The CheA histidine kinase initiates the signal transduction pathway of bacterial chemotaxis by auto-phosphorylating a conserved histidine on its phospho-transferase domain (P1). Site-directed mutations of neighboring conserved P1 residues (Glu-67, Lys-48, and His-64) show that a hydrogen-bonding network controls the reactivity of the phospho-accepting His (His-45) in *Thermotoga maritima* CheA. In particular, the conservative mutation E67Q dramatically reduces phospho-transfer to P1 without significantly affecting the affinity of P1 for the CheA ATP-binding domain. High resolution crystallographic studies revealed that although all mutants disrupt the hydrogen-bonding network to varying degrees, none affect the conformation of His-45. *^{15}N*-NMR chemical shift studies instead showed that Glu-67 functions to stabilize the unfavored N^2H tautomer of His-45, thereby rendering the N^2 imidazole unprotonated and well positioned for accepting the ATP phosphoryl group.

Histidine phosphorylation is a key feature of bacterial signaling. Histidine kinases transduce information from the extracellular environment to regulate many cellular processes including gene expression, membrane permeability, energy production, and motility (1–3). Mammals do not contain histidine kinases homologous to those found in prokaryotes. Thus, these enzymes are attractive targets for the development of new antibiotics, and thus, their catalytic mechanisms hold great interest.

The dimeric histidine kinase CheA initiates the phosphorylation cascade that regulates the directed movement of bacteria toward and away from chemical stimulants. All necessary elements for histidine phosphorylation are found in the phospho-transfer (P1) and kinase (P4) domains of the five-domain CheA protein (4). Conserved residues in the P4 domain bind ATP in a pocket that optimally positions the γ-phosphoryl for transfer to a specific histidine on the N-terminal P1 domain (4, 5). Although P1 residues other than the active histidine have been shown to impact phospho-transferase activity, their chemical functions have not been assigned (6).

The structure of CheA domain P4 revealed no similarity to eukaryotic Ser/Thr or Tyr kinases but significant resemblance to ATPases from the functionally divergent GHL family (for GyrB, Hsp90, MutL) (4, 7). Instead of using ATP as a phospho-donor to histidine, the GHL ATPases effectively transfer the ATP γ-phosphoryl group to a water molecule. CheA and the GHL family of ATPases share similar topologies, a deep cavity for ATP binding, and four regions of sequence conservation surrounding the ATP-binding site: the N, G1, F, and G2 boxes (4, 7).

Despite the similarities, there are also interesting differences in residue composition between histidine kinases and ATPases that likely delineate their divergent functions. CheA does not contain an analog of an essential glutamate in the GHL ATPases (7), presumed to be the general base involved in water activation for ATP hydrolysis (8). However, the CheA phosphotransfer domain (P1) contains a conserved glutamate adjacent to the phospho-accepting histidine (Glu-67 in *T. maritima* and Glu-70 in *Escherichia coli* and *Salmonella typhimurium*) (9). In *S. typhimurium*, mutation of Glu-70 to Ala abolishes CheA activity (6). Thus, this glutamate may tune the reactivity of the phospho-accepting histidine in a manner similar to the water-activating glutamate of the GHL-ATPases (7).

The properties of the CheA phospho-accepting histidine (residue 48 in *E. coli* or 45 in *T. maritima*) deviate from those of typical solvent-exposed histidines. NMR spectroscopy showed His-48 to have a higher than normal pK_a of 7.8 (9). Furthermore, a hydrogen bond to His-48 N^2H locks the imidazole ring in the N^2H unfavored tautomeric form, even at pH values above its pK_a (9). Crystal structures of the phosphotransfer domain, P1, implicated a glutamate residue (67 and 70 in *T. maritima* and *S. typhimurium*, respectively) as a hydrogen bond acceptor for the phospho-accepting histidine (6, 10). A group of conserved residues (Glu-67, Lys-48, and His-64 in *T. maritima*) also forms a hydrogen bond network with His-45 that could modulate its reactivity (10). Herein, we present biochemical studies of mutants of the *T. maritima* CheA P1 domain that disrupt hydrogen-bonding networks surrounding the phospho-accepting histidine. Biochemistry, crystallography, and NMR spectroscopy were combined to define how local hydrogen bonds activate the CheA histidine for phosphotransfer.

