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Characterization of Phosphorylated Histidine-Containing Protein (HPr) of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System†

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ABSTRACT: The histidine-containing phosphocarrier protein (HPr) of the phosphoenolpyruvate:sugar phosphotransferase system, when phosphorylated, contains a 1-phosphohistidinyl (1-P-histidinyl) residue (His-15). The properties of this 1-P-histidinyl residue were investigated by using phospho-HPr (P-HPr), P-HPr-1, and P-HPr-2. HPr-1 and HPr-2 are deamidated forms of HPr produced by boiling. In addition, HPr-1 produced during frozen storage was investigated. Both pH and temperature dependencies of the rate of hydrolysis of the phosphoryl group of the 1-P-histidinyl residue were investigated. The results show that the 1-P-histidinyl residue in HPr and HPr-1 has significantly different properties from free 1-P-histidine and that these differences are attributable to the active-site residues Glu-66 and Arg-17 and the pK of the imidazole group of the 1-P-histidinyl residue in P-HPr. The 1-P-histidinyl residue in P-HPr and P-HPr-1 shows a greater lability at physiological pH than the free amino acid. A proposal for the active site of P-HPr is made on the basis of these results and the recently obtained tertiary structure. In contrast, the hydrolysis properties of the 1-P-histidinyl residue in P-HPr-2 were similar to those obtained for either free 1-P-histidine or denatured P-HPr. The loss of activity that is associated with boiling HPr was shown to be due to HPr-2 formation as HPr-1 was found to be fully active.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] in *Escherichia coli* and *Salmonella typhimurium* is responsible for the concomitant phosphorylation and translocation of a number of sugars. The PTS was initially described by Kundig et al. (1964) and has now been more fully described by Waygood et al. (1984). The sugar-specific phosphotransferases for glucose, mannose, fructose, and glucitol have been shown to be comprised of two enzymes and two phosphocarrier proteins as shown in Figure 1. The PTS specific for mannitol and *N*-acetylglucosamine do not have a factor III^{sugar} protein but do have a phosphorylated enzyme II^{sugar}. In addition, no factor III^{sugar} can be detected for the galactitol and dihydroxyacetone PTS (K. G. Peri and E. B. Waygood, unpublished results). Begley et al. (1982) concluded from stereochemical observations that there should be five phosphoryl transfer steps in the reaction mechanism of the glucose-specific PTS. This predicted the phosphorylation of

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system(s); HPr, histidine-containing phosphocarrier proteins of the PTS; FPr, fructose-induced HPr-like phosphocarrier protein; HPr-1_{frozen}, HPr with one deamidation produced during frozen storage; HPr-1_{boil}, HPr with one deamidation produced by boiling; HPr-2, HPr with two deamidations produced by boiling; III^{sugar} or factor III^{sugar}, sugar-specific phosphocarrier protein of the PTS; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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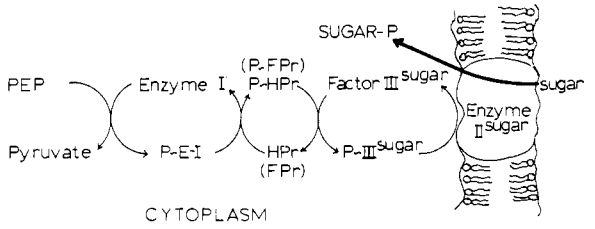

FIGURE 1: Model of the phosphoenolpyruvate:sugar phosphotransferase system. The PTS carries out the concomitant phosphorylation and translocation across a membrane for various sugars. The process involves five phosphoryl transfer steps in which two common components, enzyme I and HPr, are found. HPr is replaced by FPr for a fructose-specific system. The sugar specificity is conferred by the membrane-bound enzyme II^{sugar} which may interact with a separate phosphocarrier protein, factor III^{sugar} (e.g., PTS specific for glucose, mannose, fructose, or glucitol), or the enzyme II^{sugar} may incorporate both its own functions and the factor III^{sugar} functions (e.g., PTS specific for mannitol and *N*-acetylglucosamine). The evidence for such a model is best presented by Saier et al. (1985).

enzyme $II^{glucose}$, and it has been subsequently described (Peri et al., 1984). Thus, it would seem likely that when a separate factor III^{sugar} is found, the enzyme II^{sugar} would also be phosphorylated. Various considerations have led Saier et al. (1985) to predict that for the PTS in which no factor III^{sugar} is found (e.g., mannitol and *N*-acetylglucosamine PTS), the enzyme II^{sugar} serves a dual function in that it has a factor III^{sugar} function as well as an enzyme II^{sugar} function.

Except for the fructose PTS, HPr is common to all these systems and has been shown to contain a 1-phosphohistidinyl (1-P-histidinyl) residue when phosphorylated. 1-P-histidinyl residues have been described only in HPr from various bacterial species (Anderson et al., 1971; Simoni et al., 1973; Kalbitzer et al., 1982), nucleoside-diphosphate kinase (Edlund et al., 1969), FPr (Waygood et al., 1984), and tentatively in enzyme $II^{glucose}$ (Peri et al., 1984), despite the fact that such a high-energy compound (Weigel et al., 1982a) would appear to be ideal as an intermediate in phosphoryl transfer reactions. The initial characterization of 1-P-histidine was by Hultquist (1968), who pointed out that the greater instability of 1-P-histidine with respect to 3-P-histidine (Hultquist et al., 1966) was in part due to an interaction of the N-1 phosphoryl group with the positively charged α-amino group. This suggests that, in general, a 1-P-histidinyl residue in a peptide chain should have different properties from the free amino acid. The 1-P-histidinyl residue in phospho-HPr (P-HPr) was found to be similar to the free amino acid (Anderson et al., 1971), but the majority of the characterization appears to have been carried out on P-HPr-2. HPr-1 and HPr-2 are deamidated products of HPr; HPr-1 has one deamidation while HPr-2 has a second subsequent deamidation (glutamine to glutamate). The HPr-1 was described as having 50% of HPr activity while the HPr-2 had only 30% activity.

