Escherichia coli Phosphoenolpyruvate Dependent Phosphotransferase System. NMR Studies of the Conformation of HPr and P-HPr and the Mechanism of Energy Coupling
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The polysaccharide could also originate in the membrane where it may have a specific role in the binding of P-HPr to membrane-bound EII. This would correlate with its apparent activation effect which we find in the phosphotransferase activity measurements. (3) If one assumes that the α-1–6 glucan is a form of sugar storage not previously detected, one could suggest that the polysaccharide may function as a mechanism for controlling the activity of the PTS. By considering that the PTS appears to be involved in regulating a number of other systems (adenylate cyclase, transport of non-PTS sugars (Saier, 1977)), it is possible that the polysaccharide has a more general role in the control of these processes. (4) The polysaccharide could be an intermediate product of the sugar transport process itself. Its discovery, then, would suggest that the PTS might transport sugar in the form of long polymers. In this respect it is tempting to speculate whether the structure of the polymer, being an α-1–6 helix, has a special function. α-1–6 helices should be much longer than the usual α-1–4 amylase helices which are involved in the storage of sugar. These various possibilities are currently under investigation.

Recent experiments isolating HPr from Salmonella typhimurium grown and harvested under a variety of conditions consistently result in an HPr preparation which contains the α-1–6 glucan homopolymer.

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References


Escherichia coli Phosphoenolpyruvate Dependent Phosphotransferase System. NMR Studies of the Conformation of HPr and P-HPr and the Mechanism of Energy Coupling†

G. Dooijewaard, F. F. Roossien, and G. T. Robillard* 1

ABSTRACT: 1H and 31P nuclear magnetic resonance investigations of the phosphoprotein intermediate P-HPr and the parent molecule HPr of the E. coli phosphoenolpyruvate dependent phosphotransferase system (PTS) show that HPr can exist in two conformations. These conformations influence the protonation state of the reactive histidine residue, thereby determining the reaction pathway in the phosphoryl group transfer step. A general mechanism is proposed for the energy-coupling process in the PTS.

The transport of sugars by the bacterial phosphoenolpyruvate dependent phosphotransferase system is coupled to the energy source, phosphoenolpyruvate, via a series of phosphoryl group transfer steps involving phosphoenzyme intermediates (Roseman, 1969; Postma & Roseman, 1976; Hengstenberg, 1977).

1 Abbreviations used: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate dependent phosphotransferase system; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; DTT, dithiothreitol; P (as in P-histidine), phosphoprotein.
Depending on the system under investigation, either \( P\text{-HPr} \) or \( P\text{-F}_{\text{III}} \) serves as the phosphoryl group donor at the membrane-bound \( E_{\text{II}} \) level.

\[
P\text{-HPr} + \text{hexose (out)} \xrightarrow{E_{\text{II}}} \text{hexose-P (in)} + P\text{-F}_{\text{III}}
\]

Since purification procedures for all proteins of the PTS have been developed (Anderson et al., 1971; Kundig & Roseman, 1971; Dooijewaard et al., 1979; Robillard et al., 1979; Hays et al., 1973; Schrecker & Hengstenberg, 1971) and the number of components is limited, we are now in a position to ask what are the molecular details of energy coupling? What mechanism drives the phosphoryl group transfer process? What physical events describe sugar transport?

Nuclear magnetic resonance is capable of probing the structure of macromolecules and furnishing data concerning the environment of various groups, their ionization state, and changes in these parameters. We have applied this technique to the phosphoprotein intermediate, \( P\text{-HPr} \), and its parent molecule, \( HPr \). The information obtained has provided a mechanism for the formation and breakdown of the \( P\text{-HPr} \) intermediate. This mechanism may be generally applicable to all the phosphoprotein intermediates in the energy-coupling process.

Materials and Methods

\( HPr \) was purified according to the procedure presented in the previous publication of this series (Dooijewaard et al., 1979).

