(Collatz et al., 1977), and L13′ and L18′ (Tsurugi et al., 1978). Some, if not all, of these proteins may have been produced from genuine ribosomal proteins as similar artifacts by aggregation or degradation.

Itoh and Wittmann-Liebold (unpublished data) recently determined the sequence of YP 55 almost completely and compared it with amino-terminal sequences of rat liver proteins. The highest homology (53% in the first 30 amino acid residues of the amino-terminal region) was obtained between YP 55 and rat liver L37.

Amons et al. (1977) reported the amino-terminal sequence of YP A1 and YP A2, which revealed a considerable sequence homology with an acidic ribosomal protein not only from *A. salina* but also from *Halobacterium cutirubrum*. This suggests the general occurrence of these proteins in eucaryotes and at least in one bacteria. Using our data, Tsurugi et al. (1978) compared the amino acid compositions of YP A1 and YP A2 with acidic protein(s) from other organisms, but as yet there is no conclusive evidence indicating homology between eucaryotic acidic proteins and *E. coli* L7/L12.

In the course of this study we have purified 23 proteins, which represent nearly half of some 40 protein species generally reported for the eucaryotic large subunit. Purification of the remaining proteins is now under way.

Acknowledgments

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The *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Observation of Heterogeneity in the Amino Acid Composition of HPr†

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

ABSTRACT: Resonances of the aromatic protons of tyrosine have been observed in the proton nuclear magnetic resonance (^{1}H NMR) spectrum of purified HPr from *Escherichia coli*. Analysis of the NMR spectrum of native HPr suggests that the tyrosine is located in a single position in the secondary structure and that this position is on the interior of the molecule inaccessible to solvent. Previous reports suggested that *E. coli* HPr contained no tyrosine [Anderson, B., Weigel, N., Kundig, W., & Roseman, S. (1971) *J. Biol. Chem. 246*, 7023–7033]. In contrast, we find, by amino acid analysis and ultraviolet and NMR spectroscopy, that *E. coli* HPr does contain tyrosine but at a subintegral level of 0.5 ± 0.1 mol of tyrosine per mol of HPr.

HPr is a phosphoryl group carrying protein which functions as an intermediate in the transfer of a phosphoryl group during the energized transport of sugars according to eq 1.[1]

$$\begin{aligned} \text{PEP} + \text{E}_\text{I} &\xrightarrow{\text{Mg}^{2+}} \text{P–E}_\text{I} + \text{pyruvate} \\ \text{P–E}_\text{I} + \text{HPr} &\leftrightarrow \text{P–HPr} + \text{E}_\text{I} \\ \text{P–HPr} + \text{hexose}_{(\text{out})} &\xrightarrow[\text{Mg}^{2+}]{\text{E}_\text{II}} \text{HPr} + \text{hexose–P}_{(\text{in})} \end{aligned} \quad (1)$$

The phosphoryl group transfer and sugar transport processes are catalyzed by the enzymes E_I and E_{II}, respectively (Kundig & Roseman, 1971). *Escherichia coli* HPr was first isolated and characterized by Roseman and co-workers (Roseman,

† From the Department of Physical Chemistry, University of Groningen, 9747 AG Groningen, The Netherlands. *Received August 30, 1979.* This research and the 360-MHz NMR facility at the University of Groningen have been supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

‡ Present address: Gaubius Institute, Health Research Organization, TNO, 2313 AD, Leiden, The Netherlands.

[1] Abbreviation used: PEP, phosphoenolpyruvate.

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1969; Anderson et al., 1971). It is a small protein, 9600 daltons, whose published amino acid composition is notable particularly for its lack of tyrosine and tryptophan. Four moles of phenylalanine and two moles of histidine per mole of HPr are the only aromatic amino acids reported (Anderson et al., 1971). The UV spectrum reflects this aromatic amino acid composition inasmuch as it is dominated by an absorption profile characteristic of phenylalanine. Nevertheless, a distinct shoulder is present in the absorption profile with a maximum between 275 and 285 nm which cannot be attributed to phenylalanine.

