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Identification of the Binding Interfaces on CheY for Two of its Targets, the Phosphatase CheZ and the Flagellar Switch Protein FliM

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CheY is the response regulator protein serving as a phosphorylation-dependent switch in the bacterial chemotaxis signal transduction pathway. CheY has a number of proteins with which it interacts during the course of the signal transduction pathway. In the phosphorylated state, it interacts strongly with the phosphatase CheZ, and also the components of the flagellar motor switch complex, specifically with FliM. Previous work has characterized peptides consisting of small regions of CheZ and FliM which interact specifically with CheY. We have quantitatively measured the binding of these peptides to both unphosphorylated and phosphorylated CheY using fluorescence spectroscopy. There is a significant enhancement of the binding of these peptides to the phosphorylated form of CheY, suggesting that these peptides share much of the binding specificity of the intact targets of the phosphorylated form of CheY. We also have used modern nuclear magnetic resonance methods to characterize the sites of interaction of these peptides on CheY. We have found that the binding sites are overlapping and primarily consist of residues in the C-terminal portion of CheY. Both peptides affect the resonances of residues at the active site, indicating that the peptides may either bind directly at the active site or exert conformational influences that reach to the active site. The binding sites for the CheZ and FliM peptides also overlap with the previously characterized CheA binding interface. These results suggest that interaction with these three proteins of the signal transduction pathway are mutually exclusive. In addition, since these three proteins are sensitive to the phosphorylation state of CheY, it may be that the C-terminal region of CheY is most sensitive for the conformational changes occurring upon phosphorylation.

Keywords: protein-protein interactions; chemotaxis; CheY; CheZ; FliM

Introduction

Motile bacteria respond to their environment with changes in their swimming behavior in order to result in a more favorable environment. The chemotaxis signal transduction system is the pathway used to regulate the events leading to changes in the swimming behavior of the cell (for a review, see Blair, 1995; Falke *et al*., 1998). Central to this signal transduction pathway is the response regulator CheY, which serves as a phosphorylation-dependent switch. When phosphorylated, CheY interacts strongly with the flagellar motor to result in a change in the rotational bias of the motor (Barak & Eisenbach, 1992; Welch *et al*., 1993). The cellular levels of phosphorylated CheY are controlled at several stages. Initially, in response to chemical attractants or repellents, the transmembrane receptors signal to the receptor-associated kinase CheA to adjust its rate of autophosphorylation. CheA serves as the phosphate donor for CheY, and thereby the levels of CheA ~ P limit the potential pool of phosphorylated CheY. CheY itself has an intrinsic dephosphorylation activity which

Abbreviations used: CheA~P, phosphorylated CheA; CheY~P, phosphorylated CheY; HSQC, heteronuclear single-quantum coherence.

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results in an *in vitro* phosphorylation half-life of approximately 20 seconds (Hess *et al.*, 1988; Lukat *et al.*, 1991). The cytosolic phosphatase CheZ strongly accelerates the dephosphorylation of CheY (Hess *et al.*, 1988).

In the chemotaxis signal transduction system, the interactions of many of the proteins are regulated by phosphorylation events. The kinase CheA interacts sixfold more strongly with CheY in its unphosphorylated state (Li *et al.*, 1995). Phosphorylated CheY (CheY ~ P) shows enhanced affinity for both the phosphatase CheZ and the flagellar switch protein FliM compared to the non-phosphorylated form (Blat & Eisenbach, 1994; Welch *et al.*, 1993).

We are interested in understanding the mechanisms of specificity and regulation of the interactions of the components of the chemotaxis pathway on a molecular level. Currently, we are focused on examining the interactions of CheY with the phosphatase CheZ and the flagellar switch protein FliM to determine the affinities and regions of association. Full-length CheZ and FliM proteins form higher-order complexes in solution, which compounds the difficulty of studying the interaction of these proteins with CheY. Thus the intact proteins are poorly suited for structural analysis by high-resolution NMR.

In order to determine the regions of CheY which interact with CheZ and FliM, we made use of previously characterized peptides of CheZ and FliM which interact specifically with CheY. Distinct regions of CheZ and FliM that are necessary and sufficient to interact with CheY have been identified by mutational analyses and biochemical characterization (Blat & Eisenbach, 1996; Bren & Eisenbach, 1998; Sockett *et al.*, 1992; Toker & Macnab, 1997). A peptide consisting of the conserved C terminus of CheZ, residues 196-214, has been shown to bind specifically to phosphorylated CheY, though it does not catalyze dephosphorylation (Blat & Eisenbach, 1996). Deletion and truncation analyses have demonstrated that the N-terminal region of FliM is important for binding to CheY (Toker & Macnab, 1997). The region of FliM which binds to CheY has been further narrowed down to the N-terminal 16 residues, and it has been demonstrated that a peptide consisting solely of this region will bind to CheY (Bren & Eisenbach, 1998). In addition, Bren & Eisenbach (1998) have demonstrated the specificity of CheY for this peptide by showing that a single amino acid change at position 7 from S to Y eliminates binding. Both the CheZ and FliM peptides have been reported to have a higher affinity for the phosphorylated form of CheY than for the non-phosphorylated form, though these affinities have not been quantitatively measured (Blat & Eisenbach, 1996; Bren & Eisenbach, 1998).

Using these previously characterized peptides of CheZ and FliM, we have now studied their interactions with CheY using fluorescence and NMR spectroscopy. The use of these peptides offer a number of advantages for this study, including low molecular mass and therefore minimal linebroadening in the NMR spectra. NMR resonances are sensitive to the local structural and electronic environment, and therefore conformational or electrostatic changes can affect the chemical shift of a given resonance, though these effects can be direct or indirect. This approach makes use of the fact that the resonance assignments for CheY are available (Bruix *et al.*, 1993; Moy *et al.*, 1994). Chemical shift changes observed in the ^{1}H-^{15}N correlation spectrum of CheY as a function of the addition of peptide provide a sensitive means of simultaneously monitoring the environment of every amide nitrogen during the course of the titration. One of the strengths of this approach is that the wild-type protein is used, and therefore the method does not depend on the stability or conformational changes which may be present in site-specific mutants.