Recently, it had been noted that the stability of the phosphoryl group in P-HPr-1 was greater than P-HPr on isoelectric focusing gels (Mattoo et al., 1984) even though the p*I* of P-HPr-1 was more acidic than P-HPr. This suggested either that the 1-P-histidinyl residue in HPr has stability properties that were substantially different from the free amino acid or that there were significant differences between P-HPr and P-HPr-1.

This paper will show that the first deamidation which produces HPr-1 affects neither the kinetic properties nor the 1-P-histidinyl hydrolysis properties, while the second deamidated form, HPr-2, has impaired kinetic and 1-P-histidinyl hydrolysis properties. The 1-P-histidinyl residue in P-HPr and P-HPr-1 has properties that are significantly different from free 1-P-histidine, and these differences have been used to interpret the active site of P-HPr.

Materials and Methods

Materials. DEAE-cellulose paper (DE-81) was obtained from Whatman. [^{32}P]Phosphoenolpyruvate was synthesized from [γ-^{32}P]ATP obtained from NEN as previously described (Mattoo & Waygood, 1983). G50 Sephadex was from Pharmacia. Ampholytes were obtained from LKB and Pharmacia. Urea, ultrapure, was from Schwarz/Mann. All other reagents were reagent grade or better.

Protein Preparation. HPr and enzyme I were purified from *Escherichia coli* P650 as has previously been described (Waygood & Steeves, 1980). Some of the experiments were carried out with HPr purified from *E. coli* pAB65 which contained a cloned plasmid for HPr (Lee et al., 1982). HPr-1 was isolated by two methods. HPr-1_{frozen} was formed during storage of HPr in 0.01 M Tris-HCl, pH 7.5, at −25 °C for periods up to 5 years. HPr-1_{boil} and HPr-2 were produced by boiling HPr (0.1–2 mg/mL) for 15 min in 0.05 M potassium phosphate buffer, pH 7.5, as previously described (Anderson et al., 1971). HPr, HPr-1_{boil}, and HPr-2 were separated from each other by DEAE-cellulose chromatography, equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, with 1 mM EDTA, and eluted with a 0–0.3 M NaCl gradient in buffer. All preparations which had been concentrated by lyophilization were applied to a G50-Sephadex column (2.5 × 100 cm) equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, with 0.1 M NaCl, and in all cases, a single peak eluted at the HPr position.

Preparation of ^{32}P-Labeled HPr, HPr-1, and HPr-2. [^{32}P]P-HPr (or HPr-1 or HPr-2) was prepared by incubation of HPr (various amounts) in the presence of 1 mM [^{32}P]-phosphoenolpyruvate (1×10^7 cpm/μmol), 5 mM $MgCl_2$, 0.01 M Hepes buffer, pH 7.0, and enzyme I (5 units/mL incubation volume). The incubations were for 5 min at 37 °C and were then cooled rapidly and applied to a G50 Sephadex column (2.5 × 100 cm) which was equilibrated with 2 mM Na_2CO_3 and 20 mM KCl (Weigel et al., 1982a). The fractions containing [^{32}P]P-HPr were stored at −76 °C.

Assay of HPr, HPr-1, and HPr-2. The amount of HPr, HPr-1, and HPr-2 was determined by coupling the enzyme I reaction to lactate dehydrogenase, and they were also assayed by the sugar phosphorylation reaction as previously described (Waygood et al., 1979).

Rate of 1-P-histidine Hydrolysis in Protein. The rates of hydrolysis were determined by using a refrigerated water bath, which controlled temperature to ±0.1 °C. Phosphoprotein samples were preincubated for 1 min with the appropriate buffers and additions at the required temperature in thin-walled glass tubes before samples were taken. Samples, 0.05 mL, containing about 5000 cpm were taken at the appropriate times and mixed with 0.05 mL of ice-cold 0.2 M Na_2CO_3. The 0.1-mL mixtures were then applied to DEAE-cellulose paper (Whatman DE81) strips, and ascending chromatography was carried out as previously described (Weigel et al., 1982a).

After 1–1.5 h, the strips were dried and cut into two pieces: the upper piece contained [^{32}P]P_i at the solvent front and the lower piece [^{32}P]P-HPr at the origin. The radioactivity (cpm) was determined by counting the strips, fully immersed in scintillation fluid, using a liquid scintillation spectrophotometer. The amount of [^{32}P]P-HPr was determined as a proportion of the total ^{32}P on each strip. For the determination of the rate constants k_1 and k_2, between 7 and 9 samples were taken at different times. $k_2 = (k_{observed} - k_1)/(\text{molarity of compound added})$.

Buffers. The buffers used for pH dependency of the hy-

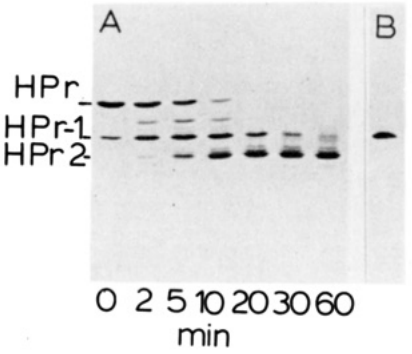

FIGURE 2: Isoelectric focusing of HPr species. (A) HPr (0.1 mg/mL) which had been stored frozen in buffer and thus contained some HPr-1 (zero time point) was boiled in 0.05 M potassium phosphate buffer, pH 7.5, and samples (0.05 mL) were taken at various times up to 60 min. The samples were immediately frozen and were subsequently applied to polyacrylamide isoelectric focusing gels (75% pH 4–6 and 25% pH 3–10 ampholytes) and focused over 10 cm for 1.5 h (12 W and 1500 V limiting for 10 × 20 cm gels). (B) Purified HPr-1_{frozen} (5 μg). The gels were stained as previously described (Mattoo et al., 1984).