Because HPr is an intermediate in the energy-transfer process, we have been carrying out detailed solution structural studies on HPr and P–HPR and the conversion between the two in an effort to gain insight into the mechanism of transfer and conservation of chemical energy. Part of these studies involved the application of high-resolution proton magnetic resonance, in which we have monitored the histidine and aromatic amino acid proton resonances as well as selected aliphatic proton resonances of HPr and P–HPr (Dooijewaard et al., 1979a). During these measurements, we have observed resonances in the aromatic proton region of the NMR spectrum which cannot be attributable to phenylalanine. The subsequent investigations, reported below, demonstrate that the resonances arise from the aromatic protons of tyrosine, previously not observed in *E. coli* HPr.

Materials and Methods

Bacteria. Both the *E. coli* K235 and *Salmonella typhimurium* LT2 cultures were obtained from the American Type Culture Collection, Rockville, MD. Single colonies of each culture were used to prepare the inocula for the large-scale fermentations which provided the cells for these experiments.

HPr. E. coli HPr was purified according to the procedure of Dooijewaard et al. (1979b). *S. typhimurium* HPr was purified according to the same procedure; however, a second DEAE-cellulose chromatography, using a gradient elution, was performed following the Sephadex G-75 gel filtration step. The resulting HPr was pure by polyacrylamide disc gel electropheresis. *E. coli* K235 was grown and harvested as stated previously (Dooijewaard et al., 1979b). *S. typhimurium* LT2 was grown on the same media as the *E. coli* but under two conditions, with and without the addition of extra glucose during the fermentation. After being harvested, each batch of cells was split in two. One half was washed by normal procedures, and the other half was treated, after being washed, with phenol to kill the bacteria. The cells were stored as frozen cell paste at −20 °C until use.

Amino Acid Analysis. Fifty to one hundred and fifty micrograms of HPr was dissolved in doubly distilled 6 N HCl. After addition of 25 nmol of norleucine as an internal standard, the tubes were sealed under vacuum and heated for 12–72 h at 110 °C. Amino acid analyses were performed on a Kontron Liquimat III amino acid analyzer.

Isoelectric focusing was performed on gels in narrow tubes in the presence of urea and mercaptoethanol by the method of O'Farrell (1975), as modified by Ames & Nikaido (1976).

Pronase Treatment of HPr. A 1.5-mg amount of HPr, lyophilized in 0.05 M KCl, was dissolved in 200 μL of 0.1 M NaOH and heated at 37 °C for 1 h. Twenty microliters of Tris-HCl (0.1 M, pH 7.7) was added and the pH adjusted to 7.5. A fresh stock solution consisting of 2 mg of Pronase (Calbiochem B grade) dissolved in 0.1 M Tris-HCl and 20 mM $CaCl_2$, pH 7.8, was prepared for each new addition of Pronase to HPr. Three additions were made at 12-h intervals, 5 μL/addition, followed by incubation of the sample at 37 °C.

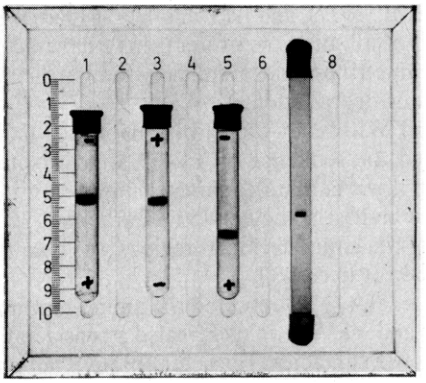

FIGURE 1: Standard polyacrylamide disc gel electrophoresis of pure HPr with (1) 15% polyacrylamide gel at pH 9.5, (3) 15% polyacrylamide gel at pH 4.5, and (5) 15% polyacrylamide gel at pH 9.5 in the presence of $NaDodSO_4$. (7) Isoelectric focusing between pH 4 and 6 of pure HPr in the presence of urea and mercaptoethanol (see Materials and Methods).

After the third incubation, the sample was heated for 5 min at 85 °C in a water bath. After the solution was cooled, a final 5-μL volume of Pronase was added and the sample was incubated at 37 °C for 6 h. It was subsequently lyophilized after addition of 20 μL of 20 mM EDTA. Deuterium exchange was carried out by repeated lyophilization of the sample from D_2O (99.8% D, Merck).

