

FOR THE RECORD

Tautomeric state and pK_a of the phosphorylated active site histidine in the N-terminal domain of enzyme I of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system

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Abstract: The phosphorylated form of the N-terminal domain of enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system of *Escherichia coli* has been investigated by one-bond and long-range ¹H-¹⁵N correlation spectroscopy. The active site His 189 is phosphorylated at the Nε2 position and has a pK_a of 7.3, which is one pH unit higher than that of unphosphorylated His 189. Because the neutral form of unphosphorylated His 189 is in the Nδ1-H tautomer, and its Nε2 atom is solvent inaccessible and accepts a hydrogen bond from the hydroxyl group of Thr 168, both protonation and phosphorylation of His 189 must be accompanied by a change in the side-chain conformation of His 189, specifically from a χ_2 angle in the g^+ conformer in the unphosphorylated state to the g^- conformer in the phosphorylated state.

Keywords: histidine phosphorylation; N-terminal domain of enzyme I; pK_a ; tautomeric state

Transport of some hexose sugars across the cytoplasmic membrane of bacterial cells is coupled to a phosphorylation cascade involving several protein intermediates (see Herzberg & Klevit, 1994 and Postma et al., 1996 for reviews). *Escherichia coli*

Enzyme I, the first protein in the phosphoryltransfer pathway, is autophosphorylated by phosphoenolpyruvate at His 189. Phosphorylated EI acts as the phosphoryl donor to His 15 of the histidine-containing phosphocarrier protein, HPr. Phosphorylated HPr in turn donates the phosphoryl group to sugar transporters, collectively known as enzymes II. EI is a 64-kDa protein consisting of N- and C-terminal domains (LiCalsi et al., 1991; Lee et al., 1994). The N-terminal domain of EI terminates in the linker region from Glu 252 to Leu 264 (Lee et al., 1994) and can be phosphorylated on His 189 in a fully reversible manner by phosphorylated HPr, although it has lost its ability to become phosphorylated by PEP (Chauvin et al., 1996; Seok et al., 1996). In addition, EIN is capable of functioning in phosphotransfer reactions with a variety of acceptor proteins that cannot be phosphorylated by intact EI (Seok et al., 1996).

Recently, crystal (Liao et al., 1996) and solution NMR (Garrett et al., 1997a) structures of EIN (1–258 + Arg) have been determined and the interaction surface between EIN and HPr has been mapped (van Nuland et al., 1995; Garrett et al., 1997b). EIN consists of an α domain (residues 33–143) and an α/β domain (residues 1–20 and 148–230) connected by linkers (residues 21–32 and 144–147), and a C-terminal helix (residues 233–250) (Fig. 1). The active site His 189 is located at the interface of the α and α/β domains. In the unphosphorylated state, His 189 has a pK_a of 6.3 (Garrett et al., 1997a). At neutral pH, His 189 exists as the Nδ1-H tautomer (Garrett et al., 1997a) and its Nε2 atom accepts a hydrogen bond from the hydroxyl of Thr 168 (Liao et al., 1996; Garrett et al., 1997a). The pK_a and tautomeric state of His 189 remain unchanged upon complex formation with HPr (in the absence of phosphorylation) (Garrett et al., 1997b).

Figures 2 and 3 show the one-bond and long-range ¹H-¹⁵N correlation spectra, respectively, of ¹⁵N-labeled EIN at pH 6.9 in an approximately 1:1 mixture of phosphorylated and unphosphor-

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Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system of *E. coli*; EI, enzyme I of the PTS; EIN, N-terminal domain (residues 1–258 + Arg) of EI; EII, enzymes II of the PTS; HPr, histidine-containing phosphocarrier protein of the PTS; PEP, phosphoenolpyruvate; HSQC, heteronuclear single quantum coherence.