Results

Binding affinities of CheY and the CheZ or FliM peptides

Using fluorescence spectroscopy we measured the affinity of CheY for the CheZ and FliM peptides by monitoring the emission spectrum from the single tryptophan residue on CheY. Addition of either the CheZ or FliM peptide causes a quenching of the tryptophan emission as a function of the amount of peptide added (Figure 1). The binding constants for CheY and either the CheZ or FliM peptide are similar, 440(±10) μM for CheZ and 680(±10) μM for FliM. The dissociation constants for CheY ~ P and the CheZ or FliM peptides

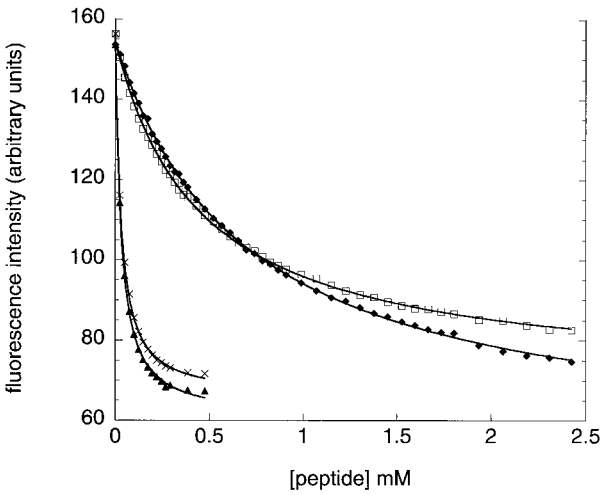


Figure 1. Fluorescence intensity at 340 nm of CheY with (□) CheZ peptide or (◆) FliM peptide and CheY ~ P with (×) CheZ peptide or (▲) FliM peptide. The fluorescence intensity of CheY ~ P has been normalized to that of CheY. The calculated binding curves are shown.

were measured by phosphorylating CheY with phosphoramidate in the presence of magnesium chloride under otherwise identical conditions (Figure 1). Phosphorylation itself quenches about 65 % of the tryptophan fluorescence intensity, which has been previously observed for tryptophan fluorescence measurements of phosphorylated CheY (Lukat *et al.*, 1992). The fluorescence of CheY ~ P is further quenched by the addition of either peptide. A random control peptide (CALLQSR) does not cause appreciable quenching of the tryptophan fluorescence of CheY in either the phosphorylated or unphosphorylated state (data not shown). The binding of both the CheZ and FliM peptides was approximately 20-fold stronger for CheY ~ P than CheY, having binding constants of 26(±1) μM for the CheZ peptide and 27(±1) μM for the FliM peptide.

Peptide binding sites on CheY

CheY with CheZ peptide

To identify the binding interface on CheY for the CheZ peptide, NMR spectra were recorded of labeled CheY protein titrated with the CheZ peptide. ^{1}H-^{15}N correlation (HSQC) spectra were recorded after each addition of peptide. By following the peaks through the course of the titration, the resonance assignments of the peptide-bound form of CheY were determined. Combined proton and nitrogen chemical shift changes larger than 95 Hz are noted for 15 residues of CheY in the presence of CheZ peptide (Figure 2(a)). These residues are, in decreasing order of chemical shift change, Y106, V107, T87, A99, V86, M85, K91, A98, S104, V108, N94, K119, I96, D57 and N59. Two peaks (corresponding to Y106, V107) that have very large overall chemical shift changes show exchange broadening in the NMR spectra at intermediate points in the titration, and then sharpen as saturation is reached.

The residues with large chemical shift changes in the presence of the CheZ peptide are located primarily in the fourth helix and the fourth and fifth β-strands of CheY (Figure 3(a)). The phosphorylation site of CheY, D57, located at the end of the third β-strand in the tertiary structure of CheY, also shows a large chemical shift change in the presence of CheZ. Some chemical shift changes are likely to be induced by propagating effects in the local structure rather than direct interactions, since some of the residues in the fourth β-strand showing chemical shift changes are not accessible from the surface of the protein.

CheY with FliM peptide

The interaction surface on CheY for the FliM peptide was determined from NMR spectra of CheY in the presence of the FliM peptide, in a manner similar to that described for CheY and the CheZ peptide. Large chemical shift changes, greater than 95 Hz, are observed for 15 residues in CheY upon the addition of FliM peptide (Figure 2(b)). These residues are, in decreasing order of chemical shift change, V107, K109, Y106, A99, M85, V86, D57, L116, V108, N121, T87, E118, Y21, K91, and K126. These residues are located chiefly in the fourth and fifth helices of CheY, and in the fourth and fifth β-strands (Figure 3(b)), though the phosphorylation site, D57, also shows a chemical shift effect.

