Biochemistry

 Volume 32, Number 49 December 14, 1993

Accelerated Publications

Structure of the Mg^{2+}-Bound Form of CheY and Mechanism of Phosphoryl Transfer in Bacterial Chemotaxis[†,‡]

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Received August 20, 1993; Revised Manuscript Received October 15, 1993[⊛]

ABSTRACT: The response regulator protein of bacterial chemotaxis, CheY, is representative of a large family of signal transduction proteins that function as phosphorylation-activated switches to regulate the activities of associated effector domains. These regulators catalyze the metal ion-dependent phosphoryl transfer and dephosphorylation reactions that control the effector activities. The crystal structures of *Salmonella typhimurium* CheY with and without Mg^{2+} bound at the active site have been determined and refined at 1.8-Å resolution. While the overall structures of metal-bound and metal-free CheY are similar, significant rearrangements occur within the active site involving the three most highly conserved residues of the response regulator family. Conservation of the cluster of carboxylate side chains at the active site of response regulator domains can be rationalized in terms of their role in coordinating the catalytically essential divalent metal ion. The Mg^{2+} coordination geometry provides insights to the mechanism of phosphoryl transfer.

A common signal transduction mechanism is found in over 60 different bacterial regulatory systems that mediate adaptive responses to changing conditions (Bourret et al., 1991; Parkinson & Kofoid, 1992; Stock, J. B., et al., 1989). The signal transduction pathway utilizes two conserved protein components: a histidine protein kinase and a response regulator protein. Environmental signals regulate the activity of the histidine kinase which autophosphorylates at a specific histidine residue. This high-energy phosphoryl group is transferred from the histidine residue of the kinase to an aspartate residue within the N-terminal domain of the response regulator protein, resulting in activation of an effector function of either an attached C-terminal domain or a separate target protein. The recent observation of phosphorylation of response regulator proteins by small molecule phospho-donors such as phosphoramidate, acetyl phosphate, and carbamoyl phosphate (Feng et al., 1992; Lukat et al., 1992) indicates that the response regulator itself catalyzes the phosphoryl-transfer reaction. Three-dimensional structure information is available for only one response regulator protein, CheY, which regulates the direction of flagellar rotation in bacterial chemotaxis (Stock, A. M., et al., 1989; Volz & Matsumura, 1991). However, comparison of the amino acid sequences of the

[†] Supported by NIH Research Grants GM26788 to G.A.P. and D.R., GM47958 to A.M.S., and AI20980 to J.B.S.; grants from the Lucille P. Markey Charitable Trust to G.A.P., D.R., and A.M.S.; and a postdoctoral research fellowship from the Jane Coffin Childs Memorial Fund for Medical Research to A.H.W.

[‡] Atomic coordinates of the models have been deposited with the Brookhaven Protein Data Bank under accession numbers 1CHE and 1CHF.

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[⊛] Abstract published in *Advance ACS Abstracts*, November 15, 1993.

0006-2960/93/0432-13375$04.00/0

proteins of the response regulator family suggests that all regulatory domains share a similar α/β fold with four highly conserved residues clustered at the C-terminal edge of the parallel β-sheet (Stock et al., 1990; Volz, 1993). This cluster of conserved residues contains the aspartic acid side chain, Asp^{57} in CheY, that is phosphorylated (Sanders et al., 1989). We report here the crystal structures of CheY in the presence and absence of the required catalytic metal ion Mg^{2+}. These structures allow us to address the roles of the highly conserved residues with respect to their participation in the catalysis of phosphoryl transfer.