\( E_{\text{I}} \). At the time that this research was performed, the hydrophobic interaction chromatography procedure for the purification of \( E_{\text{I}} \) (Robillard et al., 1979) had not been completed; therefore, the \( E_{\text{I}} \) used in this research was partially purified in the following manner. After suspending 100 g of \textit{Escherichia coli} cells (wet weight) in 500 mL of 20 mM \( K_{2}HPO_{4} \), 1 mM DTT, 1 mM EDTA, pH 7.6, they were ruptured by passage through a French press at 10000 psi, 5 \( ^\circ \)C, and the suspension was centrifuged at 48000g for 30 min at 5 \( ^\circ \)C. The supernatant was brought to 0.33% protamine sulfate by dropwise addition of a 2% protamine sulfate stock solution, with stirring. The suspension was stirred for 30 min and then centrifuged at 48000g for 30 min. The supernatant was brought to 0.27 M KCl, loaded on a DEAE-23 cellulose column (5 \( \times \) 60 cm), and eluted with a linear gradient of 0.27 M KCl to 0.47 M KCl in 10 mM Tris-HCl buffer containing 1 mM DTT and 1 mM EDTA, pH 7.6. The total volume of the gradient was 6 L. The peak of \( E_{\text{I}} \) activity was pooled, concentrated on an Amicon ultrafiltration apparatus equipped with a UM 20 membrane, and lyophilized.

Phosphoenolpyruvate (potassium salt) was purchased from Sigma. All other chemicals were reagent grade.

\textit{NMR spectra} were recorded at 360 MHz for proton and 145.7 MHz for phosphorus on a Bruker HX 360 spectrometer operating in either the Fourier or correlation mode. The temperature of the sample was regulated by a constant temperature accessory to \( -1 \) \( ^\circ \)C. The intensity of the HOD resonance was decreased during data acquisition in the Fourier mode by selective saturation at the HOD frequency. All proton spectra were recorded at 37 \( ^\circ \)C. The phosphorus spectra were recorded at 25 \( ^\circ \)C. The stated pH values of the samples are the values measured with a combination glass electrode. They are not corrected for the presence of D.O.

The chemical shift parameters are reported relative to DSS for proton and concentrated \( H_{3}PO_{4} \) for the phosphorus NMR spectra. Proton spectra were recorded in 5-mm cylindrical microcells (Wilmad). Phosphorus spectra were recorded by using 10-mm tubes with a microcell insert (Wilmad).

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**FIGURE 1:** \( ^{1}\text{H} \) NMR spectra of the aromatic absorption region of \textit{E. coli} \( HPr \) in 50 mM sodium phosphate buffer.

**FIGURE 2:** (1) pH 6 spectrum and (2) pH 5 spectrum from Figure 1. [(1)-(2)] Computer subtraction of spectrum 2 from spectrum 1.

**Results**

\textit{\( ^{1}\text{H} \) NMR Studies on the Histidine Residues of Native \( HPr \).}

Figure 1 shows the aromatic absorption region of the \( ^{1}\text{H} \) NMR spectrum of \( HPr \) recorded at several pH values. The two lowest field resonances shifting belong to the C-2 protons of the imidazole rings of the two histidines which this protein contains. In addition, there are two resonances between \(-6.8 \) and \(-7.5 \) ppm which also shift upfield with increasing pH; they belong to the C-4 protons of the two histidines. The remainder of the resonances arise from the protons of the aromatic amino acids (unpublished data), some of which will be discussed in more detail later. Since the C-4 proton resonances overlap with resonances from aromatic protons, the pH-dependent chemical shifts were clarified by difference spectra as shown in Figure 2. The pH-dependent titration curves, derived in this manner for the C-2 and C-4 protons, are presented in Figure 3. The resonances of the imidazole protons titrating with a pK of 5.6 were arbitrarily assigned to His A (the amino acid sequence of \textit{E. coli} \( HPr \) is unknown). Those titrating with a pK of 6.0 were assigned to His B. The titration curves drawn through the points are theoretical curves for a single protonation of the imidazole rings with a Hill coefficient of 1.

The most noteworthy feature of the curves in Figure 3 is the abnormally small change in chemical shift which the C-4
FIGURE 3: pH dependence of the histidine resonances in HPr as determined from spectra, some of which are shown in Figure 1. Circles represent the C-2 proton resonances; triangles represent the C-4 proton resonances. Open symbols are assigned to His A, the histidine which becomes phosphorylated. Closed symbols are assigned to His B. The pH was determined as described under Materials and Methods.

