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The structures of T87I phosphono-CheY and T87I/Y106W phosphono-CheY help to explain their binding affinities to the FliM and CheZ peptides ☆,☆☆

Kenneth McAdams [a], Eric S. Casper [a], R. Matthew Haas [a], Bernard D. Santarsiero [b], Aimee L. Eggler [b], Andrew Mesecar [b], Christopher J. Halkides [a,*]

[a] Department of Chemistry and Biochemistry, University of North Carolina Wilmington, 601 S. College Road, Wilmington, NC 28403, USA
[b] Center for Pharmaceutical Biotechnology and Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S. Wood Street, Chicago, IL 60612-7231, USA

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ABSTRACT

CheY is a response regulator in bacterial chemotaxis. *Escherichia coli* CheY mutants T87I and T87I/Y106W CheY are phosphorylatable on Asp57 but unable to generate clockwise rotation of the flagella. To understand this phenotype in terms of structure, stable analogs of the two CheY-P mutants were synthesized: T87I phosphono-CheY and T87I phosphono-CheY. Dissociation constants for peptides derived from flagellar motor protein FliM and phosphatase CheZ were determined for phosphono-CheY and the two mutants. The peptides bind phosphono-CheY almost as strongly as CheY-P; however, they do not bind T87I phosphono-CheY or T87I/Y106W phosphono-CheY, implying that the mutant proteins cannot bind FliM or CheZ tightly *in vivo*. The structures of T87I phosphono-CheY and T87I/Y106W phosphono-CheY were solved to resolutions of 1.8 and 2.4 Å, respectively. The increased bulk of I87 forces the side-chain of Y106 or W106, into a more solvent-accessible conformation, which occludes the peptide-binding site.

Prokaryotes sense and respond to a variety of environmental signals through the use of two-component systems. In these systems, an autohistidine kinase reversibly transfers a phosphoryl group to a conserved aspartate residue at the active site of the response regulator, controlling its signaling state. CheY[1] is a single domain response regulator that functions in the chemotaxis response [1]. The histidine kinase CheA transfers a phosphoryl group from its catalytic His residue to Asp57 of CheY, creating CheY∼P. CheY∼P binds to the switch protein FliM at the base of the flagellar motor and changes its direction of rotation from counterclockwise, generating smooth-swimming behavior, to clockwise, generating tumbly behavior [2]. The cellular level of CheY∼P is reduced by dephosphorylation, through its own autophosphatase activity as well as by the phosphatase CheZ, limiting the *in vivo* half-life of CheY∼P to less than a second [3]. Changes in the concentration of CheY∼P determine how frequently the periods of smooth-swimming are punctuated by tumbles; these changes can create a biased-random walk toward a better chemical environment. Mutations in CheY that bring about a greater tendency toward smooth-swimming or toward tumbly behavior impair chemotaxis [4]. CheY and CheY∼P bind a divalent metal ion that necessary for both phosphorylation and dephosphorylation reactions [5]. In the structure of $Mn{\cdot}BeF_3$–CheY the residues that bind to the metal ion are Asp13, Asp57, the backbone carbonyl oxygen of Asn59, and one fluorine atom [6].

Escherichia coli CheY mutants T87I and T87I/Y106W CheY are phosphorylatable but cannot generate clockwise rotation of the flagella [4]. In addition, both I87 mutants have ∼5-fold lower autodephosphorylation rates and are completely resistant to CheZ activity [7]. Thus, the presence of an isoleucyl side-chain at position 87 renders CheY unable to perform its chemotactic functions.

Residue 106 is an aromatic residue (tyrosine or phenylalanine) in 80% of known response regulators [8]. Matsumura and collaborators confirmed that an aromatic amino acid at position 106 is

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* Corresponding author. Fax: +1 910 962 3013.
E-mail address: halkidesc@uncw.edu (C.J. Halkides).

