Structures of Active Site Histidine Mutants of III\text{Glc}, a Major Signal-transducing Protein in \textit{Escherichia coli}

EFFECTS ON THE MECHANISMS OF REGULATION AND PHOSPHORYL TRANSFER*  

(Received for publication, July 30, 1996, and in revised form, October 8, 1996)

Jeffrey G. Pelton**, Dennis A. Torchia**, S. James Remington*, Kenneth P. Murphy***, Norman D. Meadow****, and Saul Rosenman*****

From the *Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892, the **Institute of Molecular Biology and Department of Physics, University of Oregon, Eugene, Oregon 97403, the ***Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242, and the *****Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

III\text{Glc} (also called II\text{A\text{Glc}}), a major signal-transducing protein in \textit{Escherichia coli}, is also a phosphorylcarrier in glucose uptake. The crystal and NMR structures of III\text{Glc} show that His\text{75}, the phosphoryl acceptor, adjoins His\text{75} in the active site. Glutamine was substituted for His\text{75}, giving H75Q\text{IIIGlc} and H90Q\text{IIIGlc}, respectively (Presper, K. A., Wong, C.-Y., Liu, L., Meadow, N. D., and Roseman, S. (1989) \textit{Proc. Natl. Acad. Sci. U. S. A.} 86, 4052–4055), but the mutants showed unexpected properties. H90Q\text{IIIGlc} loses regulatory functions of III\text{Glc}, and the phosphoryltransfer rates between HPr/H75Q\text{IIIGlc} are 200-fold less than HPr/III\text{Glc} (Meadow, N. D., and Roseman, S. (1996) \textit{J. Biol. Chem.} 271, 33440–33445). X-ray crystallography, differential scanning calorimetry, and NMR have now been used to determine the structures of the mutants (phospho-H75Q\text{IIIGlc} was studied by NMR). The three methods gave completely consistent results. Except for the His to Gln substitutions, the only significant structural changes were in a few hydrogen bonds. H90Q\text{IIIGlc} contains two structured water molecules (to Gln\text{90}), which could explain its inability to regulate glyceral kinase.

Phospho-III\text{Glc} contains a chymotrypsin-like, hydrogen bond network (Thr\text{19}-His\text{75}-O\text{-phosphoryl}), whereas phospho-H75Q\text{IIIGlc} contains only one bond (Gln\text{75}-O\text{-phosphoryl}). Hydrogen bonds play an essential role in a proposed mechanism for the phosphoryltransfer reaction.

The protein III\text{Glc} (or II\text{A\text{Glc}}) plays a key role in many bacterial cell functions (see Refs. 1–6 for reviews), and these are effected by its interactions with other proteins, both covalent (the transfer of the phosphoryl group) and non-covalent (6). Two of many unresolved questions concerning this important signal-transducing protein are: (a) the mechanism of phosphoryl transfer to other proteins of the phosphoenolpyruvate-glucose phosphotransferase system (PTS), such as HPr; and (b) how III\text{Glc} "recognizes" or binds to its target proteins, despite the fact that there is no apparent common sequence in these targets.

III\text{Glc} from \textit{Escherichia coli} is 18.1 kDa, and contains two His residues, His\text{75} and His\text{90}, but not Trp, Cys, or Tyr. The structure of III\text{Glc} has been extensively studied and has been established by NMR and x-ray crystallography (7–11), as has the structure of the cytoplasmic (or IIA) domain of the \textit{Bacillus subtilis} II\text{Glc} membrane protein (12–18). \textit{E. coli} III\text{Glc} and the \textit{B. subtilis} IIA domain exhibit many similarities.

In \textit{E. coli} III\text{Glc}, the N-terminal domain (residues 1–18) is unstructured both in solution and in the crystal. The remainder of the molecule, residues 19–168, is a compact structure, shaped approximately like a barrel, about 30 Å on a side, and much thinner between the two faces. The major structural unit in each face of the protein is a β-sheet with six antiparallel strands.

Both His\text{90} and His\text{75} lie in a depression located off center of one of the faces, and His\text{90} is the residue that accepts the phosphoryl group from phospho-HPr. A space-filling model of the protein is shown in Fig. 1. The depression is surrounded by a very hydrophobic group of amino acids, and it is these residues that comprise the principal binding sites of III\text{Glc} to one of its target proteins, glyceral kinase (19–21). It also has been
suggested that the hydrophobic patch, which is about 18 Å in diameter, is intimately involved in the binding of *B. subtilis* phospho-HPr to the IIA\(^{Glc}\) domain (22). Within the active site depression of *E. coli* III\(^{Glc}\), the N-3 (or N\(^{3}\)) atoms of each imidazole ring in His\(^{75}\) and His\(^{90}\) lie so close that they are cross-linked by chloroplatinate (7).

In an attempt to understand the pleiotropic effects of III\(^{Glc}\), two mutant proteins were constructed by substituting glutamine either for His\(^{38}\) or for His\(^{95}\), yielding proteins designated HIIQ(38)III\(^{Glc}\) and HIIQ(95)III\(^{Glc}\), respectively (23). The HIIQ(38)III\(^{Glc}\) mutant could not be phosphorylated, as expected, but showed only some regulatory properties of the wild type protein, which we did not expect. Thus, His\(^{38}\) is critical in both glucose translocation and regulation.

HIIQ(95)III\(^{Glc}\) also behaved unpredictably. While it accepts the phosphoryl group from phospho-HPr, phospho-HIIQ(95)III\(^{Glc}\) is inactive in sugar phosphorylation (23), presumably because it does not transfer the phosphoryl group to the membrane protein II\(^{Glc}\). We suggested (23) that His\(^{75}\) may be directly involved in the phosphoryl transfer sequence: PEP \(\rightarrow\) Enzyme I \(\rightarrow\) HPr \(\rightarrow\) II\(^{Glc}\)(His\(^{90}\)) \(\rightarrow\) II\(^{Glc}\)(His\(^{75}\)) \(\rightarrow\) II\(^{Glc}\)(Cys\(^{421}\)) \(\rightarrow\) glucose.