EXPERIMENTAL PROCEDURES

Salmonella typhimurium CheY protein was purified from *Escherichia coli* HB101 (Bolivar & Backman, 1979) carrying a pUC12-derived CheY expression vector, pME124 (Messing, 1983; Stock, A. M., et al., 1989), by a modification of previously described procedures (Stock et al., 1985). Orthorhombic crystals of CheY ($P2_12_12_1$, a = 45.7 Å, b = 47.0 Å, c = 53.9 Å) were grown at 4 °C by vapor diffusion in 20-μL drops using equal volumes of 1 mM CheY protein in 50 mM sodium acetate, pH 6.0, and 1.9 M ammonium sulfate, 50 mM sodium acetate, and 1–2% poly(ethylene glycol) (molecular weight 400), pH 4.7, equilibrated against 500 μL of the 1.9 M ammonium sulfate–sodium acetate solution. Prior to mounting and data collection, crystals were transferred at 20 °C to 40% poly(ethylene glycol) (molecular weight 4000), 0.6 M sucrose, and 50 mM sodium acetate, pH 5.5, with or without 10 mM $MgCl_2$. Unit cell dimensions of crystals in poly(ethylene glycol) are within 0.5% of those observed for crystals in ammonium sulfate. Average diffraction intensities of crystals in poly(ethylene glycol) were approximately 80% of those observed for crystals of comparable size in ammonium sulfate. Single-crystal X-ray intensity data sets were measured at −10 °C using a multiwire twin area detector system (Hamlin, 1985) and graphite monochromated Cu Kα radiation from a Rigaku RU-200 rotating anode. Data were processed with the program of Howard *et al.* (1985). The CheY·Mg^{2+} data included 49 659 reflections that were merged to give 10 659 unique reflections with R_{sym} ($\Sigma_{hkl}|I_{obs} - I_{av}|/\Sigma_{hkl}I_{av}$) = 0.069, representing 95% completeness for all data from ∞ to 1.8 Å; the CheY without Mg^{2+} data included 41 469 total reflections that were merged to give 10 532 unique reflections with R_{sym} = 0.076 and 94% completeness to 1.8 Å. In the highest resolution shell (1.79–1.81 Å) average I/σ = 2.5 and 2.0 for the data sets with and without Mg^{2+}. Electron density maps were calculated using the program package PHASES (Furey & Swaminathan, 1990). Model building and graphics display were performed on a Silicon Graphics IRIS 4D workstation using the programs "O" (Jones et al., 1991), Ribbons (Carson, 1987), and MOLSCRIPT (Kraulis, 1991).

RESULTS AND DISCUSSION

Most phosphoryl-transfer reactions involve divalent cations (Cooperman, 1976; Knowles, 1980), although a few enzymatic phosphoryl-transfer reactions are known to be metal ion independent (Meadow et al., 1990). In the case of phosphoryl transfer between the histidine kinase, CheA, and the response regulator, CheY, the requirement for divalent cations has been established (Lukat et al., 1990). The recent observation that phosphoryl transfer from small molecule phosphoryl donors to CheY is also metal ion dependent supports the notion that CheY is the locus for the metal requirement (Lukat et al., 1992). X-ray diffraction analysis of CheY bound to divalent metal ions has been hindered by conditions required for stability of the protein crystals. Crystals of CheY are typically grown and maintained in solutions of 1.8–2.9 M ammonium sulfate (Stock, A. M., et al., 1989). In solution, and in the crystalline state, high ionic strength dramatically reduces both metal binding and phosphoryl transfer (A. West, R. Saxl, and A. Stock, unpublished results). When Mg^{2+} or Mn^{2+} is introduced in the presence of high concentrations of ammonium sulfate, either during or subsequent to crystal growth, electron density maps indicate low metal occupancy.[1] To increase metal binding, conditions were determined for transfer of crystals to solutions of low ionic strength.

Data were collected from crystals equilibrated in solutions of low ionic strength in the presence or absence of 10 mM $MgCl_2$. Atomic coordinates were refined by the method of restrained parameter least-squares using the program PROLSQ (Hendrickson, 1985), starting with atomic coordinates from a previously determined 1.7-Å resolution structure of *S. typhimurium* CheY from crystals in ammonium sulfate (A. Stock, B. Rasmussen, E. Martinez-Hackert, and G. Petsko, unpublished results) from which all side chains and solvent molecules at the active site were omitted. The bound metal ion and its associated ligands were built into the structure on the basis of difference electron density maps, which were clear and unambiguous. The CheY·Mg^{2+} structure, which includes 980 protein atoms, 96 water molecules, and 1 Mg^{2+}, has been refined to a crystallographic R factor of 0.19 for all data from 5- to 1.8-Å resolution. Average bond lengths, interbond angles, and planarities deviate from ideal values by less than 0.020 Å, 2.9°, and 0.044 Å, respectively. Refinement of the metal free structure, which includes 95 water molecules, yielded similar values (R = 0.18; average deviations 0.019 Å, 2.8°, and 0.041 Å).