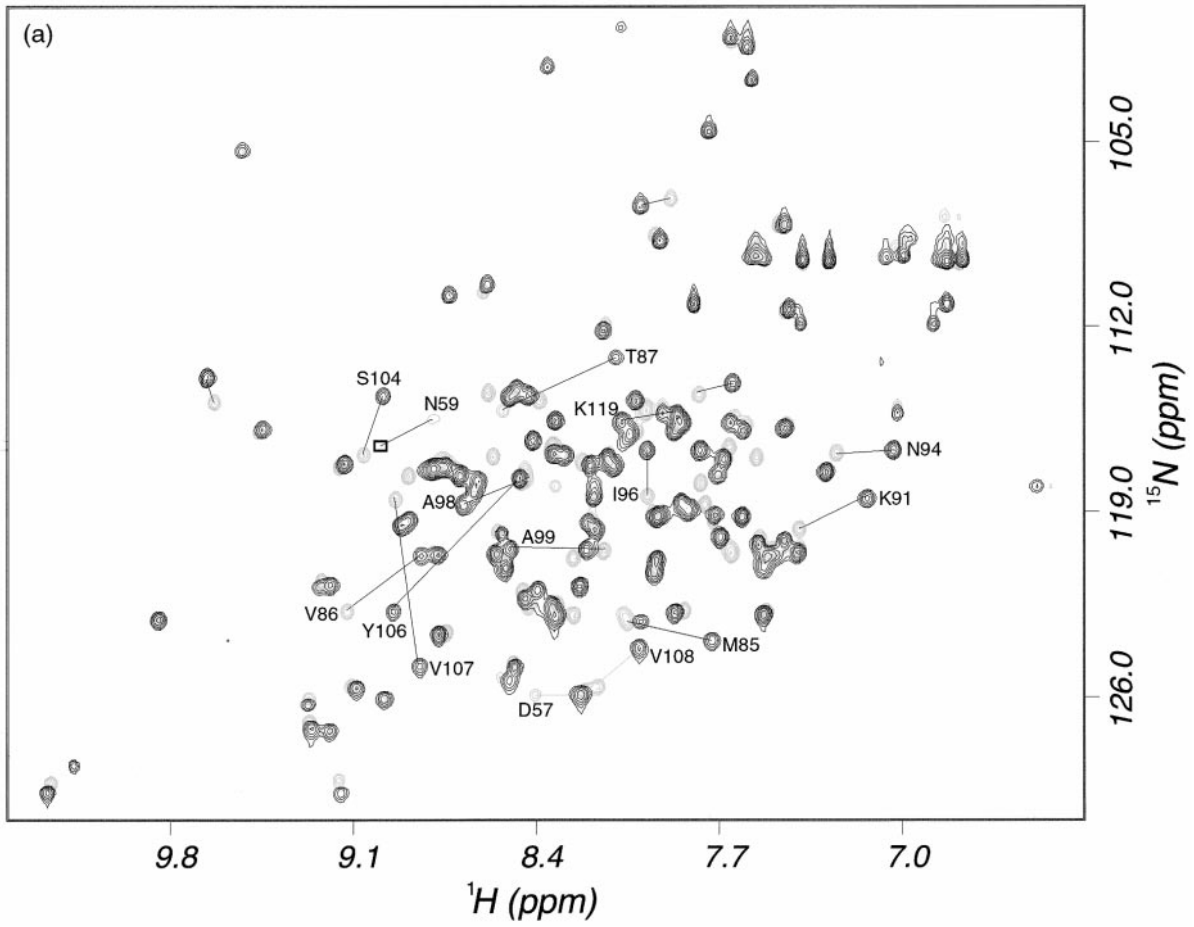
The CheZ and FliM binding sites identified by the chemical shift changes are localized to similar regions, primarily in the C-terminal region of CheY. These binding interfaces are overlapping, but not identical. Some residues in CheY are affected strongly by the binding of either peptide. These residues are D57, M85, V86, T87, K91, A99, Y106, V107, and V108. These residues are not necessarily affected to the same extent by the binding of the peptides. For example, D57 is more strongly affected by the FliM peptide than the CheZ peptide, judging from the combined chemical shift change of 184 Hz upon binding of the FliM peptide, and 101 Hz upon binding of the CheZ peptide.

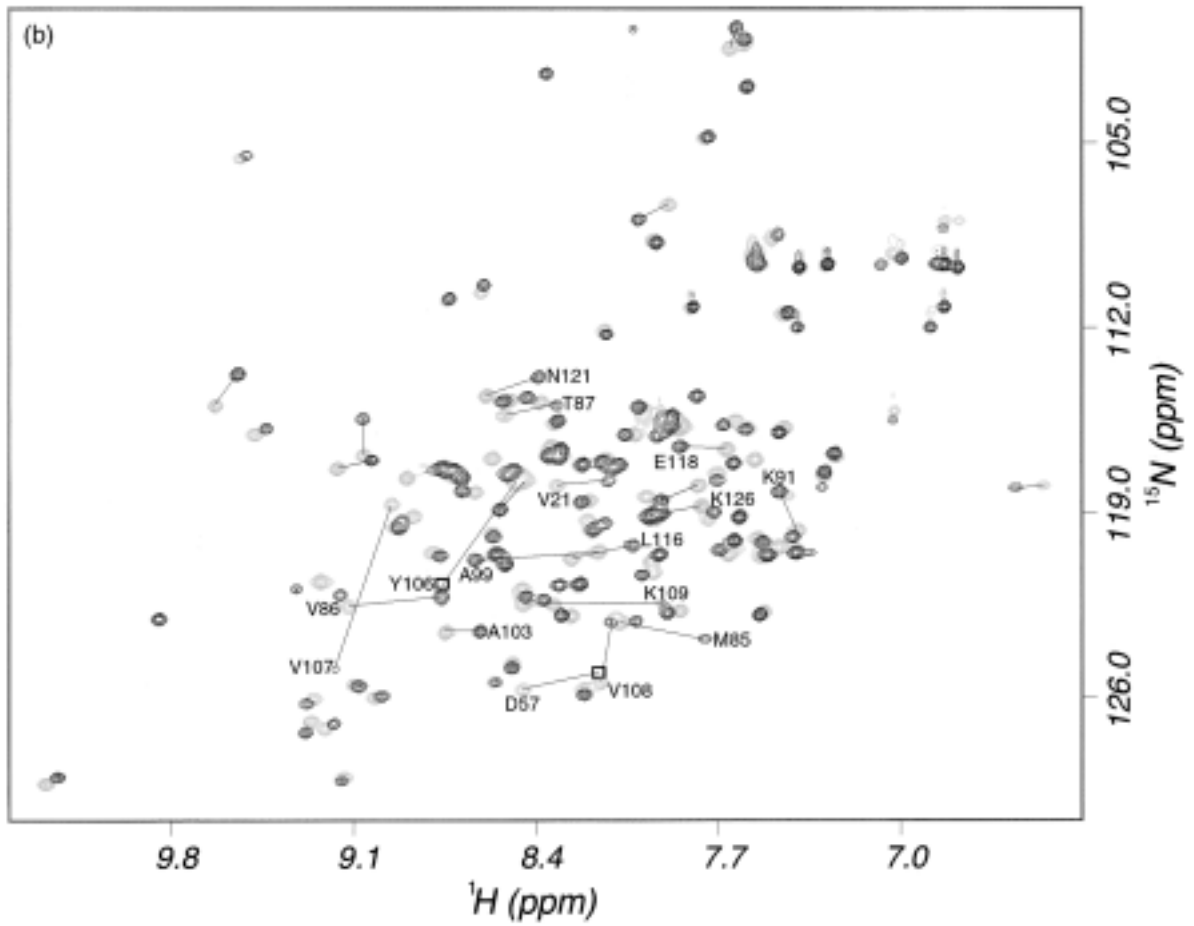
The CheZ peptide uniquely affects residues N59, N94, I96, A98, S104, and K119, while the FliM peptide affects residues V21, K109, L116, E118, N121, and K126.