Alternatively, the rate of phosphoryl transfer to the membrane protein II\(^{Glc}\) (and then to sugar) is too slow to be detected. That is, phosphoryl transfer proceeds from III\(^{Glc}\)(His\(^{90}\)) directly to II\(^{Glc}\)(Cys\(^{421}\)), and His\(^{75}\) plays an essential role in determining the rate of the reaction. In the accompanying paper (24), we report that replacing His\(^{75}\) with Gln has a 200-fold effect on the rate of phosphoryl transfer between HPr and III\(^{Glc}\).

The results obtained with the mutants are not easily explained by the structural information on the wild type protein. We have therefore conducted detailed analyses of the consequences of the two mutations in the active site of III\(^{Glc}\), and these provide information on the mechanism of action of this important signal-transducing protein.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mutant Proteins—**The III\(^{Glc}\) mutants HIIQ(38)III\(^{Glc}\) and HIIQ(95)III\(^{Glc}\) were overexpressed as described in the accompanying report (24). For NMR studies the cells were grown as described (9, 25) in the minimal medium of Neidhardt *et al.* (26) using 15NH4Cl as the source of nitrogen. In addition, the medium was supplemented with 0.2% glucose, 2 mg/ml thiamine, and 50 mg/ml ampicillin. Approximately 50 mg of HIIQ(38)III\(^{Glc}\) and 20 mg of HIIQ(95)III\(^{Glc}\) were obtained from 1.5 liters of medium. The purity of each sample was found to be greater than 97%, based on SDS-PAGE followed by quantitative densitometric scanning of the Coomassie-stained gel. Enzyme I and HPr were obtained as described (27, 28).

**Crystallization of Proteins and X-ray Diffraction Analysis—**Crystals of HIIQ(38)III\(^{Glc}\) and HIIQ(95)III\(^{Glc}\) were prepared as reported previously for wild type III\(^{Glc}\) (7). Diffraction data were collected using a Xeung-Hamlin multwire detector and reduced using the supplied software (29). Electron density maps were inspected, and model building was performed using the FRODO program (30). In some instances, electron density was not apparent for some or all side chain atoms, so these residues were truncated. The initial electron density maps were calculated using the refined model of wild type III\(^{Glc}\) for phase calculation, after removal of the mutated side chain. Crystallographic refinement was performed using the TNT program suite with the conjugate direction option for function minimization (31, 32).

**Calorimetry—**Differential scanning calorimetry experiments were performed in a DSC92 which is a DASM1 calorimeter modified at the Biocalorimetry Center (The Johns Hopkins University, Baltimore, MD). The calorimeter is interfaced to a PC computer via a Data Translation DT-2801 A to D board. The cell volume was 1.10 ml. Samples were scanned from 25 °C to 100 °C at a scan rate of 1 °C/min. Recovery of structure upon cooling was checked by rescanning the samples.

**Fig. 1.** CPK representations of III\(^{Glc}\) and phospho-III\(^{Glc}\). One face of the protein is shown with emphasis on the hydrophobic patch and the active site. The orientation is similar to those shown in Fig. 9. A, a view of III\(^{Glc}\) derived from the x-ray coordinates of the crystal structure. The hydrophobic patch is black, the imidazole ring of His\(^{75}\) lies above that of His\(^{90}\) (carbon atoms red, N atoms green), the hydrogen bonding atoms are *white:* Thr\(^{73}\) O, top, next to N\(^{3}\) of His\(^{75}\); Gly\(^{92}\) carbonyl O, next to N\(^{5}\) of His\(^{90}\); Asp\(^{94}\) N is to the right of Gly\(^{92}\); B, simulation of phospho-III\(^{Glc}\). The coordinates of the phosphoryl group (P atom, yellow; O atoms, blue) were used to construct the model, where it is linked to N\(^{2}\) of His\(^{90}\). The simulation is only an approximation (a crystal structure of phospho-III\(^{Glc}\) is not available), and does not permit, for example, a hydrogen bond to form between Asp\(^{94}\) and a phosphoryl O atom, as indicated by the NMR spectrum (see Fig. 9). The NMR data also suggest that Val\(^{96}\) may hydrogen-bond to an O atom of the phosphoryl group.
Table I

| Mutant       | H75QIIIGlc | H90QIIIGlc |
|--------------|------------|------------|
| **Data collection** |            |            |
| No. of crystals | 1          | 1          |
| Cell a (Å)     | 79.8       | 79.8       |
| Cell c (Å)     | 87.3       | 86.8       |
| Resolution (Å) | 2.2        | 2.1        |
| Number of observations | 16522      | 21831      |
| Unique reflections | 7950       | 10462      |
| Completeness (%) | 73         | 81         |
| \( R_{merge} \) a | 0.047      | 0.030      |

**Atomic model**

- R-factor b: 0.181, 0.182
- Protein atoms: 1104, 1114
- Solvent atoms: 19

**Deviations from ideality**

- r.m.s. bond lengths (Å): 0.013, 0.014
- r.m.s. bond angles (°): 2.2, 2.3
- Restained B factors (Å²): 5.8, 5.6

---

Data were analyzed using the CPPLUS6 program for the Macintosh developed at the Biocalorimetry Center. After normalizing for concentration, the melting temperature, \( T_m \), the enthalpy change, \( \Delta H \), and the heat capacity change, \( \Delta C_p \), for unfolding were determined by fitting the endotherms to a two-state transition.

