipitate at millimolar concentrations of most of these metals, but at concentrations where both proteins remained soluble, phosphotransfer from phospho-CheA to CheY occurred in the presence of Cd^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Ca^{2+}. In the case

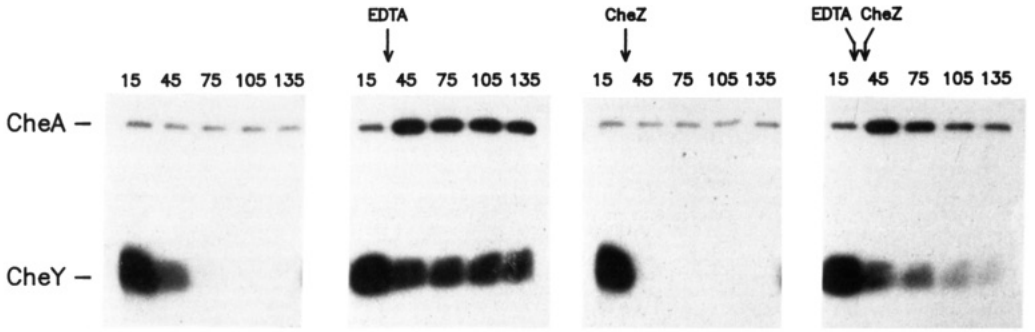

FIGURE 2: Effect of $EDTA^{4-}$ on CheY dephosphorylation. Phosphorylation of CheY was examined in reaction mixtures containing 0.25 μM [^{32}P]phospho-CheA, 1.25 μM CheY, 25 mM potassium phosphate, 12 mM sodium phosphate, 0.25 mM $EDTA^{4-}$, and 0.62 mM $MgCl_2$, pH 7.0, at 20 °C. Phosphotransfer was initiated by addition of phospho-CheA, and at the indicated times (seconds), 20-μL aliquots were removed to tubes containing 7 μL of 4× concentrated sodium dodecyl sulfate sample buffer. In the second reaction, 0.5 M $EDTA^{4-}$ was added to a final concentration of 10 mM at 25 s. In the third reaction, 17 μM CheZ was added to a final concentration of 0.5 μM at 35 s. In the fourth reaction, 0.5 M $EDTA^{4-}$ was added to a final concentration of 10 mM at 25 s, and 17 μM CheZ was added to a final concentration of 0.5 μM at 35 s. Samples were analyzed by gel electrophoresis and autoradiography.

of Mn^{2+}, Zn^{2+}, and Co^{2+} as well as Mg^{2+}, transfer was complete within 15 s. The rate of transfer was substantially lower, however, with Cd^{2+} and Ca^{2+}.

Mg^{2+} is also involved in the hydrolysis of phospho-CheY to inorganic phosphate plus CheY. The native phospho-CheY protein exhibits a first-order rate constant for hydrolysis of approximately 2 min^{-1} under physiological conditions of temperature and pH (Hess et al., 1988; Stock et al., 1988). Phospho-CheY denatured in sodium dodecyl sulfate or guanidine hydrochloride exhibits a rate constant for hydrolysis that is over 100-fold lower and corresponds to the rate expected for an acyl phosphate (Stock et al., 1988). From these results, it is apparent that CheY has an inherent autophosphatase activity. Mg^{2+} has an effect on the autophosphatase activity [also see Sanders et al. (1989)] (Figure 2, two left panels). Removal of the Mg^{2+} by addition of $EDTA^{4-}$ immediately after the generation of phospho-CheY caused a dramatic decrease in the rate of phospho-CheY hydrolysis. This effect was accompanied by an increased ratio of radioactivity in CheA relative to CheY, suggesting that the phosphotransfer reaction between CheA and CheY is reversible. In addition, another chemotaxis protein, CheZ, causes a dramatic stimulation in the rate of hydrolysis of native phospho-CheY (Hess et al., 1988). This CheZ-dependent phosphatase activity is also affected by Mg^{2+} (Figure 2, two right panels). When Mg^{2+} is removed from the phospho-CheY and CheZ reaction mixture, the rate of hydrolysis is retarded. However, the degree to which the hydrolysis rate is decreased cannot be determined from the data presented here. This is due to the fact that in the presence of CheZ and Mg^{2+} all of the phospho-CheY is hydrolyzed before the first time point can be obtained.