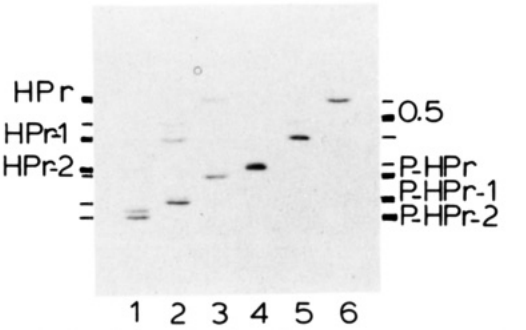

FIGURE 3: Isoelectric focusing of purified HPr, HPr-1_{boil}, and HPr-2. Fractions of HPr, HPr-1_{boil}, and HPr-2 were isolated as described in the text. Samples of these were applied to isoelectric focusing gels (conditions as described in Figure 2) following incubation with enzyme I and phosphoenolpyruvate (samples 1–3) or with no incubation (samples 4–6). The samples are HPr-2 (lanes 1 and 4), HPr-1_{boil} (lanes 2 and 5), and HPr (lanes 3 and 6). The heavy scribe marks indicate the labeled position.

drolysis rate were as follows: pH 1–2.5, HCl–KCl; pH 3.0–6.5, citrate–phosphate; pH 7.0–8.4, phosphate; pH 9.0–9.5, bicarbonate–carbonate. The buffers 2-(*N*-morpholino)ethanesulfonic acid (Mes) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) were used for the determination of rates of hydrolysis in the presence of other added compounds. In addition, acetate buffer was used for the study of P-HPr-2 hydrolysis rates. Buffers were made by using standard recommended proportions, and the final pHs were checked by using solutions that contained no HPr but all other components of the incubations. In initial experiments, overlapping buffer pH ranges were used to test for buffer effects. No effects were found.

Isoelectric Focusing. Isoelectric focusing was carried out as previously described (Mattoo et al., 1984).

Kinetic Experiments. Kinetic measurements of enzyme parameters with respect to the HPr substrates were carried out as previously described using the sugar phosphorylation assay (Waygood et al., 1979; Stock et al., 1982). In the assays, the buffer used was 50 mM Hepes, pH 7.0. Enzyme I was assayed in the presence of excess enzyme $II^{mannose}$ supplied by crude membranes from lactate-grown *S. typhimurium* strain SB2950 and 10 mM deoxy[2-U-^{14}C]glucose. Enzyme $II^{mannose}$ in membranes from lactate-grown *S. typhimurium* strain SB2950 was measured in the presence of excess enzyme I and 10 mM deoxy[2-U-^{14}C]glucose. Enzyme $II^{mannitol}$ in membranes from mannitol-grown *S. typhimurium* strain SB3507 was measured in the presence of excess enzyme I and 1 mM [U-^{14}C]mannitol.

Protein Determination. Protein was determined by the spectrophotometric method of Waddell (1956) where $OD_{215} - OD_{225} = 0.144$ mg of protein mL^{-1} $(1\ ODU)^{-1}$.

RESULTS

Protein Preparation. Anderson et al. (1971) have described the production of HPr-1 and HPr-2 by boiling solutions of HPr. In addition, long-term frozen storage of HPr in solution will result in the formation of HPr-1 as do the conditions used for the crystallization of HPr (Delbaere et al., 1982). Two forms of HPr-1 are characterized in this paper, HPr-1 produced by boiling called HPr-1_{boil} and HPr-1 produced during storage termed HPr-1_{frozen}. When HPr was boiled as described by Anderson et al. (1971), as many as 10 species of modified HPr including HPr-1_{boil} and HPr-2 were detected by isoelectric focusing (Figure 2A). HPr-1_{boil} and HPr-2 produced by boiling for 15 min were separated and purified by DEAE-cellulose and G50 Sephadex chromatography as described under Materials and Methods. HPr-1_{frozen} was similarly treated, and the preparation gave a single band on isoelectric focusing gels (Figure 2B). Isoelectric focusing gels showed that the purified preparations derived from the boiling treatment were not quite homogeneous (Figure 3); HPr (lane 6) contained some HPr-1_{boil}; HPr-1_{boil} (lane 5) contained some HPr-2 and another species labeled 0.5; HPr-2 (lane 4) was not significantly contaminated with HPr-1_{boil}, but it was comprised of two species with very similar p*I*'s. Except for the band marked 0.5, all HPr species could be phosphorylated (lanes 1–3). This was detected by the change of p*I* that occurs following phosphorylation (Mattoo et al., 1984).

Using the lactate dehydrogenase coupled assay (Waygood et al., 1979), all preparations were measured for their ability to become phosphoproteins. The measurements were in agreement with the conclusions from the isoelectric focusing gels in that they indicated that the majority of the protein in these preparations could be phosphorylated. In addition, because the 1-P-histidinyl residue is unstable, the rate at which it was hydrolyzed and rephosphorylated could be measured by this spectrophotometric assay. In the presence of excess enzyme I and phosphoenolpyruvate at pH 7.0, the rates of hydrolysis of the 1-P-histidinyl residue at room temperature (in moles per mole per minute) were found to be as follows: HPr, 0.034; HPr-1_{frozen}, 0.034; HPr-1_{boil}, 0.027; HPr-2, 0.014. This indicated that the 1-P-histidinyl residue in P-HPr-2 was more stable.