**NMR Spectroscopy**—The preparation of protein samples for NMR experiments has been reported (8, 9). Briefly, for experiments conducted in H2O, approximately 16 mg of H75QIIIGlc or 5 mg of H90QIIIGlc were dissolved in 0.5 ml of 90% H2O/10% D2O solution containing 0.15 M KCl (pH 6.4). For experiments conducted in D2O, the amide protons were exchanged for deuterons by heating for 6 h at 60 °C, followed by repeated lyophilization from D2O, and finally by dissolution in 99.99% D2O (Cambridge Isotope Laboratories, Woburn, MA). For experiments on phosphorylated H75QIIIGlc, a regeneration system was used to keep the protein in the phosphorylated state (10, 33). The regeneration system included the addition of phosphoenolpyruvate (50 mM, pH 6.4), MgCl2 (1 mM), potassium phosphate (10 mM, pH 6.4), KCl (0.15 M), and catalytic quantities of both HPr (1.6 M) and Enzyme I (5 units). To study the pH dependence of the NMR signals, the pH of the sample was adjusted by adding small aliquots of aqueous potassium hydroxide or HCl. Meter readings were not corrected for deuterium isotope effects.

1H-15N HMQC NMR experiments (34) were used to identify the imidazole \(^1J_{NH}\) couplings of the single histidine residue of each mutant protein (11). The spectra were acquired on a Bruker AMX-500 spectrometer with the sample dissolved in D2O at 36.5 °C and at pH values ranging from 6.12 to 8.85 (H75QIIIGlc), 6.12 to 8.70 (H90QIIIGlc), and 6.1 to 9.1 (H90QIIIGlc). The delay during which \(^1H\) and \(^15N\) signals become observed was set to 22 ms to refocus magnetization arising from the \(^1H\) couplings. For each spectrum, 32 scans (512 complex points) were signal-averaged for each of 64 complex \( t_1 \) points using the States-TPPI method (35) to achieve quadrature detection. The spectra were processed as described in detail (11) using commercial (NMR, Syracuse, NY) and in-house software (36). Chemical shifts are referenced to H2O (\(^1H\), 4.67 ppm from TSP at 36.5 °C) and external liquid ammonia (\(^15N\)) (37). Uncertainties in chemical shifts are ± 0.02 ppm and ± 0.2 ppm for \(^1H\) and \(^15N\) signals, respectively.

**RESULTS**

**Crystal Structures of Mutant Proteins**—H75QIIIGlc and H90QIIIGlc crystallized essentially isomorphously to wild type in space group R3. Diffraction data were collected from a single crystal of each. Data merging and atomic model statistics are summarized in Table I. The H75QIIIGlc crystal diffracted to slightly better than 2.2 Å resolution, and 73% of the theoretically possible reflections were observed. The H90QIIIGlc crystal diffracted to somewhat higher resolution, and the data set is 81% complete to 2.1 Å resolution. The initial electron density maps clearly revealed the position and orientation of the mutated side chains, so model building and refinement were straightforward. The final atomic model statistics are excellent, with good stereochemistry and crystallographic R-factors of about 0.18 in each case. Portions of the final \((2F_o - F_c)\) electron density maps in the vicinity of the mutated side chain are presented in Fig. 2.

The models in the vicinity of the mutation are shown in more detail in Fig. 3, revealing proposed hydrogen bond interactions with neighboring groups. The electron density maps do not reveal an unambiguous orientation for the terminal amidocarboxy group of glutamine. However, for the mutant H90QIIIGlc this could be deduced from the hydrogen bonding pattern. The Ne2 donates a hydrogen bond to the carbonyl of Gly32, much as His36 Nδ1 does in wild type (7, 11). The Oε1 may accept a hydrogen bond from His75 Nε2, but the distance between these atoms is 4.4 Å, which is generally considered to be too long for a hydrogen bond (38). Therefore the orientation is very likely as shown in the figures and is consistent with the chemical shift observations. This side chain makes two hydrogen bonds with well ordered solvent molecules so that its hydrogen bonding potential is nearly saturated. On the other hand, glutamine 75 does not appear to make good hydrogen bonds to neighboring groups. The Cambridge Isotope Laboratories, Woburn, MA).

**X-ray data collection and atomic model statistics, space group R3**

**RESULTS**

**Crystal Structures of Mutant Proteins**—H75QIIIGlc and H90QIIIGlc crystallized essentially isomorphously to wild type in space group R3. Diffraction data were collected from a single crystal of each. Data merging and atomic model statistics are summarized in Table I. The H75QIIIGlc crystal diffracted to slightly better than 2.2 Å resolution, and 73% of the theoretically possible reflections were observed. The H90QIIIGlc crystal diffracted to somewhat higher resolution, and the data set is 81% complete to 2.1 Å resolution. The initial electron density maps clearly revealed the position and orientation of the mutated side chains, so model building and refinement were straightforward. The final atomic model statistics are excellent, with good stereochemistry and crystallographic R-factors of about 0.18 in each case. Portions of the final \((2F_o - F_c)\) electron density maps in the vicinity of the mutated side chain are presented in Fig. 2.

The models in the vicinity of the mutation are shown in more detail in Fig. 3, revealing proposed hydrogen bond interactions with neighboring groups. The electron density maps do not reveal an unambiguous orientation for the terminal amidocarboxy group of glutamine. However, for the mutant H90QIIIGlc this could be deduced from the hydrogen bonding pattern. The Ne2 donates a hydrogen bond to the carbonyl of Gly32, much as His36 Nδ1 does in wild type (7, 11). The Oε1 may accept a hydrogen bond from His75 Nε2, but the distance between these atoms is 4.4 Å, which is generally considered to be too long for a hydrogen bond (38). Therefore the orientation is very likely as shown in the figures and is consistent with the chemical shift observations. This side chain makes two hydrogen bonds with well ordered solvent molecules so that its hydrogen bonding potential is nearly saturated. On the other hand, glutamine 75 does not appear to make good hydrogen bonds to neighboring groups. The Cambridge Isotope Laboratories, Woburn, MA).

