ARGUMENT: The phosphocarrier protein HPr of the phosphoenolpyruvate-dependent sugar transport system of Escherichia coli can exist in phosphorylated and nonphosphorylated forms. During phosphorylation, the phosphoglycerate group exchanged on a histidine residue, His15. The hydrogen-bonding state of this histidine was examined with $^{15}$N NMR. For this purpose we selectively enriched the histidine imidazole nitrogen with $^{15}$N by supplying an E. coli histidine auxotroph with the leucine acid labeled either at the N61 and Ne2 positions or at only the N61 position. $^{15}$N NMR spectra of two synthesized model compounds, phosphoimidazole and phosphomethylimidazole, were also recorded. We show that, prior to phosphorylation, the protonated His15 Ne2 is strongly hydrogen bonded, most probably to a carboxylate moiety. The H-bond structure for the mechanism of phosphorylation of HPr is discussed. The implications of the H-bond structure for the mechanism of phosphorylation of HPr are discussed.

HPr is a general component of almost all bacterial phosphoenolpyruvate-dependent (PEP)-dependent sugar transport systems (PTS). It mediates the transfer of a phosphoryl group from EI, another general PTS component, to the sugar-specific component EII or EIII (Figure 1). HPr from Escherichia coli was first characterized by Anderson et al. (1971) and has since been the subject of several reports. The enzyme can exist in phosphorylated (P-HPr) and nonphosphorylated forms.

*This research was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

1Department of Physical Chemistry and Institute BISON, State University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands, and Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Received January 30, 1990; Revised Manuscript Received May 24, 1990

FIGURE 1: Schematic representation of the mannitol- (mti) specific PTS. For some sugars additional sugar-specific component, EIII, is present between HPr and the sugar-specific EII.

It was shown by Anderson et al. (1971) that the phosphorylation very probably takes place at the N61 position of a...
Effects of Phosphorylation of HPr

histidine residue; this was later confirmed by Waygood et al. (1985). In the other proteins, EI, EI1I, and some EI1’s, the phosphoryl group is also carried on a histidine. Of the two histidine residues in *E. coli* HPr, His15 was identified as the phosphorylation site (Weigel et al., 1982). Proton NMR studies by Dooijewaard et al. (1979) showed the active center histidine to have a pKₐ = 5.6, which was raised to 7.8 upon phosphorylation. The other histidine, His76, with a pKₐ = 6.0, is unaffected by phosphorylation. This paper focuses on the details of the hydrogen-bond structure in the vicinity of the His15 which could be important in the phosphorylation process.

Witanowski et al. (1972), Blomberg et al. (1977), Alei et al. (1980), and Roberts et al. (1982) have shown that nitrogens in imidazoles can be characterized by their resonance position in the ¹⁵N NMR spectrum (Table I), revealing information on the protonation state of the nitrogen nucleus and its involvement in hydrogen bonding. The method has been used by Bachovchin and co-workers to obtain details of the catalytic triad and various enzyme intermediates which participate in the reactions catalyzed by serine esterases (Bachovchin & Roberts, 1982; Roberts et al. 1982; Bachovchin et al., 1988). In this paper we describe the application of the ¹⁵N NMR technique to the study of the active site His15 in HPr and P-HPr. In order to interpret the results of the latter, two model compounds (phosphoimidazole and phosphomethylimidazole) were synthesized and characterized with respect to their ¹⁵N chemical shifts.

Because the ¹⁵N nucleus is rather insensitive for NMR measurements compared to the ¹H nucleus (the latter is about 1000 times more sensitive) and because the natural abundance of the ¹⁵N nucleus is low (only 0.365%), it is preferable to use ¹⁵N-enriched materials for NMR measurements on proteins. For organic compounds that are soluble to high concentrations (molar range), ¹⁵N enrichment is often not necessary.