Kinetic Experiments. Measurement of HPr activity was carried out on samples taken during boiling experiments as described in Figure 3. In addition, similar experiments were carried out at 80 °C as described by Beneski et al. (1982). Assays were carried out in two ways: the first involved assays in which enzyme I was limiting, and the second was one in which enzyme $II^{mannose}$ was limiting. The former assay measures HPr as a substrate of enzyme I, and the latter assay measures HPr as P-HPr, the substrate of the enzyme $II^{mannose}$–factor $III^{mannose}$ complex (Waygood et al., 1979). The half-lives of HPr when heated at 80 and 100 °C were 65 and 25 min, respectively, when the enzyme $II^{mannose}$ assay ([EI] > [EII]) was used. This is in good agreement with the half-lives reported by Beneski et al. (1982). The enzyme I assay ([EII] > [EI]) showed that the inactivation caused by heating HPr preferentially affects the interaction with enzyme I, as heated HPr becomes a poorer substrate for this enzyme

Table I: Kinetic Experiments

enzyme assayed[a]	substrate HPr	HPr-1_{frozen}	HPr-1_{boil}	HPr-2
enzyme I				
V_{max}[c]	100[b]	100	100	36
K_m (μM)	3.0	3.5	3.6	10
enzyme $II^{mannose}$				
K_m (μM)	8	8	9	11
enzyme $II^{mannitol}$				
K_m (μM)	10	12	16	26–40

[a] As described under Materials and Methods. [b] Relative activity. [c] V_{max} was identical for all four substrate preparations.

in a shorter time. The half-lives were 32 and 12 min at 80 and 100 °C, respectively. Examination of isoelectric focusing gels similar to those in Figure 3 favored a correlation of loss of activity with HPr-2 generation.

The isolated preparations of HPr, HPr-1_{frozen}, HPr-1_{boil}, and HPr-2 were used as substrates of enzyme I, enzyme $II^{mannose}$, and enzyme $II^{mannitol}$ as described under Materials and Methods. The results of these measurements are summarized in Table I. All preparations gave essentially identical V_{max} values for the respective enzymes except for the HPr-2 preparation in its interaction with enzyme I. Although some differences from HPr were observed with respect to the K_m values for the two HPr-1 preparations, the changes were not significant. HPr-2 gave higher K_m values for both enzyme $II^{mannitol}$ and enzyme I.

Handling and Storage of [^{32}P]Phospho-HPr. [^{32}P]P-HPr produced as described under Materials and Methods was stored at −76 °C in the G50 Sephadex column elution solution, 2 mM Na_2CO_3 and 20 mM KCl, pH about 9.6. At this temperature, the rate of hydrolysis was insignificant when compared to the rate of ^{32}P decay. In a standard freezer (temperature about −25 °C), the half-life was about 30 days. In ice, at pH 4.3 or pH 7.2, the half-life was 10 h. The G50 Sephadex elution solution, 2 mM Na_2CO_3 and 20 mM KCl, must be fresh as CO_2 can dissolve and reduce the pH. Lower pH values (e.g., 7.5) result in the hydrolysis of P-HPr during fractionation at 2 °C.

pH Dependence of Hydrolysis of the 1-P-histidinyl Residue in HPr, HPr-1, and HPr-2. The stability of the 1-P-histidinyl residue was studied at 37 °C which is the usual temperature for PTS assays and for the growth of enteric bacteria. The description of free 1-P-histidine hydrolysis by Hultquist (1968) was carried out at 46 °C, as was the initial description of P-HPr, P-HPr-1, and P-HPr-2 (Anderson et al., 1971). The results of the hydrolysis experiments with P-HPr are shown in Figure 4A. The stability of the 1-P-histidinyl residue in native P-HPr was different from either the stability of the 1-P-histidinyl residue in denatured P-HPr (i.e., in 0.4 g/mL urea) or the stability of free 1-P-histidine. P-HPr-1_{frozen} yielded a very similar profile to P-HPr, while P-HPr-2 had properties that were very similar to denature P-HPr or 1-P-histidine (Figure 4B). P-HPr-1_{boil} behaved similarly to P-HPr and P-HPr-1_{frozen} (results not shown).

Dependence of P-HPr Hydrolysis on Temperature. The rate of hydrolysis of the 1-P-histidinyl residue in P-HPr and P-HPr-1_{frozen} was investigated as a function of temperature (Figures 5, 6, and 7). At the pH values 4.3, 6.3, and 7.2, considerable temperatures were reached before the rate of hydrolysis was affected by denaturation. At pH 3.3, a small inflection at about 37–46 °C was present, but otherwise, the hydrolysis rate appears to increase normally with temperature (Figure 7). At pH 8.4, the break in the increase of hydrolysis rate with increasing temperature occurs at the relatively low

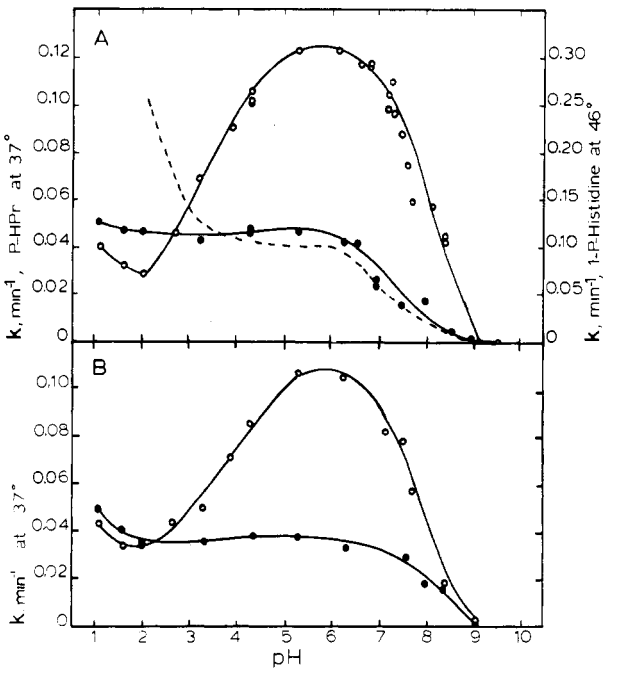

FIGURE 4: pH dependence of the rate of hydrolysis of the 1-P-histidinyl residue. (A) [^{32}P]P-HPr was incubated at 37 °C with various buffers over the pH range 1.1–9.6. Samples were taken at appropriate times and the rates of hydrolysis determined as described under Materials and Methods. Hydrolysis rates were determined for native [^{32}P]P-HPr (○) and denatured [^{32}P]P-HPr (●). Denaturation was achieved by 3–4-h incubation in urea (0.4 g/mL) at 0 °C in the G50 Sephadex elution solution. The profile of the hydrolysis of the free 1-P-histidinyl amino acid as described by Hultquist (1968) is also plotted (---). As this hydrolysis was at 46 °C, it is plotted on a different scale to give approximate equality of scale. These results are presented with permission of the author and publisher. (B) pH dependence of hydrolysis of the 1-P-histidinyl residue in deaminated HPr was determined by using conditions identical with those described above. The rates of hydrolysis for [^{32}P]P-HPr-1_{frozen} (○) and [^{32}P]P-HPr-2 (●) are shown.