**Calorimetry**—DSC melting curves for the wild type and mu-
tant proteins are shown in Fig. 5. The fitted melting parameters are listed in Table II. The fitted values of $T_m$, $\Delta H^\circ$, and $\Delta C_p$ can be used to calculate the values of $\Delta H^\circ$, $\Delta S^\circ$, and $\Delta G^\circ$ at any temperature using the following equations.

\[
\Delta H^\circ (T) = \Delta H^\circ (T_m) + \Delta C_p (T - T_m)
\]  
\text{(Eq. 1)}

\[
\Delta S^\circ (T) = \Delta S^\circ (T_m) T_m + \Delta C_p \ln (T / T_m)
\]  
\text{(Eq. 2)}

\[
\Delta G^\circ (T) = \Delta H^\circ (T_m) - T \cdot \Delta S^\circ (T_m) T_m + \Delta C_p [ (T - T_m) - T \ln (T / T_m) ]
\]  
\text{(Eq. 3)}

$T$ is the temperature of interest, and $T_m$ is the melting temperature, both in units of Kelvin. The thermodynamics of unfolding at 69.1 °C (298 K), the $T_m$ of wild type III$^{Glc}$, are given in Table III.

The energetic changes summarized in Table III can be rationalized in view of the structures of the wild type and mutant proteins. In the $H90Q$III$^{Glc}$ mutation, the glutamine side chain appears to make good hydrogen bonds to neighboring groups and is not fully desolvated. Hence, this mutation is enthalpically stabilizing ($\approx 6 \text{kcal mol}^{-1}$). However, the configurational entropy lost upon fixing the glutamine side chain is greater than that for histidine (39) so that the mutation is entropically destabilizing. The enthalpy-entropy compensation results in a marginal decrease in the stability of the protein, even though the hydrogen bonds are enthalpically stabilizing. Similar effects are seen in T4 lysozyme mutants (40), as has recently

---

**Fig. 2.** Electron density maps of mutant proteins. A, stereo view of a portion of the final $(2F_o - F_i)$ electron density map, contoured at 1.5 standard deviations of the map, for the $H75Q$III$^{Glc}$ mutant. Carbon atoms are drawn as open circles, oxygen is filled, and nitrogen is shaded. b, portion of the final $(2F_o - F_i)$ electron density map, contoured at 2.0 standard deviations of the map, for the $H90Q$III$^{Glc}$ mutant as in panel A.
been discussed (41).

In the H75QIIIGlc mutation, the glutamine side chain does not form good hydrogen bonds. This is reflected in the unfolding enthalpy, which is decreased by about 6 kcal mol\(^{-1}\). However, the destabilization in \(\Delta H^\circ\) is offset by a stabilizing change in \(\Delta S^\circ\). The stabilizing entropy probably reflects the mobility of the glutamine side chain, which is not restricted by hydrogen bonds.

The atomic models of the two mutant III\(^{Glc}\) proteins provide support for the conclusions of the thermodynamic analyses. Inspection of the vibrational parameters, or “B factors” of the two mutant side chains reveals very high values (in the range of 80–100 Å\(^2\), corresponding to an r.m.s. vibrational amplitude of approximately 1 Å and consequent poor localization) for the terminal amidocarboxyl group of Gln\(^{75}\), while the terminal group of Gln\(^{90}\) has values of 20–30 Å\(^2\), due to its rigid fixation in the active site by the hydrogen bond to the carbonyl oxygen of Gly\(^{92}\).

**NMR Spectroscopy: Tautomeric States of the Active Site Histidines**—\(^{15}\)N nuclear magnetic resonance spectroscopy can provide detailed information about the protonation states of histidine rings in proteins (42–49). The great utility of the method is based on the sensitivity of the \(^{15}\)N shift to the protonation state of the nitrogen (43–45, 48, 50). To simplify the discussion, protonated nitrogens that are incorporated into neutral and positively charged rings have been denoted type-\(\alpha\) and type-\(\alpha^+\), respectively, and unprotonated nitrogens have been denoted type-\(\beta\) (47, 50). More recently, van Dijk et al. (51) have extended the nitrogen chemical shift measurements to include phosphorylated imidazole rings. The nomenclature and characteristic \(^{15}\)N chemical shifts for the various nitrogen species are summarized in Table IV. These results have been used to characterize the tautomeric states of the histidines in \(\alpha\)-lytic protease (46), triosephosphate isomerase (52), HPr (51), and \(E.\ coli\) \(\beta\)-hydroxydecanoyl thiolester dehydrase (53). In each of these studies, however, specific incorporation of \(^{15}\)N\(^{15}\) or \(^{15}\)N\(^{15}\).

---

**Fig. 3. Stereo views of active sites of mutant proteins.** A, stereo view of details of the active site region of the H75QIIIGlc mutant. Carbon atoms are drawn as open circles, oxygen as filled, and nitrogen as shaded. The thin lines represent putative hydrogen bonds with the indicated length in A. B, stereo view of details of the active site region of the mutant H90QIIIGlc as in panel A.
labeled histidine was required to assign the nitrogen signals.

Recently van Dijk et al. (54) and we (11) have utilized relatively strong $^{2}J_{\text{NH}}$ couplings (43) to assign the histidine nitrogen signals in uniformly $^{15}$N-labeled proteins via two-dimensional $^{1}H$-$^{15}N$ HMQC spectra. In particular, the $^{15}N^{\text{e}}$ nitrogen can be assigned on the basis of strong correlations with both $^{1}H_{\text{d}}^{2}$ and $^{1}H_{\text{e}}^{1}$ via $^{2}J_{\text{N}^{\text{e}}^{2}H_{\text{d}}^{2}}$ and $^{2}J_{\text{N}^{\text{e}}^{2}H_{\text{e}}^{1}}$ couplings, and the $^{15}N^{\text{d}}$ signal can be assigned on the basis of a strong correlation with $^{1}H_{\text{d}}^{1}$, and in some cases a weak correlation with $^{1}H_{\text{d}}^{2}$ via $^{2}J_{\text{N}^{\text{d}}^{1}H_{\text{e}}^{1}}$ and $^{2}J_{\text{N}^{\text{d}}^{1}H_{\text{d}}^{2}}$ couplings, respectively. This experiment, along with the previously determined canonical $^{15}$N chemical shift data (Table IV) were used previously to characterize the active site histidines in III$^{\text{Glc}}$ (54) and both III$^{\text{Glc}}$ and P-III$^{\text{Glc}}$ (11).