**Materials and Methods**

**Materials.** Phosphoenolpyruvate (monocyclohexyl-ammonium salt) was obtained from Sigma; α-D-[¹⁴C]-glucopyranoside (140 mCi/mmol) and [1-¹⁴C]phosphoenolpyruvate (12 mCi/mmol) were obtained from Amersham. The ¹⁵NH₃ (99%), used for the synthesis of ¹⁵N-[6(1,2)] labeled histidine, was provided by the NIH Stable Isotope Resource of the Los Alamos National Laboratory. Histidine specifically labeled at the N61 position or at both the N61 and Nε2 positions. Ampicillin was added to the medium (100 µg/mL) to stabilize the plasmid. Usually, 5 L of this medium was inoculated with 15 mL of a log-phase culture of the strain grown on Luria Broth, supplemented with 100 µg/mL ampicillin. After growth at 37 °C for 36-40 h, cells were harvested by centrifugation (10000 g, 5 min, 4 °C) yielding 25-30 g of cells (wet wt).

**EI Isolation.** EI was isolated from *E. coli* as described previously (Robillard et al., 1979).

**HPr Complementation Assay.** Samples were assayed for HPr activity by examining whether they could complement the crude cell extract of the ptsH mutant of *Salmonella typhimurium* strain SB2226 as described by Kundig and Roseman (1971), with methyl α-glucopyranoside as the substrate. Sugar and sugar phosphate were separated by an ion exchange procedure described by Misset et al. (1980) with 0.2 M HCl as the eluent instead of 0.1 M LiCl.

**Determination of HPr Concentration.** HPr was phosphorylated in a buffer containing 50 mM NaP, (pH 7.0), 1 mM DTT, 0.5 mM MgCl₂, 0.23 mM El, and 12.5 mM [¹-¹⁴C]PEP. The solution was kept at 30 °C, and samples were taken at appropriate time intervals. The [¹⁴C]pyruvate was separated from the [¹-¹⁴C]PEP by ion exchange chromatography as described by Brouwer et al. (1980). The HPr concentration can be calculated from the initial [¹-¹⁴C]pyruvate burst.

**Purification of HPr.** All handlings were performed at 4 °C unless stated otherwise. The harvested cells were resuspended in TS buffer (TS = 10 mM Tris-HCl, pH 7.6, 1 mM NaN₃, and 4 mM/g of cells, wet wt) and disrupted in a French press (10000 psi) after which DNase I and RNase (both 0.02 mg/mL) were added. After 30 min, cell debris was removed by centrifugation (48000 g, 2 h). The supernatant was collected and dialyzed against 3 L of TS buffer overnight. The pellet was resuspended in TS buffer (1 mL/g of cells, wet wt) and centrifuged again as described above. This supernatant was added to the first dialyzed supernatant, and without further dialysis, the solution was loaded onto a DEAE column (1.5 x 28 cm, 1.5 mL/min). The column was eluted with a 1-L gradient of 0-100 mM NaCl in TS buffer. Fractions were collected and assayed for HPr activity with the HPr complementation assay. All fractions showing HPr activity were pooled, and the pool was concentrated to 40 mL in an Amicon ultrafiltration apparatus, with a YM2 filter. The concentrated solution was brought on a Sephadex G-75 column (5 x 70 cm, 2.5 mL/min). Fractions were collected and assayed for HPr activity as described. All fractions showing HPr activity were pooled and brought onto a second DEAE column (1.5 x 20 cm, 1.5 mL/min), after which the HPr was eluted with a 500-mL gradient of 0-100 mM NaCl in TS buffer. The activity profile of this column usually showed three peaks at 30, 50, and 70 mM NaCl, of which only the first represented native HPr, as judged by SDS–PAGE and isoelectric focusing. The other two peaks appeared to contain mainly deamidated forms of HPr, described as HPr1 and HPr2 in previous publications (Anderson et al., 1971; Waygood et al., 1985). The final HPr-containing solution was dialyzed twice against 3 L of water and finally against 3 L of KPi buffer (0.5 mM, pH 7.3) containing 15 g of Chelex. After this the HPr was freeze-dried and redissolved in 0.5-1.0 mL of 100 mM KP, buffer of the desired pH, containing 2 mM EDTA, 5 mM NaN₃, and 10% D₂O. Protein concentrations were then 3-5 mM, and these solutions were used for ¹⁵N NMR measure-