**EXPERIMENTAL PROCEDURES**

Protein Cloning, Expression, and Purification—*T. maritima* full-length CheA, Δ289 (domains P3, P4, and P5, residues 290–671), do-

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2 The abbreviations used are: GHL, GyrB, Hsp90, MutL; PEG, polyethylene glycol; AmAc, ammonium acetate; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser effect spectroscopy.
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main P1 and its mutants (residues 4–133), and the truncated P1 variants P1E67Qshort, P1K48Ashort, and P1H64Ashort (residues 4–104) were subcloned in the vector pET28a (Novagen), transformed, and expressed in E. coli strain BL21(DE3) (Novagen). Full-length P1 domain constructs were used in all biochemical assays. P1 short constructs were utilized for crystallization trials. Crystals were obtained only by removing the region corresponding to the fifth helix (residues 105–133) in S. typhimurium P1. Protein purification was achieved by affinity chromatography on nickel-nitrilotriacetic acid beads (Qiagen) and gel filtration as described previously (7). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene).

**Phosphorylation Assays**—Initial velocities of full-length CheA and its mutants CheA E67Q, CheA K48A, CheA H64A (2–10 μM), or P1 (30 μM) incubated with CheA Δ289 (2 μM) were measured in 50 mM Tris, pH 8.5, 50 mM KCl, and 2 mM dithiothreitol at 50 °C. The initial velocities of full-length CheA and CheA H64A were also compared with one another at room temperature in the same buffer over a pH range of 5.5–7.5. The same protocol was used when P1 (30 μM) was incubated with Δ289 (2 μM) in the presence of a 10-fold excess of P1 mutant (300 μM) or the control bovine serum albumin (300 μM).

**Crystallization, Data Collection, Structure Determination, and Refinement**—Orthorhombic crystals belonging to the P2221 space group of full-length CheA and its mutants CheA E67Q, CheA K48A, CheA H64A (2–10 μM), or P1 (30 μM) incubated with CheA Δ289 (2 μM) were measured in 50 mM Tris, pH 8.5, 50 mM KCl, and 2 mM dithiothreitol at 50 °C. The initial velocities of full-length CheA and CheA H64A were also compared with one another at room temperature in the same buffer over a pH range of 5.5–7.5. The same protocol was used when P1 (30 μM) was incubated with Δ289 (2 μM) in the presence of a 10-fold excess of P1 mutant (300 μM) or the control bovine serum albumin (300 μM).

**RESULTS AND DISCUSSION**

The Hydrogen-bonding Network Surrounding His-45 Optimizes Phosphotransfer—Mutations of Glu-67 to Gln, Asp, and Ala were used to probe whether the hydrogen-bonding ability or negative charge of Glu were critical for phosphorylation of P1 by the kinase domain. The phosphorylation level of wild-type protein was compared with that of mutant protein following a 30-min incubation with [γ-32P]ATP in vitro. Mutations were made both in full-length CheA and in the isolated phosphotransfer domain. P1. CheA containing the mutations E67Q, E67D, and E67A showed dramatically reduced autophosphorylation activity (5.7, 8.4, and 2.4%, respectively). The same mutants in separate P1 domains produced even larger reductions in phosphotransfer activity (~1% of wild type) when assayed with a protein containing CheA domains P3, P4, and P5 (CheAΔ289).