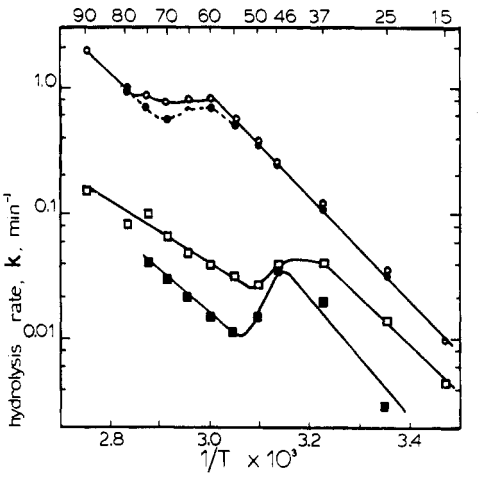

FIGURE 5: Temperature dependence of the hydrolysis of the 1-P-histidinyl residue in phospho-HPr and phospho-HPr-1_{frozen}. Hydrolysis rates of the 1-P-histidinyl residue were determined at pH 6.3 (circles) and pH 8.4 (squares) for both P-HPr (open symbols) and P-HPr-1_{frozen} (closed symbols) for the temperatures over the range 15–90 °C. The lower scale is temperature in degrees kelvin, and the upper scale is temperature in degrees centigrade.

temperature of about 46 °C. P-HPr-1_{frozen} behaved very similarly to P-HPr except that at pH 8.4 the hydrolysis rates were more substantially depressed (Figure 5). The assumption that the break in the increase of hydrolysis rate was caused by denaturation was confirmed by measurement of the rates of hydrolysis in the presence of urea (0.4 g/mL). The rates

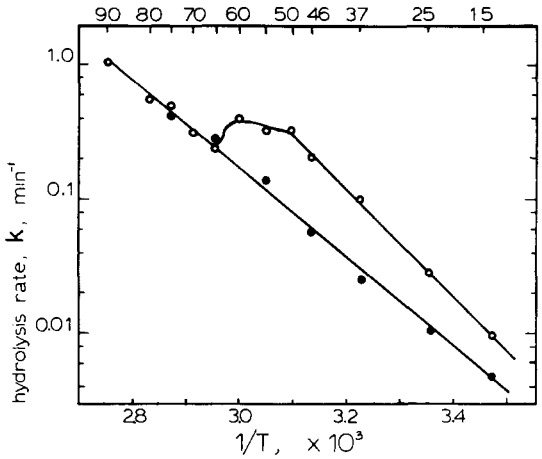

FIGURE 6: Temperature dependence of hydrolysis of native and denatured phospho-HPr. Hydrolysis rates of the 1-P-histidinyl residue in native P-HPr (○) and P-HPr in the presence of 0.4 g/mL urea (●) at pH 7.2 were determined for the temperature range 15–90 °C. The lower scale is temperature in degrees kelvin, and the upper scale is temperature in degrees centrigrade.

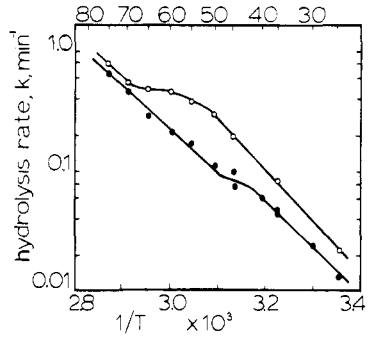

FIGURE 7: Temperature dependence of hydrolysis of phospho-HPr. Hydrolysis rates of the 1-P-histidinyl residue in P-HPr were determined at pH 4.3 (○) and pH 3.3 (●) for the temperature range 15–75 °C. The lower scale is temperature in degrees kelvin, and the upper scale is temperature in degrees centigrade.

obtained at pH 7.2 are shown in Figure 6.

Effect of Other Compounds on Phospho-HPr. The chemistry of phosphoramidates has been investigated by Jencks & Gilchrist (1965), Hultquist et al. (1966), and Hultquist (1968). The data in Table II in general show that the phosphoramidate bond in P-HPr was sensitive to nucleophilic attack by heterocyclic amines as expected. In general, the k_2 values were considerably higher than those observed by Jencks & Gilchrist (1965). Because enzyme $II^{glucose}$ appears to contain a 1-P-histidinyl residue (Peri et al., 1984) and because some sequence homologies exist between enzyme $II^{mannitol}$ and HPr (Saier et al., 1985), several sugars and simple alcohols were investigated. No indication of any transfer to glucose, fructose, glycerol, and ethanol was found. However, methanol up to about 30% (v/v) did increase the rate of hydrolysis (Table II). At 40% (v/v) methanol, the hydrolysis was inhibited presumably because of denaturation. The increased hydrolysis rates caused by methanol do not appear to be a solvent effect as neither ethanol nor dioxane was effective. Me_2SO was used to check the solvent effect, and the increased hydrolysis rates were presumably due to the production of "naked" nucleophilic ions (Gutsche & Pasto, 1975). At both pHs, 6.3 and 7.5, no indication of an increased hydrolysis rate was found for the following additions: $MgCl_2$, 10 mM; KF, 10 and 100 mM; NaCl, 0.1 M; KCl, 0.1 M; glucose, 10 mM, 0.1 M, and 0.4 M; fructose, 10 mM; dihydroxyacetone, 10 mM; glycerol, 20% (v/v); ethanol, 20% (v/v); pyridoxine, 80 mM; β-mercaptoethanol, 0.13 and 1.3 M. At both pHs, the following inhibited hydrolysis: methanol, 9.7 M (40% v/v); $MgCl_2$, 0.1 M. At pH 6.3, histidine, 0.11 M did not increase the hydrolysis rate. At pH 7.5, dioxane up to 40% had no effect on the hydrolysis rate. Neither dioxane nor Me_2SO was tested at pH 6.3.