---

¹ Abbreviations: EI (II, III), enzyme 1 (II, III); mlt; mannitol; PA, phosphoamidate; PEP, phosphoenolpyruvate; PI, phosphomimidazole; PMI, phosphomethylimidazole; PTS, phosphoenolpyruvate-dependent transport system.
Preparation of HPr and PHPr Samples for NMR Measurements. HPr solutions were brought to the appropriate pH value by dialyzing 0.5 mL of the HPr solution against 20 mL of KEND buffer of the correct pH (KEND pH 6.7) containing 50 mM KP, 2 mM EDTA, 5 mM NaN, and 10% D2O. The pH was measured before and after each measurement, and values agreed within 0.05 pH unit. After 2 weeks, isoelectrofocusing showed that the HPr was still 95% pure, with deamidation products described as HPrl and HPr2 (Anderson et al., 1971; Waygood et al., 1985). PHPr was prepared by dialyzing 0.5 mL of 5 mM HPr in KEND buffer against 5 mL of KEND buffer (pH 6.7) containing 500 mM PEP, 10 mM MgCl2, and 1 mM DTT. After 2 h of dialysis, 50 μL of E1 (250 μM) was added to the sample. The HPr was rapidly converted to PHPr and remained phosphorylated for about 10 h, during which time the NMR spectra were recorded. The length of time that HPr remained phosphorylated was determined by HPLC anion exchange chromatography. PHPr can be separated from HPr, EI, and PEP by a MonoQ anion exchange column and a buffer system consisting of (A) 50 mM Tris-HCl (pH 9.0) and (B) 50 mM Tris-HCl (pH 9.0) + 500 mM NaCl at a flow rate of 1.0 mL/min. Detection is performed by recording the UV absorbance at 214 nm. Gradient conditions as follows: 2 min at 5% B and then the percentage B is increased to 30% in 12 min during which the PHPr is eluted at 120 mL NaCl. After this, the column is rinsed by elution with 100% B and reequilibrated with 5% B in A.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 15% acrylamide gels.

Isoelectrofocusing. Iselectrofocusing was performed at room temperature with water cooling. Acrylamide gels [5% acrylamide, 0.15% bis(acrylamide)] contained 5% Servalyte T4-9, 5% glycerol, and 0.1% decylpoly(ethylene glycol) 300, a nonionic detergent.

Staining Procedures. Gels were stained by the silver staining procedure, described by Wray et al. (1981). TLC chromatograms were stained by spraying with an iodine solution in ethanol to reveal imidazoles or by spraying with the acidic molybdate reagent, described by Hanes and Isherwood (1949), to detect acid-labile phosphates and inorganic phosphate.

Synthesis of 15N-Labeled Histidine. dl-Histidine, selectively enriched (99%) with 15N at the Nα1 and Nε2 positions, was synthesized as described by Totter and Darby (1942; Totter & Darby, 1944). Purity was confirmed by TLC. 15N content was checked by looking at the 15N splitting of the histidine ring proton resonances by 1H NMR.

Synthesis of Phosphoramidate (PA). The monopotassium salt of PA was prepared as described by Stokes (1983).

Synthesis of Monophosphoimidazole (MPI). MPI was prepared by a procedure derived from the one described by Rathlev and Rosenberg (1956). PA (0.8 g) was added to 4 mL of an aqueous solution of imidazole (5 g/100 mL, pH 7.2). After 6 h at room temperature all the imidazole was converted to mono- and diphosphoimidazole (DPI), as judged by TLC. Then, the pH was raised to 12-13 by the addition of a concentrated KOH solution, and the solution was left at room temperature for 12 h. DPI is much less stable at this pH than 15N histidine enrichment should be about 99%.

PMI (Rathlev & Rosenberg, 1956) so that after this period most of the imidazole is present as MPI (about 80%), the rest being nonphosphorylated imidazole, as judged by TLC. This solution was used for NMR measurements without further purification.

Synthesis of Phospho-N-methylimidazole (PMI). PMI was prepared by adding 0.8 g of PA to 4 mL of an aqueous N-methylimidazole solution (5 g/100 mL, pH 7.2). After 2 h at room temperature, essentially all the N-methylimidazole was converted to PMI, as judged by TLC. This situation could be maintained for about 10 h, during which NMR measurements were performed.