Overall, the structure of metal-free CheY determined at low ionic strength is very similar to structures obtained from crystals in 1.8–2.9 M ammonium sulfate; the corresponding Cα atom positions are essentially superimposable with a root-mean-square deviation of less than 0.2 Å. However, the structure at low ionic strength compared to the structure at high ionic strength shows different conformations for several surface side chains, different positions for many solvent molecules, and the absence of two ordered sulfate ions.

The active site of CheY lies at the C-terminal edge of the β-sheet in a cleft between loops from β-strands β1 and β3 (Figure 1). This face of the molecule is exposed to solvent in the crystal lattice, and residues in this region are not involved in intermolecular contacts. At the active site, an electron density map calculated with data collected from crystals equilibrated with Mg^{2+} shows density for atoms with bond angles and distances consistent with an octahedrally coordinated magnesium ion (Figure 2A). Three protein oxygen atoms and three water molecules provide the six ligands. A difference electron density map between the metal-bound and metal-free CheY structures (Figure 2B) shows positive and negative density indicative of the presence of the metal ion, additional solvent molecules, and a shift in the position of the side-chain carboxylate of Asp^{57}.

[1] Difference Fourier maps calculated with data from crystals in high salt with metal show small perturbations in electron density at the active site compared to maps calculated with metal-free data sets. In both maps, the bond distances and coordination geometry to the density that occupies the presumed metal ion site are more consistent with a water molecule rather than a metal ion. The slight differences in the maps obtained with data collected in the presence of metals probably reflect an average of two structures, with the metal-bound structure represented at very low occupancy.

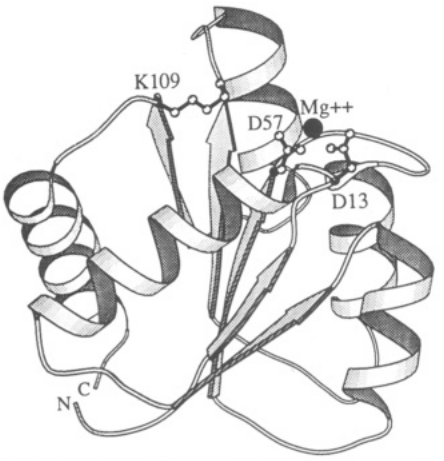


FIGURE 1: Diagram of CheY showing the α/β fold of the backbone and the locations of the conserved active site residues Asp^{13}, Asp^{57}, and Lys^{109} in the loop regions at the tops of β-strands $\beta1$, $\beta3$, and $\beta5$. Asp^{13} and Asp^{57} bind Mg^{2+}, indicated by a solid sphere.

The first coordination sphere of Mg^{2+} consists of the carboxylate oxygens of Asp^{13} and Asp^{57}, the backbone carbonyl oxygen of Asn^{59}, and three water molecules, Wat^{131}, Wat^{132}, and Wat^{133} (Figure 3A). These waters form hydrogen bonds with protein side-chain atoms in the active site and also hydrogen bond to two additional water molecules, unique to the CheY·Mg^{2+} structure, that form part of the second coordination sphere. Water molecule Wat^{132} is hydrogen bonded to the carboxylate of Asp^{12}. Bond distances between the metal ion and its ligands range from 2.1 to 2.2 Å.