FIGURE 4: $^1H$ NMR spectra of the aromatic proton resonance absorption region of (1) HPr at pH 8.4 in the absence of PEP and E$_i$ (2) P-HPr prepared in situ as described under Results. The sample in the NMR tube contained 5 mg/mL HPr, 5 mM MgCl$_2$, 5 mM PEP, 50 mM sodium phosphate buffer, and 1 mg/mL protein from a partially purified E$_i$ preparation (see Materials and Methods). The pH of the sample was 8.4. At 35°C, all HPr was converted to P-HPr within 5 min. [(1)-(2)] A computer subtraction of spectrum 2 from spectrum 1.

proton resonance of His A experiences upon deprotonation, as contrasted with the normal chemical shift range for the C-2 proton resonance of the same histidine. This will be treated after the following section.

NMR Studies on the Histidine Residues of P-HPr. Since, below pH 8.0, the half-life of the rate of hydrolysis of pure P-HPr, free in solution, becomes equal to or greater than the accumulation time needed for a single NMR spectrum (15 min for $^1H$ NMR; 30–60 min for $^{31}P$ NMR), these studies were made feasible by the enzymatic phosphorylation of HPr in situ. Spectra of P-HPr were collected under conditions where the rate of formation of P-HPr was at least ten times higher than its rate of hydrolysis. A time course of the spectra thus obtained (not shown) indicates that 5 mM PEP is sufficient to keep 0.5 mM HPr in the phosphorylated form for at least 2 h. The subsequent addition of 5 mM PEP prolonged the steady-state period of P-HPr for at least another hour, despite the fact that 5 mM pyruvate had already been formed.

FIGURE 5: pH dependence of the chemical shifts of the histidine resonances of P-HPr under conditions listed in the legend to Figure 4. Circles are the C-2 proton resonances; triangles represent the C-4 proton resonances. The open symbols are the resonances assigned to His A, the histidine which is phosphorylated in this sample. The closed symbols are assigned to His B. The dashed lines are taken directly from the titration curves presented in Figure 3.

FIGURE 6: $^{31}P$ NMR spectra of P-HPr at several pH values. The sample contained 3 mg of HPr in 0.5 mL of 20 mM Tris-HCl buffer at pH 7.8. Phosphorylation was achieved by addition of 25 µL of 0.2 M PEP, 25 µL of 0.1 M MgCl$_2$, 25 µL of a partially purified preparation of E$_i$ for a final protein concentration of 1 mg/mL. (Spectrum A) pH 7.8; (spectrum B) pH 11.8; (spectrum C) pH 12.8; (spectrum D) pH 9.0. The spectra were taken in the sequence A, B, C, and D. Thus, the sample at pH 9 in spectrum D had already been denatured by treatment at pH 12.8. The assignments of the resonances are discussed in the text.

$^1H$ NMR. Figure 4 shows the aromatic absorption region of the NMR spectra of HPr and P-HPr, and their difference spectrum, recorded at pH 8.4. In P-HPr, the C-2 proton of His A is shifted downfield and its C-4 proton upfield, indicating that His A is the residue carrying the phosphoryl group. Further, there is a small shift of two Phe protons (peak X) from -7.24 to -7.28 ppm; the remainder of the resonances is essentially unaffected. The same type of titration studies, as described above for HPr, was carried out with P-HPr. Figure 5 presents the titration curves. For comparison, the curves obtained with HPr in Figure 3 are also drawn. The pK value of His A increases from 5.6 to 7.8 upon phosphorylation, while that of His B is not affected (pK = 6.0). Data below pH 6.0 could not be collected since the rate of hydrolysis of P-HPr was too high even though P-HPr was generated, in situ, as described above.

$^{31}P$ NMR. Figure 6 shows $^{31}P$ NMR spectra of P-HPr, recorded at several pH values in the presence of excess PEP and catalytic amounts of E$_i$. The resonance at low field...
Figure 7: pH dependence of the resonances in the $^{31}$P NMR spectra of P-HPr. (Open circles) Inorganic phosphate; (open triangles) PEP; (closed circles) P-HPr. The dashed line in the P-HPr titration curve represents the chemical shift position of the resonance once the sample has been raised to pH 12.8, a spectrum collected, and then the pH lowered to pH 11 and subsequently to pH 9 for additional spectra.

Figure 8: Resolution enhanced proton NMR spectra of the aliphatic proton resonance region of HPr and P-HPr. P-HPr was prepared by using the sample which generated the pH 8.1 HPr spectrum, with the subsequent addition of Mg$^{2+}$, PEP, and E$_i$ as described in the text.