Thin-Layer Chromatography (TLC). TLC was performed with Kieselgel 60 as the stationary phase and a mixture of 0.1 M K2CO3/ethanol (35/65 v/v) as the mobile phase.

NMR Measurements. All NMR spectra were recorded on a Varian VXRS500 NMR spectrometer, operating at a frequency of 50.654 MHz. The machine is equipped with a SUN 3/160 computer. All data processing was performed with standard Varian NMR software. Protein spectra were recorded at 26 °C by use of a 1H/NH reverse-detection probe with a 5-mm bore, enabling direct measurement of either nucleus. Spectra were recorded with a 25000-Hz sweep width, a 45° flip angle, and a repetition time of 1.1 s unless stated otherwise. During acquisition, broad-band 1H decoupling was applied. An internal standard, 5 M [15NH4]ClO4 in 2 M HNO3 in a capillary, was present during all measurements.

Spectra were processed with 10-Hz line broadening. Chemical shifts are recalculated in order to be referenced to 1 M H2SO4, positive shifts being upfield. Spectra of imidazole compounds were recorded with a 10-mm 1H probe by use of 45° pulse angle and a repetition time of 11 s. Spectra were processed with a 1.0-Hz line broadening. Other conditions were as for the protein NMR spectra. Further details are given in the figure and table legends and in the text.

Results

pH Dependence of the Histidine Nα1 and Nε2 Chemical Shifts in HPr. Table I summarizes the 15N chemical shift information for the imidazole nitrogens in their various tautomeric and protonated states and the effect of H-bonding on the chemical shifts. HPr contains two histidine residues, His15 and His76. Their pKα values have been determined by 1H NMR studies (Dooijewaard et al., 1979) to be 5.6 (His15) and 6.0 (His76). His15 is known to be the site of phosphorylation in HPr. We examined the hydrogen-bonding states of both histidine imidazole rings by recording 15N NMR spectra of HPr, in which the imidazoles were specifically enriched at the Nα1 position or at both the Nα1 and Nε2 posi-
Effects of Phosphorylation of HPr

The titration of two resonances to low $\delta$ values, indicating the formation of two $\beta$-nitrogens. The other two resonances remain at high $\delta$ values, as is expected for $\alpha$-nitrogens (see Table I). Figure 2c shows a spectrum for HPr containing histidine labeled at only N61. Comparing spectrum c to spectrum b of Figure 2 shows that the resonances titrating to $\beta$-positions both belong to N61 nuclei. Thus, both histidine residues show a deprotonating N61 while N62 keeps its proton. Assignment of the resonances to His15 or His76 can be done by use of the known $pK_a$ values for these residues. Figure 3 shows the recorded data points. Assuming a conversion of the histidine residues from a single protonated state to a deprotonated one, the data can be fitted to eq 1 by a least-squares fitting procedure. In this equation $\delta_{\text{obs}}$ is the observed chemical shift position, $\delta_a$ and $\delta_b$ are the chemical shift positions of the imidazole nitrogens in the fully protonated and deprotonated states of the histidine imidazole ring, and $x$ is the $H^+$ concentration (mol/L). The fitting procedure provides the desired constants $\delta_a$, $\delta_b$, and $K_a$.

Two resonances titrate with a $pK_a$ of 6.2, demonstrating that they belong to the N61 and N62 of His 76. Of the two remaining resonances that must belong to His15, the one assigned to the His N61 titrates with a $pK_a$ of 5.8; the other one does not titrate. These $pK_a$ values are both 0.2 pH unit higher than the ones published by Dooijewaard et al. (1979), who recorded their spectra at 36 $^\circ$C. We recorded the spectra at 26 $^\circ$C; this change in temperature is probably the cause of the differences in $pK_a$ found. This was confirmed by recording the $^1H$ titration curves for the histidine residues in HPr at 26 $^\circ$C with $^1H$ NMR, analogous to the experiments of Dooijewaard et al. (1979). The $^1H$ titration curves at 26 $^\circ$C showed a $pK_a$ of 5.8 for the His15 residue and a $pK_a$ of 6.2 for the His76 residue, the same as observed for the $^15N$ titration curves, confirming that indeed the change in temperature is responsible for the observed change in $pK_a$. Very similar temperature dependence in the $pK_a$ for histidine residues has been reported by Roberts et al. (1969) for histidine residues in ribonuclease.