As with the phosphotransfer reaction, the requirement of Mg^{2+} for dephosphorylation was relatively nonspecific. Similar rates of hydrolysis were obtained with Cd^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+} added in place of Mg^{2+}. Ca^{2+}, however, did not facilitate the dephosphorylation reaction (Figure 3). In fact, in the presence of Mg^{2+}, Ca^{2+} inhibited the autophosphatase reaction, presumably by competing with Mg^{2+} (Figure 4).

Changes in the intrinsic fluorescence of CheY as a function of Mg^{2+} concentration were monitored as a direct assay for metal binding. CheY has a single tryptophan, Trp58, located near the phosphoaccepting aspartate, Asp57. Mg^{2+} binding significantly quenches the fluorescence of this residue. The position of the emission maxima for the fluorescence spectra of CheY at 346 nm is unaffected. The decreased quantum yield on Mg^{2+} binding suggests an associated alteration in the

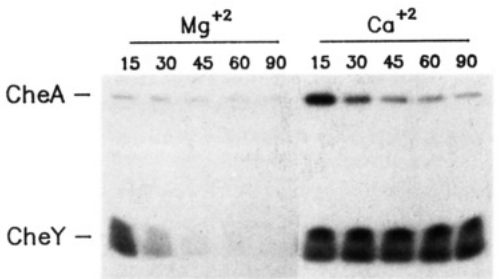

FIGURE 3: Dephosphorylation of CheY in the presence of Ca^{2+}. Phosphorylation of CheY was examined in reaction mixtures containing 0.13 μM [^{32}P]phospho-CheA, 1.9 μM CheY, 12 mM potassium phosphate, 12 mM sodium phosphate, 0.12 mM $EDTA^{4-}$, and either 1.3 mM $MgCl_2$ (left panel) or 4.4 mM $CaCl_2$ (right panel), pH 7.0, at 20 °C. At the indicated times (seconds), 20-μL aliquots were removed to tubes containing 7 μL of 4× concentrated sodium dodecyl sulfate sample buffer. Samples were analyzed by gel electrophoresis and autoradiography.

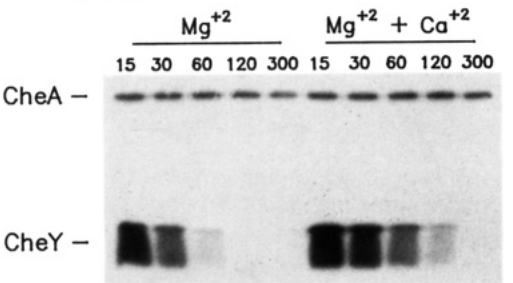

FIGURE 4: Dephosphorylation of CheY in the presence of both Ca^{2+} and Mg^{2+}. Phosphorylation of CheY was examined as described in the legend to Figure 3 except that either 3.0 mM $MgCl_2$ (left panel) or 1.0 mM $CaCl_2$ + 3.0 mM $MgCl_2$ (right panel) was present in the reaction mixture.

environment around Trp58. Although the exposure of a tryptophan residue cannot be deduced from its emission maximum alone, the 346-nm value for CheY suggests that the chromophore is in a somewhat polar environment. The environments of tryptophan residues have been classified according to their fluorescence maxima and spectral bandwidth (Burstein et al., 1973): (1) λ_{max} 330–332 nm and $\Delta\lambda$ 48–49 nm indicate residues buried in nonpolar regions; (2) λ_{max} 340–342 nm and $\Delta\lambda$ 53–55 nm indicate residues immobilized at the protein surface with limited access to water; (3) λ_{max} 350–353 nm and $\Delta\lambda$ 59–61 nm indicate residues exposed to water. The fluorescence properties of CheY (λ_{max}, 346 nm; $\Delta\lambda$, 58 nm) are between those of classes 2 and 3. This is consistent with the X-ray data (Stock et al., 1989) which