Discussion

Evaluation of Structural Data. When a histidine ring is protonated, its positive charge is delocalized over the ring through resonance stabilization. Consequently, the electron density at the C-2 and C-4 atoms is lowered and their proton resonances shift to lower fields. The effect on the C-2 proton resonance is, generally, larger because of its position between the two nitrogen atoms. Structural information can be extracted from the chemical shift and pK values of the histidines.
under certain circumstances. The chemical shift values for His A and His B of HPr and P-HPr are listed in Table I together with similar parameters published for some model compounds. The pK values are listed in Table II. The chemical shift values of the protonated and neutral forms, δ0 and δi, respectively, of His B both in HPr and P-HPr (the histidine which does not become phosphorylated) are shifted 0.2 ppm downfield for the C-2 resonance and 0.2 ppm upfield for the C-4 resonance compared with the resonance positions of the free amino acids. The titration range, δi-δ0, for both the C-2 and C-4 proton resonances of His B, 0.9 and 0.4 ppm, respectively, is similar to that of free histidine.

The one striking feature of His A, the histidine which becomes phosphorylated, is the abnormal chemical shift of -7.33 ppm for δi of the C-4 proton resonance in HPr and, in conjunction with this, the very small chemical shift titration range of this resonance. The δi-δ0 value of only 0.1 ppm instead of 0.4 ppm suggests that, on deprotonation of His A, the normal change in chemical shift position of approximately 0.4 ppm which should occur is negated by another local effect in the protein occurring in the same pH range. For instance, if a phenylalanine were to move toward the histidine ring, its ring current could deshield the C-4 proton, depending on the relative orientation of the two rings, and generate a downfield shift partially compensating the normal upfield shift which occurs on deprotonation. Since the difference spectrum in Figure 2 shows that aromatic protons change their chemical shift position in this pH range, the involvement of an aromatic amino acid in a local conformational change is reasonable. In P-HPr, the C-4 proton of His A titrates normally over the entire pH range. The presence of the phosphoryl group appears to prevent the pH-dependent conformational change found in HPr. This could be caused by a steric effect, the phosphoryl group blocking the movement of the aromatic ring, or it could arise as a result of a conformational change which occurred in the process of phosphorylating HPr itself.

Table II shows that phosphorylation of the imidazole ring as such does not change its pK value. The values for imidazole and P-imidazole are equal to 7.0 and those of histidine and 3-P-histidine are equal to about 6. The difference in pK value between imidazole and histidine is caused by the repulsion of the proton at the N-1 ring atom exerted by the positive charge of the protonated amino group of histidine (Hultquist et al., 1966; Jencks & Gilchrist, 1965). In keeping with this explanation, it has disappeared in ε-N-acetylhistidine (pK = 6.9). The negatively charged phosphoryl group in 1-P-histidine also restores the pK value of the imidazole ring to 7, probably because it is close enough to the protonated amino group to compensate for its repulsion. Thus, the pK values of histidine, 1-P-histidine, and 3-P-histidine are expected to be about 7 in a random coil protein. In a folded protein, however, their pK values may differ appreciably as a result of electrostatic forces exerted by charged groups on nearby residues or as a result of changes in the polarity of the environment. As shown in Figure 5 and Table II, phosphorylation of His A results in a shift in pK of 2.2 pH units. Since phosphorylation of the imidazole ring, as such, should not change its pK, the different pK of His A in HPr (5.6) and P-HPr (7.8) must arise from different local conformations around His A in the two proteins. Again an aromatic amino acid reports this conformational change as may be concluded from the downfield shifting phenylalanine, Figure 4, upon phosphorylation of His A.

The shift of the aliphatic resonances in response to the state of protonation and phosphorylation of HPr (see Figures 8 and 9) may be reporting the charge state of His A, by assuming the protons concerned are in the direct vicinity of this histidine. On the other hand, if these protons are more distant from His A, their resonances are probably reporting a conformational change which occurs as a result of (de)protonation of (P) His A.