Figure 2d shows the N61 resonances at pH 6.0, which is in the middle of the steep part of the titration curves. The line width of a titrating resonance is dependent on the mole fractions of the protonated and deprotonated species, the lifetime in each state, the $pK_a$, and the square of the chemical shift difference in hertz between the two resonance positions in the NMR spectrum. For the $\alpha^+ \to \beta$ conversion this difference is 73 ppm, which in our measurements is equivalent to 3698 Hz. Sudmeier et al. (1980) showed that, assuming normal diffusion-limited exchange rates, the resonance titrating from $\alpha^+$ to $\beta$ should disappear due to line broadening. Figure 2d, however, shows both N61 resonances at pH 6.0, which is close to both $pK_a$ values. The ability to observe the resonances titrating from $\alpha^+$ to $\beta$ indicates that exchange at these nitrogens must be very fast, suggesting that it is catalyzed by either a buffer component or some group on the protein. We did not try to remove the buffer components, because HPr is not stable in water alone; it easily precipitates, often accompanied by deamidation of the protein (A. van Dijk, unpublished results).

The data presented can be used to examine the hydrogen-bond state of both imidazole rings. This is done by comparing the recorded titration curves with the theoretical curves, calculated with eq 2 for an $\alpha^+ \to \beta$ transition and eq 3 for an $\alpha^+ \to \alpha$ transition. In these eqs, which are simply modified

$$\delta_{\text{exp},i} = 128 + x_i \times 73 \text{ ppm}$$

$$\delta_{\text{exp},i} = 210 - x_i \times 9 \text{ ppm}$$

forms of eq 1, $\delta_{\text{exp},i}$ is the expected chemical shift position at pH $i$, $x_i$ is the mole fraction of $\alpha^+$ at pH $i$, 128 is the $\beta$ nitrogen
volvement in H-bond formation. At pH > 7.5 the shift caused 201.0 ppm; see Table raised, Ne2 remains protonated, and it shows a growing in-

Table II: Observed versus Theoretical Chemical Shift Positions (ppm) of Imidazole Nitrogens of His15 and His76

<table>
<thead>
<tr>
<th>residue</th>
<th>nitrogen</th>
<th>pK_a</th>
<th>protonation state</th>
<th>delta_chem</th>
<th>delta_theoretical</th>
<th>delta_theoretical - delta_chem</th>
</tr>
</thead>
<tbody>
<tr>
<td>His15</td>
<td>Nδ1</td>
<td>5.8</td>
<td>protonated</td>
<td>195.8</td>
<td>201.0</td>
<td>-5.2</td>
</tr>
<tr>
<td></td>
<td>Ne2</td>
<td>5.8</td>
<td>protonated</td>
<td>200.4</td>
<td>201.0</td>
<td>-0.6</td>
</tr>
<tr>
<td></td>
<td>Nε2</td>
<td>5.8</td>
<td>deprotonated</td>
<td>130.7</td>
<td>128.0</td>
<td>+2.7</td>
</tr>
<tr>
<td></td>
<td>Nε1</td>
<td>5.8</td>
<td>deprotonated</td>
<td>200.5</td>
<td>210.0</td>
<td>-9.5</td>
</tr>
<tr>
<td>His76</td>
<td>Nδ1</td>
<td>6.2</td>
<td>protonated</td>
<td>194.4</td>
<td>201.0</td>
<td>-6.6</td>
</tr>
<tr>
<td></td>
<td>Ne2</td>
<td>6.2</td>
<td>protonated</td>
<td>198.3</td>
<td>201.0</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td>Nε2</td>
<td>6.2</td>
<td>deprotonated</td>
<td>130.4</td>
<td>128.0</td>
<td>+2.4</td>
</tr>
<tr>
<td></td>
<td>Nε1</td>
<td>6.2</td>
<td>deprotonated</td>
<td>204.0</td>
<td>210.0</td>
<td>-6.0</td>
</tr>
</tbody>
</table>