Discussion

The binding experiments using quenching of the single tryptophan residue of CheY show that the phosphorylated form of CheY binds to the CheZ peptide approximately 20-fold more strongly than the unphosphorylated form. This result demonstrates that the CheZ peptide has the determinants necessary to discriminate between the non-phosphorylated and phosphorylated states of CheY. However, this increase in binding affinity upon CheY phosphorylation is about tenfold less than that seen for full-length CheZ, which binds to CheY ~ P approximately two orders of magnitude higher than unphosphorylated CheY (Blat & Eisenbach, 1994). This difference in phosphorylation effect on binding can be explained as follows: (i) CheZ peptide does not contain all the regions of CheZ which interact with CheY; and (ii) features of CheZ important for its oligomerization and phosphatase activation (Blat *et al.*, 1998), which are missing in the peptide (which does not oligomerize and has no phosphatase activity; Blat & Eisenbach, 1996), might also contribute to the increased affinity towards CheY ~ P.

The affinity of the FliM peptide for phosphorylated CheY is also increased approximately 20-fold over the unphosphorylated form. This increase in affinity is on the order of that observed for CheY ~ P and the full-length FliM protein (Welch *et al.*, 1993). However, the possibility that full-


(a)
T87
S104
N59
K119
N94
I96
K91
A98
A99
V86
Y106
M85
V108
V107
D57
105.0
112.0
119.0
126.0
^{15}N (ppm)
9.8
9.1
8.4
7.7
7.0
^{1}H (ppm)


(b)
N121
T87
E118
K91
V21
K125
L116
A99
Y106
K109
V86
A103
M85
V107
V108
D57
105.0
112.0
119.0
126.0
^{15}N (ppm)
9.8
9.1
8.4
7.7
7.0
^{1}H (ppm)