**Implication of the Different Conformations for the Mechanism of Phosphorylation.** On the basis of the results presented in this paper, HPr exists in a minimum of two conformations. Since the changes observed are limited (i) titration behavior of the His A proton; (ii) shift of the aromatic proton resonances; (iii) shifting methyl resonances, we suggest that the conformational changes are restricted to a small area of the protein. In fact, it is sufficient to assume two different local conformations around His A, one for the deprotonated His A in HPr (conformation I) and one for protonated P-His A in P-HPr (conformation II). Conformation II equals, more or less, the conformation around the protonated His A in HPr. At physiological pH, HPr will be in conformation I with its active His A deprotonated (pK = 5.6) and P-HPr in conformation II, with P-His A protonated (pK = 7.8). Conformation I makes the deprotonated His A, with its free electron pair on the imidazole nitrogen, an excellent nucleophile for the attack on the phosphoryl group of P-Ei. In conformation II, the protonated imidazole ring of P-His A becomes an attractive leaving group, a requirement for transfer of the phosphoryl group to EI or F9H. Owing to this conformation change in the protein, both reactions are favored at physiological pH as shown in Scheme I.

**Comparison of E. coli HPr with S. aureus HPr.** A study similar to that described in this paper was carried out by Gassner et al. (1977) for HPr from Staphylococcus aureus. Since the latter protein differs from the E. coli HPr in molecular weight (7700) and amino acid composition (among other changes, it contains only one histidine and mainly tyrosine instead of phenylalanine), it is interesting to compare the structural data found for both proteins. The only histidine in S. aureus HPr obviously fulfills the same function as His A in the E. coli HPr, since it is phosphorylated by PE, from

### Table I

<table>
<thead>
<tr>
<th>Chemical shifts</th>
<th>C-2 protons</th>
<th>C-4 protons</th>
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<tr>
<td>δ0 δi</td>
<td>δ0 δi</td>
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</tr>
<tr>
<td>His</td>
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</tr>
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<td>-8.46 -7.71</td>
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</tr>
<tr>
<td>3-P-His</td>
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<td>-8.62 -7.88</td>
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</tr>
<tr>
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<td>-8.75 -8.10</td>
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</tr>
<tr>
<td>HPr B (P-HPr)</td>
<td>-8.78 -7.88</td>
<td>-8.78 -7.88</td>
</tr>
</tbody>
</table>

\( ^a \) δ0 and δi represent the chemical shifts in the protonated and neutral species. \(^b\) Gassner et al. (1977).

### Table II

<table>
<thead>
<tr>
<th>Chemical</th>
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<th>ref</th>
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<tr>
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</tr>
<tr>
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<td>a-N-acetyl-His</td>
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<tr>
<td>His A (HPr)</td>
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<tr>
<td>His B (P-HPr)</td>
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</table>

\( ^a \) Jencks & Gilchrist (1965). \(^b\) Gassner et al. (1977). \( ^c \) Hultquist et al. (1966).
S. aureus. Its pK changes from 6.0 in HPr to 8.3 in P-HPr, indicating a similar function for the pK alteration in both proteins. Moreover, evidence was found (Maurer et al., 1977) for a conformational change in S. aureus HPr on deprotonation of its histidine, since a simultaneous shift in the position of the aromatic proton resonances of a tyrosine was detected. The most striking similarity between the two proteins, however, is the abnormally small shift which the C-4 proton resonances of the reactive histidines in both HPr's experience upon titration. As in the case of the E. coli HPr, after phosphorylation occurs, the C-4 proton resonance in S. aureus P-HPr assumes a normal titration behavior.

Despite these similarities, the two HPr's do not substitute for each other in heterologous systems (Hengstenberg, 1977). This should not be surprising in light of the different amino acid compositions of the two proteins, but it is also possible that the larger HPr from E. coli (9500 daltons) fulfills a special function missing in S. aureus HPr.

One possible reason for the inability of the two proteins to substitute for one another is simply that they might carry their phosphoryl groups on different positions of their respective imidazole rings. Our 31P NMR measurements show, however, that the phosphohistidine resonance of both E. coli and S. aureus HPr, when denatured at high pH, have the same chemical shift of approximately 5.4 ppm indicating that they, most likely, carry the phosphoryl group on the same position of the imidazole ring. On the basis of the 31P NMR model compound studies of Gassner et al. (1977), one could conclude that the imidazole is phosphorylated at the N1 nitrogen.

Gassner et al. (1977) have also prepared P-HPr chemically by reacting HPr and phosphoamidate. They found a pK of 6.9 for the phosphohistidine of P-HPr (chemical) vs. a pK of 8.3 for the phosphohistidine of P-HPr (enzymatic). They conclude, from this difference in pK, that the chemically prepared intermediate is phosphorylated at the N2-position of the histidine residue.


References

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