The involvement of Asp^{12}, Asp^{13}, and Asp^{57} in metal ion binding is consistent with previous results from site-specific mutagenesis (Lukat et al., 1990, 1991) and nuclear magnetic resonance studies (Kar et al., 1992) that had implicated these residues in metal binding. Asp^{13} and Asp^{57}, which directly coordinate to the metal ion, are highly conserved within the family of response regulator domains. Asp^{12}, which coordinates through a water molecule, corresponds to a conserved carboxylate residue, either aspartate or glutamate, in the family of regulators. It is conceivable that the additional length of a glutamate side chain would allow for direct coordination of that carboxylate to the metal ion. Substitution of Asp^{12} by glutamate results in a CheY protein that is functional *in vivo* and which retains phosphoryl-transfer activity (Bourret et al., 1990). However, this protein binds Mg^{2+} weakly, with a dissociation constant approximately 100 times greater than that of wild-type CheY, which has a K_D of 0.5 mM at neutral pH (Lukat et al., 1990). Orthorhombic crystals of this mutant protein were grown in poly(ethylene glycol) and then equilibrated in the presence of 10 mM Mg^{2+}, under conditions identical to those used with the wild-type protein. Data were collected and analyzed as for the wild-type protein. In the structure of the mutant protein, no metal ion is observed in the active site, and the substituted Glu^{12} side chain is oriented away from, rather than toward, the active site acidic pocket, in contrast to the position of the Asp^{12} side chain in the structures of wild-type CheY (W. McCleary, A. West, E. Martinez-Hackert, and A. Stock, unpublished results).

The major differences between the structures of CheY with and without Mg^{2+} are localized at the active site. Outside this region, the structures are very similar. Mg^{2+}-induced perturbations of aromatic residues have been detected by nuclear magnetic resonance (Kar et al., 1992) and fluorescence studies (Lukat et al., 1990); however, no significant changes in the conformation of aromatic side chains are observed in the crystal structures. The spectroscopic alterations may reflect changes in the electrostatic environment of the aromatic residues rather than substantial structural differences.

In the metal-free structure, a water molecule occupies a position similar to that of the metal ion in the CheY·Mg^{2+} structure (Figure 3B). Coordination to the Mg^{2+} involves shorter bond distances than hydrogen bonds to the water, and the side chains of the aspartates that provide these interactions are shifted accordingly. In the CheY·Mg^{2+} structure, the side chain of Asp^{57} is rotated by approximately 65° and is moved almost 2 Å toward the metal ion. The χ_1 and χ_2 values for the Asp^{57} side chain in the Mg^{2+}-free and Mg^{2+}-bound structures are –166°, –80° and 166°, 37°, respectively, neither of which correspond to the most commonly observed rotamers of aspartate (Ponder & Richards, 1987). In this position, the salt bridge between Asp^{57} and the ϵ-amino group of Lys^{109}, present in the metal-free model, is abolished. The side chain of Lys^{109} is oriented away from the cluster of carboxylate side chains that are neutralized by the magnesium ion. The disruption of the salt bridge between Asp^{57} and Lys^{109} upon coordination of one of the carboxylate oxygens of Asp^{57} to the Mg^{2+} leaves the other carboxylate oxygen free to serve as the attacking nucleophile in the phosphoryl-transfer reaction. Recent ^{19}F nuclear magnetic resonance studies of CheY using 4-fluorophenylalanine probes have been interpreted as indicating no disruption of the Lys^{109}–Asp^{57} salt bridge upon Mg^{2+} binding (Drake et al., 1993; Bourret et al., 1993). This conclusion contrasts with what is observed in the crystal structure.