[1] *Abbreviations used:* CheA, product of the chemotaxis A gene; CheY, product of the chemotaxis Y gene; CheZ, product of the chemotaxis Z gene; CheY∼P, phosphorylated form of CheY; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid); FliM, flagellar switch protein M; AMPSO, 3-[(1, 1-dimethyl-2-hydroxyethyl)amino]-2- hydroxypropanesulfonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; RP HPLC, reversed phase high-performance liquid chromatography; PEG, polyethylene glycol; MES, 2-morpholinoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; RMSD, root mean squared deviation.

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required for proper CheY∼P signaling [9]. Mutagenesis and structure–function studies indicate that both the identity and the rotameric position of residue 106 are important for CheY∼P signaling [4,9,10]. Substitution of Tyr106 in *E. coli* CheY with tryptophan (Y106W CheY) produces a phosphorylation-dependent, hyperactive mutant that generates mainly clockwise rotational bias [9]. Replacement of Tyr106 with a nonaromatic, nonpolar residue results in completely smooth-swimming cells that are non-chemotactic [9].

In crystals of wild-type apo-CheY, Tyr106 is found at the FliM binding surface of CheY [11,12] and two rotameric conformations are evident from the electron density: an inside, solvent-inaccessible position and an outside, solvent-exposed position. Seminal work on the structure of BeF_3–CheY complexed with a peptide derived from the flagellar motor protein FliM suggested a two-state model of activation, called Y–T coupling [13]. In the uncomplexed state T87 and the β4–α4 loop are relatively distant from the active site. In the BeF_3-complexed form the hydroxyl group of T87 forms a hydrogen bond to one of the fluorine atoms, and Y106 moves to the solvent-inaccessible conformation. The Y–T coupling model has been expanded to include a form of CheY not posited in the original formulation, in which Y106 is solvent-inaccessible but T87 and the loop are not found in the same conformation as observed for BeF_3–CheY, the T-loop-Y model [14]. Structures of the complex between a peptide derived from CheZ and CheY, with and without BeF_3, demonstrate that T87 must move only a little to accommodate Y106 in the solvent-inaccessible rotamer [15].

Crystals of Y106W CheY show that tryptophan is found exclusively as the solvent-inaccessible rotamer [4]. In the structure of T87I CheY, Tyr106 is found only as the solvent-accessible rotamer [10]. The addition of the ethyl moiety at residue 87 sterically blocks residue 106 from occupying the solvent-inaccessible cavity. Combining these two mutations in T87I/Y106W CheY gives the same phenotype as T87I CheY [4], indicating that the T87I mutation is intragenically epistatic to the Y106W mutation. This result suggests that the buried rotamer of residue 106 is required for CheY∼P to induce clockwise rotation of the flagellar motor. Mutational studies of residue 106 indicate that this residue is not required for binding between CheY and FliM. Rather, residue 106 is critical in propagating the signal from the active site of CheY∼P to FliM to elicit tumbly behavior [9].

We have sought to determine the underlying mechanism for the phenotypes of T87I CheY and T87I/Y106W CheY. Does the T87I mutation bring about a defect in *binding* between CheY∼P and FliM or CheZ, or does it cause a defect in *function* without materially affecting binding? If FliM and CheZ bind to T87I CheY∼P (the second possibility), phosphorylation would presumably have altered the position of I87 thus allowing residue 106 to rotate inwards. Previous structure–function studies have examined mutant CheY proteins in their *non-phosphorylated* state; however, this study is the first structure–function examination of CheY in its *phosphorylated* state. The Y–T coupling model suggests the importance of the hydrogen bond formed between the hydroxyl group of T87 and the phosphoryl group. We wished to test the hypothesis that if the side-chain at position 87 lacks the hydroxyl group, it will be not displaced as much as T87 is upon phosphorylation and will not allow rotation of the aromatic ring of residue 106.