Table II: Hydrolysis of 1-P-histidinyl Residue in P-HPr

added compound	concn (M)	pH	$k_{observed}$	k_1	k_2[a]
no addition		6.3[b]	0.12	0.12	
methanol	4.94	6.3	0.165		0.0091
pyridine	0.016	6.3	0.31		12.7
	0.031	6.3	0.51		12.2
niacin	0.01	6.3	0.18		6.0
	0.10	6.3	0.53		4.1
no addition		7.5[c]	0.089	0.089	
methanol	2.47	7.5	0.126		0.015
	4.94	7.5	0.143		0.011
	7.40	7.5	0.192		0.014
pyridine	0.013	7.5	0.165		5.8
	0.050	7.5	0.315		4.5
niacin	0.04	7.5	0.182		2.3
	0.10	7.5	0.277		1.9
imidazole	0.04	7.5	0.131		1.1
	0.10	7.5	0.185		1.0
histidine	0.02	7.5	0.099		0.50
	0.08	7.5	0.124		0.43
Me_2SO[d]	1.41	7.5	0.112		0.016
	2.82	7.5	0.136		0.017
	5.63	7.5	0.277		0.033

[a] $k_2 = (k_{observed} - k_1)/$(concentration of added compound). [b] 25 mM Mes buffer. [c] 25 mM Hepes buffer. [d] Not measured at pH 6.3.

Discussion

The 1-P-histidinyl residue in P-HPr of *E. coli* was first characterized by Anderson et al. (1971). This phosphoamino acid has since been identified in similar phosphocarrier proteins of the PTS in other bacterial species (Simoni et al., 1973; Kalbitzer et al., 1982; Waygood et al., 1984). Anderson et al. (1971) carried out their characterization of the phosphohistidinyl residue primarily on the two deamidated forms of HPr, HPr-1 and HPr-2, and in particular HPr-2. This appears to have concealed the true nature of the stability of the 1-P-histidinyl residue in HPr. The results in this paper show that the 1-P-histidinyl residue in HPr-2 has properties very similar to those of denatured HPr and thus its hydrolysis rates more closely resemble the known properties of the free 1-P-histidine (Hultquist, 1968).

The deamidated forms of HPr were reported to have altered activities; HPr-1 and HPr-2 had about 50% and 30% PTS activities, respectively, and it was concluded that the deamidation led to the loss in activities. The kinetic experiments reported here show that P-HPr-1, either frozen or boiled, had little impairment in its interactions with the three enzymes tested. This difference from the earlier work (Anderson et al., 1971) may be due to the use of improved assay procedures or the more accurate method of measuring HPr preparations by the independent lactate dehydrogenase method (Waygood et al., 1979). Because $HPr\text{-}1_{frozen}$ is always formed during frozen storage, it is fortunate that no significant differences between it and HPr have been found.

HPr-2 with its impaired hydrolysis rates (Figure 4B) and its altered kinetic parameters (Table I) is clearly different from either HPr or HPr-1. There are seven glutamine residues in HPr, and it is not known whether the generation of HPr-1 and HPr-2 is due to unique deamidations. $HPr\text{-}1_{boil}$ and HPr-2 did not appear on isoelectric focusing gels as single proteins, but rather for each protein two bands were evident that had very close p*I* values (Figure 3). This suggests that three or four of the glutamine residues could be involved in HPr-1 (boil)

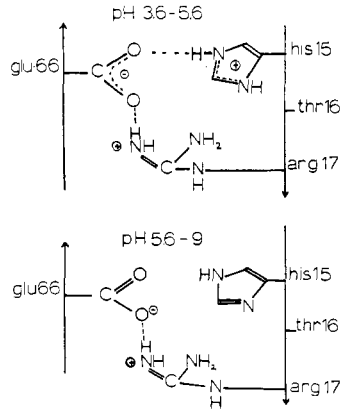

FIGURE 8: Active site of HPr. Examination of the structure of HPr by 2.5-Å resolution X-ray diffraction of HPr crystals obtained at pH 3.7 showed that Glu-66 was interacting with both His-15 and Arg-17 (El-Kabbani et al., 1984a,b). The probable arrangement of the three side groups in the crystal at pH 3.7 is shown in the upper diagram. At physiological pH, due to a pK = 5.6 for the imidazole ring of His-15, the arrangement shown in the lower part of the figure would predominate (drawings not to scale).

and HPr-2 production. HPr-2 appears to have a different conformation because (1) the 1-P-histidinyl residue is stable (Figure 4B), (2) no glutamines are near the active site and thus could not directly affect hydrolysis rates (El-Kabbani et al., 1984a,b), (3) HPr undergoes at least partial unfolding at elevated temperatures (Figures 5–7) and thus must renature when cooled, and (4) NMR measurements show that cooled HPr that had been boiled for 15 min contained about one-third of the molecules in an altered conformation (R. Klevit, unpublished results). These considerations suggest that for HPr-2 it will be difficult to separate the effects on activity by the conformational changes due to boiling from the effects of the chemical change of a glutamine becoming a glutamate. Beneski et al. (1982) have reported that the genetic conversion of Glu-4 to either serine or lysine does not affect the generation of HPr-1 and HPr-2. Examination of the tertiary structure of HPr (El-Kabbani et al., 1984a,b) shows that Gln-21 is in an obvious position to interfere with the interactions contributing to the tertiary structure of HPr. Gln-21 when converted to a glutamyl residue may interfere with a salt bridge between Glu-25 and Lys-27.