Although the E67A mutation in S. typhimurium has been shown to reduce the ATP phosphotransfer rate (6), these experiments did not distinguish whether the decrease in activity was due to the disruption of secondary structure, the loss of binding between the P1 and the kinase domain, or a catalytic defect. In 10-fold excess, P1E67Q reduces phosphate transfer from wild-type Δ289 to wild-type P1 by more than 70%. The addition of control proteins, such as bovine serum albumin, reduced activity by less than 10%. Assuming a competitive mechanism and taking a measured $K_{m}$ value for wild-type P1 of 100–250 μM (10), this level of inhibition under presaturation conditions indicates that E67Q interacts with a $K_{m}$ value within a factor of 2 when compared with wild type. This minimal defect in $K_{m}$ value cannot account for the low phosphotransfer activities of the conservative mutations E67Q and E67D. Thus, these results suggest that there are stringent requirements on the length, charge, and hydrogen-bond accepting capability of residue 67.

The phosphorylation of P1 by Δ289 has a pH dependence that suggested the phospho-accepting histidine has an elevated $pK_{a}$ when compared with that of an average solvent-exposed histidine (data not shown). Indeed, the pH dependence of P1 phosphorylation at 50 °C correlates with the fraction of deprotonated histidine present only if the histidine $pK_{a}$ is increased to 6.9 from 5.9, the estimated $pK_{a}$ value of a solvent-exposed histidine at 50 °C (9). At pH 6.5, wild-type P1 became phosphorylated only ~20% of the maximum level observed at pH 8.5. In contrast, the phosphotransfer reactions of the three Glu-67 mutants reflected a more typical $pK_{a}$ for His-45 in that they are pH-independent above pH 6.0 (data not shown).

The conserved hydrogen-bonding network involving His-45 was disrupted by the mutations K48A and H64A. The activity of CheA H64A was ~15-fold greater than K48A and roughly 270-fold greater than E67Q at 50 °C, pH 8.5. The activity of wild-type CheA was too rapid to be measured under these conditions. At pH 7.5 and room temperature, the initial velocity of wild-type CheA was ~60-fold greater than CheA H64A. A 10-fold excess of K48A reduced the phosphotransfer to wild-type P1 by 44%, indicating that this mutant still competes for the CheA kinase domain but with weaker affinity when compared with wild type or E67Q.

Diminished phosphorylated P1 (P1-P) in our assays could reflect a reduction in phosphorylation by the kinase or a decreased input.
crease in P1-P stability. However, mutations at Lys-48, His-64, or Glu-67 do not affect phosphoramidate bond stability (data not shown), and neither P1K48A nor P1E67Q affects the phosphotransfer activity to the response regulator CheY (6). Thus, there appeared to be no direct effect of the Lys-His-Glu triad on phospho-histidine stability.

**High Resolution Structures of P1 Mutants Reveal a Disrupted Hydrogen-bonding Network**—To structurally characterize the mutants P1E67Q, P1K48A, and P1H64A, we have determined the crystal structures of their truncated forms, termed short (residues 4–104) to high resolution (for details, see Table I and "Experimental Procedures"). All mutants retain the overall four-helix bundle fold of wild-type P1 (6, 10, 17) with structural changes limited to side-chain conformations near the active histidine.

The crystal structure of P1E67Q\text{short} (helices A–D) was determined to atomic (1.1 Å) resolution (Table I). The Gln-67 side-chain amide rotates around $\phi = -30^\circ$ from the position held by the Glu-67 carboxylate in wild-type P1. This movement disrupts the conserved hydrogen-bonding network involving His-45, Glu-67, Lys-48, and His-64 (Fig. 1). The N\text{1} atom of the phospho-accepting His-45 still hydrogen-bonds to the Gln-67 carbonyl oxygen (2.82 Å), but the His imidazole ring is not co-planar with the glutamine amide, unlike its alignment with the Glu-67 carboxylate in wild-type P1 (Fig. 1B). The altered position of the Gln-67 amide allowed the amide nitrogen to hydrogen-bond to two water molecules (2.83 and 3.15 Å). Note that both His-45 imidazole nitrogens should be protonated at the conditions of crystallization (pH 4.5). Although Gln-67 is within hydrogen-bonding distance to N\text{1} of the phospho-accepting histidine under these conditions, the distorted geometry and longer hydrogen bond of this interaction indicated that it has weakened relative to the wild type.