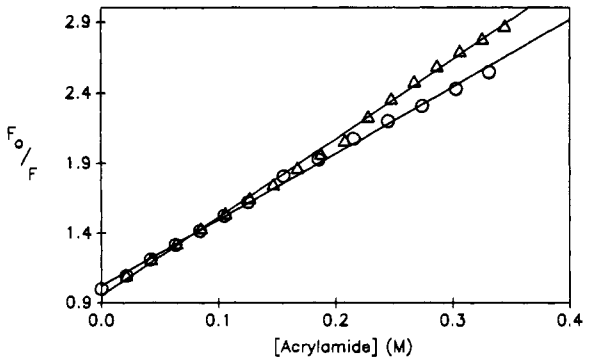

FIGURE 5: Stern–Volmer plots for acrylamide quenching for CheY in the presence (triangles) and absence (circles) of Mg^{2+}. The quenching experiments were performed in 50 mM Tris·HCl, pH 7.4. Further experimental details are listed under Materials and Methods.

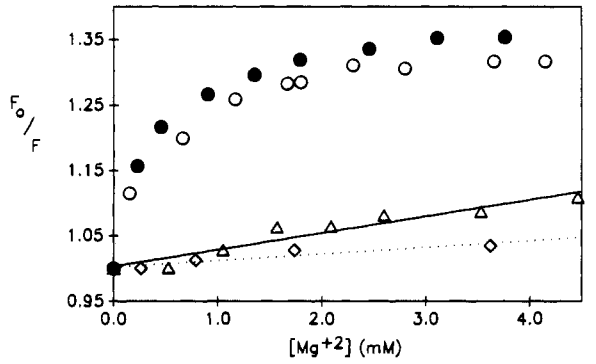

FIGURE 6: Stern–Volmer plots of wild-type CheY (open circles) and mutant CheYs D57N (diamonds), D13N (triangles), and K109R (closed circles) quenching by Mg^{2+}. The quenching experiments were performed in 50 mM Tris·HCl, pH 7.4, by addition of aliquots of a stock $MgCl_2$ solution to the protein solutions. The excitation wavelength was set at 295 nm, and emission was recorded at 346 nm. F_0 and F correspond to the fluorescence intensity at 346 nm in the absence and presence of a given concentration of Mg^{2+}, respectively.

indicate that Trp58 is near the protein surface but somewhat protected from solvent.

Acrylamide quenching experiments also indicate that Trp58 is partly solvent-exposed and that Mg^{2+} binding does not produce a large change in the environment surrounding Trp58. The dynamic quenching constant, K_{sv}, provides a measure of solvent accessibility (Eftink & Ghiron, 1976, 1981). At pH 8.5, the K_{sv} values determined for CheY and CheY/Mg^{2+} are 7.7 ± 0.2 and 7.2 ± 0.2 M^{-1}, respectively, and at pH 7.4, the corresponding K_{sv} values are 5.6 ± 0.1 and 4.7 ± 0.1 M^{-1}, respectively (Figure 5). K_{sv} values for acrylamide quenching of tryptophan fluorescence in proteins of known structure range from 13 M^{-1} for highly exposed side chains to ~0 for residues buried within the protein interior (Eftink & Ghiron, 1976).

The acidic cleft on the surface of CheY comprised of carboxylates from Asp12, Asp13, and Asp57 provides a likely site for cation binding. To test this hypothesis, we examined Mg^{2+} binding to mutant CheY proteins with Asp13 or Asp57 replaced by Asn residues (D13N and D57N). These mutant proteins are deficient in their ability to accept phosphoryl groups from phospho-CheA, and either mutation causes a complete loss in chemotactic function (Sanders et al., 1989). In both the D13N and D57N proteins, the affinity for Mg^{2+}, as measured by quenching of tryptophan fluorescence, is reduced by more than an order of magnitude (Figure 6). A mutation in CheY located outside the acidic pocket, Lys109 replaced by an Arg, is also defective in chemotactic function but is unaffected in metal binding (Figure 6). The environment of Trp58 does not appear to be dramatically altered by