The Lys^{109}–Asp^{57} salt bridge has invited speculation regarding the mechanism of activation of CheY by phosphorylation. Lys^{109} is strictly conserved in the response regulator family, and substitution of this residue by arginine or glutamine results in an inactive CheY protein (Lukat et al., 1991). It has been proposed that the Lys^{109}–Asp^{57} interaction constrains the C-terminal portion of the protein to a specific conformation and that breaking of this interaction by phosphorylation of Asp^{57} is critical to the conformational change that is associated with the active, phosphorylated form of CheY (Roman et al., 1992; Bourret et al., 1993). The structure of CheY with Mg^{2+} bound at the active site argues against this model of activation. The salt bridge observed in crystal structures in the absence of divalent cations is unlikely to exist *in vivo* where Mg^{2+} concentrations are in the millimolar range (Alatossava et al., 1985), and the metal binding site would be expected to be highly occupied. The CheY·Mg^{2+} structure also suggests that the Lys^{109}–Asp^{57} salt bridge imparts no significant constraints on the conformation of the C-terminal region of the protein, since loss of this interaction does not result in a significant perturbation of the terminal loop and helix.

The role of Lys^{109} is critical to the function of CheY, but perhaps not via an ionic interaction with Asp^{57}. Biochemical analysis of proteins with mutations at Lys^{109} (Lukat et al., 1991) together with the CheY·Mg^{2+} structure suggests that the critical interactions of Lys^{109} may occur in the phosphorylated structure. Phosphoryl transfer and autodephosphorylation activities of the mutants K109R and K109Q are only 2–5-fold less than wild type. But despite almost normal phosphorylation, the mutant proteins are unable to elicit flagellar motor responses and are not affected by CheZ, a protein which accelerates the dephosphorylation of phosphorylated wild-type CheY. Thus it appears that Lys^{109} is important for achieving the active conformation of phospho-CheY, perhaps through an ionic interaction with an oxygen of the acyl phosphate.