NMR spectra were recorded on a Bruker HX 360-MHz apparatus operating in either the Fourier or the correlation spectroscopy mode. The temperature was regulated to ±1 °C. The spectrometer was equipped with a Nicolet 1080 computer. Spectra used for intensity determinations were always collected at two different pulse repetition rates which differed by a factor of 3 in time. If saturation due to incomplete T_1 relaxation was observed, the pulse repetition rate was lengthened until no saturation effects were observed. Varied power levels were employed if the spectra were obtained by correlation spectroscopy to check for the possibility of saturation.

Integration of the NMR Spectra. Two different procedures have been employed: (1) measuring the area under a resonance on the plotted spectrum by counting square millimeters; (2) integration by computer using the integration subroutine of the Nicolet FT-75 program. A difference of less than 10% was found when both methods were used to integrate the same spectra.

Results

Purified *E. coli* HPr electrophoresed on polyacrylamide disc gels at pH 9.5 and 4.5 and in the presence of sodium dodecyl sulfate ($NaDodSO_4$) at pH 9.5 results in the gels shown in Figure 1, gels 1, 3, and 5, respectively; they have been published previously and are presented here for clarity (Dooijewaard et al., 1979b). When this HPr was subjected to isoelectric focusing over the pH range 4–6 on gels in narrow diameter tubes, the pattern in gel 7 of Figure 1 was obtained. The HPr preparation appears to be homogeneous by all of these criteria.

The UV spectrum of this purified HPr is shown in Figure 2A. It is identical with that presented by Anderson et al. (1971). It is dominated by the absorption spectrum of phenylalanine, as can be seen by comparing parts A and B of Figure 2. But, in contrast to phenylalanine, the spectrum of HPr has a shoulder of absorption between 275 and 285 nm (see the dashed line in parts A, C, and D of Figure 2). Although HPr is reported to contain 4 mol of phenylalanine and less than 0.1 mol of tyrosine and tryptophan (Anderson et al., 1971), the UV spectrum suggests higher levels of tyrosine.

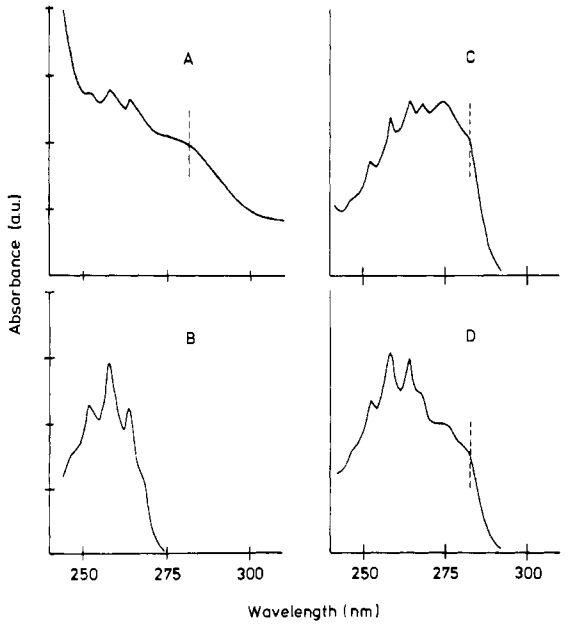

FIGURE 2: (A) UV spectrum of pure *E. coli* HPr in 50 mM KCl at pH 7. Plot of the absorbance of (B) phenylalanine, (C) a mixture of phenylalanine and tyrosine in a ratio of 4:1, and (D) a mixture of phenylalanine and tyrosine in a ratio of 9:1. The absorbances are those of the amino acids at neutral pH (*Handbook of Biochemistry*). The dotted line indicates 282.5 nm.