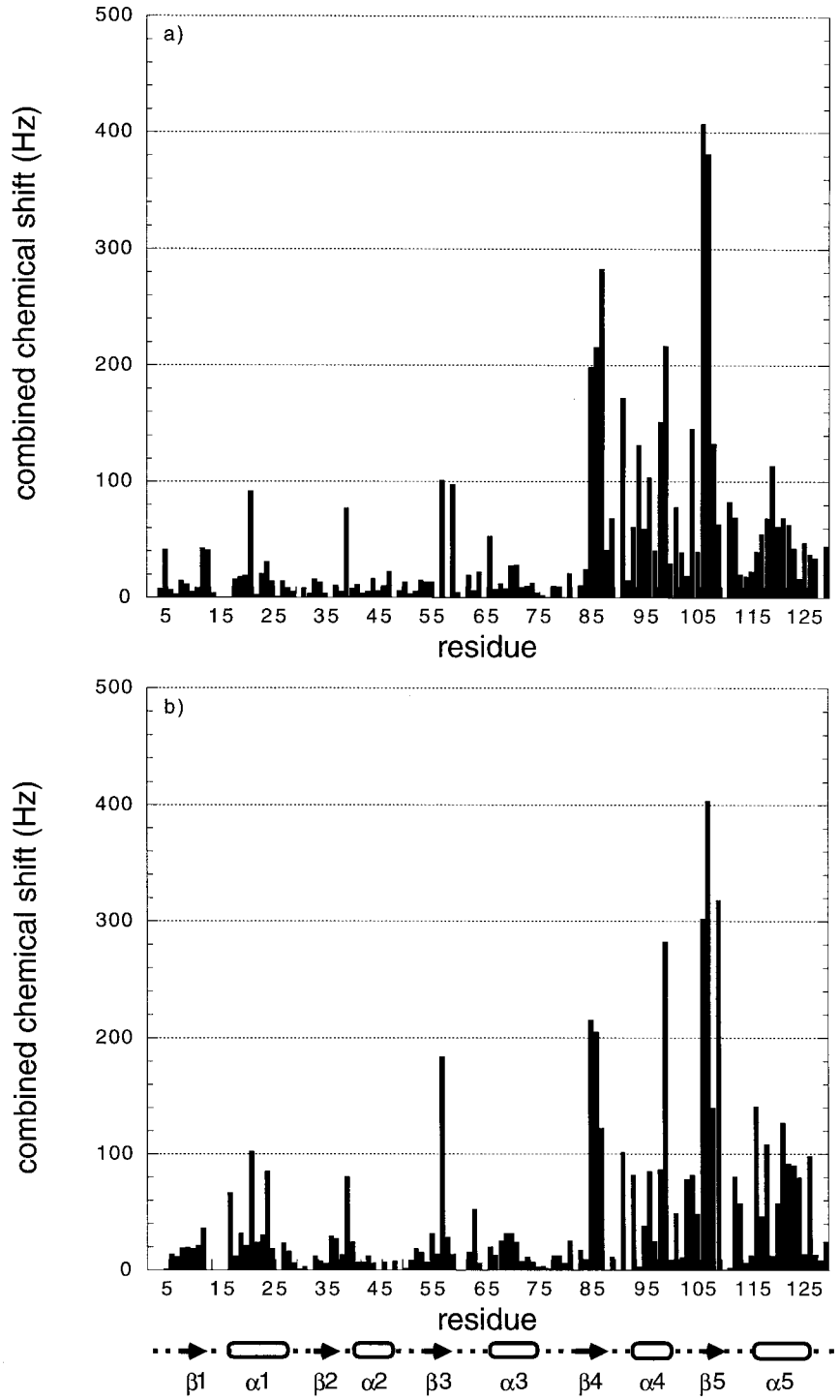


Figure 3. A plot of the combined ^{1}H-^{15}N chemical shift changes as a function of residue number observed upon the binding of (a) CheZ peptide and (b) FliM peptide. The secondary structure of CheY as reported in pdb entry 3chy (Volz & Matsumura, 1991) is shown below the plots. Turn and loop regions (···), α-helices (ovals) and β-strands (→) are indicated.

Figure 2. Overlay of the ^{1}H-^{15}N correlation spectra of (a) CheY free (red) and bound to CheZ peptide (black) and (b) CheY free (red) and bound to FliM peptide (black). Peaks that are not observed at the contour level shown, but are clear at lower contour levels, are indicated by a box. Peaks with large chemical shift changes are connected by a line from the free to the bound position. Those peaks corresponding to residues with combined chemical shift changes of greater than 95 Hz are labeled with the residue name.

length FliM may have a higher affinity than the peptide is not excluded. A higher affinity of FliM and CheY ~ P may occur if there are parts of FliM not represented by the peptide that interact with CheY as suggested by Mathews *et al.* (1998).

The relatively low affinity of the peptides for CheY ~ P can also be explained by the usual unstructured nature of peptides in solution. If the peptide is unstructured by itself, the overall free energy of binding of the peptide to CheY would represent both the folding free energy for the peptide and the intrinsic binding energy of the properly folded peptide to CheY. Thus the measured peptide affinity would be lower than the intrinsic binding affinity. For mechanistic reasons, the affinity of CheY ~ P for FliM is expected to be higher than the affinity of CheY ~ P for CheA. The suggestion that the peptide requires additional free energy for folding can explain the discrepancy in the affinity of CheY ~ P for the kinase CheA (~10 μM; Li *et al.*, 1995) and the FliM peptide affinity for CheY ~ P (~26 μM).

The binding interfaces on CheY for the CheZ and FliM peptides are localized to the C-terminal regions of CheY, but also show chemical shift effects which reach to the active site (Figure 4). Since both of these peptides show enhanced affinity for the phosphorylated form of CheY, both peptides must in some way sense the phosphorylated state of CheY, either by direct contact with the active site region, or by propagated conformational changes occurring upon phosphorylation, or both. The CheZ peptide effects on the active site may further enhance the ability of the rest of CheZ to catalyze dephosphorylation, or may make CheY ~ P a better substrate for dephosphorylation. Similarly, the FliM effect on the active site may enhance the CW promoting activity of CheY ~ P, or CheY ~ P may stabilize a conformation of FliM with enhanced CW rotation. Our experiments cannot distinguish between these possibilities.

The C-terminal regions of CheY that interact with the CheZ and FliM peptides also overlap with the binding interface for CheA determined by NMR spectroscopy (Swanson *et al.*, 1995; Figure 5). This suggests that when bound to CheA, CheY cannot bind to CheZ and FliM. Additionally, since CheA, CheZ, and FliM are sensitive to the phosphorylation state of CheY, it may be that the C-terminal region of CheY which forms the overlapping binding interfaces for these proteins are those which are most effected by phosphorylation. This view is supported by the observation that the NMR spectrum of phosphorylated CheY shows chemical shift differences from the unphosphorylated form in the C-terminal regions of the molecule (Lowry *et al.*, 1994). Although the largest of the chemical shift changes upon phosphorylation occur in the active site region, some changes are also located in the fourth and fifth β-strands and the fifth helix (Figure 5(d)).

The largest of the chemical shift changes caused by CheZ peptide binding is 407 Hz for Y106. Chemical shifts on this order are to be expected for substrate binding, which are comparable to the maximum chemical shift change (~260 Hz) in CheY induced by CheA1-233 binding (Swanson *et al.*, 1995). When CheY is phosphorylated, the largest chemical shift change is approximately 1100 Hz for D57, though other residues which are not undergoing covalent bond modification experience more modest, but significant changes, of 100 to 600 Hz (Lowry *et al.*, 1994). Chemical shift changes do not necessarily indicate a direct interaction with the substrate, but indicate a change in the local structural or electrostatic environment of a residue. Residues which show the same magni-