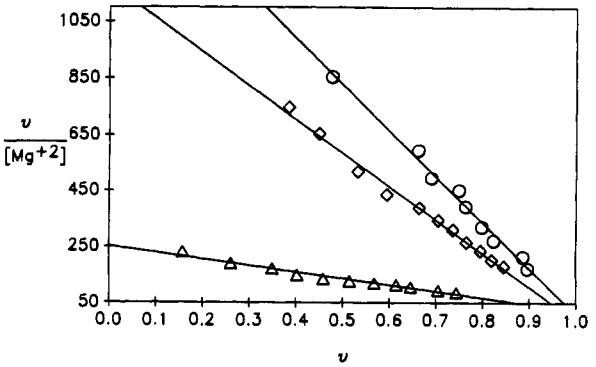

FIGURE 7: Scatchard plots for wild-type CheY titrated with magnesium ion at pHs 4.0 (triangles), 7.0 (circles), and 10.0 (squares).

Table I: Constants Determined from Binding Studies of CheY with Divalent Metal Ions at 20 °C

ligand	pH	dissociation constant, K_D	number of binding sites, n
Mn^{2+}	7.0	47 ± 7 μM	1.03 ± 0.15
Cd^{2+}	7.4	40 ± 10 μM	1.10 ± 0.12
Zn^{2+}	7.4	97 ± 9 μM	1.04 ± 0.11
Co^{2+}	7.4	123 ± 8 μM	0.95 ± 0.10
Mg^{2+}	7.4	500 ± 80 μM	1.05 ± 0.09
Ca^{2+}	7.4	6100 ± 1300 μM	1.18 ± 0.10
Mg^{2+}	4.0	4.3 ± 0.3 mM	1.10 ± 0.10
Mg^{2+}	5.0	2.2 ± 0.2 mM	0.96 ± 0.13
Mg^{2+}	6.0	0.8 ± 0.1[a] mM	1.03 ± 0.14
Mg^{2+}	7.0	0.45 ± 0.03[a] mM	1.02 ± 0.09
Mg^{2+}	8.0	0.52 ± 0.07[a] mM	1.01 ± 0.08
Mg^{2+}	10.0	0.84 ± 0.03 mM	0.99 ± 0.04

[a] These values were determined at 25 °C.

any of these mutations. The fluorescence maxima and bandwidths of emission spectra obtained with the mutant proteins are not significantly different from those obtained with wild type. Acrylamide quenching varied slightly with K_{sv} values of 5.6 ± 0.1 M^{-1} (wild type), 5.3 ± 0.2 M^{-1} (D57N), 4.9 ± 0.1 M^{-1} (K109R), and 3.9 ± 0.1 M^{-1} (D13N). Quantum yields for the mutants are less than the 0.14 for CheY at pH 7.0 (D57N, 0.11; D13N, 0.11; K109R, 0.07).

Scatchard plots of binding, as determined by fluorescence quenching, indicate 1 mol of Mg^{2+} bound per mole of CheY (Figure 7). The affinity of CheY for Mg^{2+} varies with pH, with a maximum at approximately pH 7. Decreased binding under more acidic conditions indicates that a group (or groups) with a pK_a of approximately 6 is critical to binding. Since CheY does not contain any histidine residues, it seems likely that protonation of a carboxylate side chain with a pK_a of approximately 6 interferes with binding. This would be consistent with the involvement of carboxylate side-chain anions of aspartates within the acidic pocket. An unusually high pK_a would be expected for the aspartate side chains within the acidic pocket because of the high density of negative charge in this region.