Discontinuous density surrounding the phosphorylation site of the K48A mutant indicated that Lys removal destabilizes the positions of Glu-67 and His-64. In particular, His-64 has two alternate conformations, neither of which hydrogen-bond to Glu-67 (Fig. 1, D and E). When compared with the native configuration, the two His-64 conformers swiveled about angle...
χ₁ by -42 and 121° degrees, one conformer hydrogen bonding to a water molecule (2.4 Å) and the Ala-61 residue (3.0 Å), and the other hydrogen bonding to the neighboring residue Asn-68 (3.3 Å) and another water molecule (3.4 Å). In response, the Glu-67 carboxylate rotated but still hydrogen-bonded to the N₁ atom of His-45 (2.9 Å). Although removal of Lys-48 destabilized the conformations of Glu-67 and His-64, the position of His-45 remained largely unaffected.

The structural changes incurred by the H64A mutation are not as disruptive as those observed for K48A (Fig. 1C). Glu-67 remained hydrogen-bonded to the His-45 N₁ (2.5 Å) but had a slightly altered position Lys-48 is also displaced and hydrogen-bonds to a water molecule (2.5 Å) instead of forming a salt bridge with Glu-67. However, the orientation of His-45 remained largely unchanged. Because none of these mutations perturbed the conformation of His-45 nor drastically affected binding of Δ289, reduced phosphorylation activity does not likely derive from structural effects alone.

Glu-67 Stabilizes the Reactive Protonation Tautomer of His-45—To evaluate how mutations of the residues involved in the hydrogen-bonding network affect the chemical properties of the phospho-accepting histidine, we expressed 15N-labeled P1E67Q, P1K48A, and P1H64A for NMR spectroscopy. In solution, histidine imidazole groups have three protonation states: a charged state and two neutral tautomers N₁H and N₂H, which undergo fast exchange in solution (18, 19). Chemical shift values for histidine nitrogens report on hydrogen-bonding interactions, protonation, and tautomeric states. The P1 mutants contained two or three histidines: 1) His-45, the site of phosphorylation; 2) His-64, a participant in the hydrogen bond network; and 3) a residual N-terminal histidine introduced by the expression vector. Histidine deprotonation at each site was monitored as a function of pH using two-dimensional ¹H-¹⁵N HSQC experiments (Fig. 2).

The E67Q mutation of T. maritima P1 altered the tautomeric state of the deprotonated His-45 from N₁H to N₂H. pKₐ values measured for E. coli P1 at 30 °C (9).

| P1   | His-45 | pKₐ (50 °C/30 °C) | Predominant tautomeric state | N₁H:N₂H |
|------|--------|------------------|----------------------------|---------|
| P1K48A | His-45  | 6.8/ND ⁹       | N₁H ¹   | 2.6:1    |
| P1H64A | His-45  | 6.9/ND ³       | N₁H ¹   | 2.7:1    |
| P1E67Q | His-45  | 5.8/ND ³       | N₂H ²   | 1.3:3    |
| ArcB  | His-717 | NA/6.5 ⁹, ²     | N₂H ²   | 1:4      |

⁹ Histidines have a temperature dependent heat of ionization. pKₐ values of free His are 6.5 at 30 °C degrees, but 5.9, at 50 °C (9).
³ ND, not determined.
² NA, not available.

Fig. 2. The pH titration of the His-45 imidazole ring of the CheA histidine phosphotransfer domain as determined by twodimensional NMR. Histidine imidazole groups equilibrate between the completely protonated state and the N₁H and N₂H tautomers. The chemical shifts of the His-45 N₁H (●) and N₂H (■) atoms of P1E67Q (a), P1H64A (b), and P1K48A (c) as a function of pH show that the E67Q mutation switches the dominant tautomeric state of deprotonated His-45 from N₁H to N₂H. pKₐ values were determined by curve fits to a standard expression for proton ionization, as described previously (9).
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Fig. 3. Activation of His-45 for phosphoryl transfer. The hydrogen bond formed between Glu-67 and the N$_{\text{H}}^1$ of His-45 stabilizes this otherwise unfavorable histidine tautomer. Glu-67 acts as a general base that activates the active site histidine, making its N$_2^2$ atom more nucleophilic. The P1 domain, therefore, provides a catalytic dyad: an activating glutamate and a phospho-accepting histidine that complete the CheA active site. His-64 and Lys-48 stabilize the position of the catalytic dyad. NMR studies show that His-64 deprotonates at N$_2^2$ as pH is raised; thus, to maintain hydrogen bonding with Glu-67, the His-64 imidazole ring would have to flip over relative to the conformation observed in the low pH structures.