Two-dimensional $^{1}H$-$^{15}N$ HMQC spectra for H75QIII$^{\text{Glc}}$ and phospho-H75QIII$^{\text{Glc}}$ are shown in Fig. 6A. For H75QIII$^{\text{Glc}}$ the downfield-shifted nitrogen signal (242.6 ppm) is correlated to two protons (H$^{23}$ and H$^{45}$) and can therefore be assigned to N$^{\text{e}2}$. Conversely, the upfield-shifted signal (168.6 ppm) is correlated to only H$^{41}$ and can therefore be assigned to N$^{\text{d}1}$. Comparison of the nitrogen chemical shifts with the canonical values (Table IV) shows that H90 N$^{\text{d}1}$ and N$^{\text{e}2}$ are type-$\alpha$ and type-$\beta$, respectively, and thus, H90 in H75QIII$^{\text{Glc}}$ exists predominantly as the N$^{\text{d}1}$-H$^{\text{tautomer}}$. These results are shown schematically in Fig. 6 (inset).

For phospho-H75QIII$^{\text{Glc}}$ the nitrogen assignments are complicated by the fact that both N$^{\text{d}1}$ and N$^{\text{d}2}$ correlate strongly with both protons (Fig. 6B). The additional N$^{\text{d}1}$-H$^{42}$ correlation, which results from a $^{3}J_{\text{N}^{\text{d}1}H_{\text{d}}^{2}}$ coupling, was also observed for phospho-H90 in spectra of wild type III$^{\text{Glc}}$ (11) and may be characteristic of phosphohistidine residues. To resolve the ambiguity in nitrogen assignments, the rates of hydrolysis of the

![Fig. 4. Superpositions of mutant and wild type proteins. A, stereo view of an overlay of the active site region of the mutant H75QIII$^{\text{Glc}}$ onto the model of wild type III$^{\text{Glc}}$ after optimally superimposing the backbone $\alpha$-carbons of each model. The wild type model is indicated with filled bonds, while the mutant model is drawn with open bonds. The apparent change in side chain conformational angles of Thr$^{73}$ and Thr$^{95}$ should be taken as tentative, as the electron density map is not well defined for these residues. Carbon atoms are shown as open circles, oxygen filled, and nitrogen shaded. B, stereo view of an overlay of the active site region of the H90QIII$^{\text{Glc}}$ mutant onto the wild type model as in panel A.](image-url)
phosphoproteins were determined as a function of pH. Phospho-histidinyl proteins are sensitive to acid hydrolysis, and phospho-HPr, which is found in phospho-HPr, is much more labile than phospho-N\textsuperscript{3} (55). The rate constants for the hydrolysis of phospho-I\textsuperscript{III}Glc and phospho-H\textsuperscript{75}QIIGlc are shown in Fig. 7. The shapes of the curves obtained with the two phosphoproteins are essentially the same. Furthermore, in the pH range above 5, phospho-H\textsuperscript{75}QIIGlc is hydrolyzed about 4-fold more slowly than phospho-I\textsuperscript{III}Glc, rather than much more rapidly as would be the case were the phosphoryl group linked to the His N\textsuperscript{3} atom. Thus, His\textsuperscript{90} in the H\textsuperscript{75}QIIGlc mutant is phosphorylated at the same position as the wild type protein (N\textsuperscript{2}).

It follows that the signal at 211.0 ppm, which is close to the chemical shift expected for a phosphorylated nitrogen in a charged ring (Table IV), is due to N\textsuperscript{2} and that the signal at 172.0 ppm is due to N\textsuperscript{3}. Moreover, comparison of the chemical shift of N\textsuperscript{3} with the canonical values indicates that this nitrogen is protonated and that the ring is charged at pH 8.4. These data are shown schematically in Fig. 6B (inset).

An 1\textsuperscript{H}-1\textsuperscript{5}N HMQC spectrum of the single histidine (H75) of the H\textsuperscript{75}QIIGlc mutant (pH 7.66) is shown in Fig. 8. In this spectrum it can be seen that N\textsuperscript{2} (245.0 ppm), which is coupled to two protons, is type-\(\alpha\) while N\textsuperscript{3} (164.9 ppm), which is coupled to a single proton, is type-\(\beta\). Hence, H75 exists predominantly in the N\textsuperscript{2}-H state in the H\textsuperscript{75}QIIGlc mutant as was the case in the wild type protein (11).

Effect of pH—As stated above, the protonation state of a nitrogen strongly influences its chemical shift (Table IV), and the imidazole 1\textsuperscript{5}N signals therefore serve as particularly sensitive parameters for monitoring the titration of a histidine residue. To measure the pK\textsubscript{a} values of the histidines in the mutant proteins, imidazole 1\textsuperscript{5}N and C-H chemical shifts were obtained from 1\textsuperscript{H}-1\textsuperscript{5}N HMQC spectra acquired at pH values ranging from 6.10 to 9.0. Data were not collected outside this range due to aggregation of the mutant protein at lower pH values and inefficient regeneration of the phosphorylated protein at higher pH values. The data for H\textsuperscript{75}QIIGlc, P-H\textsuperscript{75}QIIGlc, and H\textsuperscript{90}QIIGlc revealed only minimal changes in both the 1\textsuperscript{H} and 1\textsuperscript{5}N signals over the stated pH range (see Figs. S4 and S5 in supplementary material). The largest change was observed for His\textsuperscript{90} N\textsuperscript{3} in unphosphorylated H\textsuperscript{75}QIIGlc, which shifted upfield 5.0 ppm. By comparison, a type-\(\beta\) nitrogen is expected to shift upfield approximately 72 ppm (to type-\(\alpha\)+) upon protonation. The invariance of 1\textsuperscript{H} and 1\textsuperscript{5}N shifts with pH indicate that the protonation state of the single histidine in each mutant of I\textsuperscript{III}Glc does not change over the pH range 6.1 to 9.0. Furthermore, these data show that the pK\textsubscript{a} values for His\textsuperscript{90} in H\textsuperscript{75}QIIGlc and His\textsuperscript{75} in H\textsuperscript{90}QIIGlc are both less than 5.5 and that the pK\textsubscript{a} for phospho-His\textsuperscript{90} in phospho-I\textsuperscript{III}Glc is greater than 10.