Fluorescence was used to monitor binding of other divalent cations besides Mg^{2+}. As expected from the phosphotransfer experiments, metal binding to CheY is relatively nonspecific. In fact, Mg^{2+} has a relatively low affinity, with Cd^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} exhibiting tighter binding, and only Ca^{2+} binding with lower affinity (Table I). The higher affinity of CheY for divalent transition metal ions relative to Mg^{2+} or Ca^{2+} can be explained in terms of the larger thermodynamic stabilities of divalent transition metal ion complexes with carboxylate ligands. For example, the dissociation constants for Cd^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} complexes with $EDTA^{4-}$ fall in a range of 2.0×10^{-17} to 5.0×10^{-17} compared to a K_D of

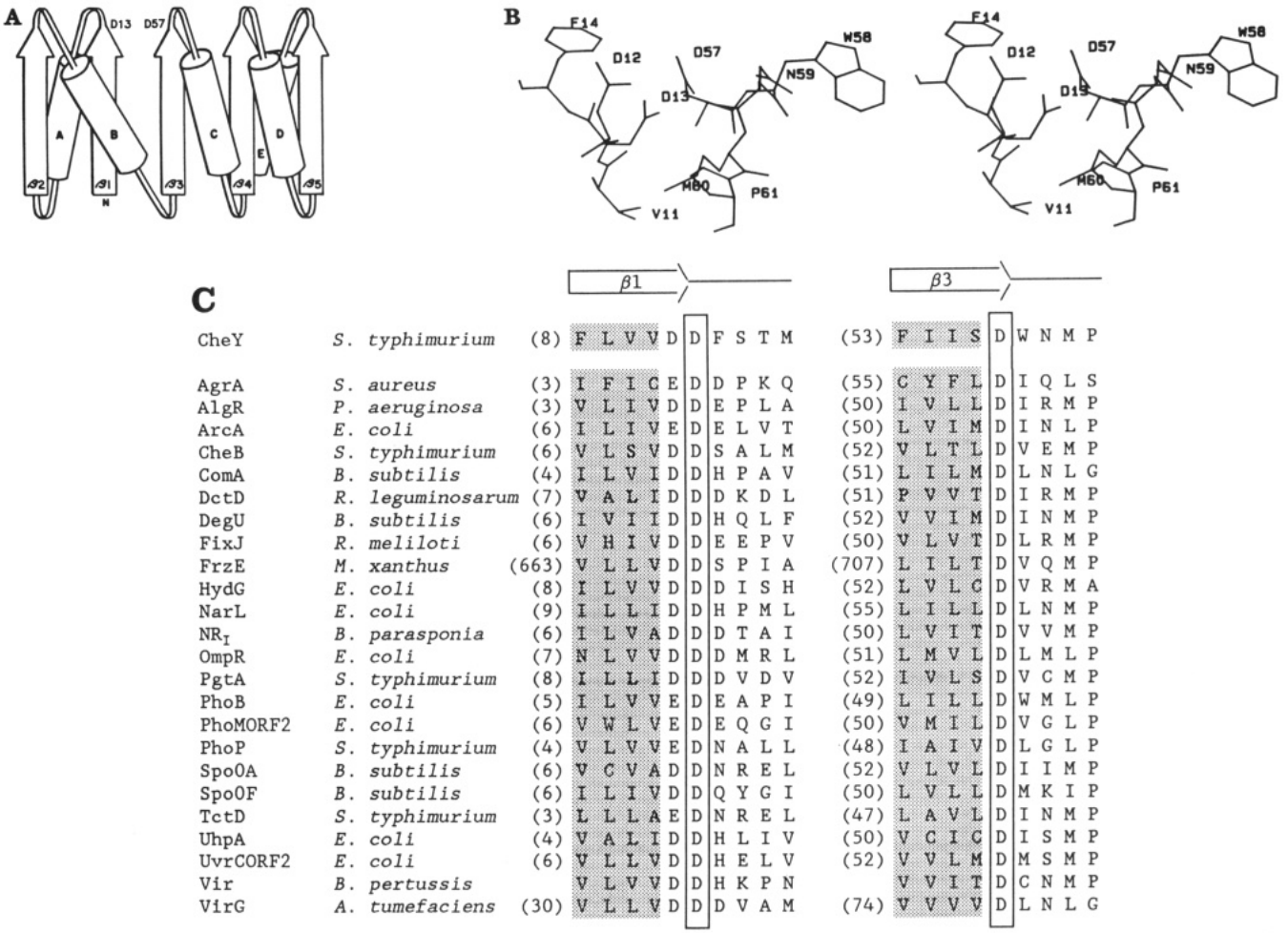

FIGURE 8: (A) Schematic diagram showing the topology of the secondary structural elements of CheY. A central parallel β sheet with β strands 1–5 depicted as arrows is flanked by five α-helical segments A–E represented as cylinders. The locations of the highly conserved aspartate residues, D13 and D57, are indicated at the C-terminal ends of β-strands 1 and 3. (B) Stereoview of the acidic pocket of CheY. Residues having atoms within a 6-Å radius of the center of the acidic cleft formed by Asp12, Asp13, and Asp57 are displayed. Trp58 which lies just outside this region has been included for clarity. The two groups of contiguous residues, Val11 to Phe14 and Asp57 to Pro61, are parts of the loops that connect β1 to αA and β3 and αC. (C) Sequences of the response regulator proteins in the regions that flank the conserved aspartates of the acidic pocket are indicated in standard one-letter code [sequence references contained in Stock et al. (1989a)]. The numbers in parentheses indicate the position of the following residue (no numbers are indicated for the *vir* gene since the sequence of the gene is incomplete and the N-terminus of the protein has not been identified). Conserved hydrophobic residues that correspond to the β strands in CheY are shaded, and the totally conserved aspartate residues are boxed.