(25). Furthermore, both ArcB and the unprotonated N$_1^1$ appropriately for nucleophilic attack onto the ATP phosphor grouping bound in the P4 kinase domain.

Chemical and Structural Determinants of Histidine Phosphorylation—CheA phosphorylation requires His-45 to have the N$_2^2$ tautomer, and Glu-67 stabilizes this otherwise unfavored protonation state. Although the three mutations studied all affect side-chain positions in the region of the active histidine, these steric changes had minor effects on reactivity when compared with switching the tautomeric state of His-45. For example, K48A is much more active than E67Q, despite this mutation disrupting the conserved hydrogen-bonding network and weakening the $K_p$ for $\Delta$289 to a greater extent. E67Q and H64A both disrupt a hydrogen bond from Lys-48 to either Glu-67 or Glu-67, respectively, but H64A is 270-fold more active. Within the catalytic triad of serine proteases, a carboxylate also stabilizes the N$_{\text{H}}^1$ tautomer of His (20).

Comparison with GHL-ATPases—In addition to sharing a common fold and ATP-binding site, CheA and GyrB also share common mechanistic features. However, CheA and the GHL family of ATPases differ in the moiety activated by a conserved glutamate residue. In GHL ATPases, a conserved glutamate likely acts as a general base to activate a water molecule for $\gamma$-phosphoryl group attack (8). Alternatively, in CheA, a conserved glutamate residue perturbs the phospho-accepting histidine $p_K_a$ and stabilizes the normally unfavored N$_{\text{H}}^1$ tautomeric state. These effects combine to make His-45 a better nucleophile for phosphate transfer (Fig. 3). The P1 domain provided a catalytic dyad consisting of the nucleophile for phosphate transfer (His-45) and the activating glutamate (Glu-67). Thus, P1 and P4 produces a catalytic center analogous to that of GyrB. Histidine-dependent peptide catalysts have been developed for the specific phosphorylation of small molecules (21, 22). Strategies employed by bacterial histidine kinases to optimize phosphotransfer may be applicable to tuning similar reactivities in such non-biological systems.

Phosphotransfer Mechanisms in Bacterial Signaling—All known histidine phosphotransferase domains display the reactive His on a four-helix bundle scaffold (6, 17, 23, 24). Within these common structural motifs, differences in amino acid composition, electrostatics, hydrogen-bonding networks, and surface properties tunes the reactivity of histidine phosphorylation sites to optimize reactions with phosphotransferase partners. For example, the chemical determinants for histidine phosphorylation differs among monomeric HPt domains from CheA, ArcB (ArcB$_1$), and Ypd1. In contrast to CheA, Ypd1 and ArcB$_1$ accepted a phosphoryl group from the aspartyl phosphate of response regulators instead of from ATP. In these proteins, a Glu hydrogen-bonds to the N$_{\text{H}}^1$ atom of the phospho-accepting histidine, which in ArcB$_1$ has the N$_{\text{H}}^1$ tautomeric state and an unaltered $p_K_a$ value (25) (Table II). An equal population of each His protonation tautomer allows for phosphorylation at N$_2^2$ (25). Furthermore, both ArcB$_1$ and Ypd1 mutations of the hydrogen-bonding Glu do not affect histidine phosphorylation (26, 27). Thus, the requirement of a hydrogen-bonded carboxylate for histidine phosphorylation depends on context. In CheA, a carboxylate at position 67 is necessary to stabilize the His-45 N$_2^2$ tautomer in the chemical environment provided to the reactive His by the entire kinase.

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