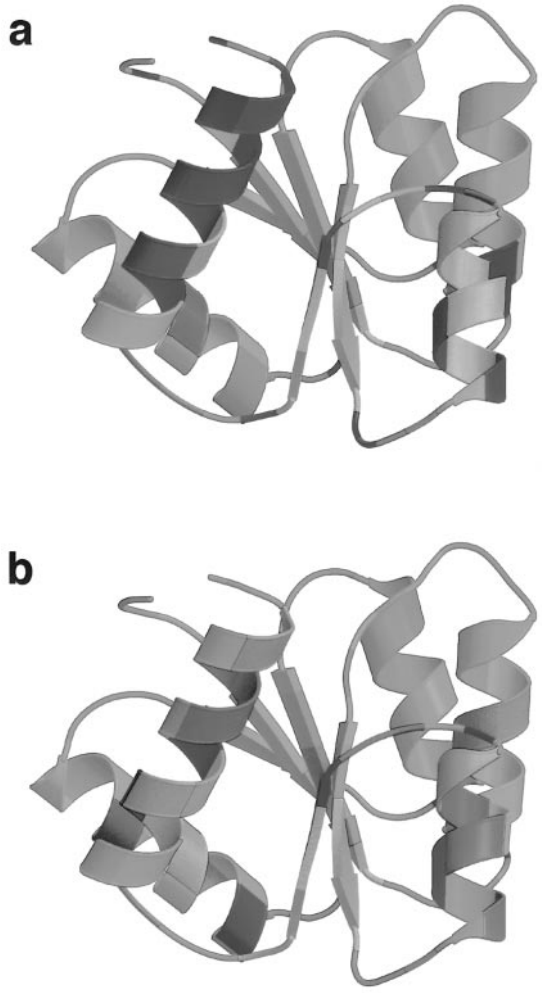


Figure 4. The chemical shift changes in the presence of (a) the CheZ peptide and (b) the FliM peptide are plotted on the structure of CheY (coordinates from pdb entry 3chy; Volz & Matsumura, 1991). Residues are shaded from red to yellow to blue based on the magnitude of the chemical shift change upon the addition of peptide. Those residues showing the greatest chemical shift change are shaded in red, and those residues with no chemical shift change are shown in blue. The ribbon representations were generated using the programs MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt & Bacon, 1997).

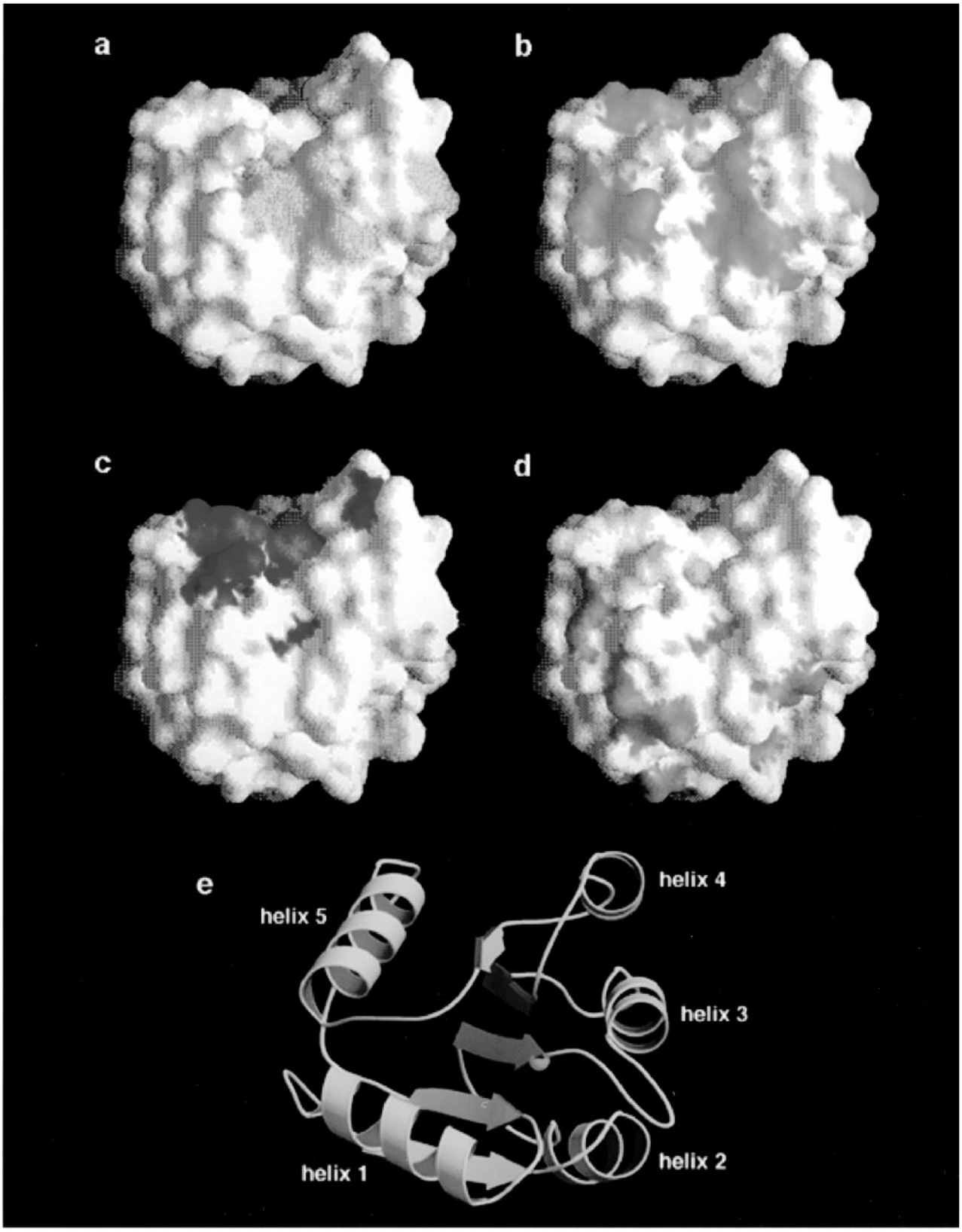


Figure 5. The interfaces of CheY. The interfaces for CheY determined by NMR chemical shift changes are shown for (a) the CheZ peptide; (b) the FliM peptide; (c) the CheY-binding region of CheA (Swanson *et al.*, 1995); and (d) phosphorylation-induced chemical shift changes (Lowry *et al.*, 1994). (e) For reference, the ribbon diagram shows the approximate orientation of CheY in the space filling models, with the site of phosphorylation, D57, shown in cyan. The coordinates of CheY are from pdb entry 3chy (Volz & Matsumura, 1991).