*Chemical shifts are referenced to 1 M HNO_3. *Chemical shifts of the protonated and deprotonated states as determined by the fitting procedure described in the text. *Chemical shifts at pH > 7.5 are calculated as explained in the text.

chemical shift position, 73 is the chemical shift difference between $\alpha^*$ and $\beta$ nitrogens, 210 is the $\alpha$ nitrogen chemical shift position, and 9 is the chemical shift difference between $\alpha$ and $\alpha^*$ nitrogens. By use of the $pK_a$ values of 5.8 and 6.2, theoretical curves were calculated and are shown in Figure 4. The information in this figure is interpreted as follows with use of the data in Table II. In the protonated imidazole ring, the Nδ1 of His15 (Figure 4a) is involved in H-bond formation as a donor in an intermediate-strength hydrogen bond; only a -5.2 ppm shift is observed whereas a shift of almost 10 ppm due to hydrogen bonding is possible (see Table II). When the pH is raised, Nδ1 deprotonates and resonates at 130.7 ppm in the neutral imidazole ring system, which is only 2.7 ppm away from a pure-type $\beta$ nucleus. Therefore, in the deprotonated state, the Nδ1 of His15 shows only very weak H-bonding. For the His15 Nε2, however, the situation is dramatically different. In the protonated imidazole ring, this nucleus is almost pure $\alpha^*$ (200.4 ppm observed compared to the theoretical 201.0 ppm; see Table II). When the pH is raised, Nε2 remains protonated, and it shows a growing involvement in H-bond formation. At pH > 7.5 the shift caused by this involvement is -9.5 ppm, which is very close to the maximal possible -10.0 ppm. This means that the His15 imidazole ring becomes strongly oriented toward a H-bond accepting group on the protein, probably a carboxylate group. We will come back to this in more detail under Discussion.

Almost the same titration behavior is observed for His76 as for His15 (Figure 4b). When the imidazole ring is protonated, the Nδ1 of His76 shows moderate involvement in H-bond formation as a donor, demonstrated by a -6.6 ppm shift relative to the theoretical curve (see Table II). In the neutral imidazole ring the H-bond character is almost completely eliminated, leaving an almost pure-type $\beta$-nitrogen as is observed for Nδ1 of His15. As with Nε2 of His15, Nε2 of His76 shows involvement in H-bonding as a donor. When the imidazole ring is protonated, the effect is rather weak, evidenced by only a -2.7 ppm shift (see Table II); the effect is stronger in the deprotonated imidazole ring (-6.0 ppm), but it does not become as strong as for the His15 Nε2.

$^{15}$N NMR of Phosphoimidazole (PI) and Phospho-N-methylimidazole (PMI). The imidazole ring of His15 carries a phosphoryl group in PHPr. In order to interpret the $^{15}$N NMR spectra of PHPr in terms of the protonation and hydrogen-bonding state of the imidazole ring, it is necessary to know the effect of the introduction of a phosphoryl group on the chemical shift positions of the imidazole nitrogens. Phosphoimidazole at pH > 12 was used to determine the influence of the phosphoryl group on the chemical shifts of the nitrogens in a neutral imidazole ring. Since protonated phosphoimidazole hydrolyzes too rapidly to allow the recording of natural abundance $^{15}$N NMR spectra, it could not be used to collect information on the effect of the phosphoryl group on the chemical shifts of the nitrogens in a protonated imidazole ring (Rathlev & Rosenberg, 1956; Jencks & Gilchrist, 1965). For this purpose phospho-N-methylimidazole was used. Due to the presence of the methyl group, the phosphorylated imidazole ring remains charged even at high pH. The results are presented in Table III. In PI the $\beta$-nitrogen resonates at 133 ppm instead of the $\beta$-N chemical shift position of 128.0 ppm (see Table I); the 5.0 ppm upfield shift is caused by the phosphoryl group. The resonance of the phosphorylated nitrogen, identified by a 25-Hz splitting due to $^{31}$P-$^{15}$N coupling, shifts from the $\alpha$-N value of 210 ppm to 175.4 ppm, a downfield shift of almost 35 ppm (Table I). In PMI the phosphoryl group causes the methyl-carrying nitrogen to shift only 1.0 ppm, compared to the protonated N-methylimidazole (Table III; 203.1 versus 204.1 ppm). But the phosphorylated nitrogen is shifted 35.7 ppm downfield, almost the same as was observed for the phosphorylated nitrogen in imidazole. The phosphoryl group attachment apparently lowers the chemical shift of the directly attached nitrogen by about 35
Effects of Phosphorylation of HPr

