A Distinct Meta-active Conformation in the 1.1-Å Resolution Structure of Wild-type ApoCheY*

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CheY is the best characterized member of the response regulator superfamily, and as such it has become the principal model for understanding the initial molecular mechanisms of signaling in two-component systems. Normal signaling by response regulators requires phosphorylation, in combination with an activation mechanism whose conformational effects are not completely understood. CheY activation involves three events, phosphorylation, a conformational change in the β4-α4 loop, and a rotational restriction of the side chain of tyrosine 106. An outstanding question concerns the nature of an active conformation in the apoCheY population. The details of this 1.08-Å resolution crystal structure of wild-type apoCheY shows the β4-α4 loop in two distinctly different conformations that sterically correlate with the two rotameric positions of the tyrosine 106 side chain. One of these conformational states of CheY is the inactive form, and we propose that the other is a meta-active form, responsible for the active properties seen in apoCheY.

The dominant mode of signal transduction in bacteria utilizes two-component systems where histidine kinases phosphorylate response regulators that control gene expression, chemotaxis, antibiotic resistance, and many other processes (1). CheY is the regulator of the chemotaxis response in Escherichia coli. The normal signaling form of CheY requires phosphorylation of the aspartyl group at position 57 (2, 3). Another intramolecular event involved in the signaling of CheY is the rotation of the tyrosine group at position 106. The side chain of Tyr106 was first shown to be a rotamer in the unactivated CheY structure, equally occupying a solvent-exposed and a solvent-inaccessible position (4). The activation mechanism of CheY was subsequently explored through structure/function studies of signaling mutants. The structure of an inactivating CheY mutant revealed that the Tyr106 side chain exclusively occupied the external position, so it was hypothesized that the side chain may undergo a conformational change during CheY activation (5). Five additional activating and inactivating CheY mutants were prepared as tests of this hypothesis, and their structural results were consistently in agreement (6–8). The aggregate work suggested a model where the Tyr106 side chain assumed the inside position when CheY was signaling and the outside position when it was not. It was further argued that conformational shifts in the β4-α4 loop assisted in the selection of either of these two rotameric positions for Tyr106 (7, 8).

Other structural facts supporting this model were as follows: 1) the location of the Tyr106 side chain on the CheY molecule correlated with the signaling surface of CheY as defined through allele-specific suppression analysis (10), 2) the CheZ-binding surface of CheY overlapped with the CheA and FliM-binding surfaces in the same region as the signaling surface of CheY (8), and 3) the 1.9-Å resolution structure of a phosphonate analogue of activated CheY showed the Tyr106 side chain exclusively occupying the internal position (11). Furthermore, results from the phosphonate analogue were in agreement with earlier x-ray structures of activation models for two other response regulators, Spo0A-P=O−−Ca2+ (12) and FixJ-P=O−− (13). Most recently, the x-ray structure of a BeF3− mimic of activated CheY·Mg2+ in complex with the cognate peptide of the target protein FliM confirmed the signaling region of CheY as the α4−β2−α5 surface, with the Tyr106 side chain exclusively occupying the internal position (14). This structure was the definitive presentation of the active conformation of CheY, revealing the hydrogen bonding network that stabilized the β4−α4 loop in its new conformation. However, the process of activation still remains obscure. The old model of a phosphorylation-induced transformation of apoCheY into the activated form slowly gave way to the concept of a population shift, where a subpopulation of apoCheY molecules assumes an active conformation and becomes stabilized by the phosphoryl group. The population shift mode was first applied to response regulators to explain the activity of the non-phosphorylatable CheY mutant D13K (15). Compelling support for the population shift model for activation of response regulators came in recent NMR studies on CheY and its homologues Spo0F and NtrC (16–19).

Despite all of the convincing data for the population shift model, there is little evidence regarding the nature of a response regulator being active in its apo-form. Here we present the atomic resolution structure of wild-type apoCheY refined to an R-factor of 11.4% at 1.08-Å resolution. The details of this structure reveal that the signaling region of unphosphorylated CheY exists in two conformational states, one of which we term the meta-active conformation.

Experimental Procedures

Crystallization, Data Collection, Structure Determination, and Refinement—Wild-type E. coli CheY was isolated, purified, and crystallized as described previously (4), with the crystallization being at room temperature in 2.2 M (NH4)2SO4, buffered in 50 mM Tris-HCl, pH 8.3. Crystals were flash-cooled in liquid nitrogen after brief soaks in the original mother liquor plus up to 20% v/v glycerol. Diffraction data were deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).†

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The atomic coordinates and structure factors (code 1JBE) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).‡

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**Table I**

Data and refinement summary for wild-type apoCheY at 1.1-Å resolution

| Parameter                  | Value       |
|----------------------------|-------------|
| Data range (Å)             | 100–1.08    |
| Unique reflections         | 42,147      |
|Completeness (%)            | 86.9 (70.2) |
| $R_{	ext{free}}$ (%)       | 4.8 (2.1)   |
| $R_{	ext{cryst}}$ (%)      | 23.2 (3.9)  |
| Redundancy                 | 3.30 (2.08) |
| Reflections ($F_o > 2\sigma$) | 33,841   |
| Reflections in $R_{	ext{free}}$ set ($F_o > 2\sigma$) | 3,757 |
| Number of protein atoms    | 1,052       |
| Number of solvent atoms    | 197         |
| r.m.s. deviations          | 0.007       |
| Bond lengths (Å)           | 0.022       |
| $R_{	ext{cryst}}$ (%)     | 11.4        |
| $R_{	ext{free}}$ (%)      | 14.4        |

*a* The statistics for the highest resolution shell (1.12 – 1.08 Å) are shown in parentheses.