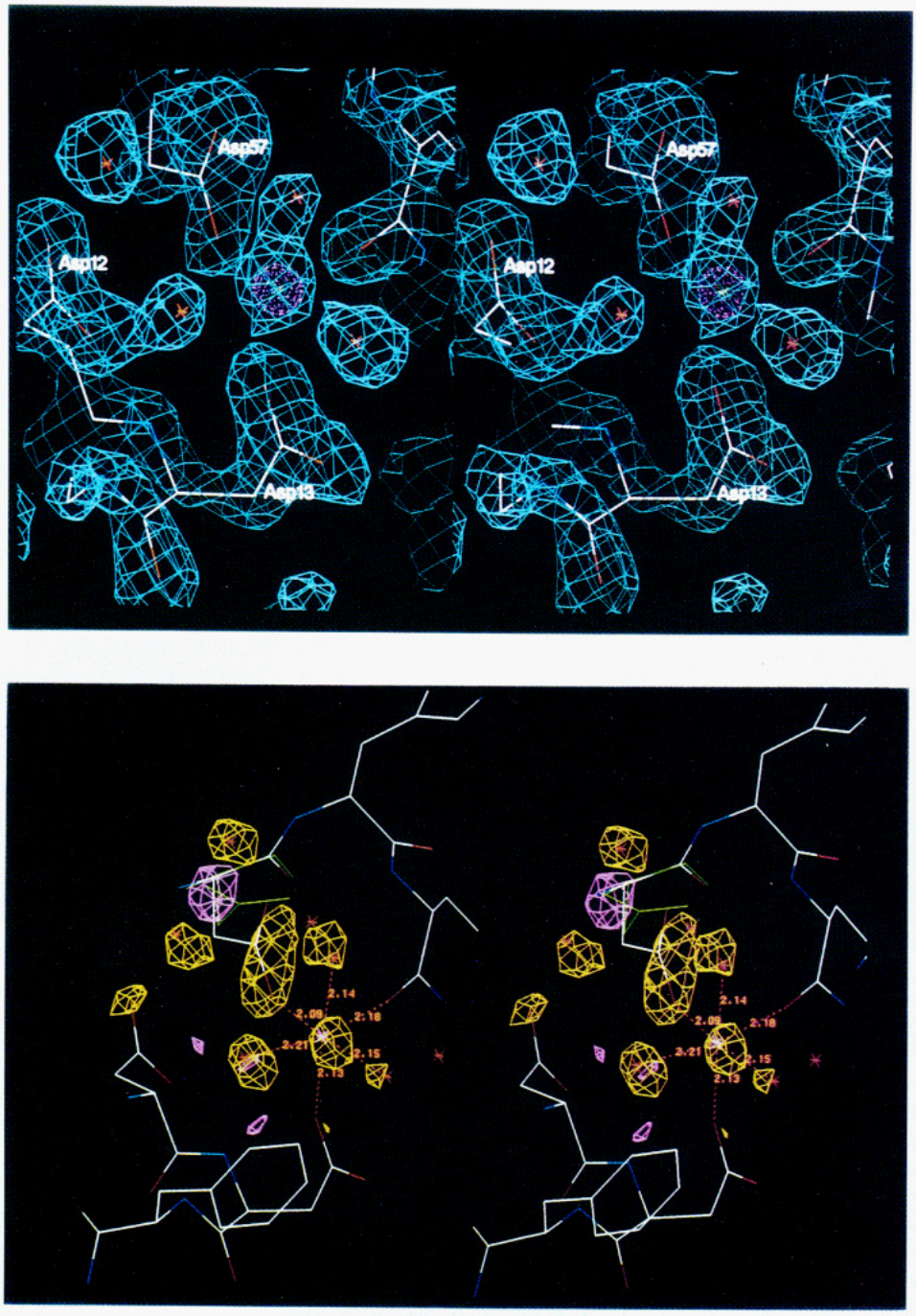


FIGURE 2: Stereoview of the Mg^{2+} binding site of CheY at 1.8-Å resolution. (A, top) $2\|F_o| - |F_c\|$ electron density map (contoured at 1σ) superimposed on the refined CheY·Mg^{2+} model. The Mg^{2+} and waters are shown as a magenta sphere and red crosses, respectively. F_o and F_c represent the measured and calculated structure factor amplitudes; phases are calculated using a refined model from which active site side chains, solvent molecules, and Mg^{2+} have been omitted. (B, bottom) Difference electron density map displayed with the refined CheY·Mg^{2+} model (colored by atom type). The map was calculated using coefficients $\|F_{CheY\cdot Mg}| - |F_{CheY}\|$, corresponding to the observed structure factor amplitudes for crystals in the presence and absence of Mg^{2+}, and calculated phases from the refined model with active site atoms omitted. Positive density (gold), corresponding to features unique to the CheY·Mg^{2+} structure, is observed at positions of the Mg^{2+}, water ligands, and the carboxylate side chain of Asp^{57}; negative density (magenta), corresponding to features of the CheY structure that are absent in the CheY·Mg^{2+} structure, superimposes with the position of the side chain of Asp^{57} in the CheY structure (shown in green). Dashed lines indicate the octahedral coordination of the Mg^{2+}, with bond distances given in angstroms.

The CheY·Mg^{2+} structure provides a basis for understanding the role of the conserved active site residues and the metal ion in catalysis of phosphoryl transfer. Much of what is known of metal ion-catalyzed phosphoryl transfer is derived from studies of nonenzymatic reactions (Cooperman, 1976; Knowles, 1980). These investigations have suggested that metal ions, because of their high charge density and multivalent coordination geometry, can facilitate phosphoryl-transfer reactions through both electrostatic interactions and transition-state stabilization.

The positive charge of a metal ion can potentially serve to activate the attacking nucleophile, stabilize the leaving group, enhance the electrophilicity of the phosphorus by polarizing the P–O bond, and provide charge shielding of the negatively charged phosphate group. These functions might equally well be provided by basic side chains at the active site of an enzyme. However, in the case of CheY, the only positively charged side chain in close proximity to the active site is Lys^{109}, and substitution of this residue by arginine or glutamine has little effect on the rate of phosphoryl transfer. It therefore seems