Table III: $^{15}$N Chemical Shifts (ppm) of Model Imidazole Compounds

<table>
<thead>
<tr>
<th>compound</th>
<th>atom type</th>
<th>imidazolium</th>
<th>imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>N$^i$-methylimidazole$^a$</td>
<td>N6$^i$</td>
<td>204.1</td>
<td>211.5</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>203.6</td>
<td>128.5</td>
</tr>
<tr>
<td>phosphomethylimidazole$^c$</td>
<td>N6$^i$</td>
<td>203.1</td>
<td>ne</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>167.9</td>
<td>ne</td>
</tr>
<tr>
<td>phosphoimidazole$^e$</td>
<td>N6$^i$</td>
<td>nd</td>
<td>175.4</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>nd</td>
<td>133.1</td>
</tr>
</tbody>
</table>

$^a$Chemical shifts are referenced as stated previously. nd = not determined; ne = not existing. $^b$Data are taken from Bachovchin (1986).

Alternative Titration Behavior of Resonances. HPr has been difficult to crystallize; until now, only one successful attempt has been reported (El-Kabbani et al., 1987). The problems may arise from the existence of more than one stable conformation of HPr. Evidence for this has been found. During our studies we have examined several preparations of $^{15}$N-labeled HPr and twice observed a slightly different titration behavior of the two N6$^i$ resonances in N6$^i$-labeled HPr. Protein dissolved twice in buffer at pH 8.0 showed the two N6$^i$ resonances at a single position at 132.0 ppm (pH 8.0), indicating involvement of the nitrogens in moderate H-bonding as donors. The conformation was stable for several days, even after the temperature was raised from 26 to 36 °C. Lowering the pH below 7.1, however, resulted in the appearance of the two resonances normally observed. Raising the pH again above 7.1 did not result in the reappearance of the single N6$^i$ resonance but continued to show the two resonances normally observed. The alternative titration behavior might indicate a second conformation of HPr. It is probably not the energetically most favorable one as it can easily be converted to the normal conformation by a moderate pH drop. At the moment, research is in progress to reveal the cause and significance of this alternative conformation.

Discussion

We have examined the hydrogen-bonding states of the two histidine residues in HPr, before and after phosphorylation of the protein. The phosphorylation was known to take place at His15 (Weigel et al., 1982) and very probably at its N6$^i$ nucleus (Waygood et al., 1985). Our aim was to examine the H-bond structure at the active site in order to reveal more details of the mechanism of phosphorylation. The results of the present study can be summarized as follows. In unphosphorylated HPr, His15 has an abnormally low pK$_a$ (5.8).
It is strongly hydrogen bonded above its pKₐ via the Ne2, which serves as a H-bond donor, but the effect diminishes below its pKₐ, leaving a non-hydrogen-bonded nucleus in the protonated imidazole ring. In phosphorylated HPr, the pKₐ of His15 shifts to 8.0, and the H-bonding via the Ne2 is disrupted.

Two different structure determinations of HPr (Klevit & Waygood, 1986; El-Kabbani et al., 1987) have led to considerably different overall structures. However, both structures show the same amino acid residues in the vicinity of His15, Arg17, and Glu85, which can be brought into the pKₐ and hydrogen-bonding considerations. The abnormally low pKₐ probably reflects the presence of the positively charged guanidino moiety of Arg17 in the vicinity of His15. Since it more readily serves as a H-bond donator rather than acceptor, it is probably not directly bonded to His15 but is close enough to it to affect the pKₐ. The carboxylate moiety of Glu85 would be a good H-bond acceptor for the Ne2 of His15. However, when H-bonded to histidine, it should raise rather than lower the pKₐ. The efficiency of the carboxylate and guanidino moieties in altering the pKₐ will depend on the charge distribution on these groups which, in turn, is dependent on the possible involvement of these residues in other hydrogen-bond interactions. Sufficient structural details are not available at the present time to fully explain the observed pKₐ of His15.