*b* $R_{	ext{cryst}} = \sum|F_o| - |F_c|/\sum|F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively, and the summations are over all unique reflections.

*c* $R_{	ext{free}}$ is calculated as for $R_{	ext{cryst}}$ except the summation is over a test set of 10% of unique reflections omitted from the refinement.

measured at the BioCARS facility at the Advanced Photon Source, Argonne National Laboratory. A single crystal of dimensions 0.1 × 0.15 × 0.4 mm was used for collection of the entire data set. Two scans were collected, the first was a 180° scan at $\lambda_1$, and the second was a 90° scan at a $\chi$ offset of 60°. A total of 327,373 observations were made of 42,174 unique reflections to a nominal resolution of 1.08 Å. Initial phases were obtained using the room temperature, 1.7-Å wild-type structure as a model (PDB accession code 3CHY). Initial refinement including data to 1.7 Å resolution was done using the program PROFFIT (20, 21). Final refinement using all 42,174 data to 1.08 Å was done with the program SHELXL (22), interspersed with ten cycles of manual rebuilding on a Silicon Graphics Indigo using the program QUANTA. Statistics for the data and refinement are shown in Table I.

**RESULTS AND DISCUSSION**

**Overall Model and Solvent Structure**—The final model of CheY refined at 1.08 Å contains 1,052 protein atoms, three SO$_4^{2–}$ ions, two glycerol molecules, and 197 spherical solvent molecules. The electron density is clear for all modeled atoms. The final model of completely activated CheY and of course their energetic differences are small. We will refer to the conformation of the loop away from the Tyr$^{106}$ residue as up and the other as down. For the Tyr$^{106}$ side chain, its two widely separated rotamer positions (g$^+$, inside; and g$^–$, outside) have excellent agreement with their equivalents in the original 1.7-Å structure (4). The occupancies for each of the two positions also refined to ~50%. We will refer to the internal position of the Tyr$^{106}$ side chain as in and the external position as out.

**Identifying the Functionally Important Conformers**—As shown schematically in Fig. 2, the conformational heterogeneities of the β$_4$–α$_4$ loop (up or down) and the Tyr$^{106}$ side chain (in or out) give four possible combinations. Are all combinations possible, or do their positions correlate? The first and simplest case to consider is the down position of the β$_4$–α$_4$ loop combined with the in position of the Tyr$^{106}$ side chain (Fig. 2A). The electron density clearly shows that these atoms do indeed occupy these positions (Fig. 1), but it is sterically impossible for them to do so simultaneously; the distance from the Tyr$^{106}$ hydroxyl oxygen to the Ala$^{90}$ C$\alpha$ carbon would be 2.15 Å. Thus, this combination does not occur. For the next two cases, it readily follows that when the β$_4$–α$_4$ loop is down, the Tyr$^{106}$ side chain must be out (Fig. 2B), and when Tyr$^{106}$ is in, the β$_4$–α$_4$ loop must be up (Fig. 2C). Hence these second and third combinations can and do exist, exclusive of each other. The only problematic interpretation is with the fourth and last combination, where the β$_4$–α$_4$ loop is up while the Tyr$^{106}$ side chain is out (Fig. 2D). This combination could possibly occur, but there is no structural evidence for or against it. We propose that in the absence of the steric barrier of the β$_4$–α$_4$ loop, the Tyr$^{106}$ side chain would adopt the internal position, as in Fig. 2C, and the fourth combination would not be functionally relevant. The end result for the four possible combinations is two meaningful apoCheY conformations, inactive (β$_4$–α$_4$ loop down and Tyr$^{106}$ out) and a state we will call meta-active (β$_4$–α$_4$ loop up and Tyr$^{106}$ side chain in). The two states are conformationally distinct.