tude of chemical shift with each peptide would be likely to experience the same perturbing interaction (residues V86 and V108, for example), though this is unlikely to be true for those residues which undergo chemical shift changes of different magnitudes (residues T87 and N59, for example).

There are two extreme mechanisms by which the phosphorylated form of CheY might be recognized by CheZ or FliM. In the first, the recognition event occurs at sites distant from the CheY active site as a result of conformational changes that propagate from the site of phosphorylation. In the other, we imagine that the phosphoaspartate moiety provides the bulk of the recognition. The data presented here suggest that both mechanisms are likely to be utilized. The perturbations of the NMR spectra by the CheZ or FliM peptides show that regions both near and distant from the active site are perturbed by peptide binding in the absence of phosphorylation. In addition, for some residues, mainly those in the fourth β-strand, the shifts introduced by fragment binding and phosphorylation induce similar structural changes in this region, which is near the active site and is not especially surface accessible.

The CheY binding interface for the CheZ peptide determined from our work does not agree very well with the interface identified by CheY mutants binding to full-length CheZ (Sanna *et al.*, 1995; Zhu *et al.*, 1997b). The mutagenic analyses suggest that resides N23, K26, E27, F111, T112, and E117 are involved in forming the CheZ binding interface of CheY. These residues are located in the first and fifth α-helices and in the loop joining β4-α4. In our NMR analysis, only one of the residues with a large chemical shift change upon CheZ binding was within the fifth helix, K119. The CheY residues with the largest chemical shifts upon binding of the CheZ peptide are in the fourth helix, with only moderate involvement of the fifth helix (Figure 4(a)). The mutational analyses suggest that residues in the fourth helix and β4-α4 are only involved in CheZ catalytic activity (Zhu *et al.*, 1997b). Therefore, while there is some overlap in the binding regions determined by both studies, our results show a stronger effect from residues in the fourth helix than in the fifth helix. These discrepancies may be due in part to the use of the full-length protein *versus* the CheZ peptide in the different studies.

The CheY binding interface for the FliM peptide agrees well with previous mutational analyses (Shukla *et al.*, 1998). To identify a flagellar switch binding surface, dominant suppressors of *fliM* mutations in *cheY* were isolated. These CheY residues which suppress the FliM mutations are E27, A90, V108, F111, T112, and E117. Mapping of these residues on the CheY structure shows that these residues are localized to the C-terminal region, in the β5-α5 region. These results agree well with the interface region determined by NMR chemical shifts in our studies, which show a strong involvement of the fifth helix of CheY in FliM peptide interactions, and also a lesser effect on residues in the first helix.

Since the binding affinities of both peptides are similar and the regions of CheY they perturb are also very similar, it is likely that a feature common to both peptides is recognized by CheY. Examination of the sequence of the peptides, shown below, suggests that the regions in bold type are likely to be the ones involved in CheY binding.

AGVVA**SQDQVDDLLD**SLGF CheZ
MGDSIL**SQAEIDALLN** FliM

These observations also argue that the simultaneous binding of both CheZ and FliM to CheY is very unlikely. This supports the conclusion that CheZ acts on CheY ~ P in solution and not when CheY ~ P is bound to the flagellar switch (Bren *et al.*, 1996).

One of the residues with the largest chemical shift change upon CheZ or FliM peptide binding is tyrosine 106 in the fifth β-strand of CheY. Residue Y106 has been implicated as important in the phosphorylation-induced structural transition of CheY from the inactive to active signaling state (Zhu *et al.*, 1997a). Solution and crystal structures of unphosphorylated CheY indicate that the side-chain of this tyrosine occupies both a solvent-exposed and a hydrophobic conformation (Moy *et al.*, 1994; Volz & Matsumura, 1991). Evidence suggests that phosphorylation stabilizes a conformation of the Y106 side-chain in a hydrophobic position (Zhu *et al.*, 1997a), whereas a solvent-exposed position is favored by the unphosphorylated CheA-bound state of CheY (McEvoy *et al.*, 1998; Welch *et al.*, 1998). Since CheZ and FliM both interact more strongly with the phosphorylated form of CheY, it is not surprising that there is a large chemical shift effect involving this residue. The large chemical shift change of the Y106 resonance in the presence of the CheZ and FliM peptides may reflect a change in the equilibrium distribution of the Y106 side-chain conformers, though the potential role of Y106 in peptide recognition remains to be determined.

Finally, increasing evidence suggests that in general the C-terminal regions of response regulators are important for transducing the effects of phosphorylation to a functional response. Phosphorylation induces structural changes that propagate away from the active site throughout CheY (Lowry *et al.*, 1994), though CheY seems to primarily use the C-terminal region for protein-protein interactions. The response regulator domain of the methylesterase CheB also uses the α4-β5-α5 region as a protein-protein interaction interface (Djordjevic *et al.*, 1998). Of other response regulators with known structures, the activating surface of NtrC seems to be the

region spanning α3 to β5 (Nohaile *et al*., 1997), NarL interacts with the DNA-binding domain with residues from the α3-β4 loop and α4 (Baikalov *et al*., 1996), and the PhoB dimerization interface involves α1, the β5-α5 loop and α5, and possibly α4-β5-α5 (Solà *et al*., 1999). Clearly these regions putatively utilizing phosphorylation-induced changes are not identical from one response regulator to the next, and therefore each two-component system may have adapted different regions with which to signal specifically within the system.