A presentation of the proposed active site of HPr as determined by X-ray diffraction studies (2.5-Å resolution) will facilitate further discussion. The active-site HPr has a structure which involves the interaction of Glu-66, His-15, and Arg-17 as shown in Figure 8 (El-Kabbani et al., 1984a,b). As the crystals were obtained at pH 3.7, the imidazole ring of His-15 is protonated, and both it and Arg-17 appear to interact with Glu-66. His-15 and Arg-17 are conserved in HPr from *E. coli*, *S. typhimurium*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Bacillus subtilis* (Beyreuther et al., 1977; Weigel, 1978; Weigel et al., 1982b; Muss, 1982; Powers & Roseman, 1984). Glu-66 is conserved in *E. coli*, *S. typhimurium*, and *S. pyogenes* and is displaced to Glu-62 in *S. aureus*, while the *B. subtilis* sequence is not known completely. The absence of homology in sequences other than these active-site regions argues for the importance of these three residues. His-15 has been identified as the active-site histidine which becomes a 1-P-histidinyl residue (Anderson et al., 1971; Simoni et al., 1973; Kalbitzer et al., 1982), and Arg-17 has been implicated by chemical modification studies and NMR studies (Kalbitzer et al., 1982).

At physiological pH, the imdiazole ring of His-15 should not be protonated as its pK has been determined to be 5.6

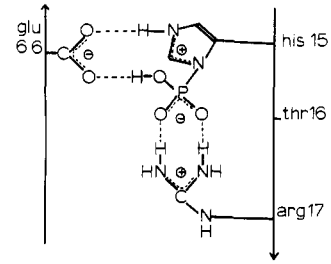

FIGURE 9: Active site of P-HPr. The diagram shows the probable arrangement of the 1-P-histidinyl residue with the Arg-17 and Glu-66 residues.

(Dooijewaard et al., 1979). Thus, Glu-66 would preferentially interact with Arg-17, and His-15 would be freer to move as no other restraints appear to affect the N-terminal region of HPr (El-Kabbani et al., 1984a,b). When His-15 is phosphorylated, two things are known to occur: the pK of the imidazole ring increases to 7.8, and there is a conformational change (Dooijewaard et al., 1979). The change in pK results in protonation of the imidazole ring of His-15 at physiological pH, and hence an ability to interact with Glu-66. Thus, the two conformations may be simply due to the change in protonation of the imidazole ring, and they are shown in Figure 8.

The results in this paper are consistent with the active-site arrangement of phospho-HPr as shown in Figure 9. While the symmetry of the Glu-66 interaction with both the N-3 imidazole position and the hydroxyl group of the phosphate is appealing, the interaction may only be with the N-3 imidazole position. The net effect of the interaction of Glu-66 with the N-3 imidazole position and the interaction of the Arg-17 guanidino group with the N-1 phosphate is to weaken the phosphoramidate bond at the N-1 position. This would imply that this phosphate would be removed more easily by nucleophilic agents including histidine. The data in Figure 4 show that the hydrolysis rates of P-HPr in its native conformation are higher than the rates for either denatured P-HPr or the free 1-P-histidine. In particular, the increase in rates occurs over the physiological pH range. The data in Table II confirm that the 1-P-histidinyl residue is more sensitive to the action of nucleophilic agents as the k_2 values are significantly higher than those determined for phosphoramidate bonds (Jencks & Gilchrist, 1965).

The action of histidine, when the imidazole ring is not protonated, was to be expected as P-HPr transfers its phosphate to either factor III^{sugars} or enzyme II^{sugars} which have 3-P-histidines. Free 1-P-histidine will phosphorylate free histidine, resulting in the formation of 3-P-histidine (Hultquist, 1968). In factor III^{Glc}, the hydrophobic sequence around the active-site His-91 (Dorschug et al., 1984; Nelson et al., 1984) suggests that the transfer between P-HPr and factor $III^{glucose}$ occurs in the absence of water. Some experiments have been carried out by using dioxane (20% v/v) and histidine, but the rate of hydrolysis was not increased greatly.

The shape of the curve for the pH dependence of hydrolysis rates is compatible with the model in Figure 9. The shift of the decline in rates at alkaline pH is consistent with the difference in pK between free 1-P-histidine (or 1-P-histidine in denatured P-HPr) and the 1-P-histidinyl residue in native P-HPr. The decline between pH 4.5 and 2.6 is consistent with the protonation of Glu-66, thus eliminating its influence on His-15. The similarity of hydrolysis rates between the 1-P-histidinyl residue in denatured P-HPr and free 1-P-histidine must be due to the interaction of the guanidino group of Arg-17 with the N-1 phosphate of the 1-P-histidinyl residue

in a manner similar to the interaction of the positively charged α-amino group with the N-1 phosphate in free 1-P-histidine (Hultquist, 1968). A bidentate arrangement for the interaction of phosphate with arginine residues has been proposed for staphylococcal nuclease (Cotton et al., 1979).

The denaturation of P-HPr by either urea or heat unfolds the HPr structure, or at the very least separates Glu-66 from Arg-17 and His-15. However, it appears that Arg-17 and His-15, being close to each other, continue to interact. The heat denaturation of P-HPr shows that between pH 4.3 and 7.3 a high temperature had to be reached before the interaction between Glu-66 and His-15 was broken (Figures 5–7). This is interpreted as reflecting the strong interaction between deprotonated Glu-66 and the protonated phosphohistidine-15 at these pH values. When either Glu-66 becomes essentially protonated (e.g., pH 3.3) or 1-phosphohistidine-15 becomes essentially deprotonated (e.g., pH 8.4), the interactions were disturbed at much lower temperatures because they are weaker interactions. Thus, both the pH dependence of hydrolysis rates and the temperature-dependent denaturation are consistent with the active-site model proposed in Figure 9.