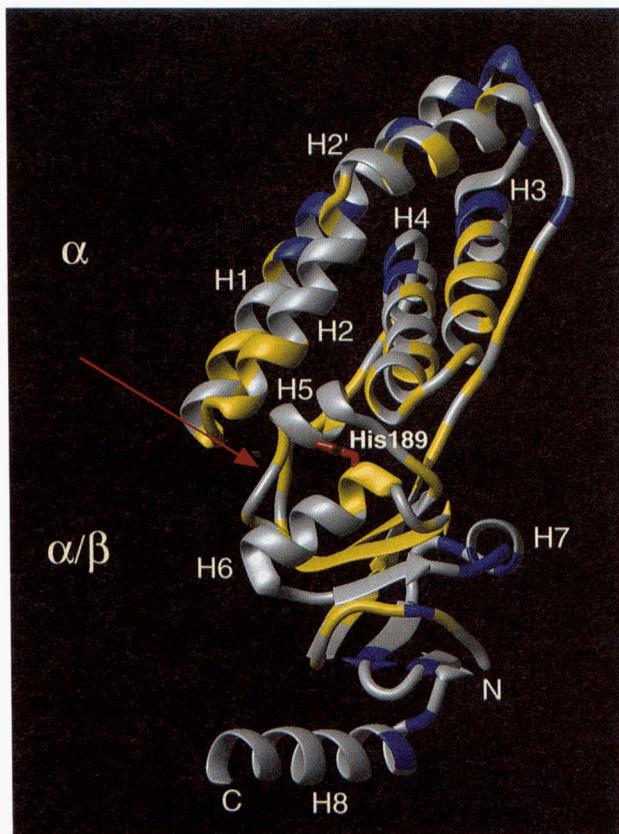


Fig. 1. Schematic ribbon drawing of EIN showing the location of the active site His 189 at the interface of the α and α/β domains. The location of the shallow depression at the interface of the two domains that provides access to His 189 is indicated by the red arrow. The ribbon is color coded, based on the results of the one-bond ^1H - ^{15}N HSQC spectrum of a 1:1 mixture of phosphorylated and unphosphorylated EIN (Fig. 2), as follows: yellow, residues whose ^1H - ^{15}N cross-peaks are clearly split into two corresponding to phosphorylated and unphosphorylated protein; blue, residues whose ^1H - ^{15}N cross-peaks are unaffected by phosphorylation; grey, residues for which we cannot ascertain with certainty whether they are affected or unaffected by phosphorylation owing to spectral overlap. The coordinates are taken from Garrett et al. (1997a) and the figure was generated with the program MOLMOL (Koradi et al., 1996).

ylated forms. The phosphorylated and unphosphorylated forms of EIN are in slow exchange on the chemical shift time scale. Two major classes of cross-peaks are observed in the one-bond ^1H - ^{15}N HSQC spectrum (Fig. 2): those that are split into two corresponding to the phosphorylated and unphosphorylated protein, and whose intensities are consequently reduced by a factor of two relative to a sample of unphosphorylated EIN; and those that are unaffected by phosphorylation. The majority of residues whose ^1H - ^{15}N cross-peaks are perturbed by phosphorylation are located at the interface between the α and α/β domains and within the α/β domain. This primarily reflects the location of the phosphorylated His 189 at the interface of the two domains, protruding upward from the α/β domain toward the α domain (Fig. 1). Interestingly, whereas the backbone NH and ^{15}N chemical shifts of a large number of residues in the α/β domain are affected by phosphorylation of His 189, HPr binding results in backbone chemical shift perturbations of only a small number of residues in the α/β domain but a large number in the α domain (Garrett et al., 1997b).

The long-range ^1H - ^{15}N HSQC spectrum (Fig. 3) correlates the imidazole nitrogens with the carbon-attached ring protons of histidine. The $\text{N}\epsilon 2$ and $\text{N}\delta 1$ resonances are readily distinguished because cross-peaks of approximately equal intensity are observed for the $\text{N}\epsilon 2$ - $\text{H}\epsilon 1$, $\text{N}\epsilon 2$ - $\text{H}\delta 2$, and $\text{N}\delta 1$ - $\text{H}\epsilon 1$ two-bond correlations ($^2J_{\text{NH}} \sim 6$ – 10 Hz; Blomberg et al., 1977), whereas either a very weak or absent cross peak is observed for the $\text{N}\delta 1$ - $\text{H}\delta 2$ three-bond correlation ($^3J_{\text{NH}} \sim 2$ – 3 Hz; Blomberg et al., 1977). For a neutral histidine, the protonated ring nitrogen resonates at ~ 168 ppm, whereas the unprotonated nitrogen resonates at ~ 250 ppm (referenced relative to the ^{15}N chemical shift of liquid ammonia; Pelton et al., 1993). For a positively charged fully protonated histidine, the two nitrogens resonate around 175 ppm, with the $\text{N}\delta 1$ atom generally resonating about 1 ppm to lower field than the $\text{N}\epsilon 2$ atom (Pelton et al., 1993). The pattern and positions of the cross-peaks of His 76, His 97, and His 105 are either unaffected (His 97 and His 105) or minimally perturbed (His 76) upon phosphorylation, indicating that their $\text{p}K_a$ values (< 6 , ~ 7.3 , and ~ 6.4 , respectively) and tautomeric states for the neutral species ($\text{N}\epsilon 2$ -H tautomer) remain unchanged (Garrett et al., 1997a). In contrast, two distinct sets of cross-peak patterns are observed for His 189. One set, corresponding to the unphosphorylated form, displays a pattern of $\text{N}\delta 1$ - $\text{H}\epsilon 1$, $\text{N}\epsilon 2$ - $\text{H}\epsilon 1$, and $\text{N}\epsilon 2$ - $\text{H}\delta 2$ cross-peaks, with $\text{N}\delta 1$ and $\text{N}\epsilon 2$ chemical shifts of 191 and 220 ppm, respectively, characteristic of the neutral $\text{N}\delta 1$ -H tautomeric state (Garrett et al., 1997a). The second set, corresponding to the phosphorylated form, is characterized by a pattern of $\text{N}\delta 1$ - $\text{H}\epsilon 1$, $\text{N}\epsilon 2$ - $\text{H}\epsilon 1$, and $\text{N}\epsilon 2$ - $\text{H}\delta 2$ cross-peaks, with $\text{N}\delta 1$ and $\text{N}\epsilon 2$ chemical shifts of 203 and 207 ppm, respectively.