Parts C and D of Figure 2 are UV absorption profiles calculated by adding the molecular absorptions (*Handbook of Biochemistry*), at each wavelength, for tyrosine and phenylalanine in different ratios. No attempt has been made to calculate the contributions of the amide absorption which would be present in a protein spectrum. Therefore, the intensities below 250 nm in the calculated absorption profiles, parts B, C, and D of Figure 2, differ substantially from what is found for the protein in Figure 2A. In the region above 250 nm, the absorption profile for the ratio 1 Tyr/9 Phe, Figure 2D, more closely approximates the spectrum of HPr than does the profile for the ratio 1 Tyr/4 Phe, Figure 2C. This would also continue to be true even if a strong absorption below 250 nm were included. The plateau at ~270 nm in the spectrum of HPr is also found in the absorption profile of 1 Tyr/9 Phe (compare parts A and D of Figure 2), but it is not found in the absorption profile of 1 Tyr/4 Phe (Figure 2C). No amount of absorption intensity at 250 nm and below will alter these features. Since only 4 mol of phenylalanine has been found per mol of HPr (Anderson et al., 1971), a preliminary interpretation of the UV spectrum suggests that the tyrosine level in HPr is closer to 0.5 mol/mol of HPr instead of 1 mol/mol of HPr.

Tyrosine Determination. (*a*) *Amino Acid Analysis.* Time-dependent hydrolyses in 6 N HCl, followed by amino acid analysis, have been performed on *E. coli* HPr. The results are presented in Table I along with the previously determined composition of Anderson et al. (1971). Of particular interest is the level of tyrosine found in the present measurements. It is constant for the 24-, 48-, and 72-h hydrolysates (data not shown). Relative to the 2 mol of histidine per mol of HPr (Anderson et al., 1971), we consistently find 0.5 ± 0.05 mol of tyrosine per mol of HPr.

(*b*) *NMR Measurements.* The 2,6 and 3,5 protons of the tyrosine ring generate a characteristic NMR spectrum consisting of two resonances, each of which is a doublet. Figure 3A presents the NMR spectrum of 1 mg of *E. coli* HPr hydrolyzed in 6 N HCl for 12 h at 110 °C. The two doublets

Table I: Amino Acid Composition of *E. coli* HPr

amino acid	this work[a]	Anderson et al. (1971)	amino acid	this work[a]	Anderson et al. (1971)
Asp	5.2	3.6	Met	1.6	2.1
Thr	8.9	10.0	Ile	3.5	3.4
Ser	6.1	6.9	Leu	8.0	8.5
Glu	14.8	13.5	Tyr	0.50	<0.05
Pro	2.6	2.2	Phe	4.3	4.0
Gly	7.2	6.7	Lys	7.1	8.1
Ala	8.7	9.4	His	2.0	2.0
Cys	<0.05	<0.05	Arg	1.6	0.96
Val	6.4	7.1			

[a] These values are an average of the analysis of four separate 24-h hydrolyses on four different preparations of *E. coli* HPr. They are corrected to the molecular weight of HPr found by Anderson et al. (1971).

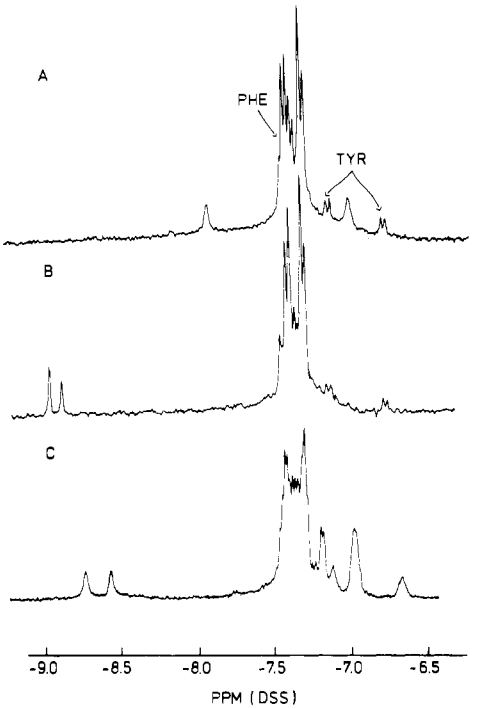

FIGURE 3: (A) 360-MHz ^{1}H NMR spectrum of 1 mg of *E. coli* HPr hydrolyzed for 12 h at +110 °C in 6 N HCl (see Materials and Methods). The pH of the NMR sample was 7.5. (B) Resolution-enhanced 360-MHz ^{1}H NMR spectrum of 1.5 mg of HPr treated with Pronase (see Materials and Methods). The pH of the sample was 1.1. (C) 360-MHz ^{1}H NMR spectrum of native *E. coli* HPr in 10 mM Tris-HCl and 0.1 M KCl, pH 4.6.

at −7.1 and −6.8 ppm occur at the same chemical-shift positions as the resonances arising from the tyrosine 2,6 and 3,5 protons, respectively, and offer strong evidence of the presence of tyrosine. The large block of resonance intensity centered at −7.35 ppm arises from phenylalanine, and the two unmarked resonances, one on the low-field side of the phenylalanines and the other between the tyrosine doublets, belong to the histidine C-2 and C-4 protons, respectively. The relative levels of tyrosine, phenylalanine, and histidine determined from the area under these peaks are approximately the same as those found by amino acid analysis (see Table II).