**Meta-active Versus Active Conformations of CheY**—How does the meta-active conformation of apoCheY compare with the phosphorylated, completely active molecule? Two models of the active form of CheY exist in the literature, a phosphonate modification of CheY at Asp$^{57}$ (see Ref. 11; PDB accession code 1C4W) and a BeF$_3$–adduct of CheY-Mg$^{2+}$ complexed with the N-terminal peptide of its target protein, FliM (see Ref. 14; PDB accession code 1F4V). These two models exhibited similar coordinate changes, which presumably are the changes that occur during true activation. The phosphate-CheY structure lacked a divalent cation, so it is possible that its transformation to the active form was not complete. Still, its conformation more closely resembles that of the meta-active apoCheY presented here; its β$_4$–α$_4$ loop is in the up position, and Tyr$^{106}$ is exclusively internal. The other activation model, the CheY-Mg$^{2+}$–BeF$_3$–peptide complex, exhibited larger conformational changes in the β$_4$–α$_4$ loop than the first and had a more stable hydrogen bonding pattern, especially including the important bonds from the invariant Thr$^{87}$ O$\gamma$ and the amide nitrogen of Ala$^{88}$ to the fluorine atoms of the BeF$_3$ moiety (14). These bonds caused a backbone rotation of the β$_4$-strand, which resulted in a large displacement of the β$_4$–α$_4$ loop. The displacement, combined with an ~90° rotation of the peptide unit between Ala$^{88}$ and Glu$^{99}$ enabled formation of a new hydrogen bond between the hydroxyl of Tyr$^{106}$ and the carbonyl oxygen of Glu$^{99}$ (14). These hydrogen bonds are the significant stabilizing forces in the model of completely activated CheY and of course are not present in our unphosphorylated, meta-active conformation of apoCheY.

**Implications for the Two-state Switch Mechanism for CheY**
Activation—Results from NMR experiments on the response regulators Spo0F (16) and NtrC (19) convincingly showed that the apo-forms of the molecules can be divided into subpopulations having different conformations. Borrowing from the general two-state model for allostery (25), the NMR results were interpreted as indicating two conformational states of apo-CheY, inactive and active, in dynamic equilibrium. It was proposed that activation is achieved by stabilizing the active conformation through phosphorylation, resulting in a population shift to an increased size of the active population. However, the NMR results were unable to provide details of the conformation of the unphosphorylated active population and only suggested that the conformation was the same as the phosphorylated, fully active form. Our present results provide an important extension of this model, where we propose that the conformation of apo-CheY in the second population of the two-state mechanism is not the active state but the meta-active state determined here.

It is tempting to speculate that in the activation pathway this meta-active conformation is the immediate antecedent to the fully active, phosphorylated CheY, but details of the CheA-mediated phosphorylation reaction must be taken into account. Importantly, what is the conformation of CheY when bound to CheA? Three structural reports of CheY in complex with the CheY-binding domain of CheA show the Tyr\textsuperscript{106} side chain out in the external, inactive conformation (26–28), indicating that this is the preferred conformation of CheY for CheA, for recognition or mechanistic purposes or both. It makes sense that the binding domain would have preferential affinity for its substrate over its product. This strongly suggests that the meta-active state is not an intermediate on the activation pathway but a more isolated population in equilibrium only with the unactive form. These observations have direct implications for the population shift model. As applied to response regulators, the population shift model invokes a phosphorylation-based stabilization of a pre-existing active conformation. In contrast, interpretation of these results calls for retention of some aspects of the old model of a phosphorylation-induced transformation of the inactive CheY conformation.

The meta-active conformation of CheY should show activity. This would be difficult to test, but the fact is that the signaling surface of the meta-active molecule is more similar to the active models than the inactive form. We propose that the meta-active conformation of CheY, and not an unphosphorylated, active conformation, is responsible for the residual activity seen for apo-CheY (9). The meta-active conformation would be active, but it would be unstable.

If a protein exists in multiple conformations in solution,
could the conformations be found in the crystalline state? These results say yes, at least for these two conformations. It is important to note that the NMR results on NtrC also showed that the response regulator interconverted between just two conformations and not among a large number of random substates (19). More often, crystallographic results for a protein suspected to have multiple states reveal only one conformation, and the results are usually presented with the attending explanation that the crystallization process selects just one population. Such an explanation is difficult to disprove, but the results here clearly show that determining multiple conformations in the crystalline state is possible. In the unsuccessful cases the structural differences may have been so subtle that they were not detectable at the resolution limits employed. The results presented here encourage investigation of other signaling proteins at very high resolution to search for conformational substates related to their activation pathways.

The Activation Mechanism of CheY—In this model of CheY activation, flexibility of the $\beta_{4-6}$ loop and rotation of the Tyr$^{106}$ side chain play key roles. In the inactive state, the backbone of the $\beta_{4-6}$ loop interconverts between two distinct conformations, whereas the Tyr$^{106}$ side chain rotates accordingly. The $\beta_{4-6}$ loop and Tyr$^{106}$ side chain alternately exchange occupation of the internal position. The absence of the phosphoryl group means a lack of stabilizing hydrogen bonds for the $\beta_{4-6}$ loop. This is the condition of apoCheY, flipping back and forth between the two populations of the completely inactive state and the meta-active state. The meta-active state is responsible for the active characteristics of apoCheY. In phosphorylation-dependent activation, the phosphoryl group provides the stabilizing bonds to Thr$^{87}$ O$^\delta$ and the Ala$^{88}$ amide nitrogen, which moves the $\beta_{4-6}$ loop up and in, which then gives the Tyr$^{106}$ side chain clear access to the interior, which finally results in the stabilizing bond between the Tyr$^{106}$ hydroxyl and the Ghu$^{89}$ carbonyl oxygen.

In conclusion, these structural results provide a useful addition to the general model of two-state activation as applied to response regulators, introducing the meta-active conformation as the subpopulation of molecules that exhibit properties of the active molecule.

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