The expected ^{15}N chemical shifts for a phosphorylated nitrogen, derived from NMR studies using model compounds such as *N*-phosphoimidazole and *N*-methyl-*N*-phosphoimidazole, are ~ 209 ppm for a neutral phosphoimidazole nitrogen and ~ 202 ppm for the cationic phosphoimidazolium moiety (Pelton et al., 1993). The titration curves for the $\text{N}\epsilon 2$ and $\text{N}\delta 1$ resonances of phosphorylated His 189 are shown in Figure 3B. Both resonances exhibit Henderson–Hasselbach behavior, with a $\text{p}K_a$ of 7.33 ± 0.05 . The ^{15}N chemical shift of the $\text{N}\epsilon 2$ atom varies only minimally with pH, with limiting values of 209.4 ppm at low pH and 200.5 ppm at high pH, typical of a phosphorylated nitrogen. The ^{15}N chemical shift of the $\text{N}\delta 1$ atom, on the other hand, ranges from 188 ppm at pH 6.1 to 241.8 ppm at pH 8.3, close to those expected for the protonated and unprotonated states, respectively, of a nonphosphorylated nitrogen in a phosphoimidazole (Pelton et al., 1993). Thus, we conclude that His 189 is phosphorylated at the $\text{N}\epsilon 2$ position, in agreement with previous biochemical results (Weigel et al., 1982).

The $\text{p}K_a$ of phosphorylated His 189 (~ 7.3) is one unit higher than that of the unphosphorylated form (~ 6.3 ; Garrett et al., 1997a). Moreover, although the neutral species of unphosphorylated His 189 is in the $\text{N}\delta 1$ -H tautomer such that the $\text{N}\epsilon 2$ nitrogen becomes protonated at low pH, in phosphorylated His 189, the $\text{N}\epsilon 2$ atom of His 189 is bonded to the phosphate group and protonation occurs at the $\text{N}\delta 1$ atom. Because the $\text{N}\epsilon 2$ atom is solvent inaccessible and accepts a hydrogen bond from the hydroxyl of Thr 168 in unphosphorylated EIN (Garrett et al., 1997a), these results imply that both protonation of unphosphorylated His 189 and phosphorylation of His 189 must be accompanied by a conformational change of the imidazole ring. In unphosphorylated EIN, the χ_1 and χ_2 angles of His 189 are in the *trans* and g^+ conformations, respectively (Liao et al., 1996; Garrett et al., 1997a). Because the