Because of our concern that the subintegral levels of tyrosine might originate from the harsh 6 N HCl treatment, a second hydrolysis procedure was employed using Pronase. This degrades the protein into smaller fragments and produces a partially random coil spectrum. The NMR spectrum of the Pronase-degraded HPr is presented in Figure 3B. Two reso-

Table II: Composition of Aromatic Amino Acids of *E. coli* HPr

method	treatment	His	Phe	Tyr
NMR[a,d]		2.0	4.8	0.49
UV[b]			4.5	0.50
amino acid analysis[c]	6 N HCl	2.0	4.3	0.50
NMR[a,d]	6 N HCl	2.0	4.6	0.50
NMR[a,d]	Pronase	2.0	5.0	0.48

[a] The intensities of the Tyr and Phe resonances are determined relative to the histidine resonances. The intensity of the histidine resonance is assumed to equal 2 (Anderson et al., 1971). [b] By comparison with the molecular absorptions of Tyr and Phe (see text). [c] See footnote *a*, Table I. [d] Intensity was determined on a non-resolution-enhanced spectrum.

nances for the histidine C-2 protons occur at low field in Figure 3B, indicating that part of the protein is in the random coil form and the remainder still retains some structure. Nevertheless, the spectrum is well enough resolved to be able to discern the two sets of doublets at −7.1 and −6.8 ppm. The integrated areas listed in Table II show that even with this chemically mild treatment only 0.5 mol of tyrosine per mol of HPr can be detected.

A tyrosine resonance can also be seen in the aromatic region of the native HPr NMR spectrum, Figure 3C. This is not a highly resolved spectrum which would enable one to see the *J* coupling in a tyrosine doublet as is observable in parts A and B of Figure 3. Presumably, aggregation leads to lower resolution. Unfortunately, increased temperature cannot be used to improve the resolution since HPr precipitates above 45 °C at these NMR concentrations. The histidine protons have been assigned by pH titrations (Dooijewaard et al., 1979a). Most of the intensity in the region of −7.5 to −7.2 ppm and in the resonance at −7.0 ppm is attributable to phenylalanine protons. The small resonance at −6.7 ppm does not arise from histidine protons and is not of the correct intensity for a phenylalanine proton resonance. However, it does not have a chemical-shift position characteristic of a tyrosine resonance in a protein but not of free tyrosine (see parts A and B of Figure 3), and furthermore it corresponds in intensity to the level of tyrosine found in the hydrolyzed HPr samples in parts A and B of Figure 3.

HPr has also been purified from *S. typhimurium* LT2 (see Materials and Methods). Its UV spectrum is identical with that presented in Figure 2A for *E. coli* HPr. The results of amino acid analysis indicate that HPr from this species also contains 0.5 mol of tyrosine per mol of HPr. The aromatic resonance region of the ^{1}H NMR spectrum of *S. typhimurium* HPr is presented in Figure 4A. It is identical with that of *E. coli* HPr even down to the low-intensity resonance at −6.7 ppm. Parts B and C of Figure 4 are spectra in which the resonances tentatively attributed to tyrosine are selectively decoupled. The two doublets for the 2,6 and 3,5 protons of free tyrosine are separated by 0.29 ppm. On the assumption that the resonance at −6.7 ppm in native HPr, Figure 4A, is a broadened doublet arising from the 3,5 protons of the tyrosine, then the doublet for the 2,6 protons should be downfield under the large resonance at approximately −7.0 ppm. Irradiation of one resonance should result in a narrowing of the line width of the second resonance since the spin–spin coupling between the 2 and 3 protons and the 5 and 6 protons will be eliminated. This is easiest to see in Figure 4B where the resonance at −7.0 ppm is irradiated, resulting in a 50% decrease in line width of the −6.7 ppm resonance. A decrease in the resonance intensity also accompanies the decrease in line width. It results from a negative nuclear Overhauser enhancement. On the upper right-hand side of Figure 4 is a vertical expansion