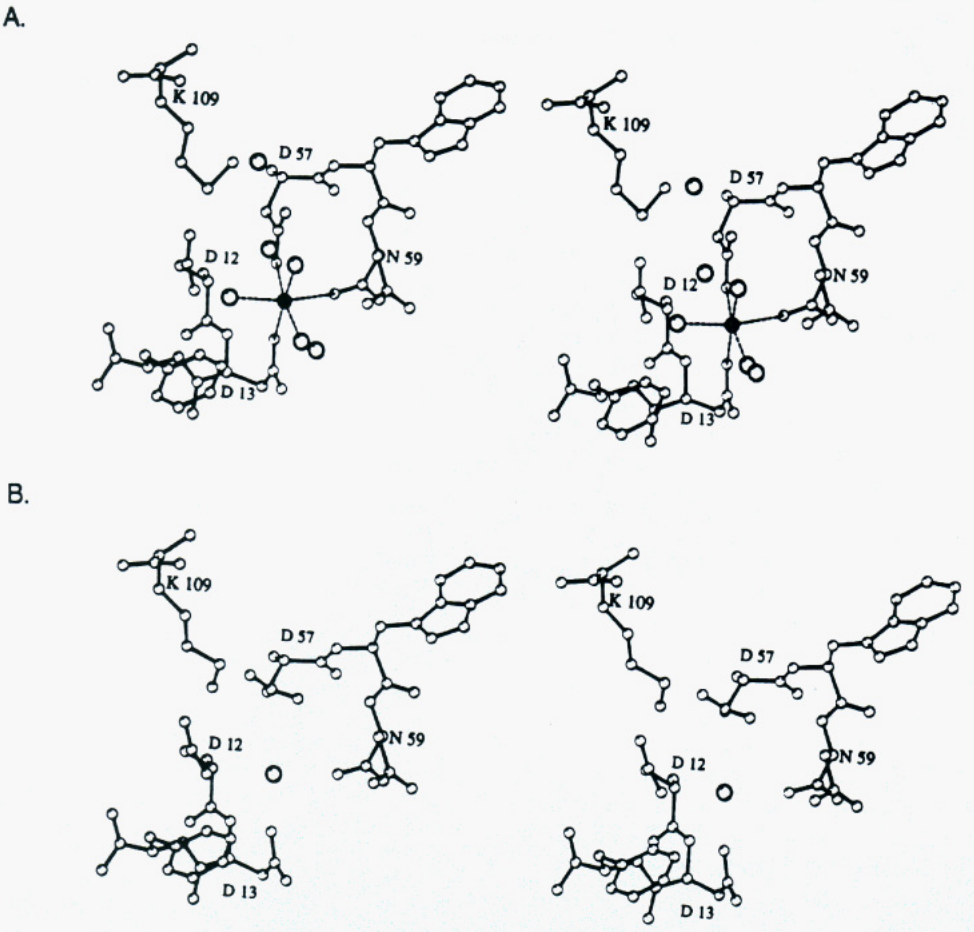
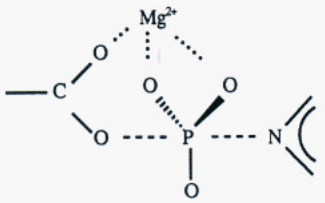


FIGURE 3: Active site of CheY (stereo). (A) Refined atomic model of CheY·Mg^{2+} showing residues 12–14, 57–59, and 109, corresponding to loops at the C-terminal ends of β-strands β1, β3, and β5. The Mg^{2+} and water molecules are indicated by solid and open spheres, respectively; dashed lines show octahedral coordination to the Mg^{2+}. (B) Similar view of the refined model of CheY determined in the absence of Mg^{2+}.

likely that electrostatic contributions to catalysis of phosphoryl transfer in CheY are largely provided by the divalent cation.

Metal ions can provide templates for phosphoryl transfer and can contribute to transition-state stabilization. Herschlag and Jencks (1990) have analyzed Mg^{2+} catalysis of phosphoryl transfer from phosphorylated pyridines to carboxylate ions, a model system that, in terms of functional groups, closely resembles phosphoryl transfer from the phosphoimidazole rings of histidine protein kinases to the aspartyl side chains of response regulator proteins like CheY. The study provided evidence for a transition state involving simultaneous coordination of Mg^{2+} to the oxygens of a pentavalent phosphorus and an oxygen of the attacking carboxylate molecule as shown:



(1)

The authors conclude that Mg^{2+} catalyzes the transfer by providing a template for the transition state.