Structural studies are difficult for CheY∼P [16], which has a half-life of 15–20 s even in the absence of CheZ [17], virtually necessitating the use of some kind of analog of the active state. Several methods of mimicking the effects of phosphorylation exist, including complexing CheY with BeF_3 and a divalent metal ion [18] or using D13K/Y106W CheY [14]. However, no chemical analog of CheY∼P has been studied with *in vivo* or *in situ* assays [19]; therefore, their biological activities are not known. Phosphono-CheY is inert to a wide variety of solution and crystallization conditions such as the presence of chelating agents, and it is nontoxic. Phosphono-CheY can be studied both in the presence and absence of a divalent metal ion [20].

In this study, stable analogs of T87I CheY-P and T87I/Y106W CheY-P were prepared similarly to phosphono-CheY [21]. Fluorescence quenching of Trp58 and isothermal titration calorimetry were used to determine the affinities of these two mutants for the FliM and CheZ peptides. The FliM peptide consists of the 16 N-terminal residues of the flagellar switch protein that compose the CheY-binding region [22], and the CheZ peptide contains the 19 C-terminal residues of the phosphatase CheZ, which binds specifically to CheY∼P, though at a lower affinity than that of intact CheZ [23]. Both peptides bind about 15- to 20-fold more strongly to CheY∼P than to CheY [24]. The peptide studies allow us to assess whether or not the I87 mutation inhibits binding to FliM or CheZ. In addition, the structures of these proteins were determined using single-crystal X-ray diffraction. We describe the changes brought about by phosphonomethylation (to make an analog of CheY-P) of T87I CheY and T87I/Y106W CheY and examine whether phosphonomethylation or the T87I mutation controls the rotameric position of residue 106 and the conformation of the loop connecting β4 to α4, the 90s loop.

Materials and methods

Protein production, purification, and modification

Escherichia coli D57C/T87I CheY and D57C/T87I/Y106W CheY cloned into separate pet24a(+) plasmid vectors (Novagen) were gifts from Phil Matsumura. Each plasmid was transformed by standard electroporation methods into *E. coli* strain B834 (DE3) (Novagen; San Diego, CA). Purification of CheY mutant proteins were performed as previously described [20] with minor modifications. Cells were lysed with a Fisher 550 sonic dismembrator, and 0.2 mg/mL lysozyme was occasionally added to assist in breaking the cell walls. In some cases a 2.5 by 54 cm column of Sephadex G-50-150 (Sigma–Aldrich; St. Louis, MO) was used to remove higher molecular weight impurities that remained after the DE52 anion-exchange column and the Affigel Blue (Bio-Rad; Hercules, CA) column.

Phosphonomethylation of purified D57C CheY variants was performed as previously described [20] with minor modifications. Prior to the reaction between CheY and phosphonomethyltriflate, the protein was reduced with 5 mM bis(2-mercaptoethyl)sulfone for up to one hour. CheY protein was exchanged into buffer consisting of 300 mM AMPSO, pH 9.10, with 1 mM EDTA, and strontium chloride or other divalent metal chloride was added to a final concentration of 150–250 mM. An aliquot of ethanol or isopropanol (equal in volume to the volume of triethylamine) was added to phosphonomethyltriflate, then an aliquot of triethylamine consisting of 2.8 mol per mole of phosphonomethyltriflate was mixed in, and this solution was quickly added to the solution of protein with divalent metal ion. The final concentration of phosphonomethyltriflate was 95–140 mM. The modification reaction proceeded for 30 min, then 10 mM DTT was added. The protein was exchanged into 50 mM BES, pH 7.1, with 2 mM EDTA. At this point the reaction mixture was sampled for RP HPLC and DTNB assays and was roughly 65% complete, though the extent of reaction was variable. Each mixture of phosphono-CheY and D57C CheY (approximately 250 μM total concentration) was reacted with 2–3 mM PEO-maleimide biotin (Pierce Biotechnology; Rockford, IL) for 5–12 h to attach a biotin group and spacer to unmodified Cys57 residues. Subsequent work has suggested that 2–3 mM PEO-iodoacetyl biotin (Pierce) in 50 mM AMPSO, pH 9.0, gives better results [21]. The mixture of biotinylated D57C CheY and phosphono-CheY was passed over immobilized monomeric avidin