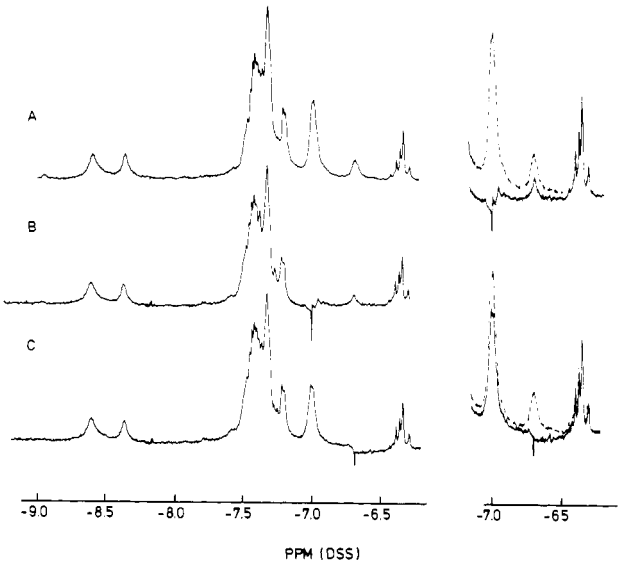

FIGURE 4: 360-MHz ^{1}H NMR spectra of *S. typhimurium* LT2 HPr in 10 mM Tris-HCl and 0.1 M KCl, pH 5.5, at 36 °C. (A) Without irradiation; (B) with irradiation at −7.0 ppm; (C) with irradiation at −6.7 ppm; (upper right-hand corner) vertical expansion of the −7.2 to −6.3 ppm region of spectrum (A) without irradiation (dashed line) and spectrum (B) with irradiation at −7.0 ppm (solid line); (lower right-hand corner) vertical expansion of the same spectral region of spectrum (A) without irradiation (dashed line) and spectrum (C) with irradiation at −6.7 ppm (solid line).

showing the results of this decoupling in the region from −7.2 to −6.3 ppm. The dashed-line spectrum is from Figure 4A (no irradiation); the solid-line spectrum is from Figure 4B, with irradiation at −7.0 ppm and collapse of the −6.7-ppm resonance. The complementary experiment is shown in Figure 4C and in the expanded region on the lower right-hand side. Since the tyrosine resonance contributes only a small amount to the intensity of the peak at −7.0 ppm, it is difficult to measure any line width decrease upon irradiation at −6.7 ppm. However, the same intensity loss occurs at −7.0 ppm upon irradiating at −6.7 ppm as occurred in Figure 4B at −6.7 ppm when irradiating at −7.0 ppm. These intensity losses cannot be attributed to a too broad decoupling pulse giving rise to a-specific saturation since the intensities of other resonances equidistant from the decoupling pulse are not altered.

(*c*) *pH Titration Studies.* During the ionization of the phenolic group, the aromatic protons of tyrosine shift upfield 0.15–0.35 ppm. In free tyrosine or in a random coil peptide the p*K* of this ionization is ~10–10.5. HPr ^{1}H NMR spectra recorded at various pH values between pH 8 and 13 showed no shift of the tyrosine resonances at −6.7 and −7.0 ppm until pH 11.8, at which point large changes in the spectrum occur indicative of a general denaturation of HPr.

Discussion

Anderson et al. (1971) reported finding less than 0.05 mol of tyrosine per mol of *E. coli* HPr by amino acid analysis and used, in confirmation of this observation, the UV spectrum which was interpreted to resemble that of phenylalanine. We have shown, on the contrary, that that same UV spectrum definitely indicates substantial levels of tyrosine in HPr; however, the levels are subintegral. Both amino acid analysis and analysis of the ^{1}H NMR spectra of *E. coli* HPr confirm these observations. In addition, the same levels of tyrosine have been observed in *S. typhimurium* HPr.

Is it possible that the tyrosine arises from a contaminating protein or peptide which copurifies with HPr? This is unlikely for the following reasons.