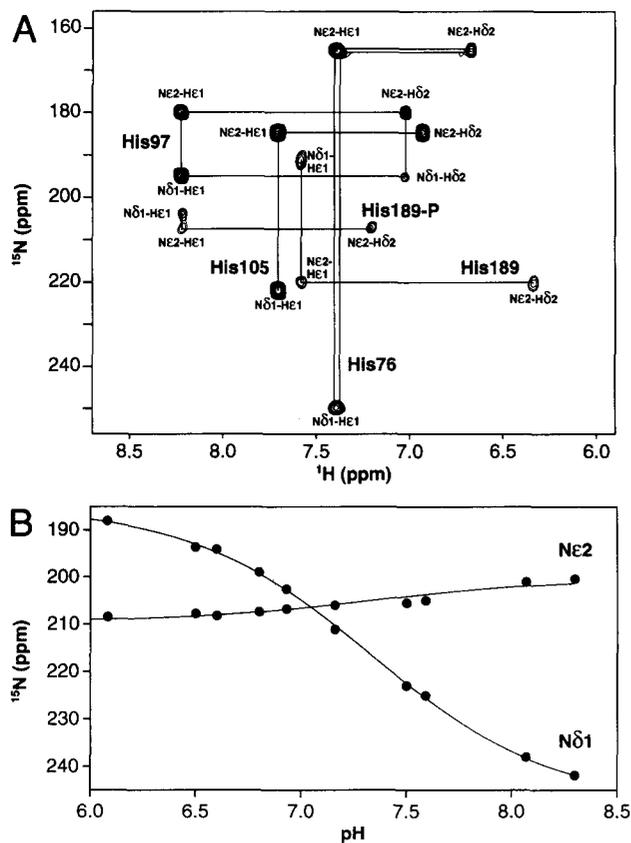


Fig. 3. **A:** Long-range ^1H - ^{15}N HSQC spectrum of an approximately 1:1 mixture of phosphorylated and unphosphorylated ^{15}N -labeled E1N at 40°C and pH 6.9. Two distinct sets of cross-peaks are observed for His 189, corresponding to the phosphorylated and unphosphorylated states of His 189; the resonances of His 76 are minimally perturbed upon phosphorylation with the $\text{Ne}2$ resonance slightly downfield, the $\text{He}1$ resonance slightly upfield, and the $\text{H}\delta 2$ resonance slightly downfield, relative to the unphosphorylated E1N; the resonances of His 97 and His 105 are unperturbed by phosphorylation. **B:** Titration of the $\text{Ne}2$ and $\text{N}\delta 1$ resonances of phosphorylated His 189 obtained by recording a series of long-range ^1H - ^{15}N correlation spectra at 30°C on a sample containing $\geq 90\%$ phosphorylated E1N. The continuous lines represent the best-fit curves for a single $\text{p}K_a$, with a $\text{p}K_a$ value of 7.33 ± 0.05 , and $\text{N}\delta 1$ and $\text{Ne}2$ ^{15}N chemical shifts of 184.9 and 209.4 ppm, respectively, for the fully protonated state, and 247.8 and 200.5 ppm, respectively, for the fully unprotonated neutral state. ^{15}N chemical shifts are referenced relative to liquid ammonia (Pelton et al., 1993).

creasing the concentration of EI to 23 nM resulted in $>90\%$ phosphorylation of E1N.

NMR spectroscopy: NMR experiments were performed at 30°C or 40°C on a Bruker AMX600 spectrometer equipped with a z-shielded gradient triple resonance probe. Two-dimensional ^1H - ^{15}N HSQC spectra were recorded using a water flip-back as described by Grzesiek and Bax (1993). Two-dimensional long-range ^1H - ^{15}N HSQC spectra to correlate the $\text{N}\delta 1$ and $\text{Ne}2$ ring nitrogens with the $\text{C}\epsilon 1\text{H}$ and $\text{C}\delta 2\text{H}$ ring protons of histidine were recorded with a 22-ms dephasing delay, during which the ^1H and ^{15}N signals become antiphase (Pelton et al., 1993). Spectra were processed with the NmrPipe package (Delaglio et al., 1995) and analyzed using the programs PIPP, CAPP, and STAPP (Garrett et al., 1991).

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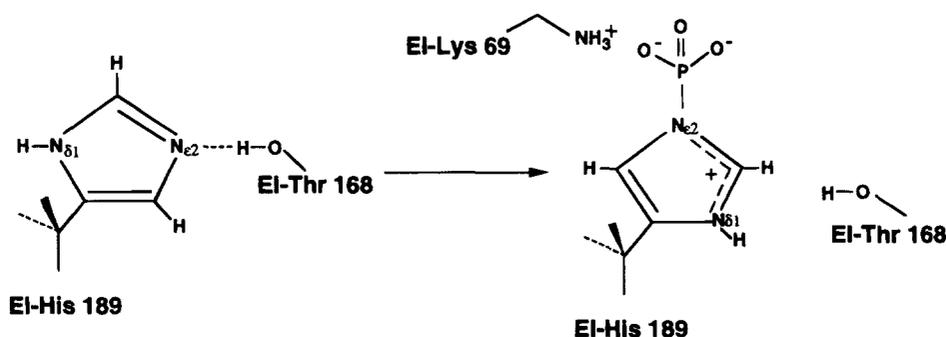


Fig. 4. Schematic of the phosphorylation reaction of E1N. In the unphosphorylated state, the χ_2 side-chain torsion angle of His 189 is in the g^+ conformation, and the $\text{Ne}2$ atom of His 189 is solvent inaccessible and accepts a hydrogen bond from the hydroxyl of Thr 168. In the phosphorylated state, the χ_2 angle of His 189 is in the g^- conformation, the $\text{Ne}2$ atom is directly bonded to the phosphate, and the negative charge on the phosphate is stabilized by an ion-pair interaction with the side chain of Lys 69. The state of His 189 depicted in the figure represents the predominant form at pH 7.

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