As pointed out above, the similarity between the hydrolysis patterns for 1-P-histidine and the 1-P-histidinyl residue in denatured HPr is probably due to the interaction of the guanidino group of Arg-17 with the phosphate. If the conclusion of Hultquist (1968) is correct, that the positively charged α-amino group of 1-P-histidine is in part responsible for its greater lability of the phosphoryl group in respect to 3-P-histidine, then the following possibilities may exist: (1) A 1-P-histidinyl residue in a peptide chain in the absence of an appropriate positively charged group should have hydrolysis rates similar to a 3-P-histidine. (2) A 3-P-histidinyl residue in a peptide chain with an appropriate amino group properly oriented should have hydrolysis rates similar to a 1-P-histidine. Recently, Hefford et al. (1985) have shown that the N-terminal histidine in glucagon and vasoactive intestinal peptide has unusual chemical properties because of the interaction between the positively charged α-amino group and the imidazole ring.

The arrangement shown in Figure 9 is not altogether without precedent. The interaction of a carboxyl group with the N-1 position of a histidinyl residue has been proposed by Vogel & Bridger (1983a) as a means of specifying phosphorylation at the N-3 position, as normally the N-1 position is the better nucleophile. Such an interaction not only would specify the position of phosphorylation but also would act to destabilize the subsequent phosphoramidate bond and make it more able to participate in a phosphoryl transfer reaction. The immobilization of the 1-P-histidinyl residue in HPr has been deduced by Vogel et al. (1982), and a similar immobilization has been reported for the phosphoseryl residue at the active site of phosphoglucomutase (Ray et al., 1977). The importance of such immobilizations to the specificity of transfer or to the rate of catalysis has been pointed out by Knowles (1980) and Mildvan (1975). In addition, the regulatory phosphoserine in glycogen phosphorylase is protected from the action of the phosphatase by the immobilization of the phosphoserine by two arginine residues (Vogel & Bridger, 1983b).

The occurrence of 1-P-histidinyl residues at active sites is not common. Winn et al. (1981) suggest that the active site of phosphoglycerate mutase with two histidine residues in parallel and in position to interact with the two phosphorylation sites of the substrate isomers may contain two phosphohistidine intermediates. Han & Rose (1979) have only been able to isolate a 3-P-histidinyl residue, and thus the double displacement mechanism is favored (Knowles, 1980). The active site of phosphoglycerate mutase has features similar to that of HPr. The two histidinyl residues are associated with two glutamyl residues, and an arginyl side chain appears to be located between the two histidinyl residues such that it could interact with a N-1 phosphohistidinyl residue if formed on either histidine. If two phosphohistidines are formed in the course of the phosphoglycerate mutase catalyzed reaction, the phosphoryl transfer reactions of the PTS would suggest that such should involve transfers from 1-P-histidine to 3-P-histidine and vice versa. By analogy to P-HPr, a 1-P-histidinyl residue formed in phosphoglycerate mutase would be extremely labile as it may have many of the structural features shown in Figure 9 and in addition would have two nucleophiles close by, either the substrate hydroxyl or the other histidinyl residue. Hence, it may prove extremely difficult to detect such an intermediate by the traditional techniques.

The structure proposed in Figure 9 cannot by proved by conventional X-ray crystallographic techniques as the 1-P-histidinyl residue is too labile. However, data for a structure of HPr at 1.9-Å resolution are being processed (unpublished results), and examination of the solution structure of HPr by two-dimensional NMR techniques appears to have been highly successful (R. Klevit and E. B. Waygood, unpublished results). It should be possible in the near future to apply the two-dimensional NMR technology to the structure of P-HPr and either confirm or improve this proposed active-site arrangement. The PTS may prove to be an interesting experimental example of phosphoryl transfer reactions in proteins as each step (or intermediate) is composed of separate components.

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Registry No. PTS, 56941-29-8; L-His, 71-00-1; L-Glu, 56-86-0; L-Arg, 74-79-3; 1-P-His, 5789-14-0; enzyme I, 37278-17-4; enzyme II, 37278-09-4.

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Physiological Roles of Zinc and Calcium Binding to α-Lactalbumin in Lactose Biosynthesis†

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ABSTRACT: Bovine apo-α-lactalbumin was shown to be severalfold more efficient than its calcium conformer as a cofactor in lactose biosynthesis. This rate enhancement was manifested in a 3.5-fold increase in V_{max}, with no differences in K_m(app) between the two α-lactalbumin forms. In the presence of zinc, which shifts Ca(II)–α-lactalbumin toward the "apo-like" conformation [Musci, G., & Berliner, L. J. (1985) *Biochemistry 24*, 3852–3856], the catalytic rate constant for lactose synthesis was identical for both the Ca(II) and apo conformers. Activity measurements at different temperatures, on the other hand, confirmed that calcium is important in stabilizing the protein (α-lactalbumin) against thermal denaturation. The stabilizing effect of calcium was independent of the presence of Zn(II), i.e., of the protein conformation. The physiological implications of these results are discussed.

The modifier protein α-lactalbumin (α-LA)[1] is the noncatalytic regulatory subunit of the "lactose" synthase complex (UDP-galactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22). The association of α-LA with galactosyltransferase (GT) imparts a change in specificity of the latter enzyme from terminal *N*-acetylglucosaminyl acceptors to glucose. Previous work with several α-LA species has described the extremely strong binding of calcium and several lanthanides ($K_d \approx 10^{-9}$–10^{-12} M) to a specific site, which also bound Mn(II) (K_d

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[1] Abbreviations: α-LA, α-lactalbumin; GT, galactosyltransferase; UDP-Gal, uridine 5′-diphosphate galactose; GlcNAc, *N*-acetylglucosamine; bis-ANS, 4,4′-bis[1-(phenylamino)naphthalene-8-sulfonate]; UDP, uridine 5′-diphosphate; Glc, glucose; PK/LDH, pyruvate kinase/lactate dehydrogenase; T_m, thermal melting temperature; Tris, tris(hydroxymethyl)aminomethane.

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