2.0×10^{-9} for $MgEDTA^{2-}$ (Peters et al., 1976).

Discussion

The crystal structure of CheY suggests that the active site of the protein is between the loops at the C-terminal ends of β strands 1 and 3 (Stock et al., 1989) (Figure 8A). In α/β proteins, the active site is typically located at the C-terminal edge of the parallel β sheet within a cleft formed by loops that connect adjacent β strands to helices on opposite faces of the sheet (Branden, 1980). Carboxypeptidase is an example of an α/β protein in which a divalent metal ion (Zn^{2+}) is bound in a cleft like that described above (Rees et al., 1983). The data presented in this paper are supportive of metal ion binding to CheY in the cleft formed between β strands 1 and 3 of CheY; this cleft contains the acidic side chains of Asp12, Asp13, and Asp57.

The current crystallographic data and some data on magnesium(II) aspartate complexes allow for some speculation about the coordination sphere of the Mg^{2+} bound to CheY. Only two magnesium(II) aspartate complexes have been studied by crystallography. The Mg–O distances were 2.057 Å in Mg(L-AspH)(D-AspH)·$4H_2O$ (Schmidbaur et al., 1989) and 2.075 and 2.066 Å in Mg(L-AspH)·$3H_2O$ (Schmidbauer et al., 1986). In the Mg(L-AspH)(D-AspH)·$4H_2O$ case, the crystals have only monodentate ligands. Consideration of the Mg–O bond distances observed in model compound structures indicates that the acidic pocket of CheY can readily accommodate a magnesium ion (Figure 8B). However, on the basis of carboxylate oxygen distances and relative geometry in the CheY metal-free case, determination of the exact metal ion coordination is difficult. Fluorescence quenching of Trp58 indicates some change occurring in the vicinity of the acidic pocket, and presently the extent to which the relative orientation of the aspartates in the acidic pocket is affected upon metal ion binding is not known. However, in solving the crystal structure, a uranyl acetate heavy-atom derivative of CheY was examined. The uranyl ion is known to bind in the acidic pocket described above (Stock et al., 1989).