(1) Polyacrylamide disc gel electrophoresis at pH 9.5 and 4.5 as well as electrophoresis in the presence of $NaDodSO_4$ shows that the HPr preparation is homogeneous. Furthermore, isoelectric focusing under denaturing conditions shows only a single band.

(2) A contaminating protein might not be detectable on the gels if its concentration were 5% of the HPr concentration. If the tyrosine which we are reporting were to originate from such a contaminant, it would have to be located in at least 10 different positions in the sequence to account for the levels found. In the NMR spectra, however, all the tyrosine is found in a single resonance at a chemical-shift position different from that for tyrosine in a random coil. Therefore, the tyrosine must be located in a single-structured environment. Since this resonance does not shift upon increasing the pH between 8 and 11.5 but only at higher pH when the entire HPr unfolds, the single environment appears to be a part of HPr.

(3) Another argument against the tyrosine originating from a contaminating protein is that the HPr from *E. coli* appears to be identical with that from *S. typhimurium*. The chance that the same contaminating protein with the same level of tyrosine would copurify with the HPr from both species is very small, especially since the cells have been grown under different conditions and the HPr's purified by different procedures.

The levels of tyrosine found are subintegral. By all the methods used and shown in Table II, we consistently find 0.5 mol of tyrosine per HPr. In addition, we find an average of 4.65 mol of phenylalanine. A number of explanations are possible. First, HPr in the cell could contain a full mole of tyrosine. During purification, as a result of limited proteolysis, a portion of the tyrosine-containing peptides could be hydrolyzed and lost. A number of observations argue against this interpretation. $NaDodSO_4$ gels show only one molecular weight species; therefore, the tyrosine peptide would have to be very close to the C or N terminal. NMR data, however, suggest that this peptide is buried and inaccessible to solvent, making it inaccessible as well to proteolytic enzymes. Furthermore, if proteolysis is the cause of this low level of tyrosine, we would expect some preparations to have higher levels, 1 mol of tyrosine per mol of HPr for instance, and others to have lower levels. Yet, out of preparations from 30 different isolations, we have always found the same level, 0.5 $\pm$ 0.1 mol of tyrosine per mol of HPr.

The second interpretation is that there are at least two forms of HPr coded for by different genes. Since this has not previously been detected by genetic mapping studies, the two genes may reside very close together on the same operon. One gene would code for HPr containing 4 Phe and 1 Tyr and the other gene would code for HPr containing 5 Phe and 0 Tyr. The net result would be 4.5 Phe and 0.5 Tyr if both genes were read with equal frequency. If two genes for two separate HPr's exist, the amino acid replacements must be minimal since only a single population of molecules can be detected by isoelectric focusing. In such a case, these other amino acid replacements will be more difficult to detect by amino acid analysis or NMR since most amino acids are already present in HPr.

Anderson et al. (1971) have described multiple forms of HPr which they named HPr 1 and 2. These forms apparently resulted from the heat-treatment step employed in their original purification procedure which, according to Anderson et al. (1971), resulted in deamination. The forms were detected by polyacrylamide gel electrophoresis, HPr, HPr 1, and HPr 2 having differing mobilities. The heterogeneity discussed above is unrelated to the HPr 1 and 2 found earlier. Our preparations are purified at 5 °C without incorporation of a heat-treatment step. Furthermore, the preparations, as shown in Figure 1, have a single mobility upon electrophoresis and isoelectric focusing.

S. aureus HPr has been sequenced (Beyreuther et al., 1977) and thoroughly studied by ^1H and ^{31}P NMR (Gassner et al., 1977; Maurer et al., 1977). In contrast to *E. coli* HPr, *S. aureus* HPr contains three tyrosines and only one phenylalanine. The NMR resonances of these residues have been assigned recently to specific residues in the primary sequence by preparation and analysis of selective nitrotyrosine HPr derivatives (Schmidt-Aderjan et al., 1979). While there does not appear to be much similarity between the *E. coli* and *S. aureus* HPr, it is worthwhile to note that the *S. aureus* protein also contains one tyrosine which does not titrate and appears to be buried in the interior of the molecule. These characteristics are similar to what we find for the tyrosine resonance in *E. coli* HPr.

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