Hydrogen Bonding Pattern Associated with Active Site Histidines—The x-ray data presented above for H\textsuperscript{75}QIIGlc suggested the presence of a hydrogen bond between His\textsuperscript{90} N\textsuperscript{3} and the carboxyl group of Gly\textsuperscript{92}. In addition, the NMR results show that this nitrogen is protonated and can therefore act as a hydrogen bond donor. To further elucidate this issue, attempts were made to identify this signal in 1\textsuperscript{H}-1\textsuperscript{5}N HMQC spectra. In general nitrogen-attached imidazole protons cannot be observed because of rapid exchange with solvent (56). In favorable cases, however, such signals can be observed in spectra that utilize a 1:1 echo H\textsubscript{2}O suppression sequence (34) if the exchange rate is reduced, for example through hydrogen bonding. Analysis of 1\textsuperscript{H}-1\textsuperscript{5}N HMQC spectra revealed peaks at nitrogen chemical shifts of 169.2 ppm (H\textsuperscript{75}QIIGlc) and 173.1 ppm (phospho-H\textsuperscript{75}QIIGlc) (see Fig. S1 in supplementary material). After correction for 2\textsuperscript{H} isotope effects (57), these signals could be assigned to His\textsuperscript{90} N\textsuperscript{3}H in the two forms of the protein. The fact that these signals can be observed provides further evidence for hydrogen bonding between His\textsuperscript{90} and Gly\textsuperscript{92} in both the phosphorylated and unphosphorylated mutant proteins.
The x-ray data for H75QIIIGlc indicate that the side chain of Gln75 points toward the active site of the protein in a similar manner to the side chain of His75 in wild type III\textsuperscript{Glc}. Because of this, it is of interest to assign the amide signals of Gln75 as a probe for interactions of this group with the phosphoryl oxygen atoms. The six pairs of H-15N signals (4 Asn, 2 Gln) for both H75QIIIGlc and phospho-H75QIIIGlc were identified by comparison of heteronuclear single quantum coherence spectroscopy (HSQC) spectra of the mutant and wild type proteins (9, 10). For H75QIIIGlc the NH\textsubscript{2} signals of Asn32, Asn57, Gln111, and Asn142 were identical in spectra of H75QIIIGlc and wild type IIIGlc, allowing for their unambiguous assignment (see Fig. S2 and Tables S2 and S3 in supplementary material). The signals for the neighboring residues Asn74 and Gln75 were also assigned by comparison with wild type \textsuperscript{15}N, 0.04 and 0.05 ppm \textsuperscript{1H} (1) than the pair of signals assigned to Gln75 (difference \textsuperscript{15}N; 0.04 ppm and 0.05 ppm \textsuperscript{1H}). Similarly, for phospho-H75QIIIGlc the pair of signals assigned to Asn74 differed significantly less from those of Asn74 in phospho-III\textsuperscript{Glc} (difference \textsuperscript{15}N; 0.04 ppm and 0.05 ppm \textsuperscript{1H}) than from the pair of signals assigned to Gln75 (difference \textsuperscript{15}N; 0.04 ppm and 0.05 ppm \textsuperscript{1H}) (see Fig. S3 and Tables S2 and S3 in supplementary material).

Strikingly, the amide nitrogen of Gln75 shifts downfield 4.6 ppm and one of the two amide protons shifts downfield 2.08 ppm upon phosphorylation of H75QIIIGlc. By comparison, no significant changes were observed in either the amide nitrogen or proton signals upon phosphorylation of wild type III\textsuperscript{Glc} (10), and the shifts in the amide resonances of the other asparagine and glutamine residues of H75QIIIGlc were much smaller (Tables S2 and S3). These data, along with the close proximity of the side chain of Gln75 to that of His75 in the x-ray structure of H75QIIIGlc suggest that the side chain Gln75 amide group interacts with the phosphoryl oxygens (see below).

**DISCUSSION**

Substituting Gln for His75 or His90 in E. coli III\textsuperscript{Glc} has virtually no effect on the structure of the protein outside of the active site. Within experimental error, the crystal structures show that all of the atoms, except Gln and His, are superposable in the wild type and mutant proteins. The only significant differences in the active sites among the three proteins and the two phosphorylated derivatives are illustrated in Fig. 9. The small changes in the thermodynamics of unfolding of wild type, H90QIII\textsuperscript{Glc}, and H75QIII\textsuperscript{Glc} proteins are explained by the differences in hydrogen bonding and are fully consistent with the crystal and NMR structures.

While H90QIII\textsuperscript{Glc} cannot accept the phosphoryl group from phospho-HPr, we predicted that it would behave similarly to wild type III\textsuperscript{Glc} in regulation. However, in vivo data (23) showed that H90QIII\textsuperscript{Glc} did not significantly inhibit utilization of non-PTS sugars, whereas H90QIII\textsuperscript{Glc} was as effective as wild type III\textsuperscript{Glc}. In vivo data are difficult to interpret, especially when they involve expression of operons, but definitive information comes from in vitro inhibition studies with one of the target proteins, glycerol kinase (GK). III\textsuperscript{Glc} binds to GK primarily via the "hydrophobic patch," and this does not involve the His residues in the absence of Zn\textsuperscript{2+}.