This transition-state model can be accommodated within the CheY active site. The structure of CheY with bound Mg^{2+} allows only one positioning of the bipyramidal phosphorus (Figure 4). The two water molecules coordinated to the Mg^{2+} would be replaced by two equatorial oxygens of the pentavalent phosphorus. Rotation of the side chain of Asp^{57}

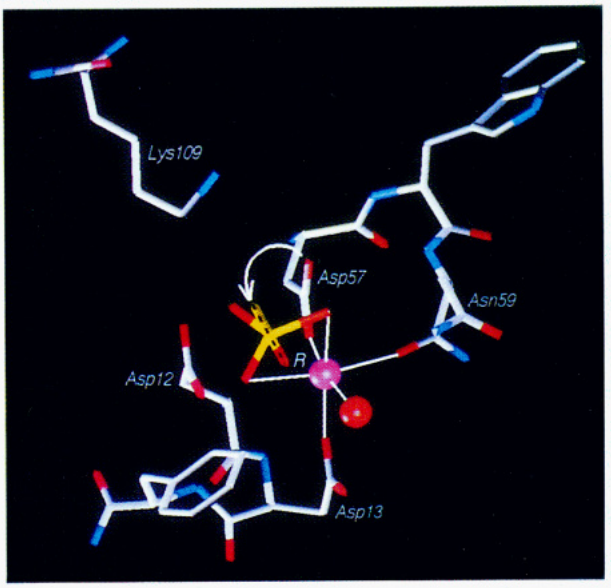


FIGURE 4: Postulated transition state for phosphorylation of CheY at Asp^{57} showing a pentavalent phosphorus positioned in the CheY·Mg^{2+} model. A transition state for phosphoryl transfer involving simultaneous coordination of Mg^{2+} to the equatorial oxygens of a bipyramidal phosphorus and an oxygen of the attacking carboxylate nucleophile (Herschlag & Jencks, 1990) can be achieved by displacing two water ligands with two trigonal planar oxygens of the pentavalent phosphorus. The arrow indicates a rotation of the Asp^{57} side chain that would position the nucleophilic carboxylate oxygen for in-line attack at the apical position of the phosphorus. An *R* marks the position that would be occupied by the leaving group.

would position the carboxylate oxygen for in-line nucleophilic attack at the axial position of the pentavalent phosphorus. This transition-state arrangement has the additional feature of placing the other axial position, the site of the leaving group, in a sterically unobstructed region of the active site cleft. A bulky leaving group such as the imidazole ring of the protein phosphoryl donor, phospho-CheA, can be positioned at this site without necessitating any movement of side chains in the current model. This transition-state model also suggests an explanation for the specificity of phosphorylation at Asp^{57}. A similar configuration is not attainable at the other conserved aspartate residue, Asp^{13}. Rearrangement of active site side chains, such as Phe^{14}, would be required to avoid steric collisions with the oxygens of the pentavalent phosphorus, and significant movement of the protein main chain would be necessary to position the carboxylate oxygen of Asp^{13} for an in-line displacement reaction.

The active site of CheY participates in three distinct functions: catalysis of phosphoryl transfer, catalysis of autodephosphorylation, and recoordination around the acyl phosphate to produce an active conformation of the regulatory domain. Determination of the structure of CheY bound to Mg^{2+} contributes to understanding the phosphoryl-transfer reaction. The conserved carboxylate cluster functions to position the metal ion within the active site, while the Mg^{2+} itself provides catalysis of phosphoryl transfer to Asp^{57}. Elucidation of the structure of phosphorylated CheY will undoubtedly suggest additional roles for active site residues in the mechanism of autodephosphorylation and induction of the activating conformational change.

ACKNOWLEDGMENT

We thank Ilme Schlichting and Joachim Reinstein for helpful discussions.

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