Alterations in the metal coordination sphere probably occur when CheY is phosphorylated. Given the metal ion location in the acidic pocket is in very close proximity to the site of phosphorylation at Asp57, it seems likely that at some point in phosphotransfer an oxygen from the phosphate is part of the metal ion coordination sphere. The dephosphorylation reacton would be expected to involve metal coordinated to the phosphoryl group. It has been shown that Mg^{2+} forms a 1:1

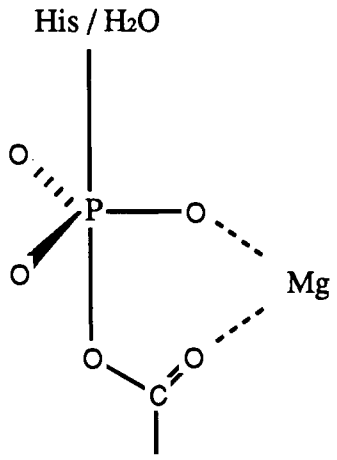

FIGURE 9: Possible metal–carboxylate–phosphoryl transition-state intermediate for phosphorylation and/or dephosphorylation at the active site of response regulator proteins.

complex with acetyl phosphate at neutral pH to facilitate its hydrolysis to acetate and orthophosphate (Koshland, 1952). A common metal–carboxylate–phosphoryl transition-state intermediate may, in fact, be involved in both the phosphorylation and dephosphorylation of CheY (Figure 9). The phosphatase activity of CheZ could result simply from an interaction between phospho-CheY and CheZ that induces a small conformational change in CheY to promote nucleophilic attack of the metal–carboxylate–phosphoryl complex by water.

An important general role for metal ion binding in the activities of phosphorylated response regulators that are homologous to CheY may explain the high degree of conservation of residues corresponding to Asp13 and other acidic groups that would be expected to form an acidic pocket surrounding the site of phosphorylation (Stock et al., 1989b). In CheY, the metal ion is essential for phosphotransfer, and it also affects the rate of dephosphorylation. One can hypothesize that the metal ion has similar functions in response regulator proteins in general. It is known, for example, that in the Ntr system the phosphorylated form of NR_1 is stabilized by the removal of divalent metal ions (Weiss & Magasanik, 1988) in the same way that Mg^{2+} removal stabilizes phospho-CheY. Cross-specificities between kinases and response regulators from different systems argue strongly that a common phosphotransfer mechanism is involved (Ninfa et al., 1988; Igo et al., 1989; Stock et al., 1989a). Furthermore, within the response regulator family, the conservation of hydrophobic residues that in CheY correspond to the central β strands and the internal faces of amphipathic helices indicates that the N-terminal domains of the response regulators share a common α/β structure (Stock et al., 1989; Stock et al., 1989b). Thus, the active-site clefts of the regulatory domains of the response regulators should be composed of residues corresponding to those flanking the Asp13 and Asp57 in CheY (Figure 8C). These regions contain an unusual density of carboxylate side chains. In addition to Asp13, there is generally an aspartic or glutamic acid residue at a position corresponding to CheY Asp12, and in half the response regulators, there is either an aspartic or a glutamic acid residue corresponding to CheY Phe14. Note also the tendency for a large hydrophobic residue followed by a proline corresponding to CheY Met60 and Pro61. There is also a lot of variation in residues in this region. These differences may be important in determining the different autophosphatase activities that have been observed for phosphorylated response regulators from different systems (Stock et al., 1989a). Slight changes in the acidic pocket regions of these proteins could have significant effects on metal ion coordination and thereby affect the stabilities of different transition complexes involved in catalysis.

Phosphoaspartyl groups are relatively uncommon in proteins. Aside from CheY and its homologues, the only well-characterized examples are the ion-translocating P-type ATPases (Jencks, 1980; Tanford, 1984). In both the Na^+/K^+- and the Ca^{2+} pumps of animal cell membranes, phosphorylation and dephosphorylation at aspartyl residues have been shown to drive a cycle of conformational changes that are responsible for the coupling of ATP^{4-} hydrolysis to ion transport. It has been proposed that the aspartyl phosphate generated in CheY causes an analogous phosphorylation-induced conformational change that regulates cellular motility to effect chemotaxis responses (Stock et al., 1989). Divalent cations play a variety of roles in the ATPases including interactions with ATP, and the ion transport channel, at least in the case of the Ca^{2+}-ATPase. Mg^{2+} is also necessary for phosphorylation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum from inorganic phosphate (Kanazawa & Boyer, 1973; Loomis et al., 1982), and for incorporation of radiolabeled inorganic phosphate into ouabain-treated Na^+/K^+-ATPase (Amar et al., 1969; Siegel et al., 1969). These reactions are simply the reversal of the phosphoaspartyl hydrolysis reaction that is the last step in Na^+/K^+- and Ca^{2+}-ATPase activities. This aspect of metal ion involvement in ATPase function may provide a further parallel between the phosphorylation-dependent conformational changes that drive ion transport and the phosphorylation activated switching mechanism that controls the regulatory activities of CheY and its homologues.