Materials and Methods

Peptide synthesis and protein preparation

The CheZ peptide, corresponding to residues 196-214 of CheZ, AGVVASQDQVDDLLDSLGF, was obtained either from Macromolecular Resources (Fort Collins, CO) or Research Genetics (Huntsville, AL). The FliM peptide corresponding to residues 1-16 of FliM, MGDSILSQAEIDALLN, was obtained from Macromolecular Resources (Fort Collins, CO) or synthesized by the Biological Services Unit of the Weizmann Institute of Science, using a 432A Peptide Synthesizer SYNERGY (Applied Biosystems).

CheY was purified according to described methods (Lowry *et al*., 1994). The unlabeled CheY protein for the fluorescence measurements was purified from cells grown in LB-H medium. The ^{13}C-^{15}N labeled CheY for the NMR experiments was purified from cells grown in M9 minimal media (Sambrook *et al*., 1989) with [^{13}C]-glucose and [^{15}N]-ammonium chloride as the sole carbon and nitrogen sources, respectively.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Hitachi F-4500 Fluorescence Spectrophotometer at room temperature. Experiments were carried out in a 3 ml quartz cuvette containing 10 μM CheY in a 2 ml solution of 50 mM sodium phosphate (pH 7.2) and 0.02% sodium azide. The excitation wavelength was 285 nm. Emission was recorded at 340 nm only, though there is a slight shift in the emission wavelength during the course of the titration. Slit widths for both excitation and emission wavelengths were 5 nm. The CheY sample was titrated with aliquots of either 10 mM CheZ peptide, FliM peptide or a random control peptide (CALLQSR) in identical buffer. The fluorescence intensity was corrected for dilution due to addition of the peptide sample. Fluorescence emission from the peptide samples alone was negligible.

To record the spectra of phosphorylated CheY, 100 mM phosphoramidate and 20 mM magnesium chloride were added to the sample of CheY in otherwise identical buffer. The phosphorylation of CheY reduces the tryptophan fluorescence emission by about 65% of the unphosphorylated value. The fluorescence quenching of CheY as a result of phosphorylation has been noted in previous studies (Lukat *et al*., 1992). After the addition of phosphoramidate to the CheY sample, the sample was allowed to come to a steady state of phosphorylation, as judged by a stabilization of fluorescence intensity, before peptide was added. Though the extent of CheY phosphorylation cannot be directly measured in the fluorescence cuvette, from NMR studies of CheY under similar conditions (Lowry *et al*., 1994) we estimate that the quantity of phosphoramidate (10,000-fold molar excess over CheY) is sufficient to result in phosphorylation of approximately 95% of the CheY population for more than two hours. This steady-state phosphorylation lifetime is well in excess of the amount of time needed to record the series of fluorescence spectra.

To calculate the binding affinities from the fluorescence data, the data were fit with a single-site binding isotherm with the dissociation constant and total fluorescence change treated as fittable parameters. Ligand concentration was corrected for amount bound.

NMR spectroscopy

Spectra were acquired on a Varian Inova 600 MHz NMR spectrometer of samples in 50 mM sodium phosphate (pH 7.2), 10% ^2H_2O, 0.02% sodium azide at 25 °C unless otherwise noted. Initial assignments of the unbound CheY spectrum are based on the assignments reported by Moy *et al*. (1994). Residue K92, which was not assigned by Moy *et al*. (1994) and residue N59, which is not apparent in the uniformly labelled samples of Moy *et al*. are based on the assignments recorded by Bruix *et al*. (1994).

Six HSQC spectra (Kay *et al*., 1992) were acquired of labeled CheY during the course of the titration with the CheZ peptide. Starting with 630 μl of 0.25 mM CheY, 12.6 μl aliquots of 5 mM CheZ peptide were added successively. Assignments of CheZ peptide-bound CheY peaks were made by following the peaks in during the course of the titration. These assignments were subsequently confirmed by analysis of a CBCA(CO)NH spectrum taken at pH 6.4, which correlates the alpha and beta carbon chemical shifts of one residue with the nitrogen and proton chemical shifts of the following residue (Grzesiek & Bax, 1992). For CheZ peptide-bound CheY, non-proline residue assignments are complete except for the following residues: A2, D3, F14, S15, T16, S56, W58, G65 and A90. The assignments of residues A88 and K92 are based on their observation in HSQC and CBCA(CO)NH spectra at pH 6.4.

Seven HSQC spectra (Kay *et al*., 1992) were obtained of labeled CheY during the course of the titration with the FliM peptide. Starting with 600 μl of 0.2 mM CheY, 12.6 μl of 20 mM FliM peptide was added, followed by five subsequent additions of 25.2 μl of 20 mM FliM peptide. Peaks assignments for the peptide-bound state were made by following the resonances during the course of the titration. The non-proline assignments of the resonances of the FliM-bound CheY spectra are complete, except for residues A2, D3, D13, F14, S15, T16, W58, M60, G65, A88, A90, and K92. Due to spectral overlap, the final positions of residues N44, T115, and K119 could not be exactly determined though this cluster of peaks does not change greatly upon the addition of peptide.

Combined proton and nitrogen chemical shift differences for CheY in the presence of either peptide were calculated using the formula:

$$((\Delta^1H)^2 + (\Delta^{15}N)^2)^{1/2}$$

where ^{1}H and ^{15}N refer to the proton and nitrogen chemical shift values in Hz, respectively.

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