Klevit and Waygood (1986) suggested that at physiological pH Glu85 and Arg17 interact with each other, leaving the His15 imidazole ring unaffected. Upon phosphorylation, one of the Glu85 carboxyls was thought to move over to interact with the positively charged phosphoimidazole ring, an interaction that they tentatively suggested to involve H-bond formation to the His15 Ne2. The present study indicates that quite the opposite is happening; a hydrogen-bond acceptor, probably a Glu or Asp, interacts with the Ne2 of His15 in the unphosphorylated HPr, but the interaction is broken upon phosphorylation.

In a study to reveal whether the α- or the γ-carboxyl group of Glu85 was involved in the phosphorylation process, Waygood et al. (1989) replaced the Glu85 by Lys, Gln, and Asp, and they also deleted the Glu altogether, leaving Leu84 as the C-terminal residue. None of these mutations noticeably affected the Kₐ or Vₘₐₓ for EI⁺ₐ. Since no details of the kinetic measurements were provided, it is impossible to judge the significance of these results since it is not clear whether the kinetics measured the phosphorylation of HPr or the dephosphorylation of PHPr. The hydrolysis characteristics of PHPr were also not significantly altered by the mutations, except for the deletion and the Lys mutants (Waygood et al., 1989). These findings led Waygood et al. (1989) to suggest that the C-terminal carboxyl is interacting with the His15 Ne2 in PHPr. The ¹⁵N data show, however, that there is no evidence for hydrogen bonding of the phosphoimidazole via Ne2.

His76 also shows a Ne2 involved in H-bonding as a donor. In this case, the adjacent Glu75 is a good candidate for the H-bond acceptor. In both the NMR structure (Klevit & Waygood, 1986) and the X-ray structure (El-Kabbani, 1987), these residues are located on an α-helix and are not present in the active center. We demonstrated that upon phosphorylation the surroundings of the His76 Nε1 change, which indicates that the phosphorylation-induced conformational change is not restricted to the active center alone but extends to a larger part of the protein. Only detailed studies of the PHPr structure can reveal the extent of these changes.

The minimum role of the H-bonding to His15 would be to fix the orientation of the histidine, a feature that may be essential for the phosphorylation. Disruption of the H-bond indicates a conformational change, as was also supported by the altered exchange rate of His76. Disruption could also lead to a more mobile histidine. Previous analysis of the ³¹P NMR line width of PHPr indicates however that this is not the case. Vogel et al. (1982) examined the frequency dependence of phosphohistidine ³¹P NMR line widths in E. coli succinyl-CoA synthetase to determine the chemical shift anisotropy factor. They also calculated the rotational correlation time, τₒ, of the phosphohistidine and showed that it was much longer than that calculated for the protein from its hydrodynamic properties. From this they concluded that the phosphohistidine was completely immobilized. A similar analysis of the ³¹P NMR line widths of HPr led to the same result. The phosphohistidine in PHPr is immobilized. This immobilization does not occur via the H-bond to the Ne2. Another possibility is the guanidino group of Arg17, which may interact directly with the phosphoryl group of P-His15 to immobilize the ring. ¹⁵N NMR studies to examine this are in progress.

The presence of the arginine moiety could also explain the high rate of hydrolysis of the phosphohistidine in HPr, reported by Waygood et al. (1985). The positively charged arginine guanidino moiety might destabilize the phosphoryl group in PHPr in a manner similar to that reported for phosphohistidine, where the positively charged amino group destabilizes the phosphoryl group on Nε1. (Hultquist, 1968). This was suggested by Waygood et al. (1985), but these authors also placed a carboxylate moiety near His15. They based this on the observation that the temperature dependence of the rate of hydrolysis of PHPr at pH 3.3 differed from the ones at pH 4.3 or higher. The difference was thought to be caused by the protonation of the carboxylate group at this pH. As already mentioned, our results do not indicate hydrogen bonding of the Ne2 of P-His15. In light of these results, the pH 3.3 dependence of the hydrolysis rate probably reflects a structural change that indirectly affects the hydrolysis rate.

References

Effects of Phosphorylation of HPr