CONCLUSIONS

Our results demonstrate that metal ion is required for the transfer of phosphate from phospho-CheA to CheY. This raises a number of questions about how the metal ion participates in the transfer process. The $MgATP^{2-}$ requirement for CheA autophosphorylation indicates a possible site for metal interaction on the CheA protein. However, it is not clear that a metal ion in this CheA site is the one necessary for phosphotransfer. As a number of divalent metal ions have been shown to bind to CheY and facilitate phosphotransfer, CheY could be the locus for the Mg^{2+} requirement in the phosphotransfer reaction. In addition, Mg^{2+} participates in the hydrolysis of phospho-CheY. Our evidence suggests that the metal binding site corresponds to an acidic pocket formed by the carboxylate side chains of Asp12, Asp13, and Asp57. This also corresponds to the site of phosphorylation. Residues that comprise the acidic pocket in CheY are highly conserved among all members of the response regulator family. This conservation in structure may reflect a common mechanism of metal-catalyzed phosphotransfer and dephosphorylation.

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Registry No. Asp, 56-84-8; Mg, 7439-95-4; Ca, 7440-70-2; histidine kinase, 99283-67-7; phosphoprotein phosphatase, 9025-75-6.

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Phosphoinositide Synthesis in Bovine Rod Outer Segments†

Barry D. Gehm‡ and David G. Mc Connell*
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824
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ABSTRACT: Phosphoinositide turnover has been implicated in signal transduction in a variety of cells, including photoreceptors. We demonstrate here the presence of a complete pathway for rapid synthesis of phosphoinositides in isolated bovine retinal rod outer segments (ROS) free of microsomal contaminants. Synthesis was measured by the incorporation of label from radioactive precursors, [γ-^{32}P]ATP and [^{3}H]inositol. [γ-^{32}P]ATP also produced large amounts of labeled phosphatidic acid. Incorporation of [^{3}H]inositol required CTP and Mn^{2+}. Mn^{2+} and Mg^{2+} increased ^{32}P incorporation into phosphatidylinositol 4-phosphate, while spermine increased phosphoinositide labeling generally. ROS that had been washed to remove soluble and peripheral proteins incorporated less label than unwashed ROS into phosphatidic acid and phosphatidylinositol. No effects of light were detected. Inhibitory effects of high concentrations of nonhydrolyzable GTP analogues were probably due to competition with ATP.

Understanding of vertebrate visual transduction has been greatly advanced by the discovery that cyclic GMP maintains the vertebrate retinal rod outer segment (ROS)[1] sodium channel in the open state (Fesenko et al., 1985). Levels of cyclic GMP are regulated by a light-driven cascade involving bleached rhodopsin, a G protein [transducin (Fung et al., 1981; Godchaux & Zimmerman, 1979)], and a cGMP phosphodiesterase (Bitensky et al., 1978; Baehr et al., 1979; Kohnken et al., 1981a,b). The possible role of calcium in transduction or light/dark adaptation is also of great interest, especially because cyclic GMP levels have been shown to depend on Ca^{2+} (Cohen et al., 1978; Kilbride, 1980; Woodruff & Fain, 1982;

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* To whom correspondence should be addressed.
‡ Completed this work in partial fulfillment of the requirements for a Ph.D. degree from MSU. Present address: Ben May Institute, University of Chicago, 5841 South Maryland, Box 424, Chicago, IL 60637.

[1] Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; ROS, retinal rod outer segment(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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