In recent experiments, Dr. Donald W. Pettigrew (Texas A&M University) did not detect inhibition of GK by H90QIII\textsuperscript{Glc}, i.e. it was less than 3% as effective an inhibitor as the wild type protein or H75QIII\textsuperscript{Glc}. From this, it appears that either H90QIII\textsuperscript{Glc} binds poorly to GK or it binds as well as wild type III\textsuperscript{Glc}, but is unable to constrain the GK polypeptide to the manner required for inhibition of catalytic activity. We predict that the binding constant of the mutant to GK will be significantly less than that of wild type III\textsuperscript{Glc}. The crystal structures appear to show that H90QIII\textsuperscript{Glc} contains two bound, structured water molecules (to the Gln amide), whereas neither wild type III\textsuperscript{Glc} nor the III\textsuperscript{Glc}/GK complex contains structured water. Thus, we suggest that the energy required for desolvation of the Gln in H90QIII\textsuperscript{Glc} leads to a decreased binding constant of
tetrahedron in phospho-IIIGlc (Fig. 10). To bring the hydrated a trigonal bipyramid in the transition state, to an inverted the P atom passes from a tetrahedron in phospho-HPr, through the required tautomers of His90 almost perfectly to accommodate a very rapid phosphoryl transfer. In wild type IIIGlc, the rates decrease 200-fold for Reaction II (the mutant). Why?

KHCO₃/K₂CO₃. Triplicate samples were taken from each reaction mixture at time intervals that ranged from 22 min to 6 h. The aliquots were immediately transferred to one third their volumes of the mixture (5 M urea, 3 M KOH) used for the rapid quench experiments described in the accompanying paper (24). Each phosphoryl between IIIGlc and HPr. The rate and equilibrium constants are (24): wild type IIIGlc, 33454 phospho-H75QIIIGlc.

The remainder of this discussion concerns the mechanism of phosphoryl transfer between III³Glc and HPr.

\[
\text{Phospho-HPr + III³Glc} \rightleftharpoons \text{phospho-III³Glc} + \text{HPr}
\]

**REACTION I**

\[
\text{Phospho-HPr + } \text{H75OIIIGlc} \rightleftharpoons \text{phospho-H75OIIIGlc} + \text{HPr}
\]

**REACTION II**

The rate and equilibrium constants are (24): wild type III³Glc, \(k_1 = 6.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\), \(k_{-1} = 4.7 \times 10^7\); H75OIII³Glc mutant, \(k_1 = 2.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\), \(k_{-1} = 2.3 \times 10^5\). The \(K_{eq}\) for both reactions are in the range 1–1.5. Thus, the rate constants for Reaction I (wild type III³Glc) are only 2–10-fold less than the rate for a diffusion limited reaction (58). However, the rates decrease 200-fold for Reaction II (the mutant). Why?

It is beyond the scope of this paper to consider all the factors involved in catalyzing this reaction, but it appears that the active sites of HPr and III³Glc must complement each other almost perfectly to accommodate a very rapid phosphoryl transfer. In wild type III³Glc, the required tautomers of His⁹⁰ and His⁷⁵ are fixed by their respective hydrogen bonds to Gly⁹² and Thr⁹³. Also, the backbone amide of Asp⁹⁴ (and possibly Val⁹⁶) is positioned to form an H bond to the phosphoryl group. Assuming an associative phosphoryl transfer mechanism, the P atom passes from a tetrahedron in phospho-HPr, through a trigonal bipyramid in the transition state, to an inverted tetrahedron in phospho-III³Glc (Fig. 10). To bring the hydrated phosphogroup of phospho-HPr⁵ into the transition state, we assume that hydrogen bonds to water are replaced by H bonds to Nε² of His⁷⁵ and the amide of Asp⁹⁴ (and possibly Val⁹⁶). Since these hydrogen bonds are presumably optimized to stabilize the transition state, they are likely to be stronger than those from water to the phosphoryl group, and this should enhance the electrophilicity of the P atom. In turn, the H bond from the carbonyl of Gly⁹² to His⁹⁰ Nε¹ should make the reactive Nε² more nucleophilic.

In short, it appears that the hydrogen bonds provide many of the factors required for optimum transition state formation (59): charge shielding and stabilization, proximity and orientation, immobilization of the reactive groups, increased nucleophilicity of the reactive Nε², and electrophilicity of the P atom. But why are the rate constants 200-fold lower when His⁷⁵ is replaced by Gln? We surmise that the relative rates are determined by differences in the number and/or strengths of hydrogen bonds in the mutant and wild type proteins. A 200-fold rate difference translates to a difference of about 3 kCal/mol in the free energies of activation \(AG^\ddagger\) for the two reactions, A and B, well within the range (1–5 kCal/mol) for formation of “normal” hydrogen bonds (60).

Hydrogen bonds can influence reaction rates by delocalizing unfavorable charge (61). As the phosphoryl group approaches bipyramidal geometry in the transition state, the total charge on the O atoms changes from −2 to a value between −2 and −3. The hydrogen bond to His⁷⁵ can serve to delocalize this increased charge by resonance stabilization to a greater extent.

Unlike His⁷⁵ and His⁹⁰ in III³Glc, which show abnormally low \(pK_a\) values, the single His residue of HPr, His⁷³, is exposed to the solvent and exhibits a \(pK_a\) of 5.6 (63). In phospho-HPr, the phosphoryl group is linked to Nε³ of the His, and the phosphoryl O atoms are undoubtedly hydrogen-bonded to water.
than is possible with Gln\textsuperscript{75}, which has less side-chain conjugation.

Hydrogen bonds can also show cooperativity (62), and it is important to note that there is a hydrogen bond network in phospho-\textsuperscript{III\textsuperscript{Glc}} (Fig. 9), Thr\textsuperscript{73}-His\textsuperscript{75}-O\textsuperscript{-phosphate}, analogous to the Ser\textsuperscript{195}-His\textsuperscript{75}-Asp\textsuperscript{102} network in chymotrypsin and related serine proteases. Thr\textsuperscript{73} does not hydrogen-bond to Gln\textsuperscript{75} in the mutant protein, which is therefore deficient in one such bond compared to the wild type. Conceivably, this network makes a significant contribution to the phosphotransfer reaction mechanism in the wild type protein.

In sum, the properties of the mutants relative to wild type \textsuperscript{III\textsuperscript{Glc}} are primarily ascribed to differences in the strength and/or number of a few hydrogen bonds within the active site.

**Fig. 9. Structures of active sites of \textsuperscript{III\textsuperscript{Glc}} proteins.** A and B are taken from Pelton et al. (11). A, wild type \textsuperscript{III\textsuperscript{Glc}}. B, phospho-\textsuperscript{III\textsuperscript{Glc}}. C, \textsuperscript{H75QIII\textsuperscript{Glc}}. D, phospho-\textsuperscript{H75QIII\textsuperscript{Glc}}. E, \textsuperscript{H90QIII\textsuperscript{Glc}}. Only the changes introduced by the mutations are shown in C, D, and E.
These theories can be tested. We are currently attempting to replace Thr37 by Ala, Val, and Ser, and His75 and His90 by Leu. A quantitative study of these mutant proteins may provide direct evidence, for or against the hypotheses offered above.

Acknowledgments—Dr. Donald W. Pettigrew kindly provided unpublished data on the inhibition studies of glycerol kinase with H90QIIIGlc. We are especially grateful to Dr. Mark Rosenman for critical comments and valuable suggestions. We also thank Dr. Bertrand Garcia-Moreno for helpful discussion and for preparing Fig. 1, Dr. Ernesto Freire for the phosphorus. In wild type IIIGlc, pyramid in the transition state with the O atoms in the same plane as R9. In H75QIIIGlc, R’ is the same as wild type, but R” is the amide N of Gin172.

Fig. 10. Schematic of phosphotransfer reactions. The tetrahedral P atom in phospho-IIIglc, and phospho-HPr forms a trigonal bi-pyramid in the transition state with the O atom of the same plane as the phosphorus. In wild type IIIglc, R’ is the amide N of Asp94 (see Fig. 1); R” is N2 of His75. In H75QIIIglc, R’ is the same as wild type, but R” is the amide N of Gin172.

REFERENCES

1. Meadow, N. D., Fox, D. K., and Roseman, S. (1990) Annu. Rev. Biochem. 59, 497–542
2. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) Biochemistry 32, 3794–3812
3. Roseman, S. (1994) in Phosphate in Microorganisms: Cellular and Molecular Biology (Torriani-Gorini, A., Yagil, E., and Silver, S., eds) pp. 335–342, American Society for Microbiology, Washington, D.C.
4. Saier, M. H., and Reizer, J. (1992) Trends Microbiol. 1, 303–317, Oxford University Press, Oxford
5. Tronrud, D. E. (1992) Acta Crystalogr. 48, 489–503
6. Witanowski, M., Stefaniak, L., Januszewski, H., Grabowski, Z., and Webb, G. (1992) J. Magn. Reson. 95, 214–220
7. Lee, K. H., Xie, D., Freire, E., and Amzel, L. M. (1994) Biochemistry 33, 8493–8499
8. Pettigrew, D. W., Feese, M., Meadow, N. D., Remington, S. J., and Roseman, S. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7023–7033
9. Fairbrother, W. J., Cavanagh, J., Dyson, H. J., Palmer, A. G., III, Sutrina, S. (1992) J. Am. Chem. Soc. 115, 2616–2623
10. Pelton, J. G., Torchia, D. A., Meadow, N. D., and Roseman, S. (1992) Biochemistry 31, 5215–5224
11. Pelton, J. G., Torchia, D. A., Meadow, N. D., and Roseman, S. (1993) Protein Sci. 2, 543–558
12. Kapadia, G., Chen, C. C. H., Reddy, P., Saier, M. H., Reizer, J., and Herzberg, O. (1991) J. Mol. Biol. 221, 1079–1080
13. Lian, D.-I., Kapadia, G., Reddy, P., Saier, M. H., Reizer, J., and Herzberg, O. (1991) Biochemistry 30, 9583–9594
14. Fairbrother, W. J., Cavanagh, J., Dyson, H. J., Palmer, A. G., III, Sutrina, S. L., Reizer, J., Saier, M. H., and Wright, P. E. (1991) Biochemistry 30, 6896–6907
15. Fairbrother, W. J., Gippert, G. P., Reizer, J., Saier, M. H., and Wright, P. E. (1992) FEBS Lett. 296, 148–152
16. Fairbrother, W. J., Palmer, A. G., III, Rance, M., Reizer, J., Saier, M. H., and Wright, P. E. (1992) Biochemistry 31, 4413–4425
17. Stone, M. J., Fairbrother, W. J., Palmer, A. G., III, Rance, M., Reizer, J., Saier, M. H., and Wright, P. E. (1992) Biochemistry 31, 4394–4406
18. Chen, Y., Reizer, J., Saier, M. H., Fairbrother, W. J., and Wright, P. E. (1993) Biochemistry 32, 32–37
19. Hurley, J. H., Faber, H. R., Worthloke, D., Meadow, N. D., Roseman, S., Pettigrew, D. W., and Remington, S. J. (1993) Science 259, 673–677
20. Feese, M., Pettigrew, D. W., Meadow, N. D., Roseman, S., and Remington, S. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3544–3548
21. Pettigrew, D. W., Feese, M., Meadow, N. D., Remington, S. J., and Roseman, S. (1994) in Phosphate in Microorganisms: Cellular and Molecular Biology (Torriani-Gorini, A., Yagil, E., and Silver, S., eds) pp. 335–342, American Society for Microbiology, Washington, D.C.
22. Herzberg, O. (1992) J. Biol. Chem